ADVANCES AND CHALLENGES OF ALLOGENEIC STEM CELL TRANSPLANTATION

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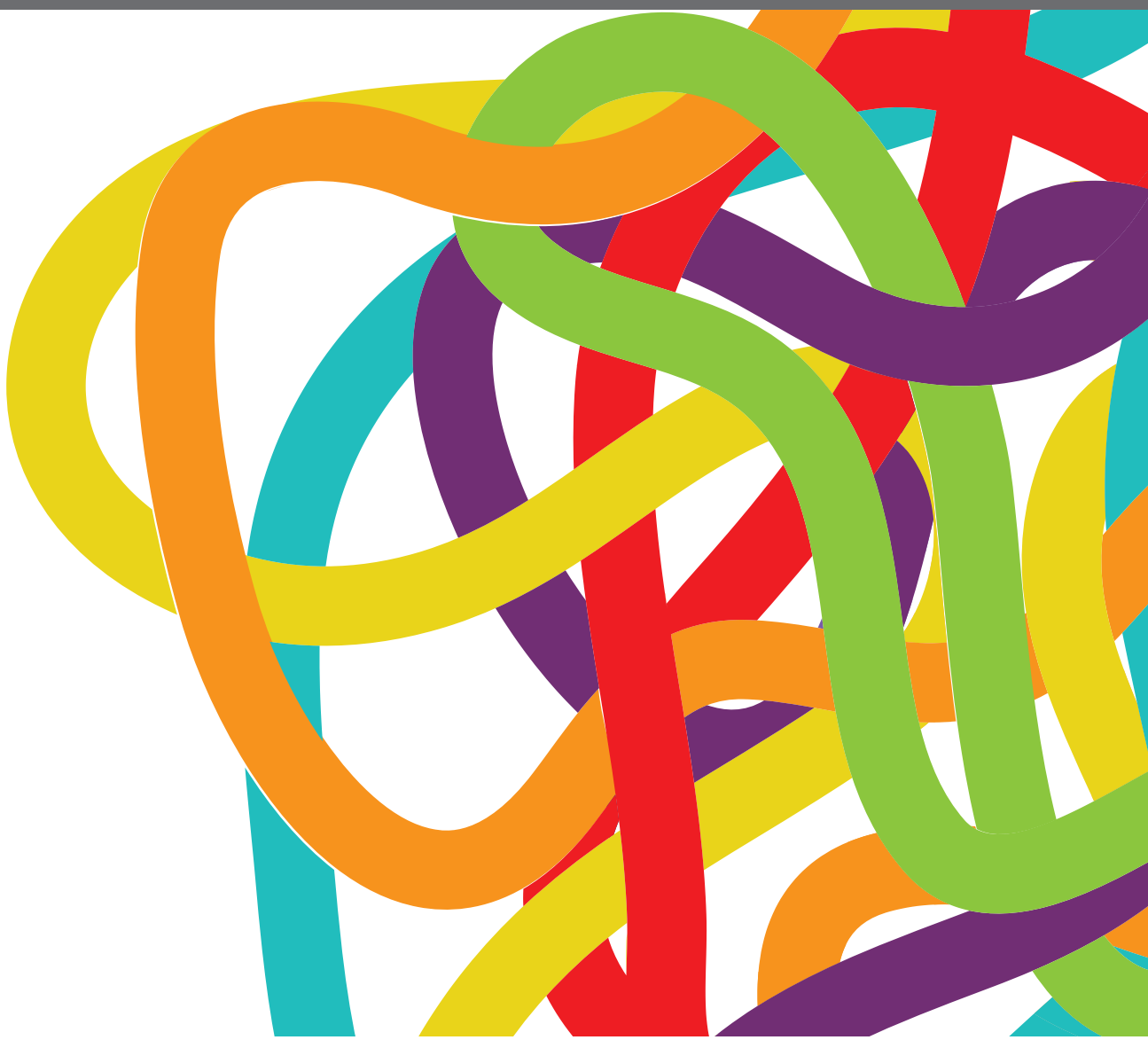

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ADVANCES AND CHALLENGES OF ALLOGENEIC STEM CELL TRANSPLANTATION

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EDITORIAL
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Editorial: Strengths and Challenges of Allo-SCT in the Modern Era

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Editorial on the Research Topic

Advances and Challenges of Allogeneic Stem Cell Transplantation

The use of allogeneic stem cell transplantation (allo-SCT) is increasing over time worldwide (1, 2) and this is the result of three major facts: i) the evidence that allo-SCT can cure a progressively higher number of patients and many hematological diseases (1, 2); ii) the increase in the upper limit of age for allo-SCT eligibility (currently up to 75 years) (3); iii) the improvement of allo-SCT platforms both in terms of efficacy and toxicity (4, 5). However, many challenges in the field of allo-SCT still need to be overcome, mainly the reduction of relapse rate and of transplant related-mortality. Currently, we can assume that approximately 50% of the patients can be cured by allo-SCT, taking into account all the favorable and unfavorable variables associated with long-term outcome after transplantation (6).

The aim of this Research Topic entitled Advances and Challenges of allo-SCT was to collect articles that highlights the most recent evolutions of the transplant platform, together with a focus on the most intriguing new insights that will be developed in the next future. Twenty-one manuscripts have been submitted and eighteen out of them have been accepted for publication.

Interestingly four of these manuscripts cover the field of relapse prevention and treatment, either with donor-lymphocyte infusion (DLI) or with de-methylating agents. Su et al. reports on two strategies on prophylactic DLI in patients with relapsed-refractory acute leukemias: infusion from day +60 irrespective of minimal residual disease (MRD) (cohort 1) and on day +60 or +90 basing on MRD (cohort 2). In summary, they showed that a delay of prophylactic DLI up to day +90 basing on MRD could be associated with lower extensive cGVHD and better graft and relapse-free survival (GRFS). In the survey by the Gruppo Italiano Trapianto di Midollo Osseo (GITMO) published by Patriarca et al. on 254 patients with acute leukemias, 73% of the cases received DLI for leukemia relapse and only 10% received DLI as pre-emptive treatment. Nevertheless, by multivariate analysis, a pre-emptive use of DLI without evident leukemia relapse and multiple infusions of lymphocytes were associated with improved overall survival. In the last 15 years, several papers have been published on the topic of DLI. However, results have been contradictory, and the risk of graft versus host disease (GVHD) following lymphocytes infusion has hampered the extensive and homogeneous use of DLI as post-transplant adoptive immunotherapy. As a consequence, DLIs have been generally used to cure overt hematological relapse. Nevertheless, the use of DLI driven by MRD assessment seems to be the challenge for the next future (7), and an early use of DLI

(from day +90) in case of MRD positivity, either by flow cytometry or by RT-qPCR on target genes at least in acute leukemias, should be explored in the context of multicentric prospective trials.

The other way to prevent relapse in acute leukemias is the use of post-transplant maintenance with molecular target drugs (e.g. FlT3 inhibitors, IDH inhibitors, BCL2 inhibitors,….) or de-methylating agents (e.g. oral azacitidine or decitabine) once again driven by MRD assessment. Antar et al. contribution was a very comprehensive review of these approaches, with a detailed algorithm for clinical use. Liu et al. report on a prospective use of low-dose decitabine as maintenance in patients with acute lymphoblastic leukemia (ALL), suggesting that this strategy could be promising in T-cell ALL. Overall, an early assessment of MRD (e.g. day +60/+90) may offer the opportunity to design a patient-specific protocol for relapse prevention, which should consider all the above-mentioned approaches. In particular, focusing on acute leukemias, the combination of immunotherapy (pre-emptive DLI) with molecular target therapies or de-methylating agents is intriguing and should be explored in multicentric trials (**Figure 1**).

Many other manuscripts of this Research Topic cover topics related to transplant toxicity and non-relapse mortality, either focusing on conditioning intensity and stem cell source, or on CMV prophylaxis with letermovir, or on microbiome assessment and preservation, or on immune reconstitution monitoring.

Li et al. showed that decitabine could be used as conditioning regimen for myelodysplastic syndromes, allowing better long-term outcome with respect to decitabine alone without allo-SCT. Ma et al. confirmed previous observations that peripheral blood stem cells could be safely used in haploidentical transplantation, leading similar outcomes in comparison to a mix of bone marrow and peripheral blood stem cells. Finally, Song et al. reviewed the available data in the literature on the topic of conditioning intensity in acute myeloid leukemia and myelodysplastic syndromes, showing that reduced intensity conditioning regimens may be as effective as conventional regimens. Overall, the issue of conditioning intensity and drugs used in the conditioning is still a matter of debate, and in the last 15 years, we observed very different approaches, moving from very low intensity reduced intensity conditioning regimens, to reduced-toxicity regimens with new drug combinations (e.g. busulfan+fludarabine, treosulfan,…) (8, 9), to very intensive treatments (sequential conditioning) (10). This point clearly highlights that the choice of the conditioning needs to be patients-based, considering the type of disease, the co-morbidities, the frailty and the type of donor.

Terao et al. report on the effects of letermovir on T-cell reconstitution following haploidentical SCT with post-transplant cyclophosphamide and suggest that it may increase the levels of HLA-DR+ T-cells that may be implicated in the development of cGVHD. The use of letermovir for CMV prophylaxis between day 0 and +100 probably represents one of the most important advances in allo-SCT in the last 10 years. Both the registrative trial and several real-life analyses confirm that the incidence of CMV clinically significant infections dropped from more than 50% in the pre-letermovir era to 10-15% in the letermovir era (11, 12). Nevertheless, the issue of CMV in allotransplanted patients still need to be investigated, in particular concerning T-cell reconstitution during letermovir prophylaxis (is it delayed)? and the impact of late CMV reactivations after day +100, when letermovir is discontinued (the results of the multicentric randomized trial with letermovir from day +100 to day +200 are highly awaited). Allo-SCT platforms using post-transplant

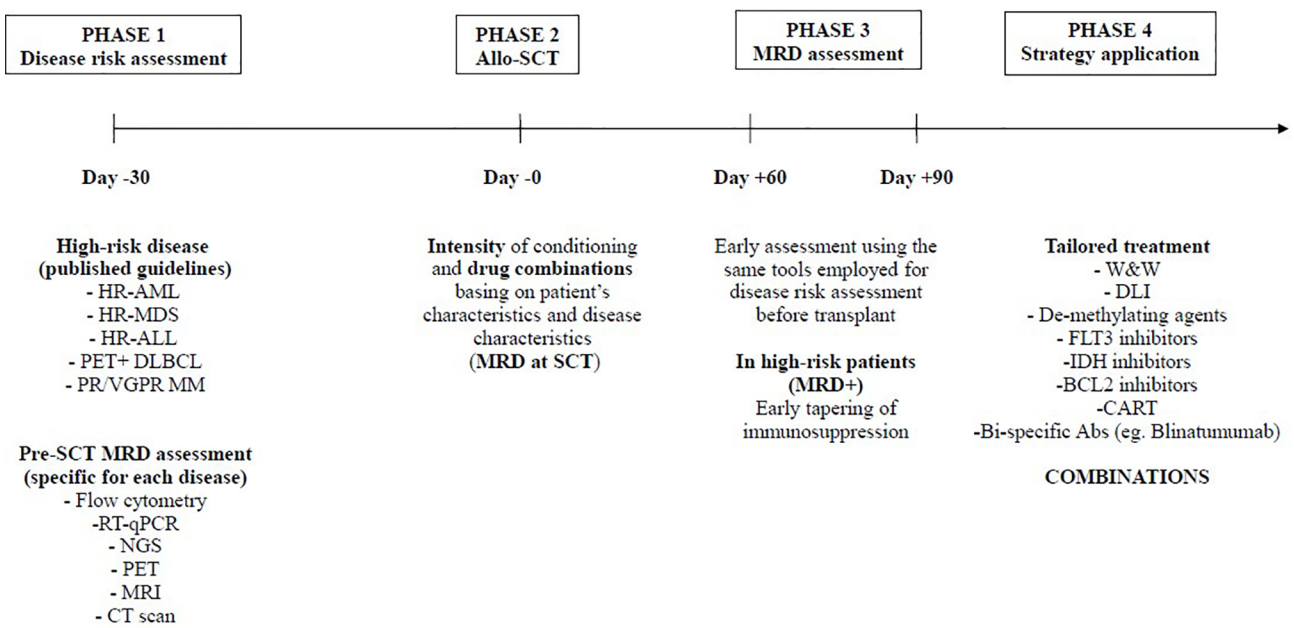

FIGURE 1 | Relapse prevention following allo-SCT.

cyclophosphamide, which has been increasingly investigated in both haploidentical and non-haploidentical settings, appears to be associated with increased viral reactivation rate (13). While letermovir introduction might be a turning point in CMV reactivation prevention, other viruses are still lacking specific management (13). Moreover, deep relationships between intestinal microbiota composition and allo-SCT outcomes have been identified (14), particularly for predicting the mortality from infectious and non-infectious causes. Furthermore, therapeutic manipulations of the gut microbiota, such as fecal microbiota transplant, have emerged as promising therapeutic approaches for restoring the intestinal microbiota post-transplantation (15).

The topic of T-cell reconstitution and its interplay with microbiome has been covered in 3 articles. Milano et al. analysed the repertoire of TCR following cord-blood transplantation and showed that high TCR diversity at day +28 is associated with better patient outcomes. Andrlová et al. approached the topic of immune reconstitution focusing on unconventional T cells, specifically mucosal-associated invariant T cells, γδ-T cells, and invariant NK T cells. Evidences from published data suggest that the near future will face the development of pre-clinical and clinical trials exploring the manipulation of unconventional T-cell compartment and the interplay between microbiota and these unconventional cells. In line with this Research Topic, Alexander et al., on behalf of the Autoimmune Diseases Working Party of the EBMT, focused on the available data on microbiome perturbance following SCT for autoimmune diseases, showing that dysbiosis may influence the outcome in this setting of patients, as observed for conventional allo-SCT for hematological malignancies. Finally, Serpenti et al. showed that immune reconstitution may be useful for predicting severity of cGVHD and long-term outcome, by calculation of a risk-score at cGVHD onset. All these manuscripts clearly underline how intriguing is the topic of immune reconstitution and how complex are the connections with other biological aspects such as microbiome.

Finally, there is an urgent need to re-think the role of allo-SCT in certain diseases (e.g. aggressive lymphomas). This topic has been covered by a review published in the Research Topic by Goldsmith et al. At present, only relapsed and refractory diffuse large B cell lymphomas (DLBCL), primary mediastinic B cell lymphoma (PMBCL) and B-cell acute lymphoblastic leukemia (B-ALL) in patients younger than 25 years have a clear access to CART-cell therapies, in presence of non-responsive residual disease (16). Following the impressive results of the third line therapy with CART, the role of allo-SCT appears even more restricted to very high risk patients in complete remission. In this view, using the CAR-T therapy as a bridge to transplant may be an attractive but questionable option, considering the very high cost of CAR-T. Recently, EBMT and EHA proposed a revised version of recommendations to guide the delivery and management of CART-cell therapies (17). In the next future, CART will be available in many Countries for many other lymphoproliferative diseases such as mantle cell lymphoma, chronic lymphocytic leukemia and multiple myeloma and moreover for acute myeloid leukemias (AMLs). In this latter case, CART could be competitive with allo-SCT or complementary as treatment able to induce complete remission in refractory AML, before eradication with allo-SCT.

In summary, considering the articles published in this Research Topic, we can say that allo-SCT is going through a very enthusiastic phase of research and integration with novel strategies. Relapse prevention with maintenance or pre-emptive treatments, immune reconstitution and microbiome monitoring, modulation of conditioning intensity and integration with CART-cell therapy are some of the active Research Topics that the transplant community will deal with in the next future. Moreover, haploidentical transplantation, in particular followed by post-transplant cyclophosphamide GVHD prophylaxis, has been proved to be as effective as or even superior to HLA-matched allo-SCT. This clearly opened a new scenario, in which a very high risk disease, such as, for example, AML with MRD persistence, can be rapidly and successfully addressed to allo-SCT in first CR (18, 19). Allo-SCT will ultimately be a tailored therapy: not one transplant for many patients, but one transplant for one patient.

AUTHOR CONTRIBUTIONS

All the authors edited the Research Topic. MMa, DR, GR, and JP wrote the editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Impact of T Cell Repertoire Diversity on Mortality Following Cord Blood Transplantation

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Introduction: Cord blood transplantation (CBT) recipients are at increased risk of mortality due to delayed immune recovery (IR). Prior studies in CBT patients have shown that recovery of absolute lymphocyte count is predictive of survival after transplant. However, there are no data on the association of T-cell receptor (TCR) and clinical outcomes after CBT. Here we retrospectively performed TCR beta chain sequencing on peripheral blood (PB) samples of 34 CBT patients.

Methods: All patients received a total body irradiation based conditioning regimen and cyclosporine and MMF were used for graft *versus* host disease (GvHD) prophylaxis. PB was collected pretransplant on days 28, 56, 80, 180, and 1-year posttransplant for retrospective analysis of IR utilizing high-throughput sequencing of TCRβ rearrangements from genomic DNA extracted from PB mononuclear cells. To test the association between TCR repertoire diversity and patient outcomes, we conducted a permutation test on median TCR repertoire diversity for patients who died within the first year posttransplant *versus* those who survived.

Results: Median age was 27 (range 1–58 years) and most of the patients (n = 27) had acute leukemias. There were 15 deaths occurring between 34 to 335 days after transplant. Seven deaths were due to relapse. Rapid turnover of T cell clones was observed at each time point, with TCR repertoires stabilizing by 1-year posttransplant. TCR diversity values at day 100 for patients who died between 100 and 365 days posttransplant were significantly lower than those of the surviving patients ($p = 0.01$).

Conclusions: Using a fast high-throughput TCR sequencing assay we have demonstrated that high TCR diversity is associated with better patient outcomes following CBT. Importantly, this assay is easily performed on posttransplant PB samples, even as early as day 28 posttransplant, making it an excellent candidate for early identification of patients at high risk of death.

Keywords: immune reconstitution, cord blood transplantation, T-cell repertoire diversity, delayed immune recovery, T-cell receptor sequencing

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INTRODUCTION

Patients undergoing hematopoietic cell transplantation (HCT) are at increased risk of transplant-related morbidity and mortality, in part due to the prolonged period of pancytopenia and immune dysregulation that results from the conditioning regimen and infusion of donor stem cells. The use of umbilical cord blood as a graft source has expanded treatment options for many patients, particularly ethnic and racial minorities (1). However, umbilical cord blood transplantation (CBT) recipients appear to be at even greater risk of non-relapse mortality (NRM) in the early posttransplant period when compared to recipients of bone marrow or peripheral blood stem cell grafts from related or unrelated HLA (human leukocyte antigen) matched adult donors (2, 3). CBT recipients have a significantly higher incidence of opportunistic infections in the first year posttransplant (4–6). Despite these challenges, when considering both NRM and relapse, CBT patients' overall mortality risk is comparable to that observed with other graft sources (7–10), and cord blood continues to be one of the graft source for patients without conventional donors (7, 9, 11).

There is a dearth of assays to accurately measure functional rather than numerical reconstitution of the adaptive immune system after transplantation. This has made it difficult to directly address the role of delayed functional immune recovery on CBT outcomes, especially in the setting of many other contributing clinical variables. Immunophenotyping by flow cytometry and quantification of thymopoiesis by detection of TCR recombination excision circles (TRECs) have demonstrated markedly reduced and prolonged T-cell recovery and thymic activity after dCBT as compared to infusion of adult stem cell grafts (12, 13). However, the period of susceptibility to infections continues after numerical recovery by these surrogate measurements, and these assays have not demonstrated substantial value in predicting infectious mortality. The ability to more accurately measure cellular immune reconstitution in patients undergoing HCT (in this case CBT), with the goal of better assessing the consequent risk of morbidity and mortality, could lead to intervention strategies aimed at reducing this risk.

We had previously investigated clonal diversity of the T cell compartment of peripheral blood is a meaningful method of assessing cellular adaptive immune reconstitution. In the blood of a healthy adult, an individual T cell primarily expresses one of millions of different T Cell Receptors (TCRs); a clone is defined as the set of T cells expressing the same TCR (14, 15). The cellular adaptive immune system plays an important role in conveying protection against pathogenic infection, in part, through the development of a highly diverse repertoire of TCR genes, which is thought to be necessary for adequate protection against pathogens. This is evident in humans with primary or acquired immunodeficiency diseases [e.g., severe combined immunodeficiency (SCIDS), common variable immune deficiency (CVID), and HIV], in aging, and following HCT where loss of TCR diversity has been implicated in the increase in morbidity and mortality from infection that is observed in these clinical settings (16–19).

Due to the extremely large number of T-cell clones present in the healthy human, estimates of total T-cell repertoire diversity must be made from subsamples of the T-cell repertoire. Herein, we apply a high-throughput DNA sequencing method to immunosequence the CDR3 regions of rearranged TCRβ genes from peripheral blood mononuclear cells (PBMC) collected from 34 recipients of myeloablative conditioning CBT at Fred Hutchinson Cancer Research Center at multiple time points after transplant.

MATERIALS AND METHODS

Study Design

Patients undergoing myeloablative single or double CBT at the Fred Hutchinson Cancer Research Center (FHCRC)/Seattle Cancer Care Alliance (SCCA) between August 2007 and 2010 on research protocols approved by the Center's Institutional Review Board were eligible for this retrospective analysis of data collected prospectively. All patients consented to collection of blood samples for studies of immune reconstitution posttransplant. In addition, healthy adults were enrolled as controls in a study, "Immunology studies of Normal Healthy Individuals," at Adaptive Biotechnologies. All subjects provided written informed consent to participate in the study, which was approved by Western Institutional Review Board.

Patients, Treatment Regimens, and Posttransplant Supportive Care

Patients were eligible for a myeloablative high-dose TBI-based CBT if they were aged ≤45 years old or treosulfan-based if they were ≤65 years old and lacked a suitably matched related or unrelated donor. The underlying disease was categorized as standard or high-risk based upon previously described criteria (20). CB donor selection was based on institutional guidelines and units were selected to optimize both HLA match and cell dose, avoiding, when possible, CB units which the patient had donor specific anti-HLA antibodies. All patients received unrelated donor CB grafts, which were 4 of 6 to 6 of 6 matched to the recipient at HLA-A, B, and DRB1 antigens. HLA typing was performed at the antigen level for HLA-A and B, and high-resolution HLA typing was performed for HLA-DRB1 alleles. The selection of two CB units was mandatory when a single CB unit did not meet the following criteria: HLA match 6 of 6 with a total nucleated cell count (TNC) dose of ≥2.5 × 107/kg or HLA match 5 of 6, 4 of 6 with a TNC dose of ≥4.0 (± 0.5) × 10^7/kg. In patients receiving a double CBT, the individual CB units were at least three of six HLA-A, B, and DRB1 matched to each other, and each contained a minimum of 1.5 × 10^7 TNC per kilogram. Of 38 patients potentially eligible, three patients without any blood samples stored for TCR analysis and one patient who died before day 28 were excluded. All patients received prophylactic antimicrobial and antifungal agents per institutional guidelines (21) and remained at our institution for a minimum of 100 days posttransplant. After discharge from our center, patients were seen as clinically indicated, with follow-up

assessments per protocol at 6 months and 1 year to include a formal graft-versus-host-disease (GvHD) assessment and PB obtained for immune reconstitution studies and basic lab work.

Sequencing Assay and Evaluation of Immune Reconstitution Posttransplant

PB was collected pretransplant and on days 28, 56, 80–100, 180, and 1-year posttransplant for retrospective analysis of immune recovery utilizing high-throughput sequencing of TCRβ rearrangements from genomic DNA extracted from PBMCs. We sequenced the CDR3 region of TCRβ from approximately 250,000 PBMCs from each time point in surviving patients, and we sequenced four PBMC samples from each of four healthy controls over a 1-year time-course. The TCRβ CDR3 region was defined according to the IMGT collaboration (22), beginning with the second conserved cysteine encoded by the 3′ portion of the Vβ gene segment and ending with the conserved phenylalanine encoded by the 5′ portion of the Jβ gene segment. TCRβ CDR3 regions were amplified and sequenced using protocols described by Robins et al. (15). Briefly, a multiplexed PCR method was employed to amplify all possible rearranged genomic TCRβ sequences using 52 forward primers, each specific to a TCR Vβ segment, and 13 reverse primers, each specific to a TCR Jβ segment. Reads of length 60 bp were obtained using the Illumina HiSeq System. Raw HiSeq sequence data were preprocessed to remove errors in the primary sequence of each read, and to compress the data. A nearest neighbor algorithm was used to collapse the data into unique sequences by merging closely related sequences, to remove both PCR and sequencing errors.

Statistical Considerations

To test the association between TCR repertoire diversity and patient outcomes, we conducted a permutation test on median TCR repertoire diversity for patients who died within the first year posttransplant *versus* those who survived by generating 10,000 permutations of mortality labels. In this case, our test is ideal because the median is robust to outliers and a permutation test makes no assumptions about the distribution of TCR repertoire diversity among patients. To maintain consistency, the same approach was used to test the association of $CD3^+$ cell counts and TREC values with patient mortality. Differences in patient characteristics according to outcome were assessed *via* a two-tailed Fisher's exact test for binary data and a two-tailed Mann-Whitney U test for continuous data. While we could not assess all possible confounding factors in a multivariate model, we did calculate and report the marginal p value associated with each possible confounding factor separately.

RESULTS

Study Cohort

Thirty-four patients were included in the final analysis. This cohort was composed of 11 pediatric and 23 adult recipients (median age, 26.5 years), who primarily had acute leukemia (n = 26). Conditioning consisted of either high dose TBI (1,320 cGy), cytoxan and fludarabine or treosulfan, fludarabine, and low dose TBI (200 cGy) with cyclosporine and mycophenolate mofetil as GvHD prophylaxis. Recipients who experienced graft failure were excluded. We analyzed PB prior to transplant, and then at 1, 2, 3, 6, and 12 months after CBT, based on sample availability. Median follow up among all patients was 370 days, range 34–1,657. **Table 1** summarizes the recipient, disease, and transplant characteristics of the patients.

Patient Mortality in the Study Cohort

Among the 34 recipients, there were 15 deaths occurring between 34 to 335 days after transplant. Seven deaths involved relapse, although one recipient died of influenza while in early relapse. Eight additional recipients experienced NRM; three died before day 56 (one of hepatic failure, one of diffuse alveolar hemorrhage and one of disseminated cytomegalovirus (CMV) infection). Five patients experienced NRM between 100 days and 1 year after transplant. Primary cause of death was multi-system organ

TABLE 1 | Patient and unit characteristics of 34 cord blood transplantation (CBT) recipients.

Characteristic	Total (n = 34)
Age, y, median (range)	27 (1–58)
Male, n (%)	15 (44.1)
Diagnosis, n (%)	
AML/MDS	18 (53)
ALL	10 (29)
Myeloproliferative disorders	4 (12)
Biphenotypic leukemia	1 (3)
Chronic lymphocytic leukemia	1 (3)
Number of units received, n (%)	
1	2 (5.9)
2	32 (94.1)
Overall CD34, median × 10^6/kg (range) †	0.21 (0.08–1.67)
Overall TNC, median × 10^7/kg (range) †	5.14 (3.5–15.9)
Total volume, ml, median (range) †	65 (43–387)
Age of CB unit, mo., median (range)	44 (8–140)
Presence of donor specific anti-HLA antibodies, n	0
HLA matching to recipients, n (%)#	
4/6	21 (62)
5/6	10 (29)
6/6	3 (9)
Conditioning intensity, n (%)	
Cytoxan, fludarabine, TBI (1320)	24 (71)
Treosulfan, fludarabine, TBI (200)	10 (29)
GvHD prophylaxis, n (%)	
CsA/MMF	34 (100)
Status at time of HCT, n (%)	
MRD+	17 (50)
CR1	13 (38.2)
CR>2	13 (38.2)
Relapsed/refractory disease	2 (5.9)
Chronic phase (CLL/CML)	4 (11.7)
Other (refractory anemia)	2 (5.9)

CBT, cord blood transplantation; AML/MDS, acute myeloid leukemia/myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; TBI, total body irradiation; GvHD, graft-versus-host-disease; CsA/MM, cyclosporine A/mycophenolate mofetil; HCT, hematopoietic cell transplantation; MRD, measurable residual disease; CR1, first complete remission; CR > 2, more than two complete remissions.

†*Pre-thaw median CD34+, TNC, and TVOL of all units.*

#*HLA matching reflects the lowest HLA-match of the unit.*

failure in three recipients (one with fungal encephalitis); respiratory failure in two recipients (one with invasive pulmonary fungal infection). Finally, a single patient died from a secondary tumor at 1,589 days post-transplant. **Table 2** summarizes features of the subjects when separated by outcome (1-year overall survival), including age, sex, CMV status at transplant, CR status (complete remission 2/3 or relapsed/refractory/progressive disease *vs*. all others), and presence of acute and chronic GVHD after transplant. None of these factors differed significantly between patients with and without NRM except for age.

Changes in T Cell Clonal Diversity Posttransplant

We utilized the distribution of T-cell clones in up to ~250,000 PBMC from each sample (subject to availability of adequate material) to estimate the species richness of unique T cell receptor beta sequences in each recipient's peripheral blood using an unseen species analysis. (15, 23) Estimated species richness was computed for each time point sampled (**Figure 1**). Myeloablative conditioning resulted in a large drop in T-cell diversity from pre-transplant values. T-cell diversity nadired at 2 months after transplant, with a slow but substantial increase in T-cell repertoire diversity by 1 year. However, T-cell diversity at 1 year in CBT recipients was still lower than that in a sample of four healthy adult subjects.

Tracking T Cell Clones Posttransplant

In order to assess the stability of the reconstituting adaptive immune system over time, we investigated the persistence of TCR clones found at early time-points in later samples. Using only patients with samples collected and sequenced at 28, 56, 100, 180, and 365 days post-transplant, we determined the top 10 TCR clones by frequency in each patient at the 28, 56, 100, and 180 day time-points and tracked their frequency over time in one representative CBT patient and one healthy subject (**Figure 2**). This comparison revealed substantial clonal turnover within the CBT patient, with large clonal expansions appearing over a short period of time and dropping to low frequency or disappearing entirely soon afterward.

We next considered all 14 patients with complete sequencing data and classified each of the top 10 T-cell receptor beta (TCRB) clones at each time-point as either persistent or transient. A top-10 TCR clone that was observed (at any frequency) at a later time-point was considered persistent, and clones that were never again observed in samples from the same patient were considered transient. **Figure 3** shows the mean number of persistent TCR clones in the top 10, at each time-point posttransplant. At 28 and 56 days posttransplant, we observed dynamic and highly unstable TCR repertoires in which many TCR clones that were present at high frequency in an early sample were never observed again. Starting at 100 days posttransplant, this pattern began to subside and patients' TCR repertoires became more stable. To confirm that this pattern is highly unusual, we sequenced PBMC samples from four healthy subjects over the same length of time. The median number of transient TCR clones in the top 10 was 0 for these healthy controls at each time-point we studied, confirming that the high prevalence of transient TCR clones following transplant is indicative of an unusually unstable TCR repertoire.

TABLE 2 | Outcomes summary.

	Alive (n = 19)	Death (n = 15)	p-value
Median age, years (range)	18 (1–58)	33 (18-56)	0.007
CMV serostatus, n (%)			
Positive	12 (63)	10 (67)	1.00
Negative	7 (37)	5 (33)	
TBI, n (%)			
1,320 cGy	14 (74)	10 (67)	0.72
200 cGy	5 (26)	5 (33)	
Acute GvHD, n (%)			
Grade 0–II	15 (83)	12 (80)	1.00
Grade III-IV	3 (17)	3 (20)	
MRD, n (%)			
+	7 (37)	10 (67)	0.17
–	12 (63)	5 (33)	
CR at transplant, n (%)*			
≤CR1	13 (68)	7 (47)	0.30
≥CR2	6 (32)	8 (53)	
Days to engraftment (WBC > 500), median (range)	19 (13–44)	25 (7–45)	0.20

Summary of patient characteristics separated by 1-year overall survival. P-values are calculated using a Mann-Whitney U test for continuous variables and a Fisher's exact test for categorical variables.

TBI, total body irradiation; GvHD, graft-versus-host-disease; CMV, cytomegalovirus; CR, complete remission; MRD, measurable residual disease; WBC, white blood cell count.

**Patients transplanted with refractory anemia or in chronic phase are considered ≤CR1 (i.e., low-risk) for this comparison. Patients not in remission are considered ≥CR2 (i.e., high-risk) for this comparison.*

Correlation of T-Cell Receptor Diversity With Patient Mortality

We found that the evolution of TCR diversity following transplant differed between patients who did and did not survive the first year posttransplant (**Figure 4**). Survivors' average TCR repertoire size reached its nadir at 28 days posttransplant followed by a period of more rapid recovery. In contrast, those who died demonstrated an average TCR repertoire size that continued to decrease until day 100 such that the median TCR repertoire size of patients who subsequently died was significantly lower than that of survivors' ($p = 0.019$ by permutation). Of the 10 patients who were alive at day 100 but died before 1-year posttransplant, median survival was 216 days, indicating that a robust statistical signal present at day 100 could allow for adequate time for the implementation of potential clinical interventions.

Other Factors Affecting Patient Mortality

Posttransplant immune recovery is influenced by many factors, most significantly by the immunologic effects of GvHD and of the IST used for its prevention and treatment. To better determine the association of TCR diversity with risk of mortality, we evaluated treatment with IST, total absolute CD3+ counts and TREC levels as potential confounders of the association between TCR diversity and patient mortality.

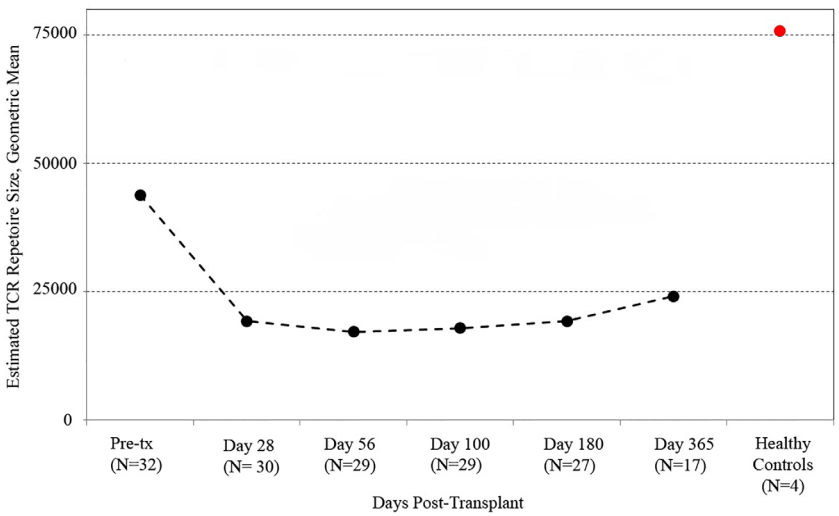

FIGURE 1 | T cell receptor (TCR) repertoire reconstitution after stem cell transplant. We obtained peripheral blood samples from each of 32 patients before transplant and five times after transplant. TCR repertoire size for each patient was estimated using high-throughput sequencing of TCR rearrangements, and the geometric mean of estimated TCR repertoire size is shown. After transplant, patients had a vastly reduced TCR repertoire which reached its minimum 56 days posttransplant before beginning a slow recovery. The value for healthy subjects is the geometric mean of sixteen samples (four samples per subject from four healthy controls). One-year posttransplant, myeloablative CBT patients still had much lower TCR repertoire sizes than healthy control subjects.

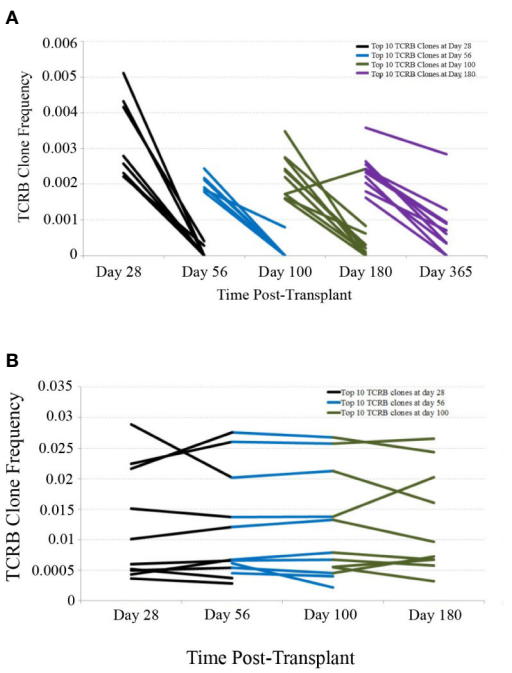

FIGURE 2 | TCRB clonal frequency over time, cord blood transplantation (CBT) patient *vs*. healthy subject. **(A)** We have charted the frequency of the 10 most frequent TCRB clones observed 28 days after transplant in one representative CBT patient. These 10 clones were tracked forward in time, and their frequencies at day 56 are plotted. Likewise, we have plotted the change in frequency of the top 10 clones for each pair of adjacent time-points in this patient. Many of the most frequent TCRB clones observed in early time-points either dropped in frequency or disappeared within weeks. By day 180, a drop-in clone frequency between time-points was still evident but most of the top 10 TCRB clones were observed again at some frequency at day 365. **(B)** We performed a similar analysis for one representative healthy subject. Very little clonal turnover was observed; many of the most frequent TCRB clones persisted across time-points, remaining at similar frequencies throughout the 6-month time-course.

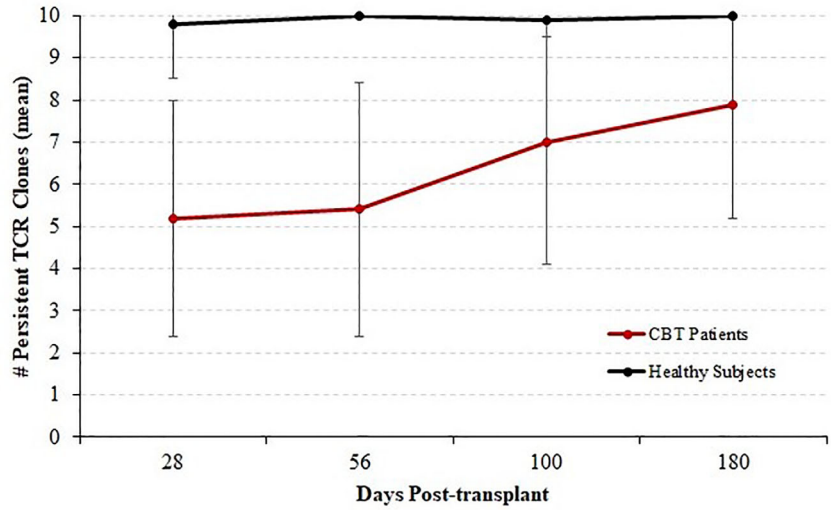

FIGURE 3 | Persistence of T cell receptor (TCR) clones during immune reconstitution. Starting with each patient who survived through day 365 and for whom samples were available for sequencing at each time-point (N = 14), each of the top 10 TCR clones by frequency was classified as either persistent (observed again in the same patient at any later time point) or transient (not observed again at any level in subsequent samples from the same patient). We report the mean and standard deviation of the number of persistent TCRB clones among patients. The number of persistent clones was highly variable, ranging from 1 to 10, but the mean number of persistent clones increased with time indicating a stabilizing TCRB repertoire by 1-year posttransplant. Four healthy subjects were analyzed in the same fashion over a similar time-course; and the number of persistent TCR clones ranged from 9 to 10 with a median of 10.

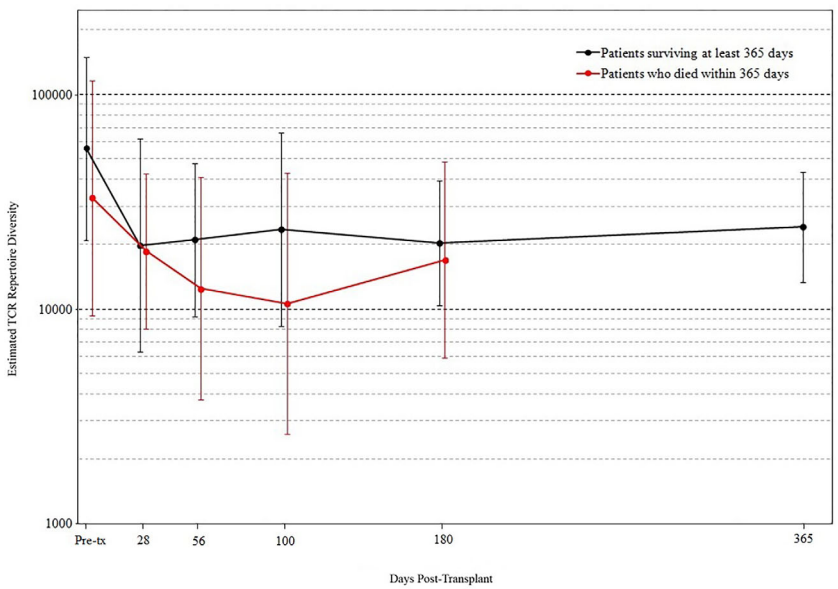

FIGURE 4 | T cell receptor (TCR) repertoire comparison by outcome. Peripheral blood samples were taken from each cord blood transplantation (CBT) patient before transplant and five times after transplant. TCR repertoire size for each sample was estimated using high-throughput sequencing of TCR rearrangements. Patients are divided into those who survived through 1-year posttransplant (black) and patients who died within 1 year (red). At each of six time-points (pre-tx, 28, 56, 100, 180, 365 days posttransplant), we report the geometric mean and standard deviation of TCR repertoire size for each group of patients. At day 100, the median TCR repertoire size of patients who died was significantly lower than that of patients who survived ($p = 0.019$ by permutation). For the six time-points in order, N = 17, 16, 18, 18, 19, 17 for patients who survived through day 365; N = 15, 14, 11, 10, 8 for patients who died before day 365. At each time-point, all surviving patients with TCR sequencing data are included.

Twenty-six patients developed acute GvHD at a median of 23 days posttransplant, including 20 patients with grade II and 6 with grade III–IV acute GvHD. These patients were initially treated with prednisone at a dose ranging from 0.5 to 2 mg/kg. Twenty-seven patients (80%) received prednisone in the first 100 days at a median time of 28 days (range, 15–91; death soon after transplant was responsible for most of the patients which did not receive prednisone). Of these 27, 23 (85%) and 10 (37%) patients remained on prednisone therapy at 1 year after transplantation, respectively. We saw no relationship between prednisone treatment and clinical outcome in this cohort.

Correlation of Absolute CD3 Counts With Patient Mortality

Another potential confounding factor in the correlation of TCR diversity measurements with clinical outcome is the recovery of total $CD3^+$ cell numbers. However, when the kinetics of T cell recovery were measured by the absolute $CD3^+$ cells/μl in peripheral blood at the same time as the measurement of TCR diversity, little of the observed difference in TCR diversity could be explained by variations in absolute T cell counts; the correlation between diversity and absolute CD3 counts was very weak in this cohort ($r = 0.05$). This finding suggests that the estimation of clonal diversity using high-throughput sequencing provides information independent from the total density of circulating T cells. Furthermore, we found that the lymphocyte count following transplant did not differ between patients who did and did not survive the first year posttransplant (**Figure 5**).

In order to assess the effect of absolute $CD3^+$ counts on patient mortality, we conducted a permutation test at 56 and 100 days posttransplant comparing median $CD3^+$ cell counts in survivors *vs.* non-survivors in the same fashion as we tested TCR repertoire diversity. In this cohort, $CD3^+$ counts do not appear to be significantly lower in non-survivors than in survivors at 56 days ($p = 0.23$) or 100 days ($p = 0.14$) posttransplant.

Correlation of T Cell Receptor Excision Circles With Patient Mortality

T cell receptor excision circles (TRECs), created during TCR rearrangement in the thymus, provide a means to quantify thymopoiesis following stem cell transplant. To investigate the relationship of TRECs to patient outcome in our cohort, we measured TREC levels using PBMC samples taken at the same five times posttransplant used for TCR diversity analysis. Overall, TREC levels differed widely between patients. Mean TREC levels were initially very low both for patients who survived and for those who died (data not shown). TREC levels decreased over time among patients who died, but recovered in surviving patients, consistent with the important role thymopoietic reconstitution is known to play in immune recovery (24). Due to the large variation between patients and the relatively late recovery of TREC values even in survivors, TREC values did not predict clinical outcome in this cohort in the first year posttransplant. We were unable to ascertain the relationship between patient outcomes and TRECs beyond the first year posttransplant, since only a single mortality (at approximately 4.5 years posttransplant, due to secondary malignancy) was observed after the first 365 days.

In addition to GvHD treatment, total $CD3^+$ counts, and TREC levels, the correlation of our TCR diversity measurement with clinical outcome may also be driven by

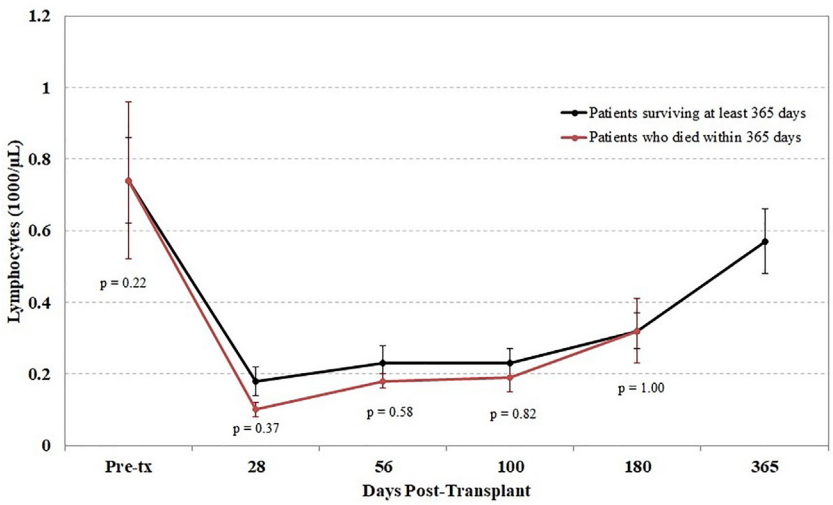

FIGURE 5 | Impact of lymphocyte counts on survival. Peripheral blood samples were taken from each cord blood transplantation (CBT) patient before transplant and five times after transplant. Patients are divided into those who survived through 1-year posttransplant (black) and patients who died within 1 year (red). At each of six time-points (0, 28, 56, 100, 180, 365 days posttransplant), we report the mean and the mean standard error of lymphocyte counts for each group of patients. No significant differences were seen at any time point between the two groups. For the six time-points in order, N = 14, 16, 17, 17, 14, 17 for patients who survived through day 365; N = 15, 11, 10, 10, 6 for patients who died before day 365. At each time-point, all surviving patients with TCR sequencing data are included.

other variables. **Table 2** presents a comparison of characteristics of the 15 patients who died within 1 year of transplant *versus* the 19 patients who survived. Most factors appeared to be unrelated to mortality. However, the 15 non-survivors were significantly older than the survivors ($p = 0.007$), which indicates a correlation to patient mortality with or without TCR diversity acting as an intermediary. In this cohort, patient age and TCR repertoire size are not significantly correlated ($r -0.28$, two-tailed $p = 0.15$ by normal approximation), suggesting that TCR repertoire and patient age may be independently correlated with mortality risk. Taken together, our results indicate that in this cohort TCR repertoire diversity is a statistically significant correlate with patient survival and among several other clinical variables measured, patient age (which is uncorrelated to TCR repertoire diversity in this cohort) is the only other statistically significant correlate.

Comparison of T Cell Receptor Diversity by High-Throughput Sequencing and Spectratype

Spectratyping is a well-established technology for the assessment of the diversity of the TCR repertoire, which uses PCR with V gene segment-specific primers coupled with an analysis of amplicon length to assess the diversity of TCRs by V gene usage and CDR3 region length. The results of our high-throughput method are expected to recapitulate those obtained with spectratype analysis, with the additional benefits of providing sequence information for each clone, the ability to distinguish a moderately diverse repertoire (with enough TCR diversity for all V gene/CDR3 length classes to be represented) from a fully diverse repertoire, and assessment of quantitative output. Spectratype analysis was performed on all patients at the same time-points used for high-throughput sequencing thereby allowing us to compare these two methods. The results of this comparison are presented for 3 patients in **Figure 6**; our sequencing data do agree with spectratype analysis in most patients, and in some patients, sequencing provides additional clinically relevant data.

DISCUSSION

In this study, we have demonstrated a significant correlation between our measurement of immune reconstitution using high-throughput TCR sequencing at day 100 posttransplant and subsequent risk of mortality in a cohort of 34 CBT patients. This result is in accordance with our initial hypothesis that delayed immune reconstitution, as measured by low diversity of TCR rearrangements in circulating T cells, puts patients at high risk. Indeed, the primary objective of this study was to evaluate whether a more direct measure of T cell clonal diversity (as measured by high-throughput sequencing) was correlated with clinical outcome, in particular an increased risk of mortality during the first year posttransplant in patients undergoing myeloablative CBT. Differently than our study, Buhler et al. recently showed that TCR diversity was not predictive of GVHD, relapse, death, or infections post-HCT in a cohort of 116 donor/recipient pairs undergoing an allogeneic HCT (unrelated = 42; related = 70; haploidentical = 4) (25). However, the latter study analyzed TCR diversity shortly before transplantation (time point 1) and at 1-year post-HCT (time point 2). Using our same approach at multiple time points after HCT, Leick et al. showed instead a significant correlation between increased clonal expansion and acute GVHD in a cohort of 99 related or unrelated donor (57 unrelated, 42 related) allogeneic HCT recipients (26).

Monitoring for risk of leukemic relapse posttransplant can be determined by DNA-based analysis of patient/donor chimerism and sensitive assays for minimal residual disease. In contrast to other risks that contribute to morbidity and mortality, the risk of infectious complications is not easy to analyze in a quantitative fashion. The development of assays which provide a direct measure of immune reconstitution could help identify those patients at higher risk of life-threatening complications and could lead to medical intervention strategies. Direct measure of hematopoietic recovery is easily accomplished by obtaining complete blood counts and measurement of TRECs is adequate to assess thymopoietic reconstitution in the first years posttransplant. However, a direct measure of early immune system recovery, especially with respect to T cell function as opposed to T cell numbers, is lacking. Existing measures of TCR diversity that might fill this role, e.g., spectratype analysis, do not provide the quantitative information necessary for robust and consistent analysis.

New methods to directly measure immune recovery in post-hematopoietic cell transplant recipients, as proposed here, are vital in tailoring the medical management of individual patients. This is particularly important if we are able to identify those patients at greatest risk of future mortality through these direct measurements in time to intervene and effectively prevent mortality. The clinical utility of such foreknowledge will rely on further study: namely, the creation of a clinically meaningful scheme for stratifying patients into risk groups and the development of effective alternative therapies for high-risk patients.

The limited size of the patient cohort did not allow for a rigorous multivariate model that is necessary to prove that TCR diversity is a significant and independent predictor of mortality. Our data are also insufficient to determine whether the association of high TCR diversity with better patient outcomes is mediated by TCR diversity *per se*, nor can our data directly address whether higher TCR diversity necessarily indicates improved clinical immunocompetence. Yet we have demonstrated that the outcomes in this study match our *a priori* hypothesis, and have further demonstrated that this result cannot be immediately explained simply by alternative measures of immune reconstitution such as peripheral blood absolute $CD3^+$ cell counts or TRECs, or by any of several other variables measured in our small cohort. It is acknowledged, however, that a thorough study of whether TCR diversity is an independent predictive measure of patient outcomes and whether low TCR diversity is directly causal of

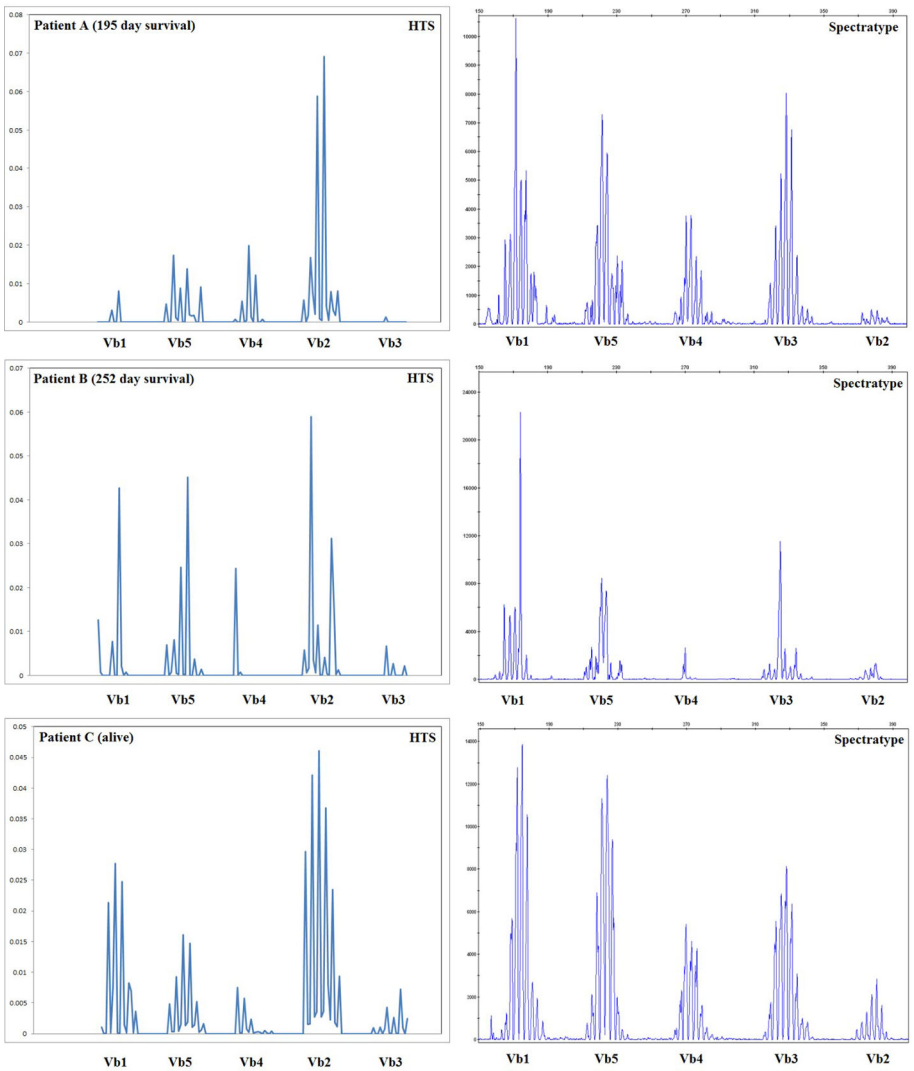

FIGURE 6 | Comparison of spectratype data with high-throughput sequencing. Here we present a subset of the data generated, including the results for Vb1–Vb5 (i.e., one spectratype reaction) for three representative patients at 56 days posttransplant, including two patients who died during the first year posttransplant and one patient who survived. For patients B and C, spectratyping and high-throughput sequencing (HTS) agree, indicating an oligoclonal repertoire in patient B and a diverse repertoire in patient C. Patient A appears much more oligoclonal in our high-throughput sequencing (HTS) data than in the spectratype data; HTS estimated a very low TCRB repertoire size for patient A, who went on to die 195 days posttransplant. Taken together, these data indicate that HTS and spectratyping data are in agreement when analyzed in a similar fashion, and HTS offers an additional depth of data and the advantage of quantitative rather than qualitative output.

inferior outcomes must await an analysis with a larger cohort of patients.

In conclusion, using a fast high-throughput TCR sequencing assay we have demonstrated that high TCR diversity is associated with better patient outcomes following myeloablative CBT. Importantly, this assay is easily performed on posttransplant peripheral blood samples, even as early as day 28 posttransplant. Currently, there are no other clinical assays available that provide information on immune reconstitution this early posttransplant. While these data confirm that T cell clonal dynamics could serve as a predictive tool to identify patients at high risk of death this will require further investigation prospectively in larger and more homogeneous patient cohorts.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Western Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FM, HR, RE, and CD participated in the study design, data analysis, and interpretation of data for the manuscript. ER and FM wrote the first draft and LT, RS, AD, HR, and CD provided revisions and critical review of the final manuscript. KG performed the statistical analyses. All authors contributed to the article and approved the submitted version.

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Donor Lymphocyte Infusions After Allogeneic Stem Cell Transplantation in Acute Leukemia: A Survey From the Gruppo Italiano Trapianto Midollo Osseo (GITMO)

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We conducted a retrospective multicenter study including pediatric and adult patients with acute leukemia (AL) who received donor lymphocyte infusions (DLIs) after allogeneic hematopoietic stem cell transplantation (HCT) between January 1, 2010 and December 31, 2015, in order to determine the efficacy and toxicity of the immune treatment. Two hundred fifty-two patients, median age 45.1 years (1.6–73.4), were enrolled from 34 Italian transplant centers. The underlying disease was acute myeloid leukemia in 180 cases (71%). Donors were HLA identical or 1 locus mismatched sibling (40%), unrelated (40%), or haploidentical (20%). The first DLI was administered at a median time of 258 days (55–3,784) after HCT. The main indication for DLI was leukemia relapse (73%), followed by mixed chimerism (17%), and pre-emptive/prophylactic use (10%). Ninety-six patients (38%) received one single infusion, whereas 65 (26%), 42 (17%), and 49 patients (19%) received 2, 3, or ≥4 infusions, respectively, with a median of 31 days between two subsequent DLIs. Forty percent of evaluable patients received no treatment before the first DLI, whereas radiotherapy, conventional chemotherapy or targeted treatments were administered in 3, 39, and 18%, respectively. In informative patients, a few severe adverse events were reported: grade III–IV graft versus host disease (GVHD) (3%), grade III–IV hematological toxicity (11%), and DLI-related mortality (9%). Forty-six patients (18%) received a second HCT after a median of 232 days (32–1,390) from the first DLI. With a median follow-up of 461 days (2–3,255) after the first DLI, 1-, 3-,

and 5- year overall survival (OS) of the whole group from start of DLI treatment was 55, 39, and 33%, respectively. In multivariate analysis, older recipient age, and transplants from haploidentical donors significantly reduced OS, whereas DLI for mixed chimerism or as pre-emptive/prophylactic treatment compared to DLI for AL relapse and a schedule including more than one DLI significantly prolonged OS. This GITMO survey confirms that DLI administration in absence of overt hematological relapse and multiple infusions are associated with a favorable outcome in AL patients. DLI from haploidentical donors had a poor outcome and may represent an area of further investigation.

Keywords: donor lymphocyte infusions, relapse, allogeneic stem cell transplantation, acute leukemia, pre-emptive treatment

INTRODUCTION

Disease relapse is the leading cause of treatment failure and mortality in patients with acute leukemia (AL) undergoing allogeneic hematopoietic stem cell transplantation (HCT). Most relapses occur within 1 year after HCT and exhibit a progressive clinical course. Two main mechanisms may be responsible for relapse after HCT: tumor cells may escape from the impact of pre-transplant conditioning chemotherapy regimens or tumor cells may evade post-transplant immune control. Many treatment strategies, including pharmaceutical and cell-based treatments, have been developed and tested to prevent and treat relapses. Donor lymphocyte infusion (DLI) is a form of adoptive immunotherapy aiming to enhance the graft-versus-leukemia (GVL) effect after HCT. DLIs were first used in patients suffering from chronic myeloid leukemia (CML) relapse after HCT. In these patients, especially in the case of cytogenetic or molecular relapse, DLIs achieved a high rate of complete responses (up to 80%) compared to patients with other hematological disorders (1–3). In patients with acute leukemia, clinical responses have been reported to be fewer, particularly in the case of overt relapse and in the presence of acute lymphoid leukemia (4–8). Moreover, clinical success is limited by the occurrence of acute and chronic graft-versus-host disease (GVHD), marrow aplasia and infections, which can be all causes of treatment-related mortality in up to 20% of patients (9, 10). To determine the efficacy and toxicity of DLIs and to identify potential factors influencing the outcome, we conducted a retrospective multicenter study including patients with AL who received DLIs after HCT from related and unrelated donors.

METHODS

Study Design and Information Collection

This was a multicenter retrospective study carried out in Italian transplant centers coordinated by the Gruppo Italiano per il Trapianto Midollo Osseo e Terapia Cellulare (GITMO) network. Criteria for patient eligibility were the following: adult and pediatric patients, without age limit; diagnosis of acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL); any stage of disease at transplant; first HCT from HLA-identical sibling or volunteer or mismatched related donor; myeloablative or reduced-intensity conditioning (RIC) regimen; and at least 1 unmanipulated DLI administered between January 1, 2010 and December 31, 2015. Exclusion criteria included: DLI treatment after second or further HCT, T-cell depleted transplant, and diagnosis other than AL. The primary endpoint was overall survival (OS). The secondary endpoints were: indications for DLI administration and the DLI schedule most commonly adopted among the GITMO centers, response rate, non-relapse mortality (NRM), hematological toxicity, and acute and chronic GVHD incidence. Thirty-four GITMO centers accredited for allogeneic HCT participated in the study. Information was collected in two phases. In the first phase, a survey was conducted to collect the data of the 34 participating centers from the GITMO registry. The data collected were the following: demographic data, relationship and HLA compatibility of patients and donors, AL type, conditioning, stem cell source, GVHD prophylaxis, acute and chronic GVHD after transplantation, relapse, patient and disease status at last follow-up, date of DLI administration, clinical indication for DLI administration, possible treatments administered before DLI, and date of possible second HCT. In the second phase, the participating centers were asked to provide data that were missing from the GITMO registry. The data provided were the following: cell doses, transfusion schedule, hematological and non-hematological toxicity, acute and chronic GVHD after DLIs, and disease response. Fifteen centers agreed to and completed the second part of the study.

Ethics Section

The study involving human participants was reviewed and approved by the Ethics Committee of the center of the national principal investigator, called "Comitato Unico Regionale Friuli-Venezia-Giulia" on 2017, 3 October (Protocol Number 26522) and by the Ethics Committees of all participating institutions. A written and informed consent was obtained from all patients according to the Declaration of Helsinki.

Definitions

DLI was defined as transfusion of unstimulated lymphocyte concentrates, collected from the original stem cell donor as buffy coat preparations, or as transfusion of unmanipulated mobilized peripheral blood stem cells (PBSC). RIC regimens were defined as described by Bacigalupo et al. (11). Acute GVHD was graded according to the 1994 Consensus Conference on acute GVHD

TABLE 1 | Characteristics of patients and allogeneic transplants.

Total number of patients	252
Sex: male	133 (53%)
Median age (range) at transplant	45.1 (1.6–73.4)
Age <18 years	13 (5%)
Diagnosis	
AML [secondary AML]	180 (71%) [32 (17%)]
ALL [Ph+ ALL]	68 (27%) [8 (12%)]
Biphenotypic AL	4 (2%)
Transplant date	
≤2005	3 (2%)
2006–2010	69 (27%)
2011–2015	180 (71%)
Disease status at transplant	
Not treated	2 (1%)
1 CR	135 (54%)
≥2 CR	61 (25%)
Primary induction	26 (10%)
Relapse	26 (10%)
Missing	2
Donor	
HLA-matched sibling	98 (39%)
1 locus mismatched related	3 (1%)
Haploidentical	49 (20%)
Unrelated	102 (40%)
Stem cell source	
Bone marrow	83 (33%)
Peripheral blood	169 (67%)
Conditioning regimen	
Myeloablative (with TBI)	42 (17%)
Myeloablative (only drugs)	137 (55%)
Reduced intensity	71 (28%)
GVHD prophylaxis	
CyA/FK + MTX	80 (32%)
Cya/FK + MTX + ATG	120 (48%)
PT-CY	13 (5%)
Other	36 (15%)
Missing	3
Acute GVHD	
Grade 0	169 (68%)
Grade I	42 (17%)
Grade II	27 (11%)
Grade III–IV	10 (4%)
Missing	4
Chronic GVHD	
Absent	109 (53%)
Mild-moderate	72 (35%)
Severe	26 (12%)
Missing	45

No, number; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; Ph+, Philadelphia chromosome positive; CR, complete remission; TBI, total body irradiation; CyA, cyclosporine; FK, tacrolimus; MTX, methotrexate; ATG, antithymocyte globulin; PT-Cy, post-transplant cyclophosphamide.

grading criteria (12), and chronic GVHD was staged according to the criteria developed by the National Institute of Health (13). Hematological relapse was defined by recurrence of blasts in PB or infiltration of bone marrow (BM) by more than 5% blasts. Pre-emptive treatment was defined as DLI administration in cases of reappearance of minimal residual disease (MRD) (any AL cytogenetic or molecular or phenotypic marker previously detected at diagnosis) in absence of hematological relapse. Prophylaxis was defined as DLI treatment to prevent hematological relapse in patients with negative MRD. Mixed chimerism was defined as failure to achieve >95% of donor cells or decreased chimerism, with evidence of AL complete remission. Targeted therapy before DLI included hypomethylating agents in patients with AML and tyrosine kinase inhibitors in patients with ALL.

Statistical Analysis

Data were collected in an XLS database and imported into Stata/SE 9.0 for Windows (StataCorp, College Station, TX) for statistical analysis. The close-out date for analysis was December 2018. The starting points of our analyses were day of first HCT and day of first DLI. NRM was defined as death due to all causes not related to leukemia and was estimated with the cumulative incidence method. OS was defined as the time (days) from the aforementioned starting points to either death or last observation and was described using the Kaplan-Meier approach.

In univariate analysis, variables considered as possible prognostic factors were: age at transplantation (years), sex, AL type (AML or ALL), conditioning intensity (myeloablative or RIC), GVHD prophylaxis (calcineurin inhibitors plus methotrexate or calcineurin inhibitors plus methotrexate plus antithymocyte serum or post-transplant cyclophosphamide or other platforms), donor type (HLA-identical plus 1 antigen mismatched related donor vs. unrelated donor vs. haploidentical donor), time between HCT and first DLI (≤180 days, 181–365 days, 366–730 days, >730 days), indication for DLI administration (relapse or mixed chimerism or pre-emptive treatment/prophylaxis), treatment administered before DLI (no pharmacological treatment or conventional chemotherapy or targeted therapy), number of infusions, and acute or chronic GVHD after HCT (yes or no). Acute and chronic GVHD were treated as time-dependent variables. Multivariate stepwise analyses included all variables found to be significant at $p \leq 0.10$ on univariate analysis. Retention in the stepwise model required the variable to be significant at $p \leq 0.05$.

RESULTS

Patient and Transplant Information (Table 1)

Two hundred fifty-two patients were enrolled from 34 Italian transplant centers. Thirty centers (86%) provided data for <10 patients. One hundred thirty-three patients (53%) were male and median age at transplant was 45.1 years (range 1.6–73.4). Only 13 patients (5%) were younger than 18 years. The underlying disease was AML in 180 patients (71%), ALL in 68 patients (27%),

TABLE 2 | Characteristics of DLIs.

	1stDLI	2ndDLI	3rdDLI	4thDLI	≥5thDLI
Total number of cases	252	156	91	49	53
Time between transplant and first DLI (days)	258 (55–3,784)				
Time between DLIs		29 (1-1015)	30 (2-636)	33 (7-623)	31 (13-441)
DLI indication					
AL relapse	172/235 (73%)	–	–	–	–
Mixed chimerism	39/235 (17%)				
Pre-emptive/prophylaxis	24/235 (10%)				
Missing	17				
Treatment before DLIs					
No treatment	41/103 (40%)	110/126 (87%)	70/78 (90%)	40/47 (85%)	28/46 (61%)
Radiotherapy	3/103 (3%)	–	1/78 (1%)	–	1/46 (2%)
Chemotherapy	40/103 (39%)	5/126 (4%)	2/78 (2%)	2/47 (5%)	–
Targeted therapy	19/103 (18%)	11/126 (9%)	6/78 (7%)	7/47 (15%)	17/46 (37%)
Missing	149	30	13	2	7
Dose ($\times 10^6$/kg)					
Median (range)	1 (0.01–10)	2 (0.01–64)	5 (0.05–100)	10 (0.05–50)	10 (0.05–50)
Missing	198	126	70	33	38
Acute GVHD					
Grade 0	141/163 (87%)	94/106 (89%)	64/69 (93%)	36/39 (82%)	43/48 (90%)
Grade I–II	17/163 (10%)	9/106 (8%)	3/69 (4%)	2/39 (5%)	4/48 (8%)
Grade III–IV	5/163 (3%)	3/106 (3%)	2/69 (3%)	1/39 (3%)	1/48 (2%)
Missing	89	50	22	10	
Grade IV hematological toxicity					
Number of cases	9/82 (11%)	4/35 (11%)	4/26 (15%)	2/18 (11%)	–
Missing	170	121	65	31	53

AL, acute leukemia.

and biphenotypic AL in 4 patients (2%). The majority of HCTs (180, 71%) were performed between 2011 and 2015, whereas the other procedures were done before 2011. Twenty percent of patients had active AL at transplant. Preparative regimens before HCT were myeloablative in 179 transplants (72%). One hundred fifty patients (60%) had a related donor, who was HLA-identical sibling, 1 locus HLA mismatched, or haploidentical in 98, 3, and 49 cases, respectively; 102 patients (40%) had an unrelated donor. An high resolution DNA typing was performed at HLA-A, -B, -C, -DRB1 loci; 65 out of 91 evalutable unrelated transplants (71%) were HLA-matched, while a single mismatch at HLA-A, -B, or -C locus was present in 10 (11%), 6 (7%), and 10 recipient and donor pairs (11%), respectively. One hundred sixty-nine patients (67%) received PBSC, and 83 (33%) received BM. GVHD prophylaxis consisted of calcineurin inhibitor (cyclosporine or tacrolimus) plus methotrexate in 80 patients (32%), calcineurin inhibitor plus methotrexate plus antithymocyte globulin (ATG) in 120 patients (48%), post-transplant cyclophosphamide-based prophylaxis in 13 patients (5%), and other platforms in the remaining 39 patients (15%). Most common miscellaneous GVHD prophylaxis regimens were used in haploidentical transplants and included rapamycin-based and ATG plus basiliximab-based platforms. Sixty-nine of the evaluable patients (32%) developed grade I-IV acute GVHD, which reached grade III–IV only in 10 cases (4%). Chronic GVHD occurred in 98 of evaluable patients (47%) and was severe in 26 cases (12%).

DLI Administration (Table 2)

All patients received at least one DLI. The first DLI was administered at a median time of 258 days (55–3,784) after HCT. The main indication for DLI was leukemia relapse after HCT (172 patients, 73%), followed by mixed chimerism (39 patients, 17%) and pre-emptive/prophylactic use (24 patients, 10%). Ninety-six patients out of 252 (38%) received one single infusion, whereas 65 (26%), 42 (17%), and 49 patients (19%) received 2, 3, or ≥4 infusions, respectively, with a median of 31 days between two subsequent DLIs. Forty percent of evaluable patients received no treatment before the first DLI, whereas radiotherapy, conventional chemotherapy or targeted treatments were administered in 3, 39, and 18%, respectively. The percentage of patients who did not receive any treatment in association with DLIs increased to 87 and 90% after the second and third DLI, respectively. The median dose of the first DLI was 1×10^6/kg (0.01–10) for the informative patients. In case of multiple infusions, an escalating schedule was mainly chosen, with median doses ranging from 1×10^6/kg CD3+ lymphocytes (0.01–10) for the first infusion to 10×10^6/kg CD3+ lymphocytes (0.05–50) for the fifth or further infusion. Median and range of CD3+ cells/kg of the first DLI were 1×10^6 (0.5–10), 1×10^6 (0.1–10), and 0.3

$\times 10^6$ (0.05–1) in recipients of DLIs from HLA identical sibling, unrelated and haploidentical donors, respectively. A sequential schedule was administered to 36/98 (37%) recipients of DLIs from HLA identical sibling donors, 32/102 (31%) recipients of DLIs from unrelated donors and to 25/52 (48%) recipients of DLIs from haploidentical donors, respectively ($p = 0.127$) After the first DLI, acute GVHD was reported in 13% of informative patients and was grade III-IV in 3% of patients. The percentage of patients who developed acute GVHD decreased to 11 and 7% after the second and third DLI, respectively. In contrast, the percentage of evaluable patients who developed chronic moderate-severe GVHD requiring treatment increased from 2% after the first DLI to 7 and 14% after the second and third DLI, respectively. Grade III-IV neutropenia and/or thrombocytopenia occurred in 11% of the evaluable patients after the first DLI and the rate was not significantly different after subsequent infusions. Severe infections were reported in 6 out 98 informative DLIs (6%) and included invasive mycoses (2 patients), viral infections (2 cases), and recurrent bacterial enteritis (2 patients). Forty-five patients who received DLI because of relapse were evaluable for response after cell infusion: 14 patients (31%) reached complete remission, 16 patients (35%) had stable disease, and 15 (33%) experienced leukemia progression. Forty-six patients (18%) received a second HCT after a median of 922 days (149–1,970) from the first HCT and after a median of 232 days (32–1,390) from the first DLI. There was no significant difference in the proportion of patients undergoing second HCT after receiving DLI according to immunotherapy indication. In fact, 35 out of 172 patients (20%) who received DLI because of relapse required a second transplant compared to 3 out of 39 (8%) and 3 out of 24 (12%) of those who were treated with DLI because of mixed chimerism or as prevention, respectively ($p = 0.136$).

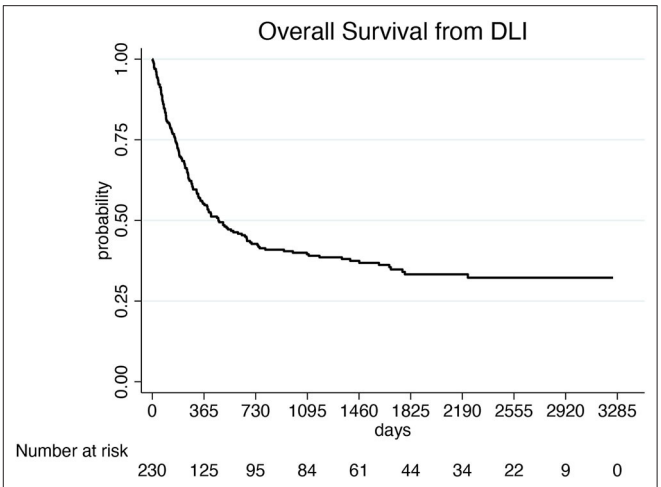

FIGURE 1 | Overall survival of the 252 patients treated with DLIs.

Outcome

With a median follow-up of 878 days (55–6,754) after the first HCT and 461 days (2–3,255) after the first DLI, 81 of the 248 evaluable patients (33%) were alive and 167 (67%) were dead. Of these latter, 141 (84%) died because of leukemia progression and 26 (16%) because of NRM. Causes of NRM were related to DLI (15 patients, 9%), second HCT (6 patients, 4%), secondary malignancy (2 cases, 1%), and to other causes (3 patients, 2%). NRM events were equally distributed between patients treated in small centers (providing data of $\leq$10 patients) and large centers (providing data of more than 10 patients): in fact, 12 out of 127 patients (9%) from small centers and 14 out of 121 patients (11%) from large centers died because of NRM ($p = 0.736$). Median survival was 915 days (55–6,754) from the first HCT and 466 days (2–3,255) from the first DLI, respectively. One-, three-, and five-year OS of the whole group from the beginning of DLI treatment was 55, 39, and 33%, respectively (**Figure 1**). Prognostic factors that were significantly ($p < 0.10$) associated with OS after DLI in the univariate proportional hazards model were: age, donor type, treatment before DLI, indication for DLI, number of DLI, time between transplant and first DLI (**Table 3**). In multivariate analysis, older recipient age and transplants from haploidentical donors significantly reduced OS (HR 1.020; 95% CI 1.008–1.033; $p = 0.001$ and HR 2.815; 95% CI 1.702–4.656; $p = 0.000$, respectively), whereas DLI for mixed chimerism or as pre-emptive/prophylactic treatment compared to AL relapse and a schedule including more than one DLI significantly prolonged OS (HR 0.379; 95% CI 0.219–0.646; $p = 0.000$; HR 0.202; 95% CI 0.098–0.415; $p = 0.000$; HR 0.876; 95% CI 0.767–1.000; $p = 0.050$, respectively). Moreover, a time between transplant and first DLI longer than 2 years significantly improved OS (HR 0.411; 95% CI 0.229–0.740; $p = 0.003$; **Table 4**). Patients who received DLI because of relapse reported a 3-year OS of 32%, which was significantly lower than the 3-year OS of 55 and 58% for those patients who were treated with DLI because of mixed chimerism ($p = 0.002$) or pre-emptive/prophylactic use ($p = 0.008$; **Figure 2**). Moreover, transplants from haploidentical donors showed a 3-year OS of 25%, which was significantly lower than that reported in transplants from unrelated donors (3-year OS 48%, $p = 0.000$; **Figure 3**). In addition, patients who received a second HCT after receiving DLI showed a trend of longer OS compared to patients who received only one transplant followed by DLI ($p = 0.077$; **Figure 4**).

Since DLIs from haploidentical donor were an independent predictor for worse OS, we compared toxicity and efficacy of DLIs among matched related, unrelated and haploidentical donors. There was no significant difference in the distribution of NRM events among the 3 groups ($p = 0.313$), while acute GVHD was significantly more frequent after DLIs from unrelated donors (21%) and haploidentical donors (28%) in comparison with DLIs from HLA-identical sibling donors (7%) (unrelated DLIs vs. HLA-identical sibling DLIs: $p = 0.041$; haploidentical DLIs vs. HLA-identical sibling DLIs: $p = 0.020$). Moreover, taking in account the 45 patients who received DLIs because of leukemia relapse and were evaluable for response, we observed a significant lower rate of leukemia control (complete remission and stable disease) after DLIs from haploidentical donors (33%) in comparison with DLIs from unrelated donors (78%) ($p =$

TABLE 3 | Univariate analysis of overall survival data from first DLI.

Factor	HR	95%CI	*p*
Age			
Modeled as continuous variable	1.012	1.001–1.024	**0.027**
Sex			
Male	1	0.683–1.403	0.623
Female	1.084		
Diagnosis			
AML	1	0.742–1.495	0.907
ALL	0.979		
Donor			
Unrelated	1		
HLA-identical sibling or 1 locus mismatched related	1.494	1.031–2.165	**0.034**
Haploidentical	1.843	1.199-2.883	**0.005**
Treatment before DLI			
No treatment/RT	1		
Chemotherapy	1.820	1.009–3.282	**0.046**
Targeted therapy	1.281	0.599–2.738	0.522
DLI indication			
AL Relapse	1		
Mixed chimerism	0.434	0.257–0.735	**0.002**
Pre-emptive/prophylaxis	0.431	0.231–0.801	**0.008**
Number of DLIs			
Modeled as continuous variable	0.883	0.782–0.997	**0.045**
Time HCT-first DLI			
≤180 days	1		
181–356 days	0.765	0.508–1.151	0.199
366–730 days	0.915	0.599–1.395	0.680
>730 days	0.556	0.324–0.955	**0.034**
Number of transplants			
1	1		
2	0.682	0.446–1.042	0.077

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; HLA-id, HLA-identical; MM, mismatched; RT, radiotherapy.

TABLE 4 | Multivariate analysis of overall survival data from first DLI.

Factor	HR	95%CI	*p*
Age			
Modeled as continuous variable	1.020	1.008–1.033	**0.001**
Donor			
Unrelated	1		
HLA-identical sibling or 1 locus mismatched	1.261	0.854–1.861	0.243
Haploidentical	2.815	1.702–4.656	**0.000**
DLI indication			
AL relapse	1		
Mixed chimerism	0.379	0.219–0.646	**0.000**
Pre-emptive/prophylaxis	0.202	0.098–0.415	**0.000**
Time HCT-first DLI			
≤180 days	1		
181–356 days	0.741	0.468–1.172	0.201
366–730 days	0.728	0.462–1.147	0.171
>730 days	0.411	0.229–0.740	**0.003**
Number of DLI			
Modeled as continuous variable	0.876	0.767–1.000	**0.050**

HLA-id, HLA-identical; MM, mismatched.

0.036), while no significant difference in efficacy was reported if DLIs from matched related donors and those from haploidentical donors were compared ($p = 0.282$).

DISCUSSION

The first aim of the present survey was to take a picture of the DLI strategy in AL patients in Italian transplant centers. We found that DLIs were administered in 73% of patients after AL clinical recurrence, whereas they represented a way of preventing hematological relapse for less than one third of cases, who received them because of mixed chimerism or MRD positivity. The median time of about 8 months between HCT and first DLI confirmed that immunotherapy was used late in the course of the disease. A few EBMT registry studies have established the efficacy of DLIs either in the setting of overt relapse or used prophylactically. In AML relapse after first HCT, DLIs prolonged OS in comparison with no DLIs (14). Comparison of DLIs and second HCT showed that the clinical benefit of DLIs was comparable to that of salvage HCT (15). Moreover, in a matched-pair analysis, prophylactic DLIs significantly improved outcome in high-risk AML, but failed to achieve an OS advantage in ALL and in standard risk AML (16). In our study, several reasons for reluctance to administer DLI earlier after HCT may be hypothesized. First, physicians may have feared life-threatening complications of DLIs such as GVHD and severe infections. Indeed, in our study, toxicity after DLI was quite low, with fatal adverse events reported in 9% of patients, confirming the NRM incidence reported in previous studies. Moreover, the incidence of severe acute and chronic GVHD was lower than that reported in other registry studies (17), although our analysis may have been limited by the small number of informative patients. Second, a prevention strategy needs standardized markers of MRD and regular monitoring after CT, which might not be available in all Italian centers. Third, contacting and preparing donors can be time-consuming, particularly if they are volunteer donors and lymphocyte donation has to be authorized by a GITMO committee, which is in charge of reviewing clinical HCT history and indication for DLI. Cryopreservation of unmanipulated mobilized PBSC instead of leukapheresis products can enhance DLI availability and accelerate infusions; however, data of the GITMO registry did not allow identification of the two different products.

As expected, we reported a significant OS benefit for patients receiving DLI because of mixed chimerism or MRD positivity in comparison with patients receiving DLI because of hematological relapse. These outcomes are in line with those reported by the EBMT and the Japanese registry studies (14, 15, 18). Moreover, multivariate analysis showed that the greater the number of DLIs

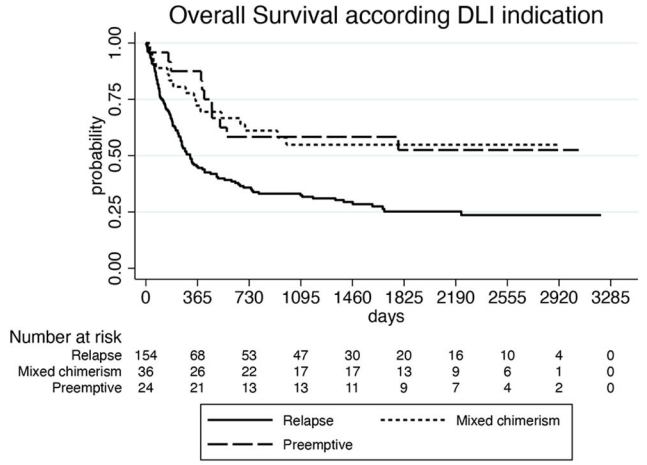

FIGURE 2 | Overall survival according to DLI indication.

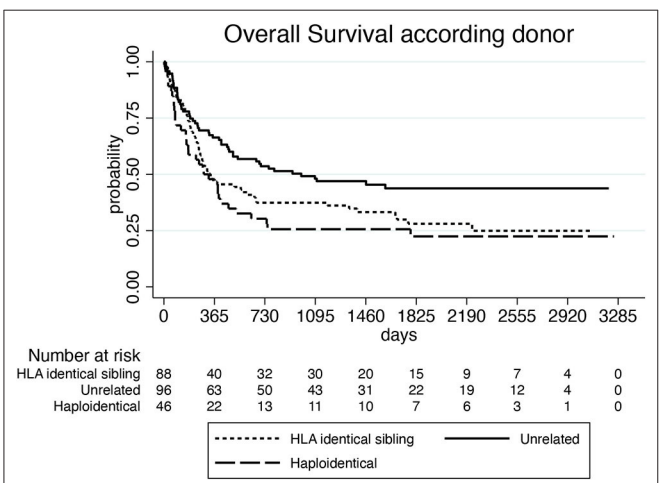

FIGURE 3 | Overall survival according to donor.

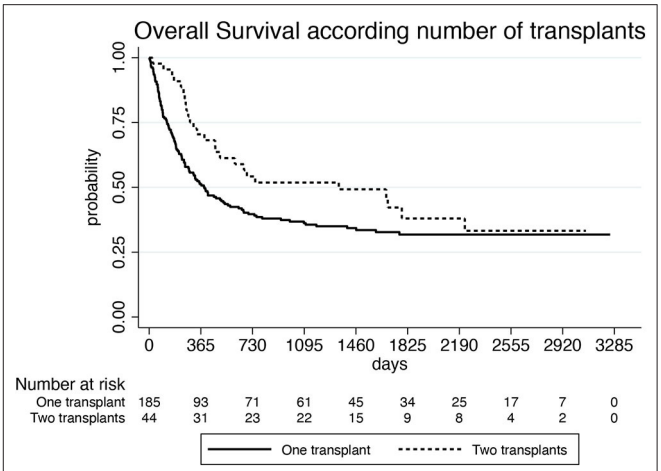

FIGURE 4 | Overall survival according to number of allogeneic transplants.

administered, the greater the OS improvement. In our study, about 60% of patients received a DLI schedule including more than one DLI at escalating doses, with a median interval of about 1 month between two subsequent infusions. A multiple DLI schedule was administered in a higher percentage of patients compared to previous registry studies, in which 49–61% of patients received one single dose. The more favorable outcome observed in our study for DLIs administered at least 2 years after HCT could reflect the greater clinical benefit of DLIs in late relapses in comparison with early recurrences after HCT, as already reported (14). Multivariate analysis showed no better outcome for patients who received chemotherapy or targeted treatments in association with DLI. These treatments were combined in 57% of patients at the time of first DLI and in a much lower percentage of patients at the following infusions. Although chemotherapy before DLI may theoretically induce leukemia debulking and improve DLI response, no advantage of chemotherapy plus DLI over DLI alone was observed in the AML relapse (18) or pre-emptive settings (19). More promising results were shown by hypomethylating agents: a few cycles of azacitidine or decitabine before DLI in relapsed patients with myeloid neoplasms could activate immune response and promoted some long-term responses, even if the latter were observed in small samples of patients and need confirmation in larger prospective studies (20–23).

In our study, older age of recipients and haploidentical donors were identified as adverse prognostic factors. Although pediatric patients were included in the study, they represented only 5% of the patients, therefore the worse outcome should be probably referred to the elderly adult patients. Moreover, a significantly shorter OS was reported by DLI from mismatched related donors, who included almost exclusively haploidentical donors. The inferior outcome seems to be caused by both lower efficacy in leukemia relapses and more toxicity, in term of acute GVHD, but these results should be interpreted with caution, because of the small number of DLIs from haploidentical donors and the heterogeneity of the GVHD prophylaxis platforms used in our study. Moreover, median dose of the first DLI was 1 log lower after haploidentical transplants in comparison with matched related and unrelated transplants and sequential doses were administered less often after haploidentical than after other HCTs: therefore, inferior doses could have impaired efficacy. Large prospective studies comparing DLIs from haploidentical and conventional donors are still lacking, particularly in the setting of the leukemia relapse. In the context of a prophylactic or pre-emptive strategy, a few small studies comparing T-repleted haploidentical or HLA-identical DLIs in refractory or very high-risk AML observed higher rates of acute GVHD and NRM (24, 25), while a large recent prospective study including 189 AL patients in first complete remission reported a prolonged graft and relapse-free survival after haploidentical HCT with an homogeneous ATG-based prophylaxis followed by DLI in comparison with HCT from matched related donors (26). Clinical trials are needed to establish the optimal timing

and cell dose in both therapeutic and prophylactic settings after haploidentical HCT and the relationship with GVHD and disease response (27).

Although the GVL effect has been reported to be lower in ALL than in myeloproliferative diseases (8, 16), in our study, ALL patients had a long-term outcome comparable to that of AML patients. Indeed, at present, other options such bispecific antibodies or chimeric antigen receptor-T cells seem to be more appealing than DLIs for the prevention and treatment of ALL relapse.

DLI administration was followed by a second HCT in 46 patients. It could be hypothesized that the second transplant was performed in patients not achieving a durable complete response after DLI. Therefore, in these patients, DLI represented a "bridge to" a second salvage HCT, allowing them to achieve a slight, but not statistically significant, OS prolongation compared to patients who received DLI alone.

We acknowledge that this study has some limitations. One is the heterogeneity of recipient and donor features of the HCTs included in the study, with 34 participating centers, the majority of which provided data for <10 patients. Another limitation is that only some of the Italian centers agreed to the second phase of the study. Therefore, evaluation of toxicity and clinical response to DLIs was based on a smaller patient population.

However, this survey presents the current "state of the art" of DLI strategy in AL in Italy and allows us to make a few practical and research considerations. From the organizational point of view, the GITMO network may promote a policy of DLI administration as pre-emptive treatment either allowing all centers to detect MRD in AL patients in centralized laboratories or accelerating authorization for leukaphereses from volunteer donors. Moreover, this survey could be the basis for further studies, either retrospective, including more homogeneous populations, or prospective, aiming to address unresolved items, such as DLI from haploidentical donors and DLI schedules according to different indications and different donors.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study involving human participants was reviewed and approved by the Ethics Committee of the center of the national principal investigator, called Comitato Unico Regionale Friuli-Venezia Giulia on 2017, 3 October (Protocol No. 26522) and by the Ethics Committees of all participating institutions. A written and informed consent was obtained from all patients according to the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

FP, AS, FL, FC, BB, and FB designed the study. EO and SM contributed clinical data from the registry. AP, WA, GS, RS, NM, IC, MM, CB, CM, and RF contributed clinical data from their site. FP, FL, and MI analyzed the data and prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

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

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REVIEW
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Pharmacologic Therapies to Prevent Relapse of Acute Myeloid Leukemia After Allogeneic Hematopoietic Stem Cell Transplantation

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Relapse is the main cause of mortality in patients with acute myeloid leukemia (AML) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Adverse cytogenetic or molecular risk factors, as well as refractory disease or persistent measurable residual disease (MRD) at the time of transplantation are associated with an increased risk of recurrence. Salvage therapy for AML relapse after allo-HSCT is often limited to chemotherapy, donor lymphocyte infusions and/or second transplants and is rarely successful. Effective post-transplant preventive intervention in high risk AML may be crucial. The most frequent and promising approach is the use of post-transplant maintenance with hypomethylating agents or with *FLT3* tyrosine kinase inhibitors when the target is present. Moreover, *IDH1/IDH2* inhibitors and *BCL-2* inhibitors in combination with other strategies are promising approaches in the maintenance setting. Here we summarize the current knowledge about the preemptive and prophylactic use of pharmacologic agents after allo-HSCT to prevent relapse of AML.

Keywords: acute myeloid leukemia, stem cell transplantation, allogeneic, relapse, prevention, hypomethylating agents

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is currently considered the optimal curative treatment option for patients with unfavorable risk acute myeloid leukemia (AML) (1–3). The implementation of non-myeloablative conditioning regimens and the improvement in supportive care has led to decrease in the transplant-related mortality (TRM) and to significant increase in the number of transplant candidates, including older patients and/or those with comorbidities (4, 5). However, reduced-intensity conditioning (RIC) is associated with higher rate of relapse (6). Allo-HSCT is generally recommended when the benefit of relapse reduction outweighs the risk of non-relapse mortality (NRM)/morbidity and this is based on the assessment of cytogenetic and molecular genetic features as well as donor, patient, and transplant-related factors (7–10). This includes intermediate or high-risk cytogenetic/molecular disease groups defined by the

2017 European Leukemia Net (ELN) guidelines, achievement of complete remission (CR) after more than one induction chemotherapy, refractory disease and the presence of pre-transplant measurable residual disease (MRD) positivity (7, 11, 12).

Disease relapse in transplanted patients in first CR (CR1) occurs in 30%–40% of cases and harbors a particular poor prognosis if it occurs in the first 6 months post-transplant (13). Relapse rates are even higher among patients who undergo allo-HSCT beyond CR1 or those with refractory disease (14, 15).

The treatment options for AML patients who relapse after transplant are very limited and highly depend on the patient performance status at the time of relapse (16). Commonly used treatment options for patients who are candidates for intensive therapy are salvage chemotherapy, often associated with donor lymphocyte infusion (DLI), allogeneic stem cell boost, or even second allo-HSCT from the same or different donor (17–24). In contrast, patients who are not eligible for intensive therapy are usually offered low intensity chemotherapy, hypomethylating agents (HMA), targeted therapies, participation in clinical trials, and withdrawal of immunosuppression or supportive care, all aiming at controlling the disease rather than achieving remission (25–27).

Salvage treatments post-allo-HSCT can induce remissions only in a minority of patients (20%) and the 2-year overall survival (OS) rates are usually below 20% (28–30). Alternatively, preventive strategies have been studied to reduce the incidence of relapse including the use of myeloablative conditioning (MAC), prophylactic DLI, graft manipulation, early withdrawal of immunosuppression or intensive surveillance. Intensification of conditioning regimen by using MAC is associated with a lower relapse rate but with higher TRM. Thus, there is no difference in OS when MAC or RIC are used in allo-HSCT for AML (31). Prophylactic DLI is associated with a decrease in the relapse rate at the expense of more graft-versus-host disease (GVHD) and therefore an increased morbidity and mortality (32).

The low efficacy of these strategies to prevent post-transplant relapse led to the introduction of alternative approaches such as prophylactic pharmacological interventions for patients with unfavorable risk, or preemptive strategies for patients with risk of imminent recurrence indicated by MRD positivity by flow cytometry, cytogenetic testing, molecular analysis or loss of donor chimerism. The ideal maintenance agent should target an active driver pathway, such as tyrosine kinase inhibitors (TKIs) targeting *FLT3* (such as sorafenib and midostaurin) or HMA (i.e., azacitidine and decitabine). These agents have an acceptable non-hematologic toxicity with manageable drug–drug interactions. Moreover, they enhance the graft-versus-leukemia effect (GVL) with non-significant effect on GVHD. For instance, *in vitro* and murine studies showed that HMAs has an important immunologic effect after transplantation in expanding circulating T regulatory (Tregs)/natural killer (NK) cells and up-regulating the expression of tumor antigens on leukemic blasts leading to increased GVL effect without increasing the risk of GVHD (33–35). Moreover, the use of *FLT3* inhibitors as maintenance post-transplant is supported by the observation of an anti-leukemic synergism between sorafenib and alloreactive donor cells (36, 37). One recent study demonstrated that sorafenib promotes GVL activity in mice and humans through interleukin-15 production in *FLT3-ITD* leukemia cells (38).

Here, we summarize the clinical data on a number of agents being studied as maintenance/preemptive therapies after allo-HSCT in AML focusing mainly on TKIs (*FLT3* inhibitors) and HMAs (azacitidine and decitabine).

MRD Assessment

There are two approaches to reduce the risk of frank AML relapse following allo-HSCT, prophylactic and preemptive strategies. Prophylactic strategies are defined as the initiation of treatment in the absence of any measurable disease after transplant. Prophylactic therapy is given to patients with high risk of relapse in the aim to eradicate residual malignant cells which are undetectable by currently available monitoring techniques. In contrast, preemptive strategies are initiated for patients with risk of imminent relapse presenting as any evidence of disease activity at MRD level to prevent frank hematological relapse.

MRD persistence at transplant has been identified as an independent and strong risk factor for post-transplant relapse that can be at least partially overcome by additional intervention such as augmented conditioning (7, 39, 40). Similarly, growing evidence strongly suggests that MRD detection by multi-parametric flow cytometry (MFC), molecular techniques, or chimerism analyses after allo-HSCT may be used as a predictor of imminent relapse (41). These should be part of routine post-transplant follow-up since MRD detection can improve outcomes by guiding subsequent therapy aiming to unleash or enhance the GVL effect (39).

Dynamic MRD monitoring after allo-HSCT may improve outcomes; however, there is a relative paucity of data and lack of clear recommendation on how we should test MRD (frequency, qualitative and/or quantitative, on peripheral blood or bone marrow), when we should react and what could be the best available MRD-directed intervention post-allo HSCT (42).

The main methods for detection of MRD in patients with AML after allo-HSCT are MFC, molecular genetics and chimerism analyses (43). MFC is the standard and most commonly used MRD method to identify residual leukemic cells reaching a sensitivity of 10^{-3} to 10^{-5} (39–44). Several studies have demonstrated a higher risk of relapse in AML patients with positive MRD detected by MFC after transplant compared to those without evidence of MRD (≤ 0.1% leukemia cells) by the same detection method (45, 46). MRD by flow cytometry has many drawbacks including the lack of standardization, its lower sensitivity, the need for high technical expertise to differentiate between leukemic from regenerating bone marrow cells, biological heterogeneity of the leukemic population and the possibility of false negative results related to sample processing, hemodilution, number of events analyzed and immunophenotypic switch (45–47).

Another method of MRD assessment is donor/recipient chimerism analysis that can detect host-derived hematopoiesis based on genomic differences between the recipient and the

donor. Decrease in donor chimerism in AML is often associated with disease relapse (48). Sensitivity of chimerism is dependent on the method applied, ranging from only 10^{-2}–10^{-3} in the conventional method using fragment analysis of short tandem repeats (STR) or in XY-FISH analysis method in sex-mismatched donor/recipient, to a high sensitivity of 10^{-4}–10^{-5} if variant-allele-specific quantitative PCR that can detect small DNA insertions or deletion or evaluation of CD34+ cell subset in AML were used (48–50). In consequence, chimerism analysis should be routinely performed after allo-HSCT on days +30, +100, +270, and +365 in conjunction with other MRD markers and clinical parameters to wisely decide on preemptive intervention (51).

The last method of MRD assessment is molecular analysis. Currently, the most widely applied strategy for molecular MRD monitoring is real-time quantitative PCR (RQ-PCR) which can detect mutated genes, fusion gene transcripts or overexpressed genes and can detect leukemic cells at 10^{-6} sensitivity (42, 43). PCR based methods are characterized by high specificity and sensitivity for leukemic cells detection and low risk of contamination; however, their use depends on identifying pretreatment AML-associated mutation at diagnosis and these molecular targets must be stable while on therapy (52, 53). For instance, some mutations like *NPM1* mutation, *RUNX1-RUNX1T1* and *CBF-MYH11* in core binding factor (CBF) AML are relatively stable during disease course hereby are suitable for PCR MRD monitoring (12). It was recently shown that *NPM1* MRD-positivity at levels >0.1% to >10% beyond Day +60 post allo-HSCT are associated with increased relapse rates and reduced survival. Hence, preemptive interventions are considered for patients with persistent *NPM1* MRD levels at >0.1%–1% and more intervention should be considered if MRD is >10% (54, 55). Persistent CBF-fusion transcripts after allo-HSCT are translated into higher cumulative relapse incidence (RI) and shorter leukemia-free survival (LFS). Thus, preemptive interventions should be considered in case of persistent MRD positivity (>1%) of *RUNX1–RUNX1T1* or *CBFB-MYH11* in two consecutive measurements or if there is >0.5 log increase in the transcripts in repeated analysis (56, 57).

Other mutations such as *FLT3* (ITD and TKD), *RAS*, *IDH1*, *IDH2*, and *MLL-PTD* may theoretically be measurable by MRD detection but are poor MRD markers and have not been integrated into routine care yet, since these mutations are relatively unstable throughout treatment. Moreover, some of these mutations are lost during disease course and treatment due to leukemia clonal evolution (58). As a result, ELN guidelines recommend against using them as single markers (39).

In contrast to the limited frequency (50%) of mutations mentioned above, over-expression of Wilms Tumor 1 (*WT1*) gene is present in almost 90% of patients with AML and can be measured in peripheral blood with better sensitivity and specificity than in bone marrow. *WT1* expression analysis in MRD assessment is recommended by ELN using a standardized and certified ELN assay (59). Several reports showed that persistent high bone marrow or continuous increase in peripheral blood *WT1* transcripts at 3 months post-transplant are associated with higher risk of relapse (60, 61). Conversely, patients with sustained low *WT1* levels after transplant have excellent outcomes (62).

Other emerging technologies like digital-droplet based PCR and next-generation sequencing (NGS) assays are expected to be particularly useful in AML (63–65).

Hypomethylating Agents as Maintenance Therapy After Allo-HSCT in AML

Table 1 summaries the studies that use HMA for relapse prevention after allo-HSCT in AML. HMAs are clinically active in AML and myelodysplastic syndromes (MDS) and represent an important new treatment modality, particularly in elderly and/or unfit patients, due to their favorable toxicity profile (77). HMA have significant antitumor activity in relapsed AML patients after allo-HSCT with a 20%–40% CR rate (78, 79). Azacitidine (AZA) which is the first reported DNMT inhibitor, appears to be well tolerated after transplantation. *In vitro* and murine studies showed that AZA has an important immunologic effect after transplantation in expanding circulating Treg cells and up-regulating the expression of tumor antigens on leukemic blasts leading to increased GVL effect without increasing the risk of GVHD (33).

Prophylactic Therapy With HMA After Allo-HSCT

AZA and decitabine have been tested in several prospective and retrospective studies as maintenance therapy to avoid relapse post-allo-HSCT. These early-phase studies generally demonstrated tolerability, feasibility and established the optimal dosage and schedule for future trials (66, 68–72, 74–76). de Lima et al. (66) reported the results of the first phase 1 dose-finding study of maintenance AZA post-transplant in 45 patients with high-risk AML (n = 37) or MDS (n = 8). The investigators examined subcutaneous AZA at different dosing schedule (8, 16, 24, 32, and 40 mg/m^2). The optimal dose was 32 mg/m^2 given for 5 consecutive days every 28 days. After a median follow up of 20.5 months, the NRM was 9%. One-year event-free survival (EFS) and 1-year OS were 58% and 77%, respectively. The rates of grade II-III acute GVHD and chronic GVHD were 27% and 37%, respectively. The authors concluded that low dose azacitidine is safe and may prolong OS and EFS in heavily pretreated AML and MDS patients as post-transplant maintenance (66).

In another report by Oshikawa and colleagues (68), AZA plus gemtuzumab ozogamicin (GO) were used in 10 patients with high-risk AML after allo-HSCT. After a median follow-up of 474 days from allo-HSCT, the NRM rate was 10% and the 1-year disease-free survival (DFS) and OS were 60% and 70%, respectively (68).

Furthermore, in a prospective trial by Craddock et al. (71), 37 AML patients received AZA at a median time of 54 days post-transplant and at a dose of 36 mg/m^2/day for 5 days every 28 days up to 12 months. AZA was well tolerated in the majority of patients. Only 17 patients had grade I–II acute GVHD. Day 100 and 1-year NRM were 0% and 8%, respectively. The 1-year and 2-year OS were 81% and 49%, respectively (71).

Moreover, El-Cheikh and colleagues (72) reported their results of an observational study on AML (n = 13) and MDS

TABLE 1 | Studies using HMA for relapse prevention after allo-HSCT in AML.

Reference	HMA	Study design	Number of patients (disease)	Median age, yrs	Schedule	Median starting time	No of cycles median	GVHD incidence	Response
de Lima et al. (66)	AZA	Phase I	45 (AML: 37; MDS: 8)	60 (24–73)	8, 16, 24, 32 and 40 mg/m^2 d1–5	+40	1–4	Acute GVHD Grades II–III: 27% chronic GVHD: 37%	- 1-yr EFS: 58% - 1-yr OS: 77%
Platzbecker et al. (67) RELAZA trial	AZA	Prospective Preemptive (detection of MRD after transplant)	20 (AML: 17; MDS: 3)	58 (20–74)	75 mg/m^2 d1–7	+169	4 (1–11)	— —	- 16 Patients (80%) responded (increase or stable CD34$^+$ with no relapse)
Oshikawa et al. (68)	AZA	Retrospective matched cohort study	10 (AML)	49 (17–65)	30 mg/m^2 d1–7 + GO 3 mg/m^2 d 8	+78	1.5 (1–4)	— —	- 1-yr OS (70% in AZA-GO group vs. 59.8% in controls) - 1-yr DFS (60% vs. 42.8%)
Pusic et al. (69)	DAC	Prospective dose finding	22 (AML:17; MDS:5)	59 (21–68)	5, 7.5, 10 and 15 mg/m^2 d1–5	+50 to +100	5 (1–8)	Acute GVHD grades I–II: 27% grades III–IV: 9%	- 2-yr OS: 56% - 2-yr DFS: 48%
Han et al. (70)	DAC	Phase I	16 (AML:5; MDS:11)	49	5 mg/m^2 d1–5 then individualized	+86	1–4	Chronic GVHD: 12.5%	— — –
Craddock et al. (71) RICAZA trial	AZA	Prospective	37 (AML)	60 (40–71)	36 mg/m^2 d1.5	+54	3–10	Acute GVHD grades I–II: 46% grades III–IV: 0%	- 1-yr OS: 81% - 2-yr OS: 49% - 1-yr RFS:57% - 2-yr RFS: 49%
El Cheikh et al. (72)	AZA	Observational	18 (AML:13; MDS:5)	58 (16–65)	32 mg/m^2 d1–5	+60	16 (1–45)	Acute GVHD $\geq$ grade II: 28%	- 1-yr OS: 70% - 1-yr DFS: 63%
Platzbecker et al. (73) RELAZA 2	AZA	Prospective Phase II Preemptive (detection of MRD after transplant)	53 (AML:48; MDS:5)	59 (52–69)	75 mg/m^2 d1–7	— —	Up to 24	Acute GVHD grade III: 2%	- 1-yr RFS: 46%
Oran et al. (74)	AZA	RCT	187 AML/MDS AZA (93) Ct (94)	57	32 mg/m^2 d1–5	+42 to +100	4 (1–12)	— —	Median RFS: AZA: 2.07 yrs Ct: 1.28 yrs p = 0.43
de Lima et al. (75)	CC-486	Prospective Phase I/II dose finding	30 (AML: 26; MDS:4)	64 (28–80)	150–300 mg d1–7 or d1–14	+42 to +84	9 (1–12)	Acute GVHD grade III: 10%	- 1-yr OS: 86% (7-day cohort) - 1-year OS: 81% (14-day cohort)
Marini et al. (76)	AZA	Retrospective	32 Pro: 21 Pre: 11	Pro: 58 (15–69) Pre: 52 (30–63)	Pro: 32 mg/m^2 d1–5 Pre: 75 mg/m^2 d1–5/7	Pro: +116 Pre: +138	Pro: 6 (1–18) Pre: 4 (4–22)	All GVHD:40%	Pro: - 1-yr OS: 100% - 1-yr EFS: 95% Pre: - 1-yr OS: 82% - 1-yr EFS: 54%

HMA, hypomethylating agent; AZA, 5-azacytidine; EFS, event-free survival; OS, overall survival; GO, gemtuzumab ozogamicin; DFS, disease-free survival; DAC, decitabine; RFS, relapse-free survival; RCT, randomized controlled study; pts, patients; Ct, control arm; yrs, years; pro, prophylaxis; pre, preemptive.

(n = 5) patients who received post-transplant reduced dose AZA of 32 mg/m^2/day for 5 days monthly, for up to five years. At the time of last follow up, 13 patients were still alive in CR, and had full donor chimerism. The 1-year DFS and OS were 63% and 70%, respectively (72).

More recently, MD Anderson Cancer Center group reported the results of first randomized controlled trial (74). In this study, 187 patients with high-risk AML or MDS who were in CR after allo-HSCT received AZA (n = 93) or placebo (n = 94) at a dose of 32 mg/m^2/day for 5 days for 12 months. However, most of the patients in the AZA arm (74.6%) did not receive the planned 12 cycles of treatment due to relapse, death, toxicity or upon patient's request. The investigators closed the study early due to slow accrual. Relapse-free survival (RFS) was comparable between both groups; however, stratification by number of AZA cycles administered showed a trend toward improved RFS in patients receiving more AZA therapy cycles (74).

In addition to injectable AZA, an oral formulation of AZA (CC-486) has been recently tested in a phase 1/2 dose-finding study on 30 patients with AML (n = 26) and MDS (n = 4) in CR as maintenance therapy after allo-HSCT (75). The study included 4 dosing schedules of 150-300 mg per day for 7 or 14 days every 28 days for up to 12 cycles. Oral AZA (CC-486) seemed safe and generally well tolerated with only 3 patients (10%) developing grade III acute GVHD. Median OS was not reached after 19 months follow-up and the 1-year OS were 86% and 81% in the 7-day and 14-day dosing cohorts, respectively (75).

Decitabine is another HMA that has been evaluated in the maintenance setting post allo-HSCT. Pusic et al. (69) tested the safety and efficacy of decitabine maintenance after allo-HSCT in 22 patients with AML (n = 17) and MDS (n = 5). Decitabine was given at a dose of 5, 7.5, 10, and 15 mg/m^2/day for 5 consecutive days every 6 weeks. The toxicity profile was acceptable. Acute GVHD grade I-II and grade III–IV occurred in 27% and 9%, respectively. The 2-year DFS and OS were 48% and 56%, respectively. The investigators concluded that the dose of 10 mg/m^2 for 5 days every 6 weeks appeared safe and optimal rather than the 15 mg/m^2 and could be administered after transplant in high-risk patients (69).

In another study, decitabine was evaluated in a phase 1 dose-finding study as maintenance therapy post allo-HSCT in 16 patients with MDS (n = 11) or secondary AML (n = 5) (70). No aggravation of preexisting acute GVHD was observed and mild/moderate chronic GVHD occurred in only 2 patients (12.5%). In conclusion, the investigators considered 5 mg/m^2/day to be the most appropriate starting dose for decitabine maintenance (70).

Preemptive Therapy With HMA After Allo-HSCT

MRD-triggered preemptive therapy with HMA is another strategy to avoid relapse of AML after transplant. The German group has tested this concept in 2 prospective studies (67, 73). The first trial was a single-center phase II study of 20 patients with MDS/AML evaluating the administration of AZA preemptively post allo-HSCT after a decrease of CD34+ donor chimerism to <80%, while still in complete hematologic remission (67). All patients received AZA for 4 cycles at a dose of 75 mg/m^2/day for 7 days. Sixteen-patients (80%) had response with either increasing CD34+ donor chimerism to >80% (n = 10; 50%) or stabilization (n = 6; 30%) with no evidence of relapse. Furthermore, 11 patients (55%) with stable disease or with subsequent drop in donor chimerism to <80% after initial response received a median of 4 (range: 1–11) additional cycles of AZA. Most patients (65%) ultimately developed hematologic relapse but their relapse was delayed by a median of 231 days after the decrease in donor chimerism.

In the second prospective trial (RELAZA-2) (73), 53 AML/MDS patients who developed MRD positivity after transplant (n = 24) or after conventional chemotherapy (n = 29) received AZA at a dose of 75 mg/m^2/day for 7 days monthly for up to 24 cycles. MRD positivity were defined by a drop of 80% or less in CD34+ donor chimerism or an increase in *NPM1* mutation, *RUNX1-RUNX1T1* and *CBFb–MYH11* >1% in the bone marrow or peripheral blood without evidence of hematological relapse. One-year RFS was 46%, and 26 (49%) patients eventually relapsed. The authors concluded that AZA could be effectively used to prevent or delay hematologic relapse in MRD-positive patients with AML/MDS (73).

Overall, these data clearly show that AML patients can tolerate maintenance therapy after allo-HSCT with HMA (azacitidine or decitabine) albeit at lower doses, with a favorable safety profile and apparently a reduction in the risk of disease relapse after transplant. Moreover, the results of preemptive studies could serve as the basis to design future studies of MRD-guided therapy using HMAs with other targeted therapies, including immuno-modulating agents.

FLT3 Inhibitors as Maintenance Therapy After Allo-HSCT in AML

Table 2 summaries the studies that use *FLT3* inhibitors for relapse prevention after allo-HSCT in AML. *FLT3*-internal tandem duplication (ITD) mutation is found in approximately 30% of patients with AML (91, 92). These patients have a high risk of relapse and low cure rates (93, 94). Patients with *FLT3-ITD* mutation also have a higher risk of early relapse after allo-HSCT compared to patients with wild type *FLT3* (38% vs. 28% in Center for International Blood and Marrow Transplant Research (CIBMTR) analysis) (94, 95). Treatment options for patients with *FLT3*-mutated AML who relapse after transplant are limited to chemotherapy, second allo-HSCT, and *FLT3* inhibitors alone or combined with DLI, all of which are rarely effective in the long term, even though, a small fraction of those patients can achieve long-standing responses with sorafenib (22, 96–99). The use of *FLT3* inhibitors as maintenance treatment after allo-HSCT is supported by the observation of an anti-leukemic synergism between sorafenib and allo-reactive donor cells (36, 37). Moreover, marrow aplasia induced by chemotherapy leads to elevated FLT3-ligand levels that may increase on-target activity of *FLT3* inhibitors (100–103).

Sorafenib was the first TKI studied in the setting of post-transplant maintenance therapy in AML with *FLT3-ITD* mutation. It showed benefit in survival and improvement of outcomes in a phase I study, several retrospective studies and two randomized studies (80–86, 104). Chen and colleagues (80) reported the results of the first phase I trial on sorafenib after transplant in 22 patients with *FLT3* mutated AML. They found that sorafenib could be safely used

TABLE 2 | Studies using FLT3 inhibitors for relapse prevention after allo-HSCT in AML.

Reference	FLT3 Inh	Study design	Patients number	Median age, yrs	Schedule	Response
Chen et al. (80)	Sorafenib	Phase 1 dose-finding	22	54 (20–67)	200–400 mg BID for 12 months	- 2-yr OS: 78% - 2-yr PFS: 72%
Antar et al. (81)	Sorafenib	Retrospective pilot study	6	50 (32–58)	400 mg BID	100% are alive after median follow-up of 16 months
Brunner et al. (82)	Sorafenib	Retrospective 2 arms	80 (Sorafenib: 26; Control: 54)	- Sorafenib: 54.5 (20–74) - Control: 53 (25–72)	200–400 mg BID for 12–24 months	- 2-yr OS: sorafenib (81%), control (62%) (S) - 2-yr PFS: sorafenib (82%), control (53%) (S)
Battipaglia et al. (83, 84),	Sorafenib	Retrospective Multi-center	28 (maintenance: 25, salvage: 3)	45 (16–57)	200–400 mg BID	- 1-yr OS: 89 ± 7% - 1-yr LFS: 91 ± 6% - 2-yr OS: 80 ± 8% - 2-yr PFS: 73 ± 9%
Bazarbachi et al. (85)	Sorafenib	Retrospective EBMT registry-based analysis	462 (Prophylaxis:19; preemptive:9; Control 434)	50 (19–75)	200–800 mg daily	Matched-pair analysis 26 sorafenib pts and 26 controls: - 2-yr LFS: 79% (sorafenib) and 54% (control) (S) - 2-yr OS: 83% (sorafenib) and 62% (control) (S)
Burchert et al. (86) SORMAIN trial	Sorafenib	Phase II prospective RCT	83 Sorafenib: 43, Placebo: 40	54 (18–75)	200–400 mg BID for up to 24 months	- 2-yr RFS: 85% (sorafenib) - 2-yr RFS: 53% (Placebo) (S)
Xuan et al. (87)	Sorafenib	Phase III randomized	202 Sorafenib: 100, Placebo: 102	18–60	400 mg BID	- 2-yr LFS: 81% (sorafenib) - 2-yr LFS: 54% (Placebo) (S) - 2-yr OS: 83% (sorafenib) - 2-yr OS: 72% (Placebo) (S)
Maziarz et al. (88) Radius trial	Midostaurin	Phase II randomized	60 Midostaurin + SOC: 30 Placebo + SOC: 30	18–70	50 mg BID for up to 12 months	- 1.5-yr RFS: 89% (midostaurin + SOC) - 1.5-yr RFS: 76% (Placebo + SOC)
Schlenk et al. (89)	Midostaurin	Phase II prospective	134 (Midostaurin: 75, Control:59)	18–70	50 mg BID for 12 months	Landmark analysis: Better EFS and OS in midostaurin pts (S)
Sandmaier et al. (90)	Quizartinib	Phase 1 Dose finding	13	43 (23–61)	40 mg daily (n = 7) 60 mg daily (n = 6)	- One relapse among 13 patients

Inh, inhibitor; yrs, years; OS, overall survival; PFS, progression-free survival; DFS, disease-free survival; S, significant; LFS, leukemia-free survival; RFS, relapse-free survival; SOC, standard of care.

after allo-HSCT with a maximum tolerated dose (MTD) of 400 mg twice daily. The 2-year progression-free survival (PFS) was 72% with a corresponding 2-year OS of 78% after allo-HSCT. Our group has reported the results of a pilot study in 6 patients with *FLT3-ITD* AML who received sorafenib (n = 5 maintenance, n = 1 salvage) after transplant. Grade II skin GVHD was observed in 5 of 6 patients shortly after sorafenib initiation, suggesting a possible immunomodulatory effect. Remarkably, all patients were alive after a median follow-up of 16 months and had sustained molecular remission (81). In a single institution observational study, sorafenib maintenance was evaluated in patients with *FLT3-ITD* AML who underwent allo-HSCT in CR1. Patients on sorafenib maintenance (n = 26) had an improved 2-year OS (81% vs. 62%, p = 0.029) and improved PFS (82% vs. 53%, p = 0.008) compared to historical controls (n = 54) (82).

In a multicenter study, single agent sorafenib was used as post-transplant maintenance in 28 adults with *FLT3* positive AML (83, 84). Twenty-five patients were given sorafenib as primary prophylaxis and three patients received it after relapse post allo-HSCT in combination with salvage chemotherapy and were then continued as maintenance after achievement of CR. At a median follow-up of 18 months, 25 patients were in CR with full donor chimerism with 1-year DFS and OS of 91% and 89%, respectively. A recent update of this study after a median follow-up of 40 months further demonstrated promising long-term outcomes with sorafenib maintenance with 2-year PFS and OS of 73% and 80%, respectively.

Recently Bazarbachi and colleagues (85) reported the results of European Society for Blood and Marrow Transplantation (EBMT) registry-based study on 462 allo-grafted *FLT3*-mutated AML patients (*FLT3-ITD*-95%) over a median follow-up of 39 months for surviving patients. Among these patients, 28 received post-transplant sorafenib maintenance as prophylactic (n = 19) or preemptive therapy (n = 9), started at a median of 55 days post-transplant (range 1–173 days) and a median dose of 800 mg/day (range 200–800 mg/day). Multivariate analysis showed that maintenance sorafenib significantly decreased RI [hazard ratio (HR) = 0.39; p = 0.05] with improvement in LFS (HR = 0.35; p = 0.01) and OS (HR = 0.36; p = 0.03). A matched-pair analysis was then performed on 52 patients (26 patients in the sorafenib group and 26 in the control group). The 2-year LFS and OS were 79% and 83%, respectively, in the sorafenib group (p = 0.02) vs. 54% and 62%, respectively, in the control group (p = 0.007).

In a recent double-blind prospective trial (SORMAIN) (86), 83 transplanted *FLT3-ITD* adult AML patients were randomized to receive either maintenance sorafenib (n = 43, up to 400 mg twice daily) or placebo (n = 40) started between days 60 and 100

after transplant for up to 24 months. The 2-year RFS was significantly improved in the sorafenib group (85%) compared to the placebo group (53%) (HR = 0.39, 95% CI, 0.18 to 0.85 p = 0.01). Sorafenib was generally well tolerated and the most common grade III–IV adverse events was acute GVHD (20%) in sorafenib group compared to (17%) in the placebo group.

More recently the Chinese group reported the results of a phase III randomized open-label multi-centers trial on 202 *FLT3-ITD* AML adult patients who underwent allo-HSCT (87). The patients received either sorafenib maintenance (n = 100; 400 mg BID) or placebo (n = 102) within 30–60 days post-transplant and for 6 months. After median follow up of 22 months, eleven and 30 patients relapsed in the sorafenib and control groups. The 2-year OS were 83% and 71%, (P = 0.025) and LFS were 81% and 54% (P < 0.001) in the sorafenib and control groups, respectively.

Acute Leukemia Working Party of the EBMT published a very recent clinical practice recommendation on allo-HSCT in AML patients with *FLT3-ITD* (105). The group recommends post-transplant maintenance with sorafenib in all cases except in patients with active acute GVHD. Sorafenib should be started as soon as possible after disease evaluation and MRD assessment at a dose of 400 mg daily in two divided doses and the dose may be increased to 800 mg daily in case of positive MRD and for a minimum of 2 years, depending on tolerance.

Midostaurin is another *FLT3* inhibitor that has activity as single agent in AML harboring *FLT3-ITD* or *FLT3* tyrosine kinase domain (TKD) mutation. It was also evaluated in the maintenance setting. Based on the RATIFY trial (106), midostaurin received FDA approval in combination with 3 + 7 induction chemotherapy for newly diagnosed *FLT3*-mutated AML. However, in this trial midostaurin maintenance was not offered for patients who underwent allo-HSCT.

The RADIUS phase II prospective trial randomized 60 patients with *FLT3-ITD* AML to standard of care (n = 30) or midostaurin (n = 30) starting 28–60 days post-transplant (88). The estimated RFS at 18-month was 76% in the standard of care arm compared to 89% in the midostaurin arm (HR = 0.46; 95% CI 0.12–1.86, P = 0.26), corresponding to relapse rates of 24% and 11%, respectively (P = 0.27).

In another phase II prospective study by Schlenk et al. (89) on 284 newly diagnosed *FLT3-ITD* AML patients, midostaurin maintenance treatment was also offered for patients receiving allo-HSCT in CR1 (56%). In a landmark analysis in patients who were event-free at day +100 after transplant (n = 116), those who received maintenance therapy within 100 days post-transplant (n = 72) had better EFS and OS (p = 0.004 and p = 0.01, respectively) than patients who did not.

Gilteritinib is another potent inhibitor of FLT3 with activity against *FLT3-ITD* and *FLT3-TKD*. In the phase 3 ADMIRAL trial, 371 adult patients with relapsed or refractory *FLT3*-mutated AML were randomly assigned in a 2:1 ratio to receive either gilteritinib or salvage chemotherapy. Patients who had a response and proceeded to allo-HSCT continued in the trial and could resume gilteritinib as maintenance therapy. Median OS in gilteritinib arm was 9.3 months compared to 5.6 months in the chemotherapy arm (107). A follow up on long-term survivors was recently presented in ASCO meeting 2020 (108). After 18 months of follow-up, gilteritinib continued to show better OS rates compared to salvage chemotherapy (27% vs. 15%). A total of 63 gilteritinib-treated patients had OS more than 18 months. A higher proportion of patients on gilteritinib achieved remission and underwent allo-HSCT. After a median of 3.5 months, 35 of 63 (56%) patients underwent allo-HSCT; 25 of these 35 patients (71%) received post-transplant gilteritinib maintenance. The authors concluded that the long-term survival in patients receiving gilteritinib is related to ongoing remission, subsequent allo-HSCT, or post-transplant gilteritinib maintenance therapy. Gilteritinib is currently being prospectively tested as maintenance therapy after allo-HSCT in *FLT3-ITD* AML patients in an ongoing randomized, double-blind, placebo-controlled phase III trial (NCT02997202) (109). This study aims to enroll and randomize 346 adult patients with AML in CR1 to receive maintenance therapy with either 120 mg gilteritinib per day or placebo for 24 months.

Quizartinib, another selective and highly potent *FLT3* inhibitor, was also evaluated in a phase I dose-finding and safety study (90). Thirteen adult patients with *FLT3-ITD* mutated AML in morphological remission following allo-HSCT received one of two quizartinib dose levels at 40 mg/day (n = 7) and 60 mg/day (n = 6), administered orally for up to 24 months. Around 77% of patients received quizartinib for at least 1 year and preliminary data indicated an acceptable tolerability and a reduced relapse rate compared with historical cohorts with only one (1/13) relapse.

Future Perspective

Based on the previously discussed trials, introducing single agent AZA as maintenance therapy can generally delay but mostly not prevent relapse after allo-HSCT. Combining AZA with DLI is a promising concept of MRD-guided post-transplant interventions since it reduces disease burden by cytotoxic therapy and reinforce an allo-immune reaction by cellular approach. This concept was evaluated in a phase II study of 30 patients with high-risk AML (n = 20) and MDS (n = 10) who were treated with prophylactic post-transplant AZA followed by escalated doses of DLI. Two-year OS and DFS were both 65.5%. Acute and chronic GVHD were reported in 31.5% and 53% of patients, respectively (110).

Many targeted agents such as isocitrate dehydrogenase (*IDH*) Inhibitors (*IDH1*, ivosidenib; *IDH2*, enasidenib), hedgehog (Hh) inhibitor (glasdegib), and BCL2 inhibitor (venetoclax) in combination therapy have been evaluated and showed encouraging results in relapsed/refractory (R/R) AML or in AML/MDS patients ineligible for intensive chemotherapy (111–117). Both *IDH* inhibitors were approved by the FDA for the treatment of R/R AML. These drugs induce cellular differentiation and may promote an allo-immunologic reaction by antigen upregulation on leukemic cells. This mode of action implies that these agents may have an interesting activity in *IDH*-mutated AML patients as salvage or even as maintenance therapy after transplant (111, 112). Currently, there are several ongoing prospective trials evaluating the role of *IDH* inhibitors in the maintenance setting after transplant in *IDH*-mutated AML (NCT03515512 and NCT03564821). The safety and efficacy of combination venetoclax plus AZA in R/R AML after allo-HSCT has been proven only in case series (113–116). The same combination is being tested in post-transplant AML patients as maintenance therapy (NCT04128501).

Although combination HMA and *FLT3* inhibitors was not investigated in the setting of maintenance therapy after allo-HSCT in AML, this combination has shown efficacy in AML. DiNardo and colleagues reported the results of the combination of venetoclax with low dose AZA in 81 elderly patients; analysis of primary and adaptive resistance was caused by an enrichment of clones harboring activated signaling pathways such as *FLT3* or *RAS* or biallelically perturbing *TP53* which helped in determining the predictors of outcome using this combination therapy (117). And we know from previous studies that combination of AZA plus sorafenib is effective and well tolerated in relapsed/refractory *FLT3-ITD* AML (118). Thus, the combination of *FLT3* inhibitor and HMA seems to be a potential strategy to prevent relapse post-transplant in high risk AML patients and it is worth being investigated.

Hedgehog inhibitor (glasdegib) has recently shown promising results in a randomized phase II study when combined with low-dose cytarabine (LDAC) as compared to LDAC alone in AML/MDS frail patients (119). A single agent glasdegib is being investigated in a phase II study as maintenance therapy following allo-HSCT for high-risk patients (NCT01841333).

Finally, despite maintenance treatment, most of the patients still relapse. Different mechanisms of resistance may emerge. For example, in patients with *FLT3-ITD* mutation, acquisition of point mutations in the *FLT3* drug binding site, or activation of alternative pathways such as mutations of the *NRAS* gene are the most described mechanism of resistance.[91] Many combinatorial strategies have evolved and probably overcome this resistance such as combination of *FLT3-TKIs* with epigenetic therapy including histone deacetylase inhibitors and HMA, which revealed promising and synergistic antileukemic *in vitro* efficacy mainly by downregulation of the *JAK/STAT* pathway (120).

Figure 1 summarizes the treatment guidelines to prevent relapse of AML after allo-HSCT.

Summary

- MRD measurement using MFC and RQ-PCR methods should be incorporated in the treatment decision process for adult AML patients after transplant.
- MRD will enable to identify high-risk patients to define patients at risk of relapse who would benefit from preemptive approaches with HMA and targeted therapies.
- Azacitidine use as maintenance therapy in high-risk AML and as preemptive MRD-triggered therapy could be considered after transplant for at least 12 months at a dose of 32 mg/m^2 for 5 days and 75 mg/m^2 for 7 days, respectively.
- In *FLT3-ITD* AML patients, post-transplant maintenance therapy with sorafenib at a dose 400–800 mg/day in two divided doses should be strongly considered for 24 months.
- Other *FLT3* inhibitors such as midostaurin and gilteritinib are attractive in the maintenance setting and warrant further investigation in larger prospective studies.
- The use of other agents (IDH inhibitors, BCL-2 inhibitors, Hedgehog inhibitors) and combination therapy with DLI are being evaluated and could have a promising result in the post-transplant maintenance setting.

AUTHOR CONTRIBUTIONS

AA reviewed the literature and wrote the manuscript. ZO reviewed the literature and wrote the manuscript. IA reviewed the literature and wrote the manuscript. JE-C reviewed the literature and wrote the manuscript. AB reviewed the literature and wrote the manuscript. All authors contributed to the article and approved the submitted version.

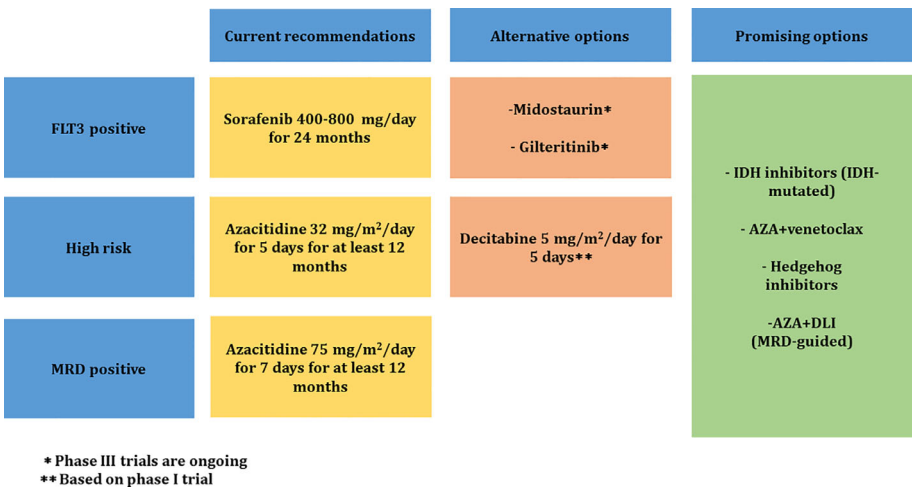

FIGURE 1 | Proposed treatment guidelines to prevent relapse of AML after allo-HSCT. MRD, measurable residual disease; IDH, isocitrate dehydrogenase; AZA, azacitidine; DLI, donor lymphocyte infusion.

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REVIEW
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Hematopoeitic Cell Transplantation and CAR T-Cell Therapy: Complements or Competitors?

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Allogeneic hematopoietic cell transplantation (allo-HCT) and chimeric antigen receptor T cell (CAR T) therapy are the main modalities of adoptive cellular immunotherapy that have widely permeated the clinical space. The advent of both technologies revolutionized treatment of many hematologic malignancies, both offering the chance at sustained remissions for patients who would otherwise invariably succumb to their diseases. The understanding and exploitation of the nonspecific alloreactivity of allo-HCT and the graft-versus-tumor effect is contrasted by the genetically engineered precision of CAR T therapy. Historically, those with relapsed and refractory hematologic malignancies have often been considered for allo-HCT, although outcomes vary dramatically and are associated with potential acute and chronic toxicities. Such patients, mainly with B-lymphoid malignancies, may now be offered CAR T therapy. Yet, a lack of prospective data to guide decisions thereafter requires individualized approaches on whether to proceed to allo-HCT or observe. The continued innovations to make CAR T therapy more effective and accessible will continue to alter such approaches, but similar innovations in allo-HCT will likely result in similarly improved clinical outcomes. In this review, we describe the history of the two platforms, dissect the clinical indications emphasizing their intertwining and competitive roles described in trials and practice guidelines, and highlight innovations in which they complement or inform one another.

Keywords: allogeneic stem cell transplantation, chimeric antigen receptor T cell therapy, cytokine release syndrome, hematologic malignancies, Allo-CAR T

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INTRODUCTION

The expanding field of immuno-oncology has unlocked the possibility of treating and potentially curing patients with the most life-threatening relapsed and refractory hematologic malignancies. The clinical benefit of allogeneic hematopoeitic cell transplantation (allo-HCT) is mediated by a graft-versus-tumor effect which results from alloreactivity of donor T cells to host major and minor histocompatibility antigens (1–3). Over the past fifty years, we have better understood and refined the process of allo-HCT, improving its success and limiting its complications (4–6). Nevertheless, disease persistence and transplant-related toxicity have driven the necessity for continued innovation.

Now, at the leading edge of immune-oncology, genetically engineered chimeric antigen receptor T (CAR T) therapies promise to advance the treatment of refractory malignancies by combining

B-cell-like target recognition with T-cell machinery and memory (7, 8). Notable responses, even among patients who had progressed after allo-HCT, led to the FDA approval of commercial CAR T therapies in young patients with acute lymphoblastic leukemia (ALL), and later in adults with relapsed refractory large cell B cell lymphomas (9). Similar to allo-HCT, disease recurrence after CAR T and treatment-related toxicities require ongoing innovation in product development and toxicity mitigation strategies.

As the novelty and success of CAR T therapies continue to escalate, many now wonder whether CAR T and allo-HCT will continue to coexist and complement one another, or whether some selective pressure, be it cost, convenience, efficacy, or toxicity, will favor only one to persist or to dominate the clinical landscape. At this point, the answer varies depending on the specific disease, practitioner perspective, and even geographic area of practice. Many still view CAR T therapy as a bridge to allo-HCT in patients with ALL, although that stance is not ubiquitous (10). Compare that to multiple myeloma (MM), in which the promise of CAR T efficacy from clinical trials has all but removed allo-HCT from the late-stage MM algorithm, although some centers continue this practice (11, 12).

While at this point it may be impossible to predict whether CAR T or allo-HCT will outlast the other, it is clear that they have been both competitive and complementary. Additionally, lessons have been translated from one platform to the other, such as the management of cytokine release syndrome (CRS), improved efficacy with lymphodepletion, and the potential for "off-the-shelf" allogeneic universal CAR T cells (UCAR T).

In this review, we will provide a historical overview of the two therapies, drawing attention to similarities and differences. We will then analyze the clinical trial data on the interplay between allo-HCT and CAR T therapy and the lessons that have been learned from each. We will describe the knowledge gaps that still exist regarding the sequencing or substitution of one platform with the other, and the ongoing preclinical and clinical work aiming to resolve them. Lastly, we will examine the future directions in which both strategies are heading, emphasizing the indications in which they will be complementary and in which one could out-compete the other.

CELLULAR THERAPIES: THE PARALLEL AND INTERTWINING HISTORIES OF ALLO-HCT AND CAR T

Hematopoeitic Cell Transplantation and the Birth of Adoptive Immunotherapy

In the middle of the 20th century, preclinical work by Jacobsen, Lorenz, and colleagues gave credence to the concept of the transplantation of bone marrow following lethal irradiation (13, 14). Over the next twenty-five years, numerous physician-scientists sought to translate this to a clinical therapy, initially for radiation-induced aplasia, but subsequently for congenital immunodeficiencies, aplastic anemia, and eventually for acute leukemias (4). Much of the initial clinical work was limited by frustrations and failures. While early reports described the feasibility of allogeneic bone marrow collection, storage, and intravenous infusion into a recipient, little progress was made regarding the impact of histocompatibility differences between donor and host; those with insufficient preparation rejected the graft and those with complete myeloablation often developed profound graft-versus-host disease (GVHD) (15). The development of canine and murine models by Thomas et al. led to a rudimentary understanding of histocompatibility, which they then translated to clinical application. Specifically in hematologic neoplasia, they initially studied syngeneic bone marrow transplantation in a small number of patients who had identical twins. While they observed normal recovery of hematopoiesis, most would relapse (1, 2). Under the hypothesis that the syngeneic immune cells lacked the ability to immunologically target the leukemic cells, they designed a regimen in which the transplant recipients would receive repeated infusions of syngeneic donor lymphocytes along with subcutaneous injections of autologous, lethally-irradiated leukemia cells in order to provide continual antigenic stimulation. This first "immunotherapy" was modestly successful at delaying leukemia relapse, and provided the initial evidence of a graft-versus-leukemia (GVL) effect.

As most patients do not have identical twins, investigators focused on HLA-identical sibling transplantation. As transplant physicians gained experience, refinements in conditioning regimens, improvements in supportive care, and the addition of post-transplant immunosuppression lessened transplant-related mortality and improved survival. One observation was that patients who developed both acute and chronic GVHD were noted to have decreased incidence of relapse, which in some cases translated to improved survival (16). Nevertheless, severe GVHD was often fatal and limited the prospects of allo-HCT, therefore investigators sought to find improved methods of GVHD prevention. T-cell depleted grafts were assessed preclinically and clinically, and while they were associated with reduced GVHD, relapse and graft failure rates were significantly higher negating any beneficial effects (17). This was especially notable in myeloid malignancies, less so in acute lymphoblastic leukemia.

These initial observations stressed the importance of the T-cell mediated GVL effect. With a deeper understanding of the adoptive immunotherapy aspect of allo-HCT, new modifications and therapies were possible. Donor lymphocyte infusions were administered to patients with mixed donor chimerism or early relapse, with durable remissions achieved especially in myeloid malignancies (18–20). Additionally, reduced-intensity conditioning regimens were designed that allowed for older and frailer patients to undergo allo-HCT, with a heavier reliance on the GVL effect (21).

The Advent of CAR T Therapy

At the same time that nonspecific adoptive cellular therapies (e.g., donor lymphocyte infusions, tumor-infiltrating lymphocytes) were being clinically deployed, novel gene-transfer techniques were allowing for the preclinical *ex vivo* engineering of T-cells harboring CARs. Initially, gene

transduction occurred *via* retroviral vectors, but methods involving lentiviral, adenoviral, and non-viral methods would be developed thereafter (7, 22). The initial CAR constructs included an extracellular antigen-specific binding moiety, usually a single chain variable fragment (scFV) of a monoclonal antibody, fused to a transmembrane segment, and an intracellular domain consisting of the CD3ζ signaling domain of the T-cell receptor (TCR) (23, 24). While these first-generation CARs could redirect the specificity of T-cells to target antigens in an HLA-independent fashion, the intracellular signaling of CD3ζ alone lacked the strength to induce proliferation and prolonged antineoplastic activity, resulting in rapid CAR T-cell exhaustion and only modest reduction in tumor growth *in vivo* (25).

In order to achieve the goal of a "living drug", in which the CAR T cells could continue to proliferate and display persistent antineoplastic activity following the *in vivo* administration, investigators examined methods in which to augment intracellular signaling. Chimeric costimulatory receptors (CCRs) were first developed and introduced into human primary T-cells. Engagement of the CCRs (specifically that with a CD28 intracellular signaling domain) led to increased IL-2 production which allowed for persistence of the T-cells in TCR-activation situations which would otherwise promote apoptosis (26). CD28 and other costimulatory domains, such as 4-1BB (CD137), were then fused with CD3ζ. These "second generation" CAR T cells utilizing one of several potential costimulatory endodomains demonstrated increased persistence, proliferation, and antitumor activity, preclinically (27, 28). In a proof-of-concept clinical pilot, Savoldo et al. treated 6 patients with relapsed non-Hodgkin's lymphoma who were simultaneously infused with a "first generation" CAR T product harboring only a CD3ζ endodomain and a "second generation" CAR T product harboring both CD3ζ and CD28 endodomains (29). Both had the same CD19-specific scFv exodomain. Peripheral blood examination demonstrated that the second-generation CAR T-cells expanded *in vivo* significantly more in the first two weeks after infusion and persisted longer. Additionally, *ex vivo* engagement of their native TCR could promote their restimulation. In contrast, the first generation CAR T-cells did not expand, could not be restimulated, and did not persist in the infused patients. With expansion and persistence demonstrated in humans, along with efficacy signals in targeting CD19+ B cell malignancies, these second-generation CAR T cells were primed for widespread clinical investigation.

CLINICAL APPLICATIONS OF CAR T-CELL THERAPY AND THE ROLE OF ALLO-HCT

Current treatment algorithms now incorporate CAR T therapy for specific hematologic malignancies. The initial target for CAR T-cell therapy was CD19, chosen for its broad and high expression on B-cell leukemias and lymphomas, as well as for restriction of its expression to the B-cell lineage which would predict limited off-target effects (30). Theoretically, targeting B-cells would also limit humorally-mediated rejection of the CAR T cells. As such, the CD19 CAR T products would be the first to obtain regulatory approval for B-cell acute lymphoblastic leukemia (B-ALL) in patients up to age 25 and in certain B-cell non-Hodgkin's lymphomas (NHL). Following a similar path, the CAR-targeting of a lymphoid/plasma cell-restricted surface antigen, B-cell maturation antigen (BCMA), has multiple myeloma on the precipice of at least one approved CAR T product.

How the role of allo-HCT has been impacted by these CAR T therapies is dependent on numerous disease, patient, and therapy factors (**Table 1**). While there is a lack of prospective data addressing the specific intertwining roles of CAR T and allo-HCT, the decisions often require individualized consideration as well as reliance on subgroup data from within existing trials and expert opinion. Hereafter, we dissect such information as it exists for these three disease groups in which CAR T therapy is part of the current treatment paradigm.

Acute Lymphoblastic Leukemia

Treatment of B-ALL has evolved tremendously over the past decade. Prolonged, intensive combination chemotherapy regimens have been very successful at curing a majority of pediatric patients with ALL (50). These pediatric-inspired regimens have been translated to young adult populations, improving relapse-free and overall survival relative to historical comparators, albeit to a lesser extent than that seen in pediatric populations (51). Even some middle-aged and older adults may be cured with front-line chemotherapy, without the need to proceed to allo-HCT.

Concurrently with the advances in therapy, there has been an evolution in the understanding of the clinical and biological heterogeneity of B-ALL. This has allowed for more precise risk stratification based on clinical factors (e.g., age, blast count at diagnosis), cytogenetic/molecular factors (e.g., *BCR-ABL* translocation, Philadelphia chromosome-like ALL, *TP53* alterations with hypodiploidy), and treatment response (e.g., minimal residual disease [MRD] post-induction) (52). Patients with high-risk features are conventionally recommended to proceed with allo-HCT in first clinical remission (CR1) (31, 32, 53). This recommendation is based on observational data suggesting a very high risk of relapse with conventional chemotherapy, and "genetically randomized" prospective trials repeatedly demonstrating a survival benefit in high-risk subsets for those who received HLA-matched sibling allo-HCT.

In both pediatric and adult patients with B-ALL, relapsed and refractory disease carries a dismal prognosis (35). Immediately prior to CAR T therapy, two immunotherapies, inotuzumab ozogamicin and blinatumomab were able to significantly prolong event-free survival and overall survival compared to salvage chemotherapy (54, 55). However, the vast majority of patients in both trials still relapsed and died within 24 months, and long-term survival was achieved only in the minority who proceeded to allo-HCT. Blinatumomab did subsequently establish a niche in converting MRD positive to MRD negative status in patients in CR1 or greater, the majority of whom are bridged to allo-HCT once MRD is no longer detected (56).

The recent advent of CD19-targeting CAR T cells (CART19) provided yet another therapy to the arsenal directed against

TABLE 1 | Comparisons of indications and outcomes of allogeneic hematopoeitic transplantation and CAR T-cell therapy.

	Allogeneic HCT	CAR-T
B-ALL		
Indications	High-risk CR1, ≥CR2, Post CAR-T (especially early loss of B-cell aplasia, no prior alloHCT), controversial in active disease	Refractory or 2nd or greater relapse in ≤25 years-old (tisagenlecleucel) Efficacy seen in post alloHCT In clinical development for adult patients: dual-targeting CAR, relapse post CD19 CAR, "off-the-shelf" allogeneic CAR T
CR	N/A	60%–80% (adults) 70%–90% (pediatrics)
OS	30%–60% at 3 years (adults) 60%–75% at 3 years (pediatrics) 20% at 3 years (alloHCT with active disease)	40%–70% at 2 years
Toxicity	aGVHD, cGVHD, graft failure and prolonged cytopenias, infections	CRS, ICANS, prolonged cytopenias, infection
References	(31–34)	See **Tables 2** and **3**
AML		
Indications	Intermediate or unfavorable risk in CR1, ≥CR2, active disease (usually on a clinical trial)	Currently in clinical development for rel/ref active disease CAR Targets: NKG2D, CD123, CLL-1, and CD33
CR/CRi	N/A	50% (early phase I data)
OS	25%–60% at 3 years (adults) 30%–70% at 3 years (pediatrics) 10%–20% at 3 years (active disease)	N/A
Toxicity	aGVHD, cGVHD, graft failure and prolonged cytopenias, infections	CRS, ICANS, marrow ablation (theoretical)
References	(33, 35)	(36–38)
DLBCL		
Indications	Relapse after ASCT – best in patients with >12 mo remission after ASCT, chemosensitive disease, lower NRM with RIC	Rel/ref after two lines of therapy (FDA indications for tisagenlecleucel and axi-cel) Allogeneic CAR T, dual-targeting CAR T, relapse post-CD19 CAR T in clinical development
CR	N/A	40%–60%
PFS	40% at 3 years	Axi-cel: 75% at 2 years in responders, 22% at 2 years in patients with SD Tisagenlecleucel: 83% at 1 year in responders
OS	54% at 3 years	Axi-cel: 50% at 2 years (ITT) Tisagenlecleucel: 40% at 1 year (ITT)
NRM	25%–30%	4% (axi-cel), 0% (tisagenlecleucel)
References	(39)	See **Table 4**
FL		
Indications	Rel/ref FL – better outcomes with chemosensitive disease and RIC	Rel/ref FL (axi-cel, in clinical development)
CR	N/A	80%
PFS	50% at 5 years	50% at 2 years
OS	60% at 5 years	90% at 2 year
NRM	20%	3%
References	(40, 41)	(42, 43)
MCL		
Indications	Rel/ref MCL – better outcomes with chemosensitive disease, RIC, earlier in disease course although controversial	Rel/ref MCL having received at least 2 lines of therapy (brexucabtagene autoleucel, approved indication)
CR	N/A	67%
PFS	40%–50% at 3 years	61% at 1 year
OS	40%–60% at 3 years	83% at 1 year
NRM	15%–25%	3%
References	(44)	(45)
MM		
Indications	Rarely indicated – usually in a younger patient or high-risk disease as part of a clinical trial	In clinical development for triple-class refractory disease and suboptimal response to ASCT
CR	N/A	30%–90%
PFS	30%–40% at 3 years	40%–50% at 1 year
OS	50% at 3 years	75%–90% at 1 year
NRM	20%–25%	2%–5%
References	(12, 46)	(47–49)

Allo-HCT, allogeneic hematopoietic cell transplantation; aGVHD, acute graft-vs-host disease; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; CR, complete response; CRS, cytokine release syndrome; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; N/A, not available; NRM, non-relapse mortality; OS, overall survival; PFS, progression-free survival; PR, partial response; rel/ref, relapsed/refractory.

relapsed/refractory B-ALL. In phase I and II trials, the second-generation CART19 had unprecedented success in achieving remissions in heavily-pretreated patients with B-ALL (**Tables 2** and **3**). With this success, new questions emerged, namely the sequencing of CART19 and allo-HCT, whether CART19 should be used as a bridge to allo-HCT or could be a "destination" in and of itself, and if there were any differences in safety or efficacy in patients who had already undergone allo-HCT prior to CART19. There is yet to be a prospective trial in which patients have been randomly assigned to allo-HCT or observation following CART19, therefore the existing data is limited to an extent by selection of patients fit to undergo allo-HCT post-CART19 and those with a suitable donor. Additionally, there is heterogeneity regarding the length of follow-up and reporting of outcomes following allo-HCT.

TABLE 2 | Response and relapse outcomes in trials assessing CD19 CAR-T therapy with CD28/CD3ζ co-stimulatory domains in B-cell acute lymphoblastic leukemia with potential bridging to allogeneic hematopoietic cell transplantation.

	Lee et al. (57)	Park et al. (58)	Curran et al. (59)
Patients, n	51	53	25
Age Category	Pediatric and YA	Adult	Pediatric and YA
Median follow-up, mo	22.5	29	7.7 (28.6 in responders)
Prior allo-HCT, %	35	36	20
CR(MRD$^-$), %	61(55)	83(60)	75 (67)
Allo-HCT post-CR,%	75	39	83
Relapse after CR: overall/after allo-HCT %	29/9.5	56/35	33/27

Allo-HCT, allogeneic hematopoietic cell transplantation; CR, complete remissions; MRD, minimal residual disease; YA, young adults.

TABLE 3 | Response and relapse outcomes in trials assessing CD19 CAR-T therapy with 4-1BB/CD3ζ co-stimulatory domains in B-cell acute lymphoblastic leukemia with potential bridging to allogeneic hematopoietic cell transplantation.

	Maude et al. (60)	Pan et al. (61)	Gardner et al. (62)	Maude et al. (63)	Hay et al. (64)	Jiang et al. (65)	Frey et al. (66)
Patients, n	30	51	45	75	53	58	35
Age Category	Pediatric and YA (25), Adult (5)	Pediatric, YA, and Adult	Pediatric and YA	Pediatric and YA	Adult	Pediatric, YA, and Adult	Adult
Median follow-up, mo	7	NA	9.6	13.1	30.9	7.7	13
Prior allo-HCT, %	60	0	62	61	43	5	37
CR/CRi(MRD$^-$), %	90 (77)	86(81)	89 (89)	81(81)	85 (85)	88 (81)	69 (57)
Allo-HCT post-CR,%	11	60	28	13	40	45	38
Relapse after CR: overall/after allo-HCT %	26/0	24/7	45/18	32/NA (50% relapse-free, 50% unknown after allo-HCT)	49/17	38/9	NA (Landmark analysis for EFS p=0.03)

Allo-HCT, allogeneic hematopoietic cell transplantation; CR, complete remissions; MRD, minimal residual disease; NA, not available; YA, young adults.

There is a lack of consensus regarding which B-ALL patients should proceed to allo-HCT after CART19, and among the clinical trials of CART19 such decisions were usually informed by patient age, institutional practice, and the expected persistence of the CAR T cell product (67, 68). A key determinant of persistence appears to be whether the co-stimulatory domain employed is CD28 or 4-1BB, with the CD28 constructs demonstrating relatively short persistence. In a phase I/II NIH study of a CD28 CART19 in children and young adults, initially 12 patients achieved a MRD negative CR, of whom 10 proceeded to allo-HCT with durable remission, whereas the two transplant-ineligible patients relapsed (69). The study expanded to 53 patients, 51 with B-ALL, and in the long-term follow-up (median 22.5 months), 60.8% achieved CR, 90% of which were MRD negative (57). Twenty-one of the 28 patients with MRD negative CR proceeded to allo-HCT, after which 2/21 (9.5%) relapsed, compared to 6/7 (85.7%) of those in MRD negative CR who did not proceed to transplant. This difference translated to significant improvement in leukemia-free survival (HR 16.9, 95% confidence interval [CI] 3.4-85.1, p=0.0006). In a large adult trial, 53 patients received a CD28 CART19 with 83% CR and 67% MRD negative CRs (58). Median event-free survival (EFS) was 6.1 months and median OS was 12.9 months. Of those who were MRD negative (n=32), half proceeded to allo-HCT and the other half did not. Allo-HCT was not associated with improved EFS or OS, although survival was poor regardless of transplant.

In the initial phase I/II trial of the 4-1BB CART19, tisagenlecleucel/CTL019, out of University of Pennsylvania and Children's Hospital of Philadelphia, Maude et al. (60) reported that only three of 30 pediatric and young adult patients underwent subsequent allo-HCT while in MRD$^-$ remission (60). Nevertheless this remission persisted 7 to 12 months after tisagenlecleucel infusion. A similarly low rate of allo-HCT after tisagenlecleucel was reported in the phase II ELIANA trial of this product in a similar population, in which only eight of 75 patients proceeded to allo-HCT while in remission (63). Two of the eight had MRD positivity and two others had lost B-cell aplasia within 6 months of the infusion. Of those eight patients, four were known to remain in remission at follow-up while the other 4 had an unknown disease status. An updated analysis of ELIANA demonstrated persistence of tisagenlecleucel with ongoing B-cell aplasia in some patients with follow-up for multiple years, which correlated with ongoing remission (70). Survival was unprecedented and irrespective of subsequent allo-HCT. Based on these results, some argue that the unique biology of pediatric B-ALL and persistence of tisagenlecleucel provide the potential for durable remission without the need to proceed to allo-HCT in this population (71).

Not all 4-1BB CART19 constructs have been associated with persistence and prolonged B-cell aplasia in children. In a phase I/II study of 45 children and young adults, Gardner et al. (62) produced 4-1BB CART19 at a defined 1:1 of CD4+:CD8+ cells, achieving 93% (40 of 43) MRD negative CRs among those who received the product (62). Median duration of B-cell aplasia, however, was relatively short, only 3 months. Loss of B-cell aplasia correlated with occurrence of relapse. Eleven patients underwent consolidative allo-HCT, two of whom were MRD$^+$ by next-generation sequencing pre-transplant and recurred following transplant. Summers et al. (72) provided an updated analysis of this trial in which there was a suggested benefit in leukemia-free survival from consolidative allo-HCT in transplant-naïve patients after CART19 as well as among patients who lose B-cell aplasia in ≤63 days, even those with a prior allo-HCT (72).

In a phase II study out of Hebei Yanda Lu Daopei Hospital, Pan et al. treated 51 children and adults with a 4-1BB CART19 which led to 85% MRD$^-$ CR/CRi in those who entered with active disease and 100% conversion of MRD$^+$ patients to MRD$^-$ (61). Sixty-percent (27/45) of these patients proceeded to allo-HCT, the majority of which were from haploidentical donors. Following allo-HCT, two died from complications of the transplant and two patients relapsed. Comparatively, nine of the 18 patients who did not proceed to allo-HCT relapsed at a median time of 64 days, although the reasons for foregoing transplant were unclear. Late relapse (90+ days after CART19) was also significantly better among allo-HCT recipients (p=0.023). A more recent Chinese study corroborated such findings in a similarly heterogeneous population (65). Jiang et al. prospectively compared outcomes of 47 4-1BB CART19 recipients who achieved MRD$^-$ CR. 21 transplant-naïve patients proceeded to allo-HCT at a median 44 days after CAR-T. Twenty-six patients did not proceed to transplant as three had previous allo-HCT, five were contraindicated, three lacked

donor, and the rest chose to forego it for personal reasons. Two patients died of complications from the transplant (chronic GVHD, infection), and two experienced CD19-negative relapse. Overall, consolidative allo-HCT was associated with improved EFS and RFS ($p<0.05$) although there was no significant difference in OS. Importantly, no patient was precluded from allo-HCT due to CAR T-related toxicities. Although these studies did not differentiate the outcomes based on patient age, they support transplanting all transplant-naïve patients who achieve MRD-negativity with 4-1BB CART19 on the basis of protection from relapse, although survival benefits are unclear.

Among United States trials of 4-1BB CART19 for B-ALL in adults, there is a suggestion that bridging to allo-HCT provides better outcomes than CAR T therapy in isolation. In a pilot study out of University of Pennsylvania, tisagenlecleucel was administered to five patients at a high dose in a fractionated schedule (HDF), all of whom achieved a CR. In the follow-up study, single infusion of a high dose 4-1BB CART19 was complicated by a high incidence of CRS-related death, and a low dose lacked efficacy, therefore the protocol underwent two amendments ultimately settling on HDF for the remaining participants (66). The MRD^- CR rate among HDF recipients (n=20) was 90%, with 2-year EFS and OS of 49.5% and 73%, respectively. Efficacy and safety outcomes were notably better in the HDF schedule than either single-infusion schema. Nine of 24 patients who had achieved CR were consolidated with allo-HCT at a median of 2.6 months after CART19. Landmark analysis by allo-HCT demonstrated a significant improvement in EFS ($p=0.029$) and nonsignificant improvement in OS ($p=0.09$). Work out of the Fred Hutchinson Cancer Research Center produced similar findings in a trial of 53 adults with B-ALL who received a 4-1BB CART19 (64, 73). 45 patients achieved MRD-negative CRs, of whom eighteen (40%) proceeded to allo-HCT. In univariate analysis, allo-HCT was associated with longer EFS compared to no allo-HCT (HR 0.31, $p=0.014$), as well as after adjusting for other factors associated with improved EFS.

In summary, until randomized controlled trials address the specific question of allo-HCT after CART19 for B-ALL, the decision to proceed to transplant must be individualized based on key patient, disease, and product factors. Most would argue that recipients of a CD28-based CART19 should proceed to allo-HCT due to lack of persistence (71). Young patients who receive tisagenlecleucel, which to date is the only FDA approved commercial product, may be able to forego allo-HCT, as sustained remissions have been seen in such patients. Prior allo-HCT or extensive prior treatment may also favor avoiding subsequent allo-HCT after CART19. Loss of B-cell aplasia, especially within 6 months of CAR T infusion in patients with B-ALL, likely warrants consideration of allo-HCT among those who initially forego it (74). Some argue that predicting persistence of 4-1BB CART19 is difficult and that relapse could occur due to lack of persistence or secondary to the loss of CD19 on leukemia cells; therefore it is reasonable to offer and prepare for allo-HCT in all patients following CART19 (75). The decision to pursue allo-HCT is only likely to become more obfuscated as CAR T therapies with new and/or multiple targets, improved persistence, and universal allogeneic off-the-shelf CAR T (UCART) are developed and deployed.

Non-Hodgkin's Lymphoma

The role of CAR T therapy is actively evolving in the treatment strategy of Non-Hodgkin's lymphoma (NHL), and varies based on NHL subtype. Likewise, the role of allo-HCT is also in flux for NHL, in large part due to the introduction and dissemination of CAR T therapy. Hereafter, we address the trial data for CAR T therapy based on NHL subtype as well as the dynamic status of allo-HCT in these diseases.

Diffuse Large B-Cell Lymphoma

The treatment paradigm for early relapsed or refractory diffuse large B-cell lymphoma (DLBCL) involves salvage chemotherapy followed by autologous stem cell transplantation (ASCT) for those that respond to the salvage regimen, in transplant-eligible patients (76, 77). Historically, allo-HCT consolidation after an initial salvage regimen was associated with decreased incidence of relapse compared to ASCT, but was more toxic resulting in comparable relapse-free and overall survival (78). Studies did posit an immunotherapeutic graft-versus-lymphoma effect that could be exploited in the event of relapse after ASCT or failure to mobilize sufficient stem cells, therefore allo-HCT was relegated to such scenarios (79). A CIBMTR analysis examining allo-HCT in the era immediately prior to the development of novel agents and CAR T therapy highlighted the limited options for and poor prognosis of patients necessitating allo-HCT for advanced DLBCL. Relapse rate was inversely correlated with conditioning intensity, although myeloablative regimens yielded non-relapse mortality of 56%, translating to similarly poor 5-year survival of around 20% (80).

While direct comparisons to allo-HCT are lacking and follow-up is still limited, data from the major trials of CD19 CAR T therapy for relapsed/refractory DLBCL and real-world registries suggest durable CR rates of 30 to 40% with treatment-related toxicities that are more benign and relegated to the acute setting (**Table 4**). In the pivotal ZUMA-1 trial, axicabtagene ciloleucel (axi-cel), a CD19-targeting CAR T cell with a CD28 co-stimulatory domain, yielded an objective response rate of 82% and CR rate of 54% (86). In an updated analysis with a median follow-up of 27.1 months, a significant number of patients had converted from SD or PR at one month to CR by 6 months (84). The estimated 24-month overall survival was 50.5%, and durable remissions were highlighted by an estimated 24-month PFS of 75.0% and 72.0% among those with a CR and PR, respectively. No patient had undergone an allo-HCT prior to axi-cel, and only two patients underwent allo-HCT while responding to axi-cel. Unlike the experience with B-ALL, loss of B-cell aplasia was not a predictor of disease recurrence, and durable responses did not appear to require prolonged persistence of functional CAR T cells. Grade 3 or worse cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) occurred in 11% and 32% of patients, respectively,

TABLE 4 | Summary of trials of CD19 CAR-T therapy with B-cell non-Hodgkin lymphoma.

	Schuster et al. (81)	Kochenderfer et al. (82)	Schuster et al. (83)	Locke et al. (84)	Abramson et al. (85)	Wang et al. (45)	Jacobson et al. (43)
Patients, n	28	22	111	108	268	74	87
Disease(s)	DLBCL, FL	DLBCL, tFL, PMBL, MCL, FL	DLBCL	DLBCL, tFL, PMBL	DLBCL, PMBL, FL3B	MCL	FL, MZL
Co-stimulatory domain	4-1BB/CD3ζ	CD28/CD3ζ	4-1BB/CD3ζ	CD28/CD3ζ	4-1BB/CD3ζ	CD28/CD3ζ	CD28/CD3ζ
Median follow-up, mo	28.6	12.5	14	27.1	10.8	13.1	11.5
Prior allo-HCT, %	7	0	0	0	3	0	NA
CR/PR, %	57/7	55/18	40/12	58/25	53/20	59/36	80/15
Allo-HCT post-CR,%	11	4	0 (6 non-responders underwent allo-HCT)	5	NA	1.3	NA
OS	DLBCL: 50% at 22 mo FL: 93% at 28.6 mo	NA	59% at 12 mo	50.5% at 24 mo	mOS 10.8 mo	83% at 12 mo	NA
PFS in CR/PR, %	DLBCL: 43/0 at 28.6 mo FL: 70/0 at 28.6 mo	63/0 at 12 mo	83% at 12 mo	75/72 at 24 mo	mDoR 13.3 mo; mPFS 6.8 mo	61% at 12 mo (all patients)	NA

Allo-HCT, allogeneic hematopoietic cell transplantation; CR, complete response; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; NA, not available; OS, overall survival; PFS, progression-free survival; PMBL, primary mediastinal B-cell lymphoma; PR, partial response; tFL, transformed FL.

but no deaths were attributed to axi-cel, and most treatment-related toxicities were confined to the peri-treatment period.

Tisagenlecleucel was examined in the pivotal single-armed, phase II JULIET trial in DLBCL and transformed follicular lymphoma (83). Patients with a prior allo-HCT were excluded. A notably higher percentage of patients had relapsed after a prior ASCT compared to the ZUMA-1 trial. The best ORR was 52%, 40% with CR and 12% with PR, with 43% conversion rate from PR/SD to CR at a median of 2 months post-infusion, but as late as 17 months. The estimated 12-month PFS was 83% among those with a CR or PR, and 12-month OS was 50% for all who received an infusion. Grade 3 or higher CRS or ICANS occurred in 22% and 12% of patients, respectively, although were mainly confined to the 8-week period after infusion. No deaths were attributed to the CAR T product. Similar to axi-cel, many patients had loss of B-cell aplasia although this was not associated with disease recurrence. Five non-responders proceeded to allo-HCT, although none of the patients proceeded to allo-HCT while experiencing a response to CAR T.

These two pivotal trials led to the commercial approval of axi-cel and tisagenlecleucel for DLBCL relapsed after or refractory to at least two lines of therapy. They demonstrated notable efficacy in a population of patients with historically poor outcomes, even in those with chemorefractory disease or high-risk features. In comparison, chemosensitivity is usually a prerequisite for proceeding to allo-HCT as chemorefractoriness portends a high risk of relapse in this setting. Such findings were corroborated by real-world reports of CD19 CAR T for DLBCL, with comparable efficacy and an improved safety profile, in part due to the learned management of acute toxicities (87, 88). With comparable or improved efficacy relative to allo-HCT and toxicities that appear generally more tolerable and limited in duration, CAR T therapy has likely supplanted allo-HCT for the treatment of multiply relapsed DLBCL.

Mantle Cell Lymphoma

Mantle cell lymphoma, although rare, is uniquely challenging in that it invariably relapses following initial therapy with induction and ASCT consolidation, its clinical course is often aggressive, and it frequently becomes refractory to chemotherapy and novel agents. Although novel therapies such as Bruton's tyrosine kinase (BTK) inhibitors have prolonged survival, progression is often inevitable and associated with poor survival (89). Allo-HCT has been offered as a potentially curative option with the advantages of providing an uncontaminated graft with theoretical GVL effect, although the advent of numerous targeted agents has been providing longer responses, and patients are more heavily treated at the point of allo-HCT consideration. Reduced-intensity conditioning (RIC) regimens have been favored due to the usual advanced age and comorbidities of MCL patients. In a retrospective registry study by the European Bone Marrow Transplant Lymphoma Working Party, MCL patients undergoing allo-HCT with RIC experienced 1-year NRM of 24%, with long-term disease-free survival of 30% at 4 years (90).

Brexacabtagene autoleucel (KTE-X19) recently garnered accelerated regulatory approval for the treatment of relapsed/

refractory MCL. KTE-X19 is the same construct as axi-cel, with a CD28 co-stimulatory domain, although it undergoes a manufacturing process that selectively removes circulating CD19-expressing malignant cells to prevent premature CAR T activation (45). In the ZUMA-2 trial, 74 patients who had relapsed after chemotherapy (anthracycline or bendamustine), an anti-CD20 monoclonal antibody, and BTK inhibitor were treated in a single arm, multicenter phase II trial of KTE-X19, with dosing based on the established dose of axi-cel. The ORR was 85% with a CR rate of 59%, with responses comparable across high risk subgroups. Among those with an initial PR or SD, 57% improved to CR. The 12-month estimated PFS was 61% and OS was 83%. Grade 3 or higher CRS occurred in 15% and ICANS in 31%. One patient had grade 4 cerebral edema. Two deaths occurred relating to the conditioning chemotherapy. One patient proceeded to allo-HCT while in a PR. There are no data comparing allo-HCT with CAR-T in these patients and we currently lack long term follow-up after CAR-T to assess true long term PFS. If CAR-T results in 30% or higher long term disease free survival, in light of lower NRM, it will likely be considered superior to allo-HCT in these patients.

Follicular Lymphoma

Follicular lymphoma (FL) is the second-most common NHL and the most common indolent lymphoma. Patients have a variable course, but generally the disease is considered incurable and many patients receive multiple lines of therapy during the course of their disease (91). While not all patients require upfront therapy, those with early treatment failure (disease progression within 24 months of chemoimmunotherapy or 12 months of rituximab monotherapy) have worse outcomes with standard therapy, and warrant more aggressive treatment (92). In the absence of transformed disease and in those who are eligible for transplantation, many will undergo salvage chemoimmunotherapy with the intent to undergo high-dose chemotherapy with ASCT if a CR is achieved. In retrospective studies, ASCT has been associated with prolonged survival compared to salvage chemo-immunotherapy alone (93). Matched-sibling donor allo-HCT provides comparable long-term survival to ASCT, with a significantly lower-risk of relapse but higher upfront NRM (94). Of those patients that survive beyond 24 months, survival was shown to be superior in those who received allo-HCT. In general, nonmyeloablative and RIC regimens are used, some incorporating immunotherapy or radioimmunotherapy (40, 95). Long term PFS may be as high as 70-80% in highly-selected patients. Anecdotally, however, the increased arsenal of novel and investigational therapies for FL, including CAR T cells, is decreasing the use and utility of allo-HCT.

Small prospective trials of CD19 CAR T cells demonstrate the promise of CAR T therapy among patients with relapsed/refractory FL, prompting ongoing larger clinical trials. Among patients treated in the initial prospective case series study of CTL019/tisagenlecleucel out of the University of Pennsylvania, 14 patients who received treatment had advanced FL, 8 of whom had double-refractory disease, three who had undergone prior ASCT and one with a prior allo-HCT (81). 10 of 14 (71%) of patients had a CR at 6 months and remained in remission at a median of 29.3 months. 70% were progression-free at 28.6 month, and 89% who responded maintained the response by the median follow-up period. Severe CRS occurred in five patients (18%) and severe ICANS occurred in three (11%), one case being fatal.

Hirayama and colleagues from the Fred Hutchison Cancer Institute included 8 patients with multiply-relapsed FL (3 with a prior ASCT and 1 with a prior allo-HCT) in their phase I/II study of CD4:CD8 ratio-defined CD19 CAR T (96). Seven (88%) achieved a CR, all of whom remained in remission and one of whom proceeded to allo-HCT. The eighth patient had SD and subsequently underwent radiation therapy with no progression at 36 months. A notable criticism was the high dose of cyclophosphamide that patients received as part of lymphodepletion (97). Importantly, while CRS and ICANS occurred in 50% of patients, no severe adverse events were reported. Axi-cel and tisagenlecleucel are actively being studied in multicenter phase II trials in FL in the ZUMA-5 and ELARA studies, respectively (42). Results from the interim analysis of ZUMA-5 reported an ORR of 95% and CR rate of 80% among 80 patients with FL. With a median follow-up of 11.5 months, 68% of patients had ongoing responses. CRS and ICANS occurred in 11% and 19% of patients (43). While the high response rates and manageable toxicities are promising in multiply recurrent FL, the length of follow-up in these trials is limited. It is therefore unknown whether CAR T therapy will compare favorably or unfavorably with allo-HCT with RIC. Although the novelty of CAR T therapy may lead physicians to lean toward it, this is an area that deserves long term analysis as CAR T should provide durable PFS of 70% or better in order to be a competitive substitute for alloHCT.

CAR T Therapy and the Waning Role of Allo-HCT in NHL

As the clinical trial data for CAR T therapy in NHL mount, the role of allo-HCT becomes more questionable. In large part, the toxicities related to CD19 CAR T therapies are acute, limited in severity, and manageable, which makes them more appealing compared to the potentially long-lasting infectious and GVHD complications seen in allo-HCT.

Unlike B-ALL, current evidence does not support consolidative allo-HCT for NHL patients responding to CAR T (98). Additionally, as responses may be delayed and evolve over a prolonged duration, active observation is generally recommended even in patients with a PR or SD post-CAR T. Patients with SD, however, are less likely to achieve a subsequent remission. Therefore, individualized consideration may be given to allo-HCT prior to progression based on the extent of disease, donor availability, and other patient-specific factors. While loss of B-cell aplasia may trigger pursuit of allo-HCT in B-ALL, it has not been associated with disease recurrence in DLBCL, and therefore should not be considered a decision point for transplant (68).

Whether allo-HCT has a role following NHL progression after CAR T therapy is also a point of controversy. While theoretically it would be the principal option that could lead to

a durable remission, in practicality it is difficult to achieve a remission pre-transplant in such patients that would justify pursuit of allo-HCT. Additionally, allogeneic transplantation likely eradicates the CAR T cells, which could otherwise potentially be stimulated through a variety of investigational methods in order to attempt to attain a response. Lenalidomide, PD-1 inhibitors, and the bispecific CD3-CD20 monoclonal antibody mosunetuzumab have all demonstrated the potential to recapture a response in patients who progressed after CAR T therapy (99–103). Therefore, pursuit of a clinical trial or off-label use of such agents may be preferred over or should be considered before proceeding with allo-HCT based on the respective risk-to-benefit ratios in such heavily pre-treated patients. As it pertains specifically to bispecific antibodies in NHL, their ease of use and promise of efficacy positions them competitively with CAR T therapy, highlighting evolving dilemmas of patient selection and sequencing of novel immunotherapies.

There is limited experience with CAR T after allo-HCT in NHL as such patients were excluded from larger clinical trials. However, a number of small reports demonstrate that it is safe and feasible to construct donor-derived CAR Ts or pseudo-donor-derived CAR Ts (104–107). In such studies, severe and active graft-versus-host disease (GVHD) was a key exclusion criterion and, while GVHD developed or worsened in a few patients, the severity was mild. Further study is needed in larger homogenous populations to determine if the safety and efficacy of donor-derived CAR T therapy is comparable.

In summary, whereas CD19 CAR T arguably has a complementary role in bridging to allo-HCT in the B-ALL algorithm, it may supplant allo-HCT in most patients with relapsed NHL based on favorable toxicity and at least comparable efficacy. Longer follow-up is needed in most of the NHL CAR T trials in order to confirm this implication.

Multiple Myeloma

While therapy for multiple myeloma (MM) has dramatically improved over the past two decades, it is generally considered incurable and most patients will die of their disease (108). During the 1990s and early 2000s, during which time novel therapies (i.e., proteasome inhibitors, immunomodulatory drugs, monoclonal antibodies) were in clinical development, allo-HCT was studied in the treatment of MM in several fashions (12). Several studies evaluated ASCT followed by RIC allo-HCT compared to tandem ASCT (109–112). Two meta-analyses of such studies yielded no differences in OS but a significantly higher risk of NRM (113, 114). Allo-HCT as a salvage therapy after relapse has been shown to provide a PFS benefit without OS benefit for a small percentage of patients, as reported in a number of retrospective series and registry studies, and outcomes have been comparable or worse than salvage ASCT in selected patients (12, 115, 116). Therefore, consensus guidelines recommend the use of allo-HCT in these settings only in the context of well-designed clinical trials (117, 118). Interestingly, there are a number of clinical trials ongoing combining allo-HCT with novel therapies as consolidation and maintenance, which may shift the paradigm at a later date. However, they are contending with the ongoing development of CAR T therapy for MM.

B-cell maturation antigen (BCMA) is a cell-surface antigen found on some mature B-cells and normal plasma cells, malignant plasma cells, and importantly not expressed on hematopoeitic stem cells, non B and plasma cell hematopoietic lineages or non-hematopoietic tissue. The first-in-human trial of a BCMA CAR T therapy with a CD28 costimulatory domain demonstrated promising anti-myeloma efficacy (119). The two patients receiving the highest dose level of 9×10^6 CAR T cells/kg body weight had at least a very good partial response, although both had severe CRS and prolonged cytopenias.

In a single-center, phase I, dose-finding clinical trial of a 4-1BB BCMA CAR T therapy, the overall response rate was 12 (48%) of 25 heavily pre-treated MM patients (120). Median duration of response was 124.5 days and 3 patients had durable responses at the time of reporting. Eight (32%) had grade 3+ CRS, one who died of candidemia following prolonged therapy for CRS, and three (12%) had grade 3+ ICANS.

The safety and preliminary efficacy of the BCMA CAR T using 4-1BB costimulatory endodomain, bb2121/idecabtagene vicleucel (ide-cel), was studied in a multicenter phase I trial (121). The ORR among 33 patients was 85%, with a 45% CR/sCR rate and a median PFS of 11.8 months. The follow-up pivotal phase II KarMMa trial of ide-cel yielded a 73% ORR and 31% CR/sCR rate with a median PFS and duration of response of 8.6 and 10.6 months, respectively, and low rate of grade 3+ CRS (5%) and ICANS (3%) (122). Based on these data, regulatory approval for ide-cel in refractory MM patients who have failed at least three independent lines of therapy is actively being pursued.

The LCAR-B38M and JNJ-4528 CAR T therapies are identical constructs comprised of a 4-1BB costimulatory endodomain and two BCMA-targeting single-domain antibodies targeting distinct BCMA epitopes. In the Chinese phase I LEGEND-2 study of LCAR-B38M, 57 patients were infused with 3 split infusions (123). Seven percent had grade 3 CRS, only one patient had ICANS. The ORR was 88%, with a 74% CR rate. Of those with CR, 39/42 were MRD-negative. The 18-month OS was 68%, with a median duration of response of 22 months, 27 months in those with CR. Another smaller trial of the LCAR-B38M products examined 17 patients with high risk features (i.e., extramedullary disease, poor cytogenetics, triple-class refractoriness). While initial responses were promising (88% ORR), factors such as extramedullary disease and the development of anti-CAR T antibodies were associated with relapse, and the 12-month PFS was only 53% (124). The phase Ib/II CARTITUDE-1 study in the United States of JNJ-4528 is ongoing with a 100% ORR and 76% sCR in the first 29 patients and similarly tolerable safety profile, albeit one delayed death from sequelae of grade 4 CRS (47, 48).

The BCMA CAR T platforms are the best positioned to break into clinical practice in the near future. Other CAR targets such as CS-1, immunoglobulin kappa light chain, and CD138 are being explored (11, 125, 126). Additionally, ongoing clinical trials are focused on the appropriate sequencing of BCMA CAR T, specifically addressing whether it should be deployed

earlier in patients who experience a suboptimal response from induction therapy and ASCT or frontline for those with high-risk features. Follow-up for BCMA CAR T-treated patients within these trials is still maturing, so it remains unclear whether a durable remission, as seen in some B-ALL and NHL patients, can be expected. The historically inconsistent survival and NRM outcomes in allo-HCT, combined with the substantial treatment burden experienced by the typical MM patient, suggest that this practice will likely be replaced by CAR T therapy, should it deliver on its promise of high responses and some durable remissions. It is unlikely that CAR T will be used as a bridge to allo-HCT, and none of the trials have reported such a practice in any participant.

THE EXPANDING FRONTIER OF CAR T AND ALLO-HCT

CAR T and Allo-HCT in Myeloid Malignancies: An Inseparable Fate

The most common indications for allo-HCT in adults are acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) (127). Despite the toxicities associated with the transplant itself, relapse remains the most significant cause of treatment failure and death after allo-HCT, highlighting the need for further disease-modifying innovation without added toxicity (128–130). Unlike B-cell malignancies, to date most CAR target antigens for myeloid malignancies have significant overlap with normal myeloid cells and hematopoietic stem/progenitor cells (HSPCs), the eradication of which would likely be poorly tolerated (131). Some of these antigens have been targeted in early clinical trials of CAR T cells with variable toxicity and success thus far, reviewed in detail in Mardiana and Gill (36).

CD123 is one such antigen already being targeted by other investigational immunotherapies (132). Preclinical work suggests that it is a viable CAR target for AML, although it could lead to myeloablation requiring allo-HCT rescue. It is also expressed on vascular endothelium, heightening the risk for toxicity such as capillary leak syndrome (133–135). One risk mitigation strategy has been the production of transient CAR T cells infused serially, a concept that was safe and feasible in a pilot study, but which was discontinued during phase I (136). An ongoing study at the City of Hope Medical Center incorporates a truncated epidermal growth factor receptor (EGFRt) into second-generation CAR T cells, allowing for inactivation with EGFR monoclonal antibodies (37). Early clinical activity has been reported, allowing two of six AML patients to proceed to a second allo-HCT after achieving CRs. Unexpectedly, myeloablation by the CD123 CAR T was not observed as hypothesized, although the intent was to bridge to allo-HCT. A "compound" CAR T cell, with two complete CAR constructs targeting those two antigens connected by a cleavable linker, was generated by Liu et al. and demonstrated promising efficacy in a phase I dose-escalation, with seven of nine participants achieving MRD-negative CR (38). Notably, given the overlap of these targets with normal hematopoietic stem cells, all experienced Grade IV pancytopenia, and six of seven responders went on to alloHCT.

As antigen overlap remains a significant challenge and the immediately perceived role of AML CAR T therapy is as a bridge to allo-HCT, one novel concept is that of genetically engineering an allograft to remove the target antigen from the normal hematopoietic system, and transplanting this allograft in sequence with donor-derived CAR T cells against the specific antigen (131). Kim et al. pioneered the preclinical work in murine and non-human primate models in which they first demonstrated that knock-out of CD33 in the donor hematopoietic stem and progenitor cell population resulted in normal hematopoiesis and myeloid function and normal multilineage engraftment (38). Subsequent administration of CART33 targeting CD33 was able to effectively eliminate CD33+ leukemia without notable off-target effects. Immunotherapeutic targeting of CD33 is already an approved AML therapy (gemtuzumab ozogamicin) with toxicity relating predominantly to the chemotherapy payload (137). The question remains as to how post-transplant immunosuppression will impact the persistence and efficacy of the donor-derived CAR T cells in this platform. Although it has yet to be clinically developed, it is hoped that the concept of combining CAR T therapy with genetically engineered allo-HCT will lead to a synergistic effect on AML with limited added toxicities.

Other approaches to improve the specificity of T-cell therapies for AML are in preclinical and clinical development, including dual-targeting CAR T cells that identify surface antigen combinations that are unique to leukemic blasts as well as T-cell receptor engineered (TCR) T cells that allow for the recognition of intracellular proteins specific to AML blasts (138–140). The existing perspective, however, is that the clinical advances to come from AML cellular therapy will likely need to be combined with allo-HCT in order to achieve the best outcomes. As more is learned about these complementary platforms, lessons from each are likely to benefit one another.

Allogeneic "Off-the-Shelf" CAR T Therapy

Numerous challenges to the widespread implementation of autologous CAR T therapy have been described (141). The products are generated from a patient's autologous T cells, which requires extensive and costly collection and manufacturing efforts. This process is time-intensive, and during the intervening period some patients have difficulty with disease control or complications from bridging chemotherapy. Additionally, the extensive pretreatment brings into question the potency and exhaustion of the cellular therapy.

Due to these limitations, numerous institutions and companies are actively developing "universal off-the-shelf" CAR T products derived from allogeneic sources (UCART), which overcome some of these hurdles although introduce new ones. Principally, alloreactivity can lead to rejection of the UCART mediated by the recipient T and NK cells, and alloreactivity from the UCART can lead to GVHD (142). Numerous studies of graft rejection and GVHD in the context of UCART have demonstrated the role of the T cell receptor (TCR) in recognizing non-self major histocompatibility complex (MHC) molecules and/or MHC molecules complexed with peptides, conferring alloreactivity (143–145). As such, the

fundamental understanding of both of these concepts, and methods to mitigate them, are derived from decades of study and observation in allo-HCT.

A unique clinical development in allo-HCT that has translated to preclinical work in the UCART space involves the isolation and therapeutic exploitation of virus-specific T cells that have a limited TCR repertoire. Initially, such allogeneic virus-specific T cells were used in allo-HCT recipients to treat and prevent severe viral infections (146–148). Despite HLA mismatches between the cellular therapy and patients, *de novo* GVHD did not occur with any significant frequency. Therefore, such virus-specific T-cells are currently being bioengineered to harbor CARs for CD19 and other targets (149, 150).

With knowledge of the TCR as the main mediator of both rejection and GVHD, disruption of the TCR through one of a number of gene editing techniques has become the predominate means of preventing GVHD by UCART. In the initial preclinical work, Torikai et al. (151) demonstrated the feasibility of knocking out the gene for the T cell receptor constant α chain (*TRAC*) using zinc finger technology in CD19 CAR T cells, without impairment of their antitumor activity (151). Subsequent methods have employed transcription activator-like effector nuclease (TALEN) technology to develop UCART products, knocking out not only the TCR but also CD52 in the products, allowing for alemtuzumab-based extended lymphodepletion in order to enhance UCART engraftment and persistence and to mitigate UCART rejection without impacting the anti-tumor efficacy of the UCART product itself. Clinical trials of UCART are ongoing in multiple hematologic malignancies (142). The advent of CRISPR/Cas9 technology has allowed for both precision knockout of *TRAC* as well as T cell-specific antigens (e.g., CD7), allowing for possible deployment in T cell ALL without the risk of fratricide (152). Another novel approach that allows for efficient production of UCART products involves adeno-associated virus (AAV)-mediated transduction of the CAR transgene into the *TRAC* locus. This process exploits a site-specific endonuclease and homology-directed repair to simultaneously knock out the native TCR and allows for the CAR to be expressed under the usual transcriptional control of *TRAC* (153, 154). Many of these UCART technologies are in clinical development and, if successful, are poised to make CAR T therapy more accessible and affordable. How they will impact the landscape of CAR T and allo-HCT remains to be seen.

DISCUSSION AND CONCLUSIONS

Allogeneic hematopoietic cell transplantation and chimeric antigen receptor T cell therapy are the two principal cellular therapies that have widely permeated the clinical space outside of clinical trials, and remain the focus of many ongoing investigations. They span the spectrum of target specificity which, in part, predicts their efficacy and toxicity. Whether the two modalities complement or compete with one another depends substantially on the disease and the patient and requires a nuanced and individualized approach. For many histologic subtypes of NHL and for relapsed refractory MM, CAR T therapy appears to provide comparable or improved outcomes to allo-HCT with potentially less long-term complications and a chance of durable remissions as a destination therapy. However, allo-HCT already had very niched indications within these diseases secondary to substantial improvements in novel therapies, so likely there will continue to be a role for allo-HCT in select patients, albeit diminished. In B-ALL much of the evidence supports CAR T therapy as a complement serving as a bridge to allo-HCT, especially in adults. However, some patients, especially pediatric patients, may enjoy sustainable remissions with CAR T alone, with active observation for loss of CAR T persistence replacing the immediate need to proceed to transplant while in remission. Should CAR T therapy become a viable treatment option for myeloid malignancies, based on current research there is a high probability that it will be used in conjunction with allo-HCT due to the antigen overlap between malignant myeloid cells and non-malignant hematopoietic stem and progenitor cells. Technologies used to build newer CAR T may be able to simultaneously modify the allografts to limit off-target effects.

The pace of innovation in the adoptive immunotherapy space is accelerating, sparked by the success of both platforms; allo-HCT and CAR T. The ongoing research in both fields is routinely translated to one another and to other forms of investigational cellular therapies, providing strategies to manage complications, such as CRS, and increase accessibility with the prospect of UCART therapy. As the technologies evolve and new therapies emerge, the challenge will continue to be in synthesizing the data in reference to the specific disease and performance status of each patient in order to provide better and more tailored treatment for each individual.

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SG wrote the manuscript. AG and JD reviewed and edited the manuscript in detail. All authors contributed to the article and approved the submitted version.

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Elevated Red Blood Cell Distribution Width as a Poor Prognostic Factor in Patients With Hematopoietic Stem Cell Transplantation

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Red cell distribution width (RDW), a measure of erythrocyte size variability, has been recently reported as an effective prognostic factor in critical illness. Hematopoietic stem cell transplantation (HSCT) has become the first choice of most patients with hematological malignancies. The aim of this study was to assess the changes of RDW in patients with HSCT and analyze the relationship between RDW and HSCT. In this study, we retrospectively enrolled 114 hematopoietic stem cell transplant patients during the period from 2015 to 2019. Logistic regression and Kaplan–Meier survival analysis were used for retrospective analysis. Multivariate analysis suggested that patients with elevated RDW (>14.5%) at three months post-transplantation have a poor clinical outcome compared with those with normal RDW ≤14.5% [odds ratio (OR) 5.12; P = 0.002]. Kaplan–Meier method analysis demonstrated that patients with elevated RDW levels (>14.5%) after hematopoietic stem cell transplantation experienced shorter progression-free survival compared to those with normal RDW levels (P = 0.008). Our study demonstrated that RDW could be an easily available and potential predictive biomarker for risk stratification in patients with HSCT. Further prospective studies are determined to confirm the prognostic value of RDW in HSCT patients.

Keywords: red blood cell distribution width, hematopoietic stem cell transplantation, prognosis, biomarker, outcome, risk factor

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) refers to a well-recognized promising procedure that treats malignant hematological diseases such as leukemia and lymphoma and restores bone marrow function in cancer patients with dysfunctional hematopoiesis, such as aplastic anemia (1). Approximately 23,000 transplants were performed each year in the United States, some of them were preceded by conditioning regimens for decreasing malignant tumor burden (2). Despite some improvements in transplantation strategies and supportive cares in recent years, transplantation still

carries a significant risk for treatment-related mortality, chemotherapy-induced toxic effects, early post-transplantation complications, and even graft-*versus*-host disease (GVHD), eventually contributing to the transplant failure (3). For these reasons, there is an urgent need for novel, more effective biomarkers that can provide the opportunity for HSCT patients to receive risk-adapted therapies to improve their outcomes.

Red cell distribution width (RDW), routinely assessed as a component of complete blood count (CBC), is a quantitative index of variability for measuring the size of peripheral blood erythrocytes with higher values showing greater homogenous sizes (4). RDW is mainly used to reflect impaired erythropoiesis and abnormal red blood cell survival but correlates also with inflammation, impaired renal function, and different types of anemia, especially identifying anemia with folate and iron deficiency (5–7). Recent cumulative evidence indicates that elevated RDW was reported to be an important prognostic biomarker for increased morbidity and mortality in patients with cardiovascular diseases and chronic kidney diseases, hepatocellular carcinoma, and rheumatoid arthritis (8–11). Although RDW appears to be a powerful and independent predictor of illness severity and clinical prognosis, the mechanism for the association between RDW and outcomes remains poorly understood. It should be noted that many patients with hematopoietic stem cell transplantation are faced with several challenging risks, such as immune activation, nutritional deficiencies, impaired iron, and inadequate production of erythropoietin (EPO), and these risks may impact RDW, finally influencing the post-transplant reconstruction of the hematopoietic system (5, 12). To address this issue, we retrospectively analyzed a cohort of hematopoietic stem cell transplantation recipients with available information about RDW levels and investigated the clinical significance of RDW increment after transplantation. Moreover, the clinical outcomes were analyzed to determine if there was an association between elevated RDW and long-term prognosis.

MATERIALS AND METHODS

Study Setting and Patients Selection

After receiving approval from our institutional review board, we reviewed the electronic medical records in our retrospectively maintained database of patients with hematologic malignancies who had undergone hematopoietic stem cell transplantation from January 2015 to December 2019 in our institution and two hospital branches in Chongqing, Southwest China. The pre-operative blood cell count from the peripheral blood was available for each patient. We excluded the patients with several conditions: (i) without available data regarding RDW at transplant, (ii) those who had acute infections or chronic active inflammatory diseases, (iii) underwent blood transfusion after post-transplantation, and/or (iv) insufficient clinical and follow-up data. Finally, 114 patients were eligible for this study (**Figure 1**).

Clinical and Laboratory Parameters

Venous blood was collected from each patient at least on admission prior to transplantation and three months after transplantation, respectively. All samples were placed in potassium ethylenediaminetetraacetic acid (EDTA-K2) anticoagulation tubes. All measurements were analyzed using XN1000 Hematology Analyzer (*Sysmex, Japan*) in which white blood cell count (WBC), hemoglobin (Hb) concentration, platelet count (PLT), mean red blood cell volume (MCV), RDW, and absolute neutrophil and lymphocyte counts were obtained directly from the blood analyzer, while albumin (Alb), alanine aminotransferase (ALT), and creatinine (CREA) were collected directly from the biochemical system database. The normal range for RDW in our hospital is defined as 11.5–14.5%.

Potential Risk Factors

We defined a "high" RDW level when the level was >14.5%. As it shown in **Table 1**, patients were divided into two groups according to their RDW levels at three months after transplantation. Two groups were compared using several indices as potential risk factors: (i) demographics (sex and age); (ii) underlying conditions or comorbidities (hypertension, diabetes mellitus, gastrointestinal or hepatic diseases, cardiovascular diseases, and renal diseases); (iii) laboratory data [RDW, red and white blood cells (RBC and WBC, respectively) PLT, hemoglobin, mean corpuscular volume (MCV), neutrophils, lymphocytes, and pre-transplantation RDW]; (iv) transplantation related data [chemotherapy times, autologous HSC transplant, human leukocyte antigens (HLA) full matched]; (v) clinical symptoms after transplantation (sepsis, electrolyte disturbance, GVHD, hemorrhagic cystitis, hepatic and renal dysfunction, mucosal herpes, respiratory tract and urinary tract infections, digestive system diseases, hypoproteinemia); and (vi) bone marrow reconstruction (13).

Definition

The following terms were defined prior to data analysis: pre-transplant RDW was defined as the RDW value of the patient's blood routine at the time of admission diagnosis for transplant. Post-transplant RDW referred to the RDW value of the patient's blood routine 3 months after HSCT. Smoking history includes patients who are smoking and those who have quit. Drinking history includes patients who are drinking alcohol and those who have stopped drinking alcohol. Sepsis was defined as a life-threatening organ dysfunction caused by host-related inflammatory response to an infection. Organic dysfunction was defined in practice as an increase in the Sequential Organ Failure Assessment (SOFA) score of at least two points from a patient's baseline (14, 15) Graft-*versus*-host disease (GVHD) might occur after allogeneic hematopoietic stem-cell transplantation, and it was regarded as an immune response mounted against the recipient of an allograft by mature donor $\alpha\beta$ T cells contained in the graft (16). Hemorrhagic cystitis was a relatively common and potentially severe complication of high-dose chemoradiotherapy, especially in conjunction with hematopoietic stem cell transplantation (17).

Statistical Analysis

All analysis was performed using SPSS version 23.0 software. Patients were divided into two groups: (i) increased RDW levels (>14.5%) and (ii) normal RDW levels (≤14.5%). Continuous variables were present as means and standard deviations and

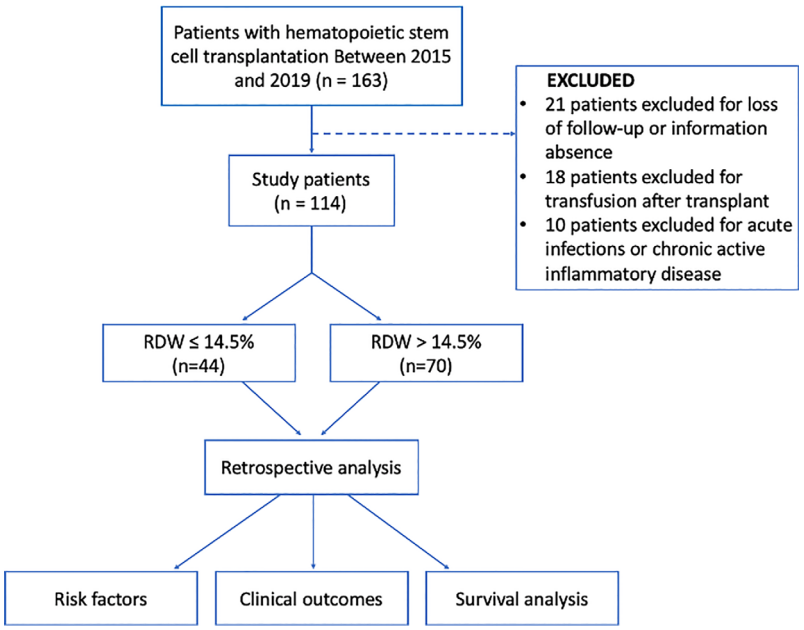

FIGURE 1 | Design of this study.

were compared using independent sample *t*-tests. Categorical variables, of which the parameters were analyzed using x^2 tests, were presented as frequencies and percentages. Logistic regression was used to investigate the relation between the clinical outcome and laboratory or clinical data. Univariate analyses were performed separately for each of variables. Variables with P <0.10 in the univariate were included in the logistic regression model for multivariate analysis. For survival analysis, the Kaplan–Meier method was used. Log-rank test was used to estimate the statistical significance between two groups. Progression-free survival (PFS) was defined as the period from stem cell transplantation to the earliest progression of disease or death.

Ethical Considerations

The data and the samples that were analyzed in the present study were obtained in accordance with the standards and approval of the Chongqing Medical University Institutional Review Board and Biomedical Ethics Committee. The ethics committee waived the need for written informed consent provided by participants due to the retrospective nature of the study. Because all patient data were analyzed in anonymity, no additional informed consent was required.

RESULTS

Patient Characteristics

The main baseline characteristics of the 114 patients studied are listed in **Table 1**. The median age was 32 years (range = 13–68 years). Most of the enrolled patients were male (70;61.4%). According to exclusion criteria, a total of 70 patients (61.4%) with a higher RDW level (>14.5%) were included in this study. A total of 44 patients with a normal RDW level (≤14.5%) were used as a control group. Additionally, the mean RDW level at three months post-transplantation was 15.2 ± 2.29%.

As shown in **Table 1**, no significant difference between the two groups in the distribution of gender, age, and complications at admission was found. Compared to patients with normal RDW levels, patients with elevated RDW levels (>14.5%) generally had unfavorable laboratory results, including significantly lower levels of RBC (P = 0.002), PLT (P = 0.001), Hb (P <0.001), and Alb (P<0.001) in addition to significantly higher levels of MCV (P = 0.049) and LDH (P = 0.003). In addition, patients with high RDW levels (>14.5%) had a higher proportion of autologous stem cell transplantation (P = 0.033) and liver dysfunction after transplantation (P = 0.023), but had a lower frequency of hemorrhagic cystitis (P = 0.016) and mucosal herpes (P = 0.019) compared with the patients having normal RDW levels.

Survival Analysis

During the median follow-up 16.5 (3–47) months period, there were 27 cases with progression or recurrence after transplantation treatment and seven deaths occurred. We defined relapse or death as a termination event. As shown in **Table 2**, univariate logistic analysis was performed on related variables to explore risk factors that may affect poor prognosis of patients with hematopoietic stem cell transplantation. RDW levels of >14.5% [odds ratio (OR) of 1.31 and 95% confidence interval (CI) 1.07–1.61; P = 0.009], WBC levels (OR 0.77; 95% CI, 0.58–1.02; P = 0.064), PLT levels (OR 0.99; 95% CI, 0.98–1.00; P = 0.004), respiratory tract infection (OR 2.77; 95% CI, 0.94–8.14; P = 0.064), and hemorrhagic cystitis after transplantation (OR 0.16; 95% CI, 0.02–1.24; P = 0.080) were proven to be potential risk factors. In the multivariate analysis

TABLE 1 | Baseline characteristics of study population divided by red cell distribution width (RDW) levels.

Variables	RDW ≤ 14.5%	RDW > 14.5%	P value
Total	**44**	**70**	
Sex (male)	30 (68.18%)	41 (58.57%)	0.328
Age (average)	32.4 ± 12.29	36.5 ± 14.38	0.168
Smoking	9 (20.45%)	23 (32.86%)	0.200
Drinking	15 (34.09%)	20 (28.57%)	0.540
Comorbidity			
Hypertension	1 (2.27%)	6 (8.57%)	0.246
Diabetes mellitus	2 (4.55%)	2 (2.86%)	0.642
Gastro/hepatic	10 (22.73%)	16 (22.86%)	1.000
Cardiovascular	2 (4.55%)	7 (10.00%)	0.479
Renal	2 (4.55%)	4 (5.71%)	1.000
Laboratory Data			
pre-transplantation RDW	13.89 ± 2.26%	15.69 ± 2.62%	**0.049**
RBC	4.01 ± 1.14	3.51 ± 1.64	**0.002**
WBC	5.03 ± 1.64	4.7 ± 1.57	0.479
PLT	149 ± 62	109 ± 55	**0.001**
HB	121 ± 18	104 ± 24	**<0.001**
MCV	93.4 ± 8.1	96.5 ± 7.8	**0.049**
Neutrophil	2.89 ± 1.64	2.42 ± 1.11	0.216
Lymphocyte	1.58 ± 0.70	1.68 ± 1.02	0.780
Alb	43.91 ± 3.62	39.77 ± 5.29	**<0.001**
ALT	44.36 ± 83.45	39 ± 49.16	0.292
LDH	234.52 ± 126.76	361.93 ± 304	**0.003**
CREA	82.34 ± 64.72	74.19 ± 24.71	0.679
Chemotherapy (times, ≥5)	10 (22.73%)	25 (35.71%)	0.105
Autologous HSC Transplantation	12 (27.27%)	32 (45.71%)	**0.033**
HLA Full Matched	31 (70.45%)	38 (54.29%)	0.115
Clinical symptoms			
Sepsis	5 (11.36%)	5 (7.14%)	0.505
Electrolyte Disturbance	8 (18.18%)	19 (27.14%)	0.366
GVHD	7 (15.91%)	9 (12.86%)	0.783
Hemorrhagic Cystitis	10 (22.73%)	4 (5.71%)	**0.016**
Hepatic Dysfunction	5 (11.36%)	21 (30.00%)	**0.023**
Renal Dysfunction	3 (6.82%)	3 (4.29%)	0.675
Mucosal Herpes	18 (40.91%)	14 (20,00%)	**0.019**
Respiratory tract infection	3 (6.82%)	13 (18.57%)	0.100
Digestive system diseases	4 (9.09%)	11 (15.71%)	0.399
hypoproteinemia	2 (4.55%)	5 (7.14%)	0.149
Urinary tract infection	1 (2.27%)	7 (10.00%)	0.705
Reconstruction			
Myeloid	12.14 ± 3.63	11.52 ± 3.58	0.167
Megakaryocyte	16.10 ± 7.75	14.42 ± 6.28	0.322

RDW, red cell distribution width; RBC, red blood cell; WBC, white blood cell; PLT, platelets; Hb, hemoglobin; MCV, mean corpuscular volume; Alb, albumin; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CREA, creatinine; HLA, human leukocyte antigen; GVHD, graft versus host disease. Data are presented as either mean ± standard deviation, median (interquartile range), or proportions, and compared using t-, log-rank, and chi-square tests, respectively. Statistically significant p-values are shown in bold font.

(**Table 3**), elevated RDW levels (>14.5%) was demonstrated as an independent risk factors which may predict poorer prognosis for patients with hematopoietic stem cell transplantation (OR 5.12; 95% CI, 1.83–14.32; P = 0.002).

Survival Curve

We used the Kaplan–Meier method to investigate the difference between the increased RDW levels (>14.5%) and normal RDW levels (≤14.5%) groups for PFS. As shown in **Figure 2**, patients with elevated RDW levels (>14.5%) after hematopoietic stem cell transplantation experienced shorter PFS compared to those without RDW levels. By using a log-rank test, it has been proven that elevated RDW levels (>14.5%) at three months post-transplantation was an independent prognostic factor for PFS (P = 0.008).

DISCUSSION

The present study aimed to clarify the prognostic value of baseline RDW in patients with hematopoietic stem cell transplantation. Our results demonstrated that elevated RDW levels was an independent predictor of disease progression or death after hematopoietic stem cell transplantation. Moreover, we provided the first evidence that patients with elevated RDW levels (>14.5%) after hematopoietic stem cell transplantation experienced shorter PFS compared to those with normal RDW levels. To our knowledge, this is the first report addressing the prognostic value of RDW in patients with hematopoietic stem cell transplantation.

Currently, hematopoietic stem cell transplantation is the only cure for acute leukemia, but leukemia relapse after transplantation is

TABLE 2 | Univariate analysis for progression-free survival.

Risk factor	OR (95% CI)	P- value
Male sex	0.81 (0.36–1.85)	0.62
Renal	2.48 (0.48–2.98)	0.281
pre-transplantation RDW	1.07 (0.71–1.27)	0.4
RDW	**1.31 (1.07–1.61)**	**0.009**
RBC	0.83 (0.53–1.28)	0.392
WBC	**0.77 (0.58–1.02)**	**0.064**
PLT	**0.99 (0.98–1.00)**	**0.004**
HB	0.99 (0.98–1.01)	0.479
MCV	1.01 (0.96–1.06)	0.704
Alb	0.96 (0.90–1.06)	0.525
LDH	1.00 (0.99–1.01)	0.491
Autologous HSC Transplantation	1.17 (0.51–2.65)	0.712
Sepsis	1.01 (0.25–4.16)	0.990
GVHD	1.08 (0.34–3.39)	0.893
hypoproteinemia	2 (4.55%)	5(7.14%)
Hemorrhagic Cystitis	**0.16 (0.02–1.24)**	**0.080**
Hepatic Dysfunction	1.33 (0.53–3.39)	0.544
Renal Dysfunction	0.46 (0.05–4.04)	0.48
Respiratory tract infection	**2.77 (0.94–8.14)**	**0.064**
Urinary tract infection	0.32 (0.04–2.67)	0.290

Factors related to the increased RDW (>14.9%) at post- transplantation 3 months. OR, odds ratio; CI, confidence interval; RDW, red cell distribution width; RBC, red blood cell; WBC, white blood cell; PLT, platelets; Hb, hemoglobin; MCV, mean corpuscular volume; Alb, albumin; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; GVHD, graft versus host disease. Statistically significant p-values are shown in bold.

TABLE 3 | Multivariate analysis for progression-free survival.

Variables	95% CI	OR	P-value
RDW	1.83–14.32	5.12	**0.002**
WBC	0.26–3.36	0.93	0.912
PLT	0.95–14.40	3.71	0.059
Hemorrhagic cystitis	0.02–1.31	0.15	0.086
Respiratory tract infection	0.67–7.79	2.29	0.185

RDW, red cell distribution width; WBC, white blood cell; PLT, platelets. Statistically significant p-values are shown in bold.

considered the biggest obstacle blocking the effects of transplantation, but the exact molecular mechanism of this relapse is not fully understood. Cumulative evidence indicates that several factors, such as leukemia cell tolerance to chemoradiotherapy, relapses of related gene mutations, and epigenetic abnormalities, could be associated with leukemia relapse (18). Despite new advances in transplantation strategies and supportive care, the efficacy of patients with relapse after hematopoietic stem cell transplantation is still poor. Therefore, effective monitoring and early intervention are especially important for reducing relapse rate and improving survival rate of patients with relapse after transplantation. For these reasons, an urgent need for novel, more effective biomarkers that can provide the opportunity to receive risk-adapted therapies to improve the outcome of HSCT patients exists.

RDW has been used for the differential diagnosis of anemia for decades. However, in recent years, numerous studies have found RDW to be a simple, robust, and convenient parameter associated with different human diseases. Initially, elevated RDW values were reported prognostic factors that were associated with cardiovascular mortality (19, 20). Some other studies have emphasized that elevated RDW levels can be used as an independent risk factor for

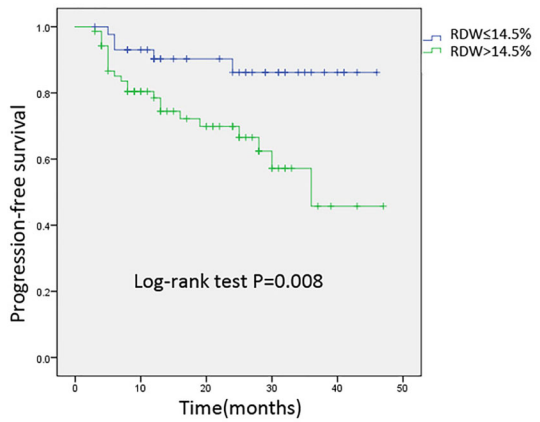

FIGURE 2 | Kaplan-Meier analysis of progression-free survival for 114 patients stratified by 3-months post-transplantation RDW levels. Cumulative survival curve of the study population; y-axis indicated the cumulative survival, and x-axis indicated the months from transplantation. The green line indicates the cumulative survival of increased red cell distribution width (RDW) (>14.5%) level at three months post-transplantation; the blue line indicates the cumulative survival of others with non-elevated RDW (≤14.5%).

poor prognosis in the hematological malignancies (21, 22). Similarly, a recent study by Yang and colleagues reported that RDW was observed to increase in colorectal cancer patients, and RDW was significantly different at each stage of colorectal cancer (23). In this present study, we focused on the prognostic value of RDW in the patients who underwent hematopoietic stem cell transplantation. Our results showed that patients with high RDW levels were more likely to have liver dysfunction after transplantation but had a lower frequency of hemorrhagic cystitis and mucosal herpes. Moreover, another important finding is the significant association between RDW and poor prognosis, specifically in hematological malignancy patients, showing RDW as a novel and powerful prognostic factor for HSCT patients.

Although the exact mechanism by which increased RDW is linked to poor prognosis for patients with HSCT is not clear, multiple factors could contribute to this association. First, elevated RDW levels may indicate impaired medullary erythropoiesis, disrupted erythrocyte metabolism, and dysregulated iron release from reticuloendothelial macrophages, thus providing opportunities for the recurrence of hematological malignancies after HSCT (24, 25). Second, inflammation could be another potential factor linking high RDW and HSCT. Some inflammatory cytokines, such as tumor necrosis alpha (TNF-α) and interleukin (IL-6), were reported to inhibit the maturation of erythrocytes through suppression of hematopoietic system in the marrow, resulting in anemia after hematopoietic stem cell transplantation (26). Third, the increased release and binding of free histones to erythrocytes increase their fragility and might contribute to the relationship between RDW and HSCT, thus finally resulting in the poor outcomes of patients with HSCT (27).

This study has some limitations. First, it was performed at our local institution with a specialized group of transplant patients, a process that potentially limits the generalizability of the results to other care settings or transplant centers. Second, the small

sample size and lack of long-term follow-up prevent us from drawing a definitive conclusion about the relationship between RDW and HSCT. Third, we did not focus on some other biomarkers whether they could be dynamically correlated with RDW levels after transplantation.

In summary, this study is the first to reveal the potential predictive role of RDW in patients with HSCT. Our results will provide a new idea for reducing relapse after HSCT and improving the prognosis of patients. A more comprehensive understanding of this routine laboratory value may influence clinical decision-making and may help to improve the quality of HSCT. RDW may be used as an economical and convenient prognostic factor for the prognosis of patients with HSCT in the future.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The data and the samples analyzed in the present study were obtained in accordance with the standards and approval of the Chongqing medical university institutional review board and biomedical ethics committee. The ethics committee waived the need for written informed consent provided by participants due to the retrospective nature of the study. Because all patient data were analyzed in anonymity, no additional informed consent was required.

AUTHOR CONTRIBUTIONS

DT and XJ designed the study, wrote the article, and consulted literature. SC, LZ, and YZ performed experiment and statistical data. HW gave key guidance for the experiment and collected the sample. JL revised manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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An Unconventional View of T Cell Reconstitution After Allogeneic Hematopoietic Cell Transplantation

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Allogeneic hematopoietic cell transplantation (allo-HCT) is performed as curative-intent therapy for hematologic malignancies and non-malignant hematologic, immunological and metabolic disorders, however, its broader implementation is limited by high rates of transplantation-related complications and a 2-year mortality that approaches 50%. Robust reconstitution of a functioning innate and adaptive immune system is a critical contributor to good long-term patient outcomes, primarily to prevent and overcome post-transplantation infectious complications and ensure adequate graft-versus-leukemia effects. There is increasing evidence that unconventional T cells may have an important immunomodulatory role after allo-HCT, which may be at least partially dependent on the post-transplantation intestinal microbiome. Here we discuss the role of immune reconstitution in allo-HCT outcome, focusing on unconventional T cells, specifically mucosal-associated invariant T (MAIT) cells, γδ (gd) T cells, and invariant NK T (iNKT) cells. We provide an overview of the mechanistic preclinical and associative clinical studies that have been performed. We also discuss the emerging role of the intestinal microbiome with regard to hematopoietic function and overall immune reconstitution.

Keywords: immune reconstitution, unconventional T cells, microbiome, allogeneic transplantation, mucosal invariant T cells (MAIT) cells, γδ T cells, invariant NK T (iNKT) cells

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INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is performed as curative-intent therapy for numerous malignant and non-malignant hematologic diseases, as well as several immunological and metabolic disorders; however, its broader implementation is limited by high rates of transplantation-related complications and a 2-year mortality that approaches 50% (1). Key early contributors to this high post-treatment mortality are infection, the multi-system immunologic complication acute graft-versus-host disease (GVHD) and relapse of underlying malignancy. The most prevalent late contributors are chronic GVHD and organ dysfunction.

The primary goal of allo-HCT for hematologic malignancies is to harness the reconstituting donor immune system to recognize and eliminate residual tumor cells, therefore decreasing the probability of relapse, a phenomenon referred to as the graft-versus-leukemia effect (GVL) (2).

Despite years of research in the field, meaningful separation of GVL effects from GVHD has been challenging, and it is thought that the same mechanisms underlie both forms of alloreactivity (3).

Acute GVHD arises when T cells in the donor graft recognize the recipient tissue as foreign. The pathology of acute GVHD is driven by direct cytotoxic effects of T cells as well as inflammatory cytokines, and commonly involves the skin, gastrointestinal tract and liver (4). Chronic GVHD is a late complication of allo-HCT and has different pathophysiology, characterized by chronic inflammation, dysregulated B cell and T cell immunity and later fibrosis (5). Research efforts in the field have improved outcomes for transplantation patients over the last several decades, but further work is required, particularly regarding post-transplantation immune recovery. Adequate reconstitution of the donor immune system—both innate and adaptive—is critical to patient outcome after allo-HCT for a number of reasons, namely, 1) early innate immunity is critical for tissue repair and infection control, 2) later restoration of adaptive immunity is key for responses to microbial and viral pathogens, 3) normal immune function is important for protective GVL effects, and 4) chronic GVHD is a syndrome best characterized by autoimmune-like dysregulation.

Successful immune reconstitution after allo-HCT depends on a number of factors, including the underlying malignancy, graft source, conditioning regimen, immune suppressive therapy for GVHD prophylaxis, GVHD itself when it occurs, and, of course, GVHD-directed therapies (6). Recipient age is another important factor, especially for *de novo* T cell generation due to age-associated thymic involution (7). In addition to these traditional modulators, evidence for the role of the gastrointestinal (GI) microbiome in shaping immune reconstitution following allo-HCT continues to emerge (8, 9) and is of growing interest specifically for microbiome-dependent unconventional T cell subsets, namely, the mucosal-associated invariant T (MAIT) cells, gamma delta (γδ) T cells, and invariant natural killer T (NKT) cells, all of which are thought to have a beneficial role in the post-transplantation setting. Therefore, in this review, we will discuss broadly the role of unconventional T cell subsets in allo-HCT and the potential relationship of the microbiota with hematopoietic function and peripheral immune reconstitution.

Reconstitution of Innate Immunity

Pre-transplantation conditioning and graft infusion are followed by a neutropenic phase. During this early phase after transplantation, the hematopoietic stem and progenitor cells infused with the graft differentiate and proliferate in the bone marrow to give rise to cells of both myeloid and lymphoid lineages (**Figure 1**). In the first 2 to 4 weeks after HCT, the descendants of myeloid progenitors, namely, neutrophils, eosinophils, basophils, and monocytes, appear in the peripheral blood and begin the reconstitution of the innate immunity. The first marker of innate immune recovery—neutrophil engraftment—is critical for anti-bacterial and anti-fungal immunity and the repair of conditioning-related tissue damage.

Natural killer (NK) cells represent the first, innate arm of the lymphoid lineage to reconstitute in the first weeks following allo-HCT (10) and comprise the majority of the peripheral blood mononuclear cells in this period. Due to their anti-tumor activity they are thought to be a crucial cell type in mediating GVL effects, which has been a subject of several recent review articles (11–14).

Reconstitution of Adaptive Immunity and the Unconventional T Cell Populations

Adaptive immunity, required for appropriate responses to microbial and viral pathogens and vaccination is much slower to recover, and even when key cell types are present in normal numbers, their function is often impaired due to the endogenous alloreactive cytokine environment and exogenous immunosuppressive drugs, administered for the prevention or treatment of GVHD (6). T cells commonly reach normal counts in the peripheral blood in the first three to six months post-transplantation ($CD8^+$ cells reconstitute faster than $CD4^+$ cells), depending on the conditioning regimen and the choice of immune suppression (15). Two different processes contribute to the long-term T cell pool in post-transplant patients: initially, the T cells transplanted in the graft proliferate in the blood and peripheral organs of the lymphopenic recipient, and subsequently, lymphoid precursors from the transplanted stem cells are generated in the bone marrow and undergo selection in the recipient thymus. The latter truly *de novo* production of T cells begins after the recovery of the thymus from conditioning induced damage, and can be influenced by the further damage that occurs if GVHD develops (16). In contrast, B cells can remain at below-normal levels for years following transplantation. Several recent review articles have focused on the restoration of conventional immune subtypes after allo-HCT and their association with clinical outcomes (6, 7, 15, 17, 18), thus we will focus on other subsets here.

In recent years, more attention has been paid to the unconventional T cell subsets and their role in transplantation immunology and anti-tumor immunity. Unconventional T cells, namely, MAIT cells, γδ T cells, and iNKT cells, share features of both innate and adaptive immunity, specifically:

- Antigen-independent activation and rapid response similar to innate immune cells
- No donor MHC restriction, similar to innate immune cells
- TCR-dependent activation similar to conventional T cells, however, the TCR is semi-invariant and does not recognize conventional peptide antigens, but molecules presented in the context of monomorphic antigen presenting molecules

MAIT cells recognize bacterial metabolites of the riboflavin pathway presented by the class I like molecule MR1, and iNKT cells react to phospholipid antigens presented by another class I-like molecule, CD1d (19). Of note, in the setting of allo-HCT, this means that they are not restricted to either donor or host. γδ T cells represent a much more diverse population (from a TCR perspective) and various ligands have been described, which are specific to the combination of γ and δ chain and organ localization (19).

Our current understanding of the relationship of the microbiota to immune reconstitution after allo-HCT will be reviewed here, with

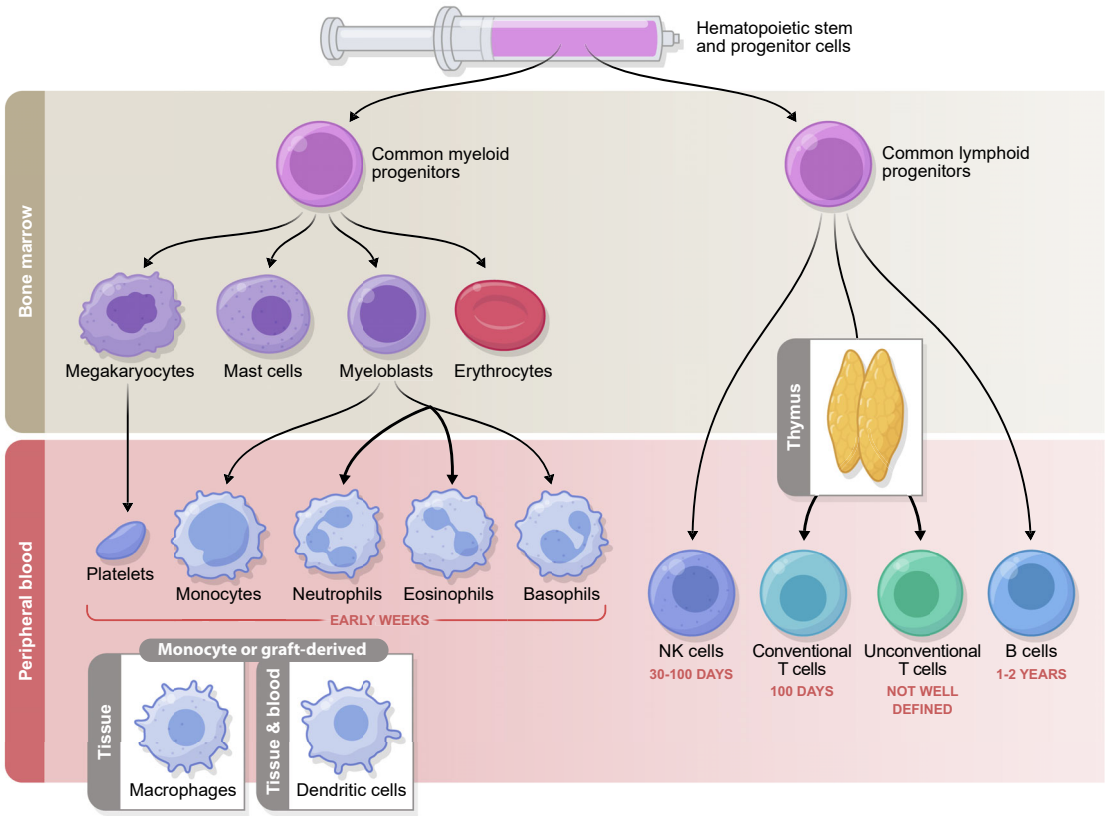

FIGURE 1 | Timeline of immune reconstitution after allo-HCT. Myeloid reconstitution takes place in the early weeks post-transplantation, followed by the lymphoid compartment. NK cells typically return to steady state number first, followed by conventional T cells which reach pre-transplantation levels during the first 3 to 6 months. B cells commonly do not fully reconstitute until years after allo-HCT. The reconstitution of the unconventional compartment differs depending on the cell type and is a subject of ongoing investigation. The immune subsets measured in the periphery reflect cells from the donor graft that have been maintained and expanded (early) followed by true reconstitution of the hematopoietic compartment via the bone marrow progenitors transferred in the graft (which can occur quite early in the case of some myeloid lineages, but on a much longer time scale with respect to T cells that must undergo thymic education). While some post-transplantation reconstitutition mimicks immune system development in early life, there are many features unique to HCT.

a focus on what is currently known regarding the relationships of unconventional T cell populations with transplantation outcomes.

THE INTESTINAL MICROBIOME, HEMATOPOIESIS, AND IMMUNE RECONSTITUTION AFTER ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION

The first indication that the commensal microbiota may influence hematopoiesis and migration of immune cells to sites of infection came from multiple studies performed in germ-free mice. Germ-free mice are known to have impaired hematopoiesis with fewer specific hematopoietic precursor cells of myeloid lineage, leading to impaired response to pathogens (20). Administration of the bacterial ligand NOD1 rescued impaired hematopoiesis in germ-free mice and induced production of hematopoietic cytokines in bone marrow mesenchymal stromal cells (21). Tada et al. observed lower neutrophil numbers in germ-free mice (22) and Inagaki et al. described impaired defenses against Listeria monocytogenes, which was attributed to defective trafficking of activated T cells to the sites of inflammation when compared to mice housed under specific pathogen free (SPF) conditions (23). Microbiota-driven myelopoiesis is dependent on functional toll-like receptor (TLR) signaling, as germ-free mice deficient in MyD88/TICAM signaling do not increase neutrophil generation upon microbial colonization (24). Similar effects have been observed with antibiotic treatment, which disrupts the normal microbiome. In mouse models of hematopoiesis and aging, oral broad-spectrum antibiotic treatment lead to impaired myelopoiesis and response to pathogens (20), depletion of circulating blood neutrophils (22, 25) and accelerated neutrophil aging (25). Josefdottir et al. demonstrated that the antibiotic-mediated microbiome changes led to decreased numbers and cell cycle activity in bone marrow progenitor cells and impaired maturation of granulocytes, and was dependent on functional Stat1 signaling in the bone marrow (26).

In addition to the potential influence on normal hematopoiesis, there is emerging preclinical evidence that the gut microbiota may

influence clonal hematopoiesis (CH)—a recently defined condition, which represents a pre-leukemic state (27). A recent study by Meisel and colleagues utilizing a mouse model of TET2 deficiency (mimicking one of the commonly identified mutations in CH) suggested that mice with this genotype require impaired GI barrier function in order to develop CH. The key evidence for this was generated using 16S rRNA sequencing of peripheral blood, mesenteric lymph nodes and spleen, which confirmed higher bacterial burden in the organs of mice with TET2 deficiency than in age-matched controls. Furthermore, germ-free TET2 deficient animals failed to develop pre-malignant myelopoiesis and antibiotic administration reversed the CH phenotype. In symptom-free mice with TET2 deletion only in the hematopoietic cells (Tet2f/fVav^{cre} mice), but not in littermate controls, a pre-leukemic CH-like state could be triggered by administration of TLR2 agonist Pam3CSK4 (28). Further to this, hematopoietic stem cells express TLRs and appear to regulate emergency hematopoiesis in response to pathogen-derived signals (29, 30). Whether dysbiosis and CH are associated in humans remains to be explored.

In the clinical transplantation setting, using a large clinical data set of 1500 patients, 446 who had daily stool samples available, as well as daily complete blood counts, Schluter et al. demonstrated a relationship between peripheral blood lymphocyte, monocyte and neutrophil dynamics after allo-HCT and the intestinal microbiota composition. Patients who received fecal microbiota transplantation (FMT) in a randomized trial exhibited significantly higher white blood cell counts, which perhaps suggests a causal relationship between gut microbiome and circulating immune cell subsets (9). Ingham et al. observed faster B and NK cell reconstitution in patients with higher abundance of the bacterial family *Ruminococcaceae*, which was associated with better clinical outcome (31). In a study from our group, Staffas et al. examined hematopoietic function in a mouse model of allo-HCT and demonstrated that the intestinal microbiota supports post-transplantation hematopoietic reconstitution in HCT recipients through its role in dietary energy uptake (8).

In addition to the potential influence at the level of bone marrow resident progenitor cells, another possible mechanism for the microbiota to influence immune reconstitution and function may lie in circulating bacterial metabolites and other small molecules (**Figure 2**), for example short chain fatty acids (SCFA), bile acids, and aryl hydrocarbon receptor (AhR) ligands (32). There is currently no evidence that these molecules influence numeric reconstitution of any immune subset, but an emerging body of literature suggests they may influence immune function. We have recently reviewed the clinical associative data and detailed mechanistic studies supporting an immunomodulatory role for the microbiota in transplantation outcome in general (33). For example, butyrate, one of the short-chain fatty acids, has been associated with immunosuppressive effects and protection against GVHD in mouse models (34, 35), and in human post-transplantation samples, lower fecal concentrations of acetate, butyrate and propionate were associated with more severe acute GVHD (36). In a recent study from our group, butyrate and propionate concentrations in plasma were decreased at day 100 post-HCT in the patients who went on to develop chronic GVHD, supporting the hypothesis that these molecules are potentially immunomodulatory (37). Bile acids are another subset of microbiota-derived molecules with immunomodulatory potential. A recent study in mice has demonstrated a protective effect of tauroursodeoxycholic acid in acute GVHD due to a decrease in intestinal antigen presentation and the prevention of intestinal epithelial apoptosis (38). Furthermore, in a metabolomic analysis of plasma from allo-HCT patients, several bile acids, plasmalogens and aryl hydrocarbon receptor ligands appeared to be decreased in samples collected prior to acute GVHD development, compared with samples from patients who did not go on to develop acute GVHD (39). The molecules thought to influence conventional T cell fate (e.g., butyrate, which promotes T regulatory cells (Tregs) in mouse models of allo-HCT) have not yet been studied with respect to unconventional T cells in allo-HCT setting.

MAIT CELLS

Introduction

MAIT cells are abundant in humans, preferentially localized in tissues and mucosa, and represent up to 10% of circulating CD3$^+$ T cells in the peripheral blood and up to 45% of liver T cells (40). They are defined by their expression of a semi-invariant TCR α-chain (Vα7.2-Jα33/20/12 in humans, Vα19-Jα33 in mice), which can combine with only a limited number of TCR β-chains: Vβ2 (TRBV20) and V$_β$13 (TRBV6) in humans and V$_β$6 (TRBV19) and Vβ8 (TRBV13) in mice (41–44). MAIT cells respond to bacterial and fungal antigens presented in the context of the monomorphic MHC-class I-related molecule, MR1 (42).

Antigens presented by MR1 are thought to be predominantly derived from microbial vitamin B biosynthesis intermediates (45). These include activating vitamin B2 (riboflavin) metabolites, such as 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) (46), as well as non-activating 6-formylpterin (6-FP), a metabolite of vitamin B9 (folic acid) (45). An alternative route of MAIT cell activation is TCR-independent and occurs in response to IL-12 and IL-18 (47, 48).

Development

MAIT cells develop in the thymus in a process that is strictly controlled by the gut microbiota. Bacterial ligand 5-OP-RU is produced by gut bacteria, circulates systemically, and is presented to MAIT cells in an MR1 dependent manner (49). This developmental process occurs during a narrow window in the early post-natal period and perturbations of this process (such as delayed bacterial colonization of the gut) lead to impaired MAIT cell development (50). The dependence of MAIT cell development on bacterial stimulation is also demonstrated by their low numbers in the thymus and the periphery of germ-free mice (42, 49, 51). During their development, MAIT cells differentiate into IFN-γ-producing T-bet$^+$ MAIT-1 cells or the IL-17A-producing RORγt$^+$ MAIT-17 cells (51, 52).

Preclinical Data in Allogeneic Hematopoietic Cell Transplantation and Anti-Tumor Immunity

In the context of allo-HCT, a preclinical mouse model has demonstrated a role for recipient MAIT cells in preventing

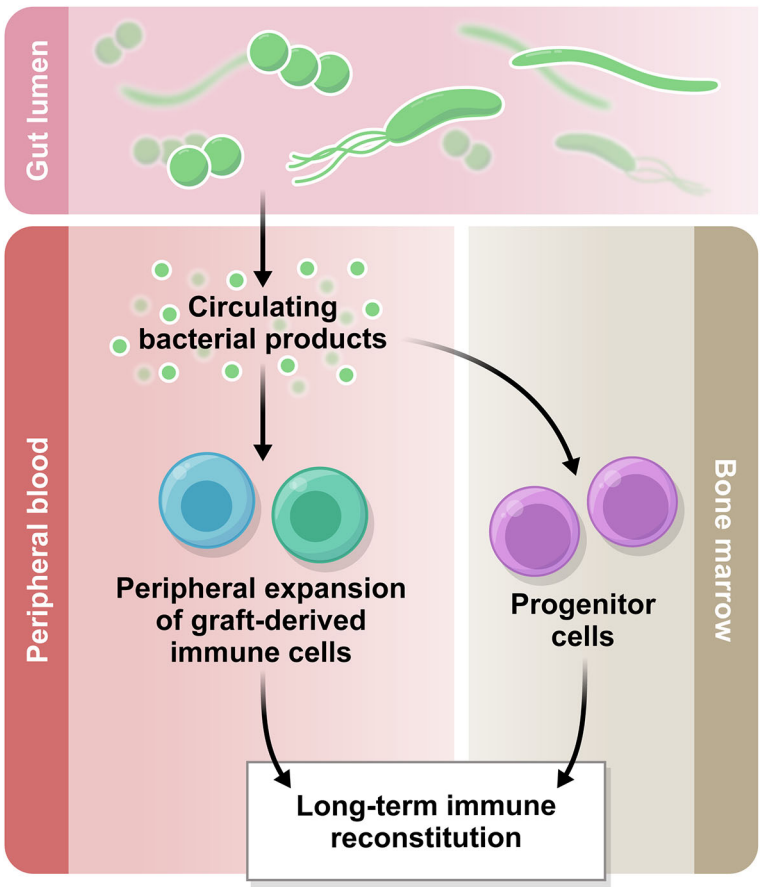

FIGURE 2 | The gut microbiome in immune reconstitution. The intestinal microbiota produces a variety of metabolites which are biologically active. Some of these products circulate systemically and there is emerging evidence regarding their immunomodulatory potential. Based on the current literature, we hypothesize that the circulating bacterial products may influence peripheral immune reconstitution after allo-HCT.

GVHD through production of IL-17A, promotion of intestinal barrier function, suppression of alloantigen presentation, and regulation of gut-microbiota composition (53). However, MAIT cells are a very rare population in mice, especially in circulation, where they represent less than 1% of $CD3^+$ cells (53). The MAIT cell population can be clearly identified in mouse tissues but also in low frequency, making mouse studies of MAIT cells in allo-HCT very technically challenging.

Recently, several studies have examined the role of MAIT cells in anti-tumor immunity. Even though they displayed tumor-lytic capacity in *in vitro* assays (54–56), the most recent study demonstrated a tumor promoting function of MAIT cells. In this study MAIT cells promoted tumor growth and metastasis by blocking the effector function of NK cells and the therapeutic blockade of MR1 suppressed tumor growth and increased the immune cell infiltration in the tumor and their function (57).

Clinical Data

In the clinical setting, several groups have now studied MAIT cell reconstitution after unmodified allo-HCT or cord blood transplantation. MAIT cell reconstitution after unmodified allo-HCT is poor and their numbers do not reach the levels seen in healthy controls, even after one to two years post-transplantation (58, 59) and their early presence in post-transplantation blood samples is at least in part dependent on the proliferation of MAIT cells transferred in the graft (58). Moreover, MAIT cells in the early post-transplantation period exhibit an altered phenotype in comparison to healthy controls with high CD69 and granzyme B expression and impaired IFN-γ and perforin response after bacterial stimulation *ex vivo* (59). Cord blood transplantation recipients exhibited even more delayed MAIT cell reconstitution (58, 60, 61) than recipients of peripheral blood stem cell (PBSC) grafts, with MAIT cell counts not reaching those of healthy controls for up to 5 years post-transplantation in children (60) and up to 10 years post-transplantation in adults (61).

Studies are emerging exploring the clinical association of MAIT cell reconstitution and acute GVHD; however, larger and more definitive studies are needed. Solders and colleagues observed a decrease in absolute MAIT cell counts in 22 patients with grade 2–4 GVHD after unmodified allo-HCT versus 16 patients with grade 0–1 GVHD, which correlated with the

decreased absolute count in other lymphocyte subsets and was attributed to ongoing immunosuppression. The proportion of MAIT cells among $CD3^+$ cells was unchanged (59). In a study of 17 pediatric cord blood transplantation recipients, subsequent MAIT cell reconstitution appeared unaffected by acute GVHD development (60). Bhattacharyya observed lower absolute MAIT cell counts on day 30 after allo-HCT in eight patients with grade 3–4 GVHD among total 105 patients. Moreover, presence of MAIT cells in a coculture assay suppressed CD4+ cell proliferation *in vitro* (58). In terms of MAIT cell reconstitution as a predictor of GVHD, lower absolute MAIT cell counts on day 60 (< 0.48 cells/ul blood) post-transplantation have been correlated with the development of acute GVHD in a multivariate analysis of 30 pediatric and adult patients receiving bone marrow (BM) transplantation (62). MAIT cell frequencies were lower in the patients with chronic GVHD following allo-HCT with unmodified grafts (63) and cord blood (61), though patient numbers were modest—22 and 98 respectively. Whether the relative loss of donor MAIT cells in acute and chronic GVHD is a biomarker for the GVHD process itself or has a functional role remains an open question.

Two studies thus far have examined the association of MAIT cell reconstitution with the post-transplantation microbiome composition. Bhattacharyya et al. observed a positive association between MAIT cell recovery and intestinal abundance of Blautia and Bifidobacterium in 54 patients with paired blood and stool samples at several timepoints after allo-HCT (58). In a separate study of 27 patients undergoing cord blood transplantation, Konuma et al. found higher microbial diversity and stool riboflavin pathway gene abundance in the early post-transplantation period in patients who exhibited MAIT cell reconstitution at six and twelve months compared to those with no reconstitution (61). Interestingly, the absolute numbers were still very low (approximately 0.1 cells/ul blood), similar to other published work regarding MAIT cells in cord blood transplantation (58, 60).

There are several factors implicated in affecting the post-transplantation reconstitution of MAIT cells. Definitive data regarding the influence of conditioning intensity on MAIT cell maintenance and development is not yet available, with conflicting results from several small studies that have been conducted thus far (58, 59, 62). With respect to the influence of GVHD prophylaxis, Bhattacharyya et al. observed markedly reduced MAIT cell number in patients receiving haploidentical transplantation with post-transplantation cyclophosphamide (PTCy), compared with expected MAIT count in the absence of PTCy (58). Furthermore, an analysis of MAIT cell reconstitution in association with cyclosporine A and glucocorticoid therapy in PBSC and cord blood transplantation recipients did not reveal any differences in MAIT cell number when patients who received these drugs were compared with those who did not (61).

Several studies have reported the presence of MAIT cells in solid tumor biopsy samples, including colorectal cancer (54, 55, 64, 65), kidney and brain cancer (66) and liver cancer (67). Results were heterogeneous, with increased intratumoral MAIT cells associated with both favorable and unfavorable clinical outcomes. Interestingly, a recent study identified a new MAIT cell subset in human colorectal carcinomas. These cells were directly stimulated by intratumoral bacterial antigens via their TCR and exhibited a distinct exhaustion phenotype, which was not observed in the MAIT cells from adjacent tissue or peripheral blood mononuclear cells (68). Regarding hematologic malignancies, MAIT cells have only been studied in multiple myeloma, where they exhibited lower counts in the blood (56, 69) and bone marrow (69) compared to healthy controls, and a higher expression of PD-1 (69). MAIT cell numbers were restored to baseline by PD-1 blockade (69). Of note, the process of mobilizing hematopoietic stem cells with G-CSF increased the numbers of IL-17-producing $CD8^+$ MAIT cells in donor grafts, but association with persistence and patient outcome were not reported, therefore these remain interesting open questions (70).

Despite some contrasting results, early MAIT cell reconstitution seems to be associated with lower rates of acute GVHD development and better long-term MAIT cell reconstitution is associated with lower rates of chronic GVHD. Both early and late MAIT cell reconstitution appears to be dependent on intestinal microbiota as well as the predicted abundance of riboflavin genes (measured using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)). Larger clinical studies are needed to clarify the association between peripheral MAIT cell reconstitution and acute GVHD. Similarly, more extensive stool data (including measured metabolites and MAIT cell-stimulating ligands) from allo-HCT recipients will be necessary to draw reliable associations between specific microbial taxa, genetic signatures and metabolite production and the reconstitution of MAIT cells after allo-HCT. The role of MAIT cells in the pro- versus anti-tumor immunity, which can be further applied to study their GVL activity is only beginning to emerge.

γδ T cells

Introduction

Gamma delta T cells are another population of unconventional T cells, characterized by use of γ and δ chains instead of α and β within the TCR, as well as the use of only a limited number of V, D and J segments created during V(D)J recombination. Similar to MAIT cells, γδ T cell activation is not MHC- restricted (19). Of note, the usage of specific γ and δ chains is different in mice and humans. The Vδ chain usage is how the subsets of γδ T cells are defined (Vδ1, 2, 3, but in practical terms, Vδ2 vs. non-Vδ2 is how most analysis is divided, at least in studies of peripheral blood populations). In humans, γδ T cells comprise 0.5–10% of $CD3^+$ T cells in the peripheral blood and the vast majority of these cells express the Vγ2Vδ9 receptor (19). These cells are activated by phosphoantigens, which are metabolites in the isoprenoid synthesis pathway. Isoprenoids are the oldest known biomolecules with numerous biochemical functions, including cell membrane synthesis, hormone synthesis and intracellular pathway regulation (71). This pathway is represented by the mevalonate pathway in mammalian cells, leading to the production of isopenthenyl pyrophosphate (IPP) (72) or the microbial 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway with the

intermediate 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which is the most potent activator of Vγ2Vδ9 cells (73). Phosphoantigens activate Vγ2Vδ9 cells by binding to butyrophilins (BTNs), particularly butyrophilin 3A1 (BTN3A1), which is ubiquitously expressed on almost all cell types and, unlike MHC class I, MHC class I–related molecule MR1 or antigen-presenting CD1 molecules, does not require presence of β2-microglobulin (74, 75). The so-called Vδ2-negative cells are predominantly located in tissues, such as skin, intestine, lungs, spleen, liver and uterus and form less than 10% of circulating γδ cells (76). In the peripheral blood, the non-Vδ2 population consists almost exclusively of Vδ1 cells, with Vδ3 cells accounting for only 0.2% of peripheral $CD3^+$ cells (77). Vδ1 cells recognize various stress-related peptides and phospholipid antigens, including MHC-class-I-related ligands, such as stress ligands EPCR, MICA, MICB and ULBP and glycoproteins CD1c, CD1d (19).

Development

In contrast to MAIT and iNKT cells, thymic γδ T cell development begins in the fetal period in both mice and humans. In mice, thymic egress occurs in waves, where each wave represents a different combination of γ and δ chains, has a different signature of surface and intracellular markers, and carries the predisposition to reside in different tissues. In humans, most of the cells developing in the fetal period are Vγ2Vδ9 cells, whereas the Vδ1 subset takes over in the postnatal period (78).

Preclinical Data in Allogeneic Hematopoietic Cell Transplantation and Anti-Tumor Immunity

The effector function of γδ T cells lies in their production of various cytokines and cytotoxic molecules. Their cytotoxic activity is mediated by production of perforin and granzyme, as well as expression of death receptor ligands, such as Fas-ligand and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (79). In mice, downstream cytokine production capacity is determined both during thymic development and by the environment (78). In humans, the majority of the thymic γδ T cells express IFNγ and TNFα but they can be polarized into IL-17 producing cells by exposure to IL-1β, IL-6, TGF-β, and IL-23 (80).

Following allo-HCT, γδ T cells mediate anti-viral, as well as anti-tumor effects and are thought to protect against relapse. Anti-tumor effects have been attributed to both Vδ1 and Vδ2 subsets via different ligands. Moreover, γδ T cells are capable of reactivity to multiple ligands on the tumor cells simultaneously using a single TCR and their early sensing of metabolic changes in the cells allows them to be among the first responders to malignant transformation (81).

Tumor cells can express an active mevalonate pathway of cholesterol synthesis and the metabolites of this pathway, such as IPP, may accumulate in the tumor cells and elicit a γδ T cell-mediated immune response (72). The enhancement of this process in the tumor cells by aminobisphoshonates and the subsequent expansion of tumor-reactive Vγ9Vδ2 cells has been demonstrated in several in vitro studies (82, 83), as well as in immunodeficient mice using human tumor cell lines and Vγ9Vδ2 cells (84). Besides the TCR, γδ T cells express several receptors also found on NK cells. Examples include NKG2D, CD94/NKG2C, DNAX accessory molecule-1 (DNAM-1), NKp30 and NKp44; and killer-inhibitory receptors (KIRs), like CD94/NKG2A, ILT2, CD161, or KIR2DL 1–3 (85).

$NKG2D^+$ γδ T cells bind to tumors expressing NKG2D ligands, such as UL16-binding proteins (ULBPs) and MHC-class I related molecules MICA and MICB (86, 87). Activation of γδ T cells by NKG2D ligands can be initiated both via TCR and the NKG2D (88). Simultaneous activation of the TCR and DNAM-1 receptor of the Vγ9Vδ2 leads to killing of acute myeloid leukemia (AML) blasts *in vitro* and these cells enhance survival in a xenotransplantation murine model of leukemia (89). Leukemic stem cells, but not healthy $CD34^+$ cells, redistribute BTN3A1 through the guanosine triphosphatase activity of RhoB, which enables their recognition by the Vγ9Vδ2 (90). Some hematological malignancies develop resistance against cytotoxic Vγ9Vδ2 via downregulation of ULBP1 (87). However, Vγ9Vδ2 are not the only subtype of γδ T cells capable of anti-tumor activity. Vδ1 cells can be cytokine-stimulated to express NKp30, NKp44 and NKp46, which are associated with cytotoxicity against lymphoid leukemia cells (91).

Another important mechanism for anti-tumor activity of γδ T cells is the expression Fc receptor FcRγIII (CD16). This receptor binds to the Fc portion of immunoglobulins and mediates anti-tumor effects via antibody-dependent cellular cytotoxicity (ADCC), similar to NK cells (92). In the case of γδ T cells, the ADCC is stimulated by phosphoantigen binding (93). Efficacy of the γδ T cell-mediated ADCC against $CD19^+$ acute lymphoblastic leukemia was demonstrated using a CD19 antibody (94), as well as a so-called "triplebody" with 2 binding sites for CD19 and 1 for CD16 (95).

In experimental models of GVHD, multiple groups have demonstrated the alloreactive potential of γδ T cells, leading to GVHD development (96–98). Blazar and colleagues demonstrated that transgenic mice expressing gamma/delta heterodimers on a high proportion of peripheral T cells reacted to nonclassical major histocompatibility complex (MHC) class lb and caused acute GVHD when used as donors in allo-HCT mouse model (96). Maeda et al. observed reduced GVHD in mice treated with anti-γδ TCR antibody or in γδ deficient mice. This was explained by reduced donor T-cell expansion and reduced allogeneic stimulatory capacity of dendritic cells (DCs) (98). However, these mouse models have limitations for the study of γδ T cells due to differences in development, tissue distribution and other characteristics between mice and humans.

Clinical Data

In contrast to αβ T cells, γδ T cells reconstitute early after allo-HCT (99–101). Whether the cells measured in patients following HCT with T cell replete grafts are generated *de novo* in the bone marrow and educated in thymus, or are a product of the *in vivo* expansion of γδ T cells transplanted in the graft has been a matter of debate. An older study from Hirokawa et al. observed common γδ TCR sequences between donor and host in selected patients in a study of 23 patients receiving allo-HCT (100). This finding has

been supported by several studies linking graft γδ T cell content to their early reconstitution and clinical outcomes after allo-HCT (102–106). However, a more recent study by Ravens et al. shows both similar and different γδ TCR clonotypes in the donor-recipient pairs, suggesting that the post-transplantation γδ T cell reconstitution may occur both de novo in the bone marrow and thymus, as well as via peripheral graft expansion (99). Vδ2 cell reconstitution was significantly impaired in the recipients of cord blood transplantation, whereas Vδ1 counts appeared to be driven by CMV reactivation and did not differ between cord blood and unmodified stem cell graft recipients (107).

The role of γδ T cells after allo-HCT can be viewed from several perspectives. First, γδ T cells exert potent anti-infectious immunity against a multitude of bacteria and viruses. In a cohort of 102 pediatric patients, higher γδ T cell counts post-transplantation were associated with lower incidence of bacterial, fungal, and viral infections (108). Serial monitoring of Vδ2 counts post-transplantation has found an association between high Vδ2 counts and lower rates of EBV reactivation (109). Patients with higher numbers of γδ T cells in the early post-transplantation period (day 30) experienced less CMV reactivation than patients with lower γδ T cell numbers (104). Similarly, patients with higher CD27 + γδ T cell counts in the graft had lower rates of CMV reactivation than those with lower numbers (103). A further study from the same group also demonstrated a role of $CD8^+$ γδ T cells, which were higher in the grafts from CMV positive donors, expressed V*γ9* and exhibited increased reactivation to cytokine and TCR/CD3 stimulation (105). In addition to these associations with less CMV reactivation, γδ T cells expand in response to CMV, which suggests an involvement in viral clearance (99, 106, 110–112). Ravens et al. demonstrated that this expansion is clonal and the clones proliferating in the context of CMV reactivation carry virus-reactive γδ TCR sequences (99). Anti-CMV activity has mostly been attributed to Vδ2-negative subsets (predominantly Vδ1) of γδ T cells (99, 110, 111). Vδ1-positive γδ T cells were also demonstrated to undergo clonal expansion in the context of EBV reactivation (113, 114).

The anti-tumor activity of γδ T cells predicts a protective role after allo-HCT with respect to relapse. The first study published in line with this hypothesis showed that among 43 patients undergoing T-cell depleted HCT from partially HLA-mismatched donors for leukemia, 10 achieved a 'high' γδ T cell proportion—as defined by reaching a proportion greater than 10% of total $CD3^+$ cells on two consecutive measurements post-transplantation. This correlated with improved disease-free survival (DFS) up to 30 months post-transplantation, where 90% of patients with increased γδ T cell proportion were disease free, compared to 31% of patients with normal proportion of γδ T cells (115). In a follow-up study, Lamb et al. confirmed the findings after 42 months of follow-up in an additional cohort of 100 patients, comparing transplantation outcomes using two different *ex vivo* α/β T cell depletion regimens. Moreover, they demonstrated that Vδ1 cells are the major subset in the patients with robust γδ T cell reconstitution and moreover, these cells exhibit anti-leukemia activity *in vitro* (116). In a follow up report, with an extension of the follow-up period to 8 years and an expansion of the cohort, the 5-year overall and disease-free survival was significantly improved in the group with higher γδ T cells (117). In the most recent study of 108 patients undergoing BM or PBSC transplantation, Minculescu et al. linked improved γδ T cell reconstitution on day 56 post-transplantation to a significantly decreased cumulative incidence of relapse and improved overall and relapse-free survival. When the counts of all γδ T cells and their subsets individually were analyzed as continuous variables, increased numbers of all γδ T cells subsets correlated with decreased risk of death and increased numbers of all γδ T cells and Vδ2 cell subset correlated with lower risk of relapse (118). The CMV-expanded clones of non-Vδ2 cells isolated from patients after unmodified allo-HCT, as well as cord blood transplantation, showed efficacy in killing leukemic blasts in vitro (111). Consistent with this finding, Dolstra et al. had previously demonstrated that Vδ1 cells isolated from a patient after allogeneic HCT exhibited an anti-tumor activity against AML blasts. Similar to NK cells, the leukemia-reactive γδ T cells expressed killer cell-inhibitory receptor (KIR) p58.2 (CD158b) (119). Not all γδ T cells harbor the same GVL efficacy. Gaballa et al. attributed this effect to $CD8^+$ γδ T cells in the graft (103), and Jin et al. to oligoclonal expansion of the *TRDV4* and *TRDV8* subfamilies in patients after allo-HCT. In contrast, *TRDV5* and *TRDV6* clones were higher in patients experiencing recurrence of the disease (120). Further to this, Arruda studied the TCR repertoire of γδ T cells in the donor graft and identified that patients without relapse more commonly received a graft containing γδ T cells with a higher proportion of 'public' TCRs in the repertoire. However, in contrast to Scheper et al. (111), γδ T cells in grafts derived from CMV positive donors displayed a more private, less diverse, skewed repertoire (121).

Early clinical studies linked higher γδ T cell counts to a higher incidence of acute GVHD, whether measured in the recipient (122) or in the graft (123), but also reported decreased γδ T cell counts in patients with chronic GVHD, specifically the CD4 and CD8 double negative subset (124). The association of γδ T cells with acute GVHD was not confirmed in further human studies, which associated γδ T cell reconstitution only with enhanced GVL effect and not higher GVHD incidence (108, 115–117). Higher γδ T cell counts on day 28 post-transplantation have been associated with lower risk of acute GVHD, when both the whole γδ T cell population is measured, or just the Vδ2 cell subset (118). Additionally, lower counts of naïve γδ T cells in the donor grafts have been associated with subsequent development of grade 2–4 acute GVHD in the recipient (125). It has been postulated that this protective effect may be due to to a regulatory subset of FoxP3 expressing γδ T cells (126, 127). Interestingly, specific subsets and clones have been proposed to be differentially responsible for GVHD and GVL effects. Gaballa et al. recently associated higher GVHD incidence with one specific subset of γδ T cells, which were $CD8^+$. In a cohort of 105 patients, those receiving grafts with higher $CD8^+$ γδ T cell numbers experienced higher incidence of grade 2–4 acute GVHD, but in parallel, a perhaps predictable lower incidence of relapse (105). Additionally, in a

2005 study of 13 patients receiving allo-HCT for multiple myeloma TCR spectratyping led to the observation of unique γδ T cell clones associated with GVHD and new dominant TCR peaks associated with clearance of the IgH clones, supportive of some tumor-specific γδ T cell responses, but not definitive (128).

Several immune profiling studies of patients transplanted with grafts depleted of αβ T cells have demonstrated an association between early γδ T cell reconstitution and positive transplantation outcome. Evidence for the minimal contribution of γδ T cells to GVHD comes from clinical success of performing transplantations with αβ T cell depleted grafts (129), as well as using these grafts as a 'stem cell boosting strategy' in the setting of graft failure (130). Airoldi and colleagues observed rapid Vδ1 and Vδ2 T cell reconstitution in 27 pediatric patients receiving haploidentical $\alpha\beta^+$ T and $CD19^+$ B cell-depleted grafts. Vδ1 cells expanded *in vivo* in the context of CMV reactivation, whereas Vδ2 cells exhibited activity against leukemia blasts *in vitro* (101).

Extensive literature describes the beneficial role of γδ T cells in the post-transplantation period but factors influencing their reconstitution, outside of viral reactivation, have not been described in a detailed fashion. Of note, their reconstitution does not appear to be dependent on conditioning intensity (118), however, γδ T cells appear extremely sensitive to PTCy in the setting of haploidentical transplantation. In this setting, γδ, and especially the $V\delta2^+$ T-cell counts were significantly lower in the early post-transplantation period (131, 132). This effect on the $V\delta2^+$ cell population persisted for up to one year post-transplantation and correlated with more frequent EBV reactivation (131).

An interaction between the intestinal microbiota and γδ T cells has been proposed in mouse models of several diseases, largely for the intraepithelial populations of γδ T cells (as opposed to the more easily measured circulating cells). Intraepithelial γδ T lymphocytes are reduced in germ-free mice and can be induced after colonization of these mice (133). In a mouse model of lung adenocarcinoma, intestinal microbiota induced intrapulmonary IL-17-producing Vδ1 cells, which promoted inflammation and tumor progression (134). In an additional mouse model of ischemic stroke, the intestinal microbiota appeared to modulate central nervous system inflammation via IL-17 producing γδ T cells (135). To our knowledge, no associations have yet been drawn between the intestinal microbiota and circulating γδ T cells. Given the previous preclinical data, as well as the reactivity of the Vδ2 subset to bacterial metabolite HMB-PP, the gut microbiome may play a role in γδ T cell reconstitution following allo-HCT.

iNKT CELLS

Introduction

The term natural killer T (NKT) cells was originally assigned to a group of $CD3^+$ cells expressing markers found on the NK cells, such as CD161. However, further research demonstrated that these markers do not fully define this population, which responds to lipid molecules presented by the MHC-I-like molecule CD1d (19, 136). There are two broad subtypes of NKT cells. Type I NKT cells, also called invariant NK T (iNKT) cells, are characterized by an invariant TCRα chain (typically Vα14-Jα18 in mice and Vα24-Jα18 in humans), accompanied by a limited number of TCRβ chains (mainly Vβ8.2, Vβ7 and Vβ2) (136–138). Type I NKT cells recognize α-galactosylceramide presented by CD1d molecule, can be recognized by α-GalCer-loaded tetramers and are the most studied subtype to date. Type II NKT cells also react to lipid molecules presented in the context of CD1d, but they are not reactive to α-GalCer and bear more diverse TCRs than type I NKT cells (138). iNKT cells are also more abundant in mice [up to 50% of the liver and bone marrow T cells (139)] than in humans [representing only about 0.1% of peripheral blood T cells (19)]. iNKT cells recognize either self-lipids or foreign lipids produced by pathogenic or commensal bacteria, fungi, viruses or present in allergens. Upon activation, they rapidly gain effector function with cytotoxic activity, with transcription factor and cytokine production dependent on tissue localization and acquire either Th1, Th2 or Th17 phenotype (140, 141). Similar to MAIT cells, aside from TCR-dependent activation, iNKT cells can be activated by cytokines, such as IL-12 (140).

Development

iNKT cells develop postnatally in the thymus where they encounter the CD1d molecule expressed by double positive cortical thymocytes, in a process that requires intracellular trafficking of lipid antigens presented by CD1d (78). In contrast to MAIT cells, the commensal microbiota are not vital for thymic iNKT cell development (142).

Preclinical Data in Allogeneic Hematopoietic Cell Transplantation and Anti-Tumor Immunity

In mouse models of allo-HCT, recipient iNKT cells ameliorate GVHD. Early studies examining the role of iNKT cells in mouse models of allo-HCT assessed for the effect of reduced intensity conditioning (RIC) together with total lymphocyte irradiation (TLI) and anti-thymocyte globulin (ATG) on GVHD development. Mice receiving this treatment regimen experienced less GVHD, as well as an expansion of iNKT cells, which was not observed in the CD1d deficient mice. The protection from GVHD was mediated through increased Th2 polarization of donor T cells (143). Later studies further explored the mechanism by which the iNKT cells reduce GVHD. They studied GVHD development in CD1d and Jα-18 deficient mice, which represent more specific models to assess for the invariant portion of NKT cells. iNKT cells reduced the expansion of alloreactive donor T cells in the GVHD target organs (144), as well as promoted the expansion of the protective Treg population in an IL-4 dependent manner (145). Protection from GVHD has also been observed upon adoptive transfer of iNKT cells in mice, both of host and donor origin, as well as third-party (146–151). Effects of adoptive transfer of human $CD4^+$ and $CD4^-$ iNKT cells into NSG mice have also been examined in a xenogeneic GVHD model. $CD4^-$ iNKT cells inhibited GVHD by decreased human T cell activation and Th1 and Th17 polarization. $CD4^+$ and $CD4^-$ iNKT cells induced dendritic cell (DC) maturation,

but $CD4^-$ iNKT cell contact with splenic and monocyte-derived DCs was more intense and associated with more iNKT cell degranulation (152). In a chronic GVHD model, adoptive transfer of iNKT cells demonstrated a protective role for this cell type, and even reversed the chronic GVHD phenotype (153). Similarly, several groups demonstrated protective effect of α-GalCer administration in acute and chronic GVHD models (146, 153–156).

Extensive studies have been performed examining the role of iNKT cells in tumor immunology. The first evidence of their anti-tumor activity comes from the study of Crowe et al. in the setting of methylcholanthrene induced sarcoma, where mice lacking iNKT cells were more susceptible to tumor development (157). Since then, numerous studies have explored the role of iNKT cells in the immune surveillance of various tumors, mostly attributing them an anti-tumor activity (158). The evidence is somewhat thinner in the context of hematologic malignancies, however, CD1d has been shown to be expressed on multiple myeloma cells (159, 160), as well as AML cells (161) and iNKT exhibited reactivity to CD1d positive tumor cells *in vitro* in a α-GalCer–dependent manner. In line with these findings, iNKT cells from donor lymphocyte infusion (DLI) could be expanded *ex vivo* and were capable of lysing leukemia cell lines and patient AML cells in CD1d-dependent manner (162).

Clinical Data

Several studies have addressed iNKT cell reconstitution post-transplantation in human subjects, largely focusing on associations with GVHD. In the first published study of a cohort of 106 patients, Haraguchi et al. observed iNKT cell reconstitution within a month after allo-HCT in PBSC graft recipients, but their numbers remained very low in the first post-transplantation year in the bone marrow (BM) graft recipients. Peripheral blood iNKT cell counts were lower in patients experiencing acute and chronic GVHD (163). In another study, iNKT cell reconstitution was examined at multiple timepoints after allo-HCT using a CD1d tetramer in a cohort of 71 patients cohort who received a mixture of reduced intensity and myeloablative conditioning regimens (RIC and MAC), either BM or PBSC grafts, and who had a variable exposure to *in vivo* T cell depletion with ATG. In both univariate and multivariate analysis, reaching a threshold of iNKT/T cell ratio higher than 10^{-3} in at least one of multiple measurements on day 15, 30, 60 and 90, was an independent predictor of lower incidence of acute GVHD and better overall survival (164). A more recent study from the same center focused on different cell populations in 117 BM and PBSC grafts (HSCs, NK cells, conventional and regulatory T cells and iNKT cells) and observed that the iNKT cells were the only population associated with lower incidence of grade 2–4 acute GVHD in a univariate analysis. In the multivariate analysis, only the lower frequency of $CD4^-$ iNKT cells could predict higher incidence of acute GVHD in patients receiving BM and PBSC grafts and higher $CD4^-$ iNKT cell *ex vivo* expansion capacity was associated with lower rates of grade 2–4 GVHD in patients receiving PBSC grafts (165). In line with these findings, Bosch et al. observed a positive correlation between graft iNKT cell numbers and peripheral iNKT cell reconstitution of the host (166). Chaidos et al. showed that higher than median CD4- iNKT cell graft content is protective against grade 2–4 GVHD. Moreover, CD4- iNKT cells were capable of contact inhibition of T cell proliferation and suppressed their IFNγ secretion *in vitro* (167). This indicates that iNKT cell reconstitution post-transplantation might be dependent on the expansion of the graft-derived population rather than de novo production in the bone marrow. Several groups have studied the association of conditioning and anti-thymocyte globulin (ATG) administration with immune reconstitution and, in addition to examining other cell subsets, also characterized iNKT cell reconstitution. Total lymphocyte irradiation (TLI) and ATG administration following RIC appeared to favor iNKT cell maintenance or development, and patients conditioned in this fashion had lower incidence of acute GVHD (168). Interestingly, these findings did not hold true in the setting of MAC, where Servais et al. did not observe any difference in iNKT cell numbers when comparing patients receiving ATG versus no ATG (169). Bosch et al. even observed an extremely slow iNKT cell reconstitution after ATG administration after MAC and a significant correlation between graft iNKT cell numbers and peripheral iNKT cell reconstitution of the host on both early and late timepoints post-transplantation (166). These results suggest extremely slow endogenous recovery of iNKT cells after MAC. PTCy in the setting of haploidentical transplantation appears to be another factor associated with the rate of iNKT cell reconstitution, with lower counts at day 30 and day 90 compared in patients receiving PTCy compared with other graft types (132). The association of early iNKT cell reconstitution and clinical outcome might be correlated with the presence of other immune subtypes. Kim et al. correlated lower frequencies of iNKT cells and monocytic myeloid derived suppressor cells measured before day 30 with higher incidence of grade 3–4 GVHD in a multivariate analysis of 119 recipients of unmodified (111 patients) and cord blood (eight patients) grafts (170).

Based on these data, a clinical trial was performed testing a liposomal formulation of α-GalCer (RGI-2001) in recipients of allo-HCT. Patients receiving this ligand on the day of transplantation exhibited improved reconstitution of $Helios^+$ Treg cells (defined as higher than 12% of $CD4^+$ cells at any timepoint after allo-HCT) and lower incidence of grade 2–4 acute GVHD (171).

Both peripheral and intratumoral iNKT cell counts have been investigated in several different malignancies (e.g., head and neck squamous cell carcinoma, lung cancer, colorectal cancer) and higher numbers have largely been associated with better prognoses (172). With regard to hematologic malignancies, iNKT cells have been most studied in multiple myeloma. Their counts inversely correlated with disease progression (159, 173–175), which was linked to decreasing CD1d expression on the tumor cells as the disease progressed (160). In a small study of 6 patients, α-GalCer-pulsed dendritic cells expanded the iNKT cell population when added to lenalidomide therapy and led to a decrease of the monoclonal immunoglobulin in asymptomatic myeloma patients (176). In AML, low iNKT cell counts were

correlated with poor overall survival (177). Despite the evidence of anti-tumor activity of iNKT cells in the context of hematologic malignancies, no difference in relapse incidence after allo-HCT has been observed in association with iNKT cell counts (164, 165).

Although commensal microbiota are not vital for thymic iNKT cell development, iNKT cells are an important intermediary in the relationship between host and microbiota, especially on the mucosal surfaces and in the liver. Examples include identification of distinct bacteria and less intestinal inflammation in the in the *Jα18*$^{-/-}$ mice versus wildtype mice in the experimental model of colitis (178). In the human colonic biopsies from patients with inflammatory bowel disease, iNKT cells produced pro-inflammatory cytokines, which was driven by exposure to mucosa-associated microbiota (179). Exposition to pathogenic bacteria or gentamicin led to decrease in hepatic NKT cells and higher degree of liver injury (180). In metastatic colon cancer, changes in the gut microbiota potentiated iNKT-cell mediated tumor control via decreased secondary bile acid production (181). To date, the influence of gut microbiota on circulating NKT cells and their reconstitution after allo-HCT has not been clarified.

Based on the literature reviewed here, iNKT cells represent a cell subset associated with protection against acute GVHD in allo-HCT patients. iNKT cells might harbor a GVL potential, which was not demonstrated in the published studies, possibly due to their extremely low numbers post-transplantation. Administration of an iNKT cell ligand proved beneficial in a small number of patients. Therefore, further exploration of iNKT cell expansion *in vivo* are needed to harness their anti-GVHD and anti-tumor potential. Their reactivity to changes in commensal microbiota in other disease models makes them a good candidate for treatments exploring microbiota modifications, however, more research is needed in this area.

DISCUSSION

Unconventional T cells represent populations are emerging as likely important for the field of transplantation immunology with a potential to reduce the risk of acute and chronic GVHD without impairing, or perhaps improving in the case of γδ T cells, GVL effects. Thus far, published studies linking unconventional T cell subtypes to favorable clinical outcomes are limited by low patient numbers, and variations in type of malignancy, conditioning regimens, graft types and immune suppressive drugs. Therefore, further studies are needed. Manipulation of unconventional T cell compartment may improve clinical outcomes in the future - for example, selecting grafts with high unconventional T cell numbers, exogenous administration of specific ligands, using adoptive transfer approaches or microbiota manipulation strategies. Some of these approaches are currently being investigated in clinical trials, others still require more mechanistic studies to gain deeper understanding. Moreover, the interplay of different types of unconventional cells with each other and their conventional counterparts might also play a role in reconstitution. For example, the Vδ2 subset shares a number of characteristics with MAIT cells, i.e. they can both undergo cytokine-dependent activation and share similar transcription profiles, thus they have some shared post-transplantation requirements for function and maintenance (182).

As described above, for each unconventional cell subtype there is evidence for their function being modulated by intestinal microbiota. However, it is unclear whether the bacteria-derived ligands of unconventional T cells circulate in the blood or execute their functions only in the context of cell contact with antigen presenting cells in the tissues. However, there has been emerging evidence in recent years demonstrating that circulating bacterial metabolites can be associated with disese outcomes. Although more evidence is needed, it is possible that these metabolites influence circulating immune cells, both numerically and functionally. Similarly, the number of studies linking the intestinal microbiome to hematopoiesis is increasing. Given that bone marrow is a remote organ to the gut, circulating bacterial metabolites might be one explanation for this phenomenon. Nevertheless, circulating immune cells likely reflect, in some fashion, the cell distribution within the organs, specifically the unconventional T cells, which are unique regarding their immediate reactivity to bacterial metabolites. MAIT cell reconstitution post-transplantation has been linked to higher abundance of certain bacteria in the gut, as well as the presence of vitamin B2 metabolic pathways in the gut bacteria in a small number of patients. For γδ T cells and iNKT cells, the evidence of their dependence on gut microbiota or organ specific microbial flora is only beginning to emerge in mouse models of diseases unrelated to allo-HCT. Further studies are needed to clarify the dependence of the reconstitution of not only of MAIT cells, but of all unconventional subsets on the presence of distinct microbial taxa and metabolites. We hypothesize that one of the reasons that microbiota damage is associated with poor overall transplantation outcome (183) is due to the influence of microbial communities on the reconstitution of robust immunity, a hypothesis we hope will be studied in detail.

AUTHOR CONTRIBUTIONS

HA reviewed the literature, designed the figures and wrote the manuscript. KM contributed to the figure design and wrote the manuscript. MB critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Comparison of Two Strategies for Prophylactic Donor Lymphocyte Infusion in Patients With Refractory/ Relapsed Acute Leukemia

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Prophylactic donor lymphocyte infusion (pDLI) could reduce relapse in patients with refractory/relapsed acute leukemia (RRAL) undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), but optimal timing of pDLI remains uncertain. We compared the outcomes of two strategies for pDLI based on time from transplant and minimal residual disease (MRD) status in patients with RRAL. For patients without grade II–IV acute graft-versus-host disease (aGVHD) on day +60, pDLI was given on day +60 regardless of MRD in cohort 1, and was given on day +90 unless MRD was positive on day +60 in cohort 2. A total of 161 patients with RRAL were enrolled, including 83 in cohort 1 and 78 in cohort 2. The extensive chronic GVHD (cGVHD) incidence in cohort 2 was lower than that in cohort 1 (10.3% vs. 27.9%, $P = 0.006$) and GVHD-free/relapse-free survival (GRFS) in cohort 2 was superior to that in cohort 1 (55.1% vs. 41.0%, $P = 0.042$). The 2-year relapse rate, overall and leukemia-free survival were comparable between the two cohorts (29.0% vs. 28.2%, $P = 0.986$; 63.9% vs. 64.1%, $P = 0.863$; 57.8% vs. 61.5%, $P = 0.666$). Delaying pDLI to day +90 based on MRD for patients with RRAL undergoing allo-HSCT could lower extensive cGVHD incidence and improve GRFS without increasing incidence of leukemia relapse compared with pDLI on day +60.

Keywords: prophylactic donor lymphocyte infusion, refractory/relapsed acute leukemia, relapse, allogeneic hematopoietic stem cell transplantation, minimal residual disease

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is accepted as the optimal choice for patients with refractory/relapsed acute leukemia (RRAL) (1, 2). However, relapse remains a barrier for the survival of these refractory patients post-transplant, with incidences of relapse of over 50% and leukemia-free survival (LFS) of about 25% (3, 4). Some studies have demonstrated that

prophylactic donor lymphocyte infusion (pDLI) is effective for preventing relapse in patients with RRAL post-transplant (5–8), but its complication of graft-versus-host disease (GVHD) has limited its application (9, 10). The morbidity and mortality of GVHD post-pDLI are related with the time interval between pDLI administration and transplantation as well as the doses and donor source of pDLI (11–13), but optimal timing of pDLI remains unknown. Our previous prospective multicenter study showed that pDLI on day +60 post-transplant regardless of minimal residual disease (MRD) could reduce relapse for patients with RRAL undergoing allo-HSCT, but the 2-year cumulative incidences of extensive chronic GVHD (cGVHD) and mortality of GVHD reached up to 21.1% and 14.1% (7).

In order to reduce the morbidity and mortality of GVHD post-pDLI, we modified our pDLI strategy by delaying the time to day +90 unless MRD was positive on day +60. We aimed at evaluating whether this new strategy for pDLI could reduce the morbidity and mortality of GVHD post-pDLI but not affect relapse and survival in patients with RRAL undergoing allo-HSCT compared with our history strategy.

MATERIALS AND METHODS

Study Population

This study was based on two prospective, independent and non-parallel cohorts. Cohort 1 came from a non-registered prospective multicenter study (7), and cohort 2 from a registered prospective multicenter clinical trial (NCT02673008). Patients undergoing allo-HSCT between January 2012 and December 2017 were enrolled in this study if they met the following criteria: (1) patients with RRAL without complete remission (CR) pre-transplant, including patients with acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), and acute biphenotypic leukemia (ABL); (2) achieving CR at 30 days post-transplant; (3) with available donor lymphocytes; (4) no evidence of relapse, uncontrolled infection, or serious organ failure at the time of the planned pDLI. RRAL was defined as primary induction failure after two or more cycles of chemotherapy or relapse refractory to salvage chemotherapy (14, 15). Enrolled patients who were not treated with pDLI due to factors such as GVHD were also included in this study. This study was approved by respective ethical review boards before study initiation, and written informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Transplantation

The sequential intensified conditioning regimen was administered in all patients: fludarabine 30 mg/m^2/day and cytarabine 2 g/m^2/day (on days −10 to −6), 4.5 Gy total body irradiation/day (on days −5 and −4), and cyclophosphamide 60 mg/kg/day and etoposide 15 mg/kg/day (on days −3 and −2). All patients undergoing HLA-matched sibling donor (MSD) or HLA-matched unrelated donor (MUD) transplant received peripheral blood stem cell (PBSC) grafts whereas patients undergoing HLA-haploidentical donor (HID) transplant received a combination of bone marrow (BM) and PBSC grafts.

Graft-Versus-Host Disease Prophylaxis and Immunosuppressant Withdrawal

Ciclosporin A (CsA) alone or CsA + methotrexate (MTX) were administered in patients undergoing MSD transplant, and CsA + MTX + antithymocyte globulin and/or mycophenolate were used in patients receiving MUD or HID transplant for GVHD prophylaxis (16, 17). Immunosuppressant was withdrawn gradually in patients without acute GVHD (aGVHD) by day +30, and was stopped at 90 days after MSD transplant or 120 days after HID or MUD transplant if patients had no GVHD. For patients receiving pDLI before day +90 after allo-HSCT, immunosuppressant was continued for another 2 weeks after pDLI, then tapered and stopped within 4 weeks if no DLI-associated GVHD occurred. If patients had GVHD, immunosuppressant was reduced by 50% when GVHD was controlled and then stopped 2 weeks later.

pDLI

pDLI used granulocyte colony-stimulating factor (G-CSF)-mobilized PBSCs (G-PBSCs), which were derived from previously cryopreserved or newly collected G-PBSCs. The CD3$^+$ T cell count for each pDLI was 3.0 × 10^7/kg of the recipient weight. pDLI strategies of the two cohorts are conducted as shown in **Figure 1**. In cohort 1, pDLI was given once on day +60 regardless of MRD for all patients without grade II–IV aGVHD, and then administered based on MRD and GVHD status. If patients were MRD negative, pDLI was not given again; if patients were MRD positive and without grade II–IV aGVHD, pDLI was given monthly until GVHD occurred or MRD became negative or for a total of four times. For patients with grade II or above aGVHD by day +60 post-transplant, the application of pDLI was based on MRD and GVHD status by day +90. If patients remained MRD positive and had no GVHD on day +90, pDLI was given once on day +90 and then administered based on MRD and GVHD status. In cohort 2, for patients who were MRD negative on day +60 and did not experience grade II–IV aGVHD by day +90, pDLI was given once on day +90 post-transplant and then administered based on MRD and GVHD status. For patients with positive MRD and without grade II–IV aGVHD on day +60, pDLI was given once on day +60 and then administered based on MRD and GVHD status. For patients with positive MRD and grade II–IV aGVHD on day +60, the application of pDLI was based on the MRD and GVHD status by day +90. If patients remained MRD positive and had no GVHD on day +90, pDLI was given once on day +90 and then administered based on MRD and GVHD status.

Surveillance and Intervention for Relapse

BM samples were analyzed pre-transplant and then once a month in the first 6 months post-transplant, once every 2 months from 6th to 12th, once every 3 months from 13th to 24^{th,} and once every 4 months from the 25th to 36th post-transplant for the monitoring of morphology and MRD. If MRD was positive, it was monitored once a week until MRD became negative. Aberrant leukemia-associated immune phenotypes detected by 8-color flow cytometry (FCM) and

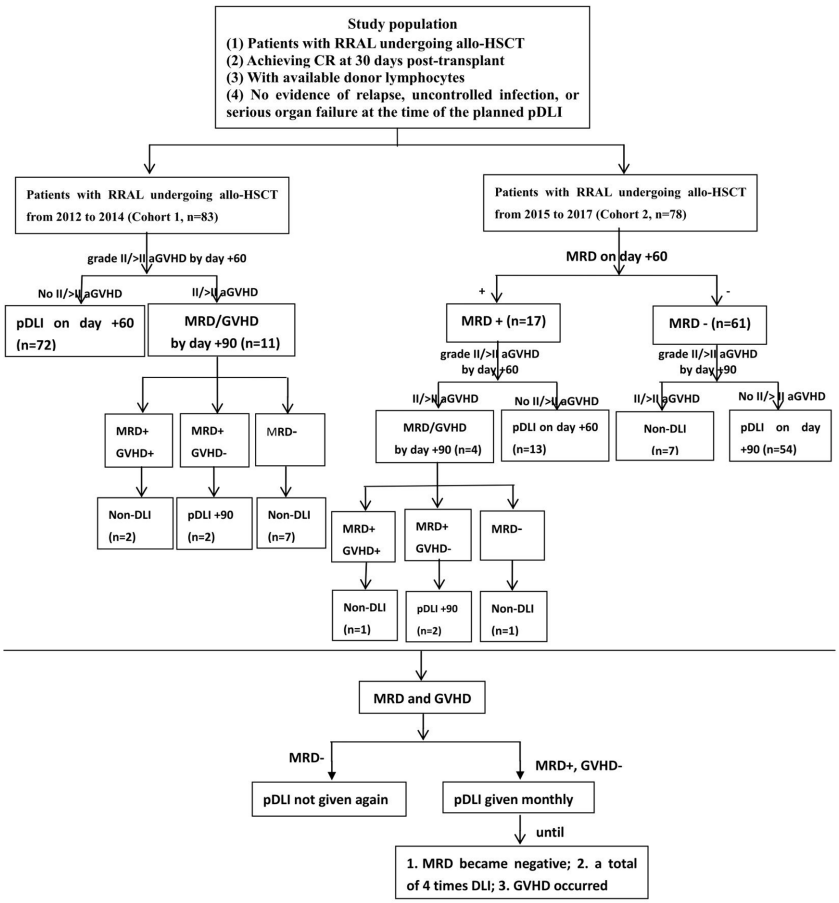

FIGURE 1 | Protocol of two pDLI strategies for patients with RRAL undergoing allo-HSCT. RRAL, refractory/relapsed acute leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation; CR, complete remission; pDLI, prophylactic donor lymphocyte infusion; aGVHD, acute graft-versus-host disease; MRD, minimal residual disease.

leukemia-related genes detected by polymerase chain reaction (PCR) were used for MRD test. FCM positive was defined as >0.01% of cells with leukemia-associated aberrant immune phenotypes. Leukemia-related fusion genes including AML1/ETO, CBFβ/MYH11 and BCR/ABL were tested and the threshold for PCR positivity was ≥ 0.001%. Subjects were scored as MRD positive if they had two consecutive positive results using FCM or PCR or were both FCM and PCR positive in a single BM sample (7, 18).

Evaluation Points and Definitions

The primary endpoint was cGVHD. Secondary endpoints included aGVHD, relapse, overall survival (OS), LFS, GVHD-free/relapse-free survival (GRFS), and non-relapse mortality (NRM). aGVHD and cGVHD were graded as described previously (19, 20). CR was defined as <5% blasts in the BM and no persistence of extramedullary disease. Relapse was defined as reappearance of leukemic blasts in peripheral blood or ≥5% blasts in BM or reappearance or new appearance of extramedullary leukemia. OS was defined as the time from transplantation until death from any cause. LFS was defined as the time from transplantation until relapse or death from any cause. NRM was defined as death from any cause not subsequent to relapse. GRFS was a composite endpoint of allo-HSCT, comprising grade III–IV aGVHD, cGVHD requiring systemic immunosuppressive treatment, NRM and relapse, and represented real recovery after transplantation.

Statistics

Our study data were analyzed on June 30, 2019. Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA) and R version 3.3.0 (R Development Core Team, Vienna, Austria). The chi-square and Mann-Whitney U tests were used for categorical and continuous variables, respectively. OS, LFS, and GFRS were estimated using Kaplan-Meier method and compared using log-rank test. Cumulative incidences of relapse, NRM and GVHD were calculated by accounting for competing risks. Competing risks for GVHD included death without GVHD and relapse. Relapse and NRM were competing risks for each other. The Cox proportional hazards model was used for the analysis of risk factors for time-to-event variables. Strategy, number, and donor source of pDLI were included in the

multivariable analyses for GVHD in pDLI recipients. The following variables were included in the univariable analyses for relapse and survival: gender, patient age, disease category, genetic status, BM blasts on day 0, transplant modality, strategy and number of pDLI, aGVHD, and cGVHD. Only variables with $P < 0.10$ were included in the multivariable analyses for relapse and survival. All statistical tests were two-tailed with a significance level of 0.05.

RESULTS

Patient Characteristics

A total of 161 patients with RRAL undergoing allo-HSCT from January 2012 to December 2017 were eligible for the study, including 69 patients with AML, 76 with ALL, and 16 with ABL. Eighty-three patients undergoing allo-HSCT from January 2012 to December 2014 and adopting previous pDLI strategy were enrolled in cohort 1, and 78 patients who underwent allo-HSCT from January 2015 to December 2017 and adopted modified pDLI strategy were enrolled in cohort 2. There were no significant differences between the two cohorts in sex, age, disease category, genetics, BM blasts at transplantation, transplant modality, and condition of tapering immunosuppressants (all $P > 0.05$) (**Table 1**).

pDLI

Of the 161 patients included, 9 patients in cohorts 1 and 2 did not receive pDLI, respectively. In cohort 1, 74 patients (72 on day +60; 2 on day +90) underwent a total of 112 courses of pDLI, including 47 patients with 1 course, 19 with 2 courses, 5 with 3 courses and 3 with 4 courses, while 69 patients (13 on day +60; 56 on day +90) in cohort 2 received 102 courses of pDLI, including 46 patients with 1 course, 15 with 2 courses, 6 with 3 courses and 2 with 4 courses ($P = 0.764$). The median number of pDLI was 1 (range: 1–4) per patient, with no difference between the two cohorts ($P = 0.170$). The median $CD3^+$ T cells of per pDLI was 3.0 (1.8–5.2) $\times 10^7$/kg and 3.0 (2.0–4.5) $\times 10^7$/kg in cohorts 1 and 2 ($P = 0.317$). In addition, the positive rates of MRD on day +60 and +90 post-transplant in cohort 1 were 19/83 (22.9%) and 10/83 (12.0%), compared with 17/78 (21.8%) and 11/78 (14.1%) in cohort 2 ($P = 0.867$, $P = 0.699$). The leukemia relapse rate from day +60 to +90 had no significant difference between the two cohorts (3.6% vs. 3.8%, $P = 1.000$).

Graft-Versus-Host Disease

The 1-year overall cumulative incidence of grade II–IV aGVHD was 42.2% (95% confidence interval (CI): 31.4%–52.6%) and 37.2% (26.5%–47.8%; $P = 0.635$), and grade III–IV aGVHD was 13.3% (95% CI: 7.0%–21.5%) and 14.1% (7.5%–22.9%; $P = 0.847$) in cohorts 1 and 2, respectively (**Figures 2A, B**). The 2-year extensive cGVHD incidence in cohort 2 [10.3% (95% CI: 4.8%–18.2%)] was lower than that in cohort 1 [27.9% (18.7%–37.9%)] ($P = 0.006$, **Figure 2C**). The 2-year overall cGVHD incidence was 60.2% (95% CI: 48.7%–69.9%) and 52.6% (40.8%–63.0%; $P = 0.232$), and GVHD mortality was 10.8% (95% CI: 5.3%–18.6%) and 5.2% (1.7%–11.8%; $P = 0.183$) in cohorts 1 and 2, respectively (**Figures 2D, E**).

TABLE 1 | Patients' clinical and transplant characteristics.

Patient characteristics	Cohort 1 (n = 83)	Cohort 2 (n = 78)	*P* value
Sex, Female/Male	25 (30.1%) /58 (69.9%)	34 (43.6%) /44 (56.4%)	0.076
Age, median (range), years	30 (12–57)	26 (14–51)	0.328
Disease category			0.215
AML	36 (43.4%)	33 (42.3%)	
ALL	42 (50.6%)	34 (43.6%)	
ABL	5 (6.0%)	11 (14.1%)	
Genetic			0.941
Favorable	4 (4.8%)	4 (5.1%)	
Intermediate	32 (38.6%)	28 (35.9%)	
Unfavorable	47 (56.6%)	46 (59.0%)	
Median BM blasts before conditioning (range)	32.0% (9.0%–91.0%)	35.0% (12.0%–93.0%)	0.725
Donor source			0.431
MSD	48 (57.8%)	39 (50.0%)	
MUD	14 (16.9%)	12 (15.4%)	
HID	21 (25.3%)	27 (34.6%)	
Stem cell source			0.197
PBSCs	62 (74.7%)	51 (65.4%)	
PBSCs + BM	21 (25.3%)	27 (34.6%)	
Median CD34+ cells per graft, 10^6/kg (range)	9.01 (4.79–17.37)	8.64 (5.86–12.00)	0.815
Tapering immunosuppressants			
Withdrawing on day +30	62 (74.7%)	59 (75.6%)	0.890
Discontinuing on day +90	14 (16.9%)	16 (20.5%)	0.553
Discontinuing on day +120	27 (32.5%)	27 (34.6%)	0.779

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; ABL, acute biphenotypic leukemia; BM, bone marrow; MSD, HLA-matched sibling donor; MUD, HLA-matched unrelated donor; HID, HLA-haploidentical donor; PBSCs, peripheral blood stem cells.

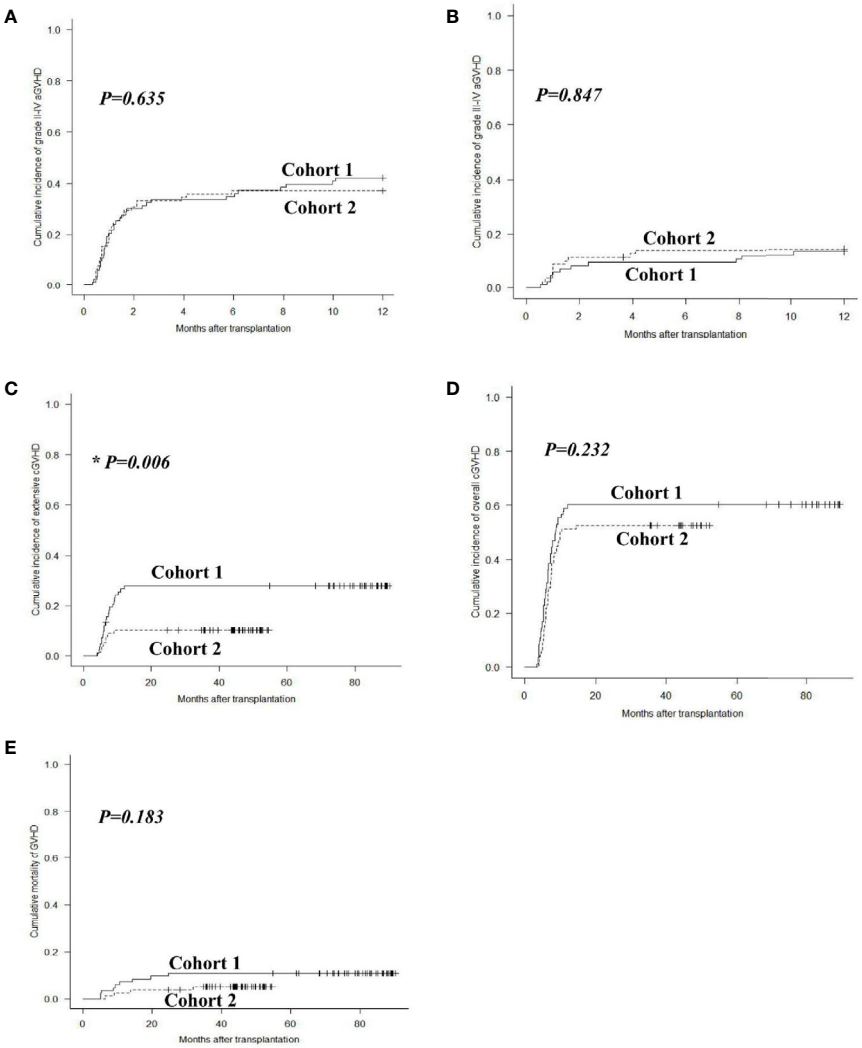

FIGURE 2 | GVHD after allo-HSCT. Cumulative incidences of grade II–IV aGVHD **(A)**, grade III–IV aGVHD **(B)**, extensive cGVHD **(C)**, overall cGVHD **(D)** and mortality of GVHD **(E)** in cohorts 1 and 2.

The incidences of grade II–IV and III–IV aGVHD after pDLI showed no significant differences between the two cohorts (P = 0.428, P = 0.887). The extensive cGVHD incidence after pDLI in cohort 2 was lower than that in cohort 1 (9.0% vs. 28.6%, P = 0.004). The overall cGVHD incidence and GVHD mortality after pDLI were similar between the two cohorts (P = 0.177, P = 0.146). In multivariable analysis, increasing numbers of pDLI predicted higher incidences of grade II–IV and III–IV aGVHD (P = 0.028, hazard risk (HR) = 2.046; P = 0.020, HR = 3.690), and a trend toward a higher incidence of extensive cGVHD (P = 0.054). Additionally, the modified pDLI strategy was associated with a lower risk of extensive cGVHD compared with previous pDLI strategy (P = 0.011, HR = 0.306). Donor source of pDLI was not associated with the incidence of aGVHD or cGVHD (all P > 0.05) (**Table 2**).

Relapse

In cohort 1, 24 patients experienced relapse at a median time of 243 (range: 71 to 1988) days post-transplant, including 17 hematological, 3 extramedullary, and 4 both hematological and extramedullary relapse. In cohort 2, 22 patients relapsed at a median time of 232 (range: 77 to 654) days post-transplant, with 16 hematological, 4 extramedullary and 2 both hematological and extramedullary relapse. The 2-year cumulative incidence of leukemia relapse was 29.0% (95% CI: 19.6%–39.0%) and 28.2% (18.7%–38.5%) in cohorts 1 and 2 (P = 0.986, **Figure 3A**). In multivariable analysis, HID transplant and cGVHD were protective factors for relapse (P = 0.038, HR = 0.476; P = 0.041, HR = 0.526), and the percentage of BM blasts ≥3% on day 0 was the only risk factor for relapse (P = 0.001, HR = 4.340) (**Table 3**).

TABLE 2 | Multivariable analyses for risk factors of GVHD in pDLI recipients.

Parameters	Grade II–IV aGVHD		Grade III–IV aGVHD		Overall cGVHD		Extensive cGVHD	
	Hazard risk (95% CI)	*P* Value	Hazard risk (95% CI)	*P* Value	Hazard risk (95% CI)	*P* Value	Hazard risk (95% CI)	*P* Value
Strategy of pDLI modified vs. previous	0.798 (0.417–1.527)	0.496	1.095 (0.378–3.168)	0.868	0.835 (0.539–1.295)	0.420	0.306 (0.123–0.758)	*0.011
Number of pDLI >1 vs. 1	2.046 (1.079–3.879)	*0.028	3.690 (1.233–11.040)	*0.020	0.894 (0.561–1.425)	0.638	2.597 (0.983–6.866)	0.054
Donor source of pDLI MSD MUD HID	0.894 (0.611–1.307)	0.562	0.712 (0.390–1.300)	0.269	0.978 (0.754–1.269)	0.868	0.862 (0.556–1.337)	0.507

*pDLI, prophylactic donor lymphocyte infusion; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; MSD, HLA-matched sibling donor; MUD, HLA-matched unrelated donor; HID, HLA-haploidentical related donor; CI, confidence interval; *P < 0.05.*

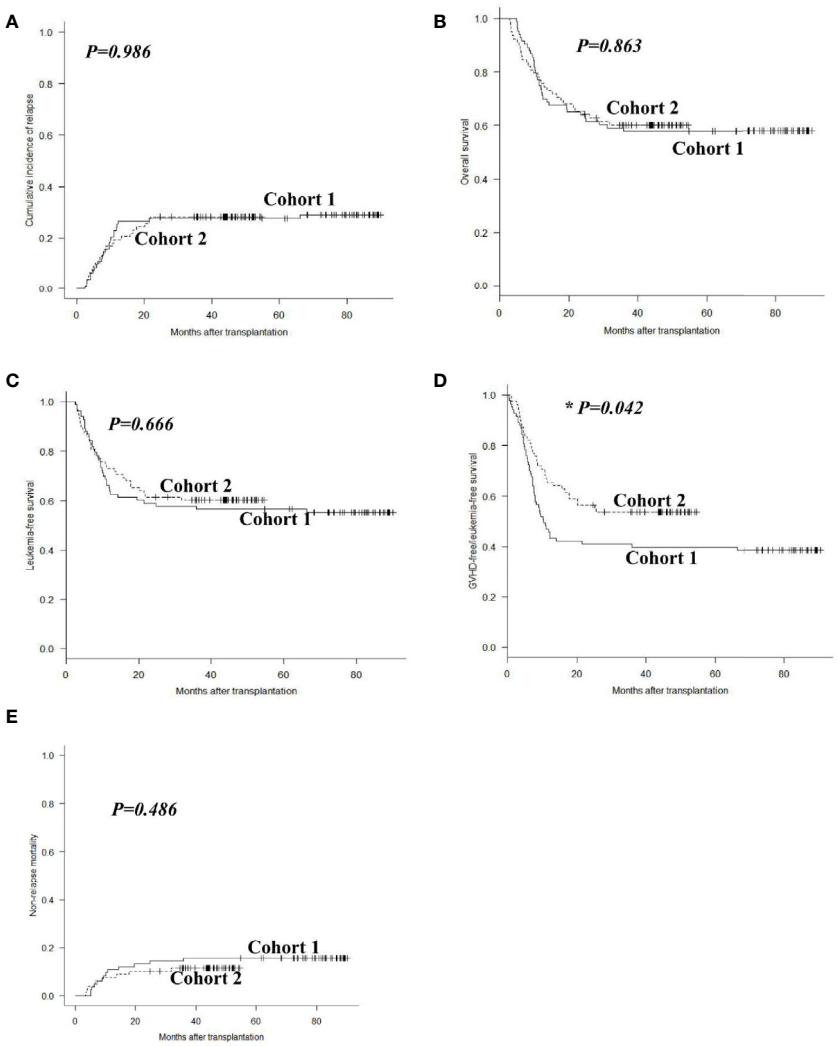

FIGURE 3 | Outcomes after allo-HSCT. Cumulative incidences of relapse **(A)**, overall survival **(B)**, leukemia-free survival **(C)**, GVHD-free/relapse-free survival **(D)**, and non-relapse mortality **(E)** in cohorts 1 and 2.

Survival

In cohort 1, 48 patients survived and 35 died with a median follow-up of 2,164 (range, 148 to 2,712) days post-transplant. Causes of death included leukemia relapse (n = 20), GVHD (n = 10), infections (n = 4), and others (n = 1). In cohort 2, 47 patients survived and 31 died with a median follow up of 1,108 (range, 91 to 1637) days post-transplant. Causes of death included leukemia relapse (n = 20), infections (n = 6), GVHD (n = 4), and others (n = 1). The 2-year OS and LFS were 63.9% (95% CI: 52.5%–73.1%) and 57.8% (46.5%–67.6%) in cohort 1, compared with 64.1% (95% CI: 52.4%–73.6%) and 61.5% (49.8%–71.3%) in cohort 2 ($P = 0.863$, $P = 0.666$, **Figures 3B, C**). However, the 2-year GRFS in cohort 2 was superior to that in cohort 1 (55.1% vs. 41.0%, $P = 0.042$, **Figure 3D**). The 2-year NRM was 13.2% (95% CI: 7.0%–21.5%) and 10.3% (4.8%–18.2%) in cohorts 1 and 2 ($P = 0.486$, **Figure 3E**). Multivariable analysis revealed that cGVHD was the only protective factor for OS and LFS ($P = 0.002$, $HR = 0.454$; $P = 0.010$, $HR = 0.524$), and modified pDLI strategy was the only protective factor for GRFS ($P = 0.010$, $HR = 0.459$). The percentage of BM blasts ≥3% on day 0 was the only risk factor for OS and DFS ($P = 0.001$, $HR = 2.861$; $P = 0.001$, $HR = 3.016$); the percentage of BM blasts ≥3% on day 0 and grade II–IV aGVHD were risk factors for GRFS ($P = 0.001$, $HR = 3.656$; $P = 0.020$, $HR = 1.679$) (**Table 3**).

DISCUSSION

Several studies including ours have shown that pDLI could prevent relapse in patients with RRAL undergoing allo-HSCT (5–8, 21). However, the high morbidity and mortality of GVHD post-pDLI have limited its application (9, 10). The morbidity and mortality of GVHD post-pDLI are related with the time of pDLI post-transplant (5, 11, 13). In this study, we compared the outcomes of two strategies for pDLI based on time from transplant and MRD status post-transplant in patients with RRAL undergoing allo-HSCT. Our results revealed that delaying pDLI time to day +90 based on MRD could lower extensive cGVHD incidence and improve GRFS without increasing incidence of leukemia relapse.

Currently, timing of pDLI is typically based on post-transplant MRD status (5, 6, 22, 23). For patients at high risk of relapse, some centers including ours have conducted pDLI without considering MRD status (7, 21, 24). Schmid et al. adopted

TABLE 3 | Univariable and multivariable analyses for risk factors of relapse and survival.

Parameters	Relapse		Overall survival		Leukemia-free survival		GVHD-free/relapse-free survival (GRFS)	
	Univariable *P* value	Multivariable *P* value; HR (95% CI)	Univariable *P* value	Multivariable *P* value; HR (95% CI)	Univariable *P* value	Multivariable *P* value; HR (95% CI)	Univariable *P* value	Multivariable *P* value; HR (95% CI)
Female vs. male	0.160	–	0.866	–	0.627	–	0.283	–
Patient age ≥29 vs. <29 years (median)	0.207	–	0.133	–	0.172	–	0.111	–
Disease category AML vs. ALL vs. ABL	0.644	–	0.759	–	0.657	–	0.736	–
Genetic status Other vs. unfavorable	0.201	–	0.451	–	0.318	–	0.253	–
BM blasts on day 0 ≥3% vs. <3% (median)	*0.001	*0.001; 4.340 (2.359–7.987)	*0.001	*0.001; 2.861 (1.744–4.693)	*0.001	*0.001; 3.016 (1.843–4.936)	*0.001	*0.001; 3.656 (2.328–5.743)
Transplant modality HID vs. MSD/ MUD	*0.040	*0.038; 0.476 (0.237–0.959)	0.125	–	0.061	0.076; 0.610 (0.354–1.053)	0.222	–
Strategy of pDLI modified vs. previous	0.456	–	0.863	–	0.666	–	*0.044	*0.010; 0.459 (0.292–0.722)
Number of pDLI 0 vs. 1 vs. >1	0.552	–	0.514	–	0.375	–	0.776	–
aGVHD II–IV vs. 0–I	0.359	–	* 0.012	0.061; 1.604 (0.978–2.630)	*0.023	0.096; 1.517 (0.928–2.480)	*0.009	*0.020; 1.679 (1.086–2.596)
cGVHD vs. No cGVHD	*0.030	*0.041; 0.526 (0.294–0.939)	*0.002	*0.002; 0.454 (0.279–0.739)	*0.005	*0.010; 0.524 (0.321–0.855)	0.677	–

*AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; ABL, acute biphenotypic leukemia; BM, bone marrow; HID, HLA-haploidentical related donor; MSD, HLA-matched sibling donor; MUD, HLA-matched unrelated donor; pDLI, prophylactic donor lymphocyte infusion; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; HR, hazard risk; CI, confidence interval; *P < 0.05.*

the strategy of intensive chemotherapy, reduced-intensity conditioning and pDLI from day +120 in 12 patients with high-risk AML and myelodysplastic syndrome, with incidences of relapse and GVHD of 16.7% and 33.3% (21). Huang et al. demonstrated that pDLI was given at the median of 70 (range, 20–314) days post-transplant in 33 patients with advanced leukemia, with incidences of relapse and cGVHD of 45.5% and 62.5%, respectively (24). However, optimal timing of pDLI is uncertain. We previously adopted the strategy of pDLI on day +60 regardless of MRD test and then based on MRD and GVHD status from day +90 post-transplant in patients with RRAL, which was demonstrated to reduce relapse rate and improve survival (7). Nevertheless, the high incidence of extensive cGVHD after pDLI hindered survival and quality of life of patient (7). Consequently, in order to reduce the morbidity and mortality of GVHD, we modified our strategy of pDLI by postponing the infusion time to day +90 unless MRD was positive on day +60 and compared with previous pDLI strategy. Our results revealed that pDLI on day +90 post-transplant had a lower incidence of extensive cGVHD (10.3% vs. 27.9%) and superior GRFS (55.1% vs. 41.0%) than pDLI on day +60.

Except for the time interval from transplant to pDLI, other factors might also influence the morbidity and mortality of GVHD after pDLI such as the doses, HLA compatibility and donor source of pDLI (11, 12, 25). In general, risk of GVHD is lower in patients receiving pDLI from MSD, and higher in those receiving pDLI from MUD or HID (26, 27). However, some domestic studies including ours have shown that there are no significant differences in the morbidity and mortality of GVHD between patients receiving G-CSF-mobilized pDLI from MSD and HID (5, 28). It might be due to that the use of G-CSF might modulate polarization of T cells from Th1 to Th2 phenotype and indirectly induce T cell hypo-responsiveness and down-regulation of co-stimulatory signal of CD28/B7 (29, 30). In this study, we also observed that the morbidity and mortality of GVHD did not differ in the patients receiving pDLI from MSD and HID, which was consistent with our former finding (7).

Relapse is the major cause of death in patients with RRAL following transplant. Recently, some studies showed that the strategy of sequential intensified conditioning followed by pDLI could reduce leukemia relapse in patients with RRAL undergoing allo-HSCT (7, 21, 31). Schmid et al. reported that a sequential regimen of Flu/Ara-c/amsacrine chemotherapy and reduced-intensity conditioning along with immunosupressant withdrawal and pDLI were used for refractory AML undergoing allo-HSCT, with 2-year OS and leukemia mortality of 40.0% and 39.3% (21). In this study, we adopted the strategy of Flu/Ara-C salvage chemotherapy and TBI/CY/VP-16 myeloablative conditioning followed by early rapid tapering of immunosuppressant and modified pDLI, with 2-year OS and relapse rate of 64.1% and 28.2%. The favorable efficacy might be attributed to two aspects: salvage chemotherapy and myeloablative conditioning regimen decreased leukemia burden at the time of transplantation; early tapering of immunosuppressant combined with pDLI accelerated the GVL effect. In addition to disease status pre-transplant, genetics was another major cause of relapse post-transplant (32–34). Interestingly, in this study, unfavorable genetics was not a risk factor for relapse, which might be due to the fact that only patients with RRAL were enrolled in this study and most of them were accompanied by unfavorable genetics. Moreover, we also found that HID transplant was the protective factor for relapse, which was coherent with other studies (25, 35, 36).

There were some limitations in this study. Although this study was based on two prospective cohorts, they were non-parallel, which could not exclude the influence of factors such as improvement in medical technology and supportive treatment. Besides, no randomized studies have shown that pDLI is superior to non-pDLI. Therefore, large-scale and randomized controlled trials are needed to validate outcomes of patients undergoing non-pDLI and different pDLI strategies.

In conclusion, our study demonstrated that delaying pDLI to day +90 based on MRD can lower extensive cGVHD incidence and improve GRFS without increasing incidence of leukemia relapse for patients with RRAL undergoing allo-HSCT. This finding provides evidence for exploring optimal timing of pDLI in patients with RRAL undergoing allo-HSCT.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Nanfang Hospital-Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

QS, QL, and LX wrote the report and did the analysis. QL, LX, DN, DL, ZG, and ZJ designed the protocol. All authors contributed patients, provided clinical data, and revised and corrected the report. QL, DN, DL, ZG, and ZJ approved and recommended the protocol within each institute. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Asymmetric Bone Marrow Involvement in Patients With Acute Leukemia After Allogeneic Hematopoietic Stem Cell Transplantation

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After allogeneic hematopoietic stem cell transplantation (allo-HSCT), acute leukemia relapse is common, and asymmetric bone marrow recurrence hasn't been reported. Because the anatomical distribution of acute leukemia clones in the bone marrow after allo-HSCT is presumed to be diffuse, bone marrow aspirations are performed in single site. The first case was a 20-year-old man who was diagnosed with acute myelomonocytic leukemia and received haploidentical allo-HSCT. Routine bone marrow biopsy of his left posterior iliac bone marrow showed 52% leukemia blasts, while the right side had 0% blasts 10 days later. The second case was a 23-year-old woman who was diagnosed with acute B lymphoblastic leukemia and received HLA-identical sibling allo-HSCT. Although 62% of blasts were found in her left iliac marrow on day +122, 0% of blasts were found on a sample obtained from the right iliac crest on day +128. Bilateral iliac bone marrow pathology and whole-body ^{18}F-FDG PET/CT scans confirmed that the leukemic infiltration in her bone marrow was asymmetric. To our knowledge, these are the first case reports of asymmetric bone marrow infiltration of blasts in acute leukemia patients after allo-HSCT. Bilateral posterior iliac crest aspirations or ^{18}F-FDG-PET/CT scans may help distinguish such involvement.

Keywords: asymmetric, blasts, relapse, acute leukemia, allogeneic hematopoietic stem cell transplantation, ^{18}F-FDG-PET/CT, case report

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative option for patients with acute leukemia. Post-HSCT recurrence still represents the major cause of treatment failure and up to 50% of acute leukemia patients will relapse (1). Periodic bone marrow aspiration is performed to monitor for early relapse (2). Although the anatomical distribution of acute leukemia

TABLE 1 | The aspiration results of case one during follow-up.

NO.	Date	Morphology (%blasts)	MFC (%blasts)	STR (% recipient)	Status
Case 1	April 20–21,2016				HSCT
	November 16, 2017	0	0	0	Right
	Mar 7, 2018	52	6.58	N	Left
	Mar 17, 2018	0	0	0	Right
	Mar 27, 2018	52	23.4	N	Left

STR, short tandem repeat mismatches; N, no detection; Right, aspirates from the right posterior iliac crest; Left, aspirates from the left posterior iliac.

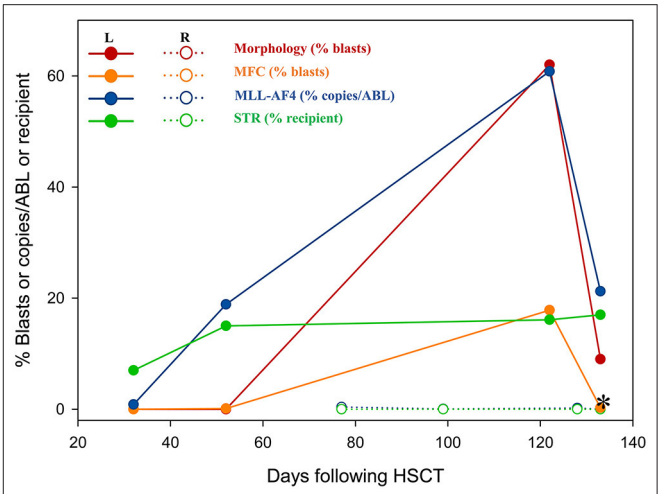

FIGURE 1 | The residual disease was detected by morphology, MFC, RT-PCR and STR. *, the day +133 for a bilateral posterior iliac crest bone marrow biopsy. STR, short tandem repeat mismatches; R, aspirates from the right posterior iliac crest; L, aspirates from the left posterior iliac.

clones after allo-HSCT has not been studied (3), blasts are generally considered to be uniformly infiltrated throughout the bone marrow system. Bone marrow aspirations are performed in one site for patients with acute leukemia (4). For patients with relapse or suspected relapse, aspirations may be conducted at 0.5 to 3-month intervals according to different therapy protocols or attending physician preference.

In serial aspirations, it is not uncommon to see inconsistent residual disease burden in different iliac crests. Differences are usually attributed to providers' operational errors, blood-diluted bone marrow, incorrect enumeration by pathologists, incorrect machine measurement, or variable graft vs. leukemia (GVL) response. However, the following two cases reveal a rare cause of inconsistent bone marrow aspiration results. All aspirations were performed on the posterior iliac crests according to published protocols and were conducted by the same providers (5).

MANUSCRIPT FORMATTING

Case Description

Case 1

In August 2015, a 20-year-old man was diagnosed as acute myelomonocytic leukemia. The cytogenetic risk stratification was intermediate. His bone marrow showed complete remission (CR) after two courses of induction. Following three courses of consolidation, granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood stem cell (PBSC) from his human leukocyte antigen (HLA) 4/6 matched father was infused on April 20–21, 2016. The conditioning regimen consisted of decitabine (20 mg/m2/day, −11 to −7), cytarabine (3 g/m2/day, −9 to −7), busulfan (3.2 mg/kg/day, −5 and −4), cyclophosphamide (1.8 g/m2/day, −3 and −2). A regimen of tacrolimus, short-term methotrexate (MTX) and mycophenolate mofetil was given for graft vs. host disease (GVHD) prophylaxis. His neutrophils and platelets were engrafted on day +10 and +13, respectively. On day +30, his bone marrow showed CR and complete donor chimerism, and the results were normal until November 16, 2017. Because of repeated mild liver chronic GVHD, tacrolimus was not withdrawn until day +350. Despite no physical symptoms and normal peripheral blood counts, his left posterior iliac bone marrow aspirate smear revealed a blast percentage of 52% during a routine follow-up on Mar 7, 2018. The blasts expressed CD34 dim, CD117, CD33 strong, HLA-DR, CD13, and did not express CD7, CD19, CD56, CD11b. The immunophenotype was the same as the first diagnosis. He declined all treatment recommendations and insisted on repeating bone marrow aspiration as soon as possible. Ten days later, as shown in **Table 1**, sampling of the right posterior bone marrow aspirates not only showed a blast percentage of 0%, but also a recipient chimerism percentage of 0%. No pathologic genetic abnormalities or meaningful mutations were found. The patient and his parents declined further treatment and only agreed to reexamination after 2 weeks. Two weeks later, with normal peripheral blood cells, the blast percentage of his left posterior iliac bone marrow smear was still 52%. The blasts expressed the same immunophenotype as before. No blast forms were seen in the peripheral blood smear. Unfortunately, due to the inconsistent results, the patient declined further intervention and died of high leukocyte syndrome 4 months later.

Case 2

In July 2017, a 23-year-old woman was diagnosed with acute B lymphoblastic leukemia with t (4, 11) (q13; q21). Bone marrow aspirate smear from her right posterior iliac crest revealed the presence of 85.5% blasts, and reverse transcription polymerase chain reaction confirmed the presence of *MLL-AF4* fusion transcripts. After a cycle of induction chemotherapy, her bone

Abbreviations: Allo-HSCT, allogeneic hematopoietic stem cell transplantation; GVL, graft vs. leukemia; CR, complete remission; G-CSF, granulocyte-colony stimulating factor; PBSC, peripheral blood stem cell; HLA, human leukocyte antigen; MTX, methotrexate; GVHD, graft vs. host disease; DLI, donor lymphocyte infusion; 18F-FDG-PET/CT, fluorine-18-fluorodeoxyglucose positron emission tomography/computed tomography; SUVmax, maximal Standardized uptake value; MRD minimal residual disease; MFC, multi-parameter flow cytometry; ALL acute lymphoblastic leukemia.

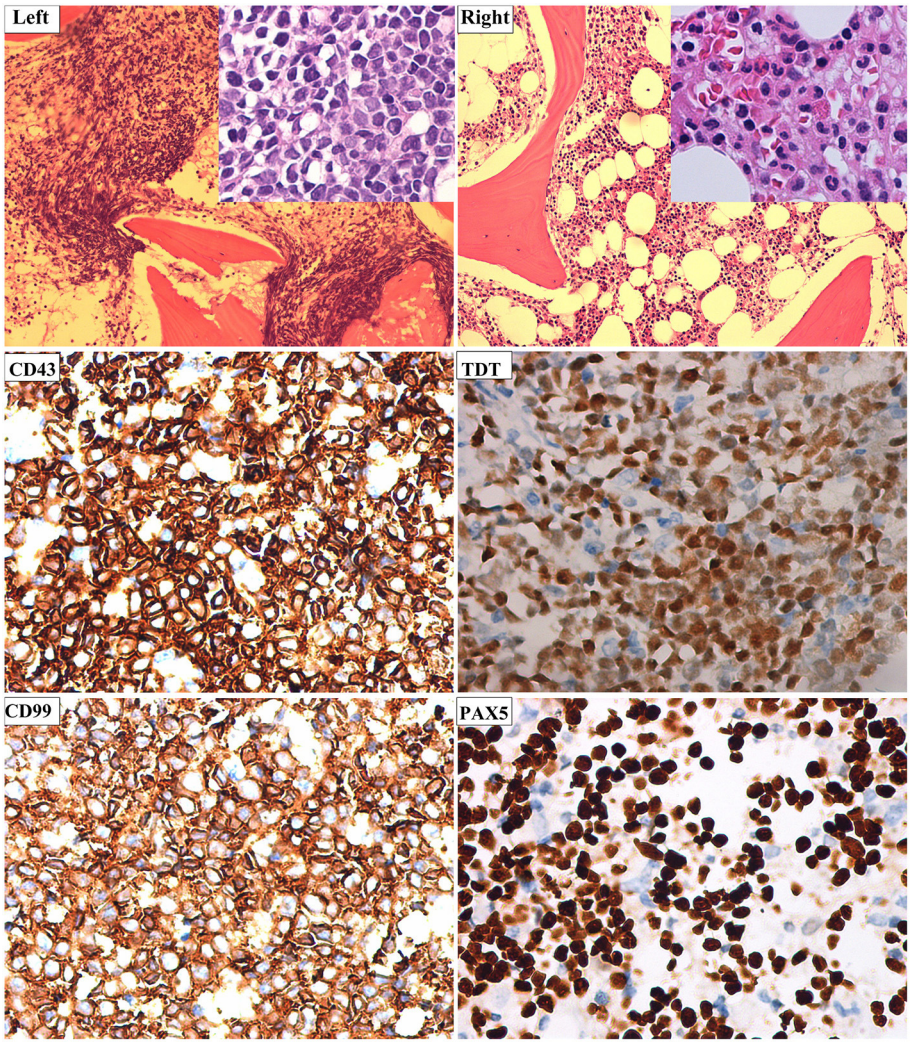

FIGURE 2 | The bone marrow biopsy was performed in bilateral posterior iliac crests. **Left**, left posterior iliac bone marrow (hematoxylin and eosin, ×100) was filled with B lymphocyte leukemia cells (inset, ×400); **Right**, right posterior iliac bone marrow (hematoxylin and eosin, ×100) was filled with normal cells (inset, ×400). Immunohistochemical studies showed that CD43, TDT, CD99, and PAX 5 were positive in the left posterior iliac bone marrow cells (×400).

marrow showed CR. Following two cycles of consolidation, she received G-CSF-mobilized PBSC from her HLA-identical sister on December 9–11, 2017. The conditioning regimen consisted of busulfan (3.2 mg/kg/day, −9 to −7), etoposide (10 mg/kg/day, −6 to −4) and cyclophosphamide (60 mg/kg/day, −3 and −2). Cyclosporine and short-term MTX were used to prevent GVHD. Her neutrophils and platelets engrafted on day +9 and +11, respectively. The bone marrow aspiration schedule is shown in **Figure 1**. On day +30, the bone marrow specimens from her left posterior iliac crest showed 3–9% recipient chimerism and low copy of *MLL-AF4* transcript (0.86%, %copies/*ABL*). The *MLL-AF4* copies almost increased 20 times to 18.87% on day +51. Oral cyclosporine was tapered quickly and discontinued on day +77, followed by stage 2 acute GVHD of skin. Biopsy of her right posterior iliac crest bone marrow showed two times full donor chimerism and the copy of *MLL-AF4* was decreased to 0.05% on day +100. Unfortunately, on day +122, up to 62% of blasts were found in her left posterior iliac crest bone marrow smear and no blast forms were seen in the peripheral blood smear. However, on day +128, the blast percentage was 0% in the right (**Figure 1**). On day +133, a bone marrow biopsy from bilateral posterior iliac crests showed that the left bone marrow was replaced with lymphoblasts and the right was normal (**Figure 2**). Immunohistochemical studies showed that the lymphoblasts expressed CD43, TDT, CD99 and PAX5, and did not express CD3, CD20, MPO, CD34, CyclinD1 and Ki67 (**Figure 2**). Facing this rare leukemia progression, the patient and her donor agreed to pursue chemotherapy combined with donor lymphocyte infusion (DLI) as soon as possible. In order to evaluate the distribution of

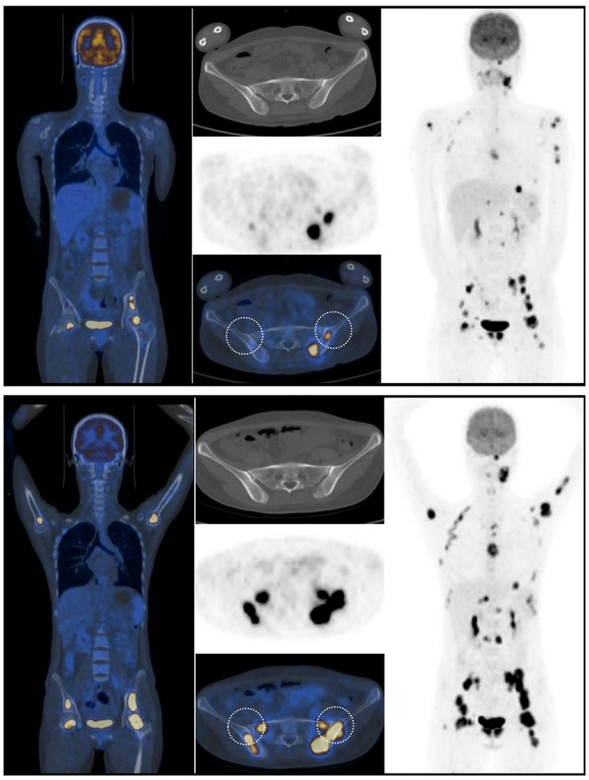

FIGURE 3 | ^{18}F-FDG-PET/CT scans showed that asymmetrical hypermetabolic foci were noted across the bone marrow system. **(A)** on the day +143. **(B)** on the day +203. Both aspirations and biopsies were performed in the circle areas.

the disease before treatment, fluorine-18-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG-PET/CT) was performed on day +143. Unexpectedly, asymmetric metabolic abnormalities were found throughout the bone marrow system, and there was no corresponding anatomical change on CT imaging (**Figure 3A**). The maximal Standardized uptake value (SUV_{max}) of left rambus mandibulae, humerus and ilium were up to 18.5, 18.4 and 21, respectively. Considering the higher leukemic burden in the left, we chose the left posterior iliac crest site for further response assessment. There was no apparent active GVHD, she achieved transient hematologic CR after chemotherapy combined with DLI. On day +203, subsequent ^{18}F-FDG-PET/CT scan revealed similar metabolic abnormalities in the bone marrow system (**Figure 3B**). On day +229, an aspiration from bilateral posterior iliac crests showed that the blast percentage of the left bone marrow smear was 62% and the right was 3.5%. Unfortunately, she died of septic shock with heart failure on day +258.

DISCUSSION

Given the well-known localized infiltrating characteristics, chronic lymphoid leukemia, lymphoma, multiple myeloma, and neuroblastoma, bilateral bone marrow aspiration and/or biopsies are usually performed for staging. Acute leukemia is characterized by a uniform bone marrow infiltration of leukemic cells (6) so bone marrow aspirations in acute leukemia patients are always performed on a unilateral iliac crest post-HSCT. Compared with classical morphology enumeration methods, minimal residual disease (MRD) detecting tools such as multi-parameter flow cytometry (MFC), cytogenetics or molecular studies are far more sensitive to detect leukemic blasts. Tetsuo Maeda and colleagues reported a case that presented as an apparent discrepancy in the DEK-CAN fusion transcript levels between the left and right iliac bone marrow sites during hematological CR, and attributed it to anatomic differences in the potency of the GVL response of DLI (7). As shown in the **Table 1**, for our patient, unlike the left ilium where a high burden of leukemic cells was detected, the immunophenotype, cytogenetics and recipient chimerism of the right ilium were completely negative. The patient had been off immunosuppression agents for more than a year with no symptoms of GVHD, and he refused all therapies during the three bone marrow aspirations in a month. Although we did not perform concurrent bilateral bone marrow aspirations/biopsies, case 1 reminds us that patients without immune antitumor effects may develop different leukemia reservoirs in the bone marrow system.

Because MRD was detected early after allo-HSCT, cyclosporine was rapidly reduced and discontinued on day +77 in case 2. On day +100, the active GVL effect appeared to significantly control the MRD of her right posterior iliac bone marrow. Due to repeated right posterior iliac crest aspirations, completely wrong conclusions were almost drawn. On day +122, the blast percentage of her left posterior iliac crest bone smear had increased to 62%, while on the day +128, the right still showed CR. A subsequent bone marrow biopsy from bilateral posterior iliac crests revealed that blasts almost completely infiltrated only in the left. This case reminds us that unilateral/single-site bone marrow aspiration may have deficiencies in the detection of residual disease or the evaluation of treatment effects. However, the number of cases is too small to draw convincing conclusions.

Two whole-body ^{18}F-FDG-PET/CT scans showed that hypermetabolism in the bone marrow system was significantly asymmetric. There is no consensus on the diagnostic and predictive value of PET/CT for intramedullary acute leukemia. Several papers reported that PET/CT incidentally detected acute lymphoblastic leukemia (ALL) (8–11), and a few studies have reported the evaluation and predictive value of PET/CT in patients with leukemia (12, 13). The prospective PETAML trial reported that the specificity of ^{18}F-FDG-PET/CT for diagnosis of extramedullary acute myeloid leukemia was 97%, despite hematological remission (14). In a retrospective study of 28 children suspected of leukemia progression or recurrence during / after chemotherapy or allo-HSCT, 14 cases with positive ^{18}F-FDG-PET/CT scans were associated with increased blasts in bone marrow biopsies, and the mean SUV_{max} was significantly higher than what is seen with infectious diseases (15). In addition, G-CSF induced high uptake of FDG in bone marrow system

was always diffusely distributed (16, 17). In the second case, the adjacent bilateral bone biopsy (day +133) and PET/CT scan (day +143) demonstrated that the focal bone marrow hypermetabolism of ^{18}F-FDG was caused by the asymmetric distribution of the blasts.

In 1972, a $3\frac{1}{2}$ year-old-boy was diagnosed with ALL in the St. Louis Children's Hospital. The bone marrow of both iliac crests was found to be replaced with lymphoblasts, however, a bone marrow aspirate from the spinous process of the first lumbar vertebra was almost normal (18). Such morphologic discordance reminds us that leukemia cells do not always evenly infiltrate through the bone marrow system. Endo, T. and colleagues occasionally found the first case of localized relapse of ALL in bone marrow extremities after allo-HSCT because of extremities pain (19). Golembe, B. and colleagues found discordant bone marrow specimens in an 11-year-old ALL patient who had been in complete remission for 6 years and off chemotherapy for $2\frac{1}{2}$ years. Three months later, bone marrow samples of three sites showed leukemic involvement (20). The final hematology relapse of the case and our case 1 indicates that there may be a precursor state of relapse in focal bone marrow sites before general relapse. To the best of our knowledge, with the combination of bilateral bone marrow aspiration/biopsy and whole body ^{18}F-FDG-PET/CT, our case report first illustrates the asymmetric bone-marrow infiltration of leukemic cells after allo-HSCT. Bone marrow aspirations were performed more frequently than usual, which may be why these incredible results were observed. However, the underlying mechanism and the exact interval between the asymmetric bone marrow recurrence and the subsequent systemic bone marrow relapse need to be confirmed by further studies.

CONCLUSIONS

Because the number of cases is still too small, it is not appropriate to perform bilateral posterior iliac crest aspiration or ^{18}F-FDG-PET/CT scan for every acute leukemia patient after allo-HSCT. However, if discordant bone marrow specimens are observed, providers need to consider these rare cases in addition to the quality control issues of bone marrow aspiration. ^{18}F-FDG-PET/CT scans or bilateral posterior iliac crest aspirations may help distinguish the asymmetric bone marrow distribution of blasts, and then further aspirations should be conducted on the side with more blasts to avoid inaccurate efficacy assessments.

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.

ETHICS STATEMENT

The work has been evaluated and approved by the ethical committee of Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HY, WS, YW, ZZhon, and YY obtained and analyzed the clinical data. ZZhou and XX made the figures. JY and LX performed the MFC and RT-PCR. WC provided morphology analysis. All authors contributed to caring for the patient, editing the figures, and writing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.626018/full#supplementary-material

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G-CSF-Primed Peripheral Blood Stem Cell Haploidentical Transplantation Could Achieve Satisfactory Clinical Outcomes for Acute Leukemia Patients in the First Complete Remission: A Registered Study

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G-CSF-mobilized peripheral blood (G-PB) harvest is the predominant graft for identical sibling donor and unrelated donor allogeneic hematopoietic stem cell transplantation (HSCT) recipients, but it was controversial in haploidentical related donor (HID) HSCT. In this registry study, we aimed to identify the efficacy of HID G-PB HSCT (HID-PBSCT) for acute leukemia (AL) patients in first complete remission (CR1). Also, we reported the outcomes for the use of G-PB grafts in comparison with the combination of G-BM and G-PB grafts in HID HSCT recipients. Sixty-seven AL patients in CR1 who received HID-PBSCT were recruited at Institute of Hematology, Peking University. Patients who received haploidentical HSCT using the combination of G-BM and G-PB harvests in the same period were enrolled as controls (n=392). The median time from HSCT to neutrophil and platelet engraftment was 12 days (range, 9–19 days) and 12 days (range, 8–171 days), respectively. The 28-day cumulative incidence of neutrophil and platelet engraftment after HSCT was 98.5% and 95.5%, respectively. The cumulative incidences of grade II–IV and grade III–IV acute graft-versus-host disease (GVHD) were 29.9% (95%CI 18.8–40.9%) and 7.5% (95%CI 1.1–13.8%), respectively. The cumulative incidences of total and moderate-severe chronic GVHD were 54.9% (95%CI 40.9–68.8%) and 17.4% (95%CI 6.7–28.0%), respectively. The cumulative incidences of relapse and non-relapse mortality were 13.9% (95%CI 5.4–22.5%) and 3.4% (95%CI 0–8.1%), respectively. The probabilities of overall survival (OS) and leukemia-free survival (LFS) were 84.7% (95%CI 74.7–94.7%) and 82.7% (95%CI 73.3–92.1%) respectively. Compared with the HID HSCT recipients using the combination of G-BM and G-PB

grafts, the engraftments of neutrophil and platelet were both significantly faster for the G-PB group, and the other clinical outcomes were all comparable between the groups. In multivariate analysis, graft types did not influence the clinical outcomes. Overall, for the patients with AL CR1, G-PB graft could be considered an acceptable graft for HID HSCT recipients. This study was registered at https://clinicaltrials.gov as NCT03756675.

Keywords: haploidentical donor, acute leukemia, stem cell transplant (SCT), peripheral blood (PB), complete remission (CR)

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the most important curative option for patients with acute leukemia (AL). The graft was one of the critical factors for allo-HSCT. Both peripheral blood (PB) and bone marrow (BM) harvests could be used as the graft sources, and cord blood cells could also be used as the graft source. Many studies had compared the clinical outcomes between patients using PB and BM grafts. In patients with human leukocyte antigen identical sibling donors (ISDs), engraftment was faster (1–3), the relapse rate was lower (4), and the leukemia-free survival (LFS) rate was better in the PB group compared with the BM group, particularly for the patients with advanced stage disease (1, 4). Similar results were also observed in patients with HLA-unrelated donors (URDs) (5–7). Considering the fact that PB stem cells (PBSCs) collection is a non-surgical procedure, PBSC transplantation (PBSCT) is more convenient and more acceptable for donors. Thus, PB is the predominant graft source for ISD and URD HSCT. Haploidentical related donors (HIDs) have become the most important alternative donors; however, whether the PB graft is suitable for haploidentical HSCT is controversial. In the HID HSCT regimen using post-transplant cyclophosphamide (PTCY), several prospective studies compared the clinical outcomes between PB grafts and BM grafts. Engraftment was also significantly faster in the PB group; but the difference of the GVHD rates between PB and BM groups was not as significant as those of ISD and URD HSCT recipients. Some studies observed that the LFS rates were significantly poorer in the PB group compared with BM group (8–10); however, the other studies observed that LFS rates of PB group were better than those of BM group (11–13).

Another important HID HSCT regimen was "Beijing protocol", which proposed by Peking University Institute of Hematology and based on antithymocyte globulin (ATG) (14). "Beijing protocol" had become the most common transplant regimen for HID HSCT in China (15–17). G-CSF primed BM (G-BM) plus G-CSF primed PB (G-PB) harvests were most commonly used in this transplant protocols, but several studies also identified the feasibility of using G-PB harvest alone. Some authors reported that the clinical outcomes of HID HSCT recipients receiving G-PB grafts were satisfactory, however, they were retrospective, single-arm designed studies (18, 19). In a retrospective single-center study, Xu et al. (9) compared the outcomes between patients using G-BM plus G-PB harvests and G-PB alone as grafts in advanced stage [i.e., most of them were beyond the third complete remission (CR3) or in non-remission] AL patients receiving haploidentical HSCT. G-PB group showed no superiority in engraftment compared with G-BM plus G-PB group. In addition, the transplant-related mortality (TRM) was significantly higher and LFS was poorer in G-PB group compared with the G-BM plus G-PB group. In a retrospective multi-center study including all types of hematologic malignancies, Zhao et al. (8) reported that the survival of G-PB groups was poorer than that of the G-BM plus G-PB group. However, this study did not compare the clinical outcomes of G-PB group and G-BM plus G-PB group in AL patients, and the center effect could not be totally excluded either. Thus far, there was no prospective registry study identifying the efficacy of PBSCT in ATG-based HID HSCT. In addition, no prospective study had directly compared the clinical outcomes between G-BM plus G-PB and G-PB alone in AL-CR1 patients receiving HID HSCT. Thus, the role of HID PBSCT in AL-CR1 patients was still unclear.

In the present registry study, we aimed to identify the clinical outcomes of HID PBSCT in AL patients in CR1. We also aimed to compare the clinical outcomes between G-PB alone and G-BM plus G-PB in HID HSCT recipients.

PATIENTS AND METHODS

Study Design

Sixty-seven AL patients in CR1 who received HID PBSCT were recruited in this prospective study at the Peking University People's Hospital between November 1, 2018, and February 29, 2020. All cases were treated according to the protocol registered at https://clinicaltrials.gov (NCT03756675). The recipients receiving HID HSCT using the combination of G-BM and G-PB harvests (i.e., BM+PB group) in the same period were collected as controls.

The inclusion criteria: 1) patients aged 2–60 years old; 2) in AL CR1; 3) donors refused the donation of BM; and 4) patients agreed to receive haploidentical PBSCT (**Figure 1**).

The primary endpoint was engraftment rates as defined by neutrophil recovery and platelet recovery. The secondary endpoints include acute graft-versus-host disease (aGVHD), chronic GVHD (cGVHD), relapse, non-relapse mortality (NRM), leukemia-free survival (LFS), and overall survival (OS).

Transplant Protocols

Conditioning regimens, immunosuppressants, and supportive care have been described in previous studies (20–22). The myeloablative busulfan (BU)-based regimen consisted of (1) cytarabine 4 g/m^2 for 2 days, busulfan 3.2 mg/kg for 3 days, cyclophosphamide 1.8 g/m^2

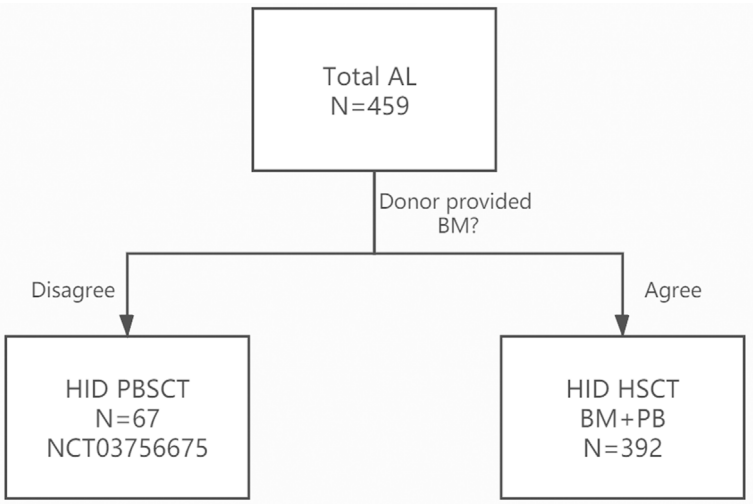

FIGURE 1 | CONSORT (the Consolidated Standards of Reporting Trials) Flow Diagram Showing the Study Design of the trial.

for 2 days, rabbit anti-thymoglobulin 2.5 mg/kg for 4 days, and semustine 250 mg/m^2 orally for one dose; or (2) cytarabine 4 g/m^2 for 2 days, busulfan 3.2 mg/kg for 3 days, cyclophosphamide 1.0 g/m^2 for 2 days, fludarabine 30mg/m^2 for 5 days, rabbit anti-thymoglobulin 2.5 mg/kg for 4 days, and semustine 250 mg/m^2 orally for one dose. Five patients received total body irradiation (TBI)-based conditioning. The immunosuppressants included cyclosporine A (CsA), mycophenolate mofetil (MMF), and short-term methotrexate (MTX). G-CSF was administered subcutaneously to patients at 5 ug/kg per day from day +6 until myeloid recovery (23–25).

Donor Specific Antibodies

Patients were tested for the presence of donor-specific antibodies (DSAs) including class I (i.e., HLA-A, -B, -C) and class II (i.e., HLA-DR) HLA antibodies. Immunoglobulin anti-HLA reactivity in the serum was tested with a bead-based screening assay. Briefly, we used the LABScreen Mixed kit (One Lambda, Canoga Park, CA, USA), which simultaneously detects class I and class II antibodies with microbeads coated with purified class I and class II HLA antigens. For HLA antibody-positive samples with a median fluorescent intensity (MFI) >500, DSAs were further tested using a LABScreen Single Antigen Kit (One Lambda). Above a cut-off value of MFI ≥2000 was considered positive. Patients with positive DSA received rituximab before transplantation, and the co-infusion of umbilical cord blood (26).

Definitions

The neutrophil engraftment was defined as the first of 3 consecutive days that neutrophils ≥0.5×10^9/L, and platelet engraftment was defined as the first of 7 consecutive days that platelets ≥20×10^9/L and transfusion independence. Relapse was defined as BM blasts >5%, or extramedullary manifestation. NRM was defined as death without evidence of leukemia. OS was the period between the date of HSCT and death. LFS was the period between the date of HSCT and relapse or death in remission. GVHD was diagnosed and graded according to internationally accepted criteria (27, 28).

Statistical Analysis

The last follow-up date was September 1, 2020. Survival was estimated with Kaplan-Meier outcome curves. The cumulative incidences of engraftment, relapse, GVHD were calculated in the completing risk model. The chi-square test, or Fisher's exact test was used for categorical variables. The non-parametric tests (Mann-Whitney test for two groups, and Kruskal-Wallis tests for more than two groups) were used for continuous variables. The multivariate Cox model was performed to determine the impact of potential prognostic factors on clinical outcomes. Factors included in the regression model were patient age (<30 years *vs.* ≥30 years), gender, donor age (<30 years *vs.* ≥30 years), underlying disease (AML *vs.* others), diagnosis to transplant (≤6 months *vs.* >6 months), HLA mismatching (1 locus vs. ≥2 loci), donor-recipient gender matching (female-male vs. others), ABO compatibility, CD34 count (using median value as a cut-off point), CD3 count (using median value as a cut-off point), and graft source (G-PB vs. G-BM+G-PB). Testing was two-sided at the P<0.05 level. Statistical analysis was performed on SPSS software (SPSS, Chicago, IL), and R software (version 2.6.1) (http://www.r-project.org).

RESULTS

Clinical Outcomes of HID PBSCT

Engraftment

One case had primary graft failure, and her DSA was negative. All the other patients achieved sustained full-donor chimerism. The median time from HSCT to neutrophil engraftment and platelet engraftment was 12 days (range, 9–19 days) and 12 days (range, 8–171 days) after HID PBSCT, respectively. The 28-day cumulative incidence of neutrophil engraftment after HSCT was

98.5% (95%CI 95.1–100%), and the 100-day cumulative incidence of platelet engraftment after HSCT was 95.5% (95% CI 90.1–100%) after HID PBSCT.

GVHD

A total of 15 and five patients showed grade II and grade III aGVHD after HID PBSCT, respectively. The 100-day cumulative incidences of grade II–IV and grade III–IV aGVHD after HSCT were 29.9% (95%CI 18.8–40.9%) and 7.5% (95%CI 1.1–13.8%), respectively.

A total of 23, 9, and 2 patients showed mild, moderate, and severe cGVHD after HID PBSCT, respectively. The cumulative incidences of total cGVHD and moderate to severe cGVHD at 1 year after HSCT were 54.9% (95%CI 40.9–68.8%) and 17.4% (95%CI 6.7–28.0%), respectively.

Virus Activation

A total of 57 patients showed CMV-DNA after HID PBSCT, and 1 of them developed CMV diseases. The 100-day incidences of CMV-DNA viremia and CMV disease after HID PBSCT were 85.1% (95% CI 76.3–93.8%) and 1.5% (95%CI 0–4.4%), respectively.

A total of five patients showed EBV-DNA viremia, and 2 of them developed posttransplant lymphoproliferative disorders (PTLD) after HID PBSCT. The 100-day cumulative incidences of EBV-DNA and PTLD was 6.0% (95% CI 0.3–11.7%) and 3.0% (95%CI 0–7.1%), respectively.

Relapse and NRM

At the last follow-up, 9 patients experienced relapse with a median time of 126 days (range, 53–202 days) after HID PBSCT. The 1-year cumulative incidence of relapse after HID PBSCT was 13.9% (95% CI 5.4–22.5%). In multivariate analysis, female donor/male recipient (FDMR) combination was the only independent prognostic factor for relapse (HR=3.141, 95%CI 1.258–7.840, P=0.014).

At the last follow-up, three patients experienced NRM with a median time of 212 days (range, 36–485 days) after HID PBSCT. The causes of death were summarized in **Supplementary Table 1**. The 1-year cumulative incidence of NRM after HID PBSCT was 3.4% (95%CI 0–8.1%). None of the variables were significantly associated with increased NRM.

Survival

The median follow-up among survivals was 341 days (range 177 to 662 days) after HID PBSCT. The probability of OS and LFS at 1 year after HID PBSCT was 84.7% (95%CI 74.7–94.7%) and 82.7% (95%CI 73.3–92.1%), respectively. In multivariate analysis, FDMR combination was the only independent prognostic factor for OS (HR=3.186, 95%CI 1.172–8.660, P=0.023) and LFS (HR=2.911, 95%CI 1.319–6.424, P=0.008).

Comparison of the Clinical Outcomes Between G-PB Alone and G-BM Plus G-PB in HID HSCT Recipients

Patients Characteristics

The characteristics between the patients in the G-PB alone group and G-BM plus G-PB group were summarized in **Table 1** and **Supplementary Table 2**. Most of the variables were comparable between the groups, except that the duration from diagnosis to HSCT was longer in the G-PB groups. As expected, the amounts of mononuclear cells, CD3+ cells, and CD34+ cells in grafts were higher in the G-PB alone groups. DSA testing was positive in 5 (7.5%) patients in the G-PB alone group and 26 (6.6%) patients in the G-PB plus G-BM group.

Clinical Outcomes

The comparison between the G-PB alone group and the G-PB plus G-BM group were shown in **Table 2**. The median time from HSCT to neutrophil engraftment and platelet engraftment was both significantly shorter in the G-PB group compared with the G-BM plus G-PB group [neutrophil: 12 days (range, 9–19 days) versus 13 days (range, 9–25 days), $P<0.001$; platelet: 12 days (range, 8–171 days) versus 15 days (range, 7–268 days), $P=0.006$]. However, all the other outcomes were comparable between the groups (**Figures 2A–D**).

TABLE 1 | Patient characteristics.

Characteristics	G-PB alone (N = 67)	G-PB+G-BM (N = 392)	*P*
Patient age, years			0.536
Median (range)	30 (2–55)	31 (3–60)	
Sex, *n*(%)			0.644
Male,	42 (62.7)	234 (59.7)	
Female	25 (37.3)	158 (40.3)	
Disease, *n*(%)			0.111
AML	26 (38.8)	200 (51.0)	
ALL	39 (58.2)	185 (47.2)	
MPAL	2 (3.0)	7 (1.8)	
Diagnosis to transplant, months, *n* (%)			0.005
≥6 months	47 (70.1)	202 (51.5)	
<6 months	20 (29.9)	190 (48.5)	
Conditioning regimen, *n*(%)			0.157
BU-based	65 (97.0)	389 (99.2)	
TBI-based	2 (3.0)	3 (0.8)	
Donor age, years			0.236
Median (range)	38 (6–68)	40 (8–65)	
Donor source, *n*(%)			0.631
Father	27 (40.3)	167 (42.6)	
Mother	6 (9.0)	22 (5.6)	
Sibling	14 (20.9)	99 (25.3)	
Child	20 (29.9)	99 (25.3)	
Collateral	0 (0.0)	5 (1.3)	
Donor-recipient ABO match, *n*(%)			0.798
Match	37 (55.2)	215 (54.8)	
Minor mismatch	16 (23.9)	77 (19.6)	
Major mismatch	12 (17.9)	80 (20.4)	
Bidirectional mismatch	2 (3.0)	20 (5.1)	
MNC, $\times10^8$/kg			0.001
Median (range)	9.78 (5.52–19.23)	8.91 (3.30–21.31)	
CD34, $\times10^6$/kg			0.001
Median (range)	2.70 (1.00–13.52)	2.19 (0.35–9.53)	
CD3, $\times10^8$/kg			<0.001
Median (range)	2.72 (1.17–5.25)	1.89 (0.33–7.06)	

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia, BM, bone marrow; BU, busulfan; HID, haploidentical donor; MNC, mononuclear cell; MPAL, mixed-phenotype acute leukemia; PB, peripheral blood; TBI, total body irradiation.

TABLE 2 | Cumulative iincidences of clinical outcomes in the G-PB group versus the G-PB plus G-BM group.

	G-PB alone group		G-PB plus G-BM group		*P**
	Cumulative incidence (%)	95% CI (%)	Cumulative incidence (%)	95% CI (%)	
100-day aGVHD					
Grade II–IV	29.9	18.8–40.9	36.5	31.7–41.2	0.269
Grade III–IV	7.5	1.1–13.8	7.4	4.8–9.9	0.991
1-year cGVHD					
Total	54.9	40.9–68.8	58.3	53.2–63.4	0.794
Moderate to severe	17.4	6.7–28.0	22.4	18.0–26.7	0.571
1-year relapse	13.9	5.4–22.5	11.8	8.5–15.1	0.455
1-year NRM	3.4	0–8.1	6.9	4.3–9.5	0.531
1-year LFS	82.7	73.3–92.1	81.3	77.2–85.4	0.828
1-year OS	84.7	74.7–94.7	87.6	84.1–91.1	0.542

BM, bone marrow; CI, confidence interval; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; NRM, non-relapse mortality; LFS, leukemia-free survival; and OS, overall survival; PB, peripheral blood.
**The criterion for statistical significance was P < 0.05.*

Multivariate Analysis

The results of the multivariate analysis were shown in **Table 3**. Multivariate analyses failed to show significant differences in clinical outcomes between G-PB alone and G-BM plus G-PB groups.

DISCUSSION

This is the first report describing the outcomes of HID PBSCT after the ATG-based conditioning regimen for AL in CR1. This study indicated that hematopoietic recovery for those using G-PB grafts was faster compared with those using G-BM plus G-PB grafts, and GVHD, relapse, NRM, and survivals were similar between groups. This study provided an opportunity for exploring the up-to-date undefined role of HID PBSCT in AL CR1 patients with the ATG-based regimen. To our knowledge, our study represented the first comparison of G-PB alone with G-BM plus G-PB as grafts for HID HSCT in a disease-specific population of patients with AL in CR1.

PBSCT was associated with better engraftment. Randomized studies showed that PB grafts were associated with faster neutrophil and platelet engraftment than BM in ISD and URD HSCT (29, 30). In HID HSCT using post-transplant cyclophosphamide, some studies reported faster engraftment in PBSCT (31, 32). Our analysis also found that neutrophil and platelet engrafted faster in the G-PB group compared with the G-BM plus G-PB group in HID HSCT based on ATG. More rapid hematopoietic recovery of G-PB grafts in HID HSCT may be due to the greater content of mononuclear cells and CD34 cells in PBSC grafts compared with G-BM grafts.

In the present analysis, we did not observe a higher rate of GVHD in the G-PB alone group. As for most studies about ISD

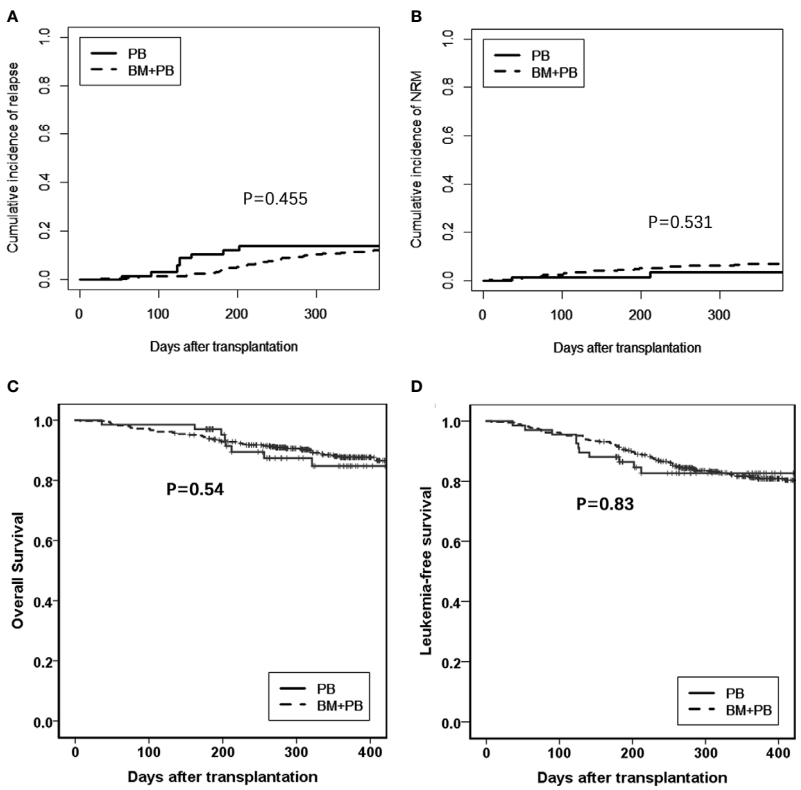

FIGURE 2 | Comparison between G-PB and G-BM plus PB groups **(A)**. Relapse; **(B)**. NRM; **(C)**. Overall survival; and **(D)**. Leukemia-free survival.

TABLE 3 | Multivariate analysis of risk factors for clinical outcomes in total population.

Outcome	HR (95% CI)	P*
Grade II to IV aGVHD		
Graft type		
PB plus BM	1	
PB	0.729 (0.452–1.175)	0.195
Other variables		
Donor age		
<30 years	1	
≥30 years	1.511 (1.019–2.242)	0.040
Grade III to IV aGVHD		
Graft type		
PB plus BM	1	
PB	0.835 (0.312–2.236)	0.720
Other variables		
Donor age		
<30 years	1	
≥30 years	3.687 (1.296–10.486)	0.014
Donor gender		
Female	1	
Male	2.312 (1.045–5.111)	0.038
CD3 count		
$\leq 2\times10^8$/kg	1	
$>2\times10^8$/kg	2.771 (1.348–5.698)	0.006
Total cGVHD		
Graft type		
PB plus BM	1	
PB	0.858 (0.455–1.618)	0.636
Other variables		
HLA mismatching		
1 loci	1	
≥2 loci	2.184 (1.030–4.631)	0.042
Moderate-severe cGVHD		
Graft type		
PB plus BM	1	
PB	0.808 (0.425–1.538)	0.517
Other variables		
Patient age		
≥30 years	1	
<30 years	1.534 (1.014–2.319)	0.043
Treatment failure as defined by overall survival		
Graft type		
PB plus BM	1	
PB	1.343 (0.655–2.750)	0.421
Other variables		
Donor type		
Others	1	
Female donor to male recipient	2.375 (1.328–4.247)	0.004
Treatment failure as defined by leukemia-free survival		
Graft type		
PB plus BM	1	
PB	1.154 (0.622–2.140)	0.649
Other variables		
Donor type		
Others	1	
Female donor to male recipient	1.771 (1.076–2.916)	0.025
Non-relapse mortality		
Graft type		
PB plus BM	1	
PB	0.687 (0.205–2.305)	0.543
Other variables		
Donor type		
Others	1	
Female donor to male recipient	2.230 (1.022–4.869)	0.044
Relapse		
Graft type		
PB plus BM	1	
PB	1.576 (0.752–3.301)	0.228

aGVHD, acute graft-versus-host disease; BM, bone marrow; CI, confidence interval; cGVHD, chronic graft-versus-host disease; HR, hazard ratio; PB, peripheral blood. None of variables was significantly associated with increased relapse.
**The criterion for statistical significance was P < 0.05.*

and URD HSCT, the rates of cGVHD were reported higher with PB grafts compared to that of BM grafts (1, 33). However, there were also several reports which showed similar rates of cGVHD between PB and BM HSCT (2–4). Our previous study on advanced diseases showed that the G-PB graft was not associated with increased cGVHD when compared with G-BM+G-PB grafts (9). In the present study on AL in CR1, we also observed similar probabilities of cGVHD in G-PB alone and G-BM plus G-PB groups. We speculated that the mature GVHD prophylaxis strategy including ATG in conditioning regimen and long-term schedules of cyclosporin for immunosuppression might reverse the risk of cGVHD with G-PB grafts (34).

Previous observations suggesting cGVHD was associated with graft versus leukemia (GVL) effect in different transplant settings (35, 36), and as mentioned above, more frequent GVHD was observed after PBSCT. Thus, PB grafts may accentuate the GVL effect. Mielcarek et al. (4) observed that among 172 ISD HSCT for hematological malignancies, the 10-year probability of relapse was 20% with PB versus 32% with BM. Bashey et al. (31) analyzed outcomes from a multicenter study comparing HID HSCT with G-CSF-primed PB versus BM and showed the lower relapse risk after PBSCT was limited to patients with leukemia. Several studies also noted that PB grafts had protection against relapse in HID HSCT with PT-CY (7, 11, 31). However, in other studies, PB grafts were not associated with lower rates of relapse (8, 9, 12, 32, 37, 38). Thus, whether more intense GVL effects could be induced in PBSCT remained controversial. In our previous study on advanced diseases, we observed a similar relapse rate between G-PB and G-BM plus G-PB groups (9). One reason may be the comparable incidences of GVHD between G-PB and G-BM plus G-BM groups in the present study, which suggested that G-PB grafts alone could induce a comparable GVL effect with G-PB plus G-BM grafts. On the other hand, because the relapse rate was relatively low among patients with AL in CR1 (20, 39, 40), we could not observe a significantly lower relapse rate in the G-PB group than the G-BM plus G-PB group.

Our previous study showed inferior results after PBSCT on advanced-stage leukemia, as compared to that receiving HID HSCT using G-BM plus G-PB (9). Differences were mostly based on a remarkably higher NRM of 62.5% for PBSCT. This might due to the higher rate of infection and early death in the refractory/relapse diseases. However, the NRM of HID PBSCT was less than 10% in the present study. In addition, the NRM rate of transplants performed in recent years appeared to be lower

(mostly less than 20%) than that of transplants done in the previous decade (20, 21). Thus, in these patients with AL-CR1, we did not observe the inferiority of HID PBSCT.

This study was not a randomized designed trial. Thus, it would be premature to derive conclusions regarding the superiority of PBSCT over HID using G-PB plus G-BM in patients with AL in CR1, and these results should be further confirmed by prospective randomized trials.

In summary, this study confirmed the safety and efficacy of HID PBSCT in patients with AL in CR1, and it also suggested that hematopoietic recovery for those using G-PB grafts was faster comparing with those using G-BM plus G-PB grafts, and other clinical outcomes were all comparable between the groups. While BM harvest needed the hospitalization of the donor, trained physicians, and specialized equipment, PBSCs were more convenient and were easy to be collected. For patients with AL in CR1, the G-PB grafts could be used as a reasonable alternative to G-BM plus G-PB grafts in HID HSCT. In the future, these results should be further confirmed by prospective randomized trials.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Peking University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

XDM and XJH designed the research. Y-RM analyzed the data and wrote the manuscript. All authors provided patient data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Decitabine With or Without Micro-Transplantation for the Treatment of Intermediate or High-Risk Myelodysplastic Syndrome: A Chinese Single-Center Retrospective Study of 22 Patients

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Li M, Li C, Geng S, Chen X, Wu P, Deng C, Chen X, Lu Z, Weng J and Du X (2021) Decitabine With or Without Micro-Transplantation for the Treatment of Intermediate or High-Risk Myelodysplastic Syndrome: A Chinese Single-Center Retrospective Study of 22 Patients. Front. Oncol. 11:628127. doi: 10.3389/fonc.2021.628127

The treatment outcomes of intermediate or high-risk myelodysplastic syndrome (MDS) remain unsatisfactory. This study was designed to evaluate the safety and efficacy of human leukocyte antigen (HLA)-mismatched hematopoietic stem cell micro-transplantation (MST) in patients with MDS. A total of 22 patients with MDS, ranging between the ages of 39 and 74, were enrolled in this study. Eleven patients were given decitabine (DAC), a DNA methyltransferase inhibitor, combined with HLA-mismatched MST (MST-DAC group), and the remaining patients were given decitabine only (DAC group). The median overall survival (OS) of the MST-DAC group was higher than that of the DAC group (24 vs. 14.3 months; HR 0.32; 95% CI: 0.11–0.96; $p = 0.04$), although it is a study with small samples. The overall response rate (ORR), marrow complete remission (mCR), plus hematological improvement (HI) rates of the MST-DAC group were higher than that of the DAC group (81.8 vs. 54.5%, $p = 0.36$; 63.6 vs. 27.3%, $p = 0.09$, respectively); however, there were no statistical differences between the two groups, which may be attributed to the limited number of cases evaluated in this study. No graft-vs.-host disease was observed in the MST-DAC group. Patients in the MST-DAC group demonstrated a slightly lower incidence of hematological and non-hematological adverse events (AEs). DAC combined with HLA-mismatched MST may provide a novel, effective, and safe treatment for use in intermediate or high-risk MDS pathologies.

Keywords: myelodysplastic syndromes, HLA-mismatched micro-transplantation, decitabine, overall survival, chronic myelomonocytic leukemia

INTRODUCTION

Myelodysplastic syndrome (MDS) represents a group of heterogeneous myeloid clonal diseases that originate from hematopoietic stem cells and are characterized as having an abnormal development of myeloid cells. Typically, MDS manifests as ineffective hematopoiesis and refractory cytopenia with the risk of transforming into acute myeloid leukemia (AML) (1). It is known that allogeneic hematopoietic stem cell transplantation (allo-HSCT) promotes curative effects in patients with

MDS; however, clinicians often face impediments to its widespread application, particularly concerning infectious and other toxicities associated with conditioning regimens, the development of significant graft-vs.-host disease (GVHD) with resultant organ dysfunction, infection from prolonged immunosuppression, and in some cases, a high rate of disease progression (2).

Hypermethylation in DNA is associated with tumor progression and differentiation arrest, which has been detected in myelodysplastic syndrome (MDS) and AML (3, 4). Decitabine (DAC), a DNA hypomethylating agent, is considered a frontline therapy for intermediate or high-risk patients who were not candidates for allo-HSCT according to National Comprehensive Cancer Network (NCCN) Guidelines (Version 2.2020) for myelodysplastic syndromes. However, the clinical efficacy of demethylation drugs to treat patients with MDS remains limited.

Currently, several studies have shown that human leukocyte antigen (HLA)-mismatched hematopoietic stem cell micro-transplantation (MST) can increase complete remission (CR) rates, improve OS, and avoid the development of graft-vs.-host disease (GVHD) in patients with AML (5–7). The term MST refers to standard chemotherapy combined with a granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cell (G-PBSCs) infusion of HLA-mismatched donor cells without the use of immunosuppressive agents (7). Although MST mediates graft-vs.-leukemia (GVL) effects and GVHD hardly occurs, it is unclear whether treatment with MST in combination with DAC can improve the outcomes of patients with intermediate or high-risk MDS compared with those treated with DAC-only. Thus, a retrospective study was designed to evaluate the safety and efficacy of DAC combined with or without MST in patients with MDS.

MATERIALS AND METHODS

Patients

Data were retrospectively collected from 22 patients with MDS [WHO 2008 classification (8)] who were treated with MST-DAC or DAC at the Department of Hematology of Guangdong Provincial People's Hospital from 2006 to 2016. All of them did not have severe infection, liver and kidney dysfunction, or an uncured secondary tumor.

Donors

Donors were selected based on the degree of HLA mismatched loci. The sex, age, and red blood cell type were not heavily considered when selecting donors.

Donor and recipient HLA-A, -B, -C, -DRB1, and-DQB1 loci were typed at intermediate resolution using polymerase chain reaction (PCR) paired with the sequence-specific primer method. Of the 11 patient/12 donor pairs, the HLA alleles of five donors were matched in 5 of 10, of three donors were matched in 0 of 10, of two donors were matched in 2 of 10, of one donor were matched in 3 of 10, and of one donor were matched in 6 of 10. The median age of the donors was 28 and included 6 adult-offspring donors, 2 relatives, and 4 unrelated donors (**Supplementary Table 1**).

The protocol was approved by the Human Ethics Committees of the Guangdong General Hospital, Guangdong Academy of Medical Science. The study was performed following ethical standards set forth by the Declaration of Helsinki. All patients and donors provided written informed consent before enrollment in the study.

Data Collection

The data collected for analysis included the clinical characteristics of the patients, such as age at diagnosis, sex, past medical history of malignancies and/or hematological diseases, family history of malignancies and/or hematological diseases, performance status (PS), complete blood counts (CBCs), blasts in peripheral blood (PB) and bone marrow (BM), cellularity of BM, chromosome abnormalities, French–American–British (FAB) and WHO classifications, risk groups in Revised International Prognostic Scoring System (IPSS-R), treatments, date of initial therapy, date of progression to AML, and date of death or that of the last follow-up.

Study Endpoints

The co-primary endpoints in this study included the overall response rate (ORR), overall survival (OS), and progression-free survival (PFS). The ORR included the rate of CR, partial response (PR), marrow complete remission (mCR), and hematological improvement (HI). The response was assessed according to the International Working Group (IWG) criteria (9). The OS was defined as the time from initiation of treatment to the date of death from any causes or until the end of the follow-up period. The PFS was defined as the time from initiation of medication to treatment failure, progression of the disease, or death from any causes. All MDS cases were confirmed based on local investigator reports and/or death certificate information. The duration of the follow-up period was measured as the date of the first treatment dose received for each patient up to 2 years.

Safety

All severe (grade 3 or higher) hematological or non-hematological adverse events (AEs) that occurred during treatment were evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.03 (10).

Assessment of Wilms' Tumor Gene (WT1)

Wilms' tumor gene transcripts were quantified by a standard European LeukemiaNet real-time quantitative PCR using the ABI 7500 real-time PCR system (11).

Mobilization and Apheresis of Donor Peripheral Mononuclear Cells

Apheresis of HLA-mismatched donor peripheral mononuclear cells was performed with a COBE-spectra 6.0 blood cell separator after the donor was subcutaneously injected with 5 μg/kg G-CSF two times a day for 5 days. The median numbers (range) of mononuclear and CD3+ cells infused per course were, respectively, 2.50 (0.97 ∼ 4.08) × 10^8 and 0.86 (0.79 ∼ 1.02) × 10^8 cells per kg. (**Supplementary Table 2**).

Statistical Analysis

Variables related to clinical characteristics between the two groups were compared using Fisher's exact test. All time-to-event analyses were performed with the use of Kaplan–Meier methods and presented by Kaplan–Meier curves. The hazard ratio (HR) and 95% confidence intervals (CIs) were estimated in comparison to a reference risk of 1.0. Statistical significance was defined with a 2-sided $p < 0.05$. SPSS (version 25.0) was used for all statistical analyses. GraphPad Prism 7.0 was used for graphing the results.

RESULTS

Patients

Of all the analyzed patients, 11 (11/22, 50.0%) were treated with MST-DAC and the rest (11/22, 50.0%) were treated with decitabine-only. In the MST-DAC group, the median patient age was 60 years (age range: 39–73 years) and 8 (72.6%) were female. The median age of the patients in the DAC group was 61 years (age range: 41–74 years) and 8 (72.6%) were female.

Treatment

The DAC group received DAC treatment, which involved infusions of 20 mg/m^2 DAC *via* intravenous drip on days 1–5. The patients in the MST group were also given an infusion of HLA-mismatched G-PBSCs within 24–72 h until the end of DAC treatment (20 mg/m^2). When ANC was $<0.5 \times 10^9$/L, G-CSF at 5 μg/kg/day was subcutaneously administered. When hemoglobin was <60 g/L, an infusion of red blood cells was given, and when platelet count was $<20 \times 10^9$/L, an infusion of platelets was administered. Notably, there were no statistical differences in the number of DAC treatment cycles between the two cohorts. The median number of treatment cycles for the DAC group was 4 (range: 2–20) and for the MST-DAC group was 5 (range: 0–11) ($p = 1.00$) (**Table 1**). The median number of treatment cycles for the MST-DAC group, those who underwent micro-transplantation, was 4 (range: 2–4).

Treatment Response

Of the nine patients (9/11, 81.8%) in the MST-DAC group who responded to treatment: seven achieved both mCR and HI (7/11, 63.6%), one had only mCR (1/11, 9.1%), and one had only HI (1/11, 9.1%). Responses were observed for all six (6/11, 54.5%) patients in the DAC group: three achieved both mCR and HI (3/11, 27.3%), and three had only mCR (3/11, 27.3%). Though no significant difference was observed between the two groups, the ORR and the ratio of achieving both mCR and HI in the MST-DAC group was higher than that for the DAC group (81.8 vs. 54.5%, $p = 0.36$; 63.6 vs. 18.2%, $p = 0.09$). The incidence of AML transformation within 12 months for the MST-DAC group was lower than that for the DAC group (0.0 vs. 27.3%, $p = 0.21$). Also, the incidence of death within 24 months for the MST-DAC group was lower than that for the DAC group (45.5 vs. 81.8%, $p = 0.18$).

Survival Analysis

Six patients were still alive at the end of the follow-up period in the MST-DAC group and two patients were alive in the DAC group. The median OS was 24 months for the MST-DAC group and 14.13 months for the DAC group. A significant difference was observed between the two groups (HR: 0.32; 95% CI: 0.11–0.96; log-rank test, $p = 0.04$) (**Figure 1A**). The median PFS was 20.8 months for the MST-DAC group vs. 9.3 months for the DAC group (HR: 0.55; 95% CI: 0.20–1.47; log-rank test, $p = 0.23$) (**Figure 1B** and **Supplementary Table 4**).

Safety and Toxicities

Safety profiles were evaluated for all patients in the cohort. During the first 12 months of treatment, one patient (1/11, 9.1%) died in the MST-DAC group, and four patients (4/11, 36.4%) died in the DAC group ($p = 0.31$). The mortality at 24 months was 5/11 (45.5%) for the MST-DAC group and 9/11 (81.1%) for the DAC group ($p = 0.18$) (**Table 2**).

The most common AEs were neutropenia, thrombocytopenia, anemia, febrile neutropenia, leukopenia, septicemia, upper respiratory tract infection, hemorrhage, and pneumonia (**Table 3**). In general, patients in the MST-DAC group demonstrated a lower incidence of hematological AEs (anemia: 27.2% in the MST-DAC group vs. 55.0%, $p = 0.39$; leukopenia: 54.5 vs. 72.7%, $p = 0.66$; neutropenia: 55.0 vs. 64.0%, $p = 1.00$) during the entire treatment, with the exception of thrombocytopenia (64% in the MST-DAC group *vs.* 27.0% in the DAC group, $p = 0.09$). The same result was found for non-hematological AEs, where a lower incidence of febrile neutropenia, hemorrhage, and upper respiratory tract infection was also observed in the MST-DAC group (**Table 3**). By comparing complete blood cell counts after micro-transplantation, we found that absolute neutrophils were partially recovered. Therefore, micro-transplantation may shorten the recovery time of the hematopoietic function and reduce the incidence of infectious complications, such as pneumonia.

Wilms' Tumor Gene in Bone Marrow (BM-WT1)

Of the 22 patients in this study, 18 had detectable BM-WT1 before and after the treatment. The results demonstrated that among the 14 patients with a clinical response, the levels of BM-WT1 in 10 patients had different degrees of decline, and the median of the decline was 85.3% (range: 52.7–99.7%). The fluctuations of BM-WT in four patients were within the normal range, and there was no significant trend in the fluctuations for both cohorts with BM-WT1. Four patients failed to respond to therapy, while the levels of BM-WT1 in three of them who achieved SD remained unchanged. The levels of BM-WT1 in one patient remained to be higher than the normal range. Four patients with detectable BM-WT1 had a significant increase in BM-WT1 when progressed to AML (**Supplementary Table 3**).

DISCUSSION

Recently, Ai Huisheng et al. (6, 12–14) explored the application of "micro-transplantation" to treat several hematological malignancies, included MDS, AML, and Philadelphia chromosome-positive acute lymphoblastic leukemia. The

TABLE 1 | Patient demographics and clinical characteristics.

Characteristic	MST-DAC group (n = 11)		DAC group (*n* = 11)		Fisher exact test value	Exact Sig. (2-sided) *P-value*
	No. of patients	%	No. of patients	%		
Sex					–	1.00
Male	8	72.6	8	72.6		
Female	3	27.4	3	27.4		
Age, years					–	1.00
Median	60		61			
Range	39–73		41–74			
≥60 years	7	63.6	7	63.6		
<60 years	4	36.4	4	36.4		
FAB classification					4.32	0.41
CMML-1	0	0	2	18.2		
CMML-2	1	9.1	1	9.1		
RAEB-1	4	36.4	6	54.5		
RAEB-2	2	18.2	1	9.1		
RCMD	4	36.3	1	9.1		
IPSS-R					1.14	1.00
Mediate (3–4.5 points)	3	27.3	4	36.4		
High (4.5–6 points)	7	63.6	7	63.6		
Very high (6 points)	1	9.1	0	0.0		
Therapy Times	DAC times %		DAC times%		1.24	0.73
Median	5		4			
Range	0–11		2–20			
<4	5	45.5	5	45.5		
≥4	6	54.5	6	54.5		
Donor/recipient with HLA mismatched loci						
6/10	1	9.1	–	–		
5/10	5	45.4	–	–		
3/10	1	9.1	–	–		
2/10	1	9.1	–	–		
NA	3	27.3	–	–		
WHO PS					–	1.00
1	11	100	11	100		

rate of CR in patients with AML who received induction chemotherapy with mitoxantrone and cytarabine combined with HLA-mismatched G-PBSCs was 80%. The probabilities of the 2-year DFS and OS in an entire population were 38.9 and 39.3%, respectively (6). As reported by other published work that compared the efficacy of MST-DAC in treating MDS and transformed acute myelogenous leukemia (tAML), the ORR of patients with MDS treated with HLA mismatched MST-DAC combined with DAC and cytarabine was significantly higher than that of patients with tAML treated with HLA mismatched MST-DAC combined with DAC (81 vs. 50%; $p = 0.03$); the PFS and OS of 2 years were 42.7 and 84.7% in patients with MDS, respectively. No sign of acute and chronic GVHD was observed in patients during MST-DAC treatment (7). In another study of patients with MDS ($n = 72$) treated with MST (Microtransplantation-group, $n = 28$) or treated with two doses of DAC (DAC group, $n = 27$; low-dose DAC group, $n = 17$), the total CR rate was 42.9 vs. 14.8% and 29.4% in the three groups, respectively. The CR rate of the MST-group was significantly higher than that of the other groups ($p = 0.02$) (15). Results from clinical trials showed that the ORR of patients with MDS who received DAC fluctuates between 32 and 73% (16–19). In the present study, the ORR was 81.8% in the MST-DAC group, which was compared with 54.5% in the DAC group. Results from our study revealed that patients in the MST-DAC group showed a slightly higher ORR compared with the DAC group. Meanwhile, our retrospective analysis also suggested that a better OS was observed in patients who received MST-DAC

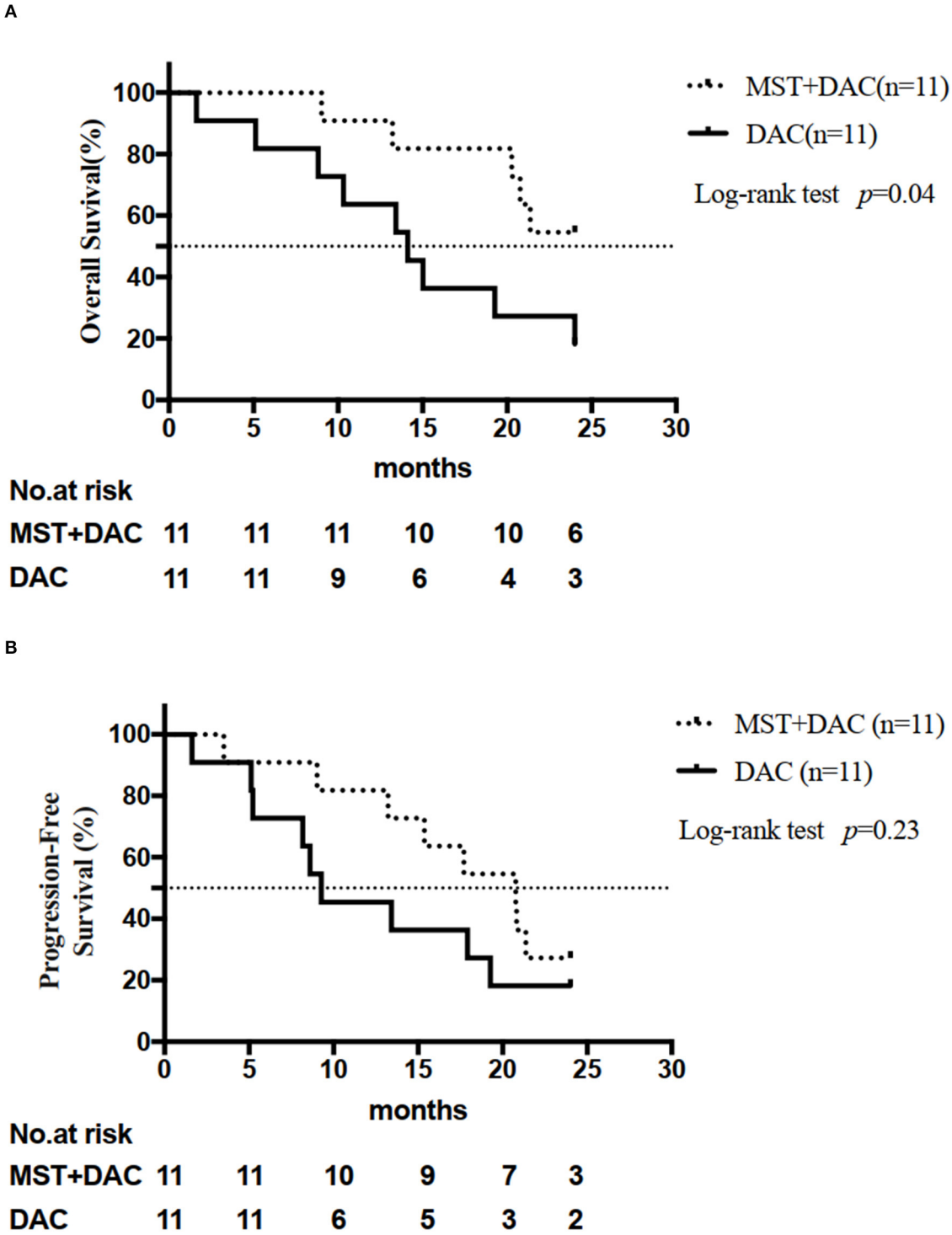

FIGURE 1 | Analysis of efficacy endpoints. **(A)** Overall survival (OS) for the two groups is shown. **(B)** Progression-free survival (PFS) for the two groups is shown.

(24.0 [9.0–24.0 months] vs. 14.3 months [1.6–24.0 months], $p = 0.04$). However, due to the small number of cases included in our retrospective study, future prospective trials with larger sample sizes are needed to verify our results.

According to the toxicities reported in our study, there was a lower incidence of severe hematological AEs in the MST-DAC group during the entire treatment period compared with the DAC only group: 27.2 vs. 54.5% for anemia ($p = 0.39$); 54.5

TABLE 2 | Patient outcomes.

Response by IWG 2006 criteria	MST-DAC group		DAC group		Fisher exact *sig.* (2-sided) *P-value*
	N	%	*N*	%	
CR	0	0.0	0	0.0	-
mCR + HI	7	63.6	3	27.3	0.09
mCR only	1	9.1	3	27.3	
HI only	1	9.1	0	0.0	-
SD	2	18.2	3	27.3	-
Failure	0	0.0	1	9.1	-
Unable to evaluate	0	9.0	1	9.1	-
ORR (mCR, HI, CR)	9	81.8	6	54.5	0.36
Cumulative incidence of ORR					
At 2nd cycle	7	63.6	3	27.3	0.09
At 4th cycle	9	81.8	5	45.5	0.18
At 6th cycle	9	81.8	5	45.5	0.18
12-month incidence of AML transformation (%)	0	0.0	3	27.3	0.21
24-month incidence of AML transformation (%)	2	18.2	3	27.3	1.00
Cumulative incidence of AML transformation (%)	2	18.2	3	27.3	1.00
12-month incidence of death (%)	1	9.1	4	36.4	0.31
24-month incidence of death (%)	5	45.5	9	81.8	0.18
Cumulative incidence of death (%)	5	45.5	9	81.8	0.18

TABLE 3 | Severe (grade 3 or higher) hematological or non-hematological adverse events (AEs) from the two therapies.

	MST-DAC				DAC				
	Grade 3	Grade 4	Grade 5	Total	Grade 3	Grade 4	Grade 5	Total	Fisher exact sig. (2-sided) *P-value*
Hematological AEs									
Anemia	1	2	-	3 (27.2%)	3	3	-	6 (54.5%)	0.39
Leukopenia	6	0	-	6 (54.5%)	5	3	-	8 (72.7%)	0.66
Neutropenia	1	5	-	6 (54.5%)	0	7	-	7 (63.6%)	1.00
Thrombocytopenia	5	2	-	7 (63.6%)	0	3	-	3 (27.3%)	0.09
Non-hematological AEs									
Febrile neutropenia	3	1	0	4 (36.4%)	6	0	0	6 (54.5%)	0.40
Pneumonia	2	0	0	2 (18.2%)	5	0	1	6 (54.5%)	0.18
Septicemia	-	1	0	1 (9.1%)	-	0	1	1 (9.1%)	1.00
Upper respiratory tract infection	2	0	0	2 (18.2%)	4	0	0	4 (36.4%)	0.64
Hemorrhage	1	1	0	2 (18.2%)	0	2	2	4 (36.4%)	0.64
Soft tissue infection	1	0	0	1 (9.1%)	1	0	0	1 (9.1%)	1.00

vs. 63.6% for neutropenia ($p = 1{,}00$); 54.5 vs. 72.7% for leukopenia, respectively ($p = 0.66$). A trend representing a lower percentage of febrile neutropenia, pneumonia, upper respiratory tract infection, and hemorrhage was also seen in the MST-DAC group. Compared with the DAC group, the incidences of AML transformation and the mortality rate were also lower in the MST-DAC group within 12 or 24 months (0.0 vs. 27.3%, 18.2 vs. 27.3%, 9.1 vs. 36.4%, 45.5 vs. 81.8%, respectively), which suggests that micro-transplantation was safe to treat patients with MDS. No signs of acute or chronic GVHD were observed in any of the patients during treatment, which reflects the same results reported in previous studies (6, 7, 13, 14). Our results illustrate the safety of the application of micro-transplantation combined with DAC treatment in patients with intermediate or high-risk MDS.

The purpose of micro-transplantation is to elicit an anti-tumor response, with little or no continuous donor cell implantation, no complete donor chimerism, and no onset of GVHD. Studies have shown that it is possible to obtain an anti-tumor response when only achieving microchimerism (<1% of

donor cells) (20–25). It is speculated that T-cell and natural killer (NK)-cell alloreactivity could generate immediate anti-leukemic effects that awaken the anti-tumor immunity of the host, change the tolerance of the host to the tumor, and allow the host to undergo an immune response to the tumor (24, 26–30).

According to some reports, the mRNA level of WT1 reflects disease changes and progression in patients with MDS (31, 32). Therefore, WT1 is a suitable marker for the detection of minimal residual disease after SCT or chemotherapy (33). Furthermore, the correlation of WT1 mRNA levels before treatment and response was evaluated in the present study. There was a trend that indicated that the reduction of WT1 mRNA levels correlated with the efficacy of MST-DAC treatments (**Supplementary Table 3**).

The MST-DAC group included four relatively young (<60 years) patients who waited for suitable donors to undergo allo-HSCT, one patient achieved both mCR and HI, one patient achieved mCR only, and the other two patients achieved SD, and there was no evidence of GVHD. Therefore, the efficiency and safety of MST-DAC in relatively young patients who waited to undergo allo-HSCT was seen in this study. Patients who are candidates for allo-HSCT may be given MST-DAC as a bridging treatment for allo-HSCT.

According to the key eligibility criteria of micro-transplantation in our center, patients with blast <5% only received micro-transplantation without DAC, or with DAC for patients with blast ≥5%. In the MST-DAC group, two patients (MST-DAC 6 and MST-DAC 10) only received micro-transplantation without DAC and received supportive care pre-MST, and had planned to be given DAC if the disease was evolution. Both patients obtained marrow complete remission (MCR) after micro-transplantation, and one of them had received allo-HSCT after 4 years post-MST. We removed the data of the two patients, and the median OS of the MST-DAC group ($n = 9$) was still higher than that of the DAC group ($n = 11$) (24 vs. 14.1 months; HR 0.36; 95% CI: 0.12–1.07; $p = 0.06$). Although not statistically significant, there was a trend toward significance ($p = 0.06$) (**Supplementary Figure 1**).

In the current study, the overall survival of patients with MDS was effectively improved, in addition to the comparatively ORR, which makes our data noteworthy. At the same time, major drawbacks of our study include its retrospective design, the limited number of patients enrolled, and the long duration of the study (10 years). Due to the wide-range time of the study, the OS of patients could have been influenced by several factors which were listed in **Supplementary Table 5**. The median of the time from diagnosis to treatment for the MST-DAC group was 33 (range, 6–320) days, and that for the DAC group was 14 (range, 0–271)days. The median time of the duration of neutropenia/cytopenias before treatment for MST-DAC was 275 (range, 31–3605) days, and that for DAC was 230 (range, 20–2926).

In conclusion, our results are promising and show that MST combined with the probable synergistic effect of DAC can achieve a better OS in patients with intermediate or high-risk MDS and cause acceptable short-term toxicities. Prospective studies are urgently needed to determine the exact role of micro-transplantation in the setting of MDS and to clarify optimal treatment modalities, such as dosage and duration.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Guangdong General Hospital, Guangdong academy of Medical Science. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to, revised, approved the manuscript content, and approved journal submission of the manuscript. The authors are fully responsible for all content and editorial decisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.628127/full#supplementary-material

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SYSTEMATIC REVIEW
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Comparisons Between Frontline Therapy and a Combination of Eltrombopag Plus Immunosuppression Therapy and Human Leukocyte Antigen-Haploidentical Hematopoietic Stem Cell Transplantation in Patients With Severe Aplastic Anemia: A Systematic Review

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Background and Aims: This study aimed at comparing the efficacy and safety of eltrombopag (EPAG) plus immunosuppressive therapies (ISTs) and haploidentical hematopoietic stem cell transplantation (haplo-HSCT) in the frontline treatment for severe aplastic anemia (SAA) patients.

Methods: Four electronic databases and Clinicaltrials.gov were comprehensively searched from January 2010 to August 2020. Studies that aimed at evaluating the efficacy and safety of EPAG+IST or haplo-HSCT in SAA patients were included. One-/2-year overall survival (OS), complete response (CR), and overall response rates (ORRs) were indirectly compared between EPAG+IST and haplo-HSCT.

Results: A total of 447 patients involved in 10 cohort studies were found to be eligible for this study. A narrative synthesis was performed due to lack of data directly comparing the outcome of EPAG+IST and haplo-HSCT. Consistent with the analysis results in the whole population, subgroup analyses in the age-matched population showed that there was no significant difference in ORR between EPAG+IST and haplo-HSCT groups. However, the CR rate was lower in the EPAG+IST group when compared with the haplo-HSCT group. The incidence rate of clonal evolution/SAA relapse ranged at 8–14 and 19–31% in the EPAG+IST group but not reported in the haplo-HSCT group. The incidence rate for acute graft vs. host disease (aGVHD) and chronic graft vs. host disease (cGVHD) ranged at 52–57 and 12–67%, respectively, for the haplo-HSCT group. The main causes

of deaths were infections in the EPAG+IST group, and GVHD and infections in the haplo-HSCT group.

Conclusion: EPAG+IST has a comparable ORR and 1-/2-year OS but lower CR rate when indirectly compared with haplo-HSCT in the frontline treatment of patients with SAA. Patients treated with haplo-HSCT may exhibit a high incidence of GVHD, whereas patients treated with EPAG+IST may experience more relapses or clone evolution.

Keywords: severe aplastic anemia, eltrombopag, immunosuppression therapy, haploidentical hematopoietic stem cell transplantation, survival

INTRODUCTION

Severe aplastic anemia (SAA) causes severe bleeding, infection, and anemia, which may be fatal. It is mainly caused by immune-mediated destruction of the hematopoietic progenitor cells (1). Currently, human leukocyte antigen (HLA)-matched sibling donor (MSD) hematopoietic stem cell (HSC) transplantation (HSCT) is recommended as the first-line therapy for young adults with SAA. In the absence of matched related donors, immunosuppressive therapy (IST) with antithymocyte globulin (ATG) plus cyclosporine A (CsA) is the recommended first-line therapy (2, 3).

IST with ATG plus CsA is an effective first-line therapeutic option with a 60–80% response rate in SAA patients (3, 4). However, it is associated with the risk of clonal evolution to myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML), hemolytic paroxysmal nocturnal hemoglobinuria (PNH), and relapse during long-term follow-up (5, 6). In addition, approximately one-third of SAA patients remain refractory to IST; this is attributed to the depletion of HSCs in the presence of ongoing immune attack (7, 8).

Transplantation including MSD HSCT, matched unrelated donor (MUD) HSCT, and haploidentical HSCT (haplo-HSCT) is a radically curative option for SAA patients (9, 10). In the absence of MSD or MUD, haploidentical transplantation has been shown to have long survival benefits and acceptable transplantation complications in young SAA patients (11–13). However, it is not widely accepted as a first-line therapeutic option due to high associated risks and a lack of convincing data (14, 15). Our previous study revealed that haplo-HSCT has comparable overall survival (OS) and better failure-free survival (FFS) when compared with IST as the frontline therapy for young patients with SAA, and the long-term OS was the same (16).

Eltrombopag (EPAG) is an oral synthetic small-molecule thrombopoietin receptor agonist that has been found to be an effective option for SAA patients refractory to IST (17, 18). Treatment with EPAG stimulates megakaryocytopoiesis as well as erythropoiesis and myelopoiesis because the thrombopoietin receptor is expressed on both megakaryocytes and HSCs (19–22). Recently, it has been shown that a combination of EPAG and IST exhibits significantly higher rates of hematologic response than IST alone (23, 24).

Haplo-HSCT is widely used in China, probably because of the rapid advances in the transplantation technique and lack of MSD. However, the efficacy and safety of EPAG plus IST have not been compared with those of haplo-HSCT. In this study, we obtained scientific publications on frontline therapy using the two regimens for SAA patients. A systematic review involving 447 patients from 10 studies was finally performed to compare the clinical outcomes and related complications of EPAG+IST and haplo-HSCT.

MATERIALS AND METHODS

Search Strategy

PubMed, Embase, Web of Science, WanFang Database, and Clinicaltrials.gov were comprehensively searched for articles that reported the efficacy and/or safety of EPAG in combination with IST and haplo-HSCT among SAA patients. This search was performed between January 2010 and August 2020. The publication language was restricted to English. The search keywords used were as follows: severe aplastic anemia/SAA, eltrombopag/EPAG/ELT, immunosuppression therapy/IST, HLA-haploidentical hematopoietic stem cell transplantation/haplo-HSCT, survival/prognosis, and progression-free survival/PFS. Moreover, we scrutinized the reference lists of the selected reports to identify additional relevant studies missed in the initial search. Our initial search query was the algorithm of "(((SAA) AND (severe aplastic anemia)) AND (((((eltrombopag) OR (EPAG)) OR (ELT)) OR ((immunosuppression therapy) OR (IST))) OR ((HLA-haploidentical hematopoietic stem cell transplantation) OR (haplo-HSCT)))) AND ((((survival) OR (prognosis)) OR (progression-free survival)) OR (PFS))."

Inclusion and Exclusion Criteria

Reports were included if they met the following criteria: (i) patients were diagnosed with SAA/very SAA (VSAA); (ii) patients underwent haplo-HSCT or EPAG plus IST (rabbit/horse ATG+CsA) as the frontline therapy; (iii) reported the OS and/or overall response rate (ORR)/complete response (CR); (iv) described the adverse events, relapse rate, clonal evolution

Abbreviations: SAA, severe aplastic anemia; EPAG, eltrombopag; ISTs, immunosuppressive therapies; haplo-HSCT, haploidentical hematopoietic stem cell transplantation; OS, overall survival; CR, complete response; ORRs, overall response rates; 95%CI, 95% confidence intervals; cGVHD, chronic graft vs. host disease; ATG, antithymocyte globulin; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; MSD, matched sibling donor; MUD, matched unrelated donor; PTCy, post-transplant cyclophosphenolate; RIC, reduced-intensity condition.

rate, and causes of treatment-related deaths; and (v) published between January 2010 and December 2020.

The exclusion criteria were as follows: (i) animal studies; (ii) review articles or meta-analysis or case reports; (iii) duplicated publications; (iv) non-English papers; (v) studies involving other hematologic malignancies (primary myelofibrosis, non-Hodgkin's lymphoma, chronic myelomonocytic leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, etc.); (vi) patients with SAA/VSAA refractory to IST; (vii) studies involving salvage HSCT; (viii) studies involving SAA patients treated with IST alone; (ix) studies involving aplastic anemia patients not eligible for the criteria of SAA; and (x) studies without OS, ORR, and CR data.

Data Extraction

Data extraction was independently performed by two investigators (YY and ZT). In case of discrepancies, they were resolved by consensus between the two investigators. The following variables were extracted: (i) study characteristics (the first author, year of publication, study design and duration, regimen, and number of participants in each study); (ii) patients' basic characteristics (gender, median age, and median follow-up); (iii) the ORR, CR, 1-/2-year OS, incidences of clonal evolution, and disease relapse in patients treated with EPAG combined with IST plus CsA; iv) ORR, 1-/2-year OS rate, the incidences of graft vs. host disease (GVHD), and mortality rates in patients subjected to haplo-HSCT. For the few reports that did not describe the 1-/2-year OS rate, we calculated their OS by using the Engauge Digitizer (Windows version 10.8) software from the Kaplan–Meier survival curve shown in the original articles.

Statistical Analysis

All the statistical analyses were performed according to the guidelines proposed by the Meta-Analysis of Observational Studies in the Epidemiology group (MOOSE) (25).

Heterogeneity among the included studies was measured using the Q tests and I^2 statistic to assess the extent of the inconsistencies (26). If a probability value of $p < 0.1$ and $I^2 > 50\%$, indicating the existence of significant heterogeneity was found, then a random pooled effect model was performed (27). Statistical heterogeneity was categorized into low (<50%), moderate (51–75%), or high (>75%) according to a predefined criteria (26). $p \leq 0.05$ was set as the threshold for statistical significance. A funnel plot and Egger's linear regression test was performed to evaluate the potential publication bias for eligible studies using ORR, CR, or OS as endpoints (28). Moreover, a $p < 0.01$ for Egger's test was considered statistically significant. The "Meta" R package was used to perform all pooled analyses. If pooled analysis cannot be performed due to high heterogeneity among included studies or lack of data directly compared the outcomes between the EPAG+IST group and haplo-HSCT group, a narrative synthesis would be performed to indirectly compare the ORR, CR, and OS between the EPAG+IST group and haplo-HSCT group. All statistical analyses were performed using R version 3.6.3.

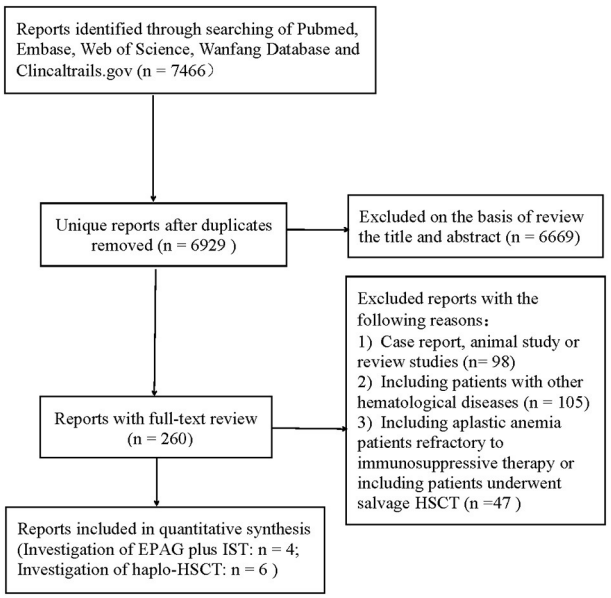

FIGURE 1 | Search flow diagram in our study.

RESULTS

Study Selection

The initial literature search yielded 7,466 articles from the four primary electronic databases. Out of these, 6,669 publications were excluded after reviewing the titles and abstracts, while 260 papers were selected for full-text review. After full-text reviews, 10 articles (3, 12, 14, 23, 29–34) were eligible for this study according to the inclusion and exclusion criteria mentioned above. The screening process was as shown in **Figure 1**.

Characteristics of the Enrolled Patients

The selected studies included three prospective and seven retrospective cohort studies. Among them, four studies used EPAG+IST (a total of 252 patients received horse ATG, while 10 patients received rabbit ATG). The other six studies used haplo-HSCT as the frontline therapy; conditioning therapies (predominantly cyclophosphamide+ATG) were used in haplo-HSCT studies. The average median ages of the EPAG+IST group and haplo-HSCT group were 40.6-years (range: 15–60-years) and 9.2-years (range: 8–28-years), respectively ($p = 0.024$). Male patients were 45.6% (range: 30.0–54.3%) in the EPAG+IST group and 61.0% (range: 56.0–70.0%) in the haplo-HSCT group ($p = 0.036$). The incidence rate of VSAA in the haplo-HSCT group was 30.1% (range: 4.3–45.0%); however, there were no data on the incidence rate of VSAA in the EPAG+IST group. The characteristics of eligible studies included in this study are presented in **Table 1**.

TABLE 1 | The characteristics of included studies.

Group	References	Disease (no. patients)	Study period	Study design	Male ratio (%)	Median age (range), years	Study protocol/ conditioning regimen	Frontline/salvage	Median follow-up (months)
EPAG+IST	(23)	SAA (92) VSAA (NR)	2012–2015	P	54.3	32 (3–82)	EPAG+horse ATG+CsA	Frontline	24 (2.8–47.4)
	(29)	SAA (21) VSAA (NR)	2012–2018	P	52.4	60 (19–84)	EPAG+horse ATG+CsA+glucocorticoid	Frontline	21 (3–49)
	(30)	SAA (39) VSAA (NR)	2012–2018	R	NR	15 (NR)	EPAG+horse ATG+CsA	Frontline	NR
	(34)	SAA (7) VSAA (NR)	2015–2016	P	30.0	55.5 (39–67)	EPAG+rabbit ATG+CsA	Frontline	88.36 (22.0–104.1)
Average	/	/	/	/	45.57 ± 13.51	40.6 ± 21.04	/	/	44.45 ± 38.05
Haplo-HSCT	(3)	SAA (11) VSAA (9)	2012–2016	R	70	13 (4–18)	CY, ATG, CY, ATG; Flu, Bu	Frontline	29 (1–47)
	(31)	SAA (52) VSAA (24)	2009–2017	R	60.5	28 (18–49)	Bu, CY, ATG	Frontline	24.7 (6.1–103.0)
	(14)	SAA (17) VSAA (11)	2007–2016	R	57.1	8 (2–17)	Bu, CY, ATG	Frontline	38 (9–108)
	(12)	SAA (23)	2007–2015	R	NR	9 (2–17)	Bu, CY, ATG	Frontline	NR
	(33)	SAA (22) VSAA (1)	1998–2012	R	60.9	9.3 (0.6–17.2)	CY, Flu, ATG; BU, TBI, CY	Frontline	NR
	(32)	SAA (18)	2010–2014	R	55.6	8 (3–14)	Flu, CY, ATG	Frontline	24 (3–52)
Average	/	/	/	/	61 ± 5.52	13.1 ± 7.308	/	/	28.93 ± 6.441
P-value	/	/	/	/	0.0357	0.0242	/	/	0.2000

SAA, severe aplastic anemia; OS, overall survival; P, prospective; R, retrospective; NR, not report; ATG, antithymocyte globulin; Elt, eltrombopag; CsA, cyclosporine A; CY, cyclophosphamide; ATG, antithymocyte globulin; Flu, fludarabine; BU, busulfan; TBI, total body irradiation.

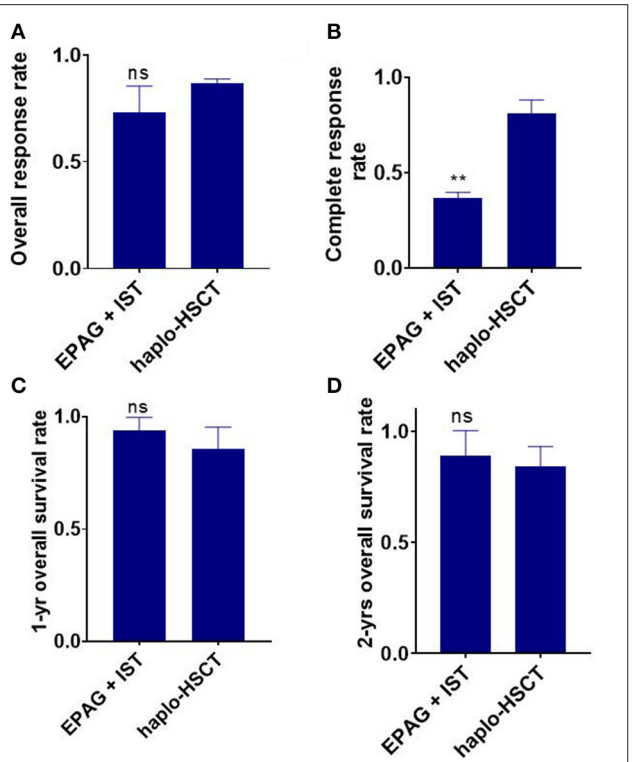

FIGURE 2 | Indirect comparison the overall response rate (ORR), complete response rate (CR), 1-/2-year overall survival (OS) between EPAG+IST and haplo-HSCT group. **(A)** Bar plot shows similar ORR between the two groups. ns, $p > 0.05$, based on the Student *t*-test. **(B)** Bar plot shows significantly lower CR rate in the EPAG+IST group compared with the haplo-HSCT group. $^{**}p < 0.01$, based on the Student *t*-test. **(C,D)** Bar plots show that the 1-/2-year OS was similar between the indicated groups. ns, $p > 0.05$, based on the Student *t*-test. EPAG, eltrombopag; IST, immunosuppressive therapy; haplo-HSCT, haploidentical hematopoietic stem cell transplantation.

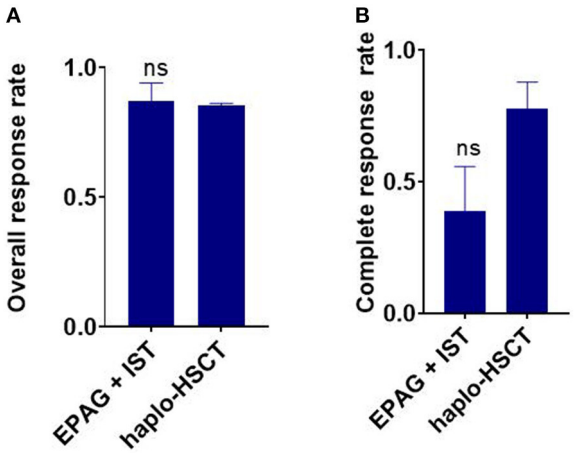

FIGURE 3 | Indirect comparison the overall response rate (ORR) and complete response (CR) rate between EPAG+IST and haplo-HSCT group in age-matched population. **(A)** Bar plots show similar ORR between the two groups. ns, $p > 0.05$, based on the Student *t*-test. **(B)** Bar plots show similar ORR between the two groups. ns, $p > 0.05$, based on the Student *t*-test. EPAG, eltrombopag; IST, immunosuppressive therapy; haplo-HSCT, haploidentical hematopoietic stem cell transplantation.

Indirect Comparison of Overall Response Rate/Complete Response at 6 Months Between the Eltrombopag Plus Immunosuppressive Therapy and Haploidentical Hematopoietic Stem Cell Transplantation Group

Since only 6 month ORR/CR data were available for EPAG+IST, we compared the ORR and CR rates of the two groups.

Four eligible studies involving a total of 159 SAA patients in the EPAG+IST group and three studies involving a total of 124 patients in the haplo-HSCT group reported the ORR. The average median age in the EPAG+IST group and haplo-HSCT group was 43.8-years (range: 15–60-years) and 13.0-years (range: 8–28-years), respectively ($p = 0.024$). Male patients were 52.4% (range: 30.0–54.3%) in the EPAG+IST group and 60.5% (range: 57.1–70.0%) in the haplo-HSCT group ($p = 0.10$). The incidence rate of VSAA in the haplo-HSCT group was 39.3% (range: 31.6–45.0%). The ORR of the EPAG+IST group was similar with that in the haplo-HSCT group ($p = 0.126$, **Figure 2A**).

Two studies involving 115 patients in the EPAG+IST group and four studies involving 142 patients in the haplo-HSCT group reported the CR rate. The average median age in the EPAG+IST group and haplo-HSCT group was 46.0-years (range: 32–60-years) and 10.5-years (range: 8–28-years), respectively ($p = 0.024$). Male patients were 53.4% (range: 52.4–54.3%) in the EPAG+IST group and 58.8% (range: 55.6–70.0%) in the haplo-HSCT group ($p = 0.13$). The incidence rate of VSAA in the haplo-HSCT group was 39.3% (range: 31.6–45.0%). The CR rate was significantly lower in the EPAG+IST group than the haplo-HSCT group ($p = 0.0012$, **Figure 2B**).

1-/2-Year Overall Survival Rate in Eltrombopag Plus Immunosuppressive Therapy and Haploidentical Hematopoietic Stem Cell Transplantation Groups

Two studies involving 113 patients in the EPAG+IST group and six studies involving 188 patients in the haplo-HSCT group reported the 1-/2-year OS. The average median ages in the EPAG+IST and haplo-HSCT groups were 46.0-years (range: 32–60-years) and 9.2-years (range: 8–28-years), respectively ($p = 0.07$). Male patients were 53.4% (range: 52.4–54.3%) in the EPAG+IST group and 60.5% (range: 55.6–70.0%) in the haplo-HSCT group ($p = 0.10$). The incidence of VSAA in the haplo-HSCT group was 35.5% (range: 4.3–45.0%).

The 1-year OS in the EPAG+IST group was similar to that in the haplo-HSCT group ($p = 0.303$, **Figure 2C**). The 2-year OS in the EPAG+IST group was similar to that in the haplo-HSCT group ($p = 0.558$, **Figure 2D**).

Comparison of Deaths and Cause of Mortality

Two studies involving 113 patients in the EPAG+IST group and five studies involving 165 patients in the haplo-HSCT group reported the causes of deaths. The median age in the EPAG+IST group and haplo-HSCT group was 46.0-years (range: 32–60-years) and 9.3-years (range: 8–28-years), respectively ($p = 0.07$). Male patients were 53.4% (range: 52.4–54.3%) in the EPAG+IST group and 60.5% (range: 55.6–70.0%) in the haplo-HSCT group ($p = 0.10$). The incidence of VSAA in the haplo-HSCT group was 35.5% (range: 4.3–45.0%). The mortality rate in the haplo-HSCT group was similar to that in the EPAG+IST group ($p = 0.098$, **Figure 3A**).

Two patients died of infections while one patient died of paraneoplastic encephalopathy at 3 months after treatment in the EPAG+IST group. Eight patients died of infections, six patients died of GVHD, four patients died of post-transplant lymphoproliferative disease, two patients died of graft failure, and the remaining two patients died of cardiogenic shock and suicide in the haplo-HSCT group (**Table 2**).

Clonal Evolution and Relapse Rate in Eltrombopag Plus Immunosuppressive Therapy and Haploidentical Hematopoietic Stem Cell Transplantation Group

Patients in the EPAG+IST group reported the rate of clonal evolution and relapse. Three studies involving 152 patients reported a clonal evolution rate, ranging at 8∼14%. The most frequent clonal evolution was loss of chromosome 7. Progression to MDSs or AML was not observed in the studies of Assi et al. or Groarke et al., nor was the development of PNH. Townsley et al. reported that one (1.1%) patient with a complex karyotype progressed to AML, while two (2.2%) patients developed PNH during follow-up. No data were available for clone evolution in the haplo-HSCT group.

Two studies involving 113 patients reported a relapse rate of 19 and 31%, respectively, for the EPAG+IST group. The study by Cheng et al. was the only one that mentioned the relapse rate in the haplo-HSCT group. They documented that there was no relapse at a median of 37.9 months of follow-up. No other relapse was reported for the rest of the studies.

The Incidence of Graft vs. Host Disease in the Haploidentical Hematopoietic Stem Cell Transplantation Group

Five studies involving a total of 165 patients reported the incidence of acute GVHD (aGVHD) in the haplo-HSCT group. The average median age in the haplo-HSCT group was 9.3-years (range: 8–28-years). Male patients were 60.5% (range: 55.6–70.0%) in the haplo-HSCT group. The incidence rate of VSAA in the haplo-HSCT group was 35.5% (range: 4.3–45.0%). The incidence of aGVHD in the haplo-HSCT was high, ranging at 52–57% (**Table 3**). The incidence of cGVHD differed considerably in included studies, ranging at 12–67% (**Table 3**).

Mycophenolate mofetil, CsA, and methotrexate were the main drug for prophylaxis against GVHD and infection, as summarized in **Table 4**.

The ORR of the EPAG+IST group was also similar to that in the haplo-HSCT group in age-matched population ($p = 0.793$, **Figure 3A**). The CR rate in the EPAG+IST group was lower than that for the haplo-HSCT group ($p = 0.064$, **Figure 3B**).

Subgroup Analyses of 6 Month Overall Response Rate/Complete Response Rate

To make the patients' baselines compatible, we picked those with a similar age [Townsley et al. (23) and Groarke et al. (30) in the EPAG+IST group and Yang et al. (3) and Xu et al. (31) in the haplo-HSCT group]. The median age was 28.5-years (range: 15–39-years) and 20.5-years (range: 13–28-years) in the EPAG+IST and haplo-HSCT groups, respectively ($p = 0.40$). The percentage of males was 55.0% (range: 53.0–55.0%) and 65.1% (range: 60.1–70.0%) in the EPAG+IST and haplo-HSCT groups, respectively ($p = 0.10$). The percentage of VSAA was 34.4% in the haplo-HSCT group.

The ORR of the EPAG+IST group was also similar to that in the haplo-HSCT group in age-matched population ($p = 0.793$, **Figure 3A**). The CR rate in the EPAG+IST group was lower than that in the haplo-HSCT group ($p = 0.064$, **Figure 3B**).

Risk of Bias Among the Included Studies

The items selected for quality assessment of studies included in the EPAG+IST and haplo-HSCT groups are shown in **Supplementary Table 1**. Overall, two studies showed a low risk of bias, while two studies showed an unclear risk of bias in the EPAG+IST group.

Bias assessment for studies in the haplo-HSCT group showed a high risk of bias in one study and an unclear risk of bias for the other five studies.

DISCUSSION

Eltrombopag (EPAG), an oral synthetic small-molecule thrombopoietin receptor agonist, was found to be effective for SAA patients that were refractory to either IST or the frontline choice (23). The development of EPAG, with its associated efficacy and safety, has greatly altered the treatment outline for SAA. However, it is associated with relapse and clonal evolution due to its stimulation on both megakaryopoiesis and hematopoiesis of other cell lineages.

Since EPAG has been used for the treatment of AA for only a short time while haplo-HSCT has been widely used in recent years, their long-term effects have not been established. In this study, we searched for all the possible related publications. After careful selection, a total of 447 patients from 10 cohort studies were enrolled. Baseline characteristics showed that patients in the EPAG+IST group were much older than those in the haplo-HSCT group. However, data on disease severity were not available in the EPAG+IST group. When the two groups were compared for ORR/CR, 1-/2-year OS, a few studies had to be excluded due to data absence.

TABLE 2 | Summary of the cause of deaths in EPAG+IST and haplo-HSCT group.

Group	References	No. of patients	No. of deaths (%)	Cause of deaths (no. of deaths)	Infection-related deaths (%)	GVHD-related deaths (%)
EPAG+IST	(23)	92	1 (1.1)	Paraneoplastic encephalopathy (1)	–	–
	(29)	21	2 (9.5)	Infections (2)	2 (100)	–
	Total	113	3 (2.7)	–	3 (66.7)	–
Haplo-HSCT	(3)	20	3 (15)	Infection (1), GVHD (1), PTLD (1)	1 (33.3)	1 (33.3)
	(31)	76	11 (14.5)	Infections (3), GVHD (2), PTLD (3), graft failure (2), suicide (1)	3 (27.3)	2 (18.2)
	(14)	28	3 (10.7)	GVHD (1), Not reported (2)	–	1 (33.3)
	(33)	23	2 (8.7)	Cardiogenic shock (2)	–	–
	Zhang (32)	18	6 (33.3)	Infection (4), GVHD (2)	4 (66.7)	2 (23.3)
	Total	165	25 (15)	–	8 (32)	6 (24)

EPAG, eltrombopag; IST, immunosuppressive therapies; HSCT, hematopoietic stem cell transplantation; GVHD, graft vs. host disease; PTLD, post-transplant lymphoproliferative disease.

TABLE 3 | Summary of the incidence of aGVHD and cGVHD in the haplo-HSCT group.

References	No. of patients	No. aGVHD (%)	No. cGVHD (%)
Yang et al. (3)	20	11 (55.0)	3 (15.0)
Xu (2018)	76	42 (55.3)	9 (11.8)
Cheng et al. (14)	28	16 (57.1)	8 (28.6)
Choi et al. (33)	23	12 (52.2)	14 (60.9)
Zhang (31)	18	9 (50.0)	12 (66.7)
Total	165	90 (54.5)	46 (27.9)

HSCT, hematopoietic stem cell transplantation; aGVHD, acute graft vs. host disease; cGVHD, chronic graft vs. host disease.

TABLE 4 | Summary the data on infection prophylaxis and GVHD prophylaxis regimens.

References	Infection prophylaxis	GVHD prophylaxis
Yang et al. (3)	–	CsA, MMF, MTX
Xu (31)	Antibiotic prophylaxis, oral trimethoprim-sulfamethoxazole, fluconazole, acyclovir	CsA, MMF, MTX
Cheng et al. (14)	Non-absorbable oral antibiotics	CsA, MMF, MTX
Choi et al. (33)	Ultrabroad spectrum Antibiotics and antifungal medications	CsA, MTX
Zhang (32)	Ultrabroad spectrum antibiotics and antifungal medications	CsA, MTX, MMF

MMF, mycophenolate mofetil; MTX, methotrexate; CsA, cyclosporine A.

Population characteristic such as age, sex, and disease severity were evenly distributed in the total patient population.

Aged patients usually exhibit poor response to treatment when compared with the younger ones, for either IST or HSCT (35–37). Under this circumstance, we found that EPAG+IST had a very similar ORR (lower in absolute number) than the haplo-HSCT (81% in the EPAG+IST group and 86% in the haplo-HSCT group, $p = 0.23$). Since age was found to be an important factor for therapeutic efficacy, we next performed subgroup analysis for patients with comparable ages. There was no significant difference in ORR between the EPAG+IST group (higher in absolute number) and the haplo-HSCT group (87% vs. 85%). However, there was a low CR rate either in the total population or in the age-matched population in the EPAG+IST group than the haplo-HSCT group, which is comparable with the findings when IST alone and haplo-HSCT were compared (12, 14, 32). As for the OS, the average 1-/2-year OS rate was 94/89% in the EPAG+IST group and 86/84% in the haplo-HSCT group. OS was higher in the EPAG+IST group compared with the haplo-HSCT group.

The mortality rate was relatively small in the EPAG+IST group, and the known causes of deaths were infections and paraneoplastic encephalopathy. In the haplo-HSCT group, the death rate was higher (although not significant), and the main causes of deaths were infections and GVHD. The high mortality rate attributed to GVHD in the haplo-HSCT group implied a relatively high treatment-related toxicity. Moreover, we found that the incidence of GVHD in the haplo-HSCT group was high. Pooled aGVHD and cGVHD were 55 and 33%, respectively. Although most of these GVHD were well-managed and not lethal, they certainly caused a longer hospitalization period, increased medical burden, and reduced the quality of life (38, 39).

Xu et al. (31) reported that donors for adult patients were younger and verified that younger donors might be associated with a lower incidence of GVHD. Furthermore, recent observational studies with small sample size (40, 41) suggested that post-transplant cyclophosphenolate (PTCy) in combination

with tacrolimus and mycophenolate is a more effective strategy than PTCy alone in preventing GVHD for older patients with hematological malignancies undergoing reduced-intensity condition (RIC) MUD SCT, but optimal GVHD prophylaxis remains need to be clarified by well-designed randomized controlled trials.

Older patients were found to respond better to EPAG+IST treatment in that they exhibited similar ORR and 1-/2-year OS to those aged younger in the haplo-HSCT group (3, 10, 23). In the age-matched subgroups of EPAG+IST, there were no significant differences in ORR and OS. However, there was a non-significant higher OS and less death rate, probably due to the small number of patients. These findings imply that EPAG+IST has comparable efficacy and OS with haplo-HSCT, even for younger patients, who are the right candidates for haplo-HSCT (35). So far, there was no head-to-head comparison for the frontline treatment of either EPAG+IST with MSD or EPAG+IST with haplo-HSCT. This study elucidates the implications for treatment choice in the era of EPAG. Of course, haplo-HSCT comes with a higher CR rate.

On the other hand, patients in the EPAG+IST group exhibited a clonal evolution rate of 9% and relapse rate of 15%, whereas no relapse or clone evolution was noticed during follow-up (median of 37.9 months) in one study. There was no other clonal evolution/relapse that was reported in the rest of the studies. These findings raised concerns about relapse and clone evolution for EPAG+IST. However, in the age-matched subgroup, in which patients were younger, there was less relapse as well as clonal evolution implying an age-related effect. Although no evidence for the increase of clone evolution rate has been identified when EPAG+IST was compared with the history controls of IST alone as the frontline therapy so far (23, 29), we do see the relapse when EPAG or IST was tapered or withdrawn (36, 42). VSAA patients usually exhibit higher chances of relapse and clonal evolution when compared with SAA patients (43). Therefore, for young VSAA patients, haplo-HSCT is an attractive option when MSD is not available (44), while for young SAA patients, treatment should be balanced depending on the related mortality and the long-term disease outcomes.

There are some limitations for our study. Due to the short period after EPAG approval for AA and the limited use of haplo-HSCT, only a few prospective/retrospective observational cohort studies with small sample sizes were included in this study. Lack of data directly comparing the therapy outcomes between EPAG+IST and haplo-HSCT groups, a narrative synthesis, rather than quantitative synthesis using meta-analysis model was applied in this study to indirectly compare the outcomes between EPAG+IST and haplo-HSCT groups. Moreover, studies on the long-term effectiveness and survival benefits of-EPAG+IST have not yet been published, making the long-term comparison impossible. Only a few of the enrolled studies reported CR/ORR in the haplo-HSCT group. Lack of a VSAA incidence in the EPAG+IST group inhibited comparisons of disease severity. Moreover, differences in treatment and supportive care in different centers, genomic background differences, imbalance of participant baseline characteristics among studies, and different treatment periods may lead to high heterogeneity, making the errors unavoidable.

Furthermore, studies on the long-term effectiveness and survival benefits of EPAG+IST have not yet been published, making the long-term comparison impossible. Only a few of the enrolled studies reported on CR/ORR in the haplo-HSCT group. Lack of a VSAA incidence in the EPAG+IST group inhibited comparisons of disease severity. Moreover, differences in treatment and supportive care in different centers, genomic background differences, imbalance of participant baseline characteristics among studies, and different treatment periods may lead to high heterogeneity, making the errors unavoidable.

In conclusion, this study elucidates the treatment options for SAA, especially in the lack of MSD. Well-designed randomized clinical trials with larger sample sizes and long-term follow-up periods are needed to confirm our findings.

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

YY and BH were responsible for the initial plan and study design, are guarantors and had full access to all of the data, including statistical reports and tables, and take full responsibility for the integrity of the data and the accuracy of the data analysis. YY, ZT, and JJ were responsible for data collection, data extraction, and statistical analyses. YY was responsible for data interpretation and manuscript drafting. 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/fonc.2021.614965/full#supplementary-material

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CASE REPORT
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Zanubrutinib Treatment of Central Nervous System Posttransplant Lymphoproliferative Disorder After Allogeneic Hematopoietic Stem Cell Transplantation: A Case Report

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Posttransplant lymphoproliferative disorder (PTLD) is a rare complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT) with poor prognosis. We report a patient with PTLD involved central nervous system (CNS) who treated with zanubrutinib, a second-generation Bruton tyrosine kinase (BTK) inhibitor. Our report supports the efficacy of bruton tyrosine kinase inhibitor zanubrutinib in the treatment of CNS-PTLD, which provides a new therapeutic option.

Keywords: hematopoietic stem cell transplantation, EBV-negative, posttransplant lymphoproliferative disorder, lymphoma, zanubrutinib

INTRODUCTION

Post-transplantation lymphoproliferative disorder (PTLD) is a spectrum of unregulated lymphoid expansion ranging from polyclonal hyperplasia to monoclonal malignant lymphoma, which normally presents with nonspecific signs such as prolonged fever and lymphadenopathy (1). Most PTLDs originate from B cells, associated with Epstein-Barr virus (EBV) reactivation. Compared with PTLD in solid organ transplantation, PTLD after hematopoietic stem-cell transplantation (HSCT) is characteristic of high invasion, early dissemination and high mortality. Currently, there is no consensus regarding the treatment of PTLD.

CASE PRESENTATION

A 39-year-old Chinese man was diagnosed with BCR-ABL-positive acute lymphoblastic leukemia in November 2018. He was treated with 8 cycles of chemotherapy combined with imatinib, resulting in complete remission (CR). He received haploidentical hematopoietic stem cell transplantation (HSCT) from his daughter on September 25, 2019 during the first CR under a myeloablative conditioning regimen (cytarabine, busulfan, cyclophosphamide, methyl-N-2-chloroethyl-N-cyclohexyl-N-nitrosourea, and anti-thymocyte globulin). Prophylaxis against graft-versus-host

disease comprised mycophenolate mofetil (MMF), cyclosporine (CsA), and short-course methotrexate (MTX). Neutrophils and platelets were engrafted on days +13 and +14, respectively. Cytogenetic studies showed complete donor chimerism on day +30. The EBV-DNA loads in the blood measured by real-time quantitative polymerase chain reaction were monitored weekly for the first 3 months after transplantation, every 2 weeks from the fourth month posttransplant. Acyclovir was used to prevent virus infection. Once EBV-DNA in the blood was positive, ganciclovir was administered until EBV-DNA turned negative on 2 consecutive measurements.

He was admitted to our center owing to headache, dizziness, and vomiting on day +90. Lumbar puncture revealed that the intracranial pressure exceeded 400 mmH_2O. Cerebrospinal fluid (CSF) next generation sequencing (NGS) was positive for *Toxoplasma gondii* (*T. gondii*). Toxoplasma serology tests were positive for IgG (16.72 IU/mL), but negative for IgM. Contrast-enhanced brain magnetic resonance imaging (MRI) demonstrated multiple enhancing hyperintense lesions surrounded by edema in bilateral cerebellar and cerebral hemispheres (**Supplementary Figures 1A–C**). He was diagnosed with cerebral toxoplasmosis based on these findings. Oral sulfamethoxazole (SMZ, 1.44 g, thrice daily) and intravenous clindamycin (400 mg, 4 times daily) were administered. His clinical signs resolved completely after 1 week of treatment. After 2 weeks, brain MRI showed a marked decrease in the size of the lesions and perifocal edema. Hence, he was discharged and switched to maintenance therapy with oral azithromycin (500 mg, once daily) and SMZ (0.96 g, twice weekly). Brain MRI performed 1 month after discharge (on day +153 after HSCT) indicated near resolution of the multiple lesions and perifocal edema (**Supplementary Figures 1D–F**).

The patient presented with a recurring headache, accompanied by reducing right-sided power and binocular diplopia on day + 203 after HSCT, which underwent gradual exacerbation. He was readmitted to our center on day +223. Physical examination revealed grade IV muscle strength in the right limb and a positive Babinski sign on the right side. Brain MRI revealed a thalamic ring-enhancing lesion surrounded by edema on the left side (**Figure 1A**). CSF analyses demonstrated elevated cerebrospinal pressure (230 mmH_2O) and elevated protein levels (1.280 g/L, reference <0.5 g/L). NGS of CSF was negative for bacterial, fungal, viral, or parasitic organisms. The serum Epstein-Barr virus (EBV)-DNA titer monitored twice a week ranged from 1.61×10^3 to 4.0×10^4copies/ml, with the treatment of ganciclovir. The distribution of T-cell subsets was examined: CD3+ T-cell count was 69 cells/μL, CD4+/CD8+ T cell was 0.57 and CD19+ B-cell count was 2 cells/μL, indicating that the patient was in a state of immunodeficiency. Systemic GVHD was not observed in the patient. Empirical treatment against Toxoplasma infection was implemented for 2 weeks but was ineffective. His clinical condition progressed on day +244 with grade II right-sided muscle strength, recurrent seizures, urinary incontinence, lethargy, memory loss, and transient consciousness disturbance. MRI was repeated, which revealed further enlargement of the lesion in the left thalamus, with a maximum diameter of approximately 53 mm (**Figure 1B**). We performed magnetic resonance spectroscopy (MRS), which depicted significant elevated lipid (Lip) and choline compounds (Cho) peaks, suggestive of lymphoma (**Figures 2A, B**). Brain biopsy, which was performed to confirm the diagnosis, identified monomorphic diffuse large B-cell lymphoma with EBV infection. The tumor cells stained positive for CD19, CD20, PAX-5, Ki-67 (approximately 65%), CD30, Bcl-2 (approximately 100%), MUM1, c-myc (approximately 25%), CD79a, and CD43 (**Figures 3A–C**). The results of EBV-encoded RNA *in situ* hybridization were positive (**Figure 3D**). Lung CT, abdominal B-ultrasound examination and bone marrow biopsy showed no evidence of systemic PTLD. Positron emission tomography-computed tomography (PET/CT) was not performed considering that the patient was critical with unstable conditions at that time. Based on these findings, the diagnosis of EBV-PTLD in central nervous system was made.

Thus, CsA administration (50mg daily) was discontinued at the diagnosis of PTLD on day +250. A single dose of rituximab 375 mg/m^2 was administrated on day +252, followed by high-dose methotrexate (MTX, 6 g) on day +256. After MTX+ rituximab, his consciousness improved and the seizure disappeared, although he still had a headache with a numerical rating scale (NRS) score of 3. Brain MRI indicated a reduction in lesion size, with the longest diameter of 36mm (**Figure 1C**). Serum EBV-DNA load decreased to 2 log_{10} within three weeks after administration of chemotherapy. The response to MTX+rituximab was stable disease. During the treatment of MTX+rituximab, the WBC and platelet counts decreased to a minimum of 0.9×10^9/L and 28×10^9/L, respectively. The patient also developed pulmonary infection. Considering that the patient's inability to tolerate chemotherapy, whole-brain radiotherapy (WBRT) was implemented on day + 280 (30 times, total dose of 30 Gy) for 47 days. During radiotherapy, the platelet and white blood cell (WBC) counts decreased to a minimum of 34 x 10^9/L and 0.9 x 10^9/L, respectively, but gradually recovered to normal. At the end of radiotherapy, his headaches were alleviated (NRS score=2) and the language impairment and dyskinesia also recovered gradually. Brain MRI showed the remained lesion with the longest diameter of 29mm (**Figure 1D**) and the serum EBV DNA load in the blood reduced to an undetectable level. After radiotherapy, the patient achieved partial response (PR) and his condition was stable, but there were still residual intracranial lesions. The patient refused further systemic chemotherapy. Considering the therapeutic activity of bruton tyrosine kinase (BTK) inhibitors for CNS lymphomas, the patient was administered oral zanubrutinib 80 mg daily (given the concurrent administration of posaconazole for preventing fungal infection) on day +382. He developed transient systemic migrating muscle soreness on the first day during zanubrutinib therapy, which was ameliorated 1 day after discontinuation of the drug. Oral administration of zanubrutinib was subsequently continued without any other side-effects. Blood counts were monitored regularly: the lowest WBC count was 2.9 x 109/L, and the hemoglobin and platelet counts were within the normal range. After starting zanubrutinib, His dizziness and headache had resolved, and the findings of neurological examination were normal. The serum EBV-DNA loads remained negative during the treatment of zanubrutinib. Follow-up brain MRI revealed that the

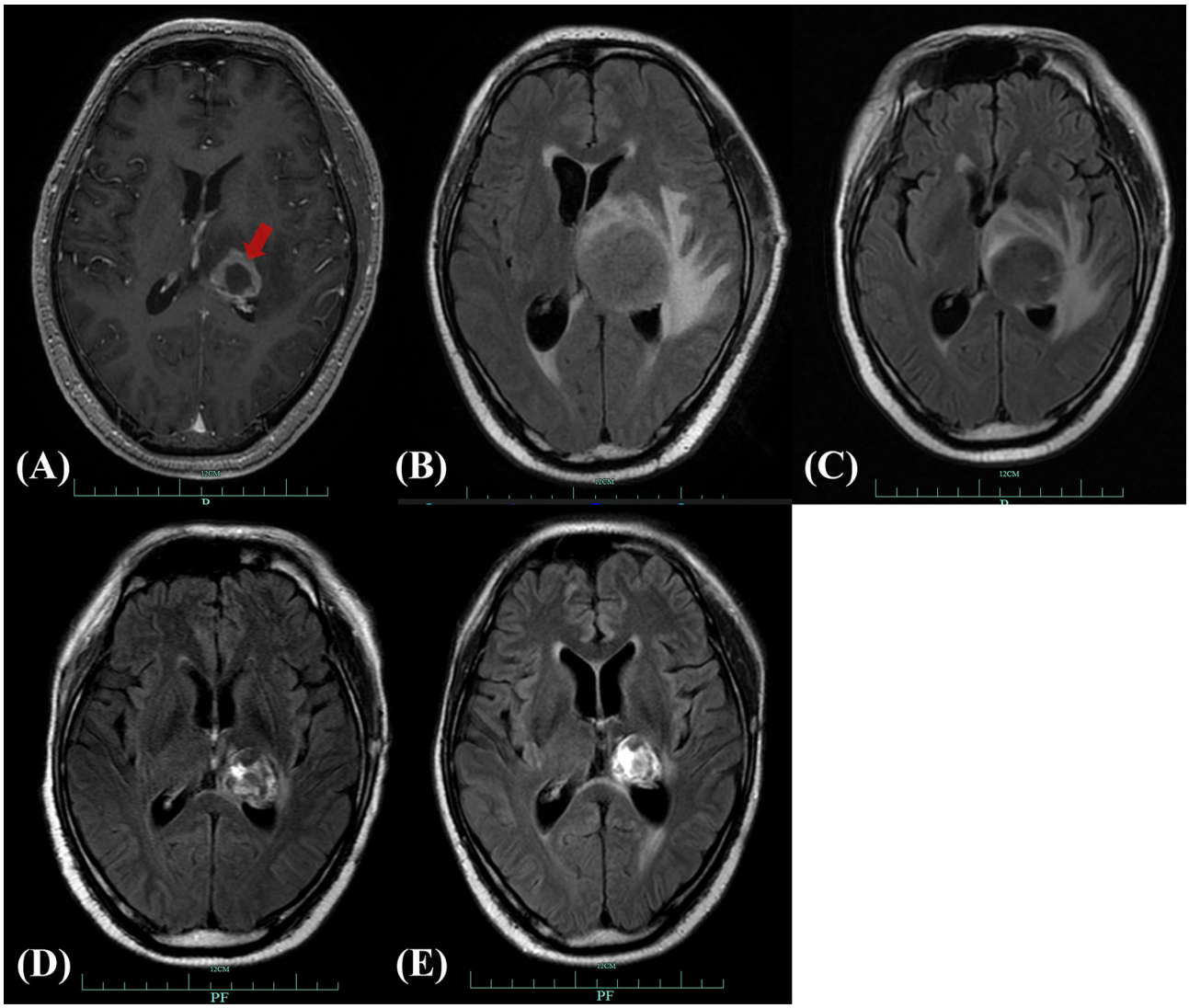

FIGURE 1 | Brain MRI of CNS-PTLD. **(A)** Emergence of CNS-PTLD on day +225. MRI revealed a hypointensity lesion in the left thalamus with ring enhancement (red arrow) on contrast-enhanced T1-weighted imagining. **(B)** Day +255 (before treatment): the enlarged lesion surrounded by significant edema with the longest diameter of about 53mm (T2 Flair). **(C)** Day +280 (three weeks after the use of rituximab and MTX): reduction in the size of the lesion and edema with the longest diameter of 36.4mm (T2 Flair). **(D)** Day +363 (after whole-brain radiotherapy completed): further reduction in the size of the lesion with the longest diameter of 29mm (T2 Flair). **(E)** Day +477(three months after the start of zanubrutinib): the reduced lesion with the longest diameter of 24mm (T2 Flair). MRI, magnetic resonance imaging; CNS-PTLD, central nervous system post-transplant lymphoproliferative disorder.

lesion's size decreased to 24 x 24 x 21 mm (**Figure 1E**) with a response of PR. Until the last follow-up in February 2021(day +516), he was alive without and clinical symptom and continued to take zanubrutinib. The treatment process and changes of serum EBV-DNA titer are summarized in **Figure 4**.

DISCUSSION

To date, there has been no definite guideline or consensus for the optimal treatment for CNS-PTLD after HSCT. The available therapeutic options include the withdrawal of immunosuppressive agents, high-dose MTX and cytarabine, WBRT and adoptive immunotherapy with EBV-specific cytotoxic T lymphocytes (2, 3). Although the patient achieved a PR with significant improvement of clinical symptoms after chemotherapy+ rituximab and WBRT, his headache remained, and MRI showed apparent residual lesions. Subsequent systemic chemotherapy was not considered because the patient refused chemotherapy. EBV-specific cytotoxic T lymphocytes(CTLs) derived from EBV-seropositive transplantation donors or the third party is effective in treat EBV- induced lymphoproliferative diseases through attacking EBV-infected cells, with durable response (4). Doubrovina et al. reviewed 19 EBV-PTLD patients who received

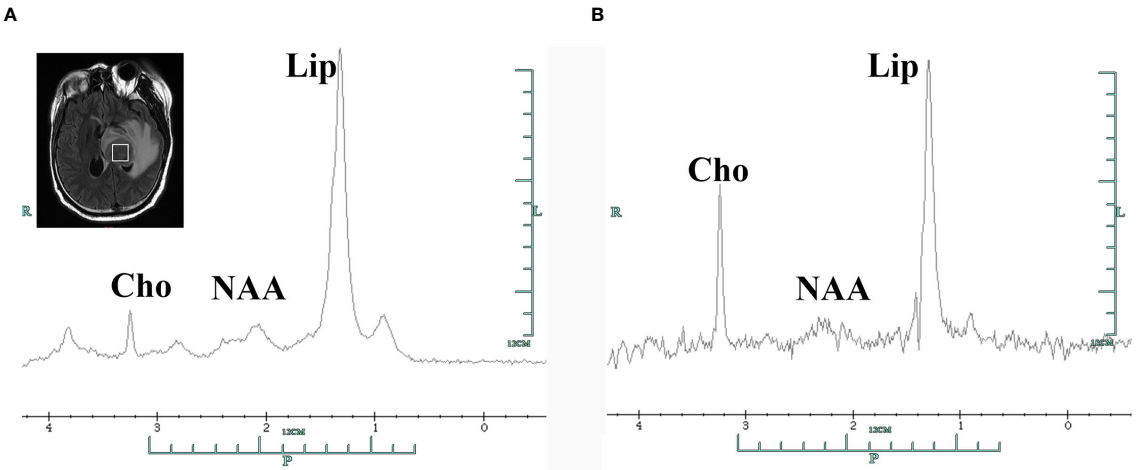

FIGURE 2 | Single-voxel 1H-magnetic resonance spectroscopy of the tumor area in the left thalamus showing elevated Cho peak in 3.2 ppm and Lip peak in 1.3ppm with decreased NAA in 2.0 ppm, with corresponding short echo time spectra (**A**, TE=35ms) and long echo time spectra (**B**, TE=144ms). Cho, choline compounds; Lip, lipid; NAA, N-acetyl-aspartate.

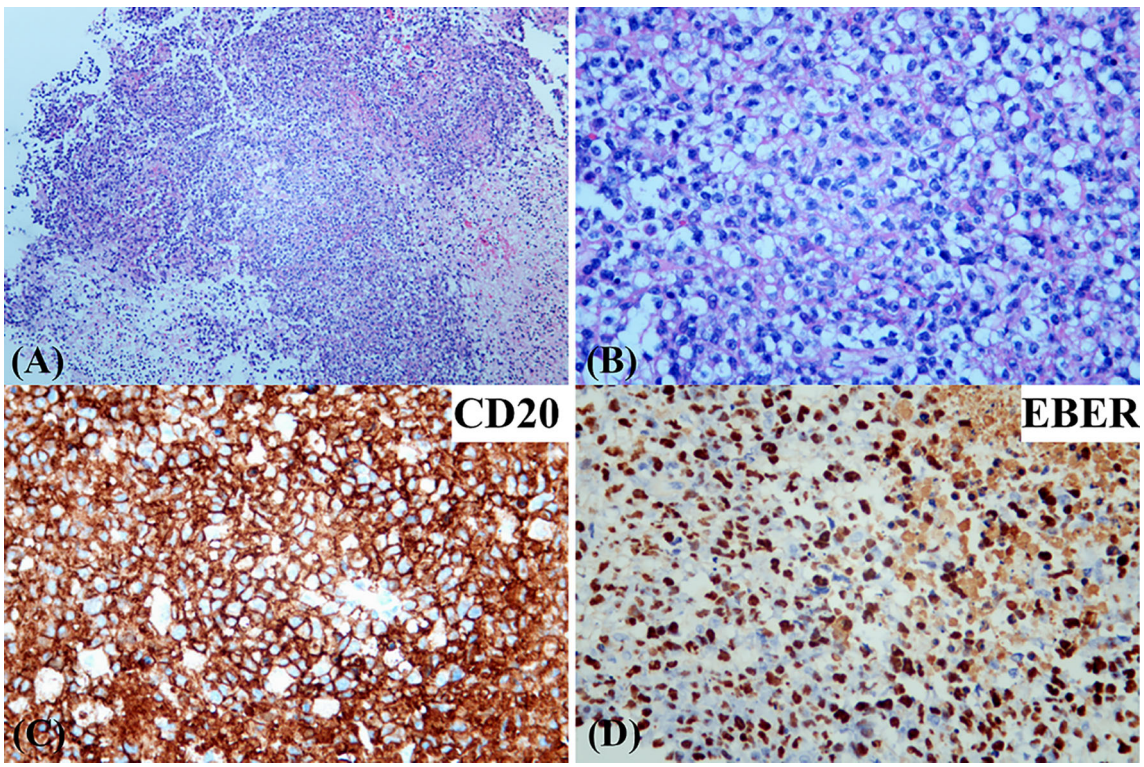

FIGURE 3 | Photomicrographs of the brain biopsy demonstrating a monomorphic diffuse large B-cell lymphoma with EBV infection. [hematoxylin and eosin (H&E) stain; original magnification **(A)** ×50; **(B)** ×200]. Immunohistochemical stains showed that the infiltrating lymphocytes were positive for CD20 **(C)**. EBV-encoded RNA *in situ* hybridization was positive in the infiltrating cells **(D)**.

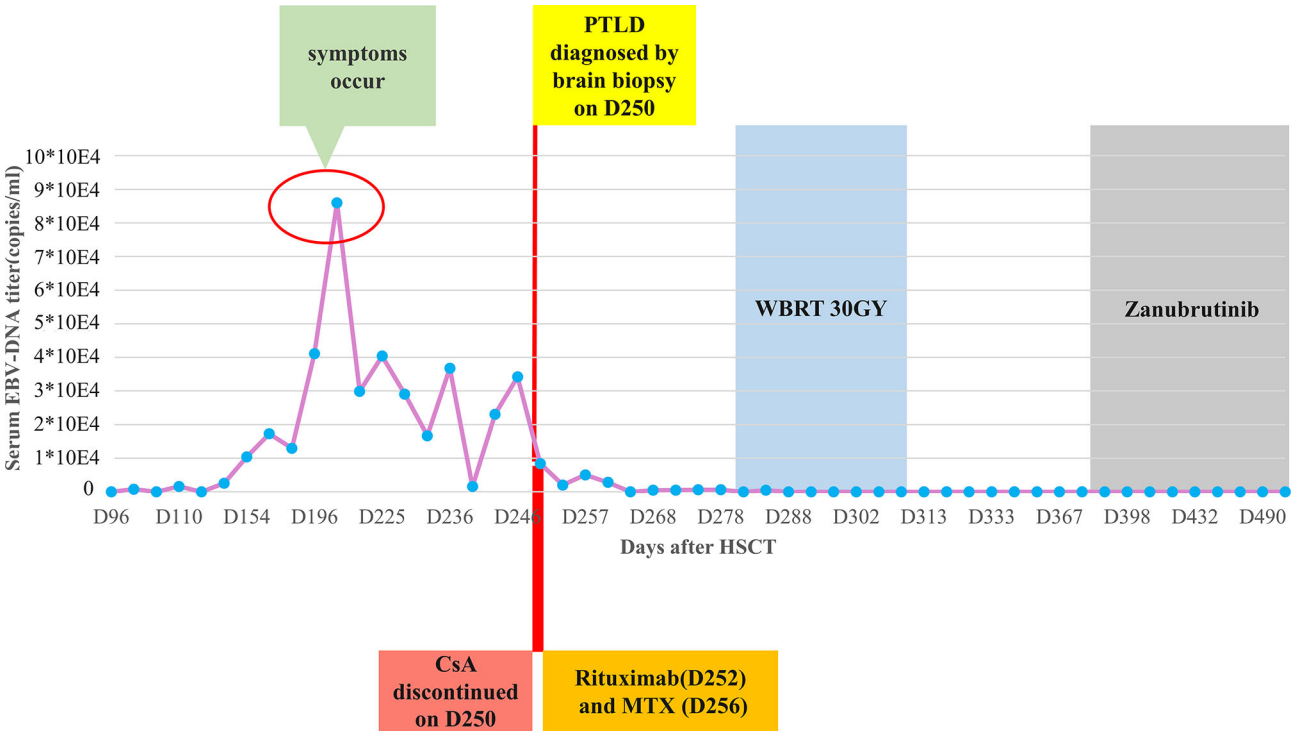

FIGURE 4 | The clinical treatment process of posttransplant lymphoproliferative disorder (PTLD) after transplantation and the changes of serum EBV-DNA titers. HSCT, hematopoietic stem cell transplantation; CsA, cyclosporine; MTX, methotrexate; WBRT, whole-brain radiotherapy.

EBV-specific CTL infusion after HSCT; in this study, 13 patients (85%) achieved CR and GVHD didn't occur in any patients (5). However, EBV-specific CTLs was unavailable in our center.

BTK, which is a tyrosine-protein kinase, is critical to B-cell maturation and proliferation, has emerged as a significant therapeutic target for various B-cell malignancies (6, 7). The first-generation BTK inhibitor ibrutinib has shown promising results for CNS lymphoma (8, 9). A phase I clinical trial conducted by Grommes et al. reported that ibrutinib showed a 77% (10/13) clinical response in patients with relapsed or refractory CNS lymphoma, including CR and partial response in 5 patients each (9). Zanubrutinib (BGB-3111), a highly-specific, irreversible second generation BTK inhibitor developed in China, has greater selectivity and higher anti-tumor activity for BTK compared to ibrutinib. It shows more restricted off-target activity for a series of kinases, such as interleukin-2-induced kinases (ITK), Scr family kinases, and epidermal growth factor receptor (EGFR), thereby limiting the toxicity and side-effects (10). It has been approved for relapsed/refractory mantle cell lymphoma and chronic lymphocytic leukemia/small lymphocytic lymphoma. Moreover, its utility for the treatment of other B-cell malignancies is also being investigated worldwide. In our case, a further decrease in the size of lesions was observed 3 months after the use of zanubrutinib, suggesting its efficacy in treating CNS PTLD. The drug is well-tolerated, and no obvious hematological toxicity or infection was observed during the period of treatment. The patient should take zanubrutinib consistently until treatment failure or the occurrence of unacceptable toxicities. To the best of our knowledge, this was the first report to describe treatment of CNS-PTLD with zanubrutinib. However, it is not sure whether the response of zanubrutinib for PTLD is durable or further improved. Longer follow-up is needed to evaluate its effect.

CONCLUSION

Our case shows that the novel BTK inhibitor zanubrutinib exhibit specific activity for CNS lymphomas. It provides a potential therapeutic option for CNS-PTLD when other attempted measures are judged to be ineffective or inappropriate.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics review committee of the First Affiliated Hospital of Zhejiang University School of Medicine. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

T-TY wrote the manuscript. W-HC, Y-MZ, H-RF and HH contributed to the patient's medical care. J-MS edited and approved the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.672052/full#supplementary-material

Supplementary Figure 1 | Brain Magnetic resonance imaging of cerebral toxoplasmosis. Multiple hyperintense enhancing lesions in bilateral cerebral hemispheres and cerebellar before treatment (on day +94 after transplantation) were observed **(A–C)**. Two months after initiation of anti-toxoplasma therapy (on day +153 after transplantation), lesions had nearly disappeared **(D, E)**. Red arrow indicated enhancing lesions on contrast-enhanced T1-weighted imaging.

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BRIEF RESEARCH REPORT
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Letermovir Administration to Prevent Cytomegalovirus Reactivation Is the Potential Risk of Chronic Graft-*Versus*-Host Disease in Patients Who Received Haploidentical Stem-Cell Transplantation With Post-Transplant Cyclophosphamide

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The prevention of chronic graft-*versus*-host disease (cGVHD) is important for recipients of hematopoietic stem-cell transplantation (HSCT). As one of the etiologies, the relationship between early T-cell recovery and subsequent cGVHD development has been the focus of attention. Recently, letermovir (LTV) was approved for preventing cytomegalovirus (CMV) reactivation in the early transplantation phase. Although CMV affects the immune reconstitution after HSCT, the impacts of LTV to prevent CMV reactivation on early T-cell recovery and cGVHD have not been fully investigated. We aimed to identify early T-cell recovery under LTV at day 30 in 15 and 33 recipients from matched related donors (MRDs) and haploidentical donors with post-transplant cyclophosphamide (PTCy-haplo), respectively. Early increases in the levels of total lymphocytes and HLA-DR$^+$ activated T-cells at day 30 were observed under CMV prophylaxis by LTV only in PTCy-haplo recipients and not in MRD recipients. Moreover, PTCy-haplo recipients with LTV showed a significantly higher incidence of cGVHD, but not acute GVHD. Our observations suggest that an early increase in the levels of HLA-DR$^+$ activated T-cells may be implicated in the development of cGVHD in patients treated with PTCy who received LTV. Further studies are warranted to validate our results and elucidate the detailed mechanisms of our new insights.

Keywords: letermovir, chronic graft-*versus*-host disease, cytomegalovirus, haploidentical stem-cell transplantation, post-transplant cyclophosphamide, HLA-DR+ activated T-cell, lymphocyte recovery

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INTRODUCTION

Recently, it has been reported that *in vivo* T-cell depletion therapy with antithymocyte globulin (ATG) or post-transplant cyclophosphamide (PTCy) is associated with the suppression of subsequent development of chronic graft-*versus*-host disease (cGVHD), suggesting the importance of regulating the early T-cell recovery after hematopoietic stem cell transplantation (HSCT) for the long-term immune tolerance (1, 2). Although the kinetics of lymphocyte recovery and its correlation with post-HSCT outcomes are well-established in the setting of matched related donors (MRDs), there are relatively few data obtained in the setting of haploidentical donors and post-transplant cyclophosphamide use (PTCy-haplo) (3). Cytomegalovirus (CMV) reactivation is an important cause of morbidity and mortality after allogeneic HSCT, which has also been reported to affect both early and long-term immune reconstitution (4). Moreover, PTCy-haplo transplant recipients show a high rate of CMV reactivation (about 70%) early after transplantation (5–7). Therefore, theoretically, a new anti-CMV prophylaxis agent letermovir (LTV) approved in 2018 in some countries including Japan could be considered for a subset of PTCy-haplo patients (8, 9). However, because LTV has been approved only recently, there is still a paucity of literature on its use for the prevention of lymphocyte recovery caused by CMV reactivation in PTCy-haplo transplant recipients (10).

In this study, we aimed to examine the early HLA-DR$^+$ activated T-cell recovery in MRDs and PTCy-haplo transplant recipients treated with LTV for CMV prophylaxis and determine the association of lymphocyte recovery with cGVHD development. All participants or their family members provided written informed consent for inclusion in retrospective studies. This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethical review board of the Kameda Medical Center.

METHODS

We retrospectively analyzed 15 MRD and 33 PTCy-haplo transplant recipients who received allogeneic HSCT as grafts of peripheral blood (PB) at our center from January 1, 2014 to August 31, 2020 (**Table 1**). The observation period ended on November 31, 2020. The haploidentical donor was defined as a relative who had two or more mismatches in human leukocyte antigen (HLA)-A, -B, -C, and -DRB1 alleles. GVHD prophylaxis was performed as follows: high-dose cyclophosphamide (50 mg/kg) on days 3 and 4, and both tacrolimus and mycophenolate mofetil (MMF) from day 5 in PTCy-haplo transplant recipients, and short-term methotrexate on day 1, 3, and 6 or MMF and calcineurin inhibitors from day -1 in MRD transplant recipients. CMV reactivation was defined as the detection of 3 or more positive cells per 50,000 cells by pp65 CMV-antigenemia assay in patients' peripheral blood without obvious end-organ dysfunction, monitored routinely weekly until day 100 or as long as clinically indicated unnecessary. CMV disease was defined by end-organ dysfunction attributable to CMV confirmed by organ biopsy because these conditions would require the administration of anti-CMV drugs (11, 12).

cGVHD diagnosis and grading were based on a previous report (13). Relapse-free survival (RFS) was defined as the time between transplantation and relapse, death, or the end of the study period. Overall survival (OS) was defined as the time between transplantation and death or the end of the study period. The probability of RFS and OS was estimated using the log-rank test. Competing events for cGVHD were death or relapse without GVHD. The groups were compared using Gray's test. All statistical analysis was conducted using R version 3.1.2 (The R Foundation for Statistical Computing, Vienna, Austria) and using the EZR software package (Saitama Medical Center, Jichi Medical University, Shimotsuke, Japan), which is a graphical user interface for R (14).

RESULTS

Baseline clinical characteristics of the patients who received PTCy-haplo or MRDs are summarized in **Table 1**. The median age at transplantation in MRD and PTCy-haplo was both 56-year-old and 12 and 25 recipients in MRD and PTCy-haplo, respectively, were male. The background hematologic malignancies were acute myeloid leukemia/myelodysplastic disorders (AML/MDS) in 7, acute lymphoblastic leukemia (ALL) in 3, and malignant lymphoma (ML) in 1 in MRD recipients, and AML/MDS in 15, ALL in 3, and 7 in ML in PTCy-haplo recipients. Since LTV was approved in Japan in 2018, patients with LTV received transplantation after 2018. Overall, the patients' backgrounds were similar between before and after LTV administration. The RFS of MRD (n=13) and PTCy-haplo (n=28) transplant recipients at 15 months was 75.5% and 55.5% (95% confidence interval [CI]: 42.6-91.4% and 31.9-73.8%), respectively. The OS of MRD and PTCy-haplo at 15 months was 66.7% and 55.1% (95% CI: 37.5–84.6% and 36.0–70.6%), respectively. The RFS and OS were not significantly different in terms of CMV reactivation and disease.

Regarding the efficacy of LTV, MRD transplant recipients prophylactically treated with LTV had a lower CMV reactivation and disease rate on day 100 than those not treated (0% *vs.* 57.1%, 95% CI: 0–0% *vs.* 26.6–90.2%; $p = 0.081$). Similarly, LTV-treated PTCy-haplo patients showed a significantly lower rate of CMV reactivation and disease on day 100 than untreated patients (12.2% *vs.* 81.2%, 95% CI: 3.4–40.5% *vs.* 59.8–95.4%; $p = 0.001$) (**Figure 1A**). Two PTCy-haplo recipients had CMV-disease (both CMV-colitis), and these patients was survived by ganciclovir treatment. No CMV-disease occurred in MRD recipients.

Next, we examined early T-cell recovery on day 30 in MRD and PTCy-haplo patients (**Table 2**). The median total lymphocyte recovery was delayed in the PTCy-haplo group compared with the MRD group (298/μL *vs.* 636/μL, $p = 0.015$). To investigate the effect of CMV prophylaxis by LTV on T-cell recovery, we further divided MRD and PTCy-haplo patients into LTV-treated and -untreated subgroups (**Table 1**); the patient backgrounds in each of the two MRD or PTCy-haplo subgroups were almost compatible.

TABLE 1 | Patients' baseline characteristics.

Characteristics	MRD			PTCy-haplo		
	LTV+ n = 8	LTV- n = 7	*p* value	LTV+ n = 17	LTV- n = 16	*p* value
Patient age at transplant (median, range)	55 (20, 60)	57 (36, 69)	0.33	55 (17, 68)	57 (20, 68)	0.69
Sex (male, %)	6 (75.0)	6 (85.7)	1	14 (82.4)	11 (68.8)	0.44
Diagnosis (n, %)			1			0.66
AML/MDS	3 (37.5)	4 (57.1)		6 (35.3)	9 (56.2)	
ALL	2 (25.0)	1 (14.3)		2 (11.8)	1 (6.2)	
ML	1 (12.5)	0		5 (29.4)	2 (12.5)	
MM/PCL	1 (12.5)	1 (14.3)		2 (11.8)	1 (6.2)	
others	1 (12.5)	1 (14.3)		2 (11.8)	3 (18.8)	
Disease status (n, %)			0.78			1
in any CR	3 (37.5)	4 (57.1)		5 (29.4)	4 (25.0)	
not CR	4 (50.0)	2 (28.6)		10 (58.8)	10 (62.5)	
other	1 (12.5)	1 (14.3)		2 (11.8)	2 (12.5)	
DRI (high/very high, %)†	4 (50.0)	2 (28.6)	0.61	10 (66.7)	8 (57.1)	0.71
MAC vs. RIC (RIC, %)	3 (37.5)	5 (71.4)	0.32	12 (70.6)	10 (62.5)	0.72
ECOG PS (<2, %)	5 (62.5)	6 (85.7)	0.57	11 (64.7)	11 (68.8)	1
CMV serostatus (n, %)††			1			0.82
D-/R+	1 (25.0)	0		4 (33.3)	5 (50.0)	
D+/R-	0	0		1 (8.3)	0	
D+/R+	3 (75.0)	2 (100)		7 (58.3)	5 (50.0)	
CMV reactivation (+, %)	1 (12.5)	4 (57.1)	0.11	3 (17.6)	13 (81.2)	<0.001
ABO match (n, %)			0.71			0.74
Matched	7 (87.5)	5 (71.4)		9 (52.9)	7 (43.8)	
Major mismatch	1 (12.5)	1 (14.3)		3 (17.6)	5 (31.2)	
Minor mismatch	0	1 (14.3)		5 (29.4)	4 (25.0)	
Infusion CD34+ cells ($\times 10^6$/kg) (median, range)	3.6 (2.0, 5.4)	3.6 (1.8, 6.1)	0.91	4.2 (2.0, 10.4)	3.7 (1.7, 5.0)	0.18
Prophylaxis of GVHD			0.28			1
PTCy + Tac + MMF	0	0		17 (100)	16 (100)	
short MTX + CNI	7 (87.5)	4 (57.1)		0	0	
MMF + CNI	1 (12.5)	3 (42.9)		0	0	
Donor age (median, range)	53 (33, 61)	54 (30, 60)	0.9	29 (15, 55)	34 (20, 59)	0.1
Donor type (n, %)			0.32			0.84
Children	1 (12.5)	0		13 (76.5)	11 (68.8)	
Parents	2 (25.0)	0		1 (5.9)	1 (6.2)	
Siblings	5 (62.5)	7 (100)		3 (17.6)	4 (25.0)	
HLA match (n, %)			NA			0.077
4/8	0	0		12 (70.6)	6 (37.5)	
5/8	0	0		5 (29.4)	7 (43.8)	
6/8	0	0		0	3 (18.8)	
aGVHD, grade II-IV (n, %)	1 (12.5)	3 (42.9)	0.28	6 (35.3)	8 (50.0)	0.49
Additional immunosuppression*before day 30†††	3 (42.9)	2 (28.6)	1	4 (28.6)	7 (43.8)	0.47

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CMV, cytomegalovirus; CNI, calcineurin inhibitor; CR, complete remission; DRI, disease risk index; ECOG PS, Eastern Cooperative Oncology Group Performance Status; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; LTV, letermovir; MAC, myeloablative conditioning; MDS, myelodysplastic syndromes; ML, malignant lymphoma; MM, multiple myeloma; MMF, mycophenolate mofetil; MRD, matched-related donor; MTX, methotrexate; NA, not applicable; PCL, plasma cell leukemia; PTCy, post-transplant cyclophosphamide; RIC, reduced-intensity conditioning; Tac, tacrolimus.

†n = 13 and 29.

††n = 6 and 22.

†††n = 14 and 30.

**Additional immunosuppression indicates additional systemic prednisolone or methylprednisolone initiation before day 30 for acute GVHD or engraftment syndrome.*

On day 30, there was no significant difference in total lymphocyte counts between LTV-treated and untreated MRD patients; however, a significant increase in total lymphocyte count was observed in LTV-treated PTCy-haplo patients (median 694/μL *vs.* 186/μL in the untreated group; $p < 0.001$) (**Figure 1B1**). We also detected an increasing trend to increase in the levels of $CD3^+HLA\text{-}DR^+$, CD4, and CD8 T-cells in the LTV-treated compared with the LTV-untreated PTCy-haplo patients (median $HLA\text{-}DR^+$ activated T-cells:150/μL *vs.* 59/μL, $p = 0.079$, $CD4^+$ and $CD8^+$ T-cells are shown in **Table 2**). However, there was no statistically significant difference in $HLA\text{-}DR^+$ activated T-cells, $CD4^+$, and $CD8^+$ T-cells between LTV-treated and -untreated MRD transplant recipients (median $HLA\text{-}DR^+$ activated T-cells: 54/μL *vs.* 130/μL, $p = 0.29$) on day 30 (**Figure 1B2**, **Table 2**).

As the early recovery of $HLA\text{-}DR^+$ activated T-cells was observed only in PTCy-haplo patients prophylactically receiving LTV, we further investigated the rate of moderate to severe cGVHD based on 3.3 months landmark analysis. PTCy-haplo transplant recipients treated with LTV showed a significant increase in the cGVHD rate at 15 months compared with those not treated (63.3% *vs.* 12.5%, 95% CI: 30.5–93.7% *vs.* 1.9–61.3%, $p = 0.025$; **Figure 1C**). However, no difference in the cGVHD rate was observed among

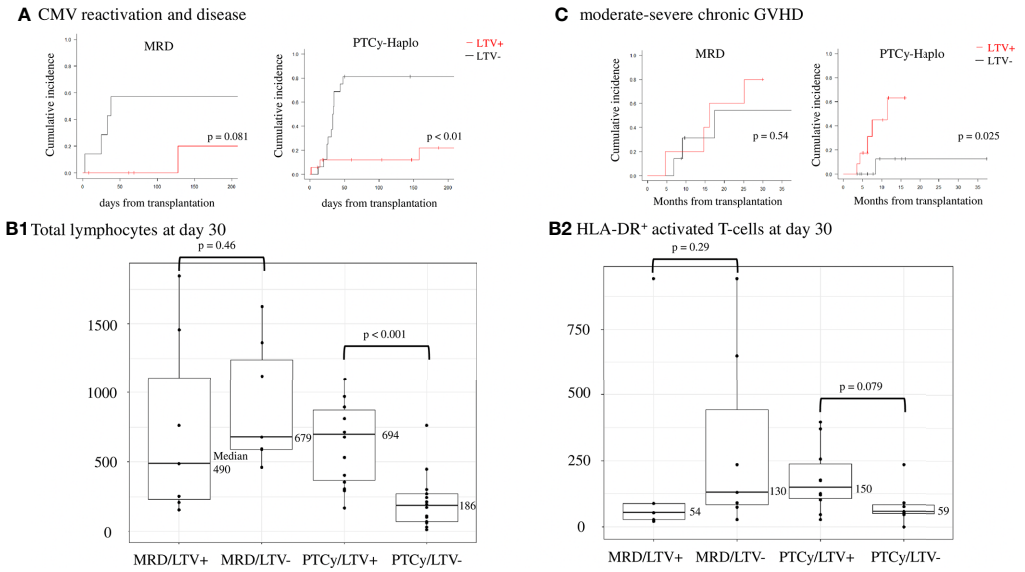

FIGURE 1 | Effects of CMV prophylaxis with LTV in matched related donor (MRD) transplantation and haploidentical transplantation with post-transplant cyclophosphamide (PTCy-haplo). **(A)** CMV reactivation and disease rate at day 100 in MRD and PTCy-haplo transplant recipients. Statistical analysis was performed using the Kaplan–Meier method with log-rank analysis and Gray's test. **(B)** Counts of total lymphocytes (left, **B1**) and HLA-DR$^+$ activated T-cells (right, **B2**) in MRD and PTCy-haplo transplant recipients treated or not with LTV. Statistical analysis was performed using the Mann–Whitney U test. **(C)** Development of moderate-to-severe chronic GVHD at 15 months in MRD and PTCy-haplo patients.

MRD patients treated or not with LTV. The cumulative incidence of grade II-IV acute GVHD (aGVHD) was not significantly different in patients with LTV and without LTV in both MRD and PTCy-cohort (**Table 1**).

DISCUSSION

These data demonstrated that only PTCy-haplo but not MRD transplant recipients subjected to CMV prophylaxis by LTV showed early recovery of total lymphocytes as well as HLA-DR$^+$ activated T-cells. These PTCy-haplo patients with LTV showed a significantly high incidence of cGVHD, but not aGVHD in this study. In general, a significant increase of acute and chronic GVHD has been reported in recipients using graft from PB which content high HLA-DR$^+$ activated T-cells (15–17). However, due to the protective effect of PTCy on regulatory T-cells (18, 19), it is considered that the incidence of aGVHD was not increased, which was compatible to the previous report (9). On the other hand, although the reason for early HLA-DR$^+$ activated T-cell expansion observed after CMV prophylaxis by LTV only in PTCy-haplo patients is still unknown, possible underlying mechanisms could include shifts in cytokine dynamics for CMV protection and changes in the integrity and heterogeneity of the T-cell repertoire (20–22).

The risk factors reported for cGVHD development in PTCy-haplo transplant recipients, including reduced-intensity conditioning regimens, older donor age, and PB as a graft source (23), are associated with increased alloreactive T-cell proliferation and exhaustion. T-cell-depleting antibodies such as ATG can suppress the development of cGVHD by removing early alloreactive T-cells (1). The other possible mechanism of increased cGVHD in PTCy-haplo recipients with LTV was insufficient T-cell suppression in the early-phase of transplantation. Therefore, to inhibit early T-cell expansion and prevent cGVHD in PTCy-haplo transplant

TABLE 2 | Lymphocytes count in MRD and PTCy-haplo.

median, μL (range)	N	MRD			N	PTCy-haplo		
		LTV+	LTV-	*p* value		LTV+	LTV-	*p* value
day 30								
Total lymphocytes	14	490 (156, 1848)	679 (460, 1625)	0.46	30	694 (168, 1100)	186 (15, 760)	<0.001
CD3$^+$	12	203 (148, 1533)	441 (276, 1173)	0.27	17	275 (58, 520)	102 (4, 302)	0.019
CD4$^+$	12	129 (92, 346)	170 (163, 346)	0.53	17	84 (15, 232)	38 (2, 83)	0.032
CD8$^+$	12	84 (42, 924)	148 (64, 982)	0.2	17	148 (31, 406)	48 (1, 205)	0.055
CD19$^+$	12	0 (0, 18)	0 (0, 11)	1	17	0 (0, 8)	0 (0, 0)	0.26
CD56$^+$	12	37 (4, 203)	190 (78, 243)	0.034	17	125 (20, 388)	81 (1, 425)	0.097
CD3$^+$HLA-DR$^+$	12	54 (22, 942)	130 (29, 941)	0.29	17	150 (29, 396)	59 (1, 235)	0.079

recipients, additional prolonged immunosuppression after PTCy administration could be considered.

The limitations of our study include the heterogeneous patient background and small sample size. The data on T-cells after day 30 and functional assay for mediating alloreactive T-cells such as interferon-gamma and tumor necrosis factor-alfa were not collected systematically. The correlation between HLA-DR+ activated T-cells and chronic but not acute GVHD might seem intriguing, however, we are unable to throw further light on the mechanistic pathways behind this association in the absence of longitudinal data. Further prospective studies on the relationship between detailed T-cell analysis and cGVHD under LTV are warranted because the use of LTV is expanding in the clinical practice. Despite these limitations, the uniformity of transplantation grafts (PB from haploidentical relatives) and GVHD prophylaxis (high-dose Cy, then tacrolimus and MMF) in PTCy-haplo patients could be considered a strength of this study.

In conclusion, our results revealed early HLA-DR$^+$ activated T-cell expansion in PTCy-haplo but not in MRD patients who received LTV for CMV prophylaxis. These LTV-treated PTCy-haplo recipients showed a higher incidence of cGVHD; thus, these patients might be subjected to prolonged immunosuppression to prevent cGVHD development. Further studies are warranted to validate our findings and elucidate the detailed mechanisms underlying the effects reported here.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethical review board of the Kameda Medical Center. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TTe conceived, designed, and initiated the study, acquired data, and wrote the manuscript. K-iM, KN, TTs, and SY participated inwriting the manuscript. TTe and KN performed statistical analyses. TTe, KN, TTs, AK, RT, DM, MT, and KM provided patient care. KM supervised the study. All authors have reviewed and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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Immune Reconstitution-Based Score for Risk Stratification of Chronic Graft-Versus-Host Disease Patients

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Introduction: Allogeneic stem cell transplantation survivors are at a relevant risk of developing chronic GvHD (cGvHD), which importantly affects quality of life and increases morbidity and mortality. Early identification of patients at risk of cGvHD-related morbidity could represent a relevant tool to tailor preventive strategies. The aim of this study was to evaluate the prognostic power of immune reconstitution (IR) at cGvHD onset through an IR-based score.

Methods: We analyzed data from 411 adult patients consecutively transplanted between January 2011 and December 2016 at our Institution: 151 patients developed cGvHD (median follow-up 4 years). A first set of 111 consecutive patients with cGvHD entered the test cohort while an additional consecutive 40 patients represented the validation cohort. A Cox multivariate model for OS (overall survival) in patients with cGvHD of any severity allowed the identification of six variables independently predicting OS and TRM (transplant-related mortality). A formula for a prognostic risk index using the β coefficients derived from the model was designed. Each patient was assigned a score defining three groups of risk (low, intermediate, and high).

Results: Our multivariate model defined the variables independently predicting OS at cGvHD onset: CD4+ >233 cells/mm^3, NK <115 cells/mm^3, IgA <0.43g/L, IgM <0.45g/L, Karnofsky PS <80%, platelets <100x10^3/mm^3. Low-risk patients were defined as having a score ≤3.09, intermediate-risk patients >3.09 and ≤6.9, and high-risk patients >6.9. By ROC analysis, we identified a cut-off of 6.310 for both TRM and overall mortality.In the training cohort, the 6-year OS and TRM from cGvHD occurrence were 85% (95% CI, 70-92) and 13% (95% CI, 5-25) for low-risk, 64% (95% CI, 44-89) and 30% (95% CI, 15-47) for intermediate-risk, 26% (95% CI, 10-47), and 42% (95% CI, 19-63) for high-risk patients (OS p<0.0001; TRM p = 0.015). The validation cohort confirmed the model with a

6-year OS and TRM of 83% (95% CI, 48-96) and 8% (95% CI, 1-32) for low-risk, 78% (95% CI, 37-94) and 11% (95% CI, 1-41) for intermediate-risk, 37% (95% CI, 17-58), and 63% (95% CI, 36-81) for high-risk patients (OS p = 0.0075; TRM p = 0.0009).

Conclusions: IR score at diagnosis of cGvHD predicts GvHD severity and overall survival. IR score may contribute to the risk stratification of patients. If confirmed in a larger and multicenter-based study, IR score could be adopted to identify patients at high risk and modulate cGvHD treatments accordingly in the context of clinical trial.

Keywords: chronic GvHD, immune reconstitution, biomarker, prognostic score, overall survival

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a recognized curative treatment for several benign and malignant disorders. Although HSCT outcomes have improved significantly over time (1), long term survivors are at a defined relevant risk of developing complications; life expectancy remains lower compared to the age- and gender-matched population (2). Acute and chronic graft-versus-host disease (aGvHD and cGvHD, respectively) represent the most detrimental complications: with standard pharmacologic prophylaxis aGvHD occurring in 20-50% of patients and cGvHD in 30-50% (3). One third of cGvHD patients dies within 5 years of cGvHD diagnosis.

For more than three decades, high dose prednisone has been the only reliable therapy for cGvHD; however new drugs are now becoming available, and some have entered clinical practice with considerable success (4–6). Considering the recent availability of more treatment choices, the need for predictive and prognostic biomarkers has emerged.

In 2014, the National Institute of Health (7) defined criteria for developing GvHD biomarkers and their clinical role: I) prognostic biomarkers - to identify patients at high risk of cGvHD, II) diagnostic biomarkers - to help diagnosis in case of clinical uncertainty, and III) predictive biomarkers - to predict outcome and response to therapy.

Identifying reliable biomarkers in cGvHD is a difficult task due to the pleiomorphism of the disease, lack of sufficient patient numbers within prospective trials, but also technical issues such as difficulties in probes selection, availability of clinical grade tests, and time-points identification (8).

For its biological implications and for its feasibility, the assessment of immune reconstitution (IR) represents a good cGvHD biomarker candidate.

Previous studies have described associations between several cellular biomarkers and cGvHD (9–18), however no cGVHD cellular biomarker has yet been qualified for use in clinical applications (7).

In this study, we evaluated CD3+, CD4+, and CD8+ cells, NK cells, and B cells as well as immunoglobulins levels as potential predictive biomarkers of cGvHD, with the aim of defining an easy, reliable, and reproducible score to stratify patients at diagnosis of cGvHD.

MATERIALS AND METHODS

The primary endpoint of the study was to assess the impact of IR in risk stratification of cGvHD patients at diagnosis. The study objective was to find a prognostic index predicting the risk of TRM and probability of OS. To this aim we included additional cGvHD prognostic factors already identified by previous studies (19–21) in addition to IR variables.

Patients

Patients aged >/= 18 years undergoing their first HSCT for any disease in indication and with any donor type or conditioning regimen, transplanted at IRCCS San Raffaele Scientific Institute between January 2011 and December 2016 were considered eligible for the study. Patients undergoing a second or third HSCT were excluded. A total of 411 patients met our inclusion criteria, among these 151 patients experienced cGvHD.

We first tested our score on a training set of consecutive patients undergoing HSCT between July 2012 and December 2016. Follow-up lasted until June 1, 2021 (or patients were censored earlier in case of a second HSCT). We then validated the scoring system retrospectively in all consecutive patients undergoing HSCT between January 2011 and June 2012 and who later developed cGvHD. Follow-up lasted until June 1, 2021. A second validation set to prospectively validate the IR score is under evaluation: patients transplanted between January 2017 and December 2019 are so far in follow-up, monitored for occurrence of cGvHD and classified according to IR score. The outcome analysis will be performed at the completion of the third year after HSCT of the last transplanted patients – December 2022 (**Supplementary Figure 1**).

Prognostic Factors

We prospectively collected IR data of all our patients at the time of cGvHD diagnosis. IR variables were CD3+, CD3+CD4+, CD3+CD8+ (T cells and subsets), CD19+ (B cells), CD3-CD16+, and/or CD56+ (NK cells) absolute cell counts and levels of IgG, IgA, and IgM. The immunophenotype evaluation was performed on EDTA whole blood samples, using a lyse-no-wash technique and a panel of directly conjugated antibodies. Ten-color flow cytometry was performed using a Navios cytometer (Flow-Count™ Fluorospheres Beckman-Coulter) and Navios software. The single platform method was used to determine

absolute counts. The analysis of lymphocyte subpopulations was performed on a lymphocyte population gate and on CD3 +lymphocytes, using quadrant dot plot statistics. Immunoglobulin titers were assessed by immunoturbidimetric assays.

NIH 2004 (22) and subsequent 2014 (23) guidelines were followed for the diagnosis and staging of GvHD. Therapy and management followed our institutional protocol.

Clinical and transplantation variables (see below) used in the analysis included age, refined disease risk index (R-DRI) (24), HCT-Comorbidity Index (HCT-CI) (25), type of donor, GvHD prophylaxis, IR values at cGvHD diagnosis, history of prior acute GvHD, Karnofsky performance status (KPS), and platelet and total lymphocyte counts. These data and sample collection were part of the routine post-transplant assessment and did not require further blood sampling.

Ethical Statement

In this non-interventional, prospective, observational cohort study, informed consent for the use of clinical data for scientific purposes was obtained from all patients undergoing HSCT in accordance with the Declaration of Helsinki.

All patients were treated according to current institutional programs upon written informed consent for transplant procedures, use of medical records, and immunological studies for patients undergoing allogenic HSCT within the non-interventional ALMON study, approved by San Raffaele Institutional Ethical Committee on October 19, 2007.

Data collection and storage were performed according to current institutional guidelines for ensuring privacy.

Statistical Analysis and Definitions

The probability of overall survival (OS) was estimated using the Kaplan-Meyer estimator (26). Cumulative incidence was estimated for TRM to accommodate relapse as a competing risk. The log-rank test was used for univariate comparisons of survival curves, while the Gray's test was conducted to compare cumulative incidences of competing risk endpoints. We built Cox multivariate models for OS in patients with cGvHD of any severity. Time was calculated from the development of cGvHD to the event of interest or last follow-up. Variables included in the models were the following: patient age (according to median value), R-DRI, type of donor (MRD – match related donor, MUD – match unrelated donor, CB – cord blood, MMRD – mismatch related donor), main GvHD prophylaxis (Anti Thymocyte Globulin [ATG]-based *vs* Post transplant Cyclophosphamide [PTCy]-based *vs* neither of the two), IR values at cGvHD diagnosis (according to median values), history of prior acute GvHD, Karnofsky performance status (KPS), platelet count $<100x10^3/mm^3$, total lymphocyte count $<1.0 \times 10^3/mm^3$, and eosinophil count $<0.5x10^3/mm^3$. A backward stepwise procedure was used for variable selection with a p-value <0.05. Once we identified the variables independently predicting OS by multivariate analysis, we derived a formula for a prognostic risk index by using the β coefficients found in the model.

Each patient, for whom we had information about all the variables found in the model, was then assigned a numeric score and three groups of risk were identified (low, intermediate, and high) by dividing the population into three classes using the first and third quartiles. This choice was based on the assumption that the proportion of patients either at low or high risk would be lower than that of patients at intermediate risk. Finally, to evaluate predictive performance of the IR score, we calculated the receiver operating characteristics (ROC) curve and the area under the curve (AUC), to summarize the IR score ability to correctly classify events and non-events.

All statistical analyses were performed with the R software (R Development Core Team, Vienna, Austria).

RESULTS

Patient Characteristics

Clinical features of patients with cGvHD are shown in **Table 1**. Among the 307 patients of the training set, 111 met the criteria for diagnosis of cGvHD according to NIH and among the 104 patients of the validation set, 40 met the criteria for diagnosis of cGvHD.

The two cohorts were similar for age, sex, disease type, graft source, R-DRI at transplant, level of mismatch, and CMV serostatus. Compared to the training cohort, the validation set included a lower proportion of patients receiving myeloablative conditioning (MAC) (52% *vs* 77% - p 0.008), a higher proportion of patients receiving ATG as GvHD prophylaxis (ATG 72% *vs* 36%) with no patients receiving PTCy, against 51% of patients in the training cohort (p <0.001). Finally, the HCT-CI score was lower in the validation cohort than in the training one (p <0.001).

Almost half of the patients received a transplant from a haploidentical family donor (47% in the training set, 40% in the validation cohort, ns).

GvHD prophylaxis in the training cohort relied mainly upon ATG in the MUD setting and on PTCy + sirolimus in haploidentical transplants, while in the validation cohort ATG was the backbone of GvHD prophylaxis both for MUD and MMRD. Peripheral blood was the preferred stem cell source in both cohorts. The proportion of MRD/MUD/MMRD was equally distributed across patients with or without cGvHD in both sets.

Median follow-up was 6 years [range 1 - 8.5] in the training set and 9.2 years [6.4 – 10] in the validation set. Median time to GvHD was 198 days [range 32-926] in the training set and 161 days [range 39-1304] in the validation set.

In the training set, the 2-year OS and 2-year cumulative incidence of TRM from cGvHD diagnosis were 71% (95% CI, 61-79) and 13% (95% CI, 7-20), respectively. In the validation set, the 2-year OS and 2-year cumulative incidence of TRM were 73% (95% CI, 56-84) and 23% (95% CI, 11-37), respectively.

Chronic GvHD Features According to NIH Classification

In the training set, the 3-year cGvHD incidence was 35% (95% CI, 29-40%) with 27% moderate-severe cGvHD (95% CI, 22-32%), while in the validation set, it was 36% (95% CI, 27-45) with 33% moderate-severe (95% CI, 24-42).

According to NIH definition, there were 69 (62.2%) classic-type cGvHD (21 mild, 26 moderate, 22 severe), and 42 (37.8%)

TABLE 1 | cGVHD patients characteristics in the training and validation cohorts.

	Training cohort N = 111	Validation cohort N = 40	p
Patient age, years, median [range]	49 [17-77]	52 [19-72]	0.91
Diagnosis, n (%)			0.51
Acute leukemia	54 (49%)	22 (55%)	
MDS or MPN	19 (17%)	9 (22%)	
Lymphoma and myeloma	36 (32%)	8 (20%)	
Aplastic anemia	2 (2%)	1 (3%)	
R-DRI at HSCT, n (%)			0.44
Low-Intermediate	63 (56%)	25 (62%)	
High	43 (39%)	11 (28%)	
Very high	4 (4%)	3 (7%)	
Not applicable	1 (1%)	1 (3%)	
HCT-CI score, median [range]	2 [0-8]	0 [0-3]	<0.001
Donor type, n (%)			0.30
MRD	25 (22%)	14 (35%)	
MUD	34 (31%)	10 (25%)	
MMRD	52 (47%)	16 (40%)	
Donor age, years, median [range]	37 (18-73)	41 (19-58)	0.83
Female donor/male recipient, n (%)	31 (28%)	17 (42%)	0.11
Host/donor CMV serostatus, n (%)			0.86
pos/pos	76 (68%)	30 (75%)	
pos/neg	19 (17%)	6 (15%)	
neg/pos	3 (3%)	1 (3%)	
neg/neg	13 (12%)	3 (7%)	
Conditioning intensity, n (%)			0.008
RIC	26 (23%)	19 (48%)	
MAC	85 (77%)	21 (52%)	
Stem cell source, n (%)			0.34
BM	6 (5%)	0	
PB	105 (95%)	40 (100%)	
GvHD prophylaxis			<0.001
ATG-based	40 (36%)	29 (72%)	
PTCy-Sirolimus-based	56 (51%)	0	
Sirolimus-MMF	10 (9%)	5 (13%)	
CSA-MMF	2 (2%)	1 (3%)	
CSA-MTX	3 (3%)	5 (12%)	

MDS, myelodysplasia; MPN, myeloproliferative neoplasms; R-DRI, revised disease risk index; HCT-CI score, Hematopoietic Cell Transplantation – specific Comorbidity Index; MRD, match related donor; MUD, match unrelated donor; MMRD, mismatch related donor; CMV, cytomegalovirus; RIC, reduced intensity conditioning; MAC, myeloablative conditioning; BM, bone marrow stem cells; PB, peripheral blood stem cells; MMF, micophenolate mofetil; CSA, cyclosporine-A; MTX, methotrexate; ATG, antithymocyte globulin; PTCy, post-transplant cyclophosphamide.

overlap cGvHD (5 mild, 15 moderate, 22 severe) in the training cohort and 21 (52.5%) classic-type cGvHD (2 mild, 9 moderate, 10 severe), and 19 (47.5%) overlap cGvHD (0 mild, 5 moderate, 14 severe) in the validation cohort. Of note, 37 patients (33%) in the training cohort and 21 (52%) in the validation cohort were previously diagnosed with acute GvHD.

All patients with a diagnosis of cGvHD were treated at our long-term follow-up clinic according to institutional guidelines and EBMT recommendations (27). All patients with a moderate to severe cGvHD received first line treatment with high-dose prednisone (0, 5-1 mg/Kg), topical therapy was added when appropriate.

Immune Reconstitution as Predictive Factor for cGvHD—Algorithm Development and Validation

The following variables independently predicting OS at cGvHD diagnosis were identified: CD4+ count >233 cells/mm^3 (β 3.09, p 0.01), NK count <115 cells/mm^3 (β 1.75, p 0.02), IgA <0.43 g/L (β 1.47, p 0.03), IgM <0.45 g/L (β 2.22, p 0.007), Karnosky PS <80% (β 5.05, p <0.001), and PLT <100x10^3/mm^3 (β 2.18, p 0.02). The multivariate Cox regression analysis of factors determining OS is reported in **Table 2**.

IR parameters at time of cGvHD onset are reported in **Table 3**. In the training cohort, the median time of IR parameters evaluation was 189 days. Overall, the median time of collection of IR parameters was 150 days.

An algorithm was created based only on variables that predicted OS significantly and independently, i.e., CD4+ count >233 cells/mm^3, NK count <115 cells/mm^3, IgM <0.45 g/L, IgA <0.43 g/L, Karnosky PS <80%, and PLT <100x10^3/mm^3. To calculate the final score, we took into account the different weight of these six variables in predicting OS, expressed by their beta coefficient. The final score was calculated as follows:

3.09 (if CD4 > 233 cells/mm^3 at time of cGvHD diagnosis) + 1.75 (if NK < 115 cells/mm^3 at time of cGvHD diagnosis) + 1.47 (if IgA < 0.43 g/L at time of cGvHD diagnosis) + 2.22 (if IgM < 0.45 g/L at time of cGvHD diagnosis) + 5.05 (if Karnofsky <80 at time of cGvHD diagnosis) + 2.18 (if PLT <100x10^3/mm^3 at time of cGvHD diagnosis).

TABLE 2 | Multivariate Cox-regression analysis of factors determining OS.

	OS		
	HR (95% CI)	**β coefficient**	**p**
CD3+CD4+ cells/mm^3 at cGvHD diagnosis			
≥233 cells/mm^3 Vs <233 cells/mm^3	21.9 (1.9-57)	3.09	0.014
NK cells/mm^{3}at cGvHD diagnosis			
<115 cells/mm^3 Vs ≥115 cells/mm^3	5.7 (1.4-23)	1.75	0.017
IgM at cGvHD diagnosis			
<0.45 g/L Vs ≥0.45 g/L	9.2 (1.8-36)	2.22	0.007
IgA at cGvHD diagnosis			
<0.43 g/L Vs ≥0.43 g/L	4.4 (1.13-16.7)	1.47	0.032
Karnofsky PS at cGvHD diagnosis			
<80% Vs ≥80%	72 (12-421)	5.05	<0.001
Platelet counts at cGvHD diagnosis			
<100 x10^3/mm^3 Vs ≥100x10^3/mm^3	8.83 (1.3-58)	2.18	0.024

Covariates included in the model: Patient age (according to median value), R-DRI, type of donor (MRD, match related donor; MUD, match unrelated donor; CB, cord blood; MMRD, mismatch related donor), main GvHD prophylaxis (Anti Thymocyte Globulin [ATG]-based vs Post transplant Cyclophosphamide [PTCy]-based vs neither of the two), IR values at cGvHD diagnosis (according to median values), history of prior acute GvHD, Karnofsky performance status (KPS), platelet count <100x10^3/mm^3, total lymphocyte count <1.0 x 10^3/mm^3, and eosinophil count <0.5x10^3/mm^3.

TABLE 3 | Immune reconstitution parameters at diagnosis of cGvHD.

Parameter	Median value [range]
CD3+	706 cells/mm^3 [53-6132]
CD3+CD4+	233 cells/mm^3 [15-1642]
CD3+CD8+	470 cells/mm^3 [13-5094]
CD19+	21 cells/mm^3 [0-1206]
IgG	4.22 g/L [0-29.83]
IgA	0.43 g/L [0-5.42]
IgM	0.45 g/L [0-5.11]
NK	115 cells/mm^3 [16-991]

Each function in the parenthesis is considered 1 if the condition is satisfied, or otherwise 0.

We then calculated the IR score for 87 patients of the training set (24 were excluded because of missing data). The 25th quartile value was 3.09, the 75th one was 6.91: low-risk patients were defined as having a score ≤3.09, intermediate as having a score >3.09 and ≤6.91, and high risk as having a score >6.91.

Patients' distribution according to NIH consensus classification and according to IR score is presented in **Table 4**. Additional information is provided in **Supplementary Figure 2**.

In the training set, the 6-year OS and TRM were stratified by both IR score and NIH consensus classification. The 6-year OS and TRM by IR score were 85% (95% CI, 70-92) and 13% (95% CI, 5-25) for low-risk patients, 64% (95% CI, 44-89) and 30% (95% CI, 15-47) for intermediate-risk patients, and 26% (95% CI, 10-47) and 42% (95% CI, 19-63) for high-risk patients (OS p<0.0001; TRM p = 0.015, **Figures 1A, B**). The 6-year OS and TRM by NIH consensus classification were 87% (95% CI, 65-96) and 9% (95% CI, 1-25) for mild cGvHD, 68% (95% CI, 51-80) and 20% (95% CI, 9-33) for moderate cGvHD, and 49% (95% CI, 33-64) and 44% (95% CI, 28-59) for severe cGvHD (OS p = 0.009; TRM p = 0.005).

In the validation set, the stratification according to IR score was confirmed to be significant, while the stratification according to NIH consensus was clearly significant for TRM and showed a trend for OS. The 6-year OS and TRM by IR score were 83% (95% CI, 48-96) and 8% (95% CI, 1-32) for low-risk patients, 78% (95% CI, 37-94) and 11% (95% CI, 1-41) for intermediate-risk patients, and 37% (95% CI, 17-58) and 63% (95% CI, 36-81) for high-risk patients (OS p = 0.0075; TRM p = 0.0009, **Figures 1C, D**). The 6-year OS and TRM by NIH consensus classification were 100% and 0% for mild cGvHD, 71% (95% CI, 41-88) and 14% (95% CI, 2-38) for moderate cGvHD, and 48% (95% CI, 27-67) and 51% (95% CI, 29-70) for severe cGvHD (OS p = 0.157; TRM p = 0.0332).

To support the validity of the IR score, the ROC curve *via* the AUC was calculated: AUC values were 81% for TRM and 88% for OS. A cut-off of 6.310 was identified with 69% sensitivity and 89% specificity for TRM, and 78% sensitivity and 90% specificity for overall mortality (**Figure 2**).

IR Score Stratifies Patients Independently From NIH Consensus cGVHD Criteria

The low-risk group included 24 and 10 patients in the training set and validation set, respectively, while the intermediate-risk group included 41 and 8 patients, and the high-risk group 22 and 22 patients.

We challenged the capability of our IR score of stratifying patients across the different NIH clinical stages (**Table 4**).

In the training cohort, the 2-year OS from cGvHD diagnosis for patients with mild cGvHD (n = 25) according to NIH classification was 100% for low and intermediate and 33% (95% CI, 1%-77%) for high-risk IR score (p <0.001). The 2-year OS for moderate cGvHD patients (n = 30) stratified according to the IR score was 100%, 83% (95% CI, 46-96%), and 62% (95% CI, 14%-89%) in low, intermediate, and high-risk groups, respectively (p 0.16). For severe cGvHD patients (n = 32), 2-year OS was 100%, 76% (95% CI, 41-92%), and 40% (95% CI, 13%-66%) in low, intermediate, and high-risk groups, respectively (p 0.02). Results therefore confirmed the independent stratification within cGVHD clinical grades.

IR Score Predicts cGVHD Mortality

We next evaluated the contribution of the IR cGVHD score in predicting TRM. Chronic GvHD was the cause of death in 2, 1,

TABLE 4 | Cross-stratification of cGvHD patients into respective risk groups by NIH consensus and IR score.

	Training cohort			Validation cohort			Total
	IR low risk	*IR int risk*	*IR high risk*	*IR low risk*	*IR int risk*	*IR high risk*	
NIH consensus mild	12	10	3	0	1	1	27
NIH consensus moderate	7	17	6	5	3	6	44
NIH consensus severe	5	14	13	5	4	15	56
Total	24	41	22	10	8	22	127

IR, immune reconstitution score; int, intermediate.

and 12 patients classified as low, intermediate, and high-risk according to IR score. High-risk patients were more likely to die from cGVHD than low and intermediate-risk patients (p<0,0001). No patients died due to infection in the low-risk group, while 9 and 3 patients died due to infectious complications in the intermediate and high-risk groups, respectively (p ns).

DISCUSSION

Chronic GvHD represents one of the major hurdles in the management of HSCT survivors. Despite progress in the optimization of conditioning regimens, ancillary measures, and pre-emptive strategies for infectious complications, we are still facing the unmet medical need of cGvHD treatment. cGVHD is responsible for 30% to 50% of non-relapse mortality in long-term survivors (28). According to data from the Fred Hutchinson Cancer Research Center (29), only approximately 50% of cGvHD patients are cured within 7 years after starting systemic treatment, 10% require continuous treatment, and 40% die within 7 years. Moreover, at 5 years from cGvHD diagnosis, only 32% of patients are alive, free of immunosuppressive therapy, and in complete remission from the primary disease (30).

The identification of valid and reproducible biomarkers for both acute and chronic GvHD is one of the most significant challenges in the field. While clinical trials investigating new drugs for the

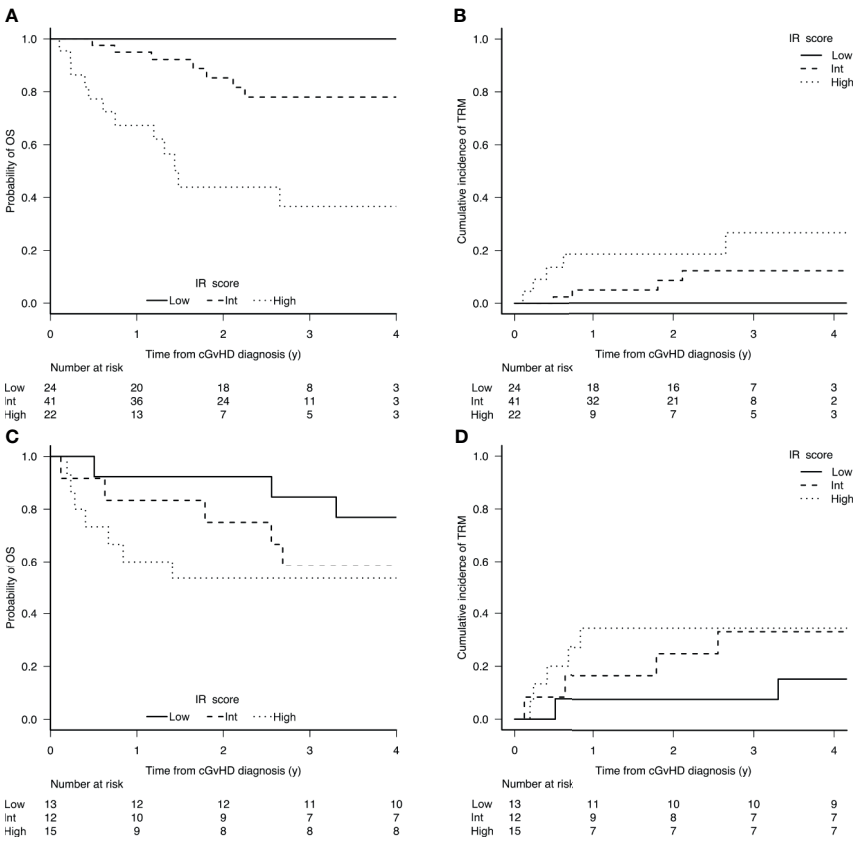

FIGURE 1 | Cumulative incidence of OS **(A)** and TRM **(B)** according to the prognostic score in the training set (n 87); and OS **(C)** and TRM **(D)** in the validation set (n 40).

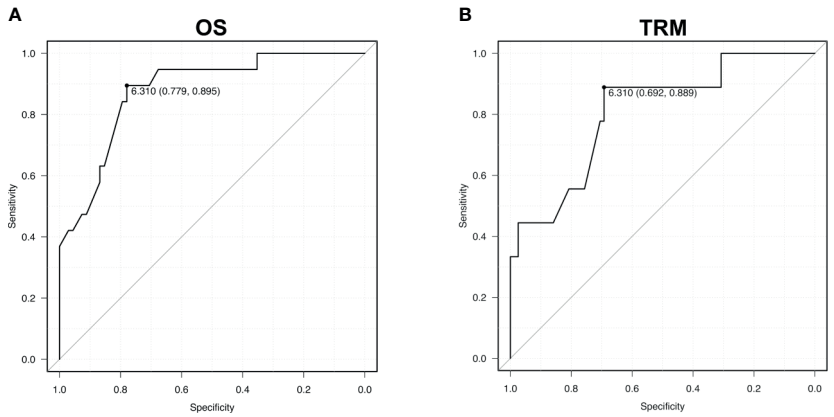

FIGURE 2 | Receiver operating characteristic (ROC) curves for OS **(A)** - area under the curve 0.88 - and TRM **(B)** – area under the curve 0.81. AUC values are reported from multivariable models.

treatment of acute GvHD nowadays are designed according to patients' stratification based on established biomarkers, this is not the case for cGvHD. cGvHD is characterized by pleiomorphic manifestation and a complex pathogenesis that elicits both inflammatory and fibrotic pathways. cGvHD affects more than one third of transplanted patients and clinical presentation at onset only partially unveils the true severity of the disease. Clinical grading, including the latest NIH consensus criteria, is not able to provide univocal prognosis of such a complication.

The identification of patients at risk is mandatory for correct cGvHD management. While innovative, highly effective, but also toxic drugs are released on the market, early identification of high-risk patients—at the time of cGvHD diagnosis—would enable an earlier and more aggressive therapy while sparing toxicity to low-risk patients. So far, biomarker studies are in progress to identify tools to enhance diagnosis and definition of prognosis, however results are still far from routine practice.

While acute GVHD is mediated by mature effector T cells from the donor (graft) that become activated after encountering alloantigens in the recipient, cGVHD is characterized by aberrant immune responses to both autoantigens and alloantigens (31, 32). Chronic GvHD arises from a failure to develop tolerance after HSCT (33). The loss of regulator-cell function appeared to be one of the critical events in the development of cGVHD: aberrant B – T – NK cells homeostasis and the inability to establish cell tolerance is a pivotal point of cGvHD (33–35). A recent international multicenter study in children and adolescents provided new insights on the immune profile peculiarity of cGvHD (33). In cGvHD, decreased transitional B cells and increased cytolytic NK cells are associated with increased activated T cells, naive helper T, and cytotoxic T cells, loss of regulatory NK cells, and increased ST2 and soluble CD13. The immune signature of cGVHD is complex with several cytokine, T-cell, NK-cell, and B-cell abnormalities (33–35). Definition of immune-based biomarker algorithms will assist in assigning patient risk for cGVHD, with the possibility of a risk-tailored treatment approach (33).

We investigated IR as a candidate biomarker, using easily collectable variables, with a high grade of reproducibility and standardization within a setting of well-known clinical grade tests. The overall incidence of cGvHD in our patient population was similar to that reported in the literature, moreover all the available HSCT platforms in terms of donor selection (MRD, CB, MUD, MMRD) and GvHD prophylaxis (ATG-based, cyclosporin-based, rapamycin-based, and PTCy-based) were represented adequately, providing an additional strength to the study.

The IR score-based algorithm provided a risk stratification power that proved independent from the nature of both GvHD prophylaxis and donor source in both the training set and in the validation cohort.

We had the opportunity to analyze over 100 consecutive cGvHD patients with an adequate follow-up. Strengths of our study were the prospective sample and data collection, the homogeneous management of post-HSCT follow-up, and the systematic clinical evaluation of patients for GvHD according to NIH guidelines. Being a single-center study, cohort size was limited and suggests the need of further validation in multicenter cohorts.

Our results showed a clear impact of immunological variables at cGvHD diagnosis: CD3+CD4+ counts, NK cells, and IgA and IgM levels were selected by our model over other clinical variables as independent predictors of patient outcome. Very few studies have demonstrated an association between biological markers and survival; more information has been found regarding biomarkers for the prediction of cGvHD risk and has been associated with the diagnosis of cGvHD (7, 36).

In addition, the IR approach has highlighted some interesting biological pathways:

- In the risk score we generated, higher CD3+CD4+ (>233 cells/mm^3) counts are linked to worse outcome. This may seem counterintuitive as the main cause of death in cGvHD patients is infection due to immunosuppression. But considering we are analyzing the cell count at the onset of cGvHD, this may reflect the pathophysiologic role of CD4+ T helper cells in cGvHD

pathogenesis. In their recent review of cGvHD pathophysiology (3), Zeiser and Blazar describe the role of CD4+ cells in orchestrating the dysregulated immune response after an initial injury. Ibrutinib, the only FDA-approved drug for steroid-resistant cGvHD, targeting Bruton's Tyrosin Kinase (in the path of B cell activation) and inducible-T cell kinase (in the path of T helper cell activation), showed good response rates [67%, in a phase II multicenter study by Miklos and colleagues (5)]. T cell depletion (linked to slower kinetics of IR) is associated with lower rate of chronic GvHD (36, 37). Evidence suggests that high CD4+ counts at GvHD diagnosis may indeed reflect a strong initial orchestrating signal for cGvHD. CD4+ counts have been investigated as prognostic biomarkers by several studies with somewhat contradictory results. However, these studies did not test CD4+ counts at onset of cGvHD. Independently from cGvHD, in transplanted patients, a fast and robust recovery of CD4+ counts at early time-points after HSCT was associated with low TRM (38, 39). This is possibly linked to the protection from opportunistic infections mediated by T cells early after transplant. High CD4+ counts have already been associated with acute GvHD (40, 41). Importantly, Podgorny and coworkers observed a persistently higher number of CD4+ counts after HSCT in patients developing cGvHD requiring systemic therapy than in cGvHD patients who did not require systemic treatment, in line with our results.

- NK cells were found to have a negative prognostic implication when lower than 115 cells/mm^3. This finding points to the protective effect that NK cells have in cGvHD pathophysiology; it was demonstrated (42) that NK cells mediate the reduction of GvHD by inhibiting activated, alloreactive T cells while retaining graft-versus-tumor effects through effector molecules such as FasL (43). Thus, similarly to T cells, NK cells display a potent anti-leukemia effector capacity, and yet, unlike them, do not mediate cGvHD (44). In the context of haploidentical transplantation performed within a PTCy regimen (45), the percentage of alloreactive mature NK cells quantified after transplant negatively correlated to relapse risk but not to cGvHD rate. Noticeably, NK cells are critical players of innate immunity against viral and bacterial infections at the mucosal barriers (46). We can thus speculate that cGvHD patients with high NK cell levels may benefit from this effect, resulting in improved outcome. In the above-mentioned study, Podgorny et al. (40) showed reduced levels of regulatory NK cells in patients with severe cGvHD compared to those not requiring systemic therapy. In several studies, high NK cell counts early after HSCT have been associated with low TRM and low aGvHD incidence, in both HLA-matched and HLA-mismatched transplant settings (47–49).
- Low IgM and IgA levels were the last IR variables significantly associated with worse prognosis in our cGvHD patient cohort. B cells reconstitution occurs relatively late after HSCT. Post-transplant B cell deficiency is—at least in part—due to insufficient B lymphopoiesis and in part, this is exerted by GvHD (50). The pathogenic role of B cells in cGvHD was first identified in murine models in 1995 (51). Recently, dysregulated B cell lymphopoiesis was proven to be associated with the onset of chronic GvHD (52). Immunoglobulin levels seem to recover in parallel to B cell reconstitution, in which recovery of Ig subclasses usually occurs in a distinctive order (53). After HSCT, Ig levels drop reflecting the absence of Ig-producing B cells. As a reflection of normal ontogeny, IgM production will reconstitute relatively early, subsequently IgG generally reaches normal levels, whereas normalization of IgA levels may take longer. Chronic GvHD is associated with significantly poorer B cell reconstitution in both function and numbers. IgM levels were consistently low in cGvHD patients and our result was in line with previous pubblications (10, 35). Khoder et al. (54) demonstrated that regulatory B cells (enriched in IgM subsets) are deficient in cGvHD patients. Abdel-Azim et al. (55) reported that IgM memory B cells were persistently lower within the first two years after HSCT in cGvHD patients, than in transplant recipients not developing cGvHD.

All these findings support the items in our prognostic score impacting cGvHD outcome. The validation step performed on the retrospective cohort is also encouraging. The score held its power in an independent cohort, despite the differences in conditioning and prophylaxis strategies. This suggests a link of the proposed score with cGvHD pathogenesis and progression, events triggered with different frequencies by different transplant platforms, but possibly similar once the disease is established.

The current study adds a new insight to a big research area on prognostication of cGvHD, going beyond scoring systems only based on clinical parameters. Clinical classification according to NIH consensus criteria displays a clear stratification for both OS and TRM; IR score was able to provide an additional stratification to implement the prognostic power at cGvHD declaration. IR score highlights among each clinical class the long-term probability of survival.

We can confirm that both IR-score stratification and NIH categorization were able to independently prognosticate TRM and OS. NIH categorization keeps its relevance but is not 100% accurate in identifying all high or low-risk patients; the IR-score biomarkers help in selection of high and low-risk patients also within their NIH risk groups. Still, in the majority of cases, there was concordance between clinical risk and IR risk, thus our results are not in contrast with the known prognostic impact of NIH categorization of cGVHD. Overall, patients with severe GvHD according to NIH classification have worse OS and TRM compared to mild GvHD, but among patients with severe GvHD those with a low-risk IR score have better prognosis in terms of OS and TRM. Similarly, patients with mild/moderate GvHD present better OS and TRM overall, but the IR score was able to predict patients at high risk of progression towards severe forms and—ultimately—worse outcome.

This suggests that the IR score can improve prognostication, especially if combined with clinical staging. Beyond the use as a definite prognostic tool, our IR score proved the important role of IR in the clinical management of cGvHD patients, suggesting further research as well as systematic clinical application of IR monitoring programs and IR-based therapeutic decisions.

Of note, we recognize that in the training cohort a consistent proportion of patients received, as GvHD prophylaxis, a

combination of pTCy and rapamycin. This combination is peculiar and is not a standard one, but also other platforms were well represented in the patient population. The current results should be confirmed in a multicenter study as well as with longer follow-up and expansion of the sample size.

We conclude that an IR-based algorithm represents a valid tool to identify high-risk patients at cGvHD onset. The algorithm predicts long-term OS and TRM, identifying subjects at high risk of death due to cGvHD through stratification into three classes of risk and the clear identification of a cut-off strongly associated with both overall mortality and TRM.

Future directions should include prospective and serial evaluations of the algorithm to define its clinical use. Our goal for the next years will be to identify tools able to shape the treatment options not only according to clinical presentation but also to risk stratification at the onset of such a detrimental transplant complication.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by San Raffaele Institutional Ethical Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conception, design, and manuscript revision: ML-S, FS, FLo, and FC. Provision of study material or patients: all authors. Collection and assembly of data: ML-S, FS, and FLo. Data analysis and interpretation: ML-S, FS, FLu, and FC. Manuscript writing: ML-S, FS, FLo, SMar, and FC. Final approval of the manuscript: all authors. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.705568/full#supplementary-material

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Maintenance Treatment With Low-Dose Decitabine After Allogeneic Hematopoietic Cell Transplantation in Patients With Adult Acute Lymphoblastic Leukemia

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Background: Post-transplant relapse remains a principal leading cause of failure after allogeneic hematopoietic stem cell transplantation (allo-HSCT) in patients with adult acute lymphoblastic leukemia (ALL). The aim of this study was to investigate the efficacy and safety of low-dose decitabine on the prevention of adult ALL relapse after allo-HSCT.

Methods: In this prospective study, we enrolled 34 patients with ALL who underwent allo-HSCT from August 2016 to April 2020 and received low-dose decitabine maintenance treatment after transplantation. The primary objectives were cumulative incidence of relapse rate (CIR), overall survival (OS), and disease-free survival (DFS). The secondary objectives were graft-*versus*-host disease (GVHD) and safety.

Results: Among the enrolled 34 patients, 6 patients relapsed and 6 patients died. The 2-year CIR, OS, and DFS were 20.2, 77.5, and 73.6%, respectively. Subgroup analysis revealed the 2-year CIR, OS, and DFS rates of 12 patients with T-ALL/lymphoblastic lymphoma (LBL) were 8.3, 90, and 81.5%, respectively. None of the seven patients with T-ALL relapsed. During maintenance treatment, only one patient (2.9%) developed grade IV acute GVHD and four (11.8%) patients had severe chronic GVHD. Thirty-two patients (94.1%) developed only grade I to II myelosuppression, and two patients (5.8%) developed grade III to IV granulocytopenia.

Conclusions: Maintenance treatment with low-dose decitabine after allo-HSCT may be used as a therapeutic option to reduce relapse in patients with adult ALL, especially in patients with T-ALL. Our findings require confirmation in larger-scale controlled trials.

Clinical Trial Registration: Chinese Clinical Trials Registry, identifier ChiCTR1800014888.

Keywords: allogeneic hematopoietic stem cell transplantation, decitabine, maintenance, prophylaxis, relapse, acute lymphoblastic leukemia

INTRODUCTION

Post-transplant relapse remains a leading cause of failure after allogeneic hematopoietic stem cell transplantation (allo-HSCT). In patients with adult acute lymphoblastic leukemia (ALL), the risk of relapse-related death is higher, up to 30–54% (1–3). At present, donor lymphocyte infusion (DLI) is the most widely used management approach to relapse after transplantation. However, the downregulation of human leukocyte antigen II (HLA-II) molecules leads to the inability of donor T cells to recognize leukemic cells, which limits the use of DLI in the treatment of relapse after transplantation in patients with acute myeloid leukemia (AML) (4, 5), and the 3-year overall survival (OS) rate of these patients is only 10–20% (6). In particular, DLI is not ideal for treatment of ALL relapse after transplantation. Given the difficulty in the treatment of post-transplant relapse, preventing relapse is more important than treatment. Therefore, it is urgent to explore novel approaches to prevent leukemia relapse after allo-HSCT in adult ALL.

Different from the relapse occurring after traditional chemotherapy, the elimination of leukemic cells after allo-HSCT mainly depends on the graft-*versus*-leukemia (GVL) effect (7). A mechanism of post-transplantation relapse involves the downregulation of HLA-class II molecules induced by epigenetic silencing to reduce the GVL effect, and the downregulation of HLA-II expression is caused by hypermethylation of the promoter of the class II major histocompatibility complex (MHC) transactivator (CIITA) (4, 5, 8). Most patients with T-ALL showed molecular loss of HLA-II (9), and only 5–17% of T-ALL expressed HLA-DR. A similar mechanism of loss of expression of HLA-II class molecules was also observed in B-cell lymphoma lines (10). In addition, many studies have shown that the degree of methylation of tumor suppressor genes is closely associated with the subtypes and prognosis of ALL (11–13). The above studies indicate the possibility of using hypomethylating agents (HMAs) as treatment in ALL after transplantation.

Both decitabine and azacitidine are HMAs and have been safely and effectively used for maintenance treatment of AML and myelodysplastic syndrome (MDS) after transplantation (14–20). The main effect of post-transplantation hypomethylation treatment is to prevent primary disease relapse and reduce graft-*versus*-host disease (GVHD). The main mechanisms include increasing the number of regulatory T (Treg) cells and inducing cytotoxic CD8+ T cells (21, 22). Thus, considering the low hematological toxicity of maintenance treatment with low-dose decitabine after AML/MDS transplantation and the advantages of preventing relapse without affecting GVHD, we administered low-dose decitabine maintenance treatment to 34 patients with ALL after allo-HSCT. This was the first prospective study with the largest number of cases to date to describe the application of decitabine prophylaxis for relapse of transplanted ALL.

MATERIALS AND METHODS

Study Design

This was a single-center, prospective, single-arm study. Informed consent was obtained from all patients, and the study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. This study is registered at www.chictr.org.cn (ChiCTR1800014888).

Patient Cohort

Eligible candidates met all of the following inclusion criteria: (1) age ≥14 years; (2) satisfied the diagnostic criteria of ALL or lymphoblastic lymphoma (LBL) in accordance with WHO 2016 guidelines (22); (3) patients underwent allo-HSCT in the First Affiliated Hospital of Zhengzhou University; (4) Eastern Cooperative Oncology Group (ECOG) performance status score ≤2; (5) morphological complete remission (CR) before maintenance treatment; (6) estimated survival ≥3 months.

The exclusion criteria included (1) concomitant diagnosis of another cancer; (2) concomitant uncontrolled fungal, bacterial, or viral infection; (3) hypersensitivity to decitabine; (4) diagnosis of human immunodeficiency virus infection or in active stage of hepatitis B or C virus infection; (5) brain dysfunction or severe mental illness; (6) concomitant disease(s) that may seriously endanger the safety of patients or affect the completion of this study; and (7) participation in another drug clinical trial(s) 1 month before the trial.

Maintenance Treatment Regimen

Maintenance treatment began more than 50 days after transplantation. This post-HSCT interval allows for adequate marrow recovery before starting decitabine. Decitabine 10 mg/d was planned for intravenous infusion 5 h on days 1 to 5, and every 4 weeks for eight cycles, based on comprehensive analysis of previously relevant studies (15, 23, 24). However, in the previous pretrial of low-dose decitabine maintenance treatment after AML/MDS transplantation at our center, patients who received decitabine for 5 days at 10 mg/d developed grade IV myelosuppression with granulocytic fever, requiring transfusion of approximately two units of platelets. Myelosuppression was alleviated, and blood products were not needed after adjusting to 10 mg/d for 3 days. Therefore, decitabine 10 mg/d (approximately 6 mg/m^2/d) was ultimately administered as an intravenous infusion for 5 h on days 1, 3, and 5 every 4 weeks for eight cycles in this study. It should be noted that the number of cycles increased by four to six cycles based on the patients' wishes, if they presented minimal residual disease (MRD) in the late period of maintenance treatment. The interval time of each cycle was also appropriately prolonged according to the recovery of the patient's hemogram.

Routine blood parameters, bone marrow (BM) smear, and MRD were examined before each cycle. MRD detection methods included flow cytometry (FCM), quantitative detection of certain genes or WT1 *via* polymerase chain reaction (PCR), and donor chimerism. In addition, patients with T-LBL also underwent regular positron emission tomography-computed tomography (PET/CT) examinations. Routine blood parameters were examined intermittently during the period of drug administration. Granulocyte colony stimulating factor (G-CSF) or blood products were administered as required according to

the hemogram. Systemic anticancer drugs and other similar experimental treatments were banned during the trial. Withdrawal criteria included (1) patients who were unable to tolerate the treatment, (2) patients with relapse of primary disease, (3) patients developing severe GVHD or unacceptable infection, and (4) subjects who decided to withdraw from the trial.

Evaluation Parameters

Patients with ALL were divided into high-risk and standard-risk groups. High-risk ALL was defined based on at least one of the following criteria: (1) age ≥35 years; (2) white blood cell (WBC) counts $>30\times10^9$/L for B-cell precursor (BCP)-ALL or $>100\times10^9$/L for thymic T-ALL; (3) pro-B-ALL (CD10−), early T-ALL or mature T-ALL, hypodiploid ALL; (4) ALL with Philadelphia chromosome (Ph), with the t(4,11) translocation, or with complex karyotype; and (5) failure to achieve CR after the first induction therapy (25). The risk classification of LBL was based on the international prognostic index (IPI) score. CR from ALL was defined as BM blasts <5%, no primitive naive lymphocytes in the peripheral blood, and no extramedullary lesions. CR of LBL was defined as PET/CT with no positive lesions and a normal BM smear. MRD-positive was defined as FCM >0.01% of cells with a leukemia-associated aberrant immune phenotype in the BM sample or BCR-ABL transcript level >0% in patients with Ph+ ALL. Acute GVHD (aGVHD) and chronic GVHD (cGVHD) were graded according to accepted international criteria (26, 27). Considering that the platelet count was lower than the normal value and the WBC count was normal in some patients before treatment, hematological adverse reactions after treatment were evaluated based on changes in WBCs and were graded according to the National Cancer Institute Common Toxicity Criteria, version 3.0.

Statistical Analysis

The follow-up deadline was July 31, 2020. The primary endpoints were cumulative incidence of relapse rate (CIR), overall survival (OS), and disease-free survival (DFS) of patients who received low-dose decitabine maintenance treatment. The secondary endpoints were the incidence of GVHD after receiving decitabine and the safety of low-dose decitabine maintenance regimen. Statistical analyses were performed using SPSS software (version 21.0), R software package (version 4.0.0), and GraphPad Prism (version 8.0). Descriptive statistics were used to describe the general clinical features of patients. Data were censored at the time of relapse, non-relapse mortality (NRM), or last available follow-up. The cumulative incidence of relapse (CIR) and NRM were performed using the competing risk model, in which death without relapse was considered a competing risk of relapse. The disease-free survival (DFS) and OS were calculated using the Kaplan-Meier method. CIR was defined as time from transplantation to relapse. OS was defined as the time from transplantation to death from any cause. DFS was defined as time from transplantation to relapse or death, whichever occurred first. Statistical significance was defined as $P < 0.05$.

RESULTS

Patient Characteristics

In total, 34 patients from our institution were enrolled between August 2016 and April 2020. The characteristics of patients are shown in **Table 1**. Our cohort comprised 34 patients with a median age of 20 years (range, 14–49 years), including 22 males and 12 females. Overall, 22 patients (64.7%) had B-ALL, 7 (20.6%) had T-ALL, and 5 (14.7%) had T-LBL. Nine patients

TABLE 1 | Patients' characteristics (N = 34).

Characteristic	Value
Age at HSCT, year, range (median)	15–49 (20)
Sex, n (%)	
Male	22 (64.7)
Female	12 (35.3)
Diagnosis, n (%)	
B-ALL	22 (64.7)
T-ALL/T-LBL	7/5 (35.3)
Risk classification, n (%)	
High risk	25 (73.5)
Standard risk	9 (26.5)
Subtype, n (%) some-positive (Ph+) ALL	
Ph^+ ALL	7 (20.6)
Ph^- ALL	27 (79.4)
MRD after the 1st induction, n (%)	
Negative	18 (64.3)
Positive	10 (35.7)
MRD at allo-HSCT, n (%)	
Negative	27 (79.4)
Positive	7 (20.6)
Disease status at allo-HSCT, n (%)	
CR1	31 (91.2)
CR2	3 (8.8)
HCI-CI score, n (%)	
0	21 (61.8)
1	12 (35.3)
2	1 (2.9)
EBMT risk score, n (%)	
0	4 (11.8)
1–2	25 (73.5)
3–4	5 (2.9)
Conditioning regimen, n (%)	
mBu/Cy	26 (76.5)
TBI/Cy	8 (23.5)
Transplant resource, n (%)	
PBSC	31 (91.2)
PBSC+BM	3 (8.8)
Donor/HLA match, n (%)	
Matched related	21 (61.8)
Mismatched related	11 (32.4)
Matched unrelated	1 (2.9)
Mismatched unrelated	1 (2.9)
$CD34^+$ cells$\times10^6$/kg, range (median)	1.52–16.3 (5.7)
MNC cells$\times10^8$/kg, range (median)	1.2–11.5 (5.3)
Time of leukocyte engraftment, d (median)	8–21 (13)
Time of platelet engraftment, d (median)	10–22 (14)

ALL, acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; Ph, Philadelphia chromosome; MRD, minimal residual disease; HCT-CI, hematopoietic cell transplantation comorbidity index; EBMT, European Society for Blood and Marrow Transplantation; Bu, busulfan; Cy, cyclophosphamide; TBI, total body irradiation; mBu/Cy, modified Bu/Cy; PBSC, peripheral blood stem cell; BM, bone marrow; MNC, mononuclear cell.

(26.5%) were at standard-risk, and 25 (73.5%) were at high-risk (including five patients with T-LBL). Seven patients (20.6%) were Philadelphia chromosome-positive (Ph^+), and 27 patients (79.4%) were Philadelphia chromosome-negative (Ph^-). Excluding six patients due to missing MRD data from other hospitals at initial treatment, 10 (35.7%) of 28 assessable patients were MRD positive (including two patients with non-CR after induction) and 18 patients (64.3%) who became MRD-negative after the first induction chemotherapy. Seven patients (20.6%) became MRD-positive, and 27 patients (79.4%) achieved MRD negativity at transplantation. All patients received myeloablative conditioning, including 26 patients (76.5%) receiving a modified busulfan (Bu)/cyclophosphamide (Cy) regimen and 8 (23.5%) patients receiving a total body irradiation (TBI)/Cy regimen (26). Prophylaxis against GVHD for all patients consisted of cyclosporine A and short-term methotrexate treatment with mycophenolate mofetil. In addition, patients without matched related donors were supplemented with anti-thymocyte globulin. The median number of infused CD34+ cells was 5.7×10^6/kg (range, $1.52–16.3 \times 10^6$/kg), and the median number of infused MNC cells was 5.3×10^8/kg (range, $1.2–11.5 \times 10^8$/kg). Neutrophils and platelets were implanted successfully in all patients. All patients achieved morphological CR and donor complete chimerism before maintenance treatment. Thirty patients (88.3%) achieved MRD negativity, and four patients (11.7%) were MRD positive before maintenance treatment (**Table 2**).

Decitabine Exposure and MRD

Outcomes of maintenance therapy with decitabine and the changes in MRD during this stage are shown in **Table 2** and **Figure 1**. All four patients with MRD-positive disease before maintenance treatment turned negative after two or two cycles. Only three patients (8.8%) had positive MRD once during maintenance therapy. The median time from transplantation to the start of maintenance treatment was 96 days (range, 51–175 days), and the median number of decitabine cycles for all patients was seven (range, 1–14). Overall, 14 patients (41.1%) completed the study and entered the follow-up phase, including 12 patients with 8 cycles, 1 patient with 14 cycles, and 1 patient with 13 cycles of treatment. Patients No. 5 and No. 6 received more than eight cycles because they were MRD positive after the completion of eight cycles of maintenance treatment, and their MRD turned negative after an additional cycle of decitabine (**Figure 1**). At the data cut-off point, eight patients (23.5%) were in the maintenance phase, including six patients who entered the study later and two patients due to the delay caused by the coronavirus disease 2019 (COVID-19) epidemic. The reasons for discontinuation included relapse (n = 4, 11.7%), GVHD (n = 3, 8.8%), and withdrawn consent (n = 5, 14.7%) (**Table 2**). Besides, as shown in **Table 3**, seven patients with Ph^+ ALL were treated with TKI maintenance during pre-transplantation chemotherapy, conditioning regimen, and post-transplantation maintenance therapy. Notably, TKI was suspended temporarily to reduce the risk of infection in patients with neutropenia after chemotherapy or transplantation.

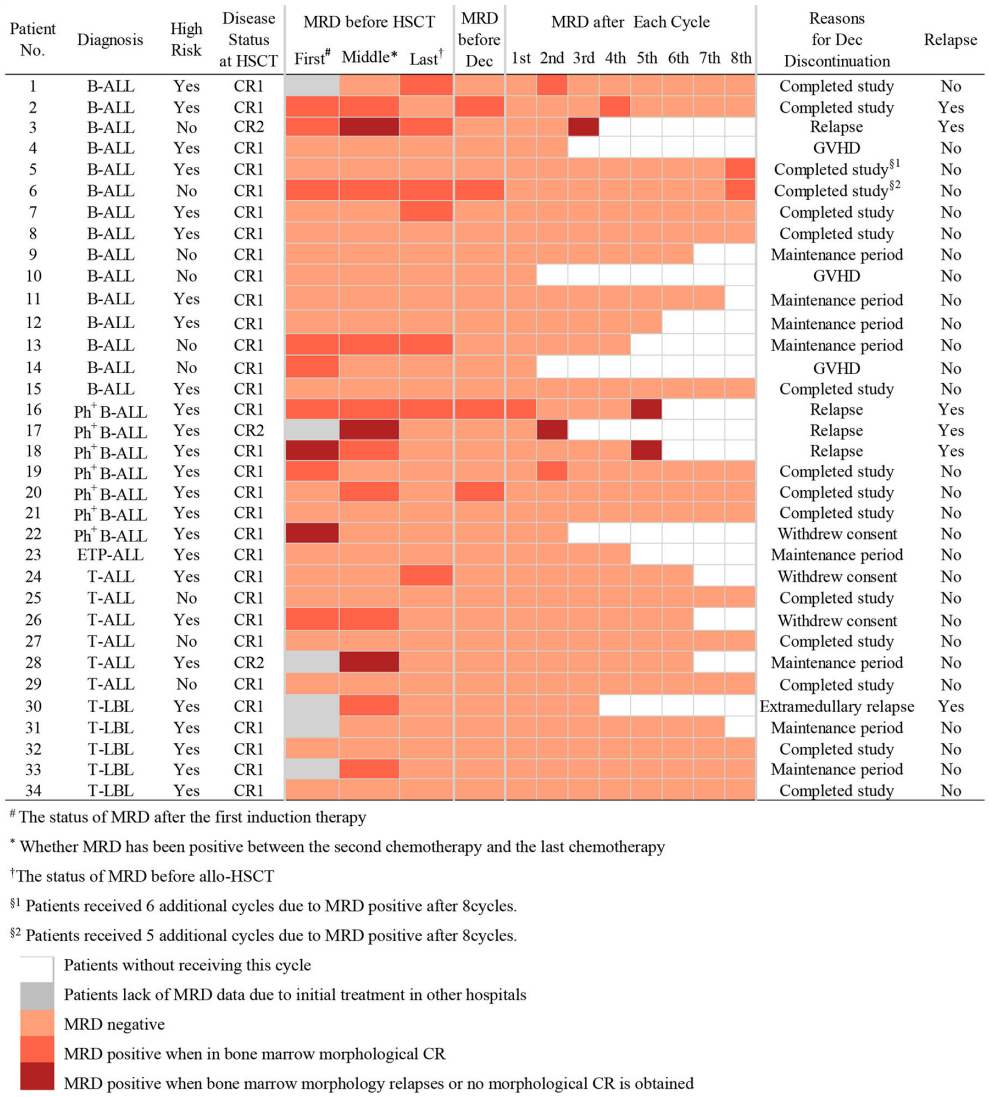
TABLE 2 | Outcomes of transplantation and maintenance treatment (N = 34).

Outcomes	Data
MRD before maintenance treatment, n (%)	
Positive	4 (11.7)
Negative	30 (88.3)
Start time of decitabine, d, median (range)	96 (51–175)
No. of cycles, median (range)	7 (1–14)
Completed study, n (%)	14 (41.1)
Maintenance period, n (%)	8 (23.5)
Reason for discontinuation, n (%)	
Withdrew consent	5 (14.7)
Relapse	4 (11.7)
GVHD	3 (8.8)
Hematological toxicity, n (%)	
I∼II	32 (94.1)
III∼IV	2 (5.8)
Acute GVHD after maintenance treatment, n (%)	1 (2.9)
I∼II	0
III∼IV	1 (2.9)
Chronic GVHD after maintenance treatment, n (%) nnnn(%), n (%)n(%)	7 (20.5)
Mild	3 (8.8)
Moderate	0
Severe	4 (11.8)
Relapse, n (%)	6 (17.6)
B-ALL	5 (14.7)
T-LBL	1 (2.9)
Cause of death, n (%)	6 (17.6)
Relapse	4 (11.7)
Infection	1 (2.9)
GVHD	1 (2.9)
Duration of follow-up, d, range (median)	154–1,629 (480.5)

GVHD, graft-versus-host disease.

Relapse

At the data cut-off point (July 2020), the median follow-up time was 480.5 days (range, 154–1629 days) (**Table 2**). A total of six patients relapsed (17.6%) with a median relapse time of 213 days (range, 156–551 days) after transplantation. Of these, five patients were at high risk (three patients with Ph^+ B-ALL, two of which had *T315I* mutation at the time of relapse; one patient with pro-B-ALL; one patient with T-LBL in the leukemic phase had a WBC count $>100\times10^9$/L at diagnosis), and one patient with B-ALL was at standard risk (**Table 4**). Among the 14 patients who completed the study, only patient No. 2 presented extramedullary recurrence at 551 days after transplantation. Patient No. 16 with Ph^+ ALL only took imatinib but stopped decitabine on his own after five cycles. Relapse happened and *T315I* mutation was detected 2 months later, whereas this patient did not take ponatinib due to economic reasons. Patient No. 17 with Ph^+ ALL relapsed for a second time, and the *T315I* mutation was detected after two cycles. Patient No. 18 with Ph^+ ALL relapsed after five cycles and refused to test for mutations in the ABL kinase domain. Patients No. 3 with CR2 at HSCT relapsed after three cycles. After the six relapsed patients, one patient received chemotherapy; one patient received chemotherapy, TBI, and DLI in turn; two patients received chemotherapy; and two patients were discharged automatically. Finally, four patients died after relapse, and two patients were still alive (**Table 4**). Interestingly, none of the seven patients with T-ALL relapsed,



Patient No.	Diagnosis	High Risk	Disease Status at HSCT	MRD before HSCT			MRD before Dec	MRD after Each Cycle								Reasons for Dec Discontinuation	Relapse
				First#	Middle*	Last†		1st	2nd	3rd	4th	5th	6th	7th	8th		
1	B-ALL	Yes	CR1													Completed study	No
2	B-ALL	Yes	CR1													Completed study	Yes
3	B-ALL	No	CR2													Relapse	Yes
4	B-ALL	Yes	CR1													GVHD	No
5	B-ALL	Yes	CR1													Completed study§1	No
6	B-ALL	No	CR1													Completed study§2	No
7	B-ALL	Yes	CR1													Completed study	No
8	B-ALL	Yes	CR1													Completed study	No
9	B-ALL	No	CR1													Maintenance period	No
10	B-ALL	No	CR1													GVHD	No
11	B-ALL	Yes	CR1													Maintenance period	No
12	B-ALL	Yes	CR1													Maintenance period	No
13	B-ALL	No	CR1													Maintenance period	No
14	B-ALL	No	CR1													GVHD	No
15	B-ALL	Yes	CR1													Completed study	No
16	Ph+ B-ALL	Yes	CR1													Relapse	Yes
17	Ph+ B-ALL	Yes	CR2													Relapse	Yes
18	Ph+ B-ALL	Yes	CR1													Relapse	Yes
19	Ph+ B-ALL	Yes	CR1													Completed study	No
20	Ph+ B-ALL	Yes	CR1													Completed study	No
21	Ph+ B-ALL	Yes	CR1													Completed study	No
22	Ph+ B-ALL	Yes	CR1													Withdrew consent	No
23	ETP-ALL	Yes	CR1													Maintenance period	No
24	T-ALL	Yes	CR1													Withdrew consent	No
25	T-ALL	No	CR1													Completed study	No
26	T-ALL	Yes	CR1													Withdrew consent	No
27	T-ALL	No	CR1													Completed study	No
28	T-ALL	Yes	CR2													Maintenance period	No
29	T-ALL	No	CR1													Completed study	No
30	T-LBL	Yes	CR1													Extramedullary relapse	Yes
31	T-LBL	Yes	CR1													Maintenance period	No
32	T-LBL	Yes	CR1													Completed study	No
33	T-LBL	Yes	CR1													Maintenance period	No
34	T-LBL	Yes	CR1													Completed study	No

The status of MRD after the first induction therapy

* Whether MRD has been positive between the second chemotherapy and the last chemotherapy

†The status of MRD before allo-HSCT

§1 Patients received 6 additional cycles due to MRD positive after 8cycles.

§2 Patients received 5 additional cycles due to MRD positive after 8cycles.

Patients without receiving this cycle

Patients lack of MRD data due to initial treatment in other hospitals

MRD negative

MRD positive when in bone marrow morphological CR

MRD positive when bone marrow morphology relapses or no morphological CR is obtained

FIGURE 1 | Changes in MDR and decitabine exposure in patients.

including one patient with early T-cell precursor ALL (ETP-ALL) and three patients at high risk. In the end, the 2-year CIR of all 34 patients was 20.0%, and the median CIR time was not reached (**Figure 2A**). Patients with T-ALL/LBL and B-ALL had a 2-year CIR of 8.3 and 25.8%, respectively (P = 0.34). Patients at high risk and standard risk had a 2-year CIR of 22.4 and 12.5%, respectively (P = 0.63). Patients with Ph^+ ALL and Ph^- ALL had a 2-year CIR of 42.8 and 14.5%, respectively (P = 0.08) (**Figure 3A**).

DFS and OS

At the data cut-off point, 28 (82.3%) of the 34 patients were alive (82.3%), and 26 patients (76.5%) were alive without relapse/progression. Causes of death included relapse (n = 4), severe infection (n = 1), and GVHD (n = 1) (**Table 2**). The 2-year NRM was 6.3% (**Figure 2A**). The 2-year OS was 77.5%, and the 2-year DFS rate was 73.6% for the 34 patients (**Figure 2B**). The 2-year OS of patients with T-ALL/LBL and B-ALL were 90 and 72.5%, respectively (P = 0.37). The 2-year OS of patients at high risk and standard risk were 73.6 and 87.5%, respectively (P = 0.57). The 2-year OS of patients with Ph^+ ALL and Ph^- ALL were 68.6 and 79.1%, respectively (P = 0.53) (**Figure 3B**). For patients with T-ALL/LBL and B-ALL, the 2-year DFS were 81.5 and 69.6%, respectively (P = 0.52). For patients at high risk and standard risk, the 2-year DFS were 68.8 and 87.5%, respectively (P = 0.36). For patients with Ph^+ ALL and Ph^- ALL, the 2-year DFS were 57.1 and 77.3%, respectively (P = 0.23) (**Figure 3C**).

GVHD

One patient (2.9%) developed grade IV aGVHD, and seven (20.5%) patients developed cGVHD (three with mild cGVHD and four severe cGVHD) during maintenance treatment phase (**Table 2**). Among the eight patients with GVHD after

TABLE 3 | Use of TKI in 7 Patients with Ph^+ ALL.

PatientNo.	TKI Before HSCT	TKI in Conditioning	TKI after HSCT			Relapse
			Starting Time of TKI (days)	Usage of TKI	Time of TKI Withdrawal (days)	
16	Dasatinib (100 mg/d) + chemotherapy	Dasatinib (100 mg/d)	60	Imatinib (400 mg/d)*	276	Yes
17	Imatinib (400 mg/d) + chemotherapy	Imatinib (400 mg/d)	61	Imatinib (400 mg/d)	170	Yes
18	Dasatinib (100 mg/d) + chemotherapy	Dasatinib (100 mg/d)	59	Dasatinib (100 mg/d)	223	Yes
19	Imatinib (400 mg/d) + chemotherapy	Imatinib (400 mg/d)	65	Imatinib (400 mg/d)	365	No
20	Imatinib (400 mg/d) + chemotherapy	Imatinib (400 mg/d)	54	Imatinib (400 mg/d)	379	No
21	Dasatinib (100 mg/d) + chemotherapy	Dasatinib (100 mg/d)	66	Dasatinib (100 mg/d)	156	No
			157	Imatinib (400 mg/d)#	365	
22	Imatinib (300 mg/d)§ + chemotherapy	Imatinib (300 mg/d)	57	Dasatinib (100 mg/d)	366	No

TKI, tyrosine kinase inhibitor; ALL, acute lymphoblastic leukemia; HSCT, hematopoietic stem cell transplantation.

**As the patient suffered from diabetes mellitus complicated with fundus disease, dasatinib was replaced with imatinib after transplantation. #Dasatinib was replaced with imatinib at 157 days after transplantation because of repeated pleural effusion after taking dasatinib.*

§The patient was intolerant to imatinib (400 mg/d), accompanied by severe nausea and vomiting, so imatinib was reduced to 300 mg/d.

TABLE 4 | Characteristics and outcomes of six patients with relapse.

Patient No.	Diagnosis	High-Risk Factor at Diagnosis	Disease Status at HSCT	Starting Time of Decitabine (days)	Cycles of Decitabine	Reason for Discontinuation of Decitabine	Bone Marrow Results at Relapse	Time From HSCT to Relapse (days)	Treatment After Relapse	Overall Survival (days)
16	B-ALL	Ph^+ WBC >100×10^9/L	CR1	63	5	Withdrew consent	Marrow blast 97.6% *T315I* mutation	276	DLI + chemotherapy	433
2	B-ALL	$CD10^-$	CR1	97	8	Completed study	Extramedullary	551	Chemotherapy; TBI; DLI	725
17	B-ALL	Ph^+ Age >35	CR2	110	2	Relapse	Marrow blast 42% *T315I* mutation	168	Automatic discharge	213
3	B-ALL	No	CR2	84	3	Relapse	Marrow blast 49.6%	205	Automatic discharge	244
30	T-LBL	Leukemic phase; WBC>100×10^9/L	CR1	93	3	Relapse	Extramedullary	156	Chemotherapy	>205
18	B-ALL	Ph^+	CR1	60	5	Relapse	Marrow blast 16.4%	221	Chemotherapy	>427

WBC, white blood cell; CR, complete remission; DLI, donor lymphocyte infusion; HSCT, hematopoietic stem cell transplantation.

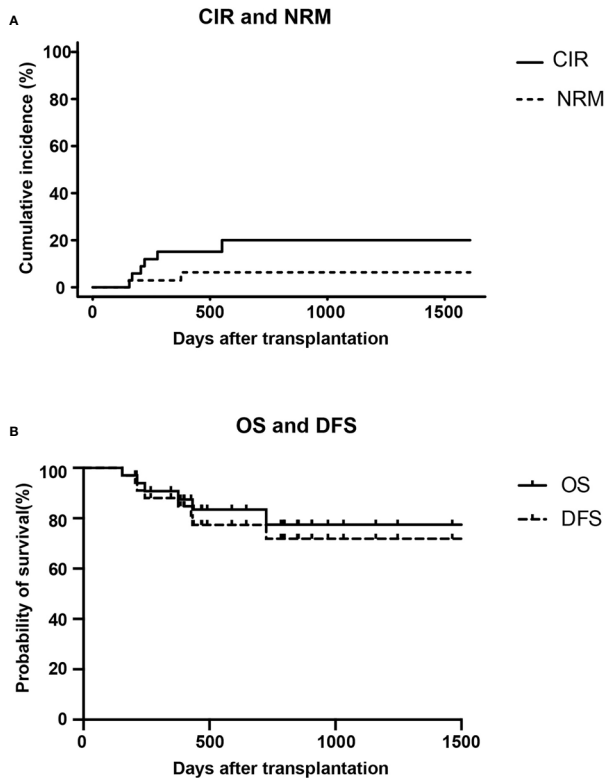


FIGURE 2 | CIR and NRM **(A)** and OS and DFS **(B)** for all patients (n = 34) after allo-HSCT.

maintenance treatment, three patients had reduced the dose of immunosuppressive drugs before developing GVHD (one withdrew immunosuppressants and developed grade IV aGVHD during the second cycle; then, the patient stopped using decitabine and received intensive immunosuppressive treatment, but the response was poor and the patient eventually died). Two patients presented cGVHD before maintenance treatment. Of the remaining three patients, two developed cGVHD after one cycle and one developed cGVHD after three cycles of maintenance treatment. Among the eight patients with GVHD, organ involvement included the skin in eight patients, the intestinal tract in two patients, the liver in three patients, the oral cavity in three patients, and the eye in one patient. No significant worsening or relief was observed in patients with GVHD due to the use of decitabine.

Adverse Events

The main adverse event caused by low-dose decitabine was hematological toxicity. Among the 34 patients, 32 (94.1%) developed grade I to II myelosuppression after maintenance treatment with low-dose decitabine (**Table 4**), and no infection occurred after timely administration of G-CSF. Only two patients (5.8%) developed grade III to IV myelosuppression. Patient No. 9 developed degree IV granulocytopenia and mild pulmonary fungal infection after one cycle, which improved after administration of G-CSF and oral voriconazole. Patient No. 22

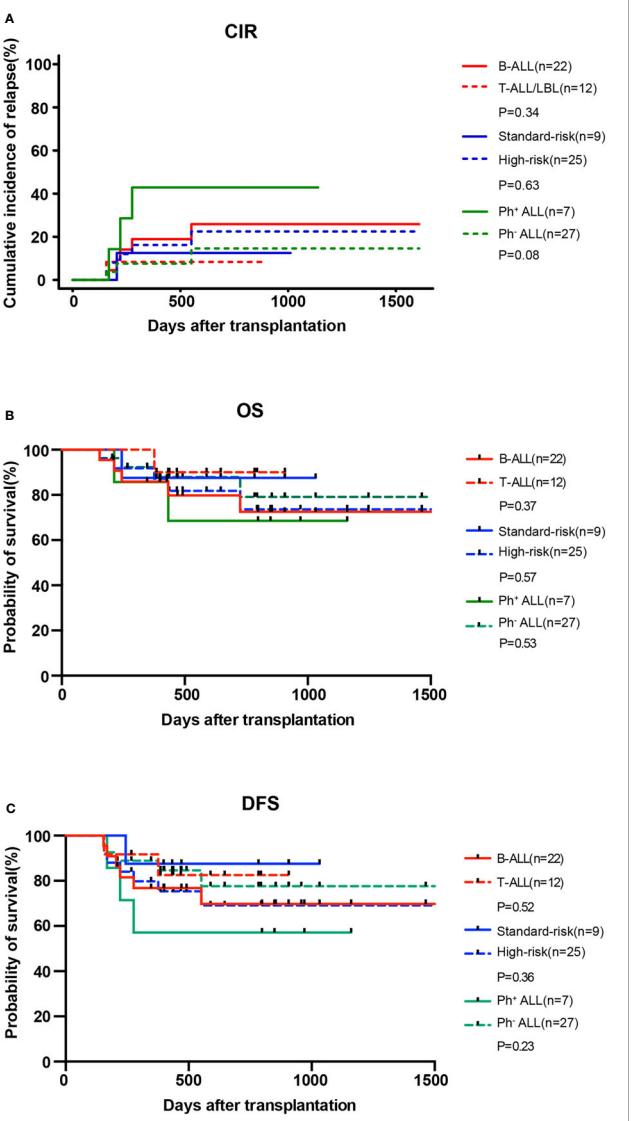


FIGURE 3 | CIR **(A)** and OS **(B)** and DFS **(C)** for patients with B-ALL (n = 22) and T-ALL/LBL (n = 12), patients at high risk (n = 25) and standard risk (n = 9), and patients with Ph^+ ALL (n = 7) and Ph^- ALL (n = 27) after allo-HSCT.

developed grade III granulocytopenia and mild pulmonary bacterial infection after one cycle, which improved after administration of G-CSF and oral azithromycin. Their granulocytes returned to normal after 14 days and 12 days of treatment, respectively. None of the patients required blood transfusion during the period of myelosuppression, and none of the patients interrupted treatment because of infection.

DISCUSSION

Disease relapse is a major therapeutic challenge in patients with adult ALL that have undergone allo-HSCT, and treatment

options are limited. The risk of relapse-related death in this population was as high as about 30–54% (1–3, 28), leaving preventing post-transplantation relapse necessary. At present, the downregulation of HLA-II molecules on leukemic cells caused by epigenetic silencing (such as the hypermethylation of CIITA) leads to immune escape of leukemic cells, which is a main mechanism of relapse post-transplantation (4, 8). Moreover, abnormalities in DNA methylation are common in ALL (11–13, 29, 30). Therefore, combined with the clear benefits of HAMs in maintenance therapy after AML/MDS transplantation (14, 15), we first evaluated the use of low-dose decitabine as maintenance therapy after allo-HSCT for adult ALL to reduce relapse and improve survival of this population. In this study, we achieved a 2-year CIR (20%) that was lower than that reported in previous studies, and the 2-year OS (77.5%) was satisfactory. Even in the high-risk group, the 2-year OS was 73.6%. To some extent, our data also indicated that maintenance therapy with decitabine may be used as a treatment option to prevent relapse after ALL transplantation.

The prognosis of adult T-ALL is unsatisfactory, with a 5-year OS of only 30–50% (31–33). Furthermore, the prognosis of patients who relapse is poorer, with a reported 5-year OS of 5% (34). Although allo-HSCT has improved the prognosis of this population, there is still a CIR of 12.4% in the low-risk group and 41.2% in the high-risk group (35). However, the 2-year CIR of patients with T-ALL/LBL in our study was only 8.3%, while OS and DFS were as high as 90 and 81.5%, respectively. Surprisingly, none of the patients with T-ALL experienced relapse, which is encouraging. Katagiri et al. (36) also reported that successful maintenance treatment was achieved with azacitidine in a patient diagnosed with myeloid/lymphoid neoplasm with FGFR1 (located on chromosome 8p11.2) rearrangement after allo-HSCT. In addition, ETP-ALL has a higher rate of remission failure and subsequent relapse than typical T-ALL (37). Meng et al. (38) reported that six patients with relapsed/refractory ETP-ALL were treated with decitabine combined with the CAG regimen (aclarubicin, cytarabine, and G-CSF), and five patients achieved CR. In this study, one patient with ETP-ALL initiated maintenance treatment with decitabine and has completed four cycles and is currently well at the date of last follow-up. The above evidence supports the feasibility of low-dose decitabine maintenance therapy in T-ALL.

Lockhart et al. (39) described a child with Ph^+ ALL having mixed donor chimerism and persistent BCR-ABL transcripts after allo-HSCT. There was no response to TKI treatment, but her clonal cytogenetic abnormalities were resolved after decitabine treatment. Cui et al. (40) also described 12 patients with relapse ALL after transplantation who were treated with decitabine alone or in combination with chemotherapy and DLI, and found that patients with Ph^+ ALL achieved higher survival than patients with Ph^- ALL. However, the effects of decitabine maintenance treatment on patients with Ph^+ ALL was not significant, and the 2-year CIR was much higher than that of patients with Ph^- ALL in this study. Although all seven patients with Ph^+ ALL received oral TKI after transplantation, three patients still relapsed. However, this may be related to the presence of the *T315I* mutation, as in two of the three relapsed patients the *T315I* mutation was detected at relapse, and one patient was not tested voluntarily. For patients with Ph^+ ALL after transplantation, exploring treatment with next-generation TKI may be more meaningful than low-dose decitabine.

HMAs can upregulate the expression of FOXP3 in CD4+CD25-T cells, thus increasing the number of Treg cells and mitigating GVHD (41, 42). In this study, only one patient developed grade IV aGVHD, while four patients presented severe cGVHD. Most cases occurred when immunosuppressants were reduced or prior to maintenance treatment. Only three patients stopped maintenance treatment because of GVHD. It is a pity that no aggravation or relief was observed in patients with GVHD due to the use of decitabine.

In this study, 94.1% patients developed grade I–II myelosuppression after receiving low-dose decitabine. Only two patients (5.8%) developed grade III–IV granulocytopenia and mild pulmonary infection. None of the patients required blood transfusion, and no one stopped this trial because of hematological toxicity. Pusic et al. (15) divided 24 patients with AML/MDS into four groups after transplantation, and each group was given different doses of decitabine for maintenance therapy. The authors found that the 10 $mg/m^2/d$ group presented fewer hematological adverse reactions and that decitabine was well tolerated, which was similar to our results. Further, our study showed that maintenance treatment with low-dose decitabine after transplantation had low hematological toxicity and is well tolerated.

Obviously, this study also has some limitations. First, our patients exhibited selection bias. Risk of disease relapse after allo-HSCT is a composite of multiple factors, including age, risk stratification at diagnosis, remission status at the time of transplantation, and duration of remission after transplantation. This study enrolled patients who were relatively young, and the sample population included 26.9% low-risk patients and several patients who were still in CR about 6 months after transplantation, which would lead to a better overall prognosis. Conversely, patients were enrolled without severe complications such as severe GVHD and were selected after day +50 of HSCT, which necessarily excluded those who relapsed early. Therefore, some transplanted patients were excluded because of early relapse or non-relapse mortality within the first 2 months. Secondly, this study did not detect changes in DNA methylation level before and after treatment, which would further support the use of HAMs. Finally, this study is limited by the small number of patients and lack of controls.

In conclusion, although the current data do not provide definitive evidence supporting the effects of low-dose decitabine maintenance treatment on the prevention of relapse after ALL transplantation, the overall results are encouraging and still indicate a positive trend. Low-dose decitabine maintenance treatment may be used as an option to prevent relapse after transplantation in patients with adult ALL, especially in patients

with T-ALL. Our findings require confirmation in larger-scale controlled trials.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RG, JL, and Z-XJ conceived, designed, and planned the study. All authors acquired the data. JL analyzed the data. JL and RG interpreted results. JL, FH, and RG drafted the report. RG, R-QL, and WL were involved in the critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Reduced Intensity Conditioning Followed by Allogeneic Hematopoietic Stem Cell Transplantation Is a Good Choice for Acute Myeloid Leukemia and Myelodysplastic Syndrome: A Meta-Analysis of Randomized Controlled Trials

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Background: Reduced intensity conditioning (RIC) before allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been reported to have the same overall survival (OS) as myeloablative conditioning (MAC) for patients with acute myeloid leukemia (AML) in complete remission (CR) and myelodysplastic syndrome (MDS). However, results from different studies are conflicting. Therefore, we conducted a systematic review and meta-analysis guided by PRISMA 2009 to confirm the efficacy and safety of RIC vs. MAC for AML in CR and MDS.

Methods: We search PubMed, Web of Science, Embase, Cochrane central, clinical trial registries and related websites, major conference proceedings, and field-related journals from January 1, 1980, to July 1, 2020, for studies comparing RIC with MAC before the first allo-HSCT in patients with AML in CR or MDS. Only randomized controlled trials (RCTs) were included. OS was the primary endpoint and generic inverse variance method was used to combine hazard ratio (HR) and 95% CI.

Results: We retrieved 7,770 records. Six RCTs with 1,413 participants (711 in RIC, 702 in MAC) were included. RIC had the same OS (HR = 0.95, 95% CI 0.64–1.4, $p = 0.80$) and cumulative incidence of relapse as MAC (HR = 1.18, 95% CI 0.88–1.59, $p = 0.28$). Furthermore, RIC significantly reduced non-relapse mortality more than total body irradiation/busulfan-based MAC (HR = 0.53, 95% CI 0.36–0.80, $p = 0.002$) and had similar long-term OS and graft failure as MAC.

Conclusion: RIC conditioning regimens are recommended as an adequate option of preparative treatment before allo-HSCT for patients with AML in CR or MDS.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=185436.

Keywords: reduced intensity conditioning (RIC), acute myeloid leukemia, myelodysplastic syndrome, overall survival, non-relapse mortality (NRM)

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has the lowest risk of relapse than any other treatment for acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) (1). However, allo-HSCT, like traditional myeloablative conditioning (MAC) regimens, has been associated with a high risk of serious adverse events and high non-relapse mortality (NRM) (2). Over the past three decades, the development of less toxic and more tolerable pre-transplantation regimens—the reduced intensity conditioning (RIC) regimen—has thus become the focus of clinical research (3). Specifically, the RIC regimens consisted of less than 8 Gray (Gy) of total body irradiation (TBI), less than 8 mg/kg PO of busulfan (Bu), or intravenous equivalent dose or other medications with high-powered immuno-suppressive effect but with less tissue toxicity to replace TBI or Bu along with fludarabine (Flu) to replace cyclophosphamide (Cy) (3). RIC reduces tissue injury and consequently reduces the incidences of acute graft versus host disease (aGVHD) and other complications but maintains graft versus leukemia effect to prevent leukemia relapse (3). Some non-randomized controlled studies reported that RIC reduced NRM but increased disease relapse, generally resulting in the same overall survival (OS) as MAC (4–6). However, these observational studies lack the benefit of random allocation, which is important to balance the baseline characteristics of patients among different treatment arms, especially to control for confounding by indication bias. Recently, several high-quality randomized controlled trials (RCTs) compared RIC with MAC for fit patients with AML in complete remission (CR) and MDS, but the results were not consistent (7–12).

The number of patients receiving RIC is rapidly increasing. In the United States, RIC accounts for more than 50% of all allo-HSCTs (13). Except for AML and MDS, there have been no prospective studies comparing RIC with MAC for other hematologic malignancies. Therefore, we undertook this systematic review (SR) and meta-analysis to clarify the efficacy and safety of RIC versus MAC for AML in CR and for MDS.

METHODS

This meta-analysis was guided by PRISMA 2009 guidelines (**Supplement 1**). The meta-analysis protocol is registered on PROSPERO with the ID of CRD42020185436.

We included only RCTs compared RIC with MAC before first allo-HSCT in patients with AML in CR or MDS, as defined by 2008 World Health Organization (14) (recruitment began after 2008) and French–American–British criteria (recruitment began before 2008). We did not restrict for age, sex, race, recruitment period, complicated diseases, or languages and allowed any aGVHD prophylaxis regimens except *in vitro* T-cell depletion. Median follow-up time should be more than 1 year.

The primary endpoint was OS, whereas the secondary endpoints were leukemia-free survival (LFS), cumulative incidences of relapse (CIR), NRM, aGVHD, and chronic (c) GVHD. Survival data were evaluated from the first day after stem cell transfusion until the first occurred event and the longest follow-up data were used. Glucksberg (15), International Bone Marrow Transplant Registry grading systems (16), and Seattle criteria (17) were used to grade aGVHD and cGVHD. Incidences of III–IV aGVHD, extensive cGVHD, graft failure (GF), overall organ toxicity, oral mucositis, specific organ toxicities, and reported infection were safety endpoints.

We electronically searched databases and hand-searched field-related articles between January 1, 1980, and July 1, 2020. **Supplement 2** showed the detailed searching strategy. Cochrane highly sensitive search filters were used for identifying RCTs in Medline and Embase (18).

YS and ZY independently screened retrieved records, extracted data of the characteristics of included studies according to **Table 1** and **Supplement 3**, and used Cochrane Collaboration-recommended tool to assess quality of included studies (**Table 2** and **Supplement 3**) (19). Only studies in the low-risk group were included and disagreement was resolved by discussion through YS, ZY, and JD. We also contacted authors if additional information was required.

Revman software (Version 5.3; Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012) was used. Hazard ratio (HR) and its 95% confidence interval (CI) were combined in the meta-analyses of OS, CIR, LFS, NRM, aGVHD, and cGVHD endpoints with generic inverse variance method (20). Statistics of log HR and variance were calculated according to Parmar et al. (21), Mantel-Haenszel (22), and DerSimonian–Laird (23) methods calculating relative risk (RR) or odds ratio (OR), and 95% CIs were used to combine dichotomous data. Two-sided $p < 0.05$ was considered significant. Heterogeneity was calculated with Q test and I^2 statistics. Fixed effect model was used if heterogeneity was not significant ($p > 0.10$ and $I^2 < 50\%$). Random effects model was used if heterogeneity was significant ($p \leq 0.10$ and/or $I^2 \geq 50\%$). Because treosulfan was less toxic than TBI/Bu (8, 24), we predefined three subgroups that were named RIC vs. TBI/Bu-based MAC, RIC vs. treosulfan 30 g/m^2-based MAC, and RIC vs. treosulfan 42 g/m^2-based MAC, respectively. In addition, in NRM and aGVHD meta-analyses, we only combined HR of every subgroup but the total HR of all included studies was not combined. Except for NRM and aGVHD, both the HR in the three subgroups and all included studies were combined. Sensitivity analyses removing included studies were used to evaluate whether quality of studies and clinical characteristics influenced results. Funnel plot and meta-regression were planned to detect publication bias.

Quality of evidence on main endpoints were evaluated with the "GRADE evidence profiles" table (25).

Abbreviations: aGVHD, Acute graft versus host disease; Allo-HSCT, Allogeneic hematopoietic stem cell transplantation; AML, Acute myeloid leukemia; Bu, Busulfan; cGVHD, Chronic graft versus host disease; CI, Confidence interval; CIR, Cumulative incidence of relapse; CR, Complete remission; Cy, Cyclophosphamide; Flu, Fludarabine; GF, Graft failure; GVHD, Graft versus host disease; Gy, Gray; HR, Hazard ratio; HSCT, Hematopoietic stem cell transplantation; LFS, Leukemia-free survival; MAC, Myeloablative conditioning; MDS, Myelodysplastic syndrome; MRD, Minimal residual disease; NRM, Non-relapse mortality; OR, Odds ratio; OS, Overall survival; RCTs, Randomized controlled trials; RIC, Reduced intensity conditioning; RR, Risk ratio; TBI, Total body irradiation.

TABLE 1 | Demographic characteristics of included studies.

Studies		Beelen et al. (8)	Bornhäuser et al. (9)	Kröger et al. (10)	MC-FludT.14/L Trial I (7)	Ringdén et al. (11)	Scott et al. (12)
Recruitment period		Jan 25th, 2013-November 16th, 2016	Nov 15th, 2004-Dec 31st, 2009	May 2004-December 2012	Nov 24th, 2008–Sep 26th, 2012	N/R	June 2nd, 2011-April 10th, 2014
Number of participants	RIC	240	99	65	168	18	137
	MAC	220	96	64	152	19	135
Median age (range), years	RIC	61.0 (56.5–64.0)	44 (18–60)	51 (22-63)	58.0 (54.0-63.0)	46 (26-61)	54.8 (21.9-65.9)
	MAC	60.0 (55.0–65.0)	45 (18–60)	50 (19-64)	59.0 (53.0-63.0)	45(22-58)	54.8 (21.9-66)
Diagnosis (number)	RIC	AML in CR (138); MDS (102)	AML in CR (99)	MDS (61); sAML in CR (4)	AML in CR (109); MDS (43)	AML in CR (14); CML in CP1 (4)	AML in CR (110); MDS (27)
	MAC	AML in CR (155); MDS (65)	AML in CR (96)	MDS (54); sAML in CR (8); missing (2)	AML in CR (130); MDS (38)	AML in CR (15); CML in CP1 (4)	AML in CR (108); MDS (27)
Number of high risk	RIC	AML in CR: 43; MDS: 55	22	7	N/R	3	71
	MAC	AML in CR: 63; MDS: 36	26	9	N/R	3	54
Donor source (number)	RIC	MRD, MUD	MRD, MUD	MRD, MUD	MRD, MUD	MRD, MUD	MRD, RUD, MUD
	MAC	MRD, MUD	MRD, MUD	MRD, MUD	MRD, MUD	MRD, MUD	MRD, RUD, MUD
Performance status before HSCT	RIC	HCT-CI Score >2, number (percentage) 140 (58%)	Participants have adequate renal, cardiac, pulmonary, and neurological function.	ECOG (number) 0 (21), 1 (29), 2 (3), 3 (2), Missing (10)	HCT-CI Score, Median (Q1, Q3) 3.0 (2.0, 5.0)	Patients who would tolerate MAC without advanced diseases.	HCT–CI Score, (number) 0 (40), 1–2 (52), ≥3 (44)
	MAC	HCT-CI Score >2, number (percentage) 131 (60%)	Participants have adequate renal, cardiac, pulmonary, and neurological function.	ECOG (number) 0 (18), 1 (32), 2 (3), 3 (0), Missing (11)	HCT-CI Score, Median (Q1, Q3) 3.0 (1.0, 4.0)	Patients who would tolerate MAC without advanced diseases	HCT–CI Score, (number) 0 (46), 1–2 (45), ≥3 (42)
Conditioning regimen	RIC	Bu 6.4 mg/kg intravenously + Flu 150 mg/m^2	TBI 8 Gy + Flu 120 mg/m^2	Bu 8 mg/kg + Flu 150 mg/m^2	Bu 6.4 mg/kg intravenously + Flu 150 mg/m^2	Bu 8mg/kg + Flu 150–180 mg/m^2	Bu 8 mg/kg + Flu (120–180 mg/m^2); Flu (120-180 mg/m^2) + Mel (≤150 mg/m^2)
	MAC	Treosulfan 30 g/m^2 + Flu 150 mg/m^2	TBI 12 Gy + Cy 120 mg/kg	Bu 16 mg/kg + Cy 120 mg/kg	Treosulfan 42 g/m^2 + Flu 150 mg/m^2	Bu 16 mg/kg + Cy 120 mg/kg	Bu 16 mg/kg or TBI (12-14.2 Gy) + Flu (120-180 mg/m^2 or Cy 120mg/kg)
Median follow-up time, months	RIC	17.4	119	72	12	40.8	50
	MAC	15.4	119	75	12	62.4	50
GVHD prophylaxis	RIC	CsA/MTX	CsA/MTX	CsA/MTX	CsA/MTX	CsA/MTX	CNI/MMF, CNI/MTX, Tac/Siro
	MAC	CsA/MTX	CsA/MTX	CsA/MTX	CsA/MTX	CsA/MTX	CNI/MMF, CNI/MTX, Tac/Siro
Withdrawn/all randomized (%)		16/476 (3.48)	0/195 (0)	0/129 (0)	10/330 (3)	0/37 (0)	0/272 (0)

N/R, not reported; RIC, reduced intensity conditioning; MAC, myeloablative conditioning; AML, acute myeloid leukemia; CR, complete remission; MDS, myelodysplastic syndrome; sAML, secondary AML; CML, chronic myeloid leukemia; CP1, the first chronic phase; MRD, matched related donor; MUD, matched unrelated donor; RUD, related mismatched donor; HCT–CI, hematopoietic cell transplantation-comorbidity index; ECOG, Eastern Cooperative Oncology Group; Q1, the first quartile; Q3, the third quartile; Bu, busulfan; Flu, fludarabine; TBI, total body irradiation; Gy, Gray; Mel, melphalan; Cy cyclophosphamide; CsA, cyclosporine; MTX, methotrexate; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil; Tac, tacrolimus; Siro, sirolimus.

RESULTS

Our search retrieved 7,770 references. After reviewing the titles and abstracts, 7,751 records were excluded for the reason that they were not relevant to RIC for AML in CR and MDS or not RCTs. After further examining full texts of the remaining 19 records, we excluded 10 references that were not RCT studies, not relevant to RIC, not compared with MAC regimens, or duplicated reports. In the end, we included 6 RCTs reported in 9 references into meta-analyses. All authors agreed to include Bornhäuser et al. (9), Kröger et al. (10), Ringdén et al. (11), Scott et al. (12), Beelen et al. (8) and MC-FludT.14/L Trial I

TABLE 2 | Quality assessment of included studies.

Studies	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Blinding of participants and personnel (performance bias) All outcomes	Blinding of outcome assessment (detection bias) All outcomes	Incomplete outcome data (attrition bias) All outcomes	Selective reporting (reporting bias)	Other bias
Beelen et al. (8)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Unclear risk
Bornhäuser et al. (9)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Kröger et al. (10)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
MC-FludT.14/L Trial I (7)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Unclear risk
Ringdén et al. (11)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Scott et al. (12)	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk

We used Cochrane Collaboration-recommended tool to assess the quality of included studies (19). The studies were classified into low-risk and high-risk groups. Studies reporting sufficient information to show low risk of bias in the sequence generation and allocation concealment were stratified into the low-risk group; otherwise, they were stratified into the high-risk group. Studies with high risk in any other domains were stratified into the high-risk group, too. Funnel plots and meta-regression would be used to assess publication bias.

studies (7) (**Figure 1**). Studies of Bornhäuser et al. (9), Kröger et al. (10), Ringdén et al. (11), and Scott et al. (12) reported the long-term follow up data (11, 26–28).

The six included studies with 1,413 participants (711 in the RIC group and 702 in the MAC group) all focused on the efficacy and safety of RIC compared with MAC, followed by allo-HSCT for AML in CR and MDS. Four studies focused on RIC vs. TBI/Bu-based MAC, whereas two studies focused on RIC vs. treosulfan-based MAC regimens. Studies used peripheral stem cell and bone marrow as stem cell sources. Donors included matched related, mismatched related, and matched unrelated donors. The demographic characteristics of the two treatment arms were similar in the included studies and are shown in **Table 1**. All included studies displayed low risk of bias. Details of quality assessment of the included studies are shown in **Table 2** and **Supplement 3**. All studies used the intention-to-treat method to analyze OS, CIR, and LFS. There was no selective reporting in all the included studies. Because funnel plots and meta-regression should only be used with more than 10 studies, we did not use them to detect publication bias in our analysis (29).

OS was not statistically ($p = 0.80$) different between RIC and MAC (HR = 0.95, 95% CI 0.64–1.4). Heterogeneity of the meta-analysis was significant ($p = 0.003$, $I^2 = 72\%$) (**Figure 2A**). The result was also similar in the RIC vs. TBI/Bu-based MAC subgroup analysis (HR = 0.84, 95% CI 0.5–1.4, $p = 0.50$) with significant ($p = 0.04$) heterogeneity ($I^2 = 65\%$). However, in the RIC vs. treosulfan 30 g/m^2-based MAC subgroup analysis, RIC was significantly ($p = 0.004$) lower than treosulfan-based MAC conditioning regimen (HR = 1.63, 95% CI 1.17–2.28). The combined long-term follow-up data also showed no difference between RIC and MAC (HR = 0.86, 95% CI 0.53–1.41, $p = 0.56$) with significant ($p = 0.01$) heterogeneity ($I^2 = 73\%$) (**Figure 3**).

We did not find a significant ($p = 0.28$) difference in CIR (HR = 1.18, 95% CI 0.88–1.59) between RIC and MAC (**Figure 2B**) and in CIR in the three subgroup analyses. Heterogeneity in the meta-analysis and in the RIC vs. TBI/Bu-based MAC subgroup was significant. Bornhäuser et al. (9), Kröger et al. (10), and Scott et al. (12) reported LFS, the combined result showed RIC had similar LFS to MAC (HR = 1.09, 95% CI 0.69–1.74, $p = 0.71$) with significant ($p = 0.05$) heterogeneity ($I^2 = 66\%$) (**Figure 2C**).

RIC significantly ($p = 0.002$) reduced NRM compared with TBI/Bu-based MAC (HR = 0.53, 95% CI 0.36–0.8) without heterogeneity ($p = 0.40$, $I^2 = 0\%$) (**Figure 4A**). However, the treosulfan 30 g/m^2-based MAC (8) significantly ($p = 0.04$) reduced NRM compared with RIC (HR = 1.67, 95% CI 1.02–2.72). RIC did not show significant difference compared with treosulfan 42 g/m^2-based MAC (MC-FludT.14/L Trial I (7); HR = 0.76, 95% CI 0.45–1.30, $p = 0.32$).

In addition, RIC showed a trend to reduce aGVHD (**Figure 4B**) and III–IV aGVHD (**Supplement 4**) compared with TBI/Bu-based MAC (HR = 0.79, 95% CI 0.60–1.03, $p = 0.08$) (RR = 0.61, 95% CI 0.36–1.04, $p = 0.07$) and with no significant ($p = 0.15$ and $p = 0.19$) heterogeneity ($I^2 = 43\%$ and $I^2 = 39\%$), respectively. Similarly, in the Beelen et al. (8) and MC-FludT.14/L Trial I (7) studies, RIC did not show a significant difference from treosulfan-based MAC (either 30 g/m^2 or 42 g/m^2).

We did not find a difference between RIC and MAC in cGVHD (**Figure 4C**) and extensive cGVHD (**Supplement 4**) (HR = 1.01, 95% CI 0.79–1.28, $p = 0.96$ and RR = 1.03, 95% CI 0.77–1.37, $p = 0.84$, respectively) with significant ($p = 0.08$ and $p = 0.09$) heterogeneity ($I^2 = 49\%$ and $I^2 = 51\%$), respectively, and no difference between RIC and MAC in the subgroup analyses was observed.

RIC showed a trend of increasing GF (OR 2.19, 95% CI 0.96–5.03, $p = 0.06$) without heterogeneity ($p = 0.34$, $I^2 = 12\%$). Moreover, GF incidence in the RIC and MAC arms was rare, 2.57% (18 events in 701 participants) and 1.16% (8 events in 690 participants), respectively. RIC did not show significant difference from MAC on overall organ toxicity and oral mucositis, with significant heterogeneity. Furthermore, RIC significantly ($p = 0.04$ and $p = 0.01$) reduced renal and urinary disorders (RR 0.61, 95% CI 0.39–0.97) and infection (RR 0.87, 95% CI 0.78–0.97) without heterogeneity (**Supplement 4**).

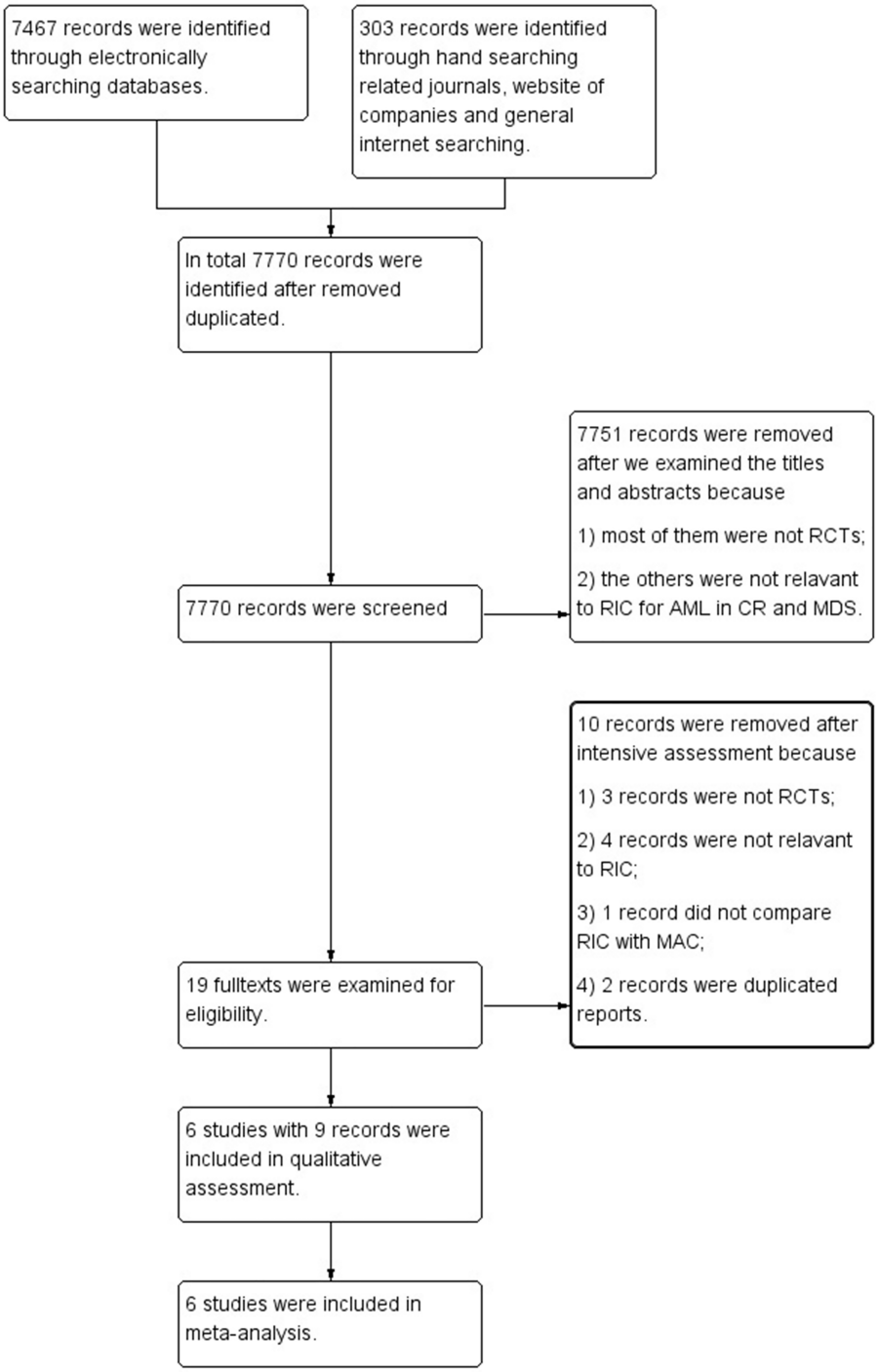

FIGURE 1 | Flow diagram of screening studies for inclusion in systematic review. AML, acute myeloid leukemia; CR, complete remission; MDS, myelodysplastic syndrome; RCTs, randomized controlled trials; RIC, reduced intensity conditioning; MAC, myeloablative conditioning.

We did subgroup analysis based on diseases (AML or MDS) for OS and CIR; however, we still could not eliminate heterogeneity. The results of subgroup analyses did not show significant difference between RIC and MAC on OS and CIR in either AML or MDS subgroups (**Supplement 5**). We repeated the meta-analyses for the OS, CIR, and long-term OS with the fixed-effect model because of their significant heterogeneity, and the results did not change the overall conclusions of these endpoints (**Supplement 6**). We also

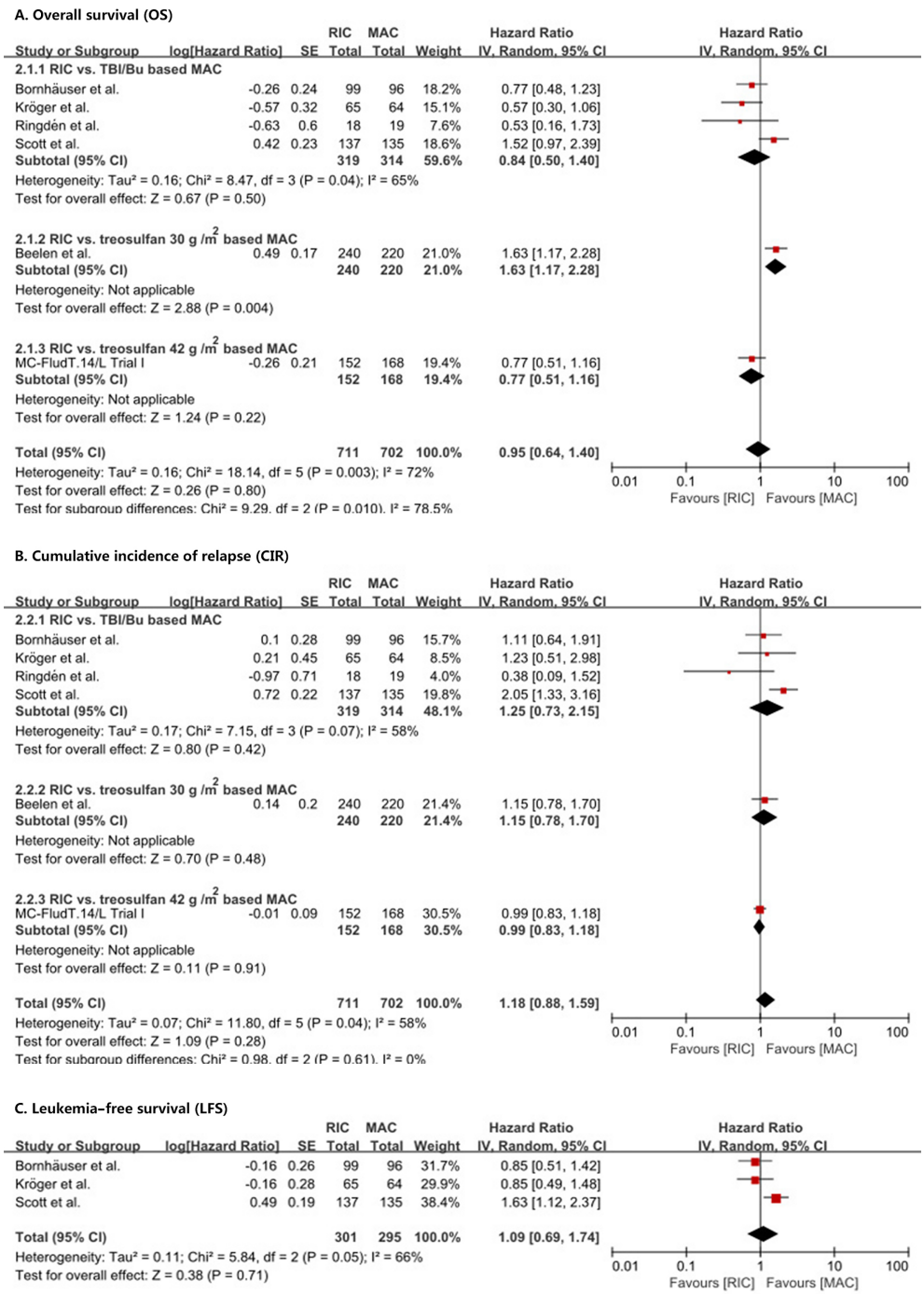

FIGURE 2 | Results of meta-analyses of OS, CIR, and LFS endpoints. The forest plots showed that RIC had the same OS **(A)**, CIR **(B)**, and LFS **(C)** as MAC. RIC, reduced intensity conditioning; MAC, myeloablative conditioning; TBI, total body irradiation; Bu, busulfan.

removed one study at a time and then repeated the meta-analysis in the sensitivity analysis. The pooled HRs ranged from 0.84 to 1.05 for OS and from 1.02 to 1.26 for CIR. Results after removing any study (including Beelen et al. (8) and Scott et al. (12) studies) were overall stable. After we removed the Scott et al. (12) study, the heterogeneity of CIR disappeared (**Supplement 7**) and the results of CIR did not change. Eight CML patients were included in the Ringdén et al. (11) study. After removing it in the sensitivity analysis, we did not observe significant changes in OS, CIR, and NRM results (**Supplement 8**).

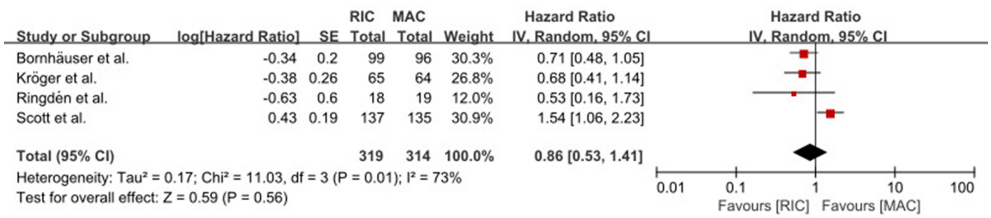

FIGURE 3 | Result of meta-analysis of long-term OS data. The forest plot showed that RIC had the same long-term OS as TBI/Bu-based MAC. OS, overall survival; RIC, reduced intensity conditioning; MAC, myeloablative conditioning; TBI, total body irradiation; Bu, busulfan.

The quality of evidence for the OS, CIR, LFS, and cGVHD endpoints was moderate. The quality of the NRM and aGVHD endpoints was high (**Supplements 9, 10**).

DISCUSSION

Retrospective studies and their meta-analyses cannot balance the baseline characteristics of patients among different treatment arms. Most patients in the RIC arm in these studies were older or had higher comorbidity burden, which might underestimate the efficacy and safety of RIC. Half of all finished RCTs [Bornhäuser et al. (9), Scott et al. (12) and Kröger et al. (10)] did not enroll enough participants as the studies had planned which limited their power to demonstrate the difference between RIC and MAC. All the finished studies cannot provide reliable evidence to evaluate RIC for AML in CR and MDS, so we need higher level of evidence on this issue. Our meta-analysis included six high-quality RCTs with 1,413 participants and published and unpublished data, which limit the risk of publication bias. It was then more powerful and covered more patients than previous studies. To date, our study is the first comprehensive meta-analysis of RCTs that combined HR value to clarify the efficacy and safety of RIC vs. MAC and provides the highest current level of evidence for this matter.

The risk that RIC may increase CIR is the main concern for physicians to prescribe these conditioning regimens. A study of Scott et al. (12) demonstrated that RIC significantly increased relapse and prompted physicians to select MAC first for fit patients. However, when we combined data from all available RCTs, we failed to show differences in CIR between RIC and MAC. The heterogeneity was reported in the Scott et al. (12) study. After we removed it in the sensitivity analysis, we did not observe heterogeneity between the remaining five studies and the results did not change (**Supplement 7**). The relapse rate is affected by many factors, including the cytogenetic and molecular biologic characteristics of diseases, minimal residual disease (MRD) before HSCT, and immunosuppressant adjustment protocol, among others (30–33). It was unfeasible that all factors before transplantation were similar in every study; hence, the CIR was expected to be heterogeneous between studies. In a large observational analysis by the EBMT that included 2,974 middle-aged AML patients, relapse incidence was higher in intermediate- or high-risk patients but not in low-risk patients in the RIC group (32, 33). Most of our included studies did not examine MRD before HSCT to stratify participants, which might influence the results as patients who were MRD-positive would have higher CIR after RIC more than after MAC (34, 35). In the Scott et al. study, nearly two-thirds of the AML participants were found to have commonly mutated genes in AML, after using next-generation sequencing techniques, and in these patients, RIC significantly increased CIR compared with MAC, whereas in the remaining third of participants in whom these genes were not detected, RIC had the same CIR as MAC (36). In addition, all of the six included studies used the same GVHD prophylaxis in RIC and MAC, but the dose-adjustment protocol of immunosuppressant that was appropriate for MAC might have increased CIR for RIC. Therefore, it was possible that there was heterogeneity between the included studies. Moreover, three RCTs demonstrated that RIC did not increase CIR in the long-term follow-up data (11, 26, 28). As there were limited long-term data reported in all the included studies, we could not combine the long-term CIR. However, as most of the relapses after HSCT occur within 2 years (35), we conclude that RIC conditioning regimens do not increase CIR more than MAC for AML in CR and MDS.

A more intensive conditioning regimen causes more serious tissue damage, which may result in more severe aGVHD (36). Therefore, RIC is expected to not only decrease organ toxicity and tissue damage but also cause less aGVHD and NRM than TBI/Bu-based MAC. Our meta-analysis showed a trend for RIC to decrease aGVHD and III–IV aGVHD compared with TBI/Bu-based MAC, but it was not statistically significant. We are still in need of more high-quality studies to confirm whether there is a difference between RIC and MAC on aGVHD and III–IV aGVHD incidences. Our results indicated that there was no difference in cGVHD between RIC and MAC and confirmed the incidence of cGVHD was not related to conditioning intensity (37). In the retrospective studies, RIC reduced NRM (4–6) but RCTs failed to demonstrate the reduction. Our meta-analysis confirmed that RIC significantly reduced NRM compared with TBI/Bu-based MAC. There was no heterogeneity, and the quality of evidence was high (**Supplement 10**). RCTs represent relatively small sample size, especially some RCTs did not include enough participants as planned, which might not be powerful enough to demonstrate the difference. We included all the RCTs, which expanded the sample size and provided more powerful evidence to clarify the difference. In addition, the four included studies in the RIC vs. TBI/Bu-based MAC subgroup analysis involved

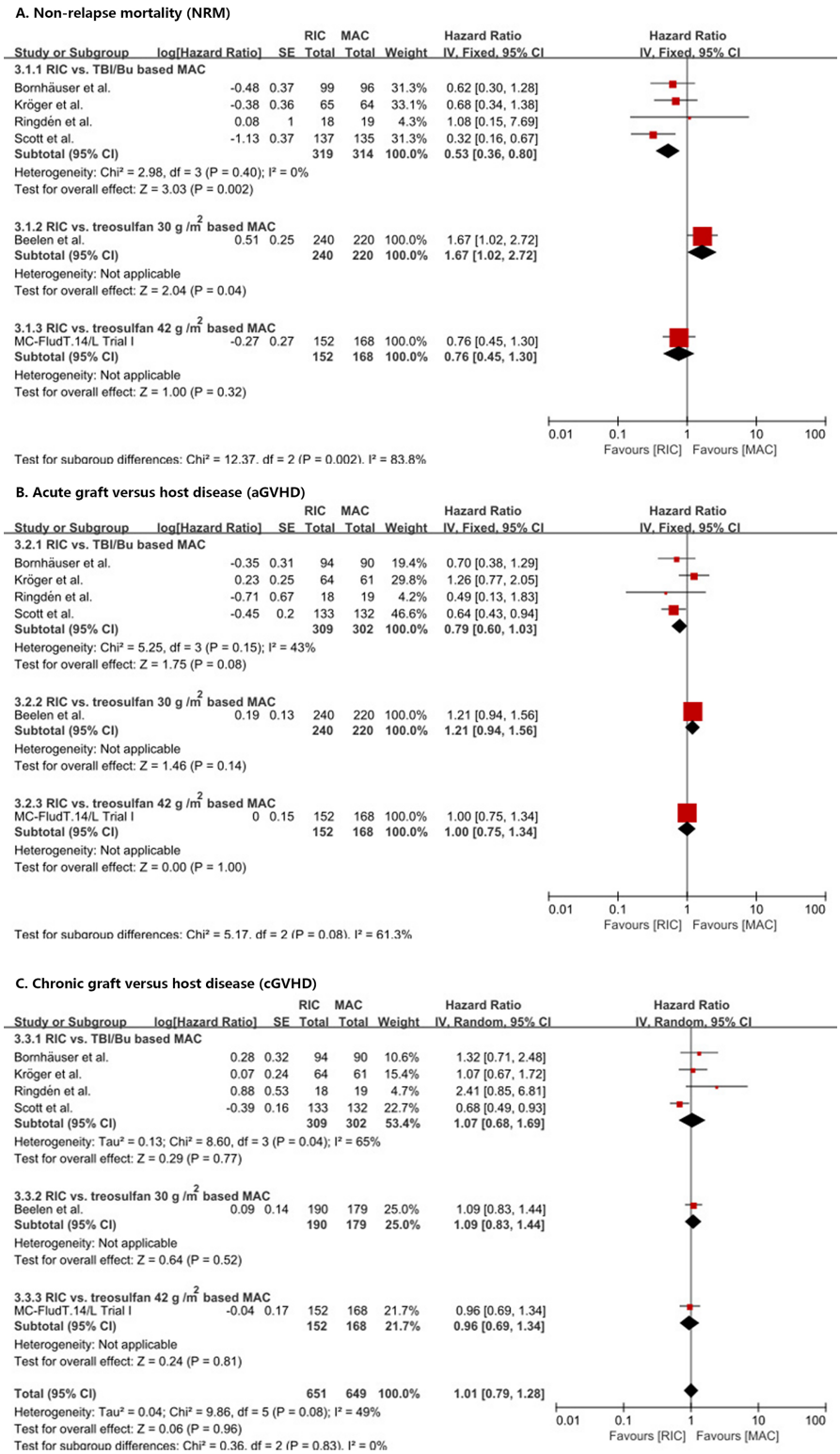

FIGURE 4 | Results of meta-analyses of NRM, aGVHD, and cGVHD endpoints. The forest plots showed that RIC significantly decreased NRM than TBI/Bu-based MAC **(A)**. RIC showed a trend to decrease aGVHD, but it was not statistically significant **(B)**. RIC had the same cGVHD as MAC **(C)**. RIC, reduced intensity conditioning; MAC, myeloablative conditioning; TBI, total body irradiation; Bu, busulfan.

relatively young and fit patients but not old patients, and in this subgroup analysis, RIC still caused less NRM. Consequently, RIC significantly reduces NRM more than TBI/Bu-based MAC for both young and old patients.

Moreover, our results showed that RIC significantly reduced some organ toxicity and infections compared with MAC, which indicated that RIC was more tolerable than MAC. On the other hand, our result did not show the difference on mucositis between RIC and MAC as generally expected. We observed that the heterogeneity of the meta-analysis was significant, so future studies are needed to clarify the issue. RIC had a trend to increase GF compared with MAC, but it was not significant. We showed only 18 GFs out of 701 patients and 8 GFs out of 690 patients reported in the RIC and MAC groups, respectively. The incidence of GF in the two groups was rare. According to the evidence available, we conclude that RIC causes marginal GF.

According to our results, RIC had the same OS as MAC, but heterogeneity was significant. In the HSCT procedure, the individualized prescriptions of different physicians will inevitably interfere with the results. Therefore, heterogeneity is common in clinical studies on HSCT, even when all the included studies are RCTs. In this regard, we used fixed-effect model to verify the results and did not find differences between RIC and MAC on OS (**Supplement 6**). In the study by Beelen et al. (8), treosulfan 30 g/m^2-based MAC, which caused less NRM than RIC, was used. Despite the fact that it was included in the meta-analysis, RIC did not increase OS compared to MAC. Moreover, RIC was still not different than MAC in OS after we excluded it in the sensitivity analysis (**Supplement 11**). A report from the Acute Leukemia Working Party of the EBMT, retrospectively included 883 RIC compared with 1,041 MAC and demonstrated that RIC increased OS for ≥50-year patients than MAC and had the same OS for ≤50-year patients as MAC (38). A large sample retrospective study also showed that there was no significant difference in long-term survival between RIC and MAC (39). Both studies also showed that RIC did not increase relapse. Our meta-analysis could not divide participants according to age, but our results also showed that RIC at least did not decrease OS compared to MAC. The RIC vs. TBI/Bu-based MAC subgroup analysis included more young patients, but RIC also showed no difference from MAC on OS. Furthermore, our long-term follow-up OS data meta-analysis showed that RIC did not decrease long-term OS compared with TBI/Bu-based MAC. Consequently, we concluded that RIC did not increase CIR but decreased NRM compared with traditional MAC regimens. It at least did not increase aGVHD and had the same cGVHD as MAC; as a result, RIC did not decrease OS. Therefore, we confirm there is no difference between RIC and MAC in OS for AML in CR and MDS.

In the RIC vs. treosulfan 30 g/m^2-based MAC subgroup analysis, treosulfan caused less NRM than RIC and increased OS (8). Treosulfan is a novel myeloablative agent with less toxicity than Bu (24) and treosulfan-based MAC was named reduced-toxicity conditioning regimen (24). The subgroup analysis confirmed that treosulfan was less toxic than Bu and suggested that treosulfan 30 g/m^2-based MAC was better than Bu- or TBI-based RIC. It was a promising result and provided new myeloablative agents that were higher than the traditional Bu or TBI. However, only one RCT finished until recently and the RIC vs. treosulfan 42 g/m^2-based MAC subgroup analysis did not show that treosulfan caused less NRM than RIC (7). Hence, we need more high-quality studies to confirm the result.

There are some limitations of our meta-analysis. Firstly, a relatively small number of clinical trials were included. Secondly, in OS, CIR, and LFS meta-analyses, there was significant heterogeneity between included studies. We tried to explore the heterogeneity with subgroup analysis based on conditioning regimens and diseases, but it could not be eliminated. We then suggest that the reason for the heterogeneity was the difference in treatment details available from the different transplantation centers and the inevitable patient heterogeneity between included studies. Thirdly, not all the included studies used blinding to personnel and patients. Allo-HSCT is a treatment with high NRM (40) and the treatment details should be individualized to every patient; therefore, blinding to patients and personnel could not be maintained. Fourthly, because we used data extracted from published reports but not individual patient data, we could not perform subgroup analysis based on diseases (AML in CR and MDS) and age. MDS patients may have less relapse than AML and young patients tolerate MAC better than old patients; thus, RIC may demonstrate better results in MDS patients and elderly patients. Despite these limitations, our meta-analysis is still reliable and can be used to guide physicians' clinical decisions.

RIC had the same OS and CIR as MAC for AML in CR and MDS and significantly decreased NRM more than TBI/Bu-based MAC. Furthermore, RIC was more tolerable and comfortable and caused marginal GF. RIC is equally effective as MAC. Therefore, RIC is also a good choice of conditioning regimen before allo-HSCT for patients with AML in CR and MDS and not only an alternative treatment to MAC for unfit patients. On the other hand, more high-quality studies should continue to focus on the OS and LFS comparing RIC with MAC. MRD, disease (AML or MDS), cytogenetic and molecular biologic characteristics, and age should be considered as classification factors in future studies to identify the factors from which patients will derive more benefit from RIC. In addition, future studies should attempt to improve GVHD prophylaxis that would be more appropriate for RIC. We also need more studies to compare treosulfan-based MAC with RIC.

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

YS conceived and designed the study, searched and selected trials for inclusion, assessed methodological quality of included trials, extracted data, performed the statistical analysis, and wrote the article. ZY searched trials, selected trials for inclusion, assessed methodological quality of included trials, and extracted data.

JD wrote and revised the review. TW wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.708727/full#supplementary-material

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Intestinal Microbiome in Hematopoietic Stem Cell Transplantation For Autoimmune Diseases: Considerations and Perspectives on Behalf of Autoimmune Diseases Working Party (ADWP) of the EBMT

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Over the past decades, hematopoietic stem cell transplantation (HSCT) has been evolving as specific treatment for patients with severe and refractory autoimmune diseases (ADs), where mechanistic studies have provided evidence for a profound immune renewal facilitating the observed beneficial responses. The intestinal microbiome plays an important role in host physiology including shaping the immune repertoire. The relationships between intestinal microbiota composition and outcomes after HSCT for hematologic diseases have been identified, particularly for predicting the mortality from infectious and non-infectious causes. Furthermore, therapeutic manipulations of the gut microbiota, such as fecal microbiota transplant (FMT), have emerged as promising therapeutic approaches for restoring the functional and anatomical integrity of the intestinal microbiota post-transplantation. Although changes in the intestinal microbiome have been linked to various ADs, studies investigating the effect of

intestinal dysbiosis on HSCT outcomes for ADs are scarce and require further attention. Herein, we describe some of the landmark microbiome studies in HSCT recipients and patients with chronic ADs, and discuss the challenges and opportunities of microbiome research for diagnostic and therapeutic purposes in the context of HSCT for ADs.

Keywords: autoimmune diseases, autoimmunity, fecal transplantation, intestinal, microbiome, stem cell transplantation, HSCT = hematopoietic stem cell transplant

INTRODUCTION

Intestinal microbiota may positively affect many aspects of the host physiology, including absorption of nutrients, prevention of overgrowth by potential pathogens, maintenance of epithelial barrier function, and shaping the immune system (1). Studies of the microbiome in the setting of hematopoietic stem cell transplantation (HSCT) demonstrated that intestinal flora are of particular importance in determining treatment outcomes, influencing immune reconstitution, and impacting complications such as infections or graft-versus-host disease (GvHD) (2, 3). In addition, changes in the microbial composition and function have been associated with various autoimmune diseases (ADs), and, although the precise mechanistic links between the microbiome and ADs remain largely unknown, increasing evidence suggests that disturbed gut microbiota contribute to pathogenesis (4). Among the potential mechanisms in the complex interplay between gut microbiota and host immune system, abnormal microbial translocation, molecular mimicry, and dysregulation of local and systemic immunity have been postulated.

This article will summarize the current evidence supporting the relationship between the microbiome and specific ADs, its impact on transplant outcomes, and potential therapeutic interventions, such as fecal microbiota transplantation (FMT). Moving forward, we propose how we may evaluate and influence the microbiome in the setting of HSCT for ADs to affect immune reconstitution and potentially improve clinical outcomes.

INTERACTION BETWEEN GUT MICROBIOTA AND THE HOST IMMUNE SYSTEM

While the primary function of the intestinal microbiota for the host has been considered to be the digestion of complex sugars and the provision of essential vitamins, it has become clear that the microbiota play an important role in the education and shaping of a functioning immune system. Evidence for this comes from the analysis of germ-free mice, which in the absence of any microbiota have underdeveloped lymph organs and reduced innate immune competence resulting in increased susceptibility to infection (5). Most likely for similar reasons, germ-free mice are resistant to genetic and induced models of autoimmunity. While the molecular mechanisms are still poorly understood, several pathways involved in the microbiota–host interaction have been identified, ranging from provision of ligands for innate receptors, such as Toll-like receptors for "trained" immunity (6), to the production of short-chain fatty acids, a product of the metabolizing of dietary fibers by certain bacteria, which have been described to enhance immune regulation (7, 8). Reciprocally, the host controls the microbiota through the production of antimicrobial peptides by intestinal epithelial cells and copious amounts of IgA antibodies, which are actively transported into the gut lumen by the intestinal epithelial cells, controlling the growth, mobility and attachment of intestinal bacteria (9). Alterations to this intricate microbiota – host interaction, e.g. genetic defects disrupting microbial sensing of the host or loss of bacterial diversity, often summarized under the term *dysbiosis*, resulting in loss of microbial functions for the host, has been associated with the development of chronic inflammatory diseases (10). Mechanistically, several pathways have been discussed by which intestinal microbiota might contribute to the development or perpetuation of autoimmune diseases (11). They include gut dysbiosis, which disrupts local gut homeostasis and may promote translocation of commensal or pathobionts to tissues where they facilitate chronic inflammation. In addition, microbiota may trigger autoimmunity directly by providing antigenic stimuli resulting in cross-reactivity of autoreactive lymphocytes and autoantibodies with bacterial orthologues. Finally, microbiota may modulate the immune system through their metabolites and may facilitate immune regulation by stimulating regulatory immune elements (summarized in **Figure 1**).

MICROBIAL PROFILING

The introduction of molecular biological methods for the characterization of the microbiota, in particular high-throughput sequencing, has greatly advanced our understanding of the diversity and function of the microbiota (22). Sequencing of the single or a combination of the 9 variable regions of the gene for the 16S ribosomal RNA of the small 30S ribosomal subunit is the mainstay to describe the composition of a microbial community. While 16S rRNA sequencing has become the method of choice due to its simplicity, it is often limited in the taxonomic resolution and is prone to bias e.g. PCR amplification and sampling depth (23). More extensive sequencing approaches include whole 16S rRNA gene sequencing allowing resolution of the microbiome to the species level, however often at the cost of sampling depth, and shot-gun metagenomics sequencing which will additionally yield information on the genetic repertoire, i.e. potential functional genes, of the bacterial community, the latter requiring

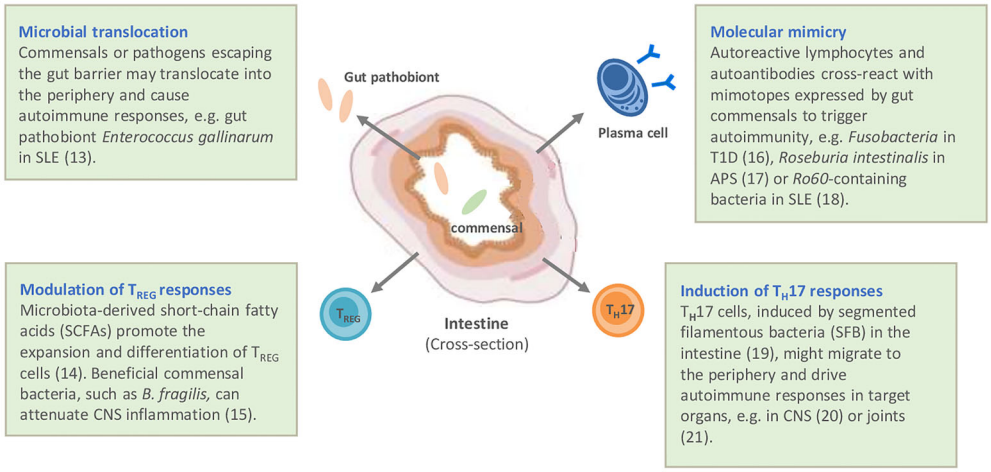

FIGURE 1 | Potential mechanisms by which autoimmune diseases are linked to gut microbiota and intestinal immunity. Modified according to (12). APS, antiphospholipid syndrome; CNS, central nervous system; SLE, systemic lupus erythematosus; T1D, type I diabetes (13–21).

extensive bioinformatics resources. Other "omics", such as metaproteomics can also be used to define the composition as well as the function of the microbiota, while metabolomic profiling identifies the mediators with which the microbiota could interact within itself and with the host [reviewed in (24)]. Recently, the combination of absolute quantification of the microbiota by flow cytometry with 16S rRNA gene profiling was shown to better reflect clinically relevant changes of the microbiome in patients with inflammatory bowel diseases (IBD) (25). Flow cytometric analysis of the microbiota also has the potential to rapidly identify alterations in the microbiota on the single cell level for monitoring purposes (26) and when combined with cell sorting and 16S rRNA gene analysis could lead to the identification of relevant bacteria in a more targeted fashion.

ROLE OF INTESTINAL MICROBIOTA IN AUTOIMMUNE DISEASES

Inflammatory Bowel Diseases

The intestinal tract, home to the largest density and diversity of microorganisms in healthy humans, is the target organ of IBD comprising Crohn's disease (CD) and Ulcerative Colitis (UC). The chronic intestinal inflammation in IBD is characterized by effector and tissue resident memory T cell responses to aspects of the intestinal microbiota (27). Despite large inter-individual variability, genetic analyses of microbial populations in stool and/or mucosal biopsies has revealed an overall decrease in diversity, a loss of symbionts and an increase in pathobionts (essentially Gram-negative proinflammatory microbes) in both UC and CD (27, 28). Whether these changes in microbial composition and IBD pathogenesis are a cause or consequence of intestinal inflammation remains a key area of study (29). Alterations in gut microbiota can disrupt epithelial and immune homeostasis, leading to increased permeability and eventual immune activation. Alternatively, the documented genetic and/or microbial-independent environmental factors associated with IBD may promote inflammation and oxidative stress, which subsequently results in a shift in microbial composition. A recent study has shown that increased fecal proteolytic activity and microbiota changes precede diagnoses of ulcerative colitis (30). In addition, the altered humoral and cellular acquired immune responses towards bacterial antigens that characterize IBD, particularly Crohn's disease (31), may predate disease onset (32). This suggests that immune responses towards microbes, rather than microbial composition itself, drives epithelial barrier disruption and altered innate responses at disease onset. Thus, in marked contrast to the impressive efficacy seen in Clostridium difficile infection (33), FMT has shown some benefit in mild UC but no impact in CD (34–36). Nonetheless, regardless of whether dysbiosis is the initial event or the result of overt inflammation, shifts in microbial composition may help perpetuate disease, as well as impact response to therapy in IBD (37, 38), and thus represent a desirable target for future therapies.

Systemic Sclerosis

In systemic sclerosis (SSc), a rare systemic autoimmune disease characterized by vasculopathy, immune activation and consequent progressive fibrosis, multiple genetic, epigenetic, and environmental factors are regarded as potential triggers for the onset and progression of the disease (39). Over the past decades, emerging evidence suggests that alterations of microbial populations colonizing epithelial surfaces (i.e., gastrointestinal tract, skin and lung), known as dysbiosis, may contribute to chronic inflammation and autoimmunity (4). Since the gastrointestinal tract is one of the organs highly affected in SSc, recent studies have aimed to investigate gastrointestinal microbiota alterations to elucidate the possible interaction with disease phenotype and clinical outcome of the disease (40).

Initial studies found that specific bacteria, particularly beneficial commensal genera (*Faecalibacterium, Clostridium* and *Rikenella*) and, conversely, more potentially pathobiont genera (*Bifidobacterium, Fusobacterium* and *Prevotella*) were decreased in SSc patients compared to healthy controls (41–43). Notably, SSc patients with more severe gastrointestinal symptoms exhibited a prevalence of the pathobiont *Fusobacterium* compared to patients with mild or no gastrointestinal symptoms (41–43). Furthermore, overabundance of opportunistic pathogenic *Clostridium* and typically oral *Streptococcus* species was recently described in SSc, while *Alistipes*, *Bacteroides*, and butyrate-producing species were depleted, congruent with findings in patients with IgG4-related disease, suggesting a common signature in both fibrosis-prone autoimmune diseases (44). Altogether, these studies confirm the existence of a shift in gut microbiota population in SSc patients. Whether these changes are causative or rather reflect the gastrointestinal involvement by inflammatory and fibrotic processes remains to be demonstrated. The role of intestinal dysbiosis in the disease pathogenesis is further complicated by the possibility that, as showed in other diseases, the intestinal microbiota in SSc might modulate local immunological mechanisms possibly responsible of local and systemic alterations (45).

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic immune-mediated disease of the central nervous system (CNS), which results from interactions of genetic and environmental factors (46). The underlying pathological process is complex but includes the abnormal activation of T and B cells targeting foreign and/or self-antigens, which could be primed within the CNS or in the periphery (47, 48). A potential source of such antigens is the gut microbiome, which exhibits a level of homology to human myelin proteins and may trigger cross-reactivity through the mechanism of 'molecular mimicry' (49, 50). Immune reconstitution studies have shown that gut Mucosal Associated Invariant T (MAIT) cells, which express chemokine receptor 6 (CCR6) to facilitate their transmigration into the CNS, are reduced following autologous HSCT, suggesting that they may play a role in crosslinking gut microbiome with the neuroaxis (51). In one experimental model, the presence of intestinal microbiota was necessary to induce CNS autoimmunity, suggesting that the gut has the ability to control systemic autoimmune responses (52, 53). Germ-free mice recipients receiving feces from patients with MS develop severe Experimental Allergic Encephalomyelitis (EAE) and the administration of *Lactobacilli* seems to suppress this process (54, 55). A number of case control studies have reported reduced gut microbiome diversity in patients with MS, particularly in those with active disease, although a consistent microbiome phenotype has not been identified (56, 57). Furthermore, some MS orally administered disease modifying therapies have been found to inhibit the growth of *Clostridium in vitro*, which may contribute to their anti-inflammatory mechanism of action (58). Given the increasing evidence that gut microbiome plays a role in the immune system homeostasis and in the pathogenesis of MS, changes in the microbiome in patients with MS undergoing HSCT warrant investigation.

ROLE OF INTESTINAL MICROBIOTA IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

Correlation With HSCT Outcomes

The intestinal microbiome undergoes profound changes during the course of transplantation. Multiple transplant-related factors (i.e. conditioning regimen, broad-spectrum antibiotics, nutrition) drive microbial shifts. At the same time, the alteration in the composition of gut flora is associated with transplant outcomes, including overall survival (OS), progression-free survival (PFS), treatment-related mortality (TRM) and GvHD (**Table 1**). Bacterial diversity largely decreases after HSCT, and is correlated with increased risk of major transplant complications such as infections or GvHD, potentially affecting the outcome of the procedure (81, 82). A large multicenter observational study has confirmed lower mortality rates in patients showing higher diversity of intestinal microbiota at engraftment (3). Recently, microbiota injury has been observed also in recipients of autologous HSCT, who undergo similar antibiotic exposures and nutritional alterations after high-dose chemotherapy and transplant procedure (80). Reduced OS and PFS have been reported in patients with lower peri-engraftment microbiome diversity.

Impact of chemotherapy, Diet, and Antibiotics on the Intestinal Microbiome in Transplant Recipients

Microbiome and transplant correlations may be influenced by local practices, antibiotic choices, hospital flora, and diet. Gastrointestinal disturbances associated with chemotherapy and radiation (83) and subsequent mucositis can also impact the composition of intestinal microbiota. A reduction in α-diversity and significant differences in the composition of the intestinal microbiota have been observed in response to chemotherapy, such as increase in *Bacteroides* and *Enterobacteriaceae* paralleled by a decrease in *Bifidobacterium*, *Faecalibacterium prausnitzii*, and *Clostridium cluster XIVa* (84), and a drastic drop in *Faecalibacterium* accompanied by an increase of *Escherichia* (85). The impact of diet on gut flora is well-recognized (86). Depletion of the intestinal microbiota reduces visceral adipose tissue and caloric uptake from diet (87), and enteral feeding may exert a beneficial effect on intestinal flora by providing the required nutrients (88). Interestingly, a lactose-free diet can prevent microbial overdominance by detrimental commensal bacteria like *Enterococcus* (72). Broad-spectrum antibiotic prophylaxis/treatment, commonly used in HSCT recipients, in the early phase after HSCT can beneficially reduce the number of transmigrated bacteria. However, their long-term effects are detrimental, because they limit microbiota diversity, by killing beneficial commensal bacteria that inhibit pathogens and promote immune defenses (81). A drastic decrease in the diversity of enteric microbiome after administration of antibiotic therapy, and the loss of obligate anaerobic commensal

TABLE 1 | Impact of microbiome on HSCT outcomes.

Study	Study Population	Microbiome Analysis	Microbiome Biomarker	HSCT Outcome
Taur et al., 2012 (59)	94 adult patients Allogeneic HSCT Single center, USA	454 pyrosequencing, V1-V3 region of the 16S RNA gene	Enterococcus domination (>30%) Proteobacteria domination (>30%)	VRE Bacteremia 9-fold increased risk Gram negative Bacteremia 5-fold increased risk
Ubeda et al., 2013 (60)	94 adult patients Allogeneic HSCT Single center, USA	454 pyrosequencing, V1-V3 region of the 16S RNA gene	Barnesiella genus* enteric colonization	Protection from VRE domination
Taur et al., 2014 (61)	80 adult patients Allogeneic HSCT Single center, USA	454 pyrosequencing, V1-V3 region of the 16S RNA gene	Low bacterial diversity at engraftment	Lower OS Higher TRM
Holler et al., 2014 (62)	31 adult patients Allogeneic HSCT Single center, Germany	Roche 454 platform sequencing, V3 region of the 16S RNA gene Strain-specific PCR of enterococci Urinary indoxyl sulfate analysis **	Enterococcus abundance > 20% Urinary indoxyl sulfate levels decrease during aplasia after HSCT	Increased frequency of GI acute GvHD –
Weber et al., 2015 (63)	131 adult patients Allogeneic HSCT Single center, Germany	Roche 454 platform sequencing, V3 region of 16S RNA gene Strain-specific PCR of enterococci Urinary indoxyl sulfate analysis **	Low urinary indoxyl sulfate levels within day +10 after HSCT (Lachnospiraceae and Ruminococcaceae *** » high urinary indoxyl sulfate levels; Bacilli » low indoxyl sulfate levels)	Low OS High TRM
Jenq et al., 2015 (64)	115 adult patients Allogeneic HSCT Single center, USA	First cohort (n=64): Roche 454 platform sequencing, V1-V3 region of the 16S RNA gene	Increased bacterial diversity	Higher OS Lower TRM Lower GvHD related mortality
		Second cohort (n=51): Illumina MiSeq platform sequencing, V4-V5 region of the 16S RNA gene	Blautia genus # abundance	Higher OS Lower GvHD related mortality Lower incidence of acute GvHD requiring systemic corticosteroids or steroid-refractory
			Lachnospiraceae abundance Clostridiales abundance Clostridia abundance	Lower GvHD related mortality
Shono et al., 2016 (65)	857 adult patients Allogeneic HSCT Single center, USA	Illumina MiSeq platform sequencing, V4-V5 region of the 16S RNA gene	Imipenem-cilastatin treatment Piperacillin-tazobactam treatment (associated to loss of Bacteroidetes and Lactobacillus ##)	Higher GvHD related mortality Higher grades 2-4 acute GvHD Higher GI acute GvHD
Harris et al., 2016 (66)	94 adult patients Allogeneic HSCT Single center, USA	454 pyrosequencing, V1-V3 region of the 16S RNA gene	Low baseline diversity Enterococcus domination (>30%) γ-Proteobacteria domination (>30%)	Higher risk of pre-engraftment pulmonary complications Higher risk of post-engraftment pulmonary complications
Peled et al., 2017 (67)	541 adult patients Allogeneic HSCT Single center, USA	Illumina MiSeq platform sequencing, V4-V5 region of the 16S RNA gene	Abundance of Eubacterium limosum and other related bacteria	Lower relapse/progression of disease risk
Mancini et al., 2017 (68)	96 adult patients Allogeneic HSCT Single center, Italy	Roche 454 platform sequencing, V3-V5 region of the 16S RNA gene	Baseline Enterobacteriaceae >5% Baseline Lachnospiraceae ≤10%	Higher risk of microbiologically confirmed sepsis, severe sepsis and septic shock Lower OS Higher infectious related mortality Higher non-infectious related mortality
Doki et al., 2017 (69)	107 adult patients Allogeneic HSCT Single center, Japan	Roche 454 platform sequencing, V1–2 region of the 16S RNA gene	Higher abundance of Firmicutes, lower abundance of Bacteroidetes, higher abundance Fecal bacterium and Eubacterium at baseline	Higher risk of acute GvHD
Lee et al., 2017 (70)	234 adult patients Allogeneic HSCT Single center, USA	Illumina MiSeq platform sequencing, V4-V5 region of the 16S RNA gene	Combined abundance of Bacteroidetes phylum, Lachnospiraceae family, Ruminococcaceae family Enterococcus faecalis at various rank designations	Protection from Clostridium difficile infection Higher risk of Clostridium difficile infection
Golob et al., 2017 (71)	66 adult patients Allogeneic HSCT Single center, USA	Illumina MiSeq platform sequencing, V3-V4 region of the 16S RNA gene	Presence of oral Actinobacteria and oral Firmicutes in stool, deficit of Lachnospiraceae at neutrophil engraftment	Higher risk of acute GvHD
Stein-Thoeringer et al., 2019 (72)	1325 adult patients Allogeneic HSCT Four centers: USA, Germany, Japan	Illumina MiSeq platform sequencing, V4-V5 region of the 16S RNA gene	Enterococcus domination (>30%) at early post-transplant period (day 0 to day +12)	Lower OS Higher GvHD related mortality Higher grades 2-4 acute GvHD incidence
Galloway-Peña et al., 2019 (73)	44 adult patients Allogeneic HSCT Single center, USA	Illumina MiSeq platform sequencing, V4 region of the 16S RNA gene	Low microbial diversity at engraftment Low Coriobacteriia, Coriobacteriaceae at engraftment	Higher risk of intestinal acute GvHD Higher TRM Higher risk of intestinal acute GvHD
Biagi et al., 2019 (74)	36 pediatric patients Allogeneic HSCT Four centers, Italy	Illumina MiSeq platform sequencing, V3-V4 region of the 16S RNA gene	Pretransplant Blautia genus abundance Pretransplant Fusobacterium abundance Abundance of Bacteroides at engraftment	Lower acute GvHD risk Higher severe GI acute GvHD risk Higher grades 2-4 acute GvHD risk

(Continued)

TABLE 1 | Continued

Study	Study Population	Microbiome Analysis	Microbiome Biomarker	HSCT Outcome
Han et al., 2019 (75)	141 adult patients Allogeneic HSCT China	Illumina MiSeq platform sequencing, V3-V4 region of the 16S RNA gene	At day 15 after HSCT: Low diversity Low Lachnospiraceae Low Peptostreptococcaceae Low Erysipelotrichaceae High Enterobacteriaceae	Higher acute GvHD risk Higher acute GvHD grades
Lee et al., 2019 (76)	211 adult patients Allogeneic HSCT Single center, Korea	16S rRNA gene sequencing	Post engraftment: Loss of diversity compared to pre transplant sample Depletion Ruminococcus Increase of Eubacterium Increase of Escherichia	Higher risk of intestinal acute GvHD
Peled et al., 2020 (3)	1362 adult patients Allogeneic HSCT Four centers: USA, Germany, Japan	Illumina MiSeq platform sequencing, V4-V5 region of the 16S RNA gene	Higher intestinal diversity in the peri-engraftment period (between days 7 and 21 after HSCT) Higher intestinal diversity before HSCT (from day –30 to –6)	Higher OS Lower TRM Lower GvHD related mortality § Higher OS Lower TRM
Payen et al., 2020 (77)	70 adult patients (n=35 with GvHD; n=35 without GvHD) Allogeneic HSCT Single center, France	Illumina MiSeq platform sequencing, V3-V4 region of the 16S RNA gene	Lower microbial diversity Depletion of Blautia Reduction of Lachnospiraceae and Ruminococcaceae Increase of Prevotella and Stenotrophomonas §§	Severe acute GvHD
Han et al., 2020 (78)	150 adult patients Allogeneic HSCT Two centers, China	Illumina MiSeq platform sequencing, V3-V4 region of the 16S RNA gene	Gut microbiota score: a formula based on selected gut microbiota features	Risk of grades 2-4 acute GvHD
Greco et al., 2021 (79)	96 adult patients Allogeneic HSCT Single center, Italy	Roche 454 platform sequencing, V3-V5 region of the 16S RNA gene	Low Diversity at day +10 after HSCT Enterococcaceae > 90% at day +10 <10% Lachnospiraceae at day +10 Staphylococcaceae >40% at day +10 	Higher grades 2-4 acute GvHD Higher grades 3-4 acute GvHD Higher risk of GI involvement Higher risk of acute GvHD with skin involvement Higher grades 2-4 acute GvHD Higher grades 3-4 acute GvHD Higher risk of acute GvHD with GI involvement Higher risk of acute GvHD with GI involvement Higher risk of acute GvHD with GI involvement Higher risk of acute GvHD with liver involvement Higher risk of steroid-refractory acute GvHD
Khan et al., 2021 (80)	534 adult patients Autologous HSCT Two centers, USA	Illumina MiSeq platform sequencing, V4-V5 region of the 16S RNA gene	Increased bacterial diversity at peri-neutrophil engraftment period Post-engraftment increased bacterial diversity Abundance of Enterococcus	Higher PFS Higher PFS and OS Lower OS

**Barnesiella genus belongs to the family Porphyromonadaceae, within the phylum Bacteroidetes.*
***Urinary indoxyl sulfate originates from the degradation of tryptophan to indole by colonic microbiota, followed by microsomal oxidation to indoxyl and sulfonation.*
****Families of Lachnospiraceae and Ruminococcaceae belong to the class of Clostridia, phylum Firmicutes. Eubacterium rectale is a prominent member of the family of Lachnospiraceae.*
#Blautia genus is classified as follows: family Lachnospiraceae, order Clostridiales, class Clostridia, and phylum Firmicutes.
##This study analyzed antibiotic treatment impact on GvHD risk, then antibiotic impact on microbiome within the same population.
§GvHD related mortality was significantly lower in patients with higher intestinal diversity in transplant from unmanipulated grafts.
§§Prevotella and Stenotrophomonas respectively belong to the Bacteroidetes and Proteobacteria families.
GvHD, Graft-versus-Host Disease; GI, gastrointestinal; HSCT, Hematopoietic Stem Cell Transplantation; OS, Overall Survival; PFS, Progression-Free survival; TRM, Transplant-related mortality; VRE, Vancomycin-resistant Enterococcus.

bacteria such as *Clostridia* and *Bacteroidetes* after piperacillin-tazobactam and meropenem administration, are recurrent in literature (89). Metronidazole administration increases enterococcal domination, whereas fluoroquinolone administration reduces domination by *Proteobacteria* (59) and represents an important variable associated with overall survival (61). Broad spectrum antibiotics, by inducing loss of bacterial diversity, are also associated with increased GvHD-related mortality (65, 90).

Intestinal Microbiome, Immune Reconstitution, and Infection Prevention

Effective and appropriate immune reconstitution is central to successful HSCT. Microbiota populations may influence immune reconstitution and cell dynamics in humans (91). The depletion of the intestinal microbiota impairs post-transplant immune reconstitution (87). Analysis of daily changes in circulating immune cell counts and extended longitudinal

microbiota analysis revealed consistent associations between gut bacteria and immune cell dynamics, paving the way for potential microbiota-targeted interventions to improve immunotherapy and treatments for immune-mediated diseases (91).

The gut microbiota play a critical role in maintaining colonization resistance against intestinal pathogens, thus preventing infections. Domination by *Enterococcus* and *Proteobacteria* are associated with the risk of bacteremia by Vancomycin-resistant *Enterococcus* and gram-negative rod respectively (59). A different baseline distribution of the gut microbiome (68) has been reported in patients at risk for microbiologically confirmed infection (high level of *Enterobacteriaceae*, low level of *Lachnospiraceae*), sepsis and septic shock (high level of *Enterobacteriaceae*). Moreover, a documented bloodstream infection may be anticipated by expansion and dominance of pathogenic strains in the gut flora (59, 68, 92, 93). Overall, a low diversity of the intestinal microbiota at engraftment has been shown to be an independent predictor of TRM from both infectious and non-infectious causes (61).

Intestinal Microbiome, GvHD, and Immunosurveillance

In the allogeneic transplant setting, a regulatory effect of the gut microbiota in the maintenance of intestinal homeostasis has been reported (94). Loss of fecal diversity, as well as increased abundance of members from *Enterococcus* or *Staphylococcus* species have been associated with the incidence and severity of acute GvHD (79), while other organisms such as *Blautia* species have a protective role (64). Metabolites produced by intestinal bacteria may promote intestinal tissue homeostasis and immune tolerance in the context of acute GvHD (95). Moreover, commensal bacteria can also play a role in tumor immunosurveillance. Increased abundance of a cluster of related bacteria including *Eubacterium limosum* was associated with decreased risk of relapse or disease-progression (67).

Altogether, these results indicate that the intestinal microbiota represent a potentially important factor in the success or failure of HSCT. As such, the microbiome can be envisioned both as a biomarker for the identification of patients at higher risk for transplant-related complications, and also a target for intervention aiming to impact clinical outcomes through enhancing microbiota recovery (96).

Modulation of Gut Microbiota by Fecal Microbiota Transplantation

FMT is a recommended therapeutic strategy for treating recurrent *Clostridioides difficile* infection (97, 98). Additionally, FMT has been investigated for treatment of steroid-resistant acute GvHD and initial positive results (99) were confirmed by several case reports (100). A small cohort study recently reported a complete response in 10 out of 14 patients (71%) with steroid-refractory or steroid-dependent acute GvHD 28 days after FMT (101). This response was accompanied by an increase in microbial α-diversity, a partial engraftment of donor bacterial species, and increased abundance of butyrate-producing bacteria, including groups in the order *Clostridiales*, namely *Blautia* species. Malard et al. recently reported the use of a next-generation FMT product "MaaT013", a standardized, pooled-donor, high-richness microbiota biotherapeutic, in the largest cohort of patients to date (n=29) with steroid-refractory or steroid-dependent intestinal acute GvHD (102). These patients had previously received and failed 1 to 5 lines of GvHD systemic treatments. The product was well tolerated and at day 28, overall response and complete remission rates were 59% and 31%, respectively. Furthermore, some studies have evaluated the role of FMT in treating dysbiosis after allogeneic HSCT. Taur et al. reported that autologous FMT after HSCT was safe and boosted microbial diversity, restoring bacterial populations lost during HSCT and reversing the disruptive effects of the broad-spectrum antibiotics (n=14) (81). Overall, FMT appears to be a promising strategy and several studies are ongoing to evaluate FMT for acute GvHD management (NCT03812705, NCGT03492502, NCT03359980, NCT03720392, NCT03678493). Regarding prevention of complications, additional studies are warranted to confirm that restoration of gut microbiota dysbiosis after FMT translates into clinical improvement after allogeneic HSCT, in particular a lower incidence of acute GvHD (96).

DISCUSSION

It is increasingly accepted that understanding the complex interactions between the microbiome and immune system will be crucial to defining the pathogenesis of ADs, whilst optimizing therapeutic interventions and clinical outcomes. HSCT is increasingly used specifically to treat severe, resistant ADs, with now more than 3000 cases being reported to the registry of the European Society of Bone and Marrow Transplantation (EBMT) (103, 104). To date very limited data is available regarding microbiome biology in the setting of HSCT for ADs, where medium to long-term clinical outcomes are considered to be due to the induction of altered (or 're-booted') immune reconstitution post-transplant. The 'immune re-boot' has been increasingly characterized in a range of ADs with a range of immunological markers, including evidence of generation of 're-educated' and regulatory populations to support re-induction of self-tolerance lasting beyond the broad immunosuppressive effects of autologous HSCT (105, 106). Changes in immune reconstitution may affect not only on disease activity, but also adverse events, such as secondary ADs (107–110).

As for ADs outside the transplant setting, and for GvHD in allogeneic HSCT, the microbiome may significantly influence the baseline status of the underlying AD *pre-transplant*, the patients general condition *peri-transplant* (which will inevitably be influenced by the treatment and supportive care, especially antibiotics), and then the dynamics of the reconstituting immune system *post-transplant*. The microbiome may therefore influence short- and long-term immune recovery and clinical outcomes following autologous HSCT. Therefore, future

TABLE 2 | Considerations for the analysis of intestinal microbiome in AD undergoing HSCT.

Summary of considerations
• Standardization of the microbiome field is complex.
• Proper documentation of sample collection, data processing, and analysis methods is crucial to be reproducible.
• The choice of method may also vary, depending on the research interests, simplicity of fecal collection procedures and presence of adequate biobanking infrastructure (63, 66).
Optimal time-points for sample collection before and after HSCT:
• pre-mobilization (usually cyclophosphamide and G-CSF)
• pre-transplant conditioning (up to 15 days before starting the conditioning regimen)
• peri-engraftment, i.e. within 7 days following stem cell engraftment
• Serial post-transplant samples at time points where other immune reconstitution samples are taken (e.g. 3 monthly in the first year, and yearly thereafter, in remission or with stable disease),
• in the event of relapse and/or progression
Collection and storage of fecal samples:
• Freshly isolated fecal samples, instantly frozen at -80°C without additives (16S rRNA, flow cytometry), widely regarded as the gold standard (69).
• Samples can be also preserved at –20°C within 15 min after collection, then transferred to a laboratory on dry ice within 24 h of collection and stored at –80°C thereafter (70).
• Sample collection in tubes containing a DNA stabilizer (e.g. OMNIgene GUT tubes or Stratec stool collection tubes) or 95% ethanol, which allows sample storage at room temperature (16S rRNA) (63, 66).
Methods of detection:
• 16S rRNA sequencing
• Shot-gun metagenomics sequencing
• Metabolic profiling
• Flow cytometric analysis
The selection of sequencing methods depends on the scientific questions and sample types:
• Amplicon sequencing: taxonomic composition of microbiota, cost effective, feasible for large-scale research.
• Shot-gut Metagenomic sequencing: more information, more expensive than amplicon sequencing.
• The integration of different methods is advisable, as multi-omics provides insights into both the taxonomy and function of the microbiome (71).
Bioinformatics analysis:
Several popular software or pipelines are available for data analysis; QIIME and USEARCH are the most largely adopted (71).

AD, autoimmune diseases; HSCT, Hematopoietic Stem Cell Transplantation; G-CSF, granulocyte colony-stimulating factor; FACS, Fluorescence-activated cell sorting.

investigations evaluating microbiome changes pre-, peri- and post-HSCT in ADs patients are warranted. **Table 2** includes proposed recommendations for studies of the microbiome (111–115) that could be compared with clinical outcome and laboratory data related to immune reconstitution in patients undergoing HSCT for various ADs. Although bio-banking and testing cannot be regarded as routine care, they could be integrated into clinical trials or observational studies with appropriate institutional approvals. In future, a greater understanding may help design of prospective studies of interventions, including FMT, to test the proof of principle of modulation of the microbiome in this setting.

In conclusion, we have summarized the current evidence supporting the relationship between the microbiome, HSCT and ADs, and speculated on the potential impact of the microbiome on clinical outcomes and immune reconstitution following HSCT for severe, resistant ADs. The evidence in this specific field is currently very limited, warranting harmonization of the microbiome monitoring and prospective studies to evaluate properly any potential impact and/or clinical benefit.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

TA, RG, and JS led on concept and design. TA and RG led on coordination and data analysis, provided expert and analytical feedback and were involved in reviewing, writing and editing the manuscript. All authors contributed to the analysis and interpretation of data, and writing sections of the manuscript. The experts on this panel are active members of the EBMT. All co-authors were involved in drafting the paper, revising it critically, and approval of the submitted and final versions.

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Comparable Outcomes and Health-Related Quality of Life for Severe Aplastic Anemia: Haploidentical Combined With a Single Cord Blood Unit *vs* Matched Related Transplants

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We retrospectively compared the outcomes and health-related quality of life (HRQoL) of severe aplastic anemia (SAA) patients who received haploidentical hematopoietic stem cell transplantation with a single unrelated cord blood unit (Haplo-cord HSCT) (n = 180) or matched related donor (MRD)-HSCT (n = 128). After propensity score matching, we were able to match 88 patients in each group and to compare the outcomes between the two matched-pair groups. Haplo-cord recipients exhibited a longer median days for neutrophil engraftment (12 *vs* 11, $P = 0.001$) and for platelet engraftment (15 *vs* 13, $P = 0.003$). Haplo-cord recipients a high cumulative incidence of grades II–IV acute graft-versus-host disease (GVHD) (29.8 *vs* 14.0%, $P = 0.006$), while similar III–IV acute GVHD, total chronic GVHD, and moderate to severe chronic GVHD at four-year (all $P < 0.05$). Among the Haplo-cord HSCT and MRD-HSCT groups, the four-year GVHD-free/failure-free survival rates were 73.5% and 66.9% ($P = 0.388$) respectively, and the overall survival rates were 81.5% and 77.2% ($P = 0.484$), respectively. Similar comparable results also were observed between the corresponding first-line, older or younger than 40 years old subgroups. The Haplo-cord HSCT group exhibited higher scores in the physical component summary, physical functioning, general health and social functioning than the MRD-HSCT group (all $P < 0.05$). In the multivariate analysis, young age and Haplo-cord HSCT were favorable factors for HRQoL, while moderate to severe cGVHD was

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associated with lower HRQoL. These results suggest that for SAA patients, Haplo-cord HSCT could achieve at least comparable efficacy and HRQoL to MRD-HSCT.

Keywords: severe aplastic anemia, transplantation, haploidentical donor, matched related donor, unrelated cord blood, health-related quality of life

INTRODUCTION

Acquired severe aplastic anemia (SAA) is a kind of bone marrow (BM) failure syndromes mainly caused by immune destruction of hemopoietic stem and progenitor cells (1, 2). For SAA, including very SAA (vSAA), once diagnosed, individuals require effective treatment as soon as possible. Otherwise, it may be life-threatening due to severe bleeding and infection. The choice of treatment for SAA is determined based on patient age, donor availability, and access to therapeutic resources. According to published guidelines, human leukocyte antigen (HLA)-matched related donor (MRD) transplantation is the preferred option for young SAA patients. However, immunosuppressive therapy (IST) using antithymocyte globulin (ATG) and cyclosporine A (CsA) is indicated for young patients who do not have a MRD and patients older than 40 years. Haploidentical-HSCT (Haplo-HSCT) has been regarded as a salvage therapy when patients fail to respond to IST (3, 4). With continued progress in transplantation techniques, the age limit of allo-HSCT in SAA patients has been cautiously expanded to 50 years of age or older (5, 6). More importantly, Haplo-HSCT has achieved survival rates comparable to MRD-HSCT for SAA patients (7). Based on these observations, the latest guideline from the Chinese Society of Hematology recommends Haplo-HSCT as the front-line treatment for SAA patients without a HLA-matched donor (8).

To further improve the efficacy of Haplo-HSCT, some experienced transplant centers have been exploring some strategies to optimize this transplant strategy, including Haplo-HSCT combined with mesenchymal stem cells (MSCs) or unrelated cord blood (CB) (9–12). Until recently, another study reported encouraging results of sequential transplantation of haploidentical stem cell and unrelated CB on ATG/post-transplantation cyclophosphamide (PTCY) basis in relapsed/refractory hematologic malignancies, possibly by preventing graft-versus-host-disease (GVHD) and anti-leukemia effect (13). Notably, Haplo-HSCT with a single unrelated CB infusion (Haplo-cord HSCT) has exhibited encouraging survival outcomes for SAA patients in our clinical center (12). Meanwhile, health-related quality of life (HRQoL) assessment could help understand the burden of disease, provide direction for future therapy, and evaluate the efficacy of treatment interventions (14, 15). Therefore, HRQoL should be considered an integral component in evaluating the medical outcome of any treatments for SAA patients (16). As we reported before, the first-line Heplo-cord HSCT achieved similar overall survival (OS) and better failure-free survival (FFS) and HRQoL with the first-line IST for SAA patients (17). However, no direct comparison was performed including the HRQoL in SAA patients treated with Haplo-cord HSCT and MRD-HSCT. Thus, we performed this retrospective multicenter study to comprehensively compare the efficacy and the HRQoL between Haplo-cord HSCT and MRD-HSCT for SAA patients.

PATIENTS AND METHODS

Patients

Between August 2003 and November 2019, 308 consecutive acquired SAA patients who underwent Haplo-cord HSCT (n = 180), or MRD-HSCT (n = 128) were enrolled in this study. Among the Haplo-cord HSCT group, previously reported 146 patients were also included (12). Inclusion criteria were as follows, (1) diagnosis of SAA (including vSAA) as defined by Camitta's criteria (18), (2) transfusion was required, and (3) the presence of a relatively intact performance status and no apparent functional damage of internal organs (heart, liver, lung, and kidney) before transplantation. MRD-HSCT was the preferred choice for SAA patients, particularly those younger ones. Patients voluntarily participated in Haplo-HSCT in combination with unrelated UB infusion with the following circumstances, (a) without a matched related or unrelated donor, (b) refused to accept the first or second IST, (c) at least with one suitable haplo-identical donor (HID). The other exclusion criteria were as follows, patients with congenital bone marrow failure syndromes (Fanconi anemia, Diamond-Blackfan anemia, congenital dyskeratosis, and so on), patients who tested positive for myelodysplastic syndrome based on BM analyses, or diagnosed with other immunological diseases. Cytogenetic analyses of the BM and flow cytometry test of paroxysmal nocturnal hemoglobinuria (PNH) clone were routinely performed for all patients.

This study was carried out in accordance with the Declaration of Helsinki and approved by the participating hospitals' Ethics Committees. All enrolled patients signed a written informed consent form prior to participation.

Donor Selection

The HLA-A, -B, -C, DRB1, and -DQB1 typing of the recipients and donors, and the HLA-A, -B, and DRB1 typing of the unrelated CB units were performed. Donors were selected based on the HLA match, age, gender, health condition, and willingness to donate stem cells. Additional aspects concerning donor selection and the unrelated CB units were consistent with our previous report (12).

Conditioning Regimen

The transplant days were numbered sequentially. The specific days preceding the transplant were indicated by a minus sign (−), such that the first day of the stem cell infusion was numbered

"day 01," the second day of infusion was "day 02". The specific days after the last stem cell infusion were indicated by a plus sign (+). Patients in the Haplo-cord HSCT group were treated with a busulfan (BU)/cyclophosphamide (CY)-based regimen that included the following drugs. BU, 0.8 mg/kg intravenous (i.v.) was given four times daily on days −7 and −6. Cy, 50 mg/kg i.v., was given once daily from days −5 to −2, and ATG (rabbit, Thymoglobuline®, Genzyme, Cambridge, MA, USA), 2.5 mg/kg i.v., was given once daily from days −5 to −2. In the MRD-HSCT group, patients were given fludarabine (Flu) + CY + ATG regimen, which included Flu 30 mg/m^2/day i.v. given for six days (days −7 to −2), Cy 50 mg/kg/day i.v. for two days (days −4 and −3), and ATG 2.5 mg/kg/day i.v., given for five days (days −8 to −4).

Graft Collection and Infusion

From day −4 to the last day of stem cell collection, the hematopoietic stem cells from HIDs and MRDs were mobilized by subcutaneous injection of recombinant human granulocyte colony-stimulating factor (rhG-CSF) at a dose of 10 μg/kg/day. BM grafts from the MRDs were collected on day 01 *via* BM aspiration in the surgery room. The target mononuclear cells (MNCs) count from the BM was 6–8 × 10^8/kg of recipient weight. If the target MNCs count was not achieved, peripheral blood stem cells (PBSCs) were collected the following day by apheresis using a COBE Spectra device (Gambro BCT, Lakewood, CO, USA). BM grafts from the HIDs were harvested on day 01 to attain a target MNCs count of 2–4 × 10^8/kg of recipient weight, and PBSCs were collected the following day. The grafts from BM and peripheral blood (PB) were expected to provide the target MNCs count of 6–8 × 10^8/kg of recipient weight. If the target count of cells was insufficient, additional PBSCs were collected on the following 1 to 2 days. Fresh unmanipulated BM and PBSCs were infused into the recipient on the day of collection. A single unrelated CB unit was infused 8 hour before the infusion of the Haploidentical grafts on day 01.

GVHD Prophylaxis and Treatment

In the Haplo-cord HSCT group, CsA, mycophenolate mofetil (MMF), and short-term methotrexate (MTX) were administered for acute graft-versus-host-disease (aGVHD) prophylaxis (19). In the MRD-HSCT group, only CsA was used to prevent GVHD (beginning on day −4). Once GVHD occurred, the procedure of treatment was as described previously (12).

Supportive Care and Post-Transplantation Surveillance

The details concerning supportive care and post-transplantation surveillance were consistent with previous experience (12).

Definitions and Post-Transplantation Evaluations

Neutrophil engraftment was defined as the first day of an absolute neutrophil count (ANC) greater than 0.5 × 10^9/L for three consecutive days. Platelet engraftment was defined as the first day of a platelet count greater than 20 × 10^9/L for seven consecutive days without transfusion support. Primary graft failure (GF) was defined as failure to achieve neutrophil engraftment after HSCT up to + 28 days. Secondary GF was defined as recurrent pancytopenia with an ANC below 0.5 × 10^9/L after a prior history of engraftment (20). Early mortality was defined as death within 60 days after HSCT. Transplantation-related mortality (TRM) was defined as death related to the transplantation and not the relapse of SAA. GVHD-free or failure-free survival (GFFS) was defined as survival without grade III–IV aGVHD, moderate to severe cGVHD, and treatment failures (including death, primary or secondary GF, and relapse) (7, 12). Poor graft function was defined as persistent cytopenia in at least two lineages (platelet < 20 × 10^9/L, neutrophil count < 0.5 × 10^9/L, hemoglobin level < 70 g/L) and/or requiring a transfusion beyond day +28, and full donor chimerism without relapse or severe GVHD (21). The Diagnosis and GVHD grade was based on the established criteria (22, 23). During the follow-up, the recipient's BM was checked monthly for three months and every three to six months for the following one to two years.

HRQoL Evaluation

Patients were excluded who had survived less than one year after transplantation, were less than 14 years old at the time of the questionnaire survey, had experienced relapse or GF, experienced any mental disorder after transplantation, or were unwilling to participate in the quality of life survey. A survey packet was mailed to every patient willing to complete a questionnaire that included a consent form, a set of questionnaires concerning their HRQoL, and a self-addressed stamped envelope. Each respondent was asked to sign the consent form and complete the HRQoL questionnaires (at 18 months after transplantation) before returning these materials to the investigators at their earliest convenience.

The 36-Item Short-Form Health Survey (SF-36) included eight subscales: physical functioning, role-physical functioning, bodily pain, general health, vitality, social functioning, role-emotional functioning, and mental health. Raw scores were transformed into standardized scores on a scale of 0–100. High scores represented high function levels. The subscales were aggregated into two summary measures, physical components and mental components.

Statistical Analysis

Because patient allocation in this study was based on the HLA-identical or HLA-haploidentical group assignments rather than by random assignment, the baseline levels of some clinical characteristics were imbalanced between the two groups. To reduce the influence of potential confounders, propensity score matching (PSM) was performed in this study. The propensity score that indicated the HLA status for each patient was calculated based on a multivariate logistic regression model. In this model, patient and donor age, female donor into male recipient, year of transplant

(from January, 2014), disease status, and graft source between the two groups were used as covariates. Patients in the HLA-identical group were matched to those in the HLA-haploidentical group using 1:1 nearest neighbor matching with a caliper width of 0.2.

After PSM, 88 pairs of patients were created, and outcomes were compared between the two matched-pair groups. For demography, disease and treatment-related factors, and the SF-36 scores, the Mann-Whitney U test and the Pearson chi-squared test were used to compare continuous variables and categorical variables, respectively. Survival analysis was conducted using the Kaplan-Meier method and the log-rank test to compare differences. Engraftment and GVHD were estimated as cumulative incidences, considering early death as a competing event. Multivariate logistic regression and Cox proportional hazard regression analyses were applied to evaluate the contribution of independent factors. $P < 0.05$ was considered statistically significant.

The final date for follow-up for all surviving patients was August 31, 2020. SPSS 22.0 statistical software (IBM, Armonk, NY, USA) was used for statistical analysis.

TABLE 1 | Patient and donor (graft) characteristics between the two groups.

Variables	*Before matching*			*After matching*		
	MRD-HSCT (n = 128)	**Haplo-cord HSCT (n = 180)**	***P***	**MRD-HSCT (n = 88)**	**Haplo-cord HSCT (n = 88)**	***P***
Median age, years (range)	29 (4–56)	24 (3–55)	**< 0.001**	29 (14–52)	29 (14–55)	0.901
Age, no. (%)			**0.004**			0.345
< 40 years	97 (75.8)	159 (88.3)		73 (83.0)	68 (77.3)	
≥ 40 years	31 (24.2)	21 (11.7)		15 (17.0)	20 (22.7)	
Gender (male/female), no.	70/58	105/75	0.524	48/40	48/40	1.000
Disease status (SAA/vSAA), no.	93/35	98/82	**0.001**	28/60	28/60	1.000
With PNH clone, no. (%)	13 (10.2)	21 (11.7)	0.677	10 (11.4)	11 (12.5)	0.816
ECOG score, median (range)	1 (0–2)	1 (0–2)	0.589	1 (0–2)	1 (0–2)	0.701
Previous transfusion						
Median units of RBC (range)	23 (3–36)	22 (2–38)	0.306	24 (4–36)	22 (2–36)	0.267
Median units of PLT (range)	22 (0–120)	18 (2–120)	0.067	22 (2–118)	20 (2–120)	0.098
Median SF, ng/mL (range)	1300 (248–4250)	1680 (180–4550)	0.059	1350 (278–4050)	1610 (180–4352)	0.072
Median time from diagnosis to HSCT, months (range)	3 (0.5–360)	2 (0.5–240)	0.440	5 (1–200)	2 (0.7–240)	0.987
HCT-CI			0.132			0.502
≤ 1	107 (83.6)	161 (89.4)		75 (85.2)	78 (88.6)	
≥ 2	21 (16.4)	18 (10.6)		13 (14.8)	10 (11.4)	
Unfront treatment, no. (%)	116 (90.4)	137 (76.1)	**0.001**	78 (88.6)	69 (78.4)	0.067
Donor median age, years (range)	31 (5–56)	41.5 (8–63)	**< 0.001**	32 (8–56)	31 (11–57)	0.742
Donor-recipient sex match, no. (%)			**0.009**			0.076
Male-male	28 (21.9)	69 (38.3)		20 (22.7)	31 (35.2)	
Male-female	32 (25.0)	41 (22.8)		24 (27.3)	18 (20.5)	
Female-male	42 (32.8)	36 (20.0)		28 (31.8)	17 (19.3)	
Female-female	26 (20.3)	34 (18.9)		16 (18.2)	22 (25.0)	
Donor sex, no. (%)			**0.013**			0.450
Male	60 (46.9)	110 (61.1)		44 (50.0)	49 (55.7)	
Female	68 (53.1)	70 (38.9)		44 (50.0)	39 (44.3)	
Blood types of donor to recipient, no. (%)			0.120			0.639
Matched	79 (62.0)	96 (53.0)		52 (59.1)	45 (51.1)	
Major mismatched	15 (11.6)	38 (21.5)		12 (13.6)	17 (19.3)	
Minor mismatched	24 (18.6)	37 (20.5)		18 (20.5)	21 (23.9)	
Major and minor mismatched	10 (7.8)	9 (5.0)		6 (6.8)	5 (5.7)	
Source of graft, no. (%)			**< 0.001**			0.089
BM	17 (13.3)	17 (9.4)		11 (12.5)	13 (14.8)	
PB	30 (23.4)	9 (5.0)		17 (19.3)	7 (8.0)	
BM + PB	81 (63.3)	154 (85.6)		60 (68.2)	68 (77.3)	
Median BM/PB MNCs, × 10^8/kg (range)	11.0 (2.3–22.4)	11.2 (3.6–33.4)	0.308	10.8 (3.6–31.2)	10.7 (2.6–22.1)	0.301
Median BM/PB $CD34^+$ cells, × 10^6/kg (range)	3.8 (1.0–16.9)	3.6 (0.7–8.9)	0.342	3.7 (0.8–8.6)	3.9 (1.2–16.4)	0.351
Median cord TNCs, × 10^7/kg (range)	–	2.1 (1.1–7.3)	–		2.0 (1.1–3.9)	–
Median cord $CD34^+$ cells, × 10^5/kg (range)	–	0.6 (0.1–2.3)	–		0.5 (0.1–2.3)	–
Median follow-up time, months (range)	51.5 (12.0–220.0)	39.0 (10.0–108.0)	**0.003**	53.0 (12.0–121.0)	48.0 (10.0–103.0)	0.134

Haplo-cord HSCT, haploidentical hematopoietic stem cell transplantation with unrelated cord blood infusion; MRD-HSCT, matched related donor hematopoietic stem cell transplantation; SAA, severe aplastic anemia; vSAA, very SAA; PNH, paroxysmal nocturnal haemoglobinuria; ECOG,eastern cooperative oncology group scale; RBC, red blood cell; PLT, paltelet; SF, serum ferritin; HCT-CI, hematopoietic stem cell transplantation-comorbidity index; BM, bone marrow; PB, peripheral blood; MNCs, mononuclear cells; TNCs, total nucleated cells.

The bold values were statistically significant (P < 0.05).

RESULTS

Patient Characteristics

As shown in **Table 1**, the proportion of vSAA at diagnosis was higher in the Haplo-cord HSCT group than the MRD-HSCT group ($P = 0.001$). The median recipient age was significantly lower in the Haplo-cord HSCT group than the MRD-HSCT group ($P < 0.001$). Similarly, the proportion of patients younger than 40 years in the Haplo-cord HSCT group was higher than the MRD-HSCT group. With respect to donors, the median age was significantly higher in the Haplo-cord HSCT group than the MRD-HSCT group ($P < 0.001$). Also, more male donors were included in the Haplo-cord HSCT group than the MRD-HSCT group ($P = 0.013$), which contributed to the difference in the donor-recipient sex match between the Haplo-cord HSCT and MRD-HSCT groups. The median follow-up time was longer in the MRD-HSCT group than that in the haplo-cord HSCT group ($P = 0.003$). All these variables were balanced between the Haplo-cord HSCT and MRD-HSCT groups after PSM (all $P > 0.05$) (**Table 1**).

The two groups were matched with respect to the ratio of males to females of recipient, the time from diagnosis to transplantation, and other characteristics whether before or after PSM.

Engraftment

85 of 88 patients in the Haplo-cord HSCT group survived more than +28 days. Among the 85 patients, two experienced primary GF, and the remaining 83 patients achieved successful HID engraftment without mixed chimerism of unrelated CB. In the MRD-HSCT group, 83 of 88 patients survived more than +28 days, and the 83 patients achieved successful MRD engraftment. The cumulative incidences of neutrophil engraftment +28 days between the Haplo-cord HSCT and MRD-HSCT were not different (97.7 ± 1.6% *vs* 100.0 ± 0.0%, $P = 0.497$), and the cumulative incidence of platelet engraftment +60 days between them were also similar (96.5 ± 2.0% *vs* 96.2 ± 2.1%, $P = 0.804$) (**Table 2**).

The median time to achieve neutrophil engraftment in the Haplo-cord HSCT and MRD-HSCT groups was 12 days and 11 days, respectively, which was significantly different ($P = 0.001$) (**Table 2**). The median time to achieve platelet engraftment in the Haplo-cord HSCT and MRD-HSCT groups was 15 days and 13 days, respectively, which was significantly different ($P = 0.003$) (**Table 2**). Based on multivariate analysis, Haplo-cord HSCT was the only unfavorable factor that affected the median time to achieve neutrophil and platelet engraftment ($P = 0.004$; $P = 0.001$, respectively) (**Table 4**).

As of the last follow-up, except for two patients with secondary GF, sustained full donor chimerism was observed in the surviving patients after the Haplo-cord HSCT. One patient experienced secondary GF in the MRD-HSCT group, and mixed chimerism was detected in another patient at 9 months after transplantation. However, this patient was restored to full donor chimerism after infusion of MSCs and donor lymphocytes.

GVHD Incidence and Severity

The cumulative incidence of grade II–IV aGVHD within 100 days in the Haplo-cord HSCT group was significantly higher than the MRD-HSCT group (29.8 ± 5.0% *vs* 14.0 ± 3.7%, $P = 0.006$) (**Figure 1A**). However, the cumulative incidence of grade III–IV aGVHD was not different between the Haplo-cord HSCT and the

TABLE 2 | Transplantation-related events between the two groups.

Variables	MRD-HSCT (n = 88)	Haplo-cord HSCT (n = 88)	*P*
Cumulative incidence of neutrophil engraftment +28 days (%)	100 ± 0.0	97.7 ± 1.6	0.497
Cumulative incidence of platelet engraftment +60 days (%)	96.2 ± 2.1	96.5 ± 2.0	0.804
Median days to ANC > 0.5 × 10^9/L (range)	11 (8–23)	12 (9–27)	0.001
Median days to PLT > 20.0 × 10^9/L (range)	13 (8–80)	15 (9–210)	**0.003**
Primary GF, no. (%)	0 (0.0)	2 (2.3)	0.477
Secondary GF, no. (%)	1 (1.1)	2 (2.3)	1.000
Infection, no. (%)	47 (53.4)	48 (54.5)	0.880
Relapse, no. (%)	0 (0.0)	0 (0.0)	1.000
Early death, no. (%)	8 (11.0)	8 (11.0)	1.000
TRM, no. (%)	20 (22.7)	16 (18.2)	0.455
Primary GF, no. (% of TRM)	0 (0.0)	1 (6.3)	0.444
Secondary GF, no. (% of TRM)	0 (0.0)	1 (6.3)	0.444
aGVHD, no. (% of TRM)	1 (5.0)	5 (31.3)	0.069
cGVHD, no. (% of TRM)	1 (5.0)	1 (6.3)	1.000
Infection, no. (% of TRM)	10 (50.0)	4 (25.0)	0.176
TA-TMA, no. (% of TRM)	4 (20.0)	2 (12.5)	0.672
Intracranial hemorrhage, no. (% of TRM)	2 (10.0)	1 (6.3)	1.000
MDS, no. (% of TRM)	0 (0.0)	0 (0.0)	–
PTLD, no. (% of TRM)	1 (5.0)	0 (0.0)	1.000
Poor graft function, no. (% of TRM)	1 (5.0)	1 (6.3)	1.000

Haplo-cord HSCT, haploidentical hematopoietic stem cell transplantation with unrelated cord blood infusion; MRD-HSCT, matched related donor hematopoietic stem cell transplantation; ANC, absolute neutrophil count; PLT, platelet; TRM, transplantation related mortality; aGVHD, acute graft-versus-host-disease, cGVHD, chronic GVHD; GF, graft failure; MDS, myelodysplastic syndrome; TA-TMA, transplantation-associated thrombotic microangiopathies; PTLD, post-transplant lymphoproliferative disease.
The bold values were statistically significant (P < 0.05).

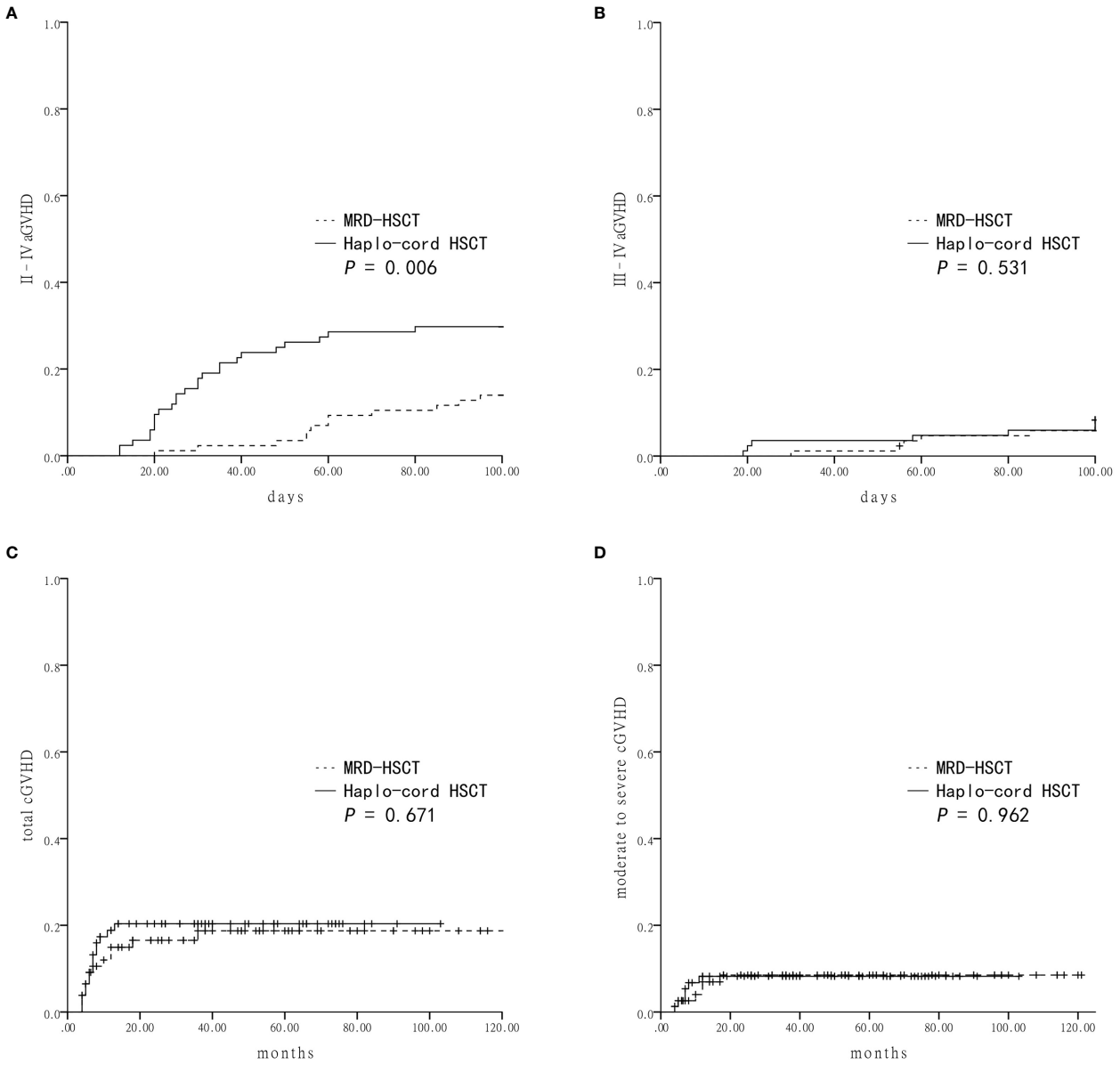

FIGURE 1 | Graft-versus-host-disease (GVHD) after Haplo-cord HSCT or MRD-HSCT **(A)** The cumulative incidence of grade II–IV acute GVHD (aGVHD). **(B)** The cumulative incidence of grade III–IV aGVHD. **(C)** The cumulative incidence of total chronic GVHD (cGVHD). **(D)** The cumulative incidence of moderate to severe cGVHD.

MRD-HSCT groups (8.3 ± 3.0% *vs* 5.9 ± 2.5%, P = 0.531) (**Figure 1B**). Multivariate analysis identified Haplo-cord HSCT as the only unfavorable factor for II–IV aGVHD (P = 0.014) (**Table 4**).

Patients who lived longer than 100 days after transplantation were evaluated for the cumulative incidence of cGVHD. There were no differences in the total cGVHD between the Haplo-cord HSCT and the MRD-HSCT groups (20.4 ± 4.7% *vs* 18.7 ± 4.8%, P = 0.671, **Figure 1C**), and in the moderate to severe cGVHD between them (8.2 ± 3.2% *vs* 8.5 ± 3.3%, P = 0.962, **Figure 1D**). Multivariate analysis identified no significant factor in the total or moderate to severe cGVHD (**Table 4**).

TRM

There was no patient with relapse in our study. The cumulative rate of transplant-related mortality between the Haplo-cord HSCT and the MRD-HSCT groups was not significantly different (18.2% *vs* 22.7%, P = 0.455). In the Haplo-cord HSCT group, four patients (25.0%) died from an infection, and six patients (37.6%) died from GVHD (five from aGVHD and one from cGVHD). In the MRD-HSCT group, ten patients (50.0%) died from infection, and two patients (10.0%) died from GVHD (one from aGVHD and one from cGVHD). Additional details of transplant-related events were shown in **Table 2**.

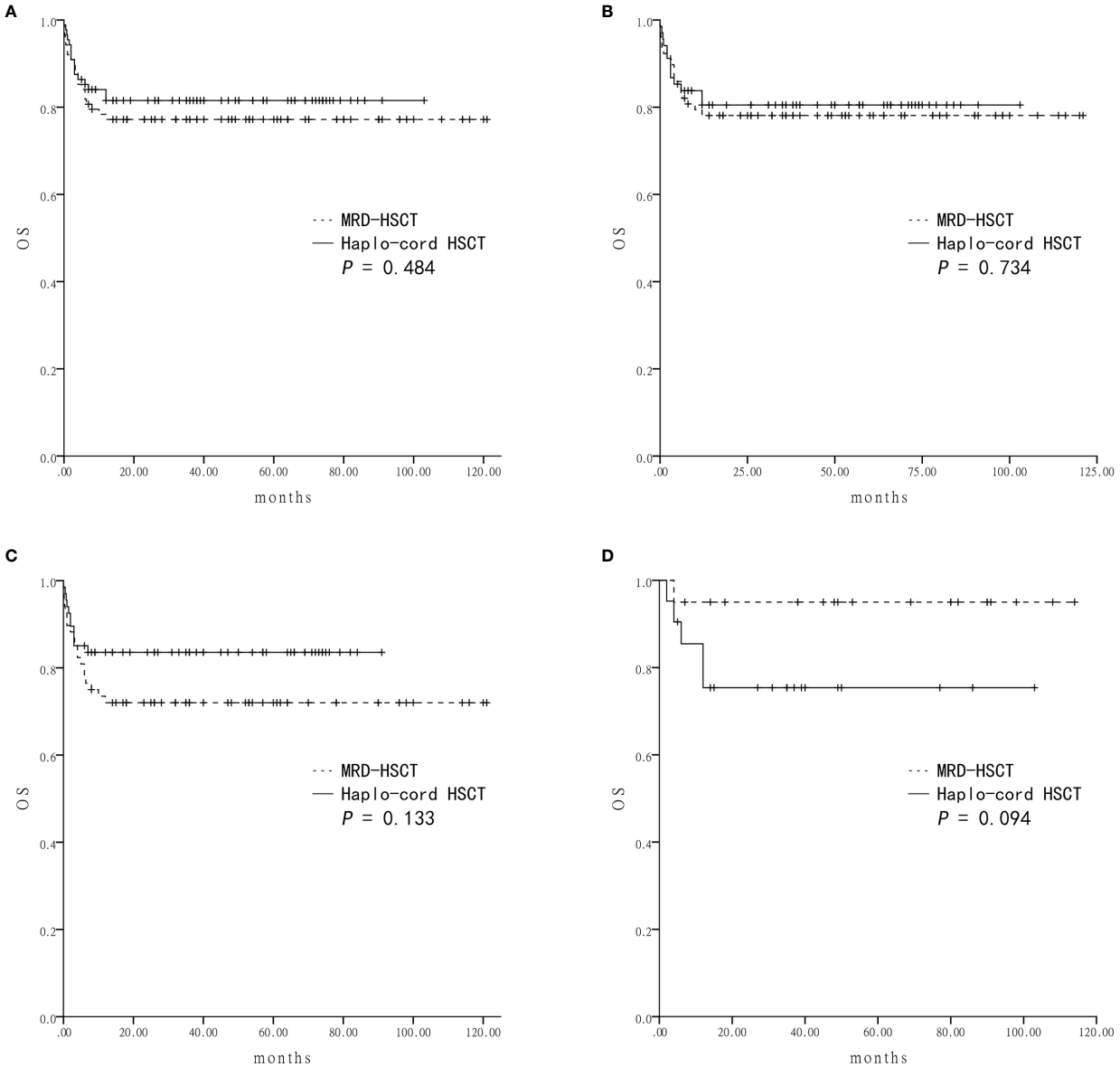

FIGURE 2 | The estimated overall survival (OS) at four-year based on donor source **(A)** The OS was 81.5 ± 4.2% in Haplo-cord HSCT and 77.2 ± 4.5% in MRD-HSCT groups as a whole. **(B)** The OS was 80.5 ± 4.9% in Haplo-cord HSCT and 78.1 ± 4.7% in MRD-HSCT subgroups as the first-line treatment. **(C)** The OS was 83.6 ± 4.5% in Haplo-cord HSCT and 72.0 ± 5.5% in MRD-HSCT subgroups with patients aged < 40 years. **(D)** The OS was 75.4 ± 9.6% in Haplo-cord HSCT and 95.0 ± 4.9% in MRD-HSCT subgroups with patients aged ≥ 40 years.

Survival

Survival was assessed four years after transplantation. The estimated OS was similar between the Haplo-cord HSCT group and the MRD-HSCT group (81.5 ± 4.2% *vs* 77.2 ± 4.5%, $P = 0.484$) (**Figure 2A**). The estimated GFFS was also similar between the two group (73.5 ± 5.0% *vs* 66.9 ± 5.0%, $P = 0.388$) (**Figure 3A**).

Subsequent subgroup analysis showed that for patients receiving HSCT as first-line treatment, the estimated OS rates were similar between the Haplo-cord HSCT group (n = 68) and the MRD-HSCT group (n = 78) (80.5 ± 4.9% *vs* 78.1 ± 4.7%, $P = 0.734$) (**Figure 2B**). The estimated GFFS rates were also similar (62.7 ± 10.7% *vs* 60.9 ± 7.2%, $P = 0.563$) (**Figure 3B**).

Next subgroup comparisons between Haplo-cord HSCT and MRD-HSCT was performed according to the patient age. Among patient less than 40 years of age, OS and GFFS tended to be better in the Haplo-cord HSCT group (n = 67) compared with the MRD-HSCT group (n = 68), although it did not reach statistical significance (OS: 83.6 ± 4.5% *vs* 72.0 ± 5.5%%; $P = 0.133$; and GFFS: 77.5 ± 5.1% *vs* 64.6 ± 5.8%; $P = 0.127$) (**Figures 2C** and **3C**.

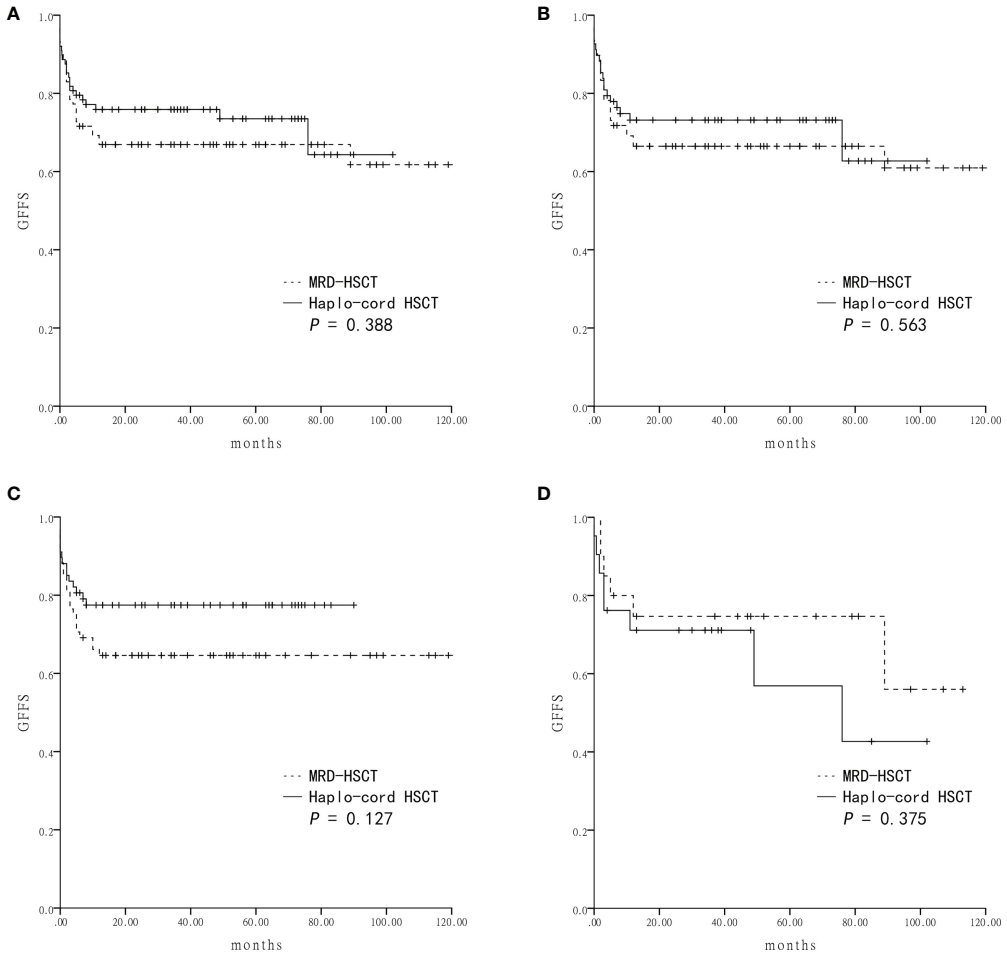

FIGURE 3 | The estimated GVHD-free and failure-free survival (GFFS) at four-years based on the donor source **(A)** The GFFS was 73.5 ± 5.0% in Haplo-cord HSCT and 66.9 ± 5.0% in MRD-HSCT groups as a whole. **(B)** The GFFS was 62.7 ± 10.7% in Haplo-cord HSCT and 60.9 ± 7.2% in MRD-HSCT subgroups as the first-line treatment. **(C)** The GFFS was 77.5 ± 5.1% in Haplo-cord HSCT and 64.6 ± 5.8% in MRD-HSCT subgroups with patients aged < 40 years. **(D)** The GFFS was 42.7 ± 16.6% in Haplo-cord HSCT and 56.0 ± 17.8% in MRD-HSCT subgroups with patients aged ≥ 40 years.

Nevertheless among patient 40 years and older, OS and GFFS tended to be higher in the MRD-HSCT group (n = 20) compared with the Haplo-cord HSCT group (n = 21), although it did not reach statistical significance (OS: 95.0 ± 4.9% *vs* 75.4 ± 9.6%; P = 0.094; and GFFS: 56.0 ± 17.8% *vs* 42.7 ± 16.6. %; P = 0.375) (**Figures 2D** and **3D**), possibly due to the small sample size. Multivariate analysis identified no significant factors that were associated with OS and GFFS (**Table 4**).

SF-36 Scores

The scores were higher for the physical component summary, physical functioning, general health, and social functioning in the Haplo-cord HSCT group than that in the MRD-HSCT group (all $P < 0.05$). No significant differences were observed for bodily pain, role-physical functioning, mental component summary, vitality, role-emotional functioning, and mental health between the two groups (all $P > 0.05$) (**Table 3**).

In the multivariate analysis, moderate to severe cGVHD was one adverse risk factor associated with general health, vitality, and social functioning (all $P < 0.05$) (**Table 4**). Younger patient at transplantation was a favorable factor for role-physical functioning, bodily pain, vitality, social functioning, role-emotional functioning, mental health, physical component summary, and mental component summary (all $P < 0.05$), and the choice of Haplo-cord HSCT was another favorable factor for physical functioning, general health, social functioning, and mental health (all $P < 0.05$) (**Table 4**).

DISCUSSION

This multicenter study was conducted to comprehensively compare the outcomes of large SAA patients cohort underwent Haplo-cord HSCT or MRD-HSCT. First of all, similar rate of

TABLE 3 | HRQoL measures of the survivors between the two groups.

SF-36 scores (IQR)	MRD-HSCT(n = 45)	Haplo-cord HSCT(n = 49)	*P*
Physical			
Physical component summary	79.3 (69.3–83.8)	84.3 (76.1–91.7)	**0.002**
Physical functioning	90.0 (80.0–95.0)	95.0 (90.0–95.0)	**0.001**
Role-physical functioning	75.0 (50.0–75.0)	75.0 (50.0–100.0)	0.096
Bodily pain	100.0 (100.0–100.0)	100.0 (95.5–100.0)	0.170
General health	57.0 (42.0–67.5)	67.0 (62.0–72.0)	**< 0.001**
Psychological			
Mental component summary	90.1 (84.4–92.4)	90.0 (84.9–96.4)	0.233
Vitality	85.0 (75.0–90.0)	85.0 (77.5–90.0)	0.651
Social functioning	87.5 (75.0–87.5)	100.0 (87.5–100.0)	**< 0.001**
Role-emotional functioning	100.0 (100.0–100.0)	100.0 (100.0–100.0)	0.950
Mental health	88.0 (84.0–92.0)	88.0 (82.0–88.0)	0.147

Haplo-cord HSCT, haploidentical hematopoietic stem cell transplantation with unrelated cord blood infusion; MRD-HSCT, matched related donor hematopoietic stem cell transplantation; HRQoL, health-related quality of life; IQR, interquartile range.
The bold values were statistically significant (P < 0.05).

hematopoietic engraftment was observed in the Haplo-cord HSCT and MRD-HSCT groups. Nevertheless, engraftment speed of neutrophil and platelet favored the MRD-HSCT group, which meant that the Haplo-cord HSCT might need more supportive care. These outcomes were similar to another study (24). GVHD was a common complication after engraftment, and it might be directly related to survival and quality of life of the survivors, especially in severe cases. In this study, although higher proportions of grade II–IV aGVHD was observed in the Haplo-cord HSCT group than the MRD-HSCT group, similar cumulative incidences were observed in grade III–IV aGVHD, total cGVHD, and moderate to severe cGVHD. Meanwhile, no differences in the aGVHD and cGVHD-related TRM were found between the two groups. The multivariate analysis determined that grade II–IV aGVHD was related to Haplo-cord HSCT. The following factors likely contributed to explain the accepted GF and GVHD in the Haplo-cord recipients. First, adequate CD34+ cells were present, including mobilized BM and PB from the HIDs. Second, additional immunosuppressant was administered due to higher incidences of aGVHD in this group.

Next, we compared the survival of SAA patients after Haplo-cord HSCT or MRD-HSCT. In general, the OS and GFFS rates were comparable between the two groups, including between the corresponding Haplo-cord HSCT and MRD-HSCT as a first-line treatment for SAA patients, which was consistent with another study (7). Considering that old age was determined to be a strong negative predictor in SAA patients receiving allo-HSCT (25), we performed subgroup analyses with the age of 40 as the cut-off. Among patients younger than 40 year, at least comparable OS and GFFS rates were observed between Haplo-cord HSCT and MRD-HSCT groups. Therefore, it was feasible to recommend the Haplo-cord HSCT for SAA patients without MRDs, which was consistent with another report (26). Patients older than 40 years had a significantly poorer prognosis in the Haplo-cord HSCT than that in the MRD-HSCT group, nevertheless, the differences were not statistically significant, probably because the number of patients in the over 40 years old subgroup were too small to draw a persuasive conclusion. Despite these limitations, our data supported 2015 edition of the British Guide for SAA, the age limit for HLA-identical HSCT in SAA patients was expanded to 50 years (3). Until recently, Yang et al. reported that the combination of MRD-HSCT with an unrelated CB unit could achieve favorable outcomes in SAA patients aged 35 to 50 years (27). Another study reported comparable survival outcomes of transplantation from HIDs and matched donors for SAA patients aged 40 years and older (28). Of course, it is necessary to perform further studies with a large sample size to confirm these outcomes of Haplo-cord HSCT for SAA patients aged 40 years and older and explore some risk factors.

With high survival rates in SAA patients with transplantation, quality of life concerns are considered equally important by physicians and patients (29). Our results suggested that scores from most subscales for physical health and social functioning subscale for psychological health were higher in SAA patients undergoing the Haplo-cord HSCT than the MRD-HSCT, while the other subscales' scores were similar between the two groups. Although similar comparative results also were reported by Mo et al. (30), our study was the first to make the comparison specifically for SAA and not multiple blood diseases. Recovery of HRQoL after allo-HSCT in most survivors is a complicated process requiring three to five years and is influenced by many factors, including age, sex, transplant type, later complications, time after transplantation, and many others (31, 32). Another study about HRQoL reported that mild and moderate cGVHD was significantly better than severe cGVHD, and patients with moderate cGVHD without multiple organ involvement and more severe organ impairment were better off than patients who experienced these conditions (33). In our multivariate analysis, moderate to severe cGVHD was a negative factor that affected most physical and psychological HRQoL of the survivors. Fortunately, the incidence of moderate to severe cGVHD was similar between the Haplo-cord HSCT and the MRD-HSCT groups. In accordance with other studies (31, 34), we also observed that a younger age was associated with a higher score for physical and psychological HRQoL. Because the median recipients' age after PSM at the time of transplantation was not different between the Haplo-cord HSCT and the MRD-HSCT groups, illustrating the effect of age on the difference in HRQoL between the two groups

TABLE 4 | Multivariate analysis of favorable factors associated with outcomes.

Outcomes	OR/HR (95% CI)	*P*
Median days to ANC > 0.5 × 10^9/L		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.758 (0.607–0.892)	**0.004**
Median days to PLT > 20.0 × 10^9/L		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.524 (0.324–0.958)	**0.001**
Grade II–IV aGVHD		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	2.613 (1.212–5.636)	**0.014**
Grade III–IV aGVHD		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.919 (0.378–2.234)	0.853
Total cGVHD		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	1.210 (0.533–2.747)	0.649
Moderate-severe cGVHD		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.974 (0.519–1.829)	0.936
Overall survival		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.792 (0.410–1.528)	0.487
GVHD-free/failure-free survival		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.790 (0.457–1.363)	0.397
Physical functioning		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	3.436 (1.402–8.422)	**0.007**
Patient age	0.977 (0.934–1.023)	0.329
Moderate to severe cGVHD	0.963 (0.390–2.378)	0.936
Role-physical functioning		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	1.373 (0.560–3.370)	0.489
Patient age	0.944 (0.900–0.990)	**0.018**
Moderate to severe cGVHD	1.615 (0.655–3.981)	0.298
Bodily pain		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.447 (0.128–1.562)	0.207
Patient age	0.896 (0.835–0.962)	**0.002**
Moderate to severe cGVHD	0.579 (0.164–2.050)	0.397
General health		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	5.760 (2.223–14.921)	**0.001**
Patient age	0.974 (0.928–1.021)	0.275
Moderate to severe cGVHD	2.593 (1.005–6.688)	**0.047**
Vitality		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	1.789 (0.703–4.555)	0.222
Patient age	0.945 (0.899–0.992)	**0.023**
Moderate to severe cGVHD	2.516 (0.987–6.412)	**0.043**
Social functioning		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	7.250 (2.544–20.666)	**0.001**
Patient age	0.923 (0.875–0.974)	**0.003**
Moderate to severe cGVHD	3.697 (1.316–10.385)	**0.013**
Role-emotional functioning		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.823 (0.251–2.701)	0.748
Patient age	0.925 (0.867–0.988)	**0.019**
Moderate to severe cGVHD	1.065 (0.323–3.511)	0.918
Mental health		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	0.353 (0.144–0.869)	**0.023**
Patient age	0.949 (0.905–0.995)	**0.029**
Moderate to severe cGVHD	1.960 (0.804–4.779)	0.139
Physical component summary		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	1.950 (0.783–4.857)	0.152
Patient age	0.929 (0.885–0.976)	**0.003**
Moderate to severe cGVHD	1.959 (0.784–4.896)	0.150
Mental component summary		
Group (**Haplo-cord HSCT** *vs* MRD-HSCT)	1.430 (0.548–3.730)	0.465
Patient age	0.925 (0.878–0.975)	**0.003**
Moderate to severe cGVHD	2.001 (0.764–5.242)	0.158

OR, odds ratio; HR, hazard risk; CI, confidence interva; vs, versus; Haplo-cord HSCT, haploidentical hematopoietic stem cell transplantation with unrelated cord blood infusion; ANC, absolute neutrophil count; PLT, platelet; aGVHD, acute graft-versus-host-disease; cGVHD, chronic graft-versus-host-disease.
The bold values were statistically significant (P < 0.05).

was limited in my study. In this case, Haplo-cord HSCT, a favorable factor of part subscales, can make a great contribution to a comparable to better physiological quality of life in the Haplo-cord HSCT group than the MRD-HSCT group. Therefore, long-term SAA survivors receiving Haplo-cord HSCT can attain desirable HRQOL comparable to better than that of patients receiving MRD-HSCT.

One limitation in this study is that some survivors did not reply to our invitation. A response rate greater than 70% is low but is not unreasonable for these cross-sectional studies. This low response rate may be related to our failure to offer a reward and to design a face-to-face questionnaire. Another disadvantage of this study is that retrospective HRQoL scores suffered from recall bias, and this phenomenon may be overcome by performing further perspective studies.

In summary, Haplo-cord HSCT for SAA patients exhibited several interesting results compared to the MRD-HSCT, (1), relatively slower engraftments of the neutrophil and platelet, (2), higher incidences of aGVHD, while similar moderate to severe cGVHD, (3), similar OS and GFFS between the whole group and the corresponding subgroups, and (4), comparable to better HRQoL. These outcomes supported the recommendation that Haplo-cord HSCT should be considered an effective alternative option for SAA patients who lack a MRD. However, this result should be supported further by a well-designed, prospective study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

M-QL, X-LL, and Y-MZ wrote the manuscript and performed the analysis. D-PW, MM, and L-ML designed the protocol. All authors contributed patients, provided clinical and laboratory data, and revised and corrected the manuscript.

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