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Immunology of allogeneic hematopoietic stem cell transplantation (HSCT)

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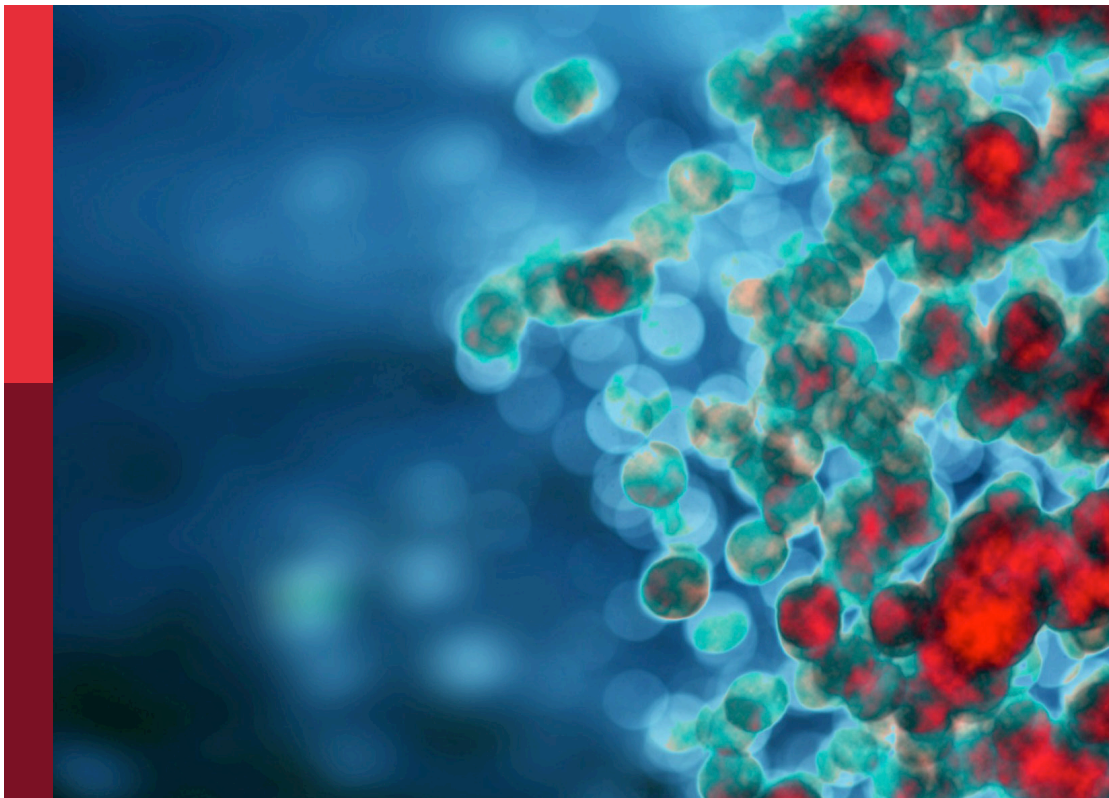

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Immunology of allogeneic hematopoietic stem cell transplantation (HSCT)

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Editorial: Immunology of allogeneic hematopoietic stem cell transplantation

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KEYWORDS

immunology, allogeneic hematopoietic cell transplant, CAR (chimeric antigen receptor) t cells, GvHD (graft-vs.-host disease), immunogenomics

Editorial on the Research Topic
Immunology of allogeneic hematopoietic stem cell transplantation

Allogeneic Hematopoietic Stem Cell Transplantation (HSCT) remains the only curative treatment for several high-risk hematologic malignancies (1). The role of the immune response in allogeneic HSCT involves both the eradication of the disease via the graft-vs.-leukemia (GvL) effect and the development of some of the major complications of the transplant procedure, such as graft rejection, graft-vs.-host disease (GvHD), and infections. Recently, there has been an increased interest in the field of cancer immunology and immunotherapy, which has also been reflected in the HSCT field. Numerous studies have focused on the understanding of the immune biology of HSCT in order to reduce adverse effects and enhance the anti-cancer efficacy. Moreover, innovative immunotherapeutic approaches such as bispecific antibodies, checkpoint inhibitors, and chimeric antigen receptor (CAR) T-cells are increasingly being combined with allogeneic HSCT to improve its therapeutic efficacy (2).

The articles published in the present Research Topic provide a glimpse of some of the critical aspects of immune biology of allogeneic HSCT and their implications in translational practice. These contributions range from retrospective cohort studies to exemplificative case reports that offer insights into managing peculiar and complex clinical scenarios.

Acute GvHD is one of the major toxicities of allogeneic HSCT. Research in this area is focused on the validation of reliable biomarkers for risk-adapted therapy (3). New therapeutic strategies aiming to avoid broad immunosuppression are under investigation (4). Sun et al. described the role of monocytes as potential biomarker for the prevention and treatment of acute GvHD, through a comparative analysis of single-cell RNA sequencing data on peripheral blood of patients with and without this complication. Monocytes showed a marked increase and activation on day 21 post-transplantation, before the onset of GvHD, which aligned with clinical cohort results obtained from routine blood examinations. Moreover, these monocytes were able to induce a significantly higher proliferation rate of T cells. Ideally, such an early GvHD

biomarker could be useful to guide clinical management of GvHD. Another subset of immune cells, namely gamma delta (γδ) T cells, though a minor fraction of the human T cell repertoire, play a crucial role in anti-infectious and anti-tumor responses in allogeneic HSCT. In a prospective study of 49 pediatric allogeneic HSCT recipients, Müller et al. identified a protective role for γδ T cells, particularly the Vδ2 + subset, against acute GvHD and Epstein–Barr virus (EBV) infection. Multivariate analyses confirmed these findings, supporting further exploration of γδ T cells as prognostic markers and potential targets for adoptive T cell transfer strategies after HSCT.

With regard to GvHD prediction from an immunogenomic standpoint, a new metric to gauge the immunopeptidome diversity, called HLA evolutionary divergence (HED), previously explored in a variety of hematological conditions (5–7), is studied in acute lymphoblastic leukemia (ALL) patients undergoing haploidentical HSCT by Cao et al.. Both class I and II HED metrics were calculated in 225 patients with ALL. While no differences were found in terms of survival outcomes, multivariate analysis indicated that a high class II HED for donor-recipient was an independent risk factor for the development of severe acute GvHD ($P = 0.007$), and that recipients with high class I HED had a more than two-fold reduced risk of relapse ($P = 0.028$).

Three studies explore the potential benefits of using umbilical cord blood (UCB) products. Niu et al. reported the outcomes of adult and pediatric patients with severe steroid-refractory acute GvHD who were treated with intravenous infusions of human umbilical cord-derived mesenchymal stromal cells (UC-MSCs). The overall response rate at day 28 was 52.3%, without serious adverse events. Poor outcomes were observed for patients with acute lower gastrointestinal and liver GvHD.

Zeng et al. described how UCB regulatory T cells (Tregs), which play a key role in immune balance, work in synergy with Ruxolitinib in GvHD treatment. This combination effectively alleviates GvHD while preserving the beneficial GvL effect, as demonstrated in xenogeneic preclinical models. Graft failure (GF) and poor graft function (PGF) are potential complications in allogeneic HSCT, particularly in recipients with donor specific antibodies (DSA). UCBs, known for their high stem cell content and low immunogenicity, have been shown to promote immune tolerance when co-infused in haploidentical HSCT. In a retrospective, single-center, controlled study, Wang et al. demonstrated that co-infusion of third-party UCBs in patients with HLA antibodies improved engraftment and reduced the incidence of chronic GvHD.

Three case reports provide valuable insights into the management of challenging clinical scenarios. Zhu et al. described an unusual case of isolated spinal cord involvement with B-cell lymphoid proliferation 11 months post-HSCT, followed six months later by EBV-positive NK/T-cell lymphoma with subcutaneous involvement. This case underscores the importance of maintaining a high suspicion for post-transplant lymphoid proliferations in the context of neurological complications after HSCT and highlights the need for early diagnosis to manage this potentially life-threatening condition. Liu et al. reported a case of relapsed/refractory ALK + anaplastic large cell lymphoma successfully treated with crizotinib and brentuximab vedotin as bridging therapy, followed by autologous HSCT and sequential anti-CD30 CAR T-cell therapy. This innovative combination therapy model offers promising guidance for managing this rare subtype of T-cell non-Hodgkin lymphoma and informs future clinical trial strategies. Finally, Miao et al. described a patient with relapsed/refractory acute myeloid leukemia receiving donor-derived CLL-1 CAR T-cell therapy for bridging to allogeneic HSCT after achieving remission, showing the promising efficacy of cellular therapies in the realm of myeloid malignancies.

The studies and case reports presented in this Research Topic underscore the dynamic interplay between immune biology and clinical practice in allogeneic HSCT. Advances in biomarker discovery, cellular therapies, and immunogenomics are shaping personalized strategies to reduce complications, enhance the quality of life, and improve outcomes. Moreover, the integration of innovative immunotherapies highlights the potential to extend curative options to even the most challenging cases. Moving forward, collaborative research is essential to optimize the therapeutic potential of allogeneic HSCT while addressing its limitations. While the studies in this Research Topic provide valuable insights, they also point to the need for prospective trials to further validate findings and refine treatment strategies. By bridging translational science and clinical application, the field is poised to offer transformative solutions for patients with high-risk hematologic malignancies.

Author contributions

CG: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. RD: Writing – original draft, Writing – review & editing. RG: Writing – original draft, Writing – review & editing. SP: Writing – original draft, Writing – review & editing.

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.

Publisher's note

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Case report: The utilization of crizotinib and brentuximab vedotin as a bridge to autologous stem cell transplantation and followed by CD30-directed CAR-T cell therapy in relapsed/refractory ALK+ ALCL

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Background: Anaplastic lymphoma kinase-positive anaplastic large cell lymphoma (ALK+ ALCL) is a rare, mature T-cell non-Hodgkin lymphoma. The prognosis of patients with relapsed or refractory ALCL following first-line chemotherapy is extremely poor. NCCN guidelines recommend intensified chemotherapy with or without ASCT consolidation for r/r ALCL, however, this is not an effective treatment for all ALK+ALCL.

Case report: Herein, we report a patient with relapsed/refractory ALK+ ALCL who received crizotinib and brentuximab vedotin as bridging therapy, followed by autologous stem cell transplantation and sequential anti-CD30 CAR T cell therapy.

Conclusion: The patient achieved complete remission and long-term disease-free survival of months and continues to be followed up. The combination therapy model in this case may provide guidance for the management of relapsed/refractory ALK+ ALCL, and further prospective trials are needed to confirm its effectiveness.

KEYWORDS

anaplastic large cell lymphoma, T-cell non-Hodgkin lymphoma, crizotinib, CAR T cell therapy, autologous stem cell transplantation

1 Introduction

Anaplastic lymphoma kinase-positive anaplastic large cell lymphoma (ALK+ ALCL) is a rare aggressive systemic T-cell non-Hodgkin's lymphoma (NHL), contributing approximately 6–7% of mature T-cell lymphomas. In 2016, the World Health Organization (WHO) classified anaplastic large cell lymphoma into four categories: ALK+ ALCL, ALK-negative ALCL (ALK– ALCL), primary cutaneous ALCL, and breast-implant-associated ALCL (BIA-ALCL). ALK+ ALCL is more common in children and young adults, with a male predominance, and is characterized by overexpression of the ALK protein because of ALK gene translocation. Most patients with systemic ALCL present with advanced stage III or IV, which is frequently associated with systemic symptoms and extranodal involvement. The systemic symptoms in patients with ALK+ ALCL include weight loss, fever, weakness, fatigue, and night sweats. Common extranodal involvement includes involvement of the skin, bone, soft tissues, lungs, and liver (1).

Compared to other peripheral T-Cell lymphoma (PTCL) subtypes, ALK+ ALCL has a significantly better prognosis after first-line treatment. Following initial treatment, patients with ALK+ ALCL demonstrate relatively favorable outcomes, with complete remission (CR) rates of up to 86% and 8-year overall survival (OS) rates of 82% in a long-term follow-up study (2). However, relapsed and refractory ALK+ ALCL is associated with a relatively poor prognosis, and established standards for the management of relapsed or refractory disease are lacking. Currently, stem cell transplantation, targeted therapy, and immunotherapy with ALK inhibitors, brentuximab vedotin (BV), histone deacetylase (HDAC) inhibitors, and programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) inhibitors are some of the most widely considered options for the treatment of relapsed or refractory ALK+ ALCL.

To date, consensus regarding the roles of autologous stem cell transplantation (ASCT) and CAR-T cell therapy in patients with relapsed/refractory ALK+ ALCL has not been achieved. In this study, we present the case of a patient who failed multiple lines of therapy and benefited from ASCT and CAR-T cell treatment. Our study provides insights into therapeutic strategies for such patients.

2 Case report

A 19-year-old man presented to our otolaryngology department in August 2017 with a table tennis-sized lump in his left chest wall and right axilla and pain in right rib that lasted for 1 month. The patient's history was free of relevant diseases. The patient's family history and genetic history are free of genetically related diseases and free of hematologic disorders such as lymphoma. Chest computed tomography (CT) showed enlargement of the right axillary lymph nodes, indicating neoplastic lesions and destruction of the right eighth rib bone. The diagnosis of ALCL was confirmed using a right axillary lymph node biopsy and immunohistochemical analysis (Figure 1). Immunohistochemical staining revealed that the tumor cells were positive for ALK and CD30. The Ki-67 proliferation index was 90%. The patient was diagnosed with stage IV ALCL and had an international prognostic index (PI) score of 3. The patient received induction chemotherapy, including 8 courses of cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisolone (CHOEP). The patient underwent clinical evaluation after completing 8 cycles of chemotherapy. Positron emission tomography–computed tomography (PET–CT) revealed bilateral neck and axillary lymph node enlargement and increased metabolism. Subsequently, the patient underwent neck and axillary sensitization radiotherapy (Dt 3960cGy/22F). Three months later, a substantial subcutaneous mass measuring approximately 1 cm was palpated in the left lower abdomen. The patient was diagnosed with relapsed/refractory ALCL after an abdominal mass biopsy (Figure 1). PET–CT (August 29, 2019) demonstrated multiple mediastinal, abdominal, and retroperitoneal lymph node enlargement that were partially fused. The largest one was approximately 4.9 ×3.3 cm, and the SUVmax was 19.6. Right pleural thickening, a slightly low-density shadow in the left kidney, multiple muscles, and subcutaneously increased metabolism were also observed. These new changes were considered lymphoma infiltration. The bilateral cervical and axillary lymph nodes increased in size, and metabolism was enhanced. Increased local metabolism of the stomach, small intestine, and transverse colon was also observed. One cycle of dexamethasone, high-dose cytosine arabinoside, cisplatin (DHAP) chemotherapy was initiated on September 4, 2019. The patient presented with abdominal pain on September 23, 2019. Abdominal imaging revealed a significant increase and enlargement of the peripancreatic, retroperitoneal, and mesenteric root lymph nodes, indicating tumor progression. New flaky low-density shadows appeared in the left kidney, indicating a potential tumor invasion. Imaging assessment of the retroperitoneal mass showed that the stable disease (SD) developed into progressive disease (PD). The patient began taking crizotinib (250 mg twice daily) on October 1, 2019, for 1 month. BV at a dose of 100 mg was administered to the patient on October 29, 2019. On the day of BV infusion, the patient underwent an abdominal CT examination. Compared with the previous abdominal images (September 25, 2019), the number of enlarged retroperitoneal and mesenteric lymph nodes was significantly reduced. The range of patchy, low-density lesions in the left kidney also decreased significantly. Abdominal imaging findings were evaluated as partial remission (PR). The patient learned of the clinical trial of tandem ASCT and CAR30 T cell infusion in r/r CD30 + lymphoma being conducted at our hospital, to further enhance the curative effect, he volunteered to participate in the clinical trial. The trial was approved by the Institutional Review Board of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and the study was registered with the Chinese Clinical Trial Registry (ChiCTR, number ChiCTR2100053662). Informed consent was obtained by the patient and her family according to the Declaration of Helsinki. On December 21, doxorubicin hydrochloride liposome with the BEAM regimen pretreatment was performed. Autologous hematopoietic stem cells (4.94×10^6/kg CD34+ cells) were infused on December 28, and CD30 (24.00×10^6/kg) CAR-T cells were infused three times. The three infusion doses of CD30 CAR-T were 5×10^6/kg, 5×10^6/kg and 1.4×10^7/kg

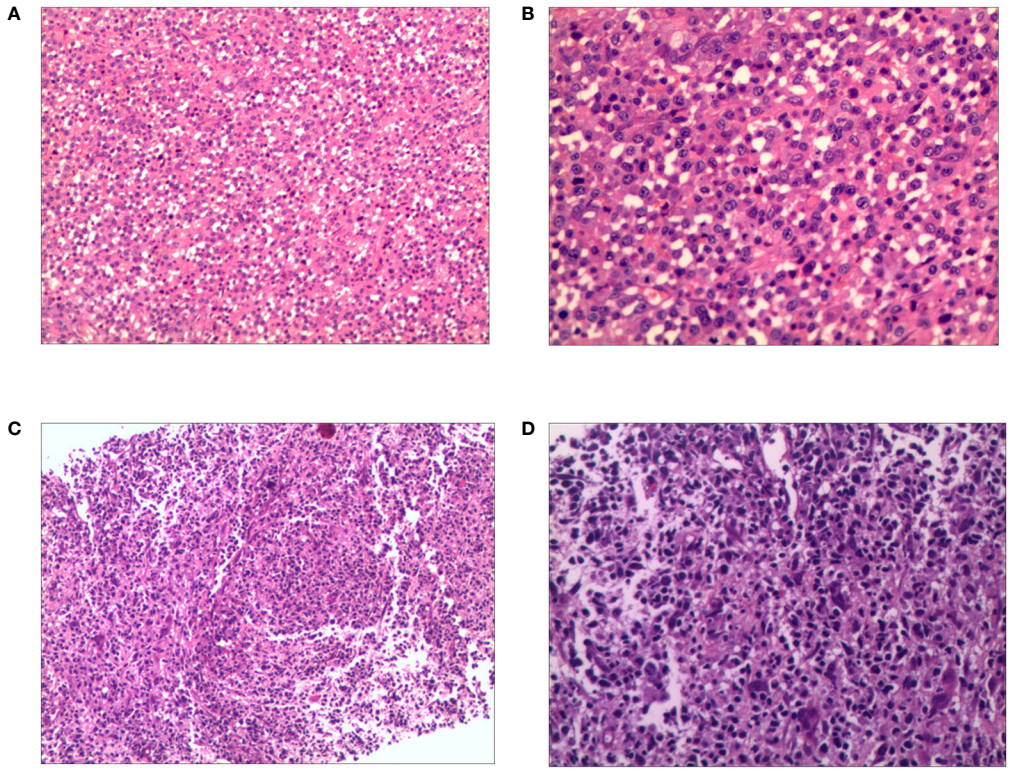

FIGURE 1
Hematoxylin and eosin (H&E) of relapsed/refractory anaplastic large cell lymphoma. The first biopsy: **(A)** Pathological HE image of the patient's right axillary lymph node (100×). **(B)** Pathological HE image of the patient's right axillary lymph node (200×). The second biopsy: **(C)** Pathological HE image of the patient's abdomen mass (100×). **(D)** Pathological HE image of the patient's abdomen mass (200×).

respectively (January 1, 2, and 3, 2020). The structure is as shown in Figure 2A. CAR30 transgene copy numbers in the peripheral blood was detected by droplet digital polymerase chain reaction (ddPCR) (Figure 2B). After infusion of anti-CD30 CAR T-cells, grade 1 cytokine release syndrome (CRS) was observed, and immune effector cell-associated neurotoxicity syndrome (ICANS) was not occur. The serum ferritin and interleukin-6 levels were assessed after cell infusion (Figure 2). The engraftment times of neutrophil and platelet after hematopoietic stem cells infusion were both 14 days. The results of the PET–CT evaluation 3 months after treatment showed that the retroperitoneal soft tissue focus was significantly reduced or had disappeared (Figure 3). PET–CT assessments at 3, 6, and 12 months after autologous stem-cell transplantation (ASCT) and CAR T-cell therapy showed sustained complete remission (CR). The latest PET–CT showed that the size of the lymph nodes in the left neck IIA region was similar, and metabolism was slightly reduced compared to previous PET–CT images (July 16, 2020). Approximately 4 years after ASCT and CAR T-cell therapy, the patient was disease-free. The timeline of clinical treatment and disease status is shown in Figure 4.

3 Discussion

It's been a long time the recommended first-line treatment options for patients with ALCL are mostly anthracycline-based cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP) or CHOP-like regimens. CHOEP is more suitable for patients aged <60 years. In a previous study, among younger patients with ALK+ ALCL (n = 78) with normal LDH levels at the time of diagnosis, adding etoposide to CHOP (-like) regimens enhanced overall response rates and resulted in superior event-free survival (EFS) (3-year EFS of 91% vs. 57% in patients treated with CHOEP vs. CHOP, respectively). Furthermore, a large analysis of 263 adult patients with ALK+ ALCL demonstrated that the integration of etoposide into primary therapy was associated with significant improvements in the 5-year progression-free survival (PFS) (83% vs. 62%) and OS (93% vs. 74%) vs. non-etoposide regimens. In patients aged ≤60 years (n = 232), the respective 5-year PFS and OS were 81% vs. 65% and 92% vs. 77%, respectively (3). In Netherlands, a nationwide population-based study assessed the impact of etoposide on overall survival (OS) among patients aged 18 to 64 years with stage II to IV ALCL, angioimmunoblastic T-cell lymphoma (AITL), or PTCL not otherwise specified (NOS) diagnosed between 1989 and 2018. In patients with ALK+ ALCL who received CHOEP, CR rate was significantly higher than in patients who received CHOP (86% vs 61%). Overall, 5-year OS for patients with ALK+ ALCL who received CHOEP was superior to that in patients who received CHOP (90% vs 61%) (4). Multiple studies have shown that etoposide being of great value in ALCL but also in other PTCL subtypes. CD30 is a transmembrane glycoprotein receptor expressed on all systemic ALCL, making it

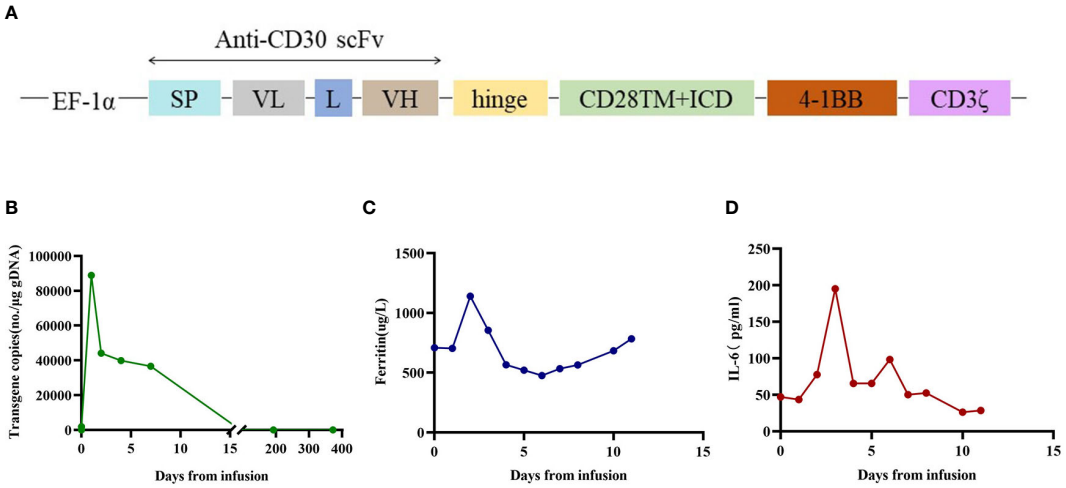

FIGURE 2
The therapeutic response after anti-CD30 CAR T-cell infusion. **(A)** Schematic diagrams of CAR construct. The third-generation CAR was composed of a single chain variable fragment (scFv), two costimulatory domains from CD28 and 4-1BB, and CD3ζ chain as activation domain. The scFv was derived from a murine monoclonal antibody against human CD30. Abbreviations: SP, signal peptide; VL, variable L chain; L, linker; VH, variable H chain. **(B)** The copies of CAR30 transgenes in the peripheral blood detected by ddPCR. **(C)** Dynamic changes in ferritin after CAR T cell infusion. **(D)** Dynamic changes in IL-6 after CAR T cell infusion.

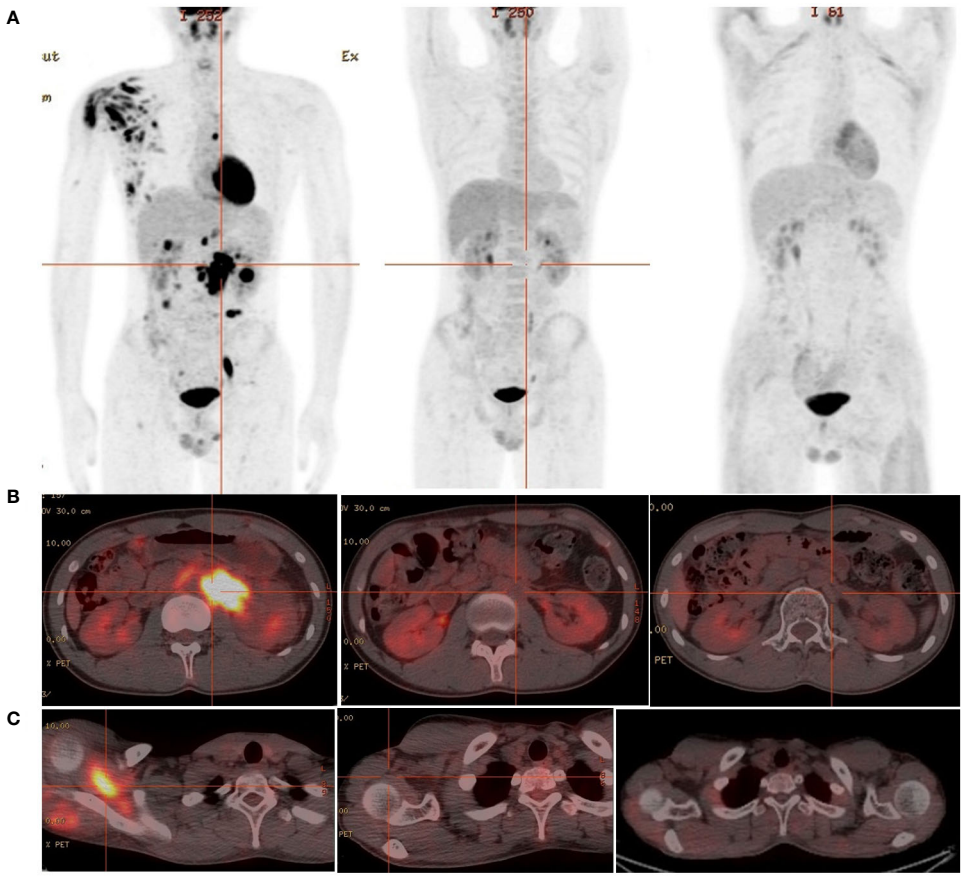

FIGURE 3
The 18F-FDG PET/CT images of relapsed/refractory ALCL. **(A)** From left to right: diagnosed as relapsed/refractory ALCL;1 month before ASCT and CAR T-cell therapy;3 months after ASCT and CAR T-cell therapy. **(B)** The metabolic images of retroperitoneal enlarged lymph nodes during disease recurrence, before ASCT and CAR T-cell treatment and 3 months after treatment (from left to right). **(C)** The metabolic images of right scapular muscle group during disease recurrence, before ASCT and CAR T-cell treatment and 3 months after treatment (from left to right).

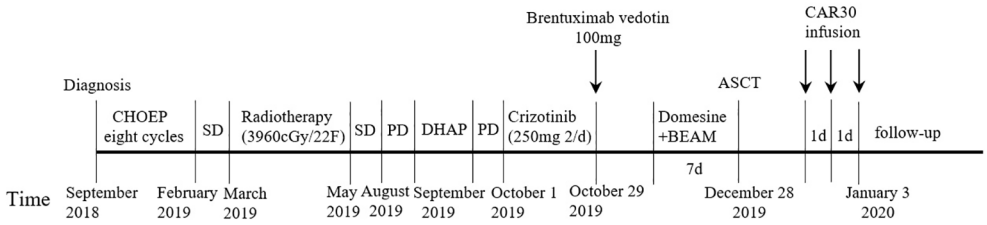

FIGURE 4
The timeline of disease status and the corresponding clinical treatment.

an ideal therapeutic target. BV is a chimeric monoclonal antibody–drug conjugate that targets CD30. The phase 3 ECHELON-2 study comparing CHOP with BV (BV substituted for vincristine; BV–CHP) regimen to CHOP in CD30+ adult PTCLs, including ALK+ ALCL with IPI ≥2, showed an improved 3-year PFS (57.1% vs. 44.4%) and OS (76.8% vs. 69.1%) in the BV group (5). The ECHELON-2 trial established the BV–CHP regimen as a new standard front-line therapy for patients with ALK+ ALCL. After 5 years of follow-up, patients with PTCL treated with BV–CHP as a frontline treatment had a survival benefit over CHOP, with a 5-year OS of 70.1% vs. 61.0%, respectively. This study further demonstrated that BV–CHP resulted in clinically significant improvements in OS compared with CHOP (6). Children's Oncology Group trial ANHL12P1 described the results of adding BV to standard chemotherapy in children with newly diagnosed ALK+ ALCL, with a 2-year EFS of 79.1% and OS of 97% (7). The addition of BV prevented relapses during therapy, and the OS and EFS estimates were relatively favorable to the results obtained using conventional chemotherapy. Thus, at present, BV-CHP (and its variations such as BV-CHEP) is the current standard of care for patients with ALCL (either ALK+ or ALK-) in the Europe and America.

Relapsed and refractory ALK+ ALCL are associated with a poor prognosis. NCCN guidelines recommend intensified chemotherapy with or without ASCT consolidation for r/r ALCL, however, this not effective treatment in all ALK+ALCL. Various therapeutic approaches, including high-dose chemotherapy regimens, new drugs such as anti-CD30 antibody drugs and ALK inhibitors, as well as autologous or allogeneic hematopoietic stem cell transplantation, have been used. ALK inhibitors are a more recently approved therapy that is often used as a stand-alone therapy for relapsed or refractory disease. Crizotinib, an inhibitor of ALK, was approved for the treatment of refractory ALK+ ALCL in pediatric patients and young adults in January 2021 (8).A small single-center study that evaluated crizotinib as a monotherapy for 25 patients with relapsed or refractory ALK+ ALCL found durable remission in almost 2/3 of patients (9). A non-controlled, open-label phase II study conducted in France enrolled 28 patients with relapsed/refractory ALK+ ALCL, of whom 25 patients receiving at least one dose of crizotinib were included in the analysis. The overall response rate at 8 weeks was 67% (95% CI: 47–82%), with 80% (95% CI: 44–97%) in children/adolescents and 57% (95% CI: 29–82%) in adults. The PFS and OS rates at 3 years were 40% (95% CI, 23–59%) and 63% (95% CI, 43–79%), respectively (10). In an open-label phase II trial, crizotinib was administered to 26 pediatric patients with relapsed or refractory ALK+ ALCL, achieving an objective response rate of 90% (11). Even though it induces CR in most cases, crizotinib has not yet been proven curative, as it may require life-long treatment. Notably, abrupt relapses of ALK+ lymphoma following crizotinib discontinuation have been reported. Crizotinib induces CR as a bridge for subsequent transplantation. Ceritinib, another ALK inhibitor, has shown a promising response as a treatment for ALK+ ALCL. The Phase I ASCEND-1 trial showed persistent responses in several adult patients with ALK+ ALCL, including three patients with relapsed ALK+ ALCL (12). Alectinib is a second-generation oral kinase inhibitor of ALK. A single-arm phase II study published in 2020 tested the efficacy of alectinib in 10 patients with relapsed/refractory ALK+ ALCL. Among these patients, eight (80%) achieved objective remission, and six (60%) achieved CR (13).

BV was approved in the USA and Europe for the treatment of relapsed ALCL in adults following the failure of at least one multi-agent chemotherapy protocol. BV has demonstrated its efficacy as a single agent (1.8 mg/kg every 3 weeks) in pediatric patients with relapsed ALCL, with objective response rates of 53–86% in phase 1 and 2 settings. A long-term follow-up of 5 years demonstrated that among a subset of patients with relapsed or refractory systemic ALCL, BV may be a potentially curative treatment option. Among all enrolled patients (n = 58), 16 (28%) had ALK+ ALCL, 10 achieved CR, and the PFS rate at 5 years was 50% among patients with ALK+ CR (95% CI, 19–81%) (14, 15). A multicenter study conducted in Italy evaluated the effectiveness of BV in 40 patients with relapsed/refractory ALCL, including 18 patients with ALK+. A total of 31 (77.5%) patients achieved a favorable response after a median of four cycles of BV monotherapy, with an overall response rate of 62.5% (16). Other immunotherapies and targeted therapies, such as PD-1/PD-L1 and HDAC inhibitors, can also be applied in ALCL. The use of PD-1/PD-L1 inhibitors in relapsed/refractory ALK+ ALCL has been demonstrated in previous case reports. Two patients with ALK + ALCL who relapsed after multiline therapy achieved sustained CR following nivolumab (3 mg/kg/2 for 2 weeks) (17, 18). Some non-randomized single-arm trials and small patient population studies have investigated the role of HDAC inhibitors, such as chidamide, romidepsin, and belinostat, combined with chemotherapy, in the first-line treatment of patients with ALCL (19–22).

Autologous hematopoietic stem cell transplantation (auto-HCT) following high-dose chemotherapy remains an option for

the treatment of relapsed/refractory ALK+ ALCL and is associated with a 5-year PFS rate of up to 56% (23). A prospective cohort study demonstrated that high-dose chemotherapy followed by ASCT achieved a remarkable long-term complete response, with a 12-year overall survival rate of 62% in patients with ALK+ ALCL (24). In a single-arm, open-label, multicenter, phase 2 study, 16 patients with relapsed/refractory ALCL who responded to BV and achieved CR and subsequently received either allogeneic or autologous consolidative SCT had a 5-year PFS rate of 69% (95% CI, 46–91%) and OS rate of 75% (95% CI, 54–96%) (14). A large cohort study performed by the Center for International Blood and Marrow Transplant Research found that patients with relapsed ALCL undergoing auto-HCT had superior outcomes to those receiving allo-HCT, with a smaller non-relapse mortality at 100 days, 1 year, and 3 years, and superior PFS and OS at 1 and 3 years for the patients who underwent auto-HCT compared with those who underwent allo-HCT (23). CAR-T therapy represents a major breakthrough in relapsed/refractory B cell NHL immunology. However, its application in T-cell malignancies is still being explored due to issues such as antigen targetability and cell fratricide. The presence of CAR-T cells that can recognize CD30 offers hope for patients with ALCL. Clinical trials of CD30-directed CAR-T cells for relapsed/refractory CD30+ ALCL are currently ongoing (NCT04653649, NCT04008394, NCT04288726, and NCT04288726). An open-label, single-center, single-arm pilot study demonstrated the combined administration of ASCT and CAR30 T-cell therapy was well-tolerate and highly effective in r/r classical Hodgkin lymphoma (cHL) and ALCL. In six r/r CD30+ lymphoma patients (five with cHL and one with ALK-negative ALCL), ORR and CR rates were 100% and 83.3%, respectively (25). Given the limited data on ASCT and sequential anti-CD30 CAR T cell therapy, it is difficult to know if the CAR-T cell therapy added any improved survival over the ASCT alone.

In the present case, the disease recurred quickly after the patient received chemotherapy and local radiotherapy. The use of crizotinib and BV as a bridge to autologous stem cell transplantation followed by CD30-directed chimeric antigen receptor T cell therapy is expected to achieve a long-term survival effect in the management of patients with relapsed/refractory CD30+ ALK+ ALCL. For patients with ALK+ ALCL with early relapse or refractory disease, targeted therapy induced remission may be considered as a bridge to transplantation or subsequent ASCT combined with CAR30 T-cell therapy as a treatment option. This is a promising approach for future clinical trials. Further prospective studies with larger sample sizes are required to demonstrate the superiority of the therapeutic strategies mentioned in this study for relapsed/refractory ALK+ ALCL.

4 Patient perspective

When discussing the feelings from diagnosis to recovery, the patient felt that everything was as surreal as a dream. His life went through significant ups and downs but turned out well. The patient's successful recovery was attributed to the combined efforts of the doctors and the wholehearted dedication and support of his parents. At present, the patient has returned to campus in good physical condition, with gratitude, optimism, and hope for the future.

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) for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

WL: Writing – original draft. JW: Data curation, Writing – original draft. XM: Data curation, Writing – original draft. QZ: Formal analysis, Writing – original draft. DZ: Formal analysis, Writing – original draft. RZ: Data curation, Writing – original draft. MZ: Writing – original draft. ZS: Writing – original draft. LC: Writing – review & editing. XZ: Writing – review & editing. YX: Funding acquisition, Writing – review & editing.

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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.

Publisher's note

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Case report: Donor-derived CLL-1 chimeric antigen receptor T-cell therapy for relapsed/refractory acute myeloid leukemia bridging to allogeneic hematopoietic stem cell transplantation after remission

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Background: Explore the efficacy and safety of donor-derived CLL-1 chimeric antigen receptor T-cell therapy (CAR-T) for relapsed/refractory acute myeloid leukemia (R/R AML) bridging to allogeneic hematopoietic stem cell transplantation (allo-HSCT) after remission.

Case presentation: An adult R/R AML patient received an infusion of donor-derived CLL-1 CAR-T cells, and the conditioning regimen bridging to allo-HSCT was started immediately after remission on day 11 after CAR-T therapy upon transplantation. Then, routine post-HSCT monitoring of blood counts, bone marrow (BM) morphology, flow cytometry, graft-versus-host disease (GVHD) manifestations, and chimerism status were performed.

Result: After CAR-T therapy, cytokine release syndrome was grade 1. On day 11 after CAR-T therapy, the BM morphology reached complete remission (CR), and the conditioning regimen bridging to allo-HSCT started. Leukocyte engraftment, complete donor chimerism, and platelet engraftment were observed on days +18, +23, and +26 post-allo-HSCT, respectively. The BM morphology showed CR and flow cytometry turned negative on day +23. The patient is currently at 4 months post-allo-HSCT with BM morphology CR, negative flow cytometry, complete donor chimerism, and no extramedullary relapse/GVHD.

Conclusion: Donor-derived CLL-1 CAR-T is an effective and safe therapy for R/R AML, and immediate bridging to allo-HSCT after remission may better improve the long-term prognosis of R/R AML.

KEYWORDS

relapsed/refractory, acute myeloid leukemia, C-type lectin-like molecule 1, donor-derived chimeric antigen receptor T cells, allogeneic hematopoietic stem cell transplantation

1 Introduction

Relapsed/refractory acute myeloid leukemia (R/R AML) has a low remission rate with chemotherapy and a high probability of relapse after salvage HSCT has been performed in the absence of remission (1–3). Therefore, it is challenging to regain remission before HSCT to achieve good conditions for successful hematopoietic stem cell transplantation (HSCT) and reduce the risk of subsequent relapse. In recent years, the success of CD19 chimeric antigen receptor T-cell (CAR-T) therapy in B-cell malignancies has led to the exploration of the feasibility of using CAR-T for the treatment of acute myeloid leukemia (AML) (4, 5).

C-type lectin-like molecule 1 (CLL-1) is a membrane protein that plays a pivotal role in the fight against infection and maintains homeostasis and self-tolerance by recognizing damage- and pathogen-associated molecular patterns that lead to the regulation of innate and adaptive immunity (6). Non-hematological tissues in humans express very low levels of CLL-1 (7). In the hematopoietic tree, CLL-1 is mainly expressed by almost all granulocytes and monocytes and by approximately 61.8% of their precursors, 41.6% of progenitors, and only 2.5% of $CD34^{+}CD38^{-}$ HSCs, but it is not expressed by T, B, and NK cells or erythrocytes or by their precursors (8). CLL-1 is also expressed by basophils, eosinophils, granulocytes, macrophages, and myeloid DCs (9). CLL-1 is also expressed in leukemic stem cells (LSCs), which have the ability to self-renew indefinitely and produce many daughter blast cells, representing one of the most important causes of leukemia relapse (10, 11). As a result, CLL-1 can serve as a marker of LSC and disease relapse. More importantly, CLL-1 is expressed by >80% of AML cells but not by normal HSC (12, 13), allowing CLL-1 to be considered an ideal druggable target for the treatment of AML.

A phase I/II clinical trial of autologous CLL-1 CAR-T therapy by Zhang et al. enrolled eight children with R/R AML, all of whom received autologous CLL-1 CAR-T therapy after a conditioning regimen with fludarabine and cyclophosphamide (Flu/Cy) (14). After Flu/Cy treatment, the patients experienced grade 1–2 cytokine release syndrome (CRS) with no fatal adverse events. Of these four children who achieved bone marrow morphology complete remission (CR) and minimal residual disease (MRD)-negative status, one child showed positive BM morphology and MRD, one child achieved CR with incomplete count recovery (CRi) with positive MRD, one child achieved partial remission (PR), and one child maintained stable disease (SD) status (14). In another phase I clinical study, Jin et al. enrolled 10 adult patients with R/R AML who received $1–2 \times 10^{6}$/kg autologous CLL-1 CAR-T cells after Flu/Cy (15). All 10 patients developed CRS (low-grade in four patients and high-grade in six patients), none of these patients developed CAR-T therapy-related encephalopathy syndrome (CRES), and 70% of these patients achieved CR/CRi. All patients presented severe pancytopenia, which was attributed to the fact that CLL-1 was also expressed in normal granulocytes. Two patients died from severe infections caused by prolonged granulocyte deficiency (15). Therefore, bridging to HSCT was considered to rescue the resulting prolonged granulocyte deficiency.

As evidenced by current studies on immunotherapy with autologous CLL-1 CAR-T cells, CLL-1 is an effective target for the treatment of R/R AML, and bridging to HSCT is required after remission to rescue the subsequent granulocyte deficiency, reduce the risk of post-HSCT relapse, and improve long-term prognosis. However, some patients with R/R AML have extremely low autologous lymphocyte counts due to a high tumor load and are unable to provide autologous lymphocytes for the preparation of CAR-T cells. Li Z et al. analyzed 12 patients with R/R T-ALL/LBL (16). These patients obtained CR or PR through donor-derived CD7-CAR-T therapy bridging to allo-HSCT, and the OS and DFS at 6 months were 91.67% and 83.33%, respectively, and the allo-HSCT-related mortality (TRM) was 8.33%. This result showed the strong anti-leukemic effect and safety of donor-derived CD7-CAR-T combined with allo-HSCT (16). Therefore, we describe an adult patient with R/R AML to explore the efficacy and safety of donor-derived CLL-1 CAR-T therapy bridging to allo-HSCT from the same donor after remission.

2 Case description

2.1 Patient characteristics before CAR-T therapy

An 18-year-old male patient was admitted to a local hospital in June 2021 with a fever. After routine blood tests, bone marrow puncture, and other related examinations, this patient was diagnosed with AML with CEBPA double mutation and normal karyotype

(classified as low risk according to the ELN 2022 risk stratification). Subsequently, the patient received four courses of chemotherapy (first course: 170 mg of cytarabine d1–d7 and 130 mg of daunorubicin d4–d6; second course: 500 mg of cytarabine q12h d1–d3; third and fourth course: 5,000 mg of cytarabine d1–d3). Bone marrow (BM) morphology showed CR and flow cytometry showed positive MRD at the end of the first course of chemotherapy. The MRD was detected based on this phenotype of $CD34^{+}CD117^{+}HLA\text{-}DR^{+}CD13^{dim+}CD33^{dim+}CD38^{+}CD123^{dim+}CD200^{+}CLL\text{-}1^{+}CD56^{+}CD7^{+}CD19^{-}$. The patient achieved CR and negative MRD since the second course of chemotherapy. During this period, a lumbar puncture was performed and no abnormalities were observed on routine cerebrospinal fluid, biochemistry, and flow cytometry examinations. The patient underwent autologous HSCT (auto-HSCT) on 6 January 2022. The conditioning regimen was busulfan/Flu/Cy/chidamide (150 mg of busulfan d1–d4, 50 mg of Flu d1–d5, 1,500 mg of cytarabine on d1–d5, and 30 mg of chidamide d1, d4, d8, d11). On 24 May 2022, routine blood tests revealed a white blood cell count of 1.14×10^{9}/L, a hemoglobin concentration of 62 g/L, a platelet count of 3×10^{9}/L, and 4% immature cells. BM morphology revealed 71% myeloid blasts, and flow cytometry showed abnormal myeloid primitive cells comprising approximately 77.74% of nucleated cells, with a phenotype of $CD34^{+}CD117^{+}HLA\text{-}DR^{+}CD13^{dim+}CD33^{dim+}CD38^{+}CD123^{dim+}CD200^{+}CLL\text{-}1^{+}CD6^{+}CD7^{+}CD19^{-}$. Chromosome karyotype analysis showed 45,XY,-9. Analysis of myeloid gene mutations identified a CEBPA double mutation. In summary, this patient was diagnosed with R/R AML with CEBPA double mutation and was classified as high risk according to the ELN 2022 risk stratification. Hence, the patient received one course of venetoclax and azacytidine chemotherapy (100 mg of venetoclax d1–d28 and 100 mg of azacytidine d1–d9) on 29 May 2022. BM morphology showed 61% primitive granulocytes on 4 July 2022. Flow cytometry showed 63.18% abnormal myeloid primitive cells suggesting a refractory disease.

2.2 CLL-1 CAR-T therapy bridging to allo-HSCT after remission

Given the refractoriness of the patient, we proposed that the patient should undergo donor-derived CLL-1 CAR-T therapy, and we obtained the patient's understanding and consent. The donor was his 21-year-old older sister. We used the COM.TEC blood component separator (Fresenius, Bad Homburg, Germany) to collect the donor's peripheral blood T cells, which subsequently were stimulated with magnetic beads coated with anti-CD3/CD28 antibodies (Thermo Fisher Scientific Massachusetts, United States of America) overnight. The patient received Flu/Cy therapy (50 mg of Flu for 3 days, 500 mg of Cy for 3 days) starting on 22 July 2022. Then, a total of 0.5×10^{6}/kg of donor-derived CLL-1 CAR-T cells were infused on 1 August 2022 and on 4 August 2022, respectively. The CAR-T manufacturing protocol was performed as follows (17). Briefly, the CLL-1 CAR lentivirus was manufactured at our center under good manufacturing practice (GMP) standards. CLL-1 CAR-T cells were manufactured with donor-derived T cells transduced with CLL-1 CAR lentivirus. Transduction efficiency, defined as the percentage of CAR^{+} cells among $CD3^{+}$ cells, and cell viability were determined just before infusion by flow cytometry and Trypan blue exclusion. Subsequently, vital signs, blood counts, cytokine levels, ferritin levels, peripheral blood CAR-T cell count, BM morphology, and flow cytometry were closely monitored. On day 8 after CAR-T therapy, the patient developed a fever with a maximum temperature of 39.2°C, which subsided on day 10 after 10 mg of intravenous dexamethasone (Figure 1A). No other adverse effects were observed, including blood pressure drop, capillary leak syndrome, CRES, gastrointestinal events, cardiovascular events, change in general conditions (fatigue, flu-like symptom, rash, and peripheral edema), laboratory values (AST increase, ALT increase, bilirubin increase, creatine increase, and LDH increase), or infection. CRS was grade 1. The patient had a severe reduction in whole blood cells (Figures 1B–D) with a mild increase in ferritin and interleukin-6 (IL-6) levels (Figures 1E, F). CAR-T cell counts in the peripheral blood are shown in Figure 1G. BM morphology on 12 August 2022 (day 11 after CAR-T therapy) showed an extreme reduction in BM proliferation with 5% primitive granulocytes. Flow cytometry revealed 0.29% abnormal myeloid primitive cells in the BM. A conditioning regimen including decitabine/cladribine/cytarabine/busulfan/semustine/ATG (300 μg of G-CSF d1–d5, 30 mg of decitabine d1–d5, 8.4 mg of cladribine d1–d5, 1,600 mg of cytarabine d1–d5, 180 mg of busulfan d1–d3, 400 mg of semustine d5, 200 mg of ATG d3, and 300 mg of ATG d4–d6) was carried out on 12 August 2022 (day 11 after CAR-T therapy), and the patient received donor stem cells [mononuclear cells (MNCs) 8×10^{8}/kg, CD34 6.6×10^{6}/kg, total nucleated cells (TNCs) 12.3×10^{8}/kg, CD3 1.3×10^{8}/kg] on 20 August 2022 (day 0 after allo-HSCT), with routine treatment of cyclosporine, mycophenolate mofetil, and short-course methotrexate (MTX) for graft-versus-host disease (GVHD) prevention. The donor was the same one described above, whose CMV status was negative and whose blood type was A+ (the patient's blood type was AB+). The HLA compatibility between them was 8/12. Leukocyte engraftment, complete donor chimerism, and platelet engraftment were observed on days +18, +23, and +26 after allo-HSCT, respectively. BM showed CR and flow cytometry was negative on day +23 after allo-HSCT. No abnormalities were observed in routine cerebrospinal fluid, biochemistry, or flow cytometry examinations on day +32 after allo-HSCT. The patient is currently at 4 months post-allo-HSCT with bone marrow morphology CR, negative flow cytometry, complete donor chimerism, and no evidence of extramedullary relapse or GVHD.

3 Timeline

The timeline of the disease and treatment course of this patient is shown in Figure 2.

4 Discussion

AML is a highly heterogeneous group of malignant hematologic diseases. Although some low-risk patients achieve prolonged survival with chemotherapy, some patients do not benefit from chemotherapy and may progress to relapsed/refractory (R/R) AML. Treatment after relapse remains a challenge, especially for AML that relapses after

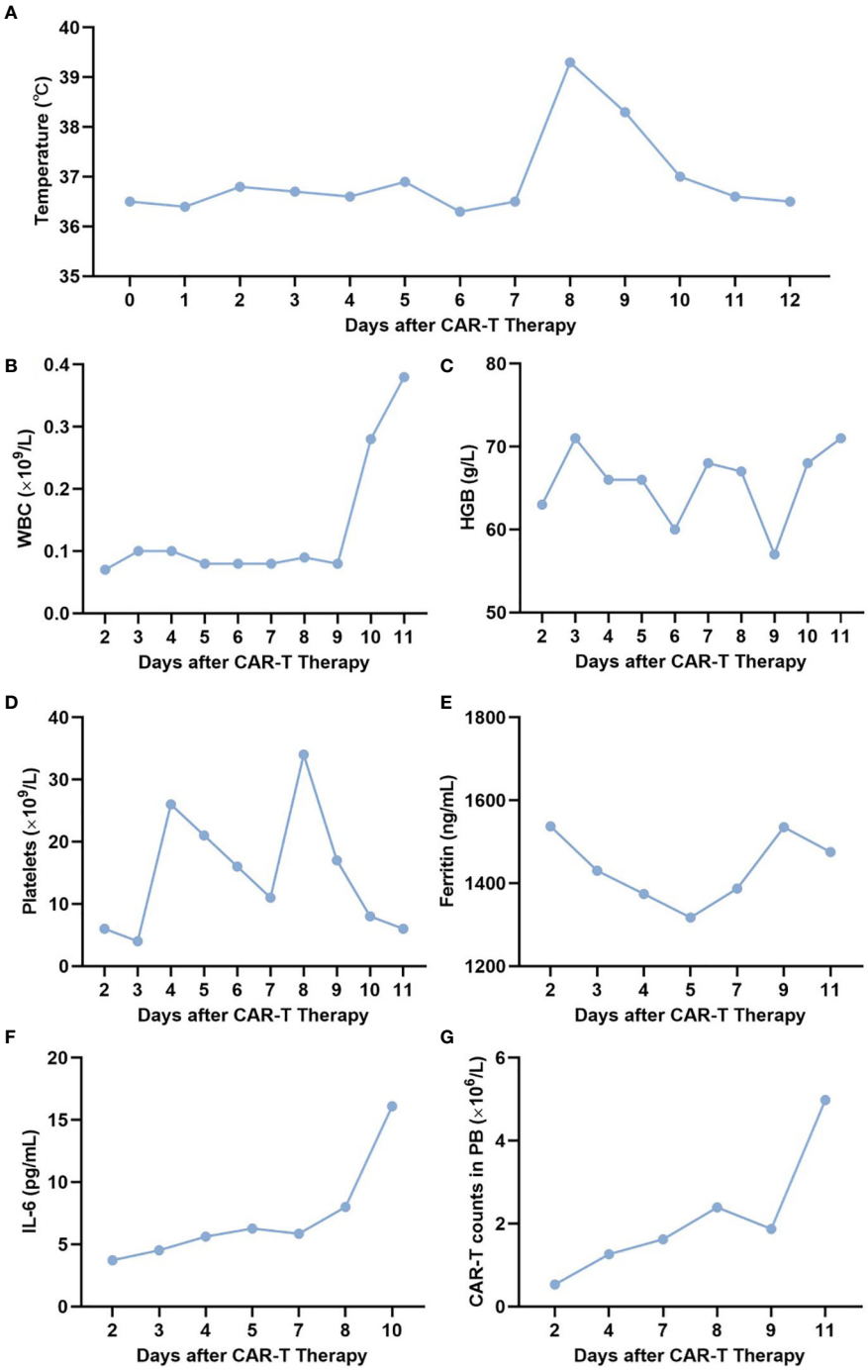

FIGURE 1
Related indexes of the patient at different time points. **(A–G)** Temperature **(A)**, WBC **(B)**, HB **(C)**, PLT **(D)**, ferritin **(E)**, IL-6 **(F)**, and CAR-T cell count **(G)** in the peripheral blood of the patient on days 2, 4, 6, 8, 10, and 12 after CAR-T therapy. WBC, white blood cell; HB, hemoglobin; PLT, platelet.

HSCT, with no standard therapies and only a series of palliative treatments (18, 19). In recent years, the success of CD19 CAR-T therapy in B-cell malignancies has led to the exploration of the efficacy and safety of CAR-T therapy in AML, the targets of which include LewisY, CD44V6, CD33, CD123, and CLL-1 (4, 8, 20–24). Although more expressed in leukemic stem cells (LSCs), CD33 and CD123 are also frequently expressed in normal HSCs, and their suppression can lead to long-term or even permanent myelosuppression (25). The fact that CLL-1 is highly expressed in AML cells but is deficient in normal HSCs makes it an attractive target in CAR-T therapy for AML (26). The efficacy and safety of autologous CLL-1 CAR-T therapy have been demonstrated in previous clinical studies (14, 15). However, to date, no cases have described allogeneic donor-derived CLL-1 CAR-T therapy in R/R AML.

The patient we describe herein was initially diagnosed with CEBPA double-mutated AML (low risk). After achieving remission from

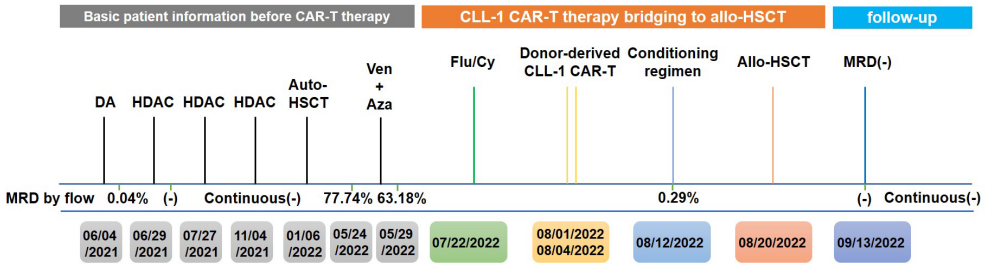

FIGURE 2
Timeline of the patient's treatment and bone marrow assessment. DA, daunorubicin and cytarabine; HDAC, high-dose cytarabine; Ven, venetoclax; Aza, azacytidine; Flu, fludarabine; Cy, cyclophosphamide.

induction chemotherapy, the patient underwent three courses of consolidation chemotherapy with sequential auto-HSCT. Relapse occurred within 6 months after auto-HSCT and then the patient was reclassified as R/R AML with CEBPA double mutation (high risk). Salvage chemotherapy with standard doses of venetoclax and azacytidine was performed after relapse; however, BM evaluation after chemotherapy indicated treatment failure. At this point, the patient was faced with the options of 1) immediate salvage allo-HSCT or 2) participation in a clinical trial of CAR-T therapy. However, the disease status at the time of allo-HSCT was closely related to the outcome after HSCT. Allo-HSCT is most effective in patients who reached CR with the lowest relapse rate since there is sufficient time to establish a strong graft-versus-leukemia (GVL) effect in such cases (2, 3). Therefore, immediate salvage allo-HSCT was not the optimal choice for this patient. The BM immunophenotyping of this patient revealed high CLL-1 expression by tumor cells on 24 May 2022. Therefore, participation in the CLL-1 CAR-T clinical trial was a feasible option. However, this patient was in a state of AML relapse and had an extremely low peripheral blood lymphocyte count, which made it difficult to collect sufficient autologous lymphocytes for the preparation of CAR-T cells. The results of Zhi Hui Li et al. showed strong anti-leukemia effect and safety of donor-derived CAR-T combined with allogenic HSCT (16). For this patient, we considered administering donor-derived CLL-1 CAR-T therapy bridging to allo-HSCT immediately after remission. After a conditioning regimen with Flu/Cy and infusion of donor-derived CLL-1 CAR-T cells, this patient developed grade 1 CRS on day 8 after CAR-T therapy. On day 11 after CAR-T therapy, BM morphology showed CR and flow cytometry showed 0.29% abnormal myeloid primitive cells. This case fully demonstrated that donor-derived CLL-1 CAR-T therapy has significant efficacy and good safety advantages, which can create favorable conditions for a transition to allo-HSCT. A routine conditioning regimen before allo-HSCT was started immediately after remission, followed by donor stem cell infusion and treatments to prevent GVHD. The patient recovered well after allo-HSCT and has a positive MRD status with no GVHD or extramedullary relapse manifestations at follow-up to date. These results demonstrated the safety and efficacy of donor-derived CLL-1 CAR-T therapy bridging to allo-HSCT immediately after remission.

The successful treatment of this patient indicates that donor-derived CLL-1 CAR-T therapy for R/R AML to achieve remission followed by immediate bridging to allo-HSCT is effective with mild and manageable adverse effects, thus providing new avenues for the treatment of R/R AML.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Ethics Committee of the People's Liberation Army The General Hospital of Western Theater Command (Chengdu, China). The studies were conducted in accordance with the local legislation and institutional requirements. The 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. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

XM: Investigation, Writing – original draft. YRS: Investigation, Writing – original draft. YH: Investigation, Writing – original draft. NZ: Investigation, Writing – review & editing. YL: Investigation, Writing – original draft. HYa: Investigation, Writing – original draft. XW: Investigation, Writing – original draft. GH: Investigation, Writing – original draft. DC: Investigation, Writing – original draft. FF: Investigation, Writing – original draft. AC: Investigation, Writing – review & editing. YS: Investigation, Writing – review & editing. HY: Funding acquisition, Investigation, Writing – review & editing.

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Conflict of interest

Author AC is a founding member of Shanghai YaKe Biotechnology Ltd., a biotechnology company focusing on research and development of tumor cellular immunotherapy.

The remaining 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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Lower incidence of grade II-IV acute Graft-versus-Host-Disease in pediatric patients recovering with high Vδ2+ T cells after allogeneic stem cell transplantation with unmanipulated bone marrow grafts: a prospective single-center cohort study

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Gamma delta (γδ) T cells represent a minor fraction of human T cell repertoire but play an important role in mediating anti-infectious and anti-tumorous effects in the context of allogeneic hematopoietic stem cell transplantation (allo-HSCT). We performed a prospective study to analyze the effect of different transplant modalities on immune reconstitution of γδ T cells and subsets. CD3, CD4 and CD8 T cells were analyzed in parallel. Secondly, we examined the impact of γδ T cell reconstitution on clinical outcomes including acute Graft-versus-Host-Disease (aGvHD) and viral infections. Our cohort includes 49 pediatric patients who received unmanipulated bone marrow grafts from matched unrelated (MUD) or matched related (MRD) donors. The cohort includes patients with malignant as well as non-malignant diseases. Cell counts were measured using flow cytometry at 15, 30, 60, 100, 180 and 240 days after transplantation. Cells were stained for CD3, CD4, CD8, CD45, TCR$\alpha\beta$, TCRγδ, TCRVδ1, TCRVδ2, HLA-DR and combinations. Patients with a MRD showed significantly higher Vδ2+ T cells than those with MUD at timepoints +30, +60, +100 ($p<0.001$, respectively) and +180 ($p<0.01$) in univariate analysis. These results remained significant in multivariate analysis. Patients recovering with a high relative abundance of total γδ T cells and Vδ2+ T cells had a significantly lower cumulative incidence of grade II-IV aGvHD after transplantation ($p=0.03$ and $p=0.04$, respectively). A high

relative abundance of Vδ2+ T cells was also associated with a lower incidence of EBV infection (p=0.02). Patients with EBV infection on the other hand showed higher absolute Vδ1+ T cell counts at days +100 and +180 after transplantation (p=0.046 and 0.038, respectively) than those without EBV infection. This result remained significant in a multivariate time-averaged analysis (q<0.1). Our results suggest a protective role of γδ T cells and especially Vδ2+ T cell subset against the development of aGvHD and EBV infection after pediatric HSCT. Vδ1+ T cells might be involved in the immune response after EBV infection. Our results encourage further research on γδ T cells as prognostic markers after HSCT and as possible targets of adoptive T cell transfer strategies.

KEYWORDS

pediatric stem cell transplantation, allogeneic stem cell transplantation, gamma delta (γδ) T cells, immune reconstitution, transplant immunobiology, Graft-versus-Host Disease (GVHD)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for various malignant and nonmalignant diseases. Delayed immune reconstitution (IR) is a main risk factor for morbidity and mortality in patients undergoing allogeneic HSCT as it is associated with higher rates of relapse, infectious complications and Graft-versus-Host-Disease (GvHD) (1). To further improve transplant-related outcomes in the future, it is critical to identify the factors that influence speed and quality of immune recovery after HSCT.

In the past two decades, more focus has been placed on the reconstitution of gamma delta (γδ) T cells as growing evidence suggests that these cells have beneficial effects in the context of HSCT, by mediating innate and adaptive immune responses independent of HLA-antigen presentation and by exerting potent antitumor activity via various receptors e.g. NKG2D or DNAM-1 (2).

While the majority of circulating CD3 lymphocytes carries an αβ T cell receptor, only 1-10% in the peripheral blood are γδ T cells. The γδ T cell receptor consists of γ and δ chains that are encoded by 6 Vγ genes on chromosome 6 respectively 8 Vδ genes on chromosome 14 (3). γδ T cells are subclassified based on their Vδ chain; Vδ2+ T cells are the predominant fraction found in the peripheral blood of healthy adults, whereas non-Vδ2+ T cells (mainly Vδ1+) are primarily found in epithelial tissue like skin or intestines (4, 5).

Nowadays, peripheral blood stem cells represent the main stem cell source for HSCT in adults. In contrast, unmanipulated bone marrow grafts among peripheral blood stem cells and cord blood are still frequently used in children. In addition to graft source and graft manipulation, donor selection has an important impact on IR.

In a recent meta-analysis of 11 studies (919 patients) on γδ T cell reconstitution after allogeneic HSCT, Arruda et al. reported that high γδ T cell counts were associated with less disease relapse, fewer viral infections and higher overall and disease-free survival (6). Most of these studies included only adult patients with partially mismatched related donors (PMRD). There is only few data on IR of γδ T cells and especially their subsets in children undergoing allogeneic HSCT from matched unrelated (MUD) or matched related donors (MRD).

In our prospective single-center cohort study, we report on the reconstitution of γδ T cells and subsets until day +240 after transplantation in a cohort of pediatric patients receiving unmanipulated bone marrow grafts. We studied the impact of transplant modalities on the IR and the effect of high versus low γδ T cells on HSCT outcomes.

Material and methods

Patients

This study includes 49 patients who underwent their first allogeneic HSCT between August 2016 and January 2019 at the Department of Pediatric Oncology and Hematology, *Charité – Universitätsmedizin Berlin*. The median patient age at HSCT was 7 years (0-19 years). All patients received unmanipulated bone marrow as graft source. The cohort consisted of 29 patients with malignant hematological disorders and 20 patients with various non-malignant HSCT-indications, mostly hemoglobinopathies. Detailed transplant characteristics of our cohort are presented in Table 1. Patients received different conditioning regimens dependent on the transplant indication (Supplementary Figure 1).

For GvHD-prophylaxis all patients received Ciclosporin A (CSA) intravenously twice daily starting from one day prior to transplantation. Patients were switched to oral CSA formulations before discharge from the hospital. In combination with CSA

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TABLE 1 Patient and transplant characteristics.

Number of patients		49
Follow-Up Time, days, median [IQR]		737 [474, 873]
Patient age, years, median [range]		7 [0 - 19]
Patient sex, n (percent)	Female	23 (46.9)
Disease, n (percent)	Acute lymphoblastic leukemia (ALL)	21 (42.9)
	Acute myeloid leukemia (AML)	6 (12.2)
	Myelodysplastic syndrome (MDS)	2 (4.1)
	Sickle cell disease (SCD)	10 (20.4)
	ß-Thalassemia	3 (6.1)
	Chronic granulomatous disease (CGD)	1 (2.0)
	Severe combined immunodeficiency (SCID)	1 (2.0)
	Severe aplastic anemia (SAA)	1 (2.0)
	Hb-Yokohama	1 (2.0)
	Fanconi anemia	1 (2.0)
	Congenital amegakaryocytic thrombocytopenia (CAMT)	1 (2.0)
	Osteopetrosis	1 (2.0)
Donor type, n (percent)	MSD	26 (53.1)
	MUD	20 (40.8)
	MFD	3 (6.1)
Antithymocyte globulin, n (percent)	Yes	33 (67.3)
Alemtuzumab, n (percent)	Yes	3 (6.1)
Methotrexat, n (percent)	Yes	36 (73.5)
Mycophenolatmofetil, n (percent)	Yes	14 (28.6)
Disease Status, n (percent)	Non-malignant disease	20 (40.8)
	Malignant disease	29 (59.2)
HLA Compability, n (percent)	9/10	3 (6.1)
	10/10	46 (93.9)
Conditioning Regimen, n (percent)	VP16/TBI	9 (18.4)
	Flu/TT/Treo	23 (46.9)
	Flu/TT/Mel	5 (10.2)
	Flu/Bu/TT	1 (2.0)
	Flu/TT	2 (4.1)
	Flu/Cy	2 (4.1)
	Flu/Bu/Cy/TT	2 (4.1)
	Flu/Bu	2 (4.1)
	Bu/Cy/Mel	2 (4.1)
	Amsacrine/Flu/Cy/Ara-C/TBI	1 (2.0)
Graft CD3, cells/kg, median [IQR]		4,9 x 10^7 [2,8 x 10^7, 7,3 x 10^7]
Graft CD34, cells/kg, median [IQR]		5,2 x 10^6 [3,4 x 10^6, 7,8 x 10^6]
Graft CD45, cells/kg, median [IQR]		3,9 x 10^8 [2,9 x 10^8, 5,8 x 10^8]
CMV Serostatus, D/R, n (percent)	+/+	19 (44.2)
	+/-	7 (16.3)
	-/-	9 (20.9)
	-/+	8 (18.6)
Donor age, years, median [range]		14 [2 - 52]
Sex (mis-)match, n (percent)	F/F	12 (25.0)
	F/M	10 (20.8)
	M/F	9 (18.8)
	M/M	17 (35.4)

patients received either Mycophenolate Mofetil (MMF) 2x600 mg/m^2/d starting on day +1 after HSCT for 30 days or Methotrexate (MTX) 10 mg/m^2 once a day on days +1, +3 and +6.

Serotherapy was administered in 36 cases, 33 patients were treated with anti-thymocyte globulin (ATG) and 3 patients received Alemtuzumab (for detailed information on dosing and administration of serotherapy see Supplementary Figure 1).

In nine patients, immunosuppression with CSA was changed to Everolimus and MMF after transplantation. For one patient, this became necessary because he developed posterior reversible encephalopathy syndrome, while the other patients had acute renal failure.

Ethics

Written informed consent was obtained from all patients or their parents/guardians before HSCT. The study was approved by the local ethics committee (EA2/144/15).

Evaluation

The day of engraftment was defined as the first of three consecutive days with an absolute neutrophil count of at least 500 cells/µl.

In patients with leukemia, relapse was defined either morphologically as more than 5% blast cells in the bone marrow or a minimal residual disease of ≥ $1x10^{-4}$ measured by flow cytometry or polymerase chain reaction. Relapse of Myelodysplastic syndrome (MDS) was defined by morphology, cytogenetics, or both.

Acute GvHD was defined and diagnosed according to the modified Glucksberg criteria based on clinical, laboratory and histological findings (7, 8).

All patients were screened twice weekly for Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Adenovirus (ADV) DNA in peripheral blood and ADV DNA in stool with polymerase chain reaction until discharge. After discharge, analysis was performed once weekly. PCR cut-off levels for detection of EBV, CMV and ADV in blood were 550, 300 and 2000 Copies/ml, respectively. A linear range of the copy numbers was provided by the local laboratory between 1000 – $2,2x10^8$ Copies/ml (EBV), 2000 – $3x10^8$ (CMV) and 2000 – $1x10^8$ (ADV). The viral load in positive stool samples was measured semi-quantitatively.

Sample collection

Whole blood samples (3 ml) from all patients undergoing HSCT were collected on ethylenediamine tetra acetic tubes and analyzed the same day at seven different timepoints: once prior to the start of the conditioning regimen and at six timepoints after HSCT on days +15, +30, +60, +100, +180, +240.

Flow cytometry

Flow cytometry was performed using Duraclone technology (Beckman Coulter) and each timepoint was analyzed using the DuraClone IM Phenotyping T cell subtypes panel containing nine conjugated antibodies. Samples were processed according to validated standard operation procedures. All analyses were performed using a NAVIOS flow cytometer (Beckman Coulter). Measurements were performed according to the validated method described previously (9) and analyzed using the FlowJo 10.4.2 software. Cell counts were conducted for cells expressing CD3, CD4, CD8, CD45, TCRαβ, TCRγδ, TCRVδ1, TCRVδ2, HLA-DR and combinations.

Statistical analysis

Wilcoxon rank test was performed for non-parametric variables, while comparing two outcomes or timepoints. For multiple strata or groups, the Kruskal test was performed. As a measure of effect size Cliffs Delta was computed for variables having two levels and Spearman correlation for continuous variables. When Spearman correlation was computed, the associated Spearman test of correlation was performed (10). Testing for confounded variables was done using a linear model with multiple variables and interaction terms followed by a log-likelihood ratio test with the null hypothesis being a single variable model. A significant log-likelihood ratio test indicates that the variable in question is confounded.

The analysis of outcomes in relation to the qualitative cell counts of interest i.e. high versus low was performed using the Cox hazard regression model via the R survival package (11) in accordance with the EBMT recommendations (12). To test for the statistical significance of the Cox fits, Rank-sum tests were performed.

The stratification of the cohort into patients with high and low cell relative abundance was done by ordering the patients by their time-averaged relative abundance of the cell type of interest, then finding the best cut-off value of this relative abundance that separates the cohort into two groups. This is defined by the largest effect size ω^2 comparing the Jaccard distances in the space of the time-averaged relative abundance and the rank of the patient based on it, Supplementary Figure 2.

In all tests, a p-value of 0.05 is considered significant for single-variable analysis. For multivariate analysis, the p-values were corrected for multiple testing using the Benjamini-Hochberg method (13) to get q-values. A corrected q-value of <0.1 is considered significant.

The t-distributed stochastic neighbor embedding (t-SNE) analysis was performed on a maximum of 200K cells randomly sampled from the samples corresponding to each timepoint and each group (grade II-IV GvHD or not). The analysis is performed using FlowJo software, with the coloring of cells corresponding to the gating performed.

Statistical analysis were performed using RStudio, Version 2023.06.2 + 561 (RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA).

Results

Patient outcomes

The median (Interquartile range) follow-up time in our cohort was 737 (474 – 873) days. Successful engraftment was achieved in 100% of the patients and none of them experienced graft rejection afterwards. During the follow-up period five patients (10%) died; three of the deaths were treatment-related mortalities (TRM); two patients died from relapse. Patients in the TRM group had severe infectious complications with consecutive multiple organ failure.

Relapse occurred in 13 of 29 cases (44.8%) in the subgroup of patients with malignant disease at a median of 171 days after transplantation. We registered 23 cases of aGvHD (46%). In eight of these patients (16%) higher grade aGvHD (grade II-IV) was diagnosed. The median onset of aGvHD was 17 (15 – 31) days after transplantation. Viral infections occurred in 34 cases (69%) and 19

patients (55% of patients with viral infection) experienced infection with more than one virus (CMV, EBV or ADV) during the course of transplantation. A total of 14 patients developed CMV infection (28.5%) at a median of 28 (9 – 40) days after transplantation. EBV infection was diagnosed in 25 patients at a median of 50 (42 – 108) days after transplant. In 13 patients ADV infection was diagnosed (26%) by positive ADV PCR from a stool sample. In eight of these patients, we also detected ADV replication in the blood. The median onset of systemic adenovirus infection was 24 (16 – 43) days after transplantation.

Descriptive analysis of γδ T cell immune reconstitution after HSCT

CD3 cells quadrupled between day +30 and day +240 after transplantation. CD8 cell counts exceeded CD4 counts during the first 240 days after transplantation with the lowest CD4/CD8 ratio of 0.3 at day +100 after transplantation. Absolute and relative cell counts of T cells and their subsets are summarized in Table 2.

Counts of γδ T cells increased gradually after HSCT until day +240 after transplantation. Between day +30 and day +240 the proportion of γδ T cells of total CD3 cells was relatively stable between 4 and 6%. At day +240 the median count of γδ T cells (68/μl) was still lower than published reference values for healthy children (14). The Vδ2/Vδ1-ratio decreased continuously from day +30 to day +240 after transplantation and was < 1.0 after day +100, Figure 1.

Impact of donor source on immune reconstitution

We analyzed the impact of pre-transplant modalities on γδ T cell reconstitution. In univariate analysis patients with a related donor (MSD or MFD) showed significantly higher absolute Vδ2+ T cell counts compared to patients with a matched unrelated donor (MUD) at timepoints +30, +60, +100 ($p<0.0001$, respectively) and +180 ($p=0.004$), Figure 2. In multivariate analyses this result remained significant for timepoints days +30, +60, +100 ($q<0.001$, respectively) and +180 ($q<0.05$), Figure 3. The correlation was also highly significant ($q<0.001$) in a multivariate time-averaged analysis, Figure 4.

The amount of total γδ T cells in patients with a related donor was higher at days +30 and +60 ($q<0.001$, respectively) after transplantation but not in the time-averaged analysis. The only timepoint with an association between elevated Vδ1+ T cells and MSD/MFD as donor source was day +60 ($q<0.001$) after transplantation, Figure 3. In summary, the donor type seems to especially influence Vδ2+ subtype reconstitution.

The donor type was also correlated with a faster recovery of the αβ T cell compartment in (uni-) and multivariate analysis. Early after transplantation (day +30) CD4 cells, HLA-DR positive CD4 cells, CD8 cells and HLA-DR positive CD8 cells showed faster recovery in patients with MSD or MFD ($q<0.001$, respectively). At day +60 after HSCT having a related donor was independently associated with higher CD4 ($q<0.001$) and CD4 HLA-DR positive cell counts ($q<0.1$), Figure 3. In the multivariate time-averaged analysis only CD4 HLA-DR positive cells showed a positive correlation with MSD/MFD donor status, Figure 4.

TABLE 2 Absolute and relative concentrations of T cells and subsets during the first 240 days after transplantation.

	Day +15	Day +30	Day +60	Day +100	Day +180	Day +240
Cell subset concentration (cells/μl), median (IQR)						
CD3	6 [0, 43]	297 [112, 593]	620 [251, 1026]	486 [335, 1121]	1011 [668, 1693]	1202 [879, 1754]
CD4	2.09 [0.20, 25.45]	112 [31, 241]	157 [75, 239]	153 [108, 224]	365 [236, 511]	469 [336, 612]
CD8	2.51 [0.08, 17.06]	156 [38, 288]	451 [102, 792]	316 [111, 827]	407 [286, 1089]	539 [326, 987]
αβ T cells	4 [0, 37]	280 [106, 565]	610 [239, 915]	459 [300, 1041]	943 [609, 1526]	1152 [791, 1551]
γδ T cells	1 [0, 5]	10 [2, 29]	17 [6, 49]	27 [14, 71]	58 [41, 94]	68 [42, 141]
Vδ1	0 [0, 1]	2 [0, 6]	4 [2, 15]	13 [3, 36]	29 [12, 53]	34 [17, 53]
Vδ2	0 [0, 3]	4 [1, 20]	4 [1, 22]	5 [2, 18]	14 [4, 23]	19 [8, 33]
nonVδ1-nonVδ2	0 [0, 0]	1 [0, 3]	3 [1, 7]	4 [1, 14]	9 [5, 17]	12 [6, 21]
Relative concentrations, %						
CD4/CD3	49 [19, 56.5]	36 [20.5, 48.2]	28 [14, 52.5]	29 [16, 48]	38 [25, 60]	41 [25, 55]
CD8/CD3	32 [19.3, 43.8]	55 [34.3, 70.3]	68.5 [38.8, 78.8]	66 [43, 73]	55 [32, 67]	52 [38.3, 64.5]
αβ T cells/CD3	84.5 [64, 94]	96 [92, 97.3]	95 [91.3, 98]	95 [90, 97.5]	94 [91, 96]	93.5 [91, 95]
γδ T cells/CD3	14.5 [4.3, 27]	4 [2.8, 8]	4.5 [2, 8]	5 [2, 10]	6 [4, 8]	6.5 [5, 9]
Vδ1/γδ T cells	20 [3.5, 35.8]	26.5 [11, 41.8]	41 [19, 48.8]	50 [27, 61.5]	48 [29, 67]	51 [35, 65.8]
Vδ2/γδ T cells	65 [39.5, 81]	60.5 [34.8, 83]	38 [16.3, 61.3]	25 [10, 51.5]	26 [8, 50]	26.5 [9, 48.5]
nonVδ1-nonVδ2/γδ T cells	5 [0, 12.5]	7 [2.6, 14.3]	13.5 [7.3, 20.8]	16 [10, 23]	14 [10, 21]	14 [9, 19]

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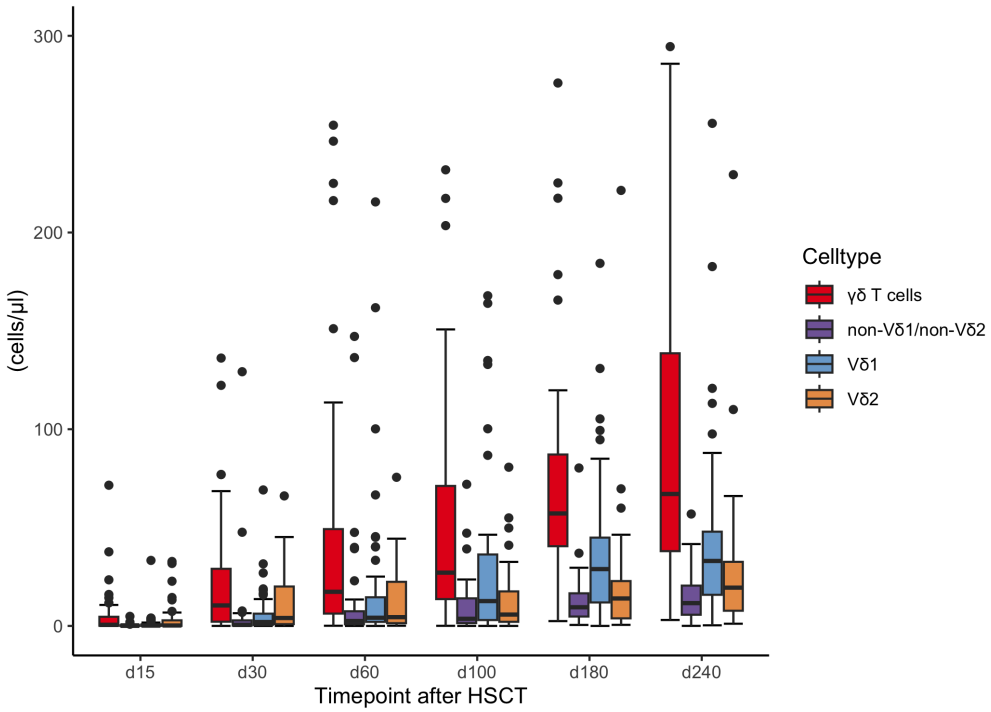

FIGURE 1
Concentrations of γδ T cells and subsets (Vδ1, Vδ2, non Vδ1-non Vδ2) during the first 240 days after transplantation. HSCT, Hematopoietic stem cell transplantation.

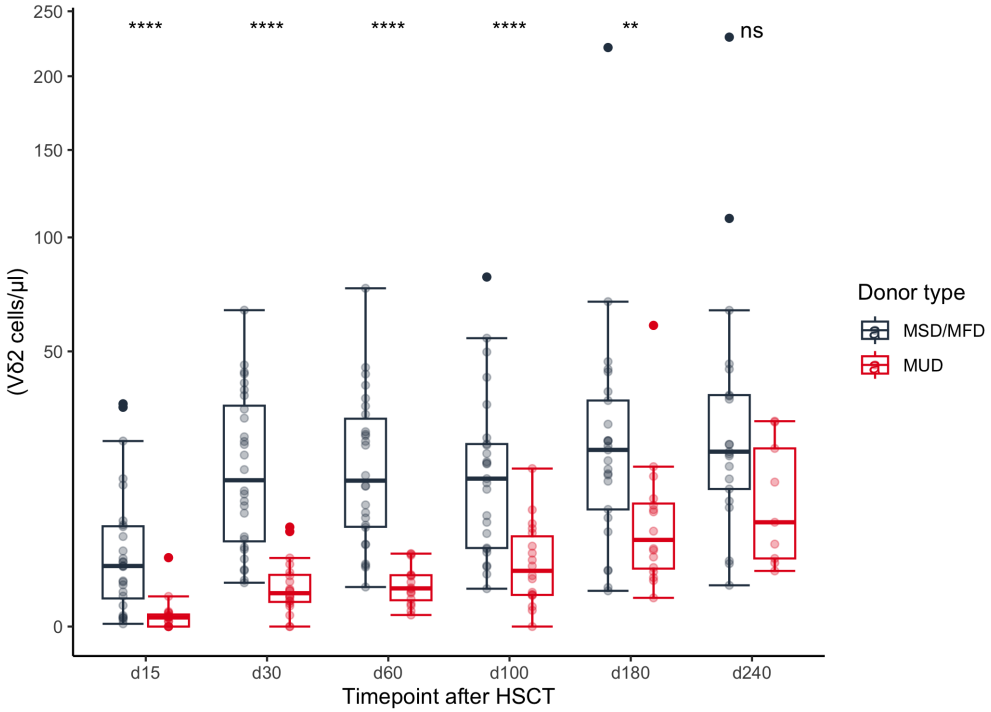

FIGURE 2
Concentrations of Vδ2 cells dependent on donor type during the first 240 days after transplantation. MSD, matched sibling donor; MFD, Matched family donor; MUD, Matched unrelated donor; HSCT, Hematopoietic stem cell transplantation. ** | p <= 0.01; **** | p <= 0.0001.

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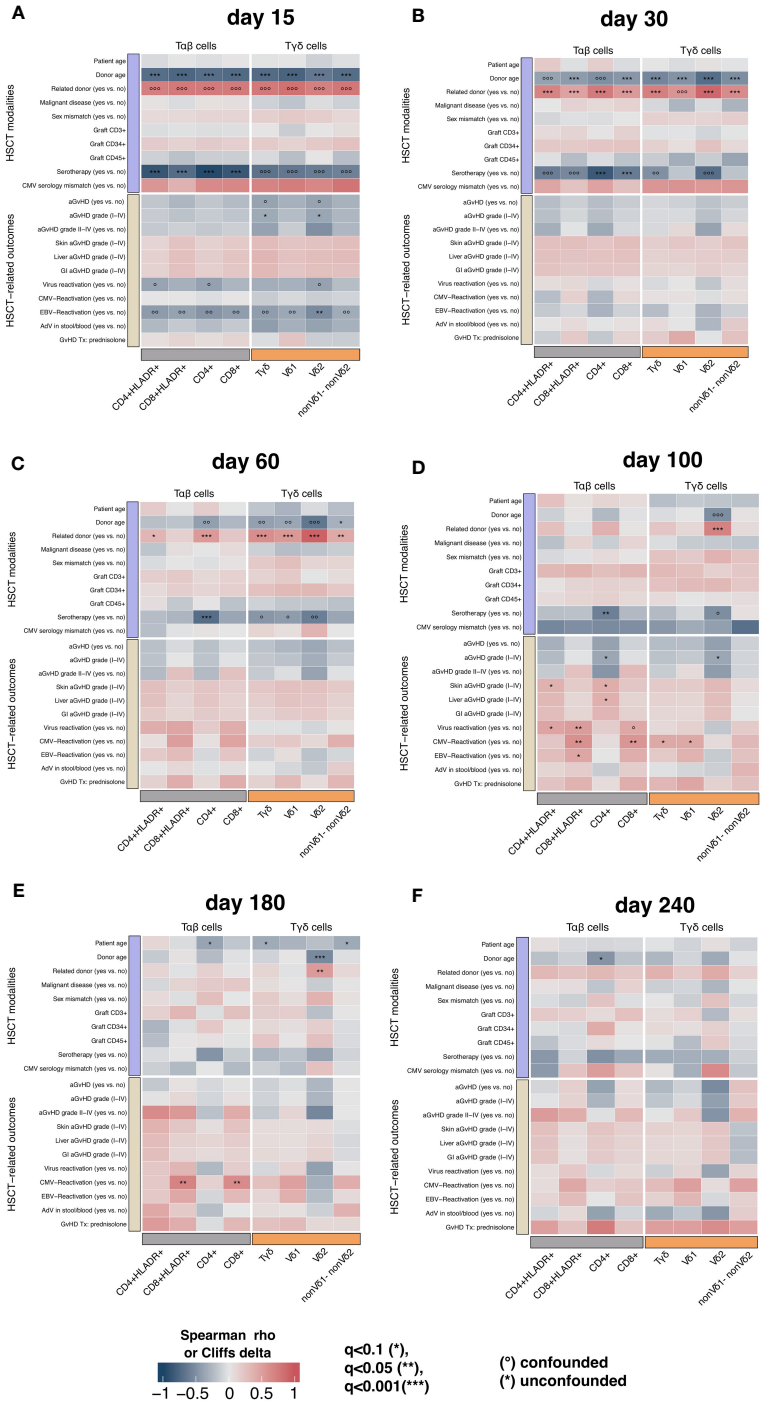

FIGURE 3
The correlation (in terms of spearman ρ or Cliffs δ) between T cell populations, transplant modalities and transplant outcomes at timepoints **(A)** day +15, **(B)** day +30, **(C)** day +60, **(D)** day +100, **(E)** day +180, **(F)** +240 with confounder analysis; confounded correlations are indicated with circles, while significant unconfounded are indicated with asterisk. A matched related donor (MSD or MFD) is the strongest transplantation modality correlating positively with γδ T cells and its subpopulations. HSCT, Hematopoietic stem cell transplantation; aGvHD, acute Graft-versus-Host-Disease; GI, gastro-intestinal.

Impact of other transplant modalities on immune reconstitution

In a multivariate analysis serotherapy with ATG or Alemtuzumab was independently associated with lower CD4 T cells at days +30, +60 (q<0.001, respectively) and +100 (q<0.05). This association was also highly significant in the multivariate time-averaged analysis (q<0,001). The same accounted for CD8 counts at day +30 (q<0.001). We saw no correlation between the use of serotherapy and the counts of total TCR γδ cells or subtypes. Supplementary Figure 3 shows a subgroup analysis of Vδ2+ T cell reconstitution in patients with malignant disease that received BM

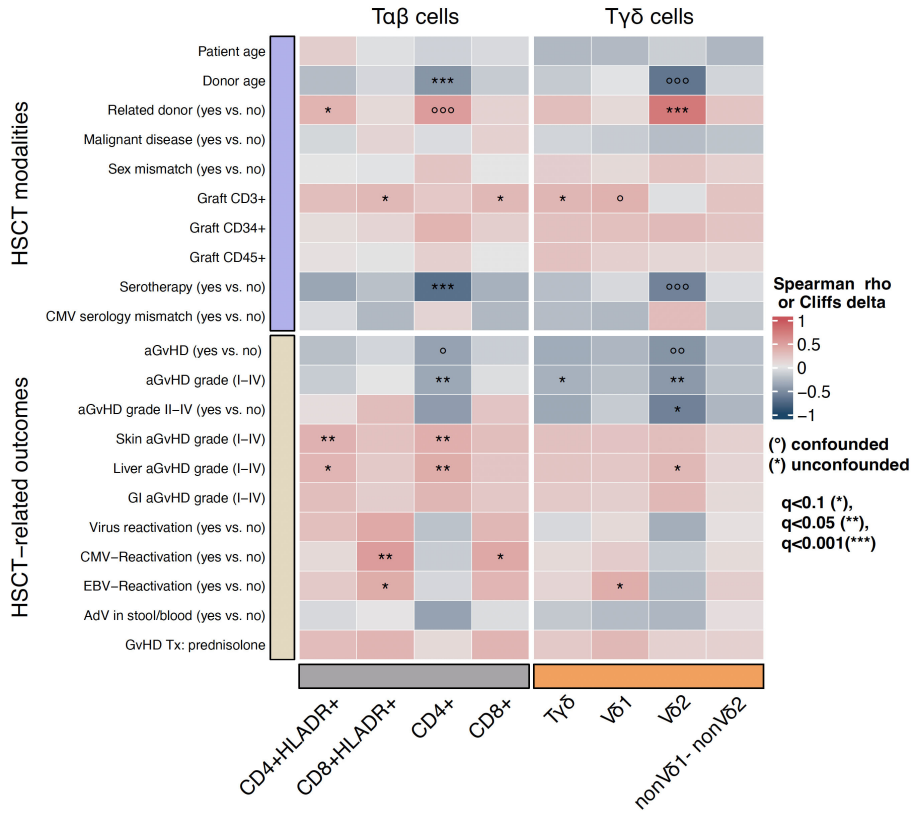

FIGURE 4
The correlation (in terms of spearman ρ or Cliffs δ) between the T cell populations, transplant modalities and transplant outcomes in a time-averaged approach, with confounder analysis, confounded correlations are indicated with circles, while significant unconfounded are indicated with asterisks. A matched related donor (MSD or MFD) is the strongest transplantation modality correlating positively with γδ T cells and its subpopulations. HSCT, Hematopoietic stem cell transplantation; aGvHD, acute Graft-versus-Host-Disease; GI, gastro-intestinal.

from a MRD without previous serotherapy compared to patients with sickle cell disease that received ATG before transplant from a MRD. Both groups presented high Vδ2+ T cell counts early after transplantation in contrast to patients with a MUD, highlighting serotherapy did not affect γδ T cell reconstitution in our cohort.

Higher donor age (age as a continuous variable) was associated with lower counts of total γδ, Vδ1+, Vδ2+, non-Vδ1/non-Vδ2 T cells (q<0,001, respectively), CD8 HLA-DR+ and CD8 cells (q<0,001, respectively) at day +30 and Vδ2+ T cells at day +180 (p<0.001). Donor age was also negatively associated with CD4 counts at day +240 (q<0.1) and in the time-averaged analysis (q<0,001), Figures 3, 4.

Immune reconstitution and clinical outcomes

Death and relapse

There was no difference in cumulative incidence of death between patients with high versus low TCR γδ cells and Vδ2+ or Vδ1+ subsets respectively. In the subgroup of patients with malignant hematological disorders we observed no statistical difference of relapse incidence between the two groups.

Acute Graft-versus-Host-Disease

In univariate analysis patients without aGvHD (grade I-IV) showed higher absolute median Vδ2+ T cell counts at day +60 (p=0.048), day +100 (p=0.023) and day +240 (p=0.027) compared to those with aGvHD (data not shown). Patients with higher grade aGvHD (grade II-IV) had higher Vδ2+ T cell counts at day +180 (p = 0.046), Figure 5. This result remained significant in multivariate time-averaged analysis (q<0.1), but not at single timepoints, Figures 3, 4. The grade of aGvHD was independently negatively correlated with the Vδ2+ T cell counts at day +100 (q<0.1) and in multivariate time-averaged analysis (q<0.05), Figures 3, 4. We observed no difference for total γδ T cells and Vδ1+ subset.

In a multivariate time-averaged analysis the grade of aGvHD was independently negatively correlated with the Vδ2+ T cell count (q<0.05), Figure 4. Patients with a high relative abundance of total γδ T cells (p=0.03) and Vδ2+ T cells (p=0.04) had a significantly lower cumulative incidence of grade II-IV aGvHD, Figures 6, 7. Patients with and without grade II-IV aGvHD showed a population inversion concerning the Vδ2/Vδ1-ratio after HSCT. While patients without grade II-IV aGvHD showed a trend towards recovering pre-HSCT Vδ2/Vδ1-ratio, those with grade II-IV aGvHD had an ongoing decline until day +240 after HSCT, Figure 8.

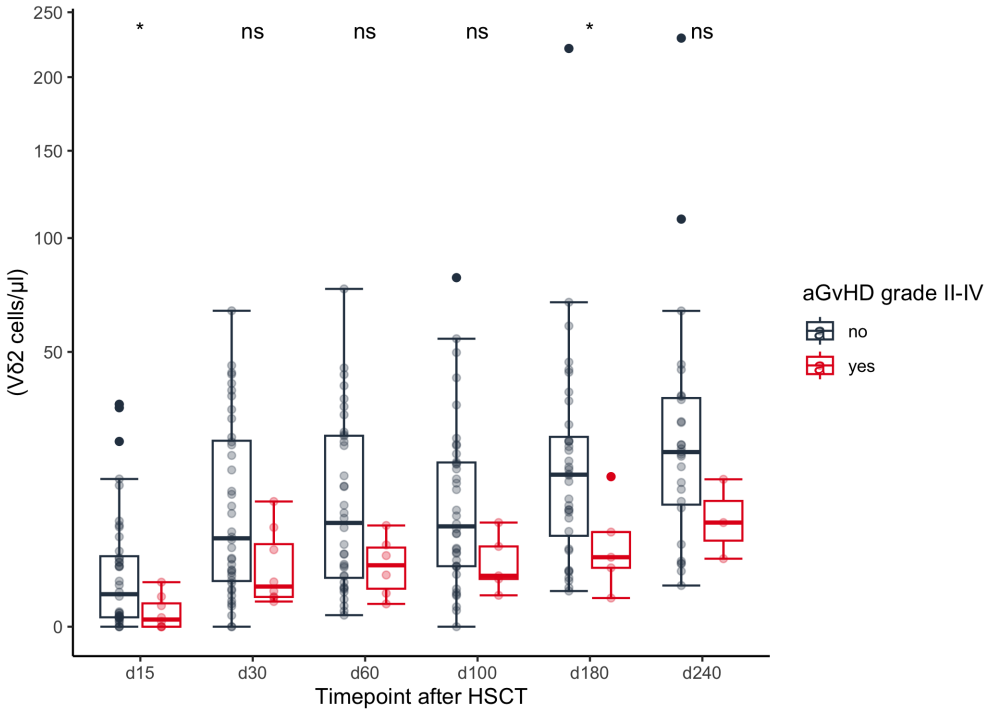

FIGURE 5
Comparison of absolute Vδ2+ T cell counts between patients with and without grade II-IV aGvHD. HSCT, Hematopoietic stem cell transplantation; aGvHD, acute Graft-versus-Host-Disease. ns | not significant; * | p <= 0.05.

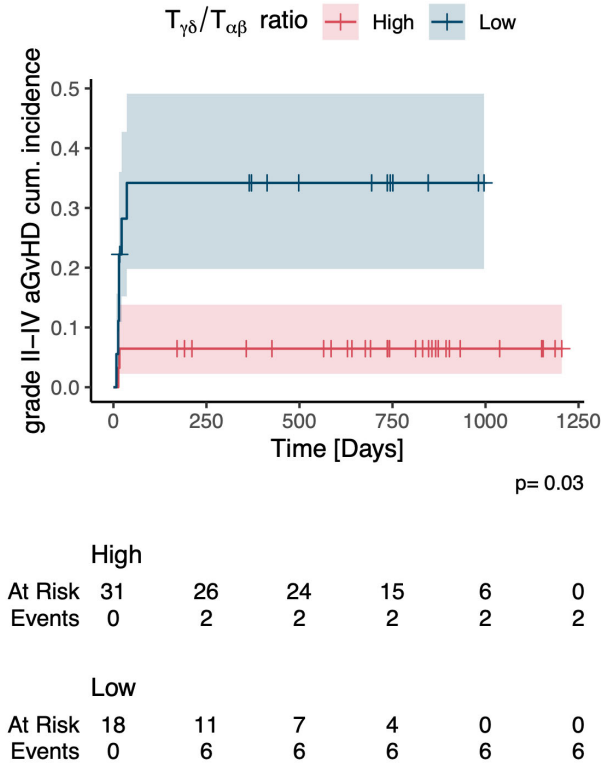

FIGURE 6
Cumulative incidence of grade II-IV acute GvHD in patients with a high vs. low relative abundance of γδ T cells. aGvHD, acute Graft-versus-Host-Disease.

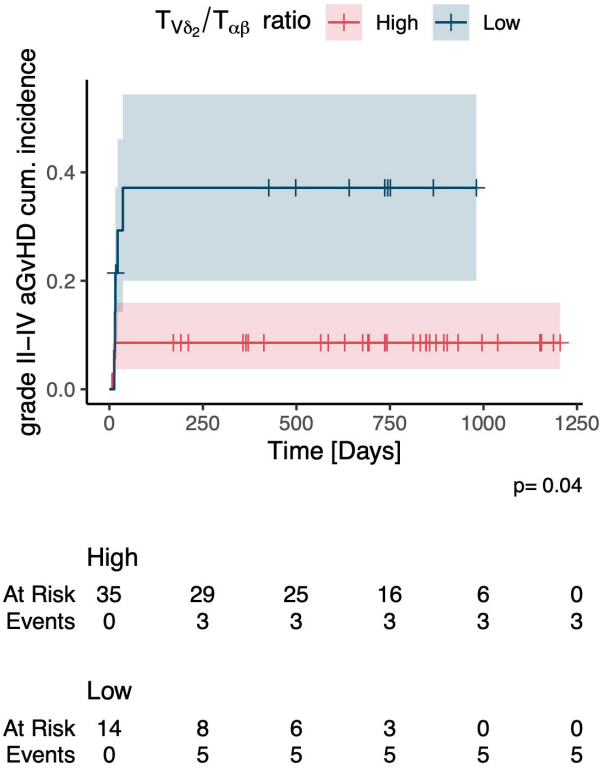

FIGURE 7
Cumulative incidence of grade II-IV acute GvHD in patients with a high vs. low relative abundance of Vδ2+ T cells. aGvHD, acute Graft-versus-Host-Disease.

We observed a lower cumulative incidence of aGvHD of the skin in patients with high total TCR γδ cells compared to those with low numbers (p=0.02), Supplementary Figure 4. No association was found for aGvHD of the liver or the gut.

Figure 9 illustrates the impact of aGvHD on immune reconstitution by using t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction and clustering of CD3 lymphocytes. Patients are stratified according to the grade of

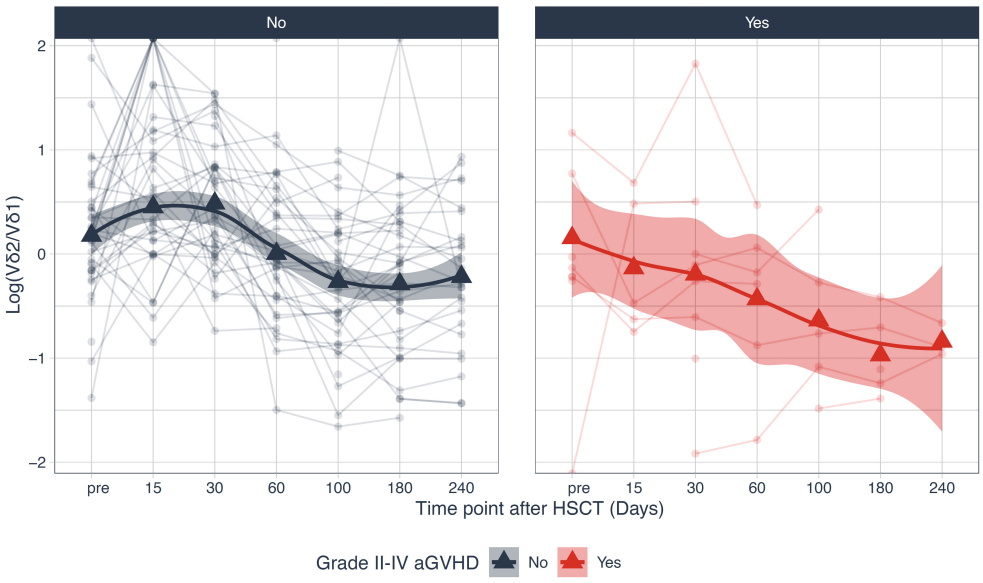

FIGURE 8
Logarithm of the Vδ2/Vδ1-ratio stratified over patients with and without grade II-IV aGvHD. HSCT, Hematopoietic stem cell transplantation; aGvHD, acute Graft-versus-Host-Disease.

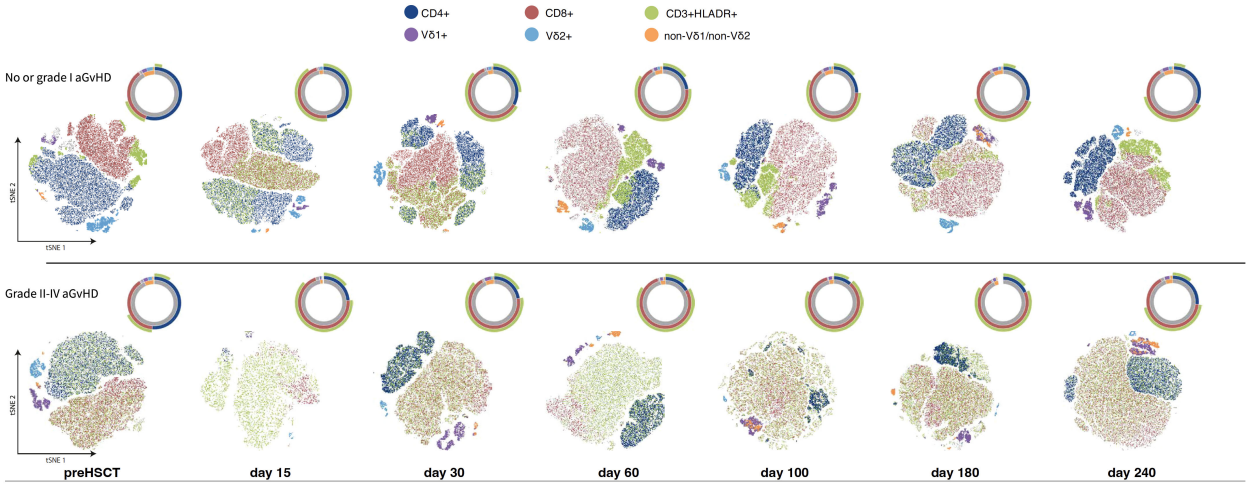

FIGURE 9
t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction and clustering of the CD3 cells, while the color indicates the gating performed on those cells. The cell populations are randomly subsampled from all the stratified samples according to the aGvHD grade status (no or grade I versus grade II-IV aGvHD) to a maximum of 200K events per time point. The counts of the gated T cell subpopulations are shown qualitatively in the sunburst diagrams.

aGvHD (no or grade I aGvHD vs. grade II-IV aGvHD). The CD4/CD8 ratio is smaller in patients with grade II-IV aGvHD and they show a higher proportion of activated HLA-DR positive lymphocytes on days +180 and +240. The fraction of Vδ2+ T cells is diminished in patients with higher grade aGvHD while the Vδ1+ and non-Vδ1/non-Vδ2 subset is present in both strata.

EBV infection

We examined the association between virus infection and γδ T cell reconstitution. In a first step we compared transplant modalities, HSCT outcomes and IR data of CD3, CD4, CD8, TCR αβ, TCR γδ, Vδ1, Vδ2 and non-Vδ1/non-Vδ2 T cells between patients with and without viral infections.

Patients with EBV infection had a higher median donor age (27.5 vs. 11 years, p=0.024) and more frequently received ATG (88% vs. 45.8%, p=0.004) or had an EBV-positive donor (100% vs. 68.2%, p=0.011) than patients without EBV infection. There was no significant difference concerning the distribution of underlying disease or conditioning regimens between the two groups. Rates of aGvHD and grade II-IV aGvHD were comparable between EBV-positive and EBV-negative patients (p=0.893 and 0.739, respectively). No significant difference was seen in CMV and ADV infection rates (p>0.2, respectively), Table 3.

There was no difference for total γδ T cells and Vδ2+ T cells in the TCR γδ cell compartment at any timepoint, but Vδ1+ T cell counts were significantly higher at day +100 and day +180 in patients with EBV infection (p=0.046 and 0.038, respectively), Supplementary Figure 5. This association was even more distinct in the analysis of relative Vδ1+ T cell counts (in percent of total TCR γδ cells). At timepoints +60 (p=0.036), +100 (p=0.006), +180 (p=0.002) and +240 (p=0.009) EBV-positive patients had a significantly higher percentage of Vδ1+ T cells than EBV-negative patients, Supplementary Figure 6.

In multivariate analysis the association between elevated Vδ1+ T cell counts and EBV infection remained significant in the time-averaged model (q<0.1) but not at single timepoints, Figures 3, 4.

As mentioned before, Vδ2+ T cell reconstitution was delayed in the whole cohort and especially in patients with grade II-IV aGvHD. We saw the same effect for patients with EBV infection. Vδ2/Vδ1-ratio decreased during the first 240 days after transplantation. In patients without EBV infection it stabilized and stayed >1, while in patients with EBV infection Vδ2/Vδ1-ratio continuously decreased from day +30 (1.5) until day +240 (0.2), Figure 10.

After stratification of the cohort into patients with high and low relative abundances of total TCR γδ, Vδ1+ and Vδ2+ T cells we registered a lower cumulative incidence of EBV-infection in patients with a high relative abundance of Vδ2+ T cells after HSCT compared to those with low Vδ2+ T cells (p=0.02), Figure 11.

CMV infection

Analogously, we studied the association of T cell subset reconstitution and the risk of CMV infection after HSCT. Patients with CMV infection had higher mortality (p=0.03) and higher rates of grade II-IV aGvHD compared to patients without CMV infection (p=0.006). The amount of CD45 positive cells in the bone marrow graft was significantly higher in the group without CMV infection (p=0.045). There was no difference concerning counts of CD3 and CD34 positive cells in the grafts and pre-transplant CMV status between the two groups, Table 4.

Patients with CMV infection had significantly higher CD8 counts at days +60, +100 and + 180 after transplantation

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TABLE 3 Distribution of transplant modalities and transplant outcomes between patients with and without EBV infection after transplantation.

		No EBV infection	EBV infection	p
n		24	25	
Diagnosis (%)	Acute lymphoblastic leukemia (ALL)	13 (54.2)	8 (32.0)	0.242
	Acute myeloid leukemia (AML)	2 (8.3)	4 (16.0)	
	Myelodysplastic syndrome (MDS)	2 (8.3)	0 (0.0)	
	Sickle cell disease (SCD)	5 (20.8)	5 (20.0)	
	ß-Thalassemia	0 (0.0)	3 (12.0)	
	Chronic granulomatous disease (CGD)	1 (4.2)	0 (0.0)	
	Severe combined immunodeficiency (SCID)	1 (4.2)	0 (0.0)	
	Severe aplastic anemia (SAA)	0 (0.0)	1 (4.0)	
	Hb-Yokohama	0 (0.0)	1 (4.0)	
	Fanconi anemia	0 (0.0)	1 (4.0)	
	Congenital amegakaryocytic thrombocytopenia (CAMT)	0 (0.0)	1 (4.0)	
	Osteopetrosis	0 (0.0)	1 (4.0)	
Donor type (%)	MSD	17 (70.8)	9 (36.0)	**0.027**
	MUD	7 (29.2)	13 (52.0)	
	MFD	0 (0.0)	3 (12.0)	
ATG (%)	No	13 (54.2)	3 (12.0)	**0.004**
	Yes	11 (45.8)	22 (88.0)	
Alemtuzumab (%)	No	22 (91.7)	24 (96.0)	0.971
	Yes	2 (8.3)	1 (4.0)	
Serotherapy (%)	No	11 (45.8)	2 (8.0)	**0.007**
	Yes	13 (54.2)	23 (92.0)	
Conditioning regimen (%)	VP16/TBI	3 (12.5)	6 (24.0)	0.198
	Flu/TT/Treo	14 (58.3)	9 (36.0)	
	Flu/TT/Mel	2 (8.3)	3 (12.0)	
	Flu/Bu/TT	0 (0.0)	1 (4.0)	
	Flu/TT	2 (8.3)	0 (0.0)	
	Flu/Cy	0 (0.0)	2 (8.0)	
	Flu/Bu/Cy/TT	0 (0.0)	2 (8.0)	
	Flu/Bu	2 (8.3)	0 (0.0)	
	Bu/Cy/Mel	1 (4.2)	1 (4.0)	
	Amsacrine/Flu/Cy/Ara-C/TBI	0 (0.0)	1 (4.0)	
Donor age (median [IQR])		11 [7, 24]	27 [11, 35]	**0.024**
EBV Status Donor (%)	negative	7 (31.8)	0 (0.0)	**0.011**
	positive	15 (68.2)	23 (100.0)	
EBV Status Patient (%)	negative	7 (31.8)	2 (9.1)	0.135
	positive	15 (68.2)	20 (90.9)	
aGVHD (%)	no	12 (50.0)	14 (56.0)	0.893

(Continued)

TABLE 3 Continued

		No EBV infection	EBV infection	p
	yes	12 (50.0)	11 (44.0)	
aGvHD grade II-IV (%)	no	21 (87.5)	20 (80.0)	0.746
	yes	3 (12.5)	5 (20.0)	
CMV (%)	no	19 (79.2)	16 (64.0)	0.391
	yes	5 (20.8)	9 (36.0)	
ADV (%)	no	20 (83.3)	21 (84.0)	1.000
	yes	4 (16.7)	4 (16.0)	

Significant p-values are indicated in bold.

(p=0.026, p=0.006, p=0.006, respectively). The same accounted for CD3 positive cells at the same timepoints (p=0.03, p=0.012, p=0.005, respectively) while there was no difference at any timepoint for CD4 positive cells. In the γδ T cell compartment we observed significantly higher amounts of total γδ T cells at day +100 (p=0.038) and of Vδ1+ T cells at days +100 and +180 (p=0.038 and p=0.041, respectively), Supplementary Figures 7-10. No difference was seen for Vδ2+ T cells.

Relative counts of γδ T cell subsets significantly differed between patients with and without CMV infection at day + 180. Counts of Vδ1 cells (in percent of total TCR γδ cells) were higher in patients with CMV infection (p=0.008), while relative counts Vδ2+ T cells were decreased consecutively (p=0.004), data not shown.

In multivariate analysis including the impact of various pre-transplant modalities and transplant outcomes CD8 cells were still significantly higher in patients with CMV reactivation at days +100 and +180 (q<0,05, respectively). Total γδ T cells and Vδ1+ T cells were higher at day +100 (q<0,1, respectively). CD8 counts were also higher at day +180 (p<0.05). In the multivariate time-averaged model the result remained significant for CD8 cells, Figures 3, 4.

After stratification into patients with high or low relative abundance of total TCR γδ, Vδ1+ and Vδ2+ T cells, we saw a lower cumulative incidence of CMV infection in patients with high total γδ T cells (p=0.02), Supplementary Figure 11. No difference was observed for patients with a high relative abundance of Vδ1+ or Vδ2+ T cells.

ADV infection

The group of patients that experienced systemic ADV infection (blood PCR positive) showed a significantly higher rate of aGvHD grade II-IV (p=0.022) and had been hospitalized significantly longer after HSCT than those patients without or with gastrointestinal ADV infection (66 days (60-88 days) vs. 48 days (44-58 days), p=0.009), data not shown.

We found no significant association between ADV infection (systemic as well as only gastrointestinal infection) and the reconstitution of any of the examined T cell subtypes.

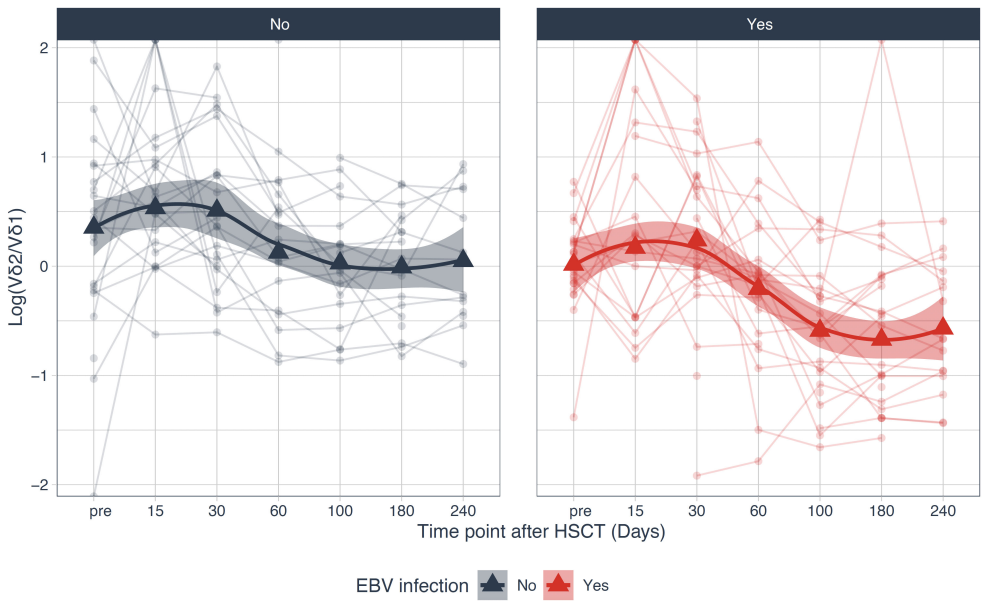

FIGURE 10
Logarithm of the Vδ2/Vδ1-ratio stratified over patients with and without EBV infection. HSCT, Hematopoietic stem cell transplantation.

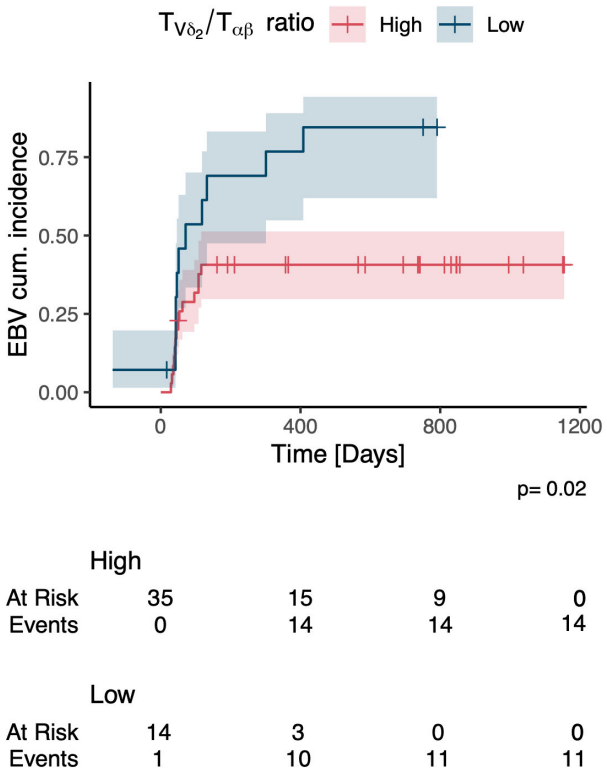

FIGURE 11
Cumulative incidence of EBV infection in patients with a high vs. low relative abundance of Vδ2 cells.

Discussion

In the past, various studies reported favorable outcomes in patients recovering with high γδ T cells after allogeneic HSCT, highlighting their potent anti-infectious and anti-tumorous abilities. Meanwhile data from pediatric cohorts and especially those with unmanipulated, HLA-matched grafts remain scarce. Despite promising results of transplant settings using haploidentical TCRab/CD19-depleted grafts in children (15), unmanipulated bone marrow grafts remain the standard of care for many transplant indications in the pediatric field. Both strategies differ fundamentally in terms of γδ T cell reconstitution due to the high content of these cells in the manipulated grafts. We performed a study on the dynamics and the clinical impact of γδ T cell reconstitution in the context of unmanipulated bone marrow transplantation.

One of the challenges in studying IR is defining thresholds for high and low γδ T cells. The definitions used in earlier studies differed widely and depended on the timepoint of assessment. Two studies defined high γδ T cell reconstitution as reaching a cut-off of 150 respectively 175 cells/μl at two consecutive timepoints in a timeframe of one year post HSCT (16, 17). Only about 10% of the included patients reached this cut-off, which bears the risk of an underestimated effect of robust γδ T cell reconstitution. A different approach is the use of median γδ T cell counts at certain timepoints for stratification. Minculescu et al. recently reported improved overall survival and relapse-free survival with lower incidence of aGvHD in adult patients with above median concentrations of γδ T cells at day +56 after HLA-matched, T cell replete stem cell transplantation (18). Other studies used relative cell counts at different single timepoints for stratification (19). Both strategies have their limitations as it is not clear which timepoint is most suitable for stratification because HCST complications occur at different stages after transplantation. Our approach to addressing the problem of stratification was using the time-averaged ratios R = TCR γδ/TCR αβ, Vδ2/TCR αβ and Vδ1/TCR αβ cells. The cut-off for high and low γδ T cells and subsets was found using clustering in the space of the ratio and the ranking of the patients based on it.

Few studies investigated the impact of graft sources on the reconstitution of γδ T cells. Perko et al. found significantly higher γδ T cell counts in patients with matched related donors (MRD) compared to matched unrelated donors (MUD) (17). Eyrich et al. described a subgroup of 12 pediatric patients that received bone marrow grafts from MSD in which some of the patients showed transient γδ T cell expansion early after transplantation, that did not occur in the other subgroup with $CD34^+$ selected PBSC grafts from unrelated donors (20). Others reported faster γδ T cell reconstitution in MSD/MUD transplantation compared to cord blood (21) or in patients with αβ-depleted PBSC grafts compared to $CD34^+$ selection (22).

In our study we investigated not only the correlation between donor source and reconstitution of total γδ T cells but also its

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TABLE 4 Distribution of transplant modalities and transplant outcomes between patients with and without CMV infection after transplantation.

		No CMV infection	CMV infection	p
n		35	14	
Diagnosis (%)	Acute lymphoblastic leukemia (ALL)	14 (40.0)	7 (50.0)	0.174
	Acute myeloid leukemia (AML)	2 (5.7)	4 (28.6)	
	Myelodysplastic syndrome (MDS)	2 (5.7)	0 (0.0)	
	Sickle cell disease (SCD)	9 (25.7)	1 (7.1)	
	ß-Thalassemia	3 (8.6)	0 (0.0)	
	Chronic granulomatous disease (CGD)	1 (2.9)	0 (0.0)	
	Severe combined immunodeficiency (SCID)	1 (2.9)	0 (0.0)	
	Severe aplastic anemia (SAA)	1 (2.9)	0 (0.0)	
	Hb-Yokohama	1 (2.9)	0 (0.0)	
	Fanconi anemia	0 (0.0)	1 (7.1)	
	Congenital amegakaryocytic thrombocytopenia (CAMT)	0 (0.0)	1 (7.1)	
	Osteopetrosis	1 (2.9)	0 (0.0)	
Conditioning regimen (%)	VP16/TBI	5 (14.3)	4 (28.6)	0.183
	Flu/TT/Treo	18 (51.4)	5 (35.7)	
	Flu/TT/Mel	4 (11.4)	1 (7.1)	
	Flu/Bu/TT	1 (2.9)	0 (0.0)	
	Flu/TT	2 (5.7)	0 (0.0)	
	Flu/Cy	1 (2.9)	1 (7.1)	
	Flu/Bu/Cy/TT	2 (5.7)	0 (0.0)	
	Flu/Bu	2 (5.7)	0 (0.0)	
	Bu/Cy/Mel	0 (0.0)	2 (14.3)	
	Amsacrine/Flu/Cy/Ara-C/TBI	0 (0.0)	1 (7.1)	
Graft CD3, cells/kg (median [IQR])		5,6 x 10^7 [3,2 x 10^7, 7,4 x 10^7]	4,3 x 10^7 [2,5 x 10^7, 5,6 x 10^7]	0.168
Graft CD34, cells/kg (median [IQR])		5,6 x 10^6 [3,5 x 10^6, 8,0 x 10^6]	4,2 x 10^6 [3,2 x 10^6, 5,9 x 10^6]	0.212
Graft CD45, cells/kg (median [IQR])		4,4 x 10^8 [3,1 x 10^8, 6,2 x 10^8]	3,1 x 10^8 [2,0 x 10^8, 4,5 x 10^8]	**0.045**
CMV Status Donor (%)	negative	14 (42.4)	3 (21.4)	0.299
	positive	19 (57.6)	11 (78.6)	
CMV Status Patient (%)	negative	14 (43.8)	3 (23.1)	0.338
	positive	18 (56.2)	10 (76.9)	
Death (%)	no death	34 (97.1)	10 (71.4)	**0.030**
	death	1 (2.9)	4 (28.6)	
NRM (%)	no	35 (100.0)	11 (78.6)	**0.030**
	yes	0 (0.0)	3 (21.4)	
aGVHD (%)	no	20 (57.1)	6 (42.9)	0.556
	yes	15 (42.9)	8 (57.1)	

(Continued)

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TABLE 4 Continued

		No CMV infection	CMV infection	p
aGvHD grade II-IV (%)	no	33 (94.3)	8 (57.1)	**0.006**
	yes	2 (5.7)	6 (42.9)	
EBV (%)	no	19 (54.3)	5 (35.7)	0.391
	yes	16 (45.7)	9 (64.3)	
ADV (%)	no	31 (88.6)	10 (71.4)	0.299
	yes	4 (11.4)	4 (28.6)	

Significant p-values are indicated in bold.

subsets Vδ1+, Vδ2+ and non-Vδ1/non-Vδ2 T cells. We could demonstrate that especially Vδ2+ T cell reconstitution is severely hampered in patients with non-related donors while patients with MSD or MFD showed fast Vδ2+ T cell recovery early after transplantation. This finding was independent of other transplant-related modalities that significantly influenced T cell reconstitution in our cohort like donor age and the use of serotherapy. Although the biological background of this finding needs further research, it bears many implications for transplant design. MSD HSCT remains the preferred donor source for many transplant indications as outcomes including GRFS (composite endpoint of graft-versus-host-disease-free and relapse-free survival), relapse-free survival and non-relapse mortality show better results compared to MUD transplantation (23). As Vδ2+ T cells are already known to have important antitumor and anti-infectious capacities, fast reconstitution of these cells might contribute to favorable outcomes in patients with matched related donors.

To what extend immune reconstitution is influenced by the method of stem cell harvest in a T cell replete setting is still unclear. As mentioned above, the study by Minculescu et al. showed superior outcomes in patients with high γδ T cells. Their cohort consisted mainly of adult patients receiving PBSC grafts from MRD/MUD donors and in contrast to our cohort, Vδ2+ T cells were the predominant subset found in the peripheral blood during the first year after transplantation. Nevertheless, they observed a shift towards Vδ1+ subset similar to the distribution in our pediatric cohort during that timeframe (18). If this difference is due to mobilization of peripheral blood stem cells is not clear and further studies are needed to identify the best transplant setting to facilitate fast γδ T cell recovery.

We report a lower cumulative incidence of grade II-IV aGvHD in patients recovering with a high relative abundance of Vδ2+ T cells. At the same time, we observed an inversed Vδ2/Vδ1-ratio after HSCT that has been described by other groups for the first few months after transplantation (22). In our cohort the population inversion persisted until day +240 after HSCT. Patients without extensive aGvHD showed a trend towards normalization of the ratio between day +100 and +240, contrary to patients with higher grade aGvHD. This evidence supports a protective effect of γδ T cells and especially Vδ2+ T cells by sustaining immune homeostasis and thereby avoiding aGvHD in the context of unmanipulated bone marrow transplantation. This hypothesis is supported by findings from pediatric studies with patients receiving TCR αβ/B cell-depleted grafts with high counts of γδ T cells (22). Although patients in this study did not receive GvHD-prophylaxis no severe aGvHD was registered. Still, other reports demonstrated an elevated risk of grade II-IV aGvHD in patients with γδ T cell-enriched graft composition (24) and several studies in mice reported that donor- as well as host-derived γδ T cells can promote aGvHD (25–27). These conflicting results emphasize the need for further research on the role of the different γδ T cell subtypes and the molecular pathways via which γδ T cells affect aGvHD pathogenesis.

Viral infections account for another large part of transplant-associated morbidity and mortality. In our cohort we observed a high EBV infection rate of 51%. One explanation of this finding might be the frequent use of serotherapy, mainly with ATG, in 73% of our patients. ATG is known to delay IR after HSCT and increase the risk of EBV infection and EBV-associated post-transplant lymphoproliferative disorder after HSCT (28).

Liu et al. reported a higher incidence of EBV infection in adult patients recovering with low absolute Vδ2+ T cell counts at day +30 after haploidentical transplantation (29). In our study we could reproduce the same significant association between EBV infection and reduced Vδ2+ counts using the time-averaged ratio R of Vδ2/TCR αβ. Our hypothesis, that early robust Vδ2+ T cell reconstitution might protect patients from developing EBV infection after HSCT is supported by groups that demonstrated the ability of Vδ2+ T cells to recognize and kill EBV-infected cells *in vitro* (29, 30).

Interestingly, we found that absolute Vδ1+ T cell counts were significantly increased at day +100 and +180 in patients with EBV infection. This was accompanied by a reversed Vδ2/Vδ1-ratio in patients with EBV infection between days +60 and +240. Like Vδ2+, Vδ1+ T cells expand upon activation with EBV-infected cells and skewing towards an oligoclonal Vδ1+ T cell repertoire after EBV infection in the context of HSCT has been observed (31). The same study reported that Vδ1+ T cells are capable of lysing EBV infected cells *in vitro*. Taking into consideration that EBV infection in our study occurred at a median of 50 days after transplantation, Vδ1+ expansion in patients with EBV infection after day +60 could be a sign of a

targeted immune reaction to clear EBV-infected cells. Furthermore, the high rate of EBV infection can serve as an explanation for the inversed Vδ2/Vδ1-ratio seen in our cohort. Whether Vδ1+ T cells detected in the peripheral blood are mobilized from epithelial tissue – its natural habitat – or proliferate upon contact with EBV-infected cells in a different location is not clear.

While expansion of Vδ1+ T cells after EBV infection is a relatively new finding, several studies have described a possible role of γδ T cells and especially Vδ1+ subset in CMV infection after HSCT (18, 22, 32, 33). Our findings support the existing evidence, adding data from a pediatric study population. We saw a lower cumulative incidence of CMV infection in patients recovering with a high relative abundance of total γδ T cells after HSCT. The Vδ1+ T cell subset expanded between day +60 and day +180 in patients with CMV infection, highlighting its capacity to recognize and kill CMV-infected cells (32). Our study cohort was too small to show any association between CMV infection and relapse rates. In 2011 Elmaagacli et al. reported lower relapse rates in adult AML patients who experienced CMV infection and Scheper et al. provided a possible explanation by showing that non-Vδ2+ T cells are able to cross-recognize leukemia cells and CMV-infected cells *in vitro* (34, 35).

In contrast to EBV and CMV disease we did not find any significant association between the reconstitution of γδ T cell subsets and ADV infection after HSCT. Interestingly, patients with systemic ADV infection had higher rates of grade II-IV aGvHD. This finding is in line with other studies that described aGvHD as a risk factor for ADV infection (36). At the same time, ADV might trigger intestinal aGvHD as hypothesized by a study that found higher rates of intestinal aGvHD in patients that had a positive ADV stool PCR before transplantation (37). Correspondingly, three of four patients in our cohort with intestinal aGvHD also presented with positive ADV PCR in stool and blood.

The results of our study are limited by the single-center design and the heterogeneity of the patient population. Still, it includes a relatively high number of patients for a pediatric HSCT study and all participants uniformly received unmanipulated bone marrow grafts, which is of great importance as graft source has a major impact on immune reconstitution.

In conclusion, we demonstrate that donor choice has a strong influence on γδ T cell reconstitution after pediatric HSCT with unmanipulated bone marrow grafts. Especially Vδ2+ T cell reconstitution was severely hampered in patients with MUD compared to those with MRD, which can contribute to the understanding of favorable outcomes after MRD transplantation. We report a lower cumulative incidence of grade II-IV aGvHD and EBV infection in patients recovering with a high relative abundance of Vδ2+ T cells. This supports a protective role of the Vδ2+ subset early after transplantation, a cell population that bears great potential as part of posttransplant adoptive T cell therapy (38, 39). First studies are currently performed exploring the potential of allogeneic γδ T cell transfer against aGvHD and the relapse of hematological malignancies after HSCT (40). Similar studies using ex vivo expansion and activation of γδ T cells are necessary to evaluate the efficacy in pediatric patients. Due to the high variability of γδ T cell reconstitution dynamics seen early after transplantation the ex vivo approach seems more promising than *in vivo* expansion methods. Establishing an effective but safe cell number for reinfusion will be a crucial point of the analysis.

Additionally, we observed expansion of Vδ1+ T cell subset in patients with CMV as well as with EBV infection. This new association in the context of EBV infection after HSCT can contribute to the process of understanding the role of γδ T cells in anti-virus immunity (41). This opens grounds for future research.

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 humans were approved by The Ethics Committee of Charité – Universitätsmedizin Berlin. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

TM: Visualization, Investigation, Data curation, Writing – original draft. LA: Writing – review & editing, Visualization, Methodology, Formal analysis. FP: Investigation, Data curation, Writing – review & editing. LZ: Formal analysis, Writing – review & editing, Investigation. MS: Methodology, Writing – review & editing. PH: Writing – review & editing, Investigation. AE: Supervision, Writing – review & editing, Investigation. JS: Writing – review & editing, Investigation. AS: Investigation, Writing – review & editing, Supervision. LO: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1433785/full#supplementary-material

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Human umbilical cord-derived mesenchymal stromal cells for the treatment of steroid refractory grades III-IV acute graft-versus-host disease with long-term follow-up

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Introduction: Mesenchymal stromal cells (MSCs) have been extensively studied as a potential treatment for steroid refractory acute graft-versus-host disease (aGVHD). However, the majority of clinical trials have focused on bone marrow-derived MSCs.

Methods: In this study, we report the outcomes of 86 patients with grade III-IV (82.6% grade IV) steroid refractory aGVHD who were treated with human umbilical cord-derived mesenchymal stromal cells (UC-MSCs). The patient cohort included 17 children and 69 adults. All patients received intravenous infusions of UC-MSCs at a dose of 1 × 106 cells per kg body weight, with a median of 4 infusions (ranging from 1 to 16).

Results: The median time between the onset of aGVHD and the first infusion of UC-MSCs was 7 days (ranging from 3 to 88 days). At day 28, the overall response (OR) rate was 52.3%. Specifically, 24 patients (27.9%) achieved complete remission, while 21 (24.4%) exhibited partial remission. The estimated survival probability at 100 days was 43.7%. Following a median follow-up of 108 months (ranging from 61 to 159 months), the survival rate was approximately 11.6% (10/86). Patients who developed acute lower GI tract and liver GVHD exhibited poorer OR rates at day 28 compared to those with only acute lower GI tract GVHD (22.2% vs. 58.8%; p= 0.049). No patient experienced serious adverse events.

Discussion: These finding suggest that UC-MSCs are safe and effective in both children and adults with steroid refractory aGVHD. UC-MSCs could be considered as a feasible treatment option for this challenging conditon. (NCT01754454).

KEYWORDS

hematopoietic stem cell transplantation, graft-versus-host disease, mesenchymal stromal cells, umbilical cord, long-term follow-up

Introduction

Acute graft-versus-host disease (aGVHD) is a severe complication following allogeneic hematopoietic stem cell transplantation (HSCT), primarily affecting skin, liver and gastrointestinal (GI) tract. Despite GVHD prophylaxis, approximately 50% of transplant recipients still develop aGVHD, and 11% develop grade III to IV aGVHD (1). The prognosis for patients with grade III to IV is dismal with a 2-year survival rate of 20% and a 5-year survival rate of 8% (2). Systemic steroids remain the standard first-line treatment for acute GVHD. Approximately 40%–50% of patients develop steroid-refractory acute GVHD, which is associated with poor OS (3, 4). Currently, ruxolitinib is the only therapy approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) for steroid refractory aGVHD. However, in the phase III randomized clinical trial, 38% of patients who received ruxolitinib did not achieve a CR or PR by day 28, and 60% of the patients required a third-line immunosuppressive therapy or had died by day 56 (5). An important clinical question is which treatment to use in patients with steroid refractory aGVHD who cannot afford ruxolitinib, not responding to ruxolitinib, with ruxolitinib toxicity (including cytopenias, infections), or contraindications.

In 2004, LeBlanc et al. pioneered the use of bone marrow-derived MSCs in the treatment of a pediatric patient with grade IV lower GI tract and liver aGVHD (6). This was followed by a phase II, multicenter clinical trial in 2008, which evaluated the efficacy of MSCs in steroid refractory severe aGVHD. Out of 55 patients, 39 responded favorably to MSCs treatment, without any side effects (7). Since then, MSCs have emerged as a promising therapeutic option for patients with steroid refractory aGVHD. In 2015, the Japanese Pharmaceuticals and Medical Devices Agency took the lead in granting approval to JR-031 (TEMCELL®) for the treatment of aGVHD in both children and adults.

Mesenchymal stem cells (MSCs) exhibit multi-lineage differentiation potential, along with nonspecific immunosuppressive and immunomodulatory effects. These cells can be isolated from various sources, including bone marrow (BM), adipose tissue, placental tissues and umbilical cord (UC) (8, 9).

Most of the large-scale analyses have focused on bone marrow-derived MSCs. However, bone marrow aspiration is an invasive procedure that may cause pain, infection or hemorrhage. And the number and function of BM-MSCs may influenced by donor age (10). Compared to BM, human umbilical cord (UC) can provide a large number of MSCs (11). It is a waste product after childbirth, and harvesting UC-MSCs does not involve invasive procedures, thus offering better ethical acceptance. What's more, UC-MSCs have demonstrated lower immunogenicity and higher proliferative capacity (12). Despite the use of UC-MSCs to treat steroid refractory aGVHD has demonstrated promising results, the OS by day 28 ranging from 59% to 80% (13–18), the number of clinical trials is relatively few, and the history of UC-MSCs application is shorter. In this study, we report the outcomes of 86 patients with grade III-IV steroid refractory acute GVHD (all involved lower GI tract) who received UC-MSCs as a salvage therapy and with a long period of follow-up. To the best of our knowledge, this is the largest cohort of 86 grade III-IV acute GVHD patients treated with UC-MSCs reported so far.

Subjects and methods

Patients

Patients of all ages experiencing steroid refractory grade III to IV acute GVHD, have lower GI tract involved, were eligible for this study. The grading and staging of acute GVHD were determined using the Modified Glucksberg Criteria (19). Steroid refractory GVHD was defined as progression of acute GVHD within 3-5 days or failure to improve within 5-7 days of treatment with 2mg/kg/day of prednisone (20). A total of 86 patients were enrolled in this study.

MSC manufacture and administration

MSCs were derived from UCs of unrelated HLA-mismatched donors. The culture and expansion of UC-MSCs were carried out by modifying methods previously published (21, 22). Briefly, UC tissues were digested with 0.05% type II collagenase (Sigma, St Louis, USA), and the cell suspension was collected by filtering through a stainless-steel mesh. The cells were resuspended in serum-free MSC culture media. After culturing, non-adherent cells were discarded. The adherent cells were detached using 0.05% Trypsin and 0.01% EDTA (Gibco, Grand Island, NY, USA). The fifth-passage cells were frozen. Each batch of UC-MSCs was characterized by flow cytometry for phenotype and, in some cases, tested for their ability to differentiate into adipocytes, osteoblasts, and chondrocytes. The UC-MSCs suspensions were cultured and tested negative for bacteria and mycoplasma contamination before infusion.

UC-MSCs therapy was initiated as soon as possible after the onset of steroid refractory grade III-IV acute GVHD. Patients received intravenous infusions of cryopreserved and freshly thawed MSCs at a dose of 1×106/kg, either once or twice a week, depending on their symptom severity. These infusions were given in conjunction with corticosteroids and cyclosporine until aGVHD showed a response.

Evaluation points

The primary endpoint of the study was to assess the efficacy of UC-MSCs therapy, which was evaluated based on complete response (CR), partial response (PR), and overall response (OR) rates. CR was defined as the complete resolution of all symptoms of aGVHD. PR was defined as a clinical improvement of at least one GVHD grade. OR encompassed both CR and PR. Response to UC-MSCs therapy was evaluated on day 28, day 56 and day 100 after the first MSC infusion, or on the date of death if it occurred before 28

days. Patients who showed no change in their disease status (stable disease, SD) or those who experienced worsening symptoms (progressive disease, PD) were classified as having no response (NR).

Statistical analysis

Response rates across different categories were compared using Fisher's exact test. This test allowed us to determine whether there were significant differences in response rated between different groups. To estimate the probability of survival, we used the Kaplan–Meier method. This method allows us to estimate the survival function from the observed data. We compare survival curves between groups using the log-rank test. A P-value of less than 0.05 was considered statistically significant, indicating that the observed differences in response rated or survival probabilities. All Statistical analyses were performed using the statistical software R.

Results

Patient characteristics

Between September 2010 and April 2018, a total of 86 patients were enrolled in this study. Patient characteristics are summarized in Table 1. The median age of these patients was 27.5 years old (ranging from 11 to 54 years). The majority of patients, 37 in total, received allogeneic HSCT due to acute myeloid leukemia (AML). Other indications for HSCT included acute lymphoblastic leukemia (ALL) in 27 patients, myelodysplastic syndrome (MDS) in 8, chronic myeloid leukemia (CML) in 4, aplastic anemia (AA) in 3, non-Hodgkin lymphoma (NHL) in 2, and other diseases in 5 patients. HSCT was performed using granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cells in 84 patients, BM in 1 patient, and a combination of both in another patient. Donors were either HLA-compatible in 19 cases or partially HLA matched in 67 cases. Myeloablative regimens were used in all cases. Forty-four patients received ATG as part of their conditioning regimen. GVHD prophylaxis consisted primarily of CsA combined with methotrexate and MMF in 44 patients. All patients developed lower GI tract aGVHD while receiving prophylactic immunosuppressive drugs. The median time from HSCT to the onset of lower GI tract aGVHD was 37 days (ranging from 7 to 216 days). The majority of patients, 71 out of 86 (82.6%), presented with grade IV acute lower GI tract GVHD. Additionally, 18 patients (20.9%) developed both acute lower GI tract and liver GVHD. None of the patients had used ruxolitinib.

Treatments of aGVHD before MSCs infusions

All patients enrolled in the study received steroids as first-line treatment for aGVHD, but none responded to this therapy.

TABLE 1 Characteristics of patients.

Sex, n(%)			
	Male	57	(66)
	Female	29	(34)
Age at HSCT, n(%)	**Median: 27.5 (11-54)**		
	<18 y	17	(20)
	18-25 y	21	(24)
	>25 y	48	(56)
Primary disease, n(%)			
	AML	37	(43)
	ALL	27	(31)
	MDS	8	(9)
	CML	4	(5)
	AA	3	(3)
	NHL	2	(2)
	Others	5	(6)
Disease status at HSCT, n(%)			
	CR	62	(72)
	PR	19	(22)
	NR	5	(6)
Type of donor, n(%)			
	MUD	52	(60)
	MSD	20	(23)
	Haploidentical	14	(16)
HLA typing, n(%)			
	HLA-identical	19	(22)
	9/10	21	(24)
	8/10	20	(23)
	7/10	7	(8)
	6/10	2	(2)
	5/10	15	(17)
	other	2	(2)
Hematopoietic stem cell source, n(%)			
	PBSC	84	(98)
	BM	1	(1)
	PBSC + BM	1	(1)
GVHD Prophylaxis, n(%)			
	CSA+MTX+MMF	44	(51)
	CSA+MTX +MMF+MVC	7	(8)
	CSA+MTX	33	(38)

(Continued)

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TABLE 1 Continued

	CSA+MTX+MVC	2	(2)
Onset of aGVHD (days)	**Median: 37 (7-216)**		
Gut aGVHD grade, n(%)			
	III	15	(17)
	IV	71	(83)
with liver aGVHD, n(%)			
	Yes	18	(21)
	No	67	(78)
	NR	1	(1)

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; AA, aplastic anemia; NHL, non-Hodgkin lymphoma; CR, complete response; PR, partial response; NR, no response; MUD, matched unrelated donor; MSD, matched sibling donor; PBSC, peripheral blood stem cell; BM, bone marrow; CsA, cyclosporine A; MTX, methotrexate; MMF, mycophenolate mofetil; MVC, maraviroc; ATG, antithymocyte globulin.

Subsequently, 30 patients (34.9%) received one or two second-line immunosuppressive drug (these patients were treated with either a CNI alone or in combination with Basiliximab), while 56 patients (65.1%) did not respond to three or more additional immunosuppressive therapies. The median time from the diagnosis of aGVHD to the first infusion of UC-MSCs was 7 days (ranging from 3 to 88 days). The cell dose per infusion was 1 x 106/kg. Each patient received a median of 4 infusions, with a range of 1 to 16 infusions. The UC-MSCs therapy was well-tolerated, and no acute or late side effects were observed during or after the infusions.

Response to MSC treatment

At day 28 post-treatment, over half of the patients (45/86, 52.3%) achieved an OR, including 24 patients (27.9%) who achieved a CR and 21 (24.4%) who achieved a PR (Figure 1). Forty-three patients did not respond to the treatment, with 21 (24.4%) showing SD, 14 (16.3%) showing PD, and 6 (7.0%) dying. Patients who developed both acute lower GI tract and liver GVHD had significantly worse OR at day 28 compared to patients who only had acute lower GI tract GVHD (22.2% vs. 58.8%; p= 0.049) (Figure 2).

There were no significant differences in clinical responsiveness based on age groups. Among children under 18 years old, 6 (37.5%) reached CR and 2 (12.5%) achieved PR on day 28. Among patients aged 18-25 years, 5 (25.0%) achieved CR, 6 (30.0%) achieved PR. Among patients above 25 years of age, 13 (26.0%) achieved CR, 13 (26.0%) PR. Similarly, there were no significant differences in OR between patients receiving once or twice weekly infusions of UC-MSCs (48.0% vs. 58.3%; p= 0.777). Furthermore, there were no differences in OR based on whether patients were in CR1 status before transplantation (50.0% vs. 54.1%, p=0.915) (Figure 2).

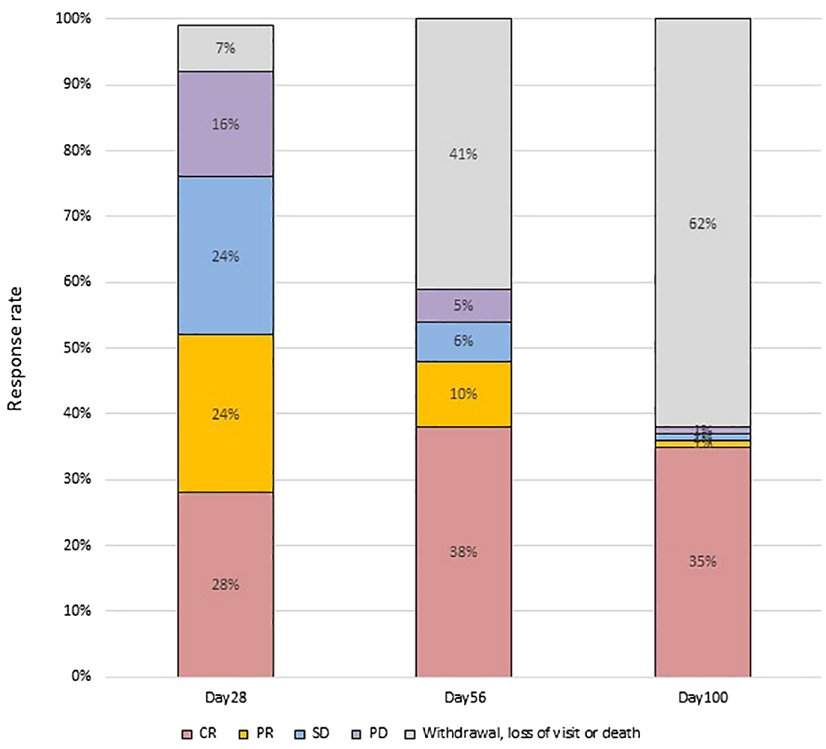

FIGURE 1
GVHD response and outcome.

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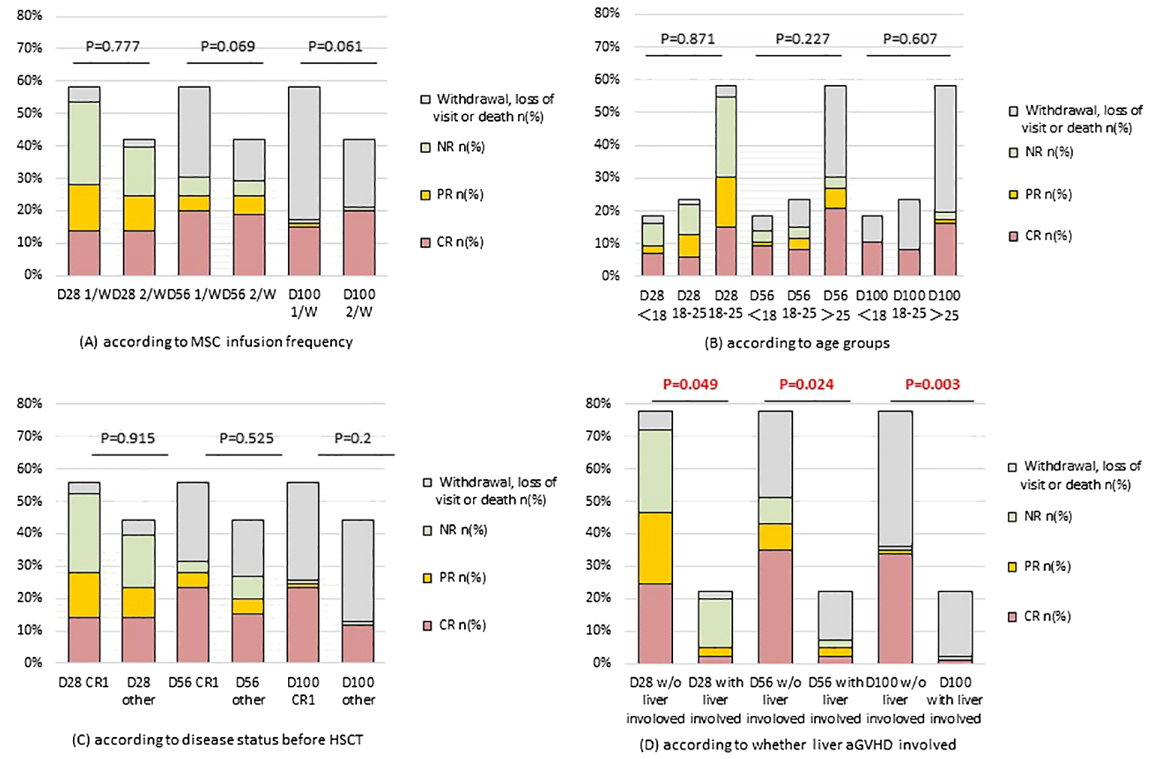

FIGURE 2
GVHD response in different subgroups. **(A)** according to MSC infusion frequency; **(B)** according to age groups; **(C)** according to disease status before HSCT; **(D)** according to whether liver aGVHD involved. D28/56/100: 28/56/100 days after the first infusion of UC-MSCs. W/O, without. NR, not response. PR, partial response. CR, complete response.

Survival

The overall survival (OS) at 100 days was 43.7% for the entire cohort of patients, and 60.0% for children specifically (P=0.297). When stratified by GVHD involvement, the OS was significantly higher for patients with only acute lower GI tract GVHD (52.6%) compared to those with liver involvement (11.1%) (P=0.003) (Figure 3).

As of the last follow-up in December 2023, 10 patients (11.6%) were alive, with a median follow-up duration of 108 months (ranging from 61 to 159 months) from the first infusion of UC-MSCs. Notably, none of the survivors experienced recurrence of their original disease or development of secondary tumors, leading to a DFS rate of 11.6%. Ten patients were out of touch and 66 had passed away: 17 due to infections, 8 due to disease relapses, 22 due to the progression of GVHD, 2 due to TMA, 1 due to bleeding, and for 16, the cause of death was indeterminate.

Discussion

The present study represents the largest single-center cohort of 86 patients treated with UC-MSCs for steroid refractory acute GVHD. It suggests the use of umbilical cord as a source of MSCs seems to have similar results with that of bone marrow. In our study, an OR of 52.3% (45 of 86 patients) was observed by day 28 following UC-MSCs infusion. This is comparable to a previous single-center study reporting an OR of 59% in 54 children treated with UC-MSCs for grades II-IV aGVHD (15). And our study indicates that UC-MSCs maintain its safety profile even after long-term follow-up.

Currently, BM-MSCs have been approved in Japan, Canada and New Zealand for the treatment of GVHD. Although clinical studies of MSCs for aGVHD have generally shown encouraging efficacy results, the response rates can vary. Many factors are likely to influence the outcomes: expansion protocols, MSC dose per infusion, number of infusions, patient age, and choice of second-line agents. The recent review concludes several trials with 30 or more patients treated with BM-MSC, the OS by day 28 ranging from 40% to 82.8% (23). In the field of UC-MSC therapy, Ding Y, et al. studied 54 patients with grade II-IV aGVHD, with the majority (74%) at grade III and 14.8% at grade IV. The median age was 12.5 years, spanning from 1 to 62 years old. The 28-day OR rate was 59.3% (15). Donadel CD, et al. presented data on 52 patients with grade II-IV aGVHD, with 25% at grade III and 71.2% at grade IV. The median age was also 12.5 years, but the age range was broader, from 0.3 to 65 years old. Their 28-day OR rate was slightly higher at 63.5% (16). Zhao, et al. reported on a less severe cohort of 25 patients with aGVHD of grade II-IV, with 44% having grade III/IV. This group had a higher median age of 37 years, ranging from 24.5 to 47 years old. They achieved a notably higher 28-day OR rate of 80% (18). The relatively inferior results reported in our study could be attributed to the severity of the disease in our

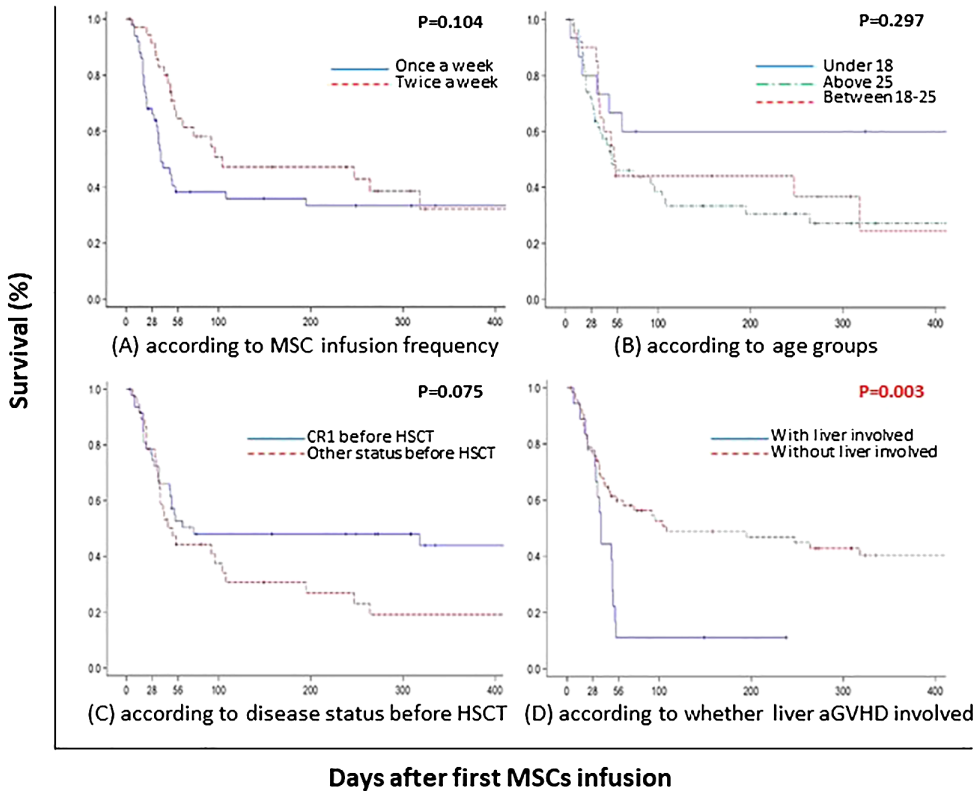

FIGURE 3
One-year survival estimates for patients with steroid-resistant and therapy-refractory III-IV acute GI GVHD from time of first MSC infusion. **(A)** according to MSC infusion frequency; **(B)** according to age groups; **(C)** according to disease status before HSCT; **(D)** according to whether liver aGVHD involved. Abbreviations: aGVHD, acute graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; CR, complete response; MSC, mesenchymal stem cell.

patient cohort. Most (82.6%) of our patients had grade IV steroid refractory aGVHD, which is much more severe than most published series (grade II to IV). Our study's emphasis is on GI aGVHD, with or without liver involvement. This is because skin-limited aGVHD (stage 4 limited-skin aGVHD are also categorized as grade IV) typically responds well to steroid therapy and is less likely to be life-threatening (24, 25). Additionally, most of our patients had received more than two additional treatments (none of the patients ever received ruxolitinib) before UC-MSCs infusion, indicating that they were receiving MSCs as a salvage therapy. It has suggested that patients with severe lymphodepletion due to GVHD and multiple immunosuppressive treatment regimens may lose responsiveness to MSCs (23). Despite these challenges, the results we obtained from such a highly challenging patient cohort (17.4% grade III and 82.6% IV) suggest the advantage of treatment of aGVHD with UC-MSCs.

The association between liver involvement and a worse response, as observed in our study, is consistent with other reports (15, 26, 27). This suggests that liver involvement is a prognostic factor for aGVHD, rather than a specific predictor for MSC therapy. However, it is noteworthy that a prospective randomized trial found that remestemcel-L led to significantly higher overall response rates in GVHD patients with liver involvement (28). This finding highlights that MSCs cannot be excluded from the treatment options for liver aGVHD patients. Further exploration of MSCs as a treatment option for aGVHD patient with liver involvement is needed.

Our study's observation of similar response rates between children and adults (P = 0.871) is consistent with recent reports on UCB-MSCs (15) and a study using Temcell (27). This suggests that MSCs may have similar efficacy in both pediatric and adult patients with aGVHD. However, this finding disagrees with most studies that have demonstrated a trend towards a better clinical response in children both in an UC-MSC report (16) or in BM-MSC reports (28–31). This discrepancy could be due to differences in patient populations, disease severity, or other confounding factors.

Regarding the frequency of MSC infusion, our study did not find a significant difference in response rates between once-weekly or twice-weekly infusions. However, Larger controlled studies are needed to definitively identify the optimal infusion schedule for MSCs in the treatment of aGVHD.

In our study, the administration of UC-MSCs did not elicit any adverse effects among patients, thus confirming its safety. As of the last follow-up in December 2023, our cohort, with a median follow-up of 108 months (ranging from 61 to 159 months) from the initial

UC-MSCs infusion, exhibited a survival rate of approximately 11.6% (10/86).

We noticed that a phase II study showed a pre-MSC six-biomarker (IL2Rα, TNFR1, HGF, IL-8, Elafin and Reg3α) panel and post-MSC ST2 levels were predictive of mortality (32). However, over the past few years, we have not made it a standard practice to monitor the cell subsets or plasma aGVHD biomarkers. In the future study, we will initiate the monitoring of biomarkers before and after administration of UC-MSCs therapy.

UC-MSCs offer a promising therapeutic potential due to their non-invasive acquisition and comparable clinical efficacy to bone marrow-derived stem cells. This study provides important insights into the clinical outcomes and safety profile of UC-MSC therapy in the treatment of aGVHD, particularly in patients with liver involvement and in both pediatric and adult populations. Future randomized, placebo-controlled, and double-blind studies are necessary to identify the most suitable patient populations, predictors of response, optimizing infusion schedules, and exploring combinations of MSCs with other immunosuppressive agents to further improve outcomes for patients with aGVHD.

Data availability statement

The datasets presented in this article are not readily available because only the editorial office could apply to the dataset during the review process. Requests to access the datasets should be directed to LH: huliangding@sohu.com.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Fifth Medical Center of PLA General Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

LH: Conceptualization, Project administration, Supervision, Writing – review & editing. J-WN: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. YL: Conceptualization, Methodology, Writing – review & editing. CX: Data curation, Methodology, Writing – review & editing. HS: Data curation, Resources, Writing – review & editing. CT: Resources, Writing – review & editing. HN: Methodology, Writing – review & editing. JH: Methodology, Writing – review & editing. JC: Methodology, Writing – review & editing. BL: Methodology, Writing – review & editing. JW: Methodology, Writing – review & editing. XL: Methodology, Writing – review & editing. NL: Methodology, Writing – review & editing. YFS: Methodology, Writing – review & editing. YS: Methodology, Writing – review & editing. ZQ: Methodology, Writing – review & editing. LW: Methodology, Writing – review & editing. YZ: Methodology, Writing – review & editing. SL: Methodology, Writing – review & editing. JX: Methodology, Writing – review & editing. JR: Methodology, Writing – review & editing. BP: Methodology, Writing – review & editing. SW: Methodology, Writing – review & editing. YS: Methodology, Writing – review & editing. LZ: Resources, Writing – review & editing. YJZ: Resources, Writing – review & editing. HC: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. BZ: Conceptualization, Data curation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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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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Human leukocyte antigen evolutionary divergence as a novel risk factor for donor selection in acute lymphoblastic leukemia patients undergoing haploidentical hematopoietic stem cell transplantation

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Introduction: The human leukocyte antigen (HLA) evolutionary divergence (HED) reflects immunopeptidome diversity and has been shown to predict the response of tumors to immunotherapy. Its impact on allogeneic hematopoietic stem cell transplantation (HSCT) is controversial in different studies.

Methods: In this study, we retrospectively analyzed the clinical impact of class I and II HED in 225 acute lymphoblastic leukemia patients undergoing HSCT from related haploidentical donors. The HED for recipient, donor, and donor-recipient pair was calculated based on Grantham distance, which accounts for variations in the composition, polarity, and volume of each amino acid within the peptide-binding groove of two HLA alleles. The median value of HED scores was used as a cut-off to stratify patients with high or low HED.

Results: The class I HED for recipient ($R\_HED^{class\ I}$) showed the strongest association with cumulative incidence of relapse (12.2 vs. 25.0%, P = 0.00814) but not with acute graft-versus-host disease. The patients with high class II HED for donor-recipient ($D/R\_HED^{class\ II}$) showed a significantly higher cumulative incidence of severe aGVHD than those with low $D/R\_HED^{class\ II}$ (24.0% vs. 6.1%, P = 0.0027). Multivariate analysis indicated that a high $D/R\_HED^{class\ II}$ was an independent risk factor for the development of severe aGVHD (P = 0.007), and a high $R\_HED^{class\ I}$ had a more than two-fold reduced risk of relapse (P = 0.028). However, there was no discernible difference in overall survival (OS) or disease-free survival (DFS) for patients with high or low HED, which was inconsistent with the previous investigation.

Discussion: While the observation are limited by the presented single center retrospective cohort, the results show that HED has poor prognostic value in OS or DFS, as well as the associations with relapse and aGVHD. In haploidentical

setting, class II HED for donor-recipient pair (D/R_HED$^{class\ II}$) is an independent and novel risk factor for finding the best haploidentical donor, which could potentially influence clinical practice if verified in larger cohorts.

KEYWORDS

human leukocyte antigen (HLA) evolutionary divergence (HED), acute lymphoblastic leukemia, haploidentical hematopoietic stem cell transplantation, donor selection, risk factor

1 Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative therapy for many hematopoietic disorders, including acute lymphoblastic leukemia (ALL) (1). The success of allo-HSCT partly depends on the recognition of tumor antigens presented to alloreactive T cells via human leukocyte antigens (HLAs). The importance of HLA matching is currently well established, resulting in a fully HLA-matched sibling or unrelated donor being the preferred source for allo-HSCT to reduce the risk of GVHD through allo-recognition of foreign HLA molecules (2, 3).

The divergence of HLA alleles may lead to an increased functional capability of the immunopeptidome, which would defend against potentially fatal opportunistic infections and leukemia cells causing relapse (4). Heterozygosity was typically used to assess the HLA allelic difference. Recently, HLA evolutionary divergence (HED), a metric reflecting the immunopeptidome diversity, has been utilized to more accurately quantify HLA allele divergence using the Grantham distance, which accounts for variations in the composition, polarity, and volume of each amino acid within the peptide-binding groove of two HLA alleles (5, 6). Previous research has linked the high heterozygosity of HLA class I loci to an improved response to immune checkpoint inhibitors in advanced cancer patients (7). Further, Chowell et al. found that the effect of HED on survival was independent of other clinically relevant variables and that a high HED in class I alleles was strongly related with response to checkpoint inhibitors in advanced cancer patients (8). These findings, however, were subsequently challenged by a study with a large cohort of cancer patients who had undergone anti-PD1 immunotherapy (9).

In the context of liver grafts, Feray et al. discovered that the donor's HED was an intrinsic feature completely independent of the recipient's characteristics and that a high class I HED of the donor was strongly related to a poor outcome (10). The influence of class I and II HED in the HSCT setting has primarily been explored in acute myeloid leukemia (AML). In AML patients, a high class I/class II HED ratio was revealed to be an independent factor for improved overall and disease-free survival (11, 12). More recently, HED was utilized to predict the outcome of children and young adults who underwent HSCT from an unrelated donor for a variety of malignant disorders (4). According to this study, patients with a high HED score of the combined HLA-B and -DRB1 loci had significantly increased overall and disease-free survival.

As an alternative donor transplant, HLA-haploidentical transplantation allows patients who do not have fully matched donors to undergo a transplant, and it has been increasingly used globally over the last two decades (13). In the haploidentical HSCT setting, almost all patients have more than one donor. As a result, the search for the best donor is a critical issue because donor selection can considerably affect the incidences of graft-versus-host, relapse, transplant-related mortality, and survival (13). Previous studies have identified a variety of characteristics that influence haploidentical outcomes, including HLA matching, donor age, donor sex, family relationships, and so on. These risk factors should be considered when selecting the best donor. However, the effects of HLA disparity on transplantation outcomes have vanished due to the improved protocols of haploidentical HSCT with anti-thymocyte globulin (ATG) or with post-transplantation cyclophosphamide (PT/Cy). If HLA disparity, either the quantity of HLA-mismatched loci or the mismatch combination of specific sites, is not a risk factor for haploidentical donor selection, it is currently unclear whether HED, which reflects HLA allele spatial epitope information, affects donor selection and clinical outcomes (4). To date, little is known about the impact of HED on outcomes in the HLA-haploidentical HSCT setting. In this study, we scored HED for donors, recipients, and donor-recipient pairs, and assessed the clinical significance of class I and II HED in 225 ALL patients who received HLA-haploidentical HSCT from a related donor. We found that the Grantham distance score of HLA evolutionary divergence was associated with acute GVHD and relapse in ALL patients undergoing HLA-haploidentical HSCT from a related donor, which may be considered a novel risk factor for donor selection in the haploidentical transplant setting.

2 Materials and methods

2.1 Patient characteristics

To investigate the influence of HED on clinical outcomes following HSCT, we conducted a retrospective analysis of consecutive Acute Lymphoblastic Leukemia patients (ALL) receiving allo-HSCT between 2012 and 2017 at Hebei Yanda Lu Daopei Hospital, Langfang City, PR

China. HED was calculated using data from all patients. The clinical data collected included graft-versus-host disease (GVHD), relapse, date of the event, survival status, and last follow-up date, etc. All patients were prepared for transplantation using modified myeloablative or reduced intensity conditioning regimens (based on total body irradiation, busulfan, or fludarabine, depending on the patient's comorbidities) (14). According to Chinese Bone Marrow Transplant Cooperative Group recommendations, GVHD prophylaxis was based on anti-thymoglobulin (ATG), cyclosporin A (CsA), methotrexate (MTX), and mycophenolate mofetil (MMF) (15–17).

This retrospective study was reviewed and approved by the Ethics Committee of Hebei Yanda Lu Daopei Hospital (DEPC-M-2023, No. 20). Before data collection, written informed consent was obtained from the patient or the patient's parents if the patient was under the age of 18. This study follows the Declaration of Helsinki.

2.2 HED calculation

HLA compatibility was determined at five loci (HLA-A, -B, -C, -DRB1, and -DQB1) using sequencing-based typing (SBT) GenDx excellerator kits (GenDX, Utrecht, Netherlands). The patient and donor two-field resolution typing of these HLA loci served as the input for the HED calculation, and the calculation was performed using a Python script according to the original Grantham distance formula presented in the literature (5).

For each donor and recipient, the HED score was determined by calculating the Grantham distance between the peptide-binding domains of the two alleles at the HLA loci (exons 2 and 3 for HLA-A, HLA-B and HLA-C, exons 2 for HLA-DQB1, HLA-DRB1) loci (6, 7). For donor-recipient pair, HED per locus was estimated for pairwise allele combinations between donors and recipients. We take HLA-A as an example to illustrate how HED between donors and recipients was calculated (Figure 1). If recipient has HLA-A allele 1 and 2, donor has HLA-A allele 3 and 4 (Figure 1). D_{ij} is Grantham distance between two alleles and calculated using the original formula (5) as follows:

$$D_{ij} = [\alpha(c_i - c_j)^2 + \beta(p_i - p_j)^2 + \gamma(v_i - v_j)^2]^{1/2}$$

Where i and j represent paired amino acids of the same position in the sequence of two alleles. c, p and v represent respective composition, polarity and molecular volume of the homologous amino-acids at a given position. α, β and γ are constants. HED between donor and recipient ($HED^{donor/recipient}$) was calculated by the sum of Grantham distance of four combinations for donor-recipient alleles, given by the formula:

$$HED^{donor/recipient} = \sum_{ij}(D_{ij}^{13} + D_{ij}^{14} + D_{ij}^{23} + D_{ij}^{24})$$

In the context of haploidentical HSCT, where donor and recipient always have one allele shared in any HLA locus, as shown in the diagram allele 1 = allele 3, the formula is:

$$HED^{donor/recipient} = \sum_{ij}(D_{ij}^{14} + D_{ij}^{23} + D_{ij}^{24}) = HED^{donor} + HED^{recipient} + \sum_{ij}(D_{ij}^{24})$$

Furthermore, if HLA-A matched (allele 1 = allele 3, allele 2 = allele 4), the formula is:

$$HED^{donor/recipient} = HED^{donor} + HED^{recipient}$$

The mean HED score of class I HLA ($HED^{class\ I}$) or class II HLA ($HED^{class\ II}$) was measured for donor, recipient, and donor-recipient, respectively. HED was denoted by the prefix R (Recipient), D (Donor), or D/R (Donor-Recipient pair). The median HED score was used as the threshold to define a high- or low-HED group.

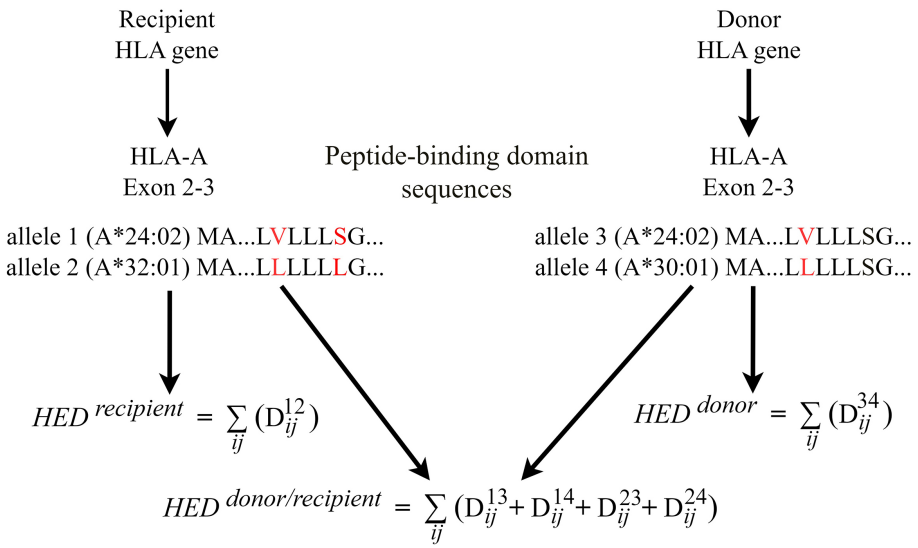

FIGURE 1
HED calculation flowchart for donors, recipients and donor-recipient pairs.

2.3 Clinical endpoints

The primary objective was to assess the impact of HED on relapse, non-relapse mortality (NRM), and acute and chronic graft-versus-host disease (GVHD). The secondary aim of the study was to assess the effect of HED on prognosis following haploidentical HSCT.

Endpoints of interest included the cumulative incidence of GVHD, relapse and NRM, overall survival (OS), and disease-free survival (DFS). aGVHD incidence was defined as time to first diagnosis of aGVHD (grade 2-4). Because acute GVHD, especially of grade 2 or higher, is probably the most suitable marker of morbidity, an additional sub-analysis for aGVHD (grades 3-4) was performed. Patients who survived more than 14 and 100 days following transplantation were evaluated for acute and chronic GVHD, respectively. The modified Keystone Criteria were used to grade aGVHD (18), while the National Institute of Health Consensus Criteria were used to evaluate cGVHD (19). Relapse incidence was defined as the time to relapse and death without prior recurrence. The NRM event was treated as a competing risk for relapse. NRM was defined as the time to death from any cause other than relapse. OS was defined as the time from transplantation to death, or the last follow-up. DFS was defined as the probability of survival without disease at any period following transplantation, with relapse or death considered events.

At the last follow-up, patients free from the event of interest were censored. The presence of 5% or more leukemic cells in the bone marrow and no indication of extramedullary localization was considered a hematological relapse.

2.4 Statistical analysis

Patient characteristics were summarized using descriptive statistics. Categorical variables are reported as counts (%), while continuous variables are described as the medians. The chi-square test, or Fisher's exact test, was used to assess differences in categorical variables across two groups. The Mann-Whitney U test was used to compare the intergroup continuous variables.

Cumulative incidences of GVHD, relapse, and NRM were estimated with the methods of Fine and Gray considering the respective competitive risks; comparisons between the high and low HED groups were performed with Gray's test. The Kaplan-Meier survival curve was used to estimate the probability of OS and DFS, and the significance was determined with a log-rank test. Potential risk factors were identified using the univariate Cox regression method to assess the hazard ratio (HR) for the various factors associated with clinical outcomes. Multivariate Cox regression analysis retained significant HED and other variables that might have been clinically meaningful or statistically significant in univariate analysis (P<0.2). The final multivariate models were built using a backward stepwise model approach.

Variables considered in the multivariate models were donor sex, donor and patient age, donor-recipient HLA disparity, donor-recipient family relationship, disease status at transplant (non-remission vs. complete remission), and donor-recipient sex matching. KIR matching and the HSCT-specific comorbidity index were not included due to insufficient data.

All tests were two-sided, and $P<0.05$ was considered statistically significant. The date collected is as of December 31, 2017. Statistical analysis was performed using the SPSS 25 package (SPSS Inc., Chicago, USA) and a graphical user interface for R language, EZR version 1.32 (20).

3 Results

3.1 Patient characteristics

The study comprised 225 ALL patients who had HSCT from a related donor between 2012 and 2017. Most of the transplants (179) were parents as donors. Thirty-nine transplants were siblings as donors. The median age was 15 years, with the range of 2 to 48 years, and the median follow-up time following transplantation was 35.8 months (range, 1-83.9). High-resolution HLA typing revealed that 146 (64.9%) of 225 donor-recipient pairs had five mismatches, 43 (19.1%) had four mismatches, and 36 (16.0%) had three or fewer HLA mismatches. Thirty-one individuals (13.8%) had active disease at the time of transplantation. Table 1 summarizes the patient demographics and characteristics.

3.2 HED scores

We estimated HED strictly following the original formula of Grantham distance. Our HED value for class I was 3.56 times higher than Pierini and Lenz's (6), and for class II, it was 1.75 times higher (Supplementary Method). This discrepancy is due to differences in data processing, but there is a clear and straightforward relationship between the two calculation methods, thus they can be considered identical in clinical investigations.

For recipients, HLA-B locus showed the highest HED variability (R_HED^{B}, median 29.7), followed by HLA-A (R_HED^{A}, median 26.8), HLA-DRB1 and -DQB1 (R_HED^{DRB1} and R_HED^{DQB1}, median 26.5 and 22.5, respectively), and HLA-C locus displayed the lowest HED variation (R_HED^{C}, median 19.8) (Figure 2A). HLA-B evolutionary divergences were greater than HLA-A and HLA-C, supporting previous findings that HLA-B is the most ancient and diverse of the three HLA-class I loci (6). Class I HLA had a slightly higher mean HED ($R\_HED^{class\ I}$) than class II HLA ($R\_HED^{class\ II}$) (median 23.9, 22.9, respectively) (Figure 2A). The variance and distribution pattern of donor HED were quite comparable to that of the recipient, with HLA-B having the highest value (D_HED^{B}, median 29.8) and HLA-C having the lowest (D_HED^{C}, median 20.3) (Figure 2B).

Despite the fact that the HED scores for donor-recipient pairs were much higher than those of the donor or recipient due to the computed divergence among the four alleles, the HED distribution and variation patterns of each locus or class were identical to those

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TABLE 1 Patient characteristics.

Variable		N	% or range
Age at transplant (yr.)	Median	15	2-48
Gender	Female	81	36%
	Male	144	64%
Donor-recipient relationship	Parent-child	179	79.6%
	Child-parent	7	3.1%
	Sibling-sibling	39	17.3%
HLA-matching	5/10	146	64.9%
	6/10	43	19.1%
	7/10	23	10.2%
	8/10	8	3.6%
	9/10	5	2.2%
Disease status at HSCT	CR1	103	45.8%
	CR2	73	32.4%
	Active disease	49	21.8%
Conditioning regimen	MAC	101	44.9%
	RIC	124	55.1%
TBI	Yes	213	94.7%
	No	12	5.3%
Acute GVHD	Yes	123	54.7%
Chronic GVHD	Yes	161	71.6%
Time from HSCT to aGVHD occurrence (days)	Median	60	4-240
Time from HSCT to cGVHD occurrence (days)	Median	180.5	28-4170
Time from HSCT to relapse (days)	Median	984.5	18-2491
CMV reactivation		144	64.0%
EBV infection		73	32.4%

CMV, cytomegalovirus; CR, complete remission; EBV, Epstein-Barr virus; GVHD, graft-versus-host disease; MAC, myeloablative conditioning; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; RIC, reduced intensity conditioning; TBI, total body irradiation.

of the donor or recipient. The highest was D/R_HED^{B} (median 84.2), followed by D/R_HED^{DRB1} (median 72.7), D/R_HED^{A} (median 72.0), and D/R_HED^{DQB1} (median 64.4), while the lowest was D/R_HED^{C} (median 55.4). D/R_HED$^{class\ I}$ was higher than D/R_HED$^{class\ II}$ (median 69.9 versus 67.4) (Figure 2C).

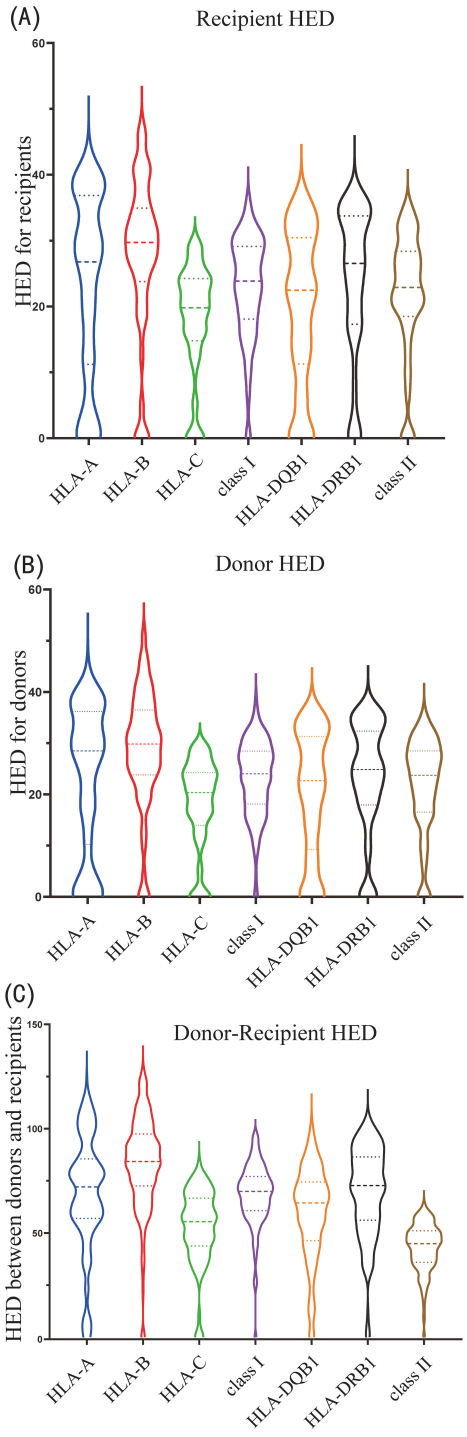

FIGURE 2
Violin plot of HLA evolutionary divergence **(HED)** distribution for **(A)** recipients, **(B)** donors, and **(C)** donor-recipient pairs.

3.3 GVHD

The overall cumulative incidences of grade 2-4 and 3-4 aGVHD at 100-day were 36.5% (95% confidence interval [CI]: 29.9-43.0%), and 15.1% (95% CI: 10.3-20.7%), respectively. Neither the donor (D_HED$^{class\ I}$, D_HED$^{class\ II}$) nor recipient HED values (R_HED$^{class\ I}$, R_HED$^{class\ II}$) had any effect on aGVHD. Surprisingly, the HED score of donor-recipient pair (D/R_HED$^{class\ II}$) was significantly associated with the cumulative incidence of grade 3-4 aGVHD at 100-day. The incidence of grade 3-4 aGVHD was 24.0% (95%CI:15.7-33.3%) in patients with high D/R_HED$^{class\ II}$ compared to 6.1% (95%CI: 2.5-12.2%) in patients with low D/R_HED$^{class\ II}$ (P = 0.0027) (Table 2,

TABLE 2 Cumulative incidences (%) of Relapse, NRM, cGVHD and aGVHD based on HED$^{class\ I}$ and HED$^{class\ II}$.

Factor	Group	Relapse*	NRM*	aGVHD§	cGVHD*
D_HED$^{class\ I}$	High	11.8 (6.6-18.6)	21.9 (14.7-30.1)	16.6 (9.7-25.2)	79.4 (69.4-86.5)
	Low	25.2 (17.5-33.7)	23.2 (15.9-31.4)	13.5 (7.5-21.3)	78.9 (68.4-86.3)
	P value	0.0123	0.808	0.928	0.666
R_HED$^{class\ I}$	High	12.2 (6.8-19.3)	25.5 (17.7-33.9)	14.7 (8.4-22.7)	82.7 (72.0-89.6)
	Low	25.0 (17.4-33.3)	19.7 (12.9-27.6)	15.4 (8.8-23.7)	76.7 (66.4-84.2)
	P value	0.00814	0.246	0.858	0.355
D/R_HED$^{class\ I}$	High	12.7 (7.3-19.7)	23.6 (16.2-31.9)	18.3 (11.1-26.8)	78.8 (68.6-86.0)
	Low	24.2 (16.7-32.5)	21.5 (14.4-29.5)	11.6 (6.0-19.1)	80.1 (69.6-87.2)
	P value	0.0232	0.631	0.331	0.533
D_HED$^{class\ II}$	High	18.1(11.5-25.9)	24.2 (16.7-32.5)	18.1 (10.8-26.9)	79.6 (69.3-86.7)
	Low	19.1(12.3-27.0)	20.9 (13.8-29.0)	12.1 (6.5-19.5)	79.0 (68.6-86.3)
	P value	0.796	0.556	0.402	0.702
R_HED$^{class\ II}$	High	15.8 (9.6-23.4)	20.2 (13.2-28.2)	15.3 (8.8-23.6)	74.8 (64.2-82.6)
	Low	21.2 (14.2-29.2)	24.9 (17.3-33.2)	14.7 (8.4-22.8)	84.3 (74.2-90.7)
	P value	0.293	0.421	0.903	0.0886
D/R_HED$^{class\ II}$	High	19.3 (12.5-27.2)	23.9 (16.3-32.2)	24.0 (15.7-33.3)	73.0 (62.0-81.3)
	Low	18.0 (11.4-25.7)	21.3 (14.3-29.4)	6.1(2.5-12.2)	85.6 (75.8-91.7)
	P value	0.775	0.674	0.0027	0.0311

*Cumulative incidence (%) at 5 years; §Incidence of grade 3-4 aGVHD at 100 days; NRM, non-relapse mortality; Numbers in parenthesis indicate 95% Confidence Interval. HED's prefix D, R, and D/R indicate donor, recipient and donor-recipient pair, respectively.

Figure 3A). The favorable impact of D/R_HED$^{class\ II}$ appears to be primarily driven by D/R_HED^{DRB1}. The higher the D/R_HED^{DRB1}, the higher the incidence of grade 3-4 aGVHD (23.4% [95%CI:15.1-32.8%] vs 7.2% [95%CI: 3.1-13.5%], P = 0.0047).

The 5-year cumulative incidence of cGVHD was unexpectedly high, at 80.4% (95%CI: 74.1-86.0%). In contrast to the results for aGVHD, the cumulative incidence of cGVHD at 5-year was significantly associated with higher D/R_HED$^{class\ II}$ (P = 0.0311), with higher D/R_HED$^{class\ II}$ being associated with lower cGVHD risk (73.0% vs. 85.6%), but not with D/R_HED$^{class\ I}$ (P = 0.533) (Table 2). D/R_HED$^{class\ II}$ was therefore included in the subsequent cox regression analysis for GVHD. Regardless of the negative association with D/R_HED^{B}, there was no significant correlation between cGVHD and D/R_HED$^{class\ I}$.

3.4 Relapse and NRM

Forty-four of 225 (19.6%) patients relapsed at a median time of 984.5 days (range 18-2491) after transplantation. The 5-year cumulative incidence of relapse (CIR) for all patients after transplantation was 18.6% (95% CI: 13.7-24.0%). The cumulative incidence of NRM at five years was 22.6% (95% CI: 17.3-28.3%), which was greater than the 5-year CIR.

When patients are stratified based on HED$^{class\ I}$ or HED$^{class\ II}$, all three HED$^{class\ I}$ (D_HED$^{class\ I}$, R_HED$^{class\ I}$, and D/R_HED$^{class\ I}$) scores show an obvious association with CIR (Table 2). Higher D_HED$^{class\ I}$ and D/R_HED$^{class\ I}$ contribute to a lower 5-year CIR (11.8 vs. 25.2%, P = 0.0123; 12.7% vs. 24.2%, P = 0.0232) (Table 2). R_HED$^{class\ I}$, in particular, exhibited the strongest association with 5-year CIR (12.2 vs. 25.0%, P = 0.00814) (Table 2, Figure 3B). Conversely, neither HED$^{class\ II}$ were correlated with 5-year CIR. Thus, the three HED$^{class\ I}$(D_HED$^{class\ I}$, R_HED$^{class\ I}$, and D/R_HED$^{class\ I}$) were used as candidate risk factors for subsequent Cox regression analysis. The cumulative incidence of NRM at five years was not associated with any HED$^{class\ I}$ or HED$^{class\ II}$. These findings suggest that genetic divergence of class I HLA, rather than class II HLA, may be responsible for the differences in CIR, but that genetic differentiation of either class I or II HLA loci has little effect on NRM.

3.5 Multivariate analysis

The impact of HED on GVHD and relapse was further investigated using the Cox proportional hazard regression analysis with consideration of other risk factors in multivariate analysis. The univariate analysis for GVHD, relapse and DFS is shown in Supplementary Table 1.

The multivariate regression analysis revealed that the low R_HED$^{class\ I}$ group had a more than two-fold greater risk of relapse (HR = 2.101 [95%CI: 1.083-4.078], P = 0.028) (Table 3, Figure 3B,

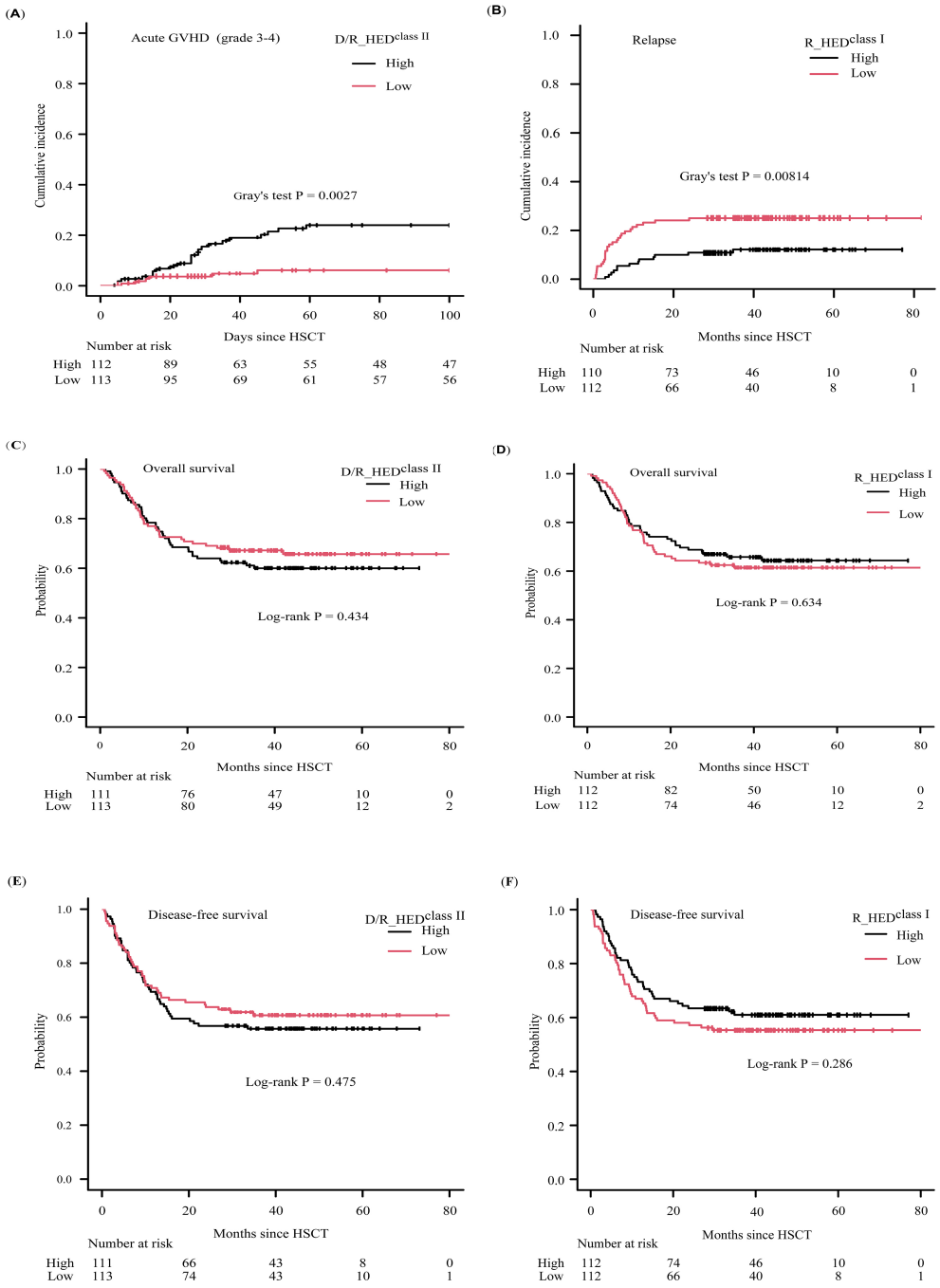

FIGURE 3
Clinical outcomes according to D/R_HED$^{class\ II}$ and R_HED$^{class\ I}$ **(A)** cumulative incidence of aGVHD (grade3-4) stratified by D/R_HED$^{class\ II}$, **(B)** cumulative incidence of relapse stratified by R_HED$^{class\ I}$, **(C)** KM curve of overall survival stratified by D/R_HED$^{class\ II}$, **(D)** KM curve of overall survival stratified by R_HED$^{class\ I}$, **(E)** KM curve of disease-free survival stratified by D/R_HED$^{class\ II}$, **(F)** KM curve of disease-free survival stratified by R_HED$^{class\ I}$.

Supplementary Table 2), whereas non-remission patients exhibited an approximately threefold risk of relapse. Therefore, R_HED$^{class\ I}$ can be considered an independent risk factor for relapse.

In the multivariate model of severe aGVHD, the low D/R_HED$^{class\ II}$ significantly reduced the risk of grade 3-4 aGVHD (HR = 0.335 [95% CI: 0.148-0.756], P = 0.009) as the only protective factor when considering donor age as a continuous variable (Table 3; Supplementary Table 2). However, when donor age was considered a dichotomous variable, it remained in the final model as a risk factor but failed to reach a statistically significant level (HR = 2.153, P = 0.068) (Table 3; Supplementary Table 2).

Regarding cGVHD, Model 1 with donor age as a continuous variable revealed that D/R_HED$^{class\ II}$ was the only independent risk factor, and low D/R_HED$^{class\ II}$ was associated with high risk of

TABLE 3 Significant factors for GVHD and relapse in multivariate analyses.

Outcomes	HR	95% CI	P value
Relapse*			
R_HED$^{class\ I}$, low vs high	2.101	1.083-4.078	0.028
Disease status, NR vs CR	2.928	1.440-5.951	0.003
aGVHD (grade 3-4) *			
D/R_HEDclass II, low vs high	0.335	0.148-0.756	0.009
cGVHD			
Model 1*			
D/R_HED$^{class\ II}$, low vs high	1.376	0.995-1.904	0.054
Model 2§			
Donor age, >=45 vs <45	1.738	1.160-2.603	0.007
aGVHD status	1.438	1.024-2.018	0.036

*Donor age was treated as continuous variable. §Donor age was treated as dichotomous variable. CR, complete remission; NR, non-remission; aGVHD and cGVHD, acute and chronic graft-versus-host disease.

cGVHD (HR = 1.376 [95% CI: 0.995-1.904]); however, this association reached marginal statistical significance (P = 0.054, Table 3; Supplementary Table 2). In Model 2, patients with a history of aGVHD or receiving transplantation from donor older than 45 years had a high risk for cGVHD development, while D/R_HED$^{class\ II}$ no longer remained significant.

3.6 Survival

The proportions of 5-year OS and DFS for the entire cohort were 62.9% (95% CI: 56.1-68.9%) and 58.2% (95% CI: 51.4-64.4%), respectively. There was no discernible difference in overall and disease-free survival (OS or DFS) between patients with high- and low-HED (Figures 3C–F; Supplementary Figures 1, 2) except D_HED$^{class\ I}$ associated with DFS, implying that HED was ineffective as a prognostic indicator for ALL patients who underwent HLA-haploidentical HSCT with related donors. Multivariate regression analysis confirmed that HED, including D_HED$^{class\ I}$, was not associated with survival.

4 Discussion

In the present investigation, we report the impact of HED scores on clinical outcomes for ALL patients underwent haploidentical HSCT. This represents the first study investigating HED in a pure cohort of haploidentical transplantations with patients affected by only one type of hematologic malignancy. While the observation are limited by the presented single center retrospective cohort, the results find that HED has poor prognostic value in OS/DFS, as well as the associations with relapse and aGVHD. In haploidentical setting, HLA disparity was once considered to have little impact on transplantation benefits, but our results showed that HED is an independent risk factor for selecting the best haploidentical donor.

Previous studies investigated the impact of HED on prognosis in mixed AML patients transplanted from either related or unrelated HLA-matched donors (11, 12). Roerden et al. examined the effect of HED on survival in an AML cohort with an HLA-identical sibling or foreign donor and found that a high class I HED had a favorable impact on OS (12). In AML patients undergoing HSCT, Daul et al. investigated the effect of class I and II HED on survival using four different donor sources: identical siblings, haploidentical donors, matched unrelated donors, and mismatched unrelated donors (11). The authors claimed that the class I/II HED ratio was an independent factor associated with better DFS/OS and could be an additive indication of GVL in addition to the major allogenic effect associated with the mismatched HLA. Recently, various hematological diseases were examined in a study by Merli et al., which supports the use of HED as a predictive marker in young adult and pediatric patients receiving transplantation from unrelated donors (4).

The ability of immune cells to interact with mismatched HLAs, minor histocompatibility antigens, and tumor-associated antigens (TAAs) on the leukemic cells is the foundation of the GVL effect (graft-versus-leukemia) (21). Compared to related patient/donor pairs, the overall genetic divergence for unrelated patient/donor pairs is higher. According to whole exome sequencing of patient-donor pairs undergoing allo-HSCT, an average of 6,445 non-synonymous SNVs were found to be mismatched, offering a sizable pool of possible miHAs (22). Genome-wide SNP array analyses revealed that the average mismatched SNVs in the coding region were 9.4% for sibling donors, rising to 17.3% for unrelated donors (23). To lessen the confounding effect of genetic background divergence, we therefore restricted the analysis to a pure cohort of haploidentical transplantation recipients who received transplantation from the related donor.

Our data showed that high R_HED$^{class\ I}$ was associated with a lower 5-year CIR, confirming the crucial function of CD8$^+$ effective T cells in the GVL immune response and thus directly reflecting the immunological benefit of high HED. Patients with high HED scores potentially exhibit more immunogenic peptides than those with low HED scores, which may be recognized by donor-derived T lymphocytes (24), thereby reducing the likelihood of relapse. This explanation can be supported by similar research conducted recently. It was found that AML patients with high class I HED tended to recover their CD8$^+$ T, B, and NK cells more quickly (11). Recently, Pagliuca et al. found that high recipient class I HED was associated with a higher diversity of TCR repertoire (25). In the first year of HSCT, a higher diversity of TCR repertoire and enhanced immune reconstitution might result in a strong defense against opportunistic infections (4).

However, our studies did not reveal any differences in OS or DFS between high and low R_HED$^{class\ I}$ALL patients, indicating that high R_HED$^{class\ I}$ was not always associated with a good prognosis as seen in AML. Patients with high R_HED$^{class\ I}$ had a relatively high incidence of NRM (25.5%) despite a low relapse rate (12.2%) (Table 2), which in turn offset the survival benefit from high R_HED$^{class\ I}$, resulting in no significant difference in OS. This

possible explanation is related to the Beijing protocol we used. The difference between our results and those of earlier studies may also be due to differences in disease type and ethnicity. Our cohort enrolled ALL patients, which has characteristics that cannot be totally extrapolated from studies of AML patients. For instance, while AML is incredibly sensitive to NK cell alloreactivity, the majority of adult ALL patients are not (26, 27). Furthermore, our homogeneous cohort is limited to Chinese, and distinct HLA alleles and HLA haplotypes are present in each ethnic group (28), emphasizing the significance of studying HED in this particular population. It's interesting to note that Chhibber et al. (2022) found that genetic diversity of class I or II HLA loci (HED, heterozygosity, genotype) was not associated with clinical outcomes (9), suggesting that this biomarker shouldn't be used for clinical decision-making for cancer patients receiving pembrolizumab. Similar studies conducted independently have also confirmed Chhibber's conclusion (29–31). To properly comprehend the overall impact of HED, therefore, more research in larger cohorts and across more centers would be required.

As an alternative donor transplant, haploidentical HSCT offers patients who lack fully matched donors the chance to receive transplant, while donor-derived alloreactive T cells elicit a strong allogeneic response and exert an immense GVL effect (32). Between 2005 and 2015, there was a roughly threefold increase of haplo-HSCT in Europe due to favorable practical aspects of using a haploidentical donor and the accumulation of data of better outcomes achieved with TCR platforms (33). The democratization of using haploidentical donors leads to a fundamental paradigm shift: while donor availability was the key challenge for years, the issue today becomes identifying the best donor among several possible ones when haplo-HSCT (34). In general, the outcome of haploidentical HSCT may be influenced by DSA (donor-specific antibody), donor age, donor sex, KIR (killer immunoglobulin-like receptor), NIMA (noninherited maternal antigen), HLA matching, as well as family relationships (35, 36). Recent studies have confirmed that neither the quantity of HLA loci nor the combination of specific sites would affect the outcome of haploidentical HSCT (35, 37–40). The Beijing protocol showed that 1, 2, or 3 mismatches of 6 HLA loci had no effect on the cumulative incidence of cGVHD or aGVHD. Additionally, the number of HLA mismatches had no influence on the cumulative incidence of relapse, overall survival, and leukemia-free survival (35, 37). The cumulative incidence of GVHD, relapse rate, NRM, and overall survival were not affected by differences in the HLA locus in the T-cell-replete (TCR) haploidentical HSCT with a low dose of anti-T lymphocyte globulin (ATG), according to a prospective multicenter study from Japan (39). In the multivariate analysis, the only significant predictive factor for increased relapse was non-CR status prior to transplantation ($P = 0.0424$), which tended to be associated with a worse survival rate ($P = 0.0524$). It was also observed that the degree of HLA mismatching had no effect on post-transplant OS, cumulative incidence of aGVHD, NRM, or 1-year cGVHD in the high-dose PT/Cy haploidentical transplantation protocol, whether in the HVG (host-versus-graft) or GVH (graft-versus-host) settings (40). According to Kasamon et al., survival following nonmyeloablative transplants with posttransplant cyclophosphamide is also not correlated with the degree of HLA disparity (41).

Technique advances in aGVHD prophylaxis, prevention of post-transplant relapse, and treatment strategies have greatly improved the outcome of haploidentical HSCT compared to the past decades. Although the team from the Beijing protocol established the notion of donor selection and the best option of donor selection is to choose youthful, male, and NIMA-incompatible donors (35, 36), the consensus of donor selection, however, is still limited within the TCD and TCR haploidentical systems at this time. New criteria for donor selection may develop as a result of an increase in haploidentical HSCT cases and updated assessments of the factors influencing transplant outcomes (33, 34, 42–44). In this study, we found a strong association between D/R_HED$^{\text{class II}}$ and aGVHD incidence, with higher D/R_HED$^{\text{class II}}$ indicating more severe aGVHD. Single locus analysis revealed that the influence of D/R_HED$^{\text{class II}}$ appears to be predominantly driven by D/R_HED$^{\text{DRB1}}$,which proves the conclusions that DRB1 has the highest diversity among all HLA class II genes and the highest cell surface expression when compared to other HLA class II antigens (45). A high D/R_HED$^{\text{class II}}$ implies great spatial structural differences between donors and recipients, as well as more targets from tissue cells being presented. As a result, the greater the effect of T-cells attacking the tissue cells, the more severe the damage to the organ. The number of mismatch loci is obviously a relatively rough indicator, although it also reflects the degree of incompatibility between recipient and donor. Therefore, previous studies and our results suggest that the amount of HLA mismatch should not be used as a criterion for the selection of family haploidentical donors. Instead, D/R_HED$^{\text{class II}}$ provides more epitope information than mismatch numbers and also indicates donor and recipient mismatches, suggesting that D/R_HED$^{\text{class II}}$ may be taken into account as a new risk factor for donor selection in related haploidentical HSCT.

There are some limitations to this study. Our research was based on single-center and retrospective data and had a limited number of patients. Independent, prospective, larger, and multicenter investigations would be needed and beneficial to further confirm the impact of HED on outcome and the clinical significance of D/R_HED$^{\text{class II}}$ in donor selection. Due to the unavailability of data or limitations of the methods themselves, other approaches such as peptide binding motifs (PBM) (46), T-cell epitope (TCE) (47) or KIR-ligand mismatches (34, 45) were not considered.

In conclusion, we conducted a retrospective analysis to investigate the correlation between HED and outcomes in ALL patients who underwent transplants from related haploidentical donors. Results revealed that only class I HED of the recipient (R_HED$^{\text{class I}}$) was associated with 5-year CIR and only D/R_HED$^{\text{class II}}$ was significantly correlated with severe aGVHD.

Multivariate Cox regression analysis did confirm that a high D/R_HED$^{class\ II}$ was an independent risk factor for grade 3-4 aGVHD, and the high R_HED$^{class\ I}$ group had a more than two-fold reduced risk of relapse. KM and multivariate regression analyses confirmed that none of HED was associated with overall or disease-free survival. These results suggest that HED$^{class\ II}$ of donor-recipient pair could be used for donor selection as a novel risk factor for grade 3-4 aGVHD and patient's HED$^{class\ I}$ for relapse in the setting of related haploidentical HSCT, but not as an independently prognostic factor for predicting OS or DFS.

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 humans were approved by Ethics Committee of Hebei Yanda Lu Daopei Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

H-FZ: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. X-JL: Conceptualization, Investigation, Methodology, Resources, Supervision, Writing – review & editing. X-YC: Data curation, Investigation, Methodology, Project administration, Resources, Writing – review & editing. X-BL: Data curation, Formal analysis, Methodology, Writing – review & editing.

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Conflict of interest

Author H-FZ, X-JL and X-BL are employed by Beijing BFR Gene Diagnostics Co., Ltd.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1440911/full#supplementary-material

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Monocytes as an early risk factor for acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation

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Acute graft-versus-host disease (aGVHD) is a major complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT) and contributes to high morbidity and mortality. However, our current understanding of the development and progression of aGVHD after allo-HSCT remains limited. To identify the potential biomarkers for the prevention and treatment of aGVHD during the early hematopoietic reconstruction after transplantation, we meticulously performed a comparative analysis of single-cell RNA sequencing data from post-transplant patients with or without aGVHD. Prior to the onset of aGVHD, monocytes in the peripheral blood of patients with aGVHD experienced a dramatic rise and activation on day 21 post-transplantation. This phenomenon is closely aligned with clinical cohort results obtained from blood routine examinations. Furthermore, *in vitro* co-culture experiments showed that peripheral blood monocytes extracted from patients with aGVHD approximately 21 days post-transplantation induced a significantly higher proliferation rate of allogeneic T cells compared to those from patients without aGVHD. Our study indicates that monocytes could be a crucial early clinical risk factor for the development of aGVHD, and this insight could potentially guide the timing of monitoring efforts, recommending assessments at the pivotal juncture of approximately day 21 post-transplantation, shedding fresh light on the significance of early hematopoietic regeneration in relation to the onset of aGVHD.

KEYWORDS

aGVHD, HSCT, monocytes, hematopoietic reconstruction, risk factor

Introduction

Despite the routine use of graft-versus-host disease (GVHD) prophylaxis, acute GVHD (aGVHD) still affects 30%–60% of patients receiving allogeneic hematopoietic stem cell transplantation (allo-HSCT) and is associated with poor clinical prognosis (1–3). In recent years, there has been an increase in the number of allo-HSCT procedures conducted annually due to technological advancements and the promotion of haploidentical allo-HSCT (4–6). It also indicates that there will be a sharp increase in the number of aGVHD patients. The development of aGVHD was initially observed as a secondary disease that appeared after the recovery from conditioning-induced toxicity in murine models of bone marrow transplantation (7). After that, Billingham formulated three conditions for the development of aGVHD: the graft comprises immunologically competent cells, the recipient expresses tissue antigens that are absent in the transplant donors, and the recipient is incapable of eradicating the transplanted cells (8). Additionally, the development of aGVHD can be conceptually separated into three phases: activation of antigen-presenting cells (APCs); donor T-cell activation, proliferation, differentiation, and migration; and target tissue damage (9).

Considering the high morbidity and mortality of aGVHD, precisely predicting the occurrence of aGVHD is of vital importance for early intervention. Current pretransplant clinical risk factors for aGVHD mainly include human leukocyte antigen (HLA) compatibility, the ages and genders of recipients and donors, and conditioning regimen intensity (10, 11). When aGVHD manifests clinically, specific biomarkers including tumor necrosis factor receptor 1 (TNFR1), interleukin-33 receptor (ST2), and regenerating islet-derived protein 3-alpha (REG3α) are found in elevated levels in blood plasma, and immune cell infiltration has been detected in affected target organs like the liver, gut, and skin (12–14). Using biomarkers or risk models to predict the risk of aGVHD onset in patients receiving allo-HSCT can assist in effective clinical intervention (15, 16). However, the risk factors for aGVHD during the early hematopoietic reconstruction require further elucidation.

During the initial stages of HSCT, hematopoietic stem and progenitor cells (HSPCs) could be regulated in several ways, including a range of inflammatory signals, which could alter their differentiation bias (17, 18). Taking advantage of the rapid development of single-cell RNA sequencing (scRNA-seq) technology, we dissected the reconstitution dynamics of transplanted HSPCs at single-cell resolution in both mice and humans in previous studies (19, 20). More importantly, we identified a cluster of neutrophil progenitors with immunoregulatory function in mobilized human grafts, which have the potential against the development of aGVHD. However, in the context of aGVHD, a deeper comprehension is necessary of the hematopoietic reconstitution dynamics and intricate regulatory mechanisms of transplanted human HSPCs.

This study involved a comparative analysis at the single-cell level for the early hematopoietic reconstitution dynamics in aplastic anemia (AA) patients with or without aGVHD after allogeneic granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cell transplantation (allo-PBSCT). We found that patients with aGVHD had an obvious increase and activation of monocytes in day 21 peripheral blood (PB) post-transplantation and verified this phenomenon with clinical cohort and *in vitro* co-culture experiments. Our findings introduce a new risk factor for early prognostication of aGVHD, and monocytes could potentially serve as an intervention target for aGVHD management following transplantation.

Methods

Sample collection

All blood samples of patients were obtained from the Blood Diseases Hospital, Chinese Academy of Medical Sciences in China, and were collected into ethylenediaminetetraacetic acid (EDTA) tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll-Paque™ Plus (Gibco, Grand Island, NY, USA). The cells were frozen in CellBanker (AMSBIO, Cambridge, MA, USA), a fetal bovine serum (FBS)-free cryoprotectant, and stored in liquid nitrogen until further use.

Single-cell RNA sequencing and data preprocessing

We included scRNA-seq data of total nucleated cells (TNCs) of PB and bone marrow (BM) from three healthy controls (HCs) and six patients, as well as scRNA-seq data of TNCs from patient-paired G-CSF-mobilized peripheral blood (donor) in our published study (20) (Table 1), and followed previous preprocessing and quality control using scanpy pipeline (Version 1.9.3) (21). Next, we normalized count data using *scanpy.pp.normalize_total* function and performed logarithmically transformation for the following analysis.

Batch effect correction and cell type annotation

Using *scanpy.pp.highly_variable_genes*, we identified 1,869 highly variable genes for the following analysis. For principal component analysis, we regressed out the total number of counts and the proportion of mitochondrial counts and used the harmony algorithm to correct batch effects (22). We generated a neighborhood graph using *scanpy.pp.neighbors* with "neighbors = 30, npcs = 10" for downstream Uniform Manifold Approximation and Projection (UMAP) visualization and clustering analysis. We performed unsupervised clustering using *scanpy.tl.leiden*, and we identified 19 clusters by setting "resolution = 0.8". Next, we identified subclusters for monocytes, neutrophils, and lymphoid cells. We repeated the data integration and unsupervised clustering for monocytes and performed batch effect correction by harmony and bbknn (23). For T, B, NK, and neutrophils, we reran neighborhood graph computation and unsupervised clustering. We identified the cell types of subclusters according to the expression of marker genes.

TABLE 1 Clinical parameters and outcomes of six AA patients undergoing allo-PBSCT.

Case ID	Age (years) (P)	Sex (P/D)	Diagnosis	Type of conditioning regimen	HLA-matched	aGVHD prophylaxis	aGVHD onset time and grade
P1	25	F/F	SAA	RIC	8/10	CSA+MMF	No
P8	17	M/M	SAA	RIC	10/10	FK506+MTX	No
P9	35	M/F	VSAA	RIC	10/10	CSA+MTX	No
P6	52	F/M	SAA	RIC	6/10	CSA+MTX+MMF	d37; grade I
P7	21	F/F	VSAA	RIC	5/10	FK506 +MTX+MMF	d21; grade II
P10	18	M/F	SAA	RIC	5/10	FK506 +MTX+MMF	d31; grade III

P, patient; D, donor; M, male; F, female; AA, aplastic anemia; SAA, severity AA; VSAA, very SAA; HLA, human leukocyte antigen; aGVHD, acute graft-versus-host disease; RIC, reduced intensity conditioning regimen; CSA, cyclosporine; MMF, mycophenolate mofetil; MTX, methotrexate; FK506, tacrolimus.

Differential gene expression analysis

Differentially expressed genes (DEGs) were detected using the *scanpy.tl.rank_genes_groups* function with the Wilcoxon rank-sum test. Genes with an absolute value of *log foldchange* more than 1 and adjusted *p*-value less than 0.05 were defined as DEGs.

Gene set enrichment analysis

For functional annotation of DEGs, we performed gene set enrichment analysis by Metascape (24) (Version 3.5.2) and used terms in *GO Molecular Functions and GO Biological Processes*. The R package pheatmap (Version 1.0.12) was used for visualizing the gene expression and functional annotation results.

Cell–cell communication analysis

CellChat (Version 1.6.1) (25) was used to assess the cell–cell interactions between monocytes and lymphoid cells. The normalized gene expression data and CellChat human database were taken as input. Genes that expressed more than 10% of the cells in one cluster and the ligand–receptor pairs with *p*-values less than 0.05 were considered significant interaction molecules among different cell types. Results were visualized using functions in CellChat.

Calculation of the signature score

The signature scores for monocytes and T cells were calculated by *scanpy.tl.score_genes* with functional gene sets from published studies.

Statistical analysis

FlowJo, version 10.8.1, was used for analysis of flow cytometry data. Statistical comparison was performed using R (Version 4.2.3). *p*-Values for the Mann–Whitney U test and Tukey–Kramer test were calculated using the "stats" package, and the significance was shown as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $**** p < 0.0001$.

Monocyte-allogeneic T-cell co-culture experiments

T cells were isolated from fresh PBMCs of healthy volunteers using human CD3 MicroBeads, following the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Similarly, monocytes were isolated from cryopreserved human PBMCs using human CD14 MicroBeads (Miltenyi Biotec). Isolated cells were confirmed to consist of >95% target cells by flow cytometry (BD Canto II flow cytometer, BD Biosciences, San Jose, CA, USA). T cells were labeled with 1 µL carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA) and were co-cultured with sorted monocytes in 96-well U-bottom plates at 37°C with 5% CO_2 at a 4:1 ratio in RPMI 1640 medium supplemented with 1%100 IU/mL penicillin, 10 µg/mL streptomycin (Gibco), 1% 2 mM L-glutamine (Invitrogen), and 10% heat-inactivated FBS (Gibco) for 5–7 days. Then, cultured cells were harvested into a FACS tube; incubated with antibodies CD4, CD8, CD25, and CD69 (BioLegend, San Diego, CA, USA); and analyzed using a BD Canto II flow cytometer.

Criteria of clinical cohorts

Inclusion criteria

Patients' ages ranged from 15 to 60 years.

Patients were diagnosed with aplastic anemia or acute leukemia and underwent allo-PBSCT.

Patients underwent conditioning regimens before allo-PBSCT.

Patients complied with study procedures and follow-up.

Exclusion criteria

Patients had co-occurring chronic diseases such as hepatitis and diabetes mellitus.

Patients were diagnosed with acute myelomonocytic leukemia.

Patients with abnormal liver function or gastrointestinal complications required further diagnostic evaluation.

Patients relapsed during 60 days post-transplantation.

Result

The early hematopoietic reconstitution is altered in transplant patients with aGVHD

To investigate the dynamics of early hematopoietic reconstruction in patients with aGVHD, we involved published scRNA-seq data of TNCs of PB and BM from three HCs and six patients, as well as scRNA-seq data of TNCs from patient-paired G-CSF-mobilized peripheral blood (donor). The case ID of six patients involved in the study corresponds one-to-one to the patients in our previous work (20). Patients included in this study were diagnosed as AA and underwent allo-PBSCT after BM conditioning. Three of them developed different grades of aGVHD and received surging immunosuppressive treatment, while the rest did not show any clinical manifestations of aGVHD within 6 months after allo-PBSCT (Table 1). The schematic workflow is depicted in Figure 1A, and the 10x Genomics platform was employed to generate single-cell transcriptome data of TNCs.

After rigorous quality control (Supplementary Figure 1A), 230,550 high-quality cells and 20,862 genes were obtained for subsequent analysis. All TNCs were visualized using UMAP and classified into eight major cell populations: Neutrophil progenitors (ProNeus), Neutrophil precursors (PreNeus), Mature neutrophils (MatureNeus), Monocytes (Monos), Megakaryocytes (MKs), B lymphocytes/Plasmas (B/Plasma), T lymphocytes, and Natural killer (NK) cells (Figure 1B; Supplementary Figures 1B, C).

Consistent with previous studies, neutrophils and monocytes emerged as the predominant cell populations during the first month after allo-PBSCT, while T cells remained largely absent until 30 to 60 days; the reconstruction state of T cells was greatly influenced by immunosuppressive therapy (20, 26), and these regulations of hematopoietic reconstruction showed consistency in both PB and BM (Figure 1C). Furthermore, the dynamics of hematopoietic reconstitution exhibited distinct characteristics and intriguing differences in PB compared to BM between the two groups. For example, the proportions of monocytes and MatureNeus in PB showed noteworthy differences between the aGVHD and non-aGVHD groups before the initial diagnosis of aGVHD (range from day 21 to day 37 after allo-PBSCT), and day 21 was a key time point of this hematopoietic reconstruction disparity. In the non-aGVHD group, MatureNeus had a higher proportion of PB within 21 days after allo-PBSCT. However, in the aGVHD group, the proportion of PB monocytes was higher within 21 days and reached the peak on day 21, and this enrichment of PB monocytes showed consistency among three aGVHD patient groups (Figure 1D; Supplementary Figure 1D). Therefore, we mainly focused on monocytes in PB on day 21 post-transplantation during subsequent analysis.

We further investigated possible explanations for the significant enrichment of day 21 PB monocytes in aGVHD patients. Monocytes from day 21 PB predominantly originated from the donors, with only 0.05% and 0.12% of monocytes being identified as recipient-derived cells by demuxlet (27) in the aGVHD and non-aGVHD groups, respectively (Supplementary Figure 1E). Considering that hematopoietic stem cells (HSCs), with the capacity of multilineage differentiation and self-renewal, are the origination of all reconstituted blood cell lineages (28), we introduced the scRNA-seq data of HSPCs in BM from HCs and patients 14 days post-transplantation involved in this study and compared the myeloid differentiation trajectory of HSPCs between the aGVHD and non-aGVHD groups (Supplementary Figures 1F, G). The ratio of monocyte/dendritic progenitors (MDs) to unipotent neutrophil progenitors (NePs) was elevated in patients with aGVHD than those without aGVHD (Figure 1E). The cell fate bias of multipotent and bipotent progenitors [estimated in a previous study (20)] on day 14 toward NePs exhibited no significant difference between the two groups while maintaining a pronounced inclination toward MDs in the aGVHD group than the non-aGVHD group (Figure 1F). Thus, the differentiation preference of HSPCs toward monocytes in the aGVHD group occurred prior to day 21, accounting for the abnormal regeneration of monocytes before the onset of aGVHD.

Taken together, the comparison analysis of scRNA-seq data systematically identified aGVHD-associated immune disturbances. A prominent enrichment of PB monocytes on day 21 after allo-PBSCT occurs in aGVHD patients, and this emergency monocytopoiesis stems from pre-existing differentiation bias of HSPCs.

Prominent activation of enriched PB monocytes in aGVHD group on day 21 post-transplantation

Considering the contribution of monocytes and monocyte-derived cells to the development of aGVHD (29, 30), we conducted further investigation into the functional variations of monocytes in patients with aGVHD. The heterogeneity of monocytes always corresponds to diverse functional specializations (31). To comprehensively characterize the functional variation of PB monocytes enriched on day 21, we compared the transcriptome profiles of monocyte subsets between the aGVHD and non-aGVHD groups. We defined three subsets in monocytes according to CD14 and CD16 expression: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$), and non-classical monocytes ($CD14^{+}CD16^{++}$). Classical monocytes exhibited the highest expression levels of *S100A8* and *S100A9*, the proinflammatory mediator released by myeloid cells in many acute and chronic inflammatory disorders (32), while non-classical monocytes upregulated the expression of antigen presentation-associated genes, like *CD74*, *HLA-DRA*, and *HLA-DRB1* (33, 34) (Figures 2A, B). On day 21, the prominent enrichment of PB monocytes in the aGVHD group was primarily attributed to classical and intermediate monocytes, while the proportion of PB non-classical monocytes was comparable between the aGVHD and non-aGVHD groups (Figure 2C; Supplementary Figure 2A).

To further investigate the possible role of monocytes in the pathophysiology of aGVHD, we performed differential expression gene analysis for day 21 monocytes in PB. In the aGVHD group,

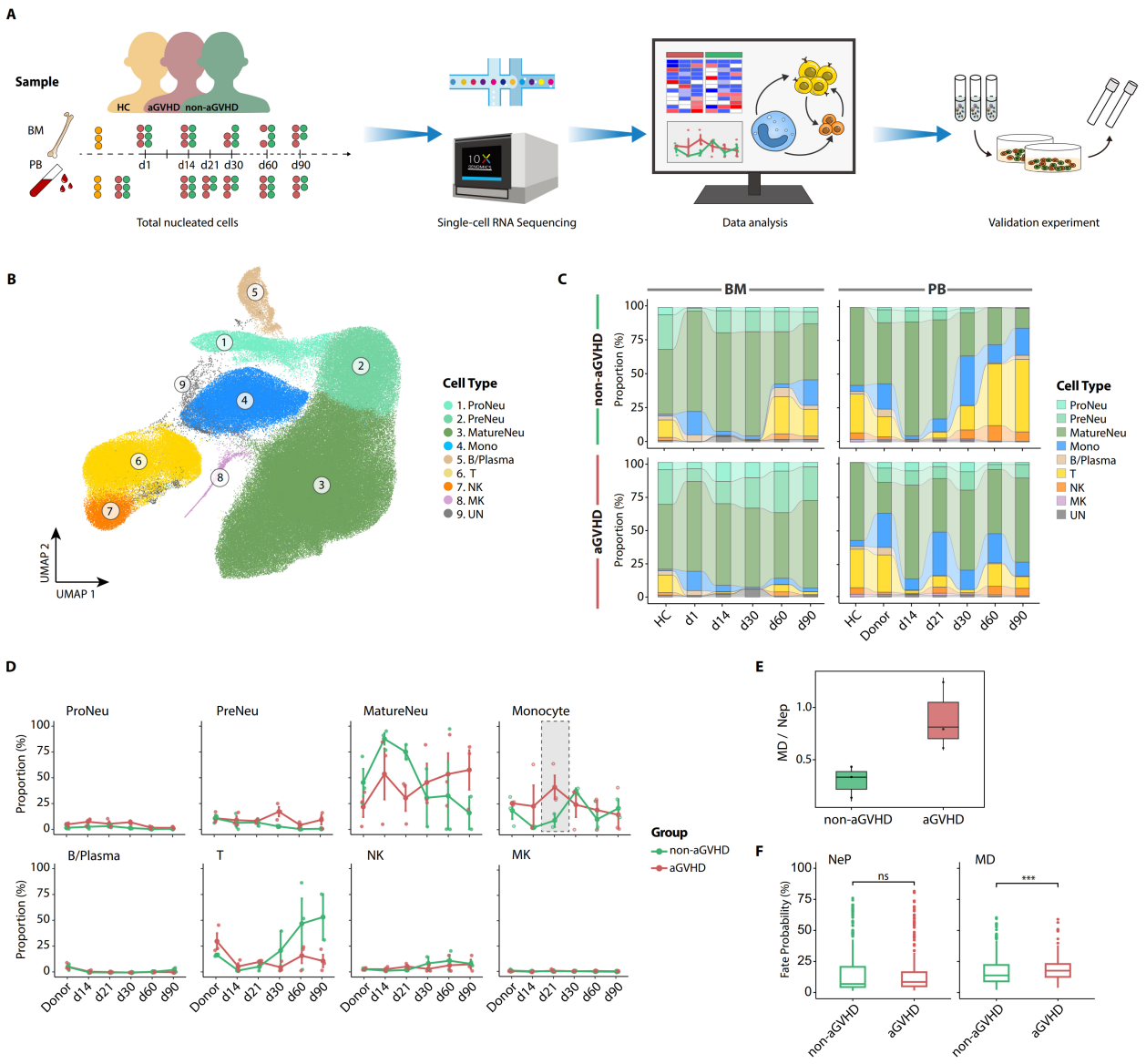

FIGURE 1
Comparison of hematopoietic reconstruction between patients with acute graft-versus-host disease (aGVHD) and those without aGVHD during early post-transplantation. **(A)** Overview of experimental design and data analysis. **(B)** Uniform Manifold Approximation and Projection (UMAP) visualization of total nucleated cells (TNCs) from healthy controls (HCs), granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood (donors), and six patients. **(C)** The post-transplant cell compositions in patients with (bottom) or without aGVHD (upper) at multiple follow-up time points. **(D)** The dynamic proportion of TNC subsets between aGVHD and non-aGVHD groups at multiple follow-up time points. The monocyte proportion from d21 PB shows a sharp increase in aGVHD group. The line plots show the means ± SEM for the proportions of each cell type. **(E)** The ratio of monocyte/dendritic progenitors (MDs) to neutrophil progenitors (NePs) in d14 hematopoietic stem and progenitor cells (HSPCs) in bone marrow (BM). **(F)** Cell fate probabilities of hemopoietic stem cell multipotent (HSC/MPP), lymphoid-primed multi-potential progenitor (LMPP), and granulocyte-monocyte progenitor (GMP) in BM on d14 post-transplantation. *p*-Values were evaluated by the two-tailed Mann–Whitney U test. ns, not significant; ***$p < 0.001$.

monocytes showed higher proliferation and chemotaxis potential with the upregulation of genes like *FOS*, *JUN*, and *CXCL8* while downregulating interferon-associated genes like *ISG15* and *IFIT3* (Figure 2D). The aGVHD group demonstrated a significantly higher number of upregulated genes across each monocyte subset when contrasted with the non-aGVHD group. This observation highlighted the pronounced functional differences among the monocyte subsets between the two groups (Figure 2E; Supplementary Figure 2B). To further profile the functional characteristics of each monocyte subset, we annotated upregulated genes in monocyte subsets in the aGVHD group by gene ontology analysis. Classical monocytes showed activation in the regulation of phagocytosis, chemotaxis, and cohesion, while non-classical monocytes demonstrated enhanced capability of antigen-presenting and T-cell viability. The higher expression of *ICAM* and *TNFSF14*, the genes associated with co-stimulatory signals for T-cell proliferation and activation (35, 36), indicates that monocyte subsets may contribute to the proliferation and activation of T cells in the aGVHD group (Figure 2F).

We additionally explored the functional disparities of lymphocytes between the aGVHD and non-aGVHD groups. We

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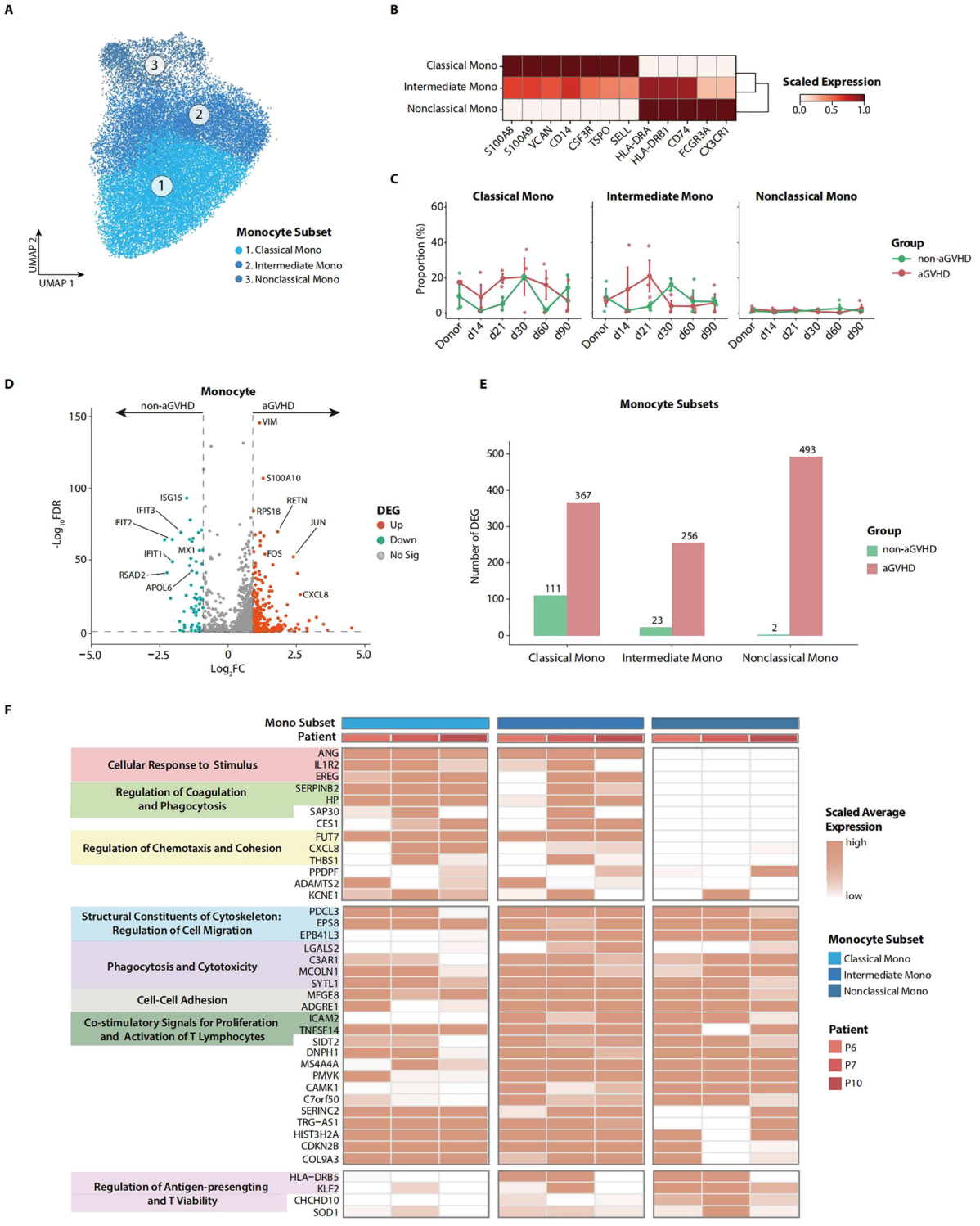

FIGURE 2
Transcriptome characteristics of peripheral blood (PB) monocytes on day 21 in patients with acute graft-versus-host disease (aGVHD). **(A)** Uniform Manifold Approximation and Projection (UMAP) of monocyte subsets. **(B)** Heatmap shows the marker genes for each monocyte subset. **(C)** The dynamic proportions of monocyte subsets in PB at multiple follow-up time points after allo-PBSCT. **(D)** Volcano plot depicts the differentially expressed genes (DEGs) in d21 PB monocytes between aGVHD and non-aGVHD groups. **(E)** Transcriptomic difference for each monocyte subset between aGVHD and non-aGVHD groups. The number of upregulated genes in the aGVHD group shows that the drastic transcriptomic variation occurred in all monocyte subsets. **(F)** Heatmap represents expression level of upregulated functional genes of each monocyte subset in d21 PB for aGVHD group. Annotations show functional attributes of genes in aGVHD group.

defined elaborate subsets of T, B, and NK cells (Supplementary Figures 2C, D). By performing differential expression gene analysis for CD8 effector T and CD16 NK cells, we found that cytotoxicity-associated genes like *GZMB* and *KLF2* (37) were upregulated in the aGVHD group, implying the highly activated functional state of CD8 effector T and CD16 NK cells in aGVHD patients (Supplementary Figure 2E).

Collectively, the transcriptome profile of monocytes provides insights into the functional characteristics of monocytes from aGVHD patients. The overstated activation of monocytes may play an essential role in inducing T-cell activation and proliferation in the context of aGVHD.

Enhanced cell–cell interactions between monocytes and cytotoxic cells occur in patients with aGVHD during the early hematopoietic reconstitution

To further explore the effect of aGVHD-associated activation of monocytes on the cell–cell regulatory network, we performed cell–cell communication analysis for PB immune cells on day 21 using CellChat (25) software. Patients with aGVHD showed the highest interaction strength and numbers among the HC, aGVHD, and non-aGVHD groups, indicating enhanced cell–cell interactions (Figure 3A; Supplementary Figure 3A). Compared with the non-aGVHD group, the augmentation of interaction strength in aGVHD patients was mainly focused on monocytes, T cells, and NK cells. The enhanced interaction strength of three monocyte subsets was primarily attributed to the outgoing signals of interaction, emphasizing the pivotal role of monocytes in regulating other cell populations in the context of aGVHD. Correspondingly, the incoming signal was obviously strengthened for CD8 effector T, CD16 NK, and CD56 NK, supporting the potential activation of lymphocytes in the aGVHD group (Figure 3B). Furthermore, the aligned interactive interplay between three monocyte subsets and CD8 effector T, CD16 NK, and CD56 NK was also remarkably augmented in the aGVHD group, indicative of the stimulation role of monocytes on T cells and NK cells. In addition, the cell–cell communication between monocyte subsets and CD8 memory T was relatively weak in the aGVHD group, supporting the initiative role of cytotoxic lymphocytes including CD8 effector T and CD16 NK in inducing target damage of aGVHD (38) (Figure 3C; Supplementary Figure 3B).

To investigate the underlying mechanism of altered cell–cell interaction, we compared the level of involvement of all detected pathways between the aGVHD and non-aGVHD groups and revealed the different enrichment paradigms of signal pathways between the two groups. The upregulation of cytokine-associated pathways including RESISTIN, IL16, and IL1 pathways reflected that immune cells in aGVHD patients had a higher proinflammatory ability and stronger signal transmission, while the upregulated CCL, CXCL, and ITGB2 pathways indicated the enhanced ability of cell migration and cohesion of immune cells in the aGVHD group. Additionally, the signal pathways, including ICAM, MHC-II, and CD45, which play crucial roles in the co-stimulation of T cells, were found to be significantly enriched in the aGVHD group (Figure 3D), which was consistent with the observation in Figure 2E.

We subsequently investigated the pivotal role of enriched pathways in facilitating enhanced cell–cell interactions among monocytes, T cells, and NK cells in patients with aGVHD. The overall enrichment state of the same signal pathway remained consistent in both outgoing signal-originated monocyte subpopulations and incoming signal-received T and NK subsets, supporting that these pathways upregulated in the aGVHD group mediate the enhancement of cell–cell interactions among monocytes, T cells, and NK cells (Figure 3E). The CXCL pathway was specifically activated between non-classical monocytes and CD8 effector T cells in aGVHD patients, and the expression of the ligand–receptor pair genes *CXCL16* and *CXCR6* was respectively upregulated in non-classical monocytes and CD8 effector T cells. The CXCL pathway plays an important role in immune cell migration (39); thus, the activation of the CXCL pathway showed enhanced migration ability of CD8 effector T cells and non-classical monocytes in patients with aGVHD. Similarly, the RESISTIN pathway showed a preference for cell–cell interactions between classical, intermediate monocytes, and CD8 effector T cells and NK cells. In the aGVHD group, the expression of the ligand gene *RETN* was elevated on classical and intermediate monocytes, while the corresponding receptor gene *CAP1* was upregulated on CD8 effector T cells and NK cells (Figures 3E, F). These results demonstrate that monocytes may regulate the functional activity of T cells and NK cells by secreting immune effectors (40). In addition, even the identical pathways could exert different roles in mediating cell–cell interaction in conditions of aGVHD or no-aGVHD. The ITGB2 pathway mediated the interactions from monocytes to naive T cells and NK cells in the non-aGVHD group, whereas the interactions between monocytes and naive T cells were absent in the aGVHD group (Supplementary Figure 3C).

To elucidate the pivotal role of augmented cell–cell interactions within the aGVHD group in the pathogenesis of aGVHD, we calculated signature scores based on published gene sets to evaluate the killing and migration ability of T cells and NK cells (Supplementary Table 1). We found that both cytotoxicity and migration scores were significantly higher in the aGVHD group (Figure 3G). In addition, we scored the migration and cytokine secretion ability of monocytes. Monocytes in the aGVHD group also had higher scores of cytokine secretion and migration ability, which were especially noticeable in intermediate and non-classical monocytes (Supplementary Figure 3D).

In conclusion, we emphasized the enhanced regulatory network from monocytes to CD8 effector T cells and NK cells in the aGVHD group. These results further support that the day 21 monocytes in PB have the potential to induce the overstated activation and proliferation of T cells and NK cells in aGVHD patients.

Insufficient immunosuppression may contribute to the development of aGVHD

Immunosuppression refers to the prevention or reduction of immune response, and insufficient immunosuppression can give

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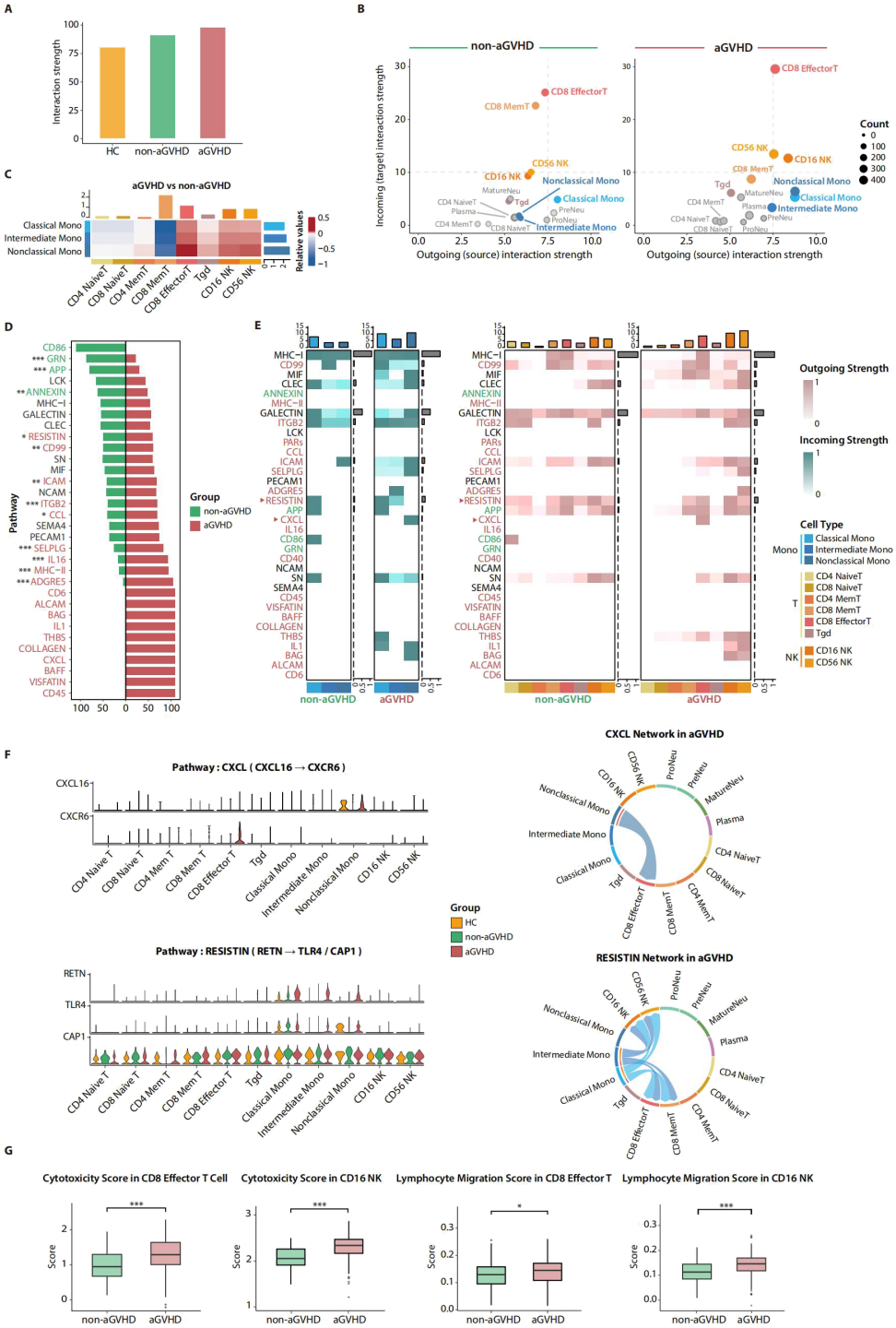

FIGURE 3
Cell–cell communication analysis for total nucleated cells (TNCs) in d21 peripheral blood (PB). **(A)** Barplot shows the strength of cell–cell interaction among healthy control (HC), acute graft-versus-host disease (aGVHD), and non-aGVHD groups. **(B)** Scatter plots show the outgoing and incoming strength for each cell type. Monocyte subsets, CD8 memory T, CD8 effector T, and NK subsets are the most variable cell types between aGVHD and non-aGVHD groups. CD4 Mem T, CD4 Memory T; CD8 Mem T, CD8 Memory T. **(C)** Relative value of interaction strength among the most variable cell types between aGVHD and non-aGVHD groups. The positive value (red) represents stronger interaction strength, and the negative value (blue) represents weaker interaction strength in aGVHD group. CD4 Mem T, CD4 Memory T; CD8 Mem T, CD8 Memory T. **(D)** Relative information flow for signaling pathways between aGVHD and non-aGVHD groups. The pathways with significantly enhanced information flow are highlighted using colors corresponding to their respective groups. *p*-Values were evaluated by the two-tailed Mann–Whitney U test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. **(E)** Heatmap shows outgoing and incoming signal intensities of most variable cell types. CD4 Mem T, CD4 Memory T; CD8 Mem T, CD8 Memory T. **(F)** Gene expression levels of ligand–receptors of CXCL and RESISTIN pathways in HC, aGVHD, and non-aGVHD groups (left). Chord plots show these two pathways mediated the cell–cell interaction patterns in aGVHD group (right). CD4 Mem T, CD4 Memory T; CD8 Mem T, CD8 Memory T. **(G)** Module scores of functional gene sets in CD8 effector T and CD16 NK. *p*-Values were evaluated by the two-tailed Mann–Whitney U test. $*p < 0.05$, $***p < 0.001$.

rise to allograft rejection and recipient-specific antibody development (41). Myeloid-derived suppressor cells (MDSCs) are renowned for their roles in exerting anti-inflammatory and immunosuppressive effects, including two major subsets: polymorphonuclear (PMN)-MDSCs and monocytic (M)-MDSCs (42). In a previous study, we identified a cluster of neutrophil progenitors S100A^{high} Neu2 with the transcriptome characteristics of PMN-MDSCs, and these cells have the potential protection against the development of aGVHD (20). In this research, we found that PreNeus in PB had similar transcriptome characteristics with S100A^{high} Neu2. By scoring neutrophils with published gene sets of MDSCs, we found the signature scores were significantly higher in PreNeus than in other neutrophil subsets, showing that PreNeus may have immunosuppressive function (Supplementary Figure 4A; Supplementary Table 1).

On day 21 post-transplantation, significant transcriptomic disparities were observed between the two groups of PreNeus, with those from the non-aGVHD group exhibiting the most pronounced upregulation of DEGs (Supplementary Figure 4B). Volcano plot further demonstrated that PreNeus in the non-aGVHD group upregulated immunosuppression-associated genes such as *ARG1* and *IL4R* (43), suggesting that PreNeus in the non-aGVHD group are more likely to exert immunosuppressive function than those in the aGVHD group (Supplementary Figure 4C). In addition, we selected published gene sets related to immunosuppression functions to evaluate the impact of PreNeus cells on immune response. The module scores of three gene sets were all significantly higher in the non-aGVHD group, further supporting that PreNeus from non-aGVHD patients exhibits a greater potential for negative regulation of immune response (Supplementary Figure 4D; Supplementary Table 1). This phenomenon also suggests that immunosuppressive cells like PreNeus may be crucial for the development and progression of aGVHD.

Functional validation and clinical value of the abnormal accumulation of day 21 PB monocytes in aGVHD monitoring during the early post-transplantation period

To validate the function of PB monocytes on day 21 in aGVHD patients, we collected PB samples from AA patients approximately day 21 after allo-PBSCT and isolated monocytes to co-culture with T cells from HCs for 5–7 days (Figure 4A). As expected, monocytes from aGVHD patients were noted to be more capable of inducing T-cell proliferation, while the T-cell activation showed no significance between the two groups (Figures 4B–D; Supplementary Figure 5). To further verify the clinical value of the enrichment of PB monocytes on day 21 in monitoring aGVHD onset, we collected the results of blood routine examination from 32 AA patients during 60 days after allo-PBSCT, including 16 aGVHD who were diagnosed as aGVHD during 21–100 days after transplantation and 16 non-GVHD patients who never manifested symptoms of aGVHD 120 days post-transplantation (Supplementary Tables 2, 3). By comparing the median cell percentage within 3 days for each post-transplantation time point, we observed similar monocyte enrichment on day 21 in aGVHD patients, which was consistent with the results concluded by transcriptome analysis (Figure 4E; Supplementary Figure 6A). In addition, we extended the observation of PB monocytes into acute leukemia (AL) patients receiving allo-PBSCT. For blood routine examination data from 33 AL patients after allo-PBSCT (18 aGVHD and 15 non-aGVHD patients), day 21 PB monocytes were also significantly enriched in the aGVHD group (Figure 4F; Supplementary Figure 6B; Supplementary Tables 2, 3). Moreover, the median time point for aGVHD onset was 36 days in the two clinical cohorts (Supplementary Table 4). These clinical data validated the phenomenon of the abnormal enrichment of PB monocytes on day 21 in aGVHD patients, supporting the potential of monocytes as an early risk factor to monitor the development of aGVHD for patients receiving allo-PBSCT clinically.

In this segment, we validated the functional superiority of day 21 PB monocytes from aGVHD patients in inducing the proliferation of T cells. In addition, aGVHD-associated aberrant accumulation of PB monocytes on day 21 after allo-PBSCT was also confirmed in clinical blood routine examination data from transplant patients with AA and AL, indicating the universality of the phenomenon. The overall findings suggest the promising potential of monocytes as an early-stage risk factor for the development of aGVHD.

Discussion

HSCT is an established procedure for various disorders of the hematopoietic, immune, and metabolic systems. However, aGVHD remains the major complication of allo-HSCT and poses a threat to good prognosis. Here, our study provides new insights into the dynamics of the early hematopoietic reconstruction for patients with aGVHD after allo-PBSCT. We focused on immune cells from PB at different periods post-transplantation and found a significant increase in monocyte proportion on day 21 in the aGVHD group. The transcriptional profiling and *in vitro* co-culture experiments confirmed that day 21 PB monocytes isolated from aGVHD patients had a stronger ability to stimulate the proliferation of T lymphocytes than those of non-aGVHD patients. Furthermore, we verified our findings with clinical blood routine data, concluding that the aGVHD-associated enrichment of PB monocytes on day 21 post-transplantation could be generalized in patients undergoing allo-PBSCT, to some extent. Based on the dynamics of early hematopoietic reconstruction after transplantation, our findings provide new insights for early monitoring and therapeutic intervention of aGVHD.

Although the reconstitution dynamics of transplanted allogeneic HSPCs in both mice and humans have been described at single-cell resolution (19, 20, 44, 45), our understanding of the

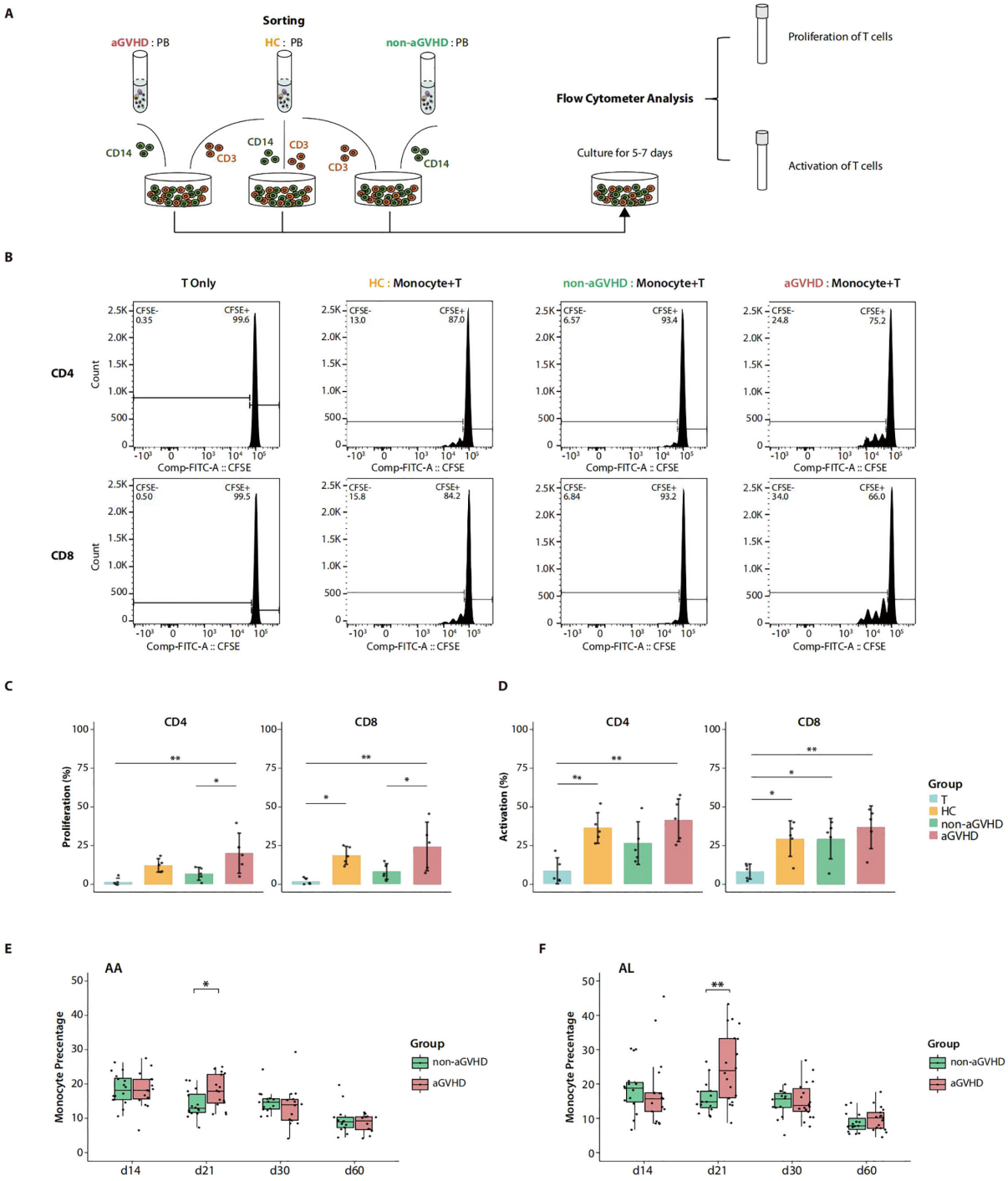

FIGURE 4
Co-culture experiments and clinical cohort. **(A)** Schematic overview of co-culture experiments of T cells and monocytes. **(B)** Flow cytometry graphs show the proliferation frequency of the allogeneic $CD4^+$ T and $CD8^+$ T cells estimated by carboxyfluorescein succinimidyl ester (CFSE) dilution after co-culture with monocytes. Monocytes were sorted from approximately day 21 peripheral blood (PB) of aplastic anemia (AA) patients undergoing allo-PBSCT with acute graft-versus-host disease (aGVHD) (aGVHD), without aGVHD (non-aGVHD), and PB of healthy controls (HCs). T cells cultured without monocytes (T Only) were used as the baseline control. **(C)** The summary of the proportion of T-cell proliferation (percentage of CFSE dilution) in co-culture experiments with five replications. *p*-Values were evaluated by the Tukey–Kramer test. $*p < 0.05$, $**p < 0.01$. **(D)** The summary of proportion of T-cell activation (percentage of $CD69^+/CD25^+$ T cells) in co-culture experiments with five replications. *p*-Values were evaluated by the Tukey–Kramer test. $*p < 0.05$, $**p < 0.01$. **(E)** Monocyte percentage from blood routine examination of AA patients with aGVHD (aGVHD group, n = 16) and without aGVHD (non-aGVHD group, n = 16) after allo-PBSCT. Each point represents the median value of monocyte percentages within 3 days around the corresponding time point. *p*-Values were evaluated by the two-tailed Mann–Whitney U test. $*p < 0.05$. **(F)** Monocyte percentage from blood routine examination of acute leukemia (AL) patients with aGVHD (aGVHD group, n = 18) and without aGVHD (non-aGVHD group, n = 15) after allogeneic peripheral blood stem cell transplant transplantation (allo-PBSCT). Each point represents the median value of monocyte percentages within 3 days around the corresponding time point. *p*-Values were evaluated by the two-tailed Mann–Whitney U test. $**p < 0.01$.

early hematopoietic reconstruction in the context of aGVHD is still severely limited. Previous studies have indicated that APCs can initialize and exacerbate GVHD in mice; however, the cell type has not been specified in humans, and associated GVHD prophylaxis regimens remain further to be developed (46–48). Clinical studies have revealed alterations in the proportions and phagocytic functions of monocyte subpopulations following complications after HSCT (49, 50). However, the biological characteristics and potential pathogenic role of monocytes in the development of aGVHD require further elucidation. Our research delineated the transcriptomic landscape of aGVHD progression during the initial phase of hematopoietic reconstitution and highlighted the abnormal accumulation and activation of day 21 PB monocytes before the occurrence of aGVHD. The abnormal accumulation of day 21 PB monocytes is close to being a universal phenomenon in patients undergoing allo-PBSCT and could be detected by clinical blood routine examination. In general, our study reveals that the abnormal accumulation of monocytes in PB on day 21 following allo-PBSCT is clinically feasible as a potential risk factor for aGVHD, and this groundbreaking discovery supports the advancement of aGVHD surveillance and intervention measures to coincide with this critical time point, specifically approximate day 21 post-transplantation.

Although we validated that monocytes in aGVHD patients have a stronger capability to induce the activation and proliferation of T cells compared with those in non-aGVHD patients, the spectrum of cytokines secreted by monocytes and T cells remains to be further explored. Of note, cytokines and related inflammatory pathways exert an essential role throughout the three phases of aGVHD pathophysiology. In the first phase, conditioning chemoradiotherapy or total body irradiation (TBI) traditionally provokes pathological tissue damage and promotes the release of proinflammatory cytokines [such as interleukin-1a, interleukin-33, tumor necrosis factor-α (TNF-α), and interleukin-1] as well as pathogen-associated molecular pattern (PAMP) molecules, which could significantly boost the antigen-presenting capacity of APCs (51–55). During the second phase of aGVHD pathophysiology, activated APCs induce the proliferation and activation of T cells by presenting alloantigens and secreting cytokines. Alloantigens are internalized, processed, and presented to T cells by APCs through the major histocompatibility complex (MHC)–peptide complex, which provides the first signal for T-cell activation. Interaction of costimulatory molecules on the APC and T-cell surface (including CD80/CD86-CD28 and CD40-CD40L) delivered a second costimulatory signal for T-cell activation. In addition, cytokines secreted by APCs are important components of the third signal of T-cell activation (56, 57). Systemic interleukin-6 (IL-6) concentration is elevated early after allogeneic transplant, and donor dendritic cell (DC)-derived IL-6 exerts a crucial regulatory role in the expansion and differentiation of T cells during aGVHD (58). Documented IL-6 signaling is critical to induce donor type-17 T (Th17) and type 22 T (Th22) cell differentiation after bone marrow transplantation (BMT) (59–61), and the anti-IL-6 receptor monoclonal antibody, tocilizumab, has been shown to effectively reduce the incidence of acute GVHD (62). Moreover, type I interferon (IFN) produced by putative DCs could enhance $CD8^+$ T cell-mediated GVHD and graft-versus-leukemia (GVL), although protecting recipients from $CD4^+$-mediated GVHD (63). Murine models also demonstrated that OX40, a molecule expressed on activated T cells and interacting with OX40L on activated APCs, stimulates effector T-cell proliferation. Furthermore, OX40 signaling in regulatory T cells (Tregs) disturbs their immunosuppressive effects (64, 65). In the third phase, differentiated effector cells, such as T cells and phagocytes—including monocytes and macrophages—secrete cytokines that contribute to the persistence and exacerbation of aGVHD. During aGVHD, Th17 and non-Th17 donor lineages are a primary source of granulocyte-macrophage colony-stimulating factor (GM-CSF), which can expand myeloid populations (66, 67). In addition, monocytes can achieve self-regulation through the secretion of GM-CFS during inflammatory response after HSCT (68). Collectively, these studies indicate that the combination of IL-6 and GM-CSF appears to establish a positive feedback mechanism, which significantly contributes to the progression of aGVHD.

Our study's transcriptome analysis revealed that NK cells from patients with aGVHD exhibited heightened functional activation. A study demonstrated that NK cells migrate to GVHD target organs following a spatial and temporal distribution extremely similar to T cells after HSCT (69). Although there are still some controversies about the role of NK cells in aGVHD (70–72), much attention should be paid to NK cells because of the first donor-derived lymphocyte subset to recover (73) and their crucial role in GVL after HSCT for hematological malignancies (74). Kordelas L. et al. reported that the proportions of donor-derived NK cells expressing the activating receptor CD94/NKG2C were lower in recipients with GVHD compared with those without GVHD after HSCT. GVHD patients presented with a lower ratio of CD94/NKG2C to CD94/NKG2A on NK cells (75). Consistently, Ghadially and coworkers suggested that NK cells inhibited or promoted GVHD development by relying on different receptor expression profiles. NK cells with NKp46 receptor stimulation mediated the elimination of APCs, thereby reducing the incidence of GVHD, while the absence of NKp46 on donor NK cells results in DC-mediated increased stimulation of donor T, thereby facilitating GVHD development (76, 77). However, pro-inflammatory cytokines derived from NK cells contribute to GVHD development, which is well-established. Xun et al. showed that *in vitro* IL-2-activated human NK cells producing IFN-γ and TNF-α were able to induce aGVHD in a xenogeneic model (78). Furthermore, higher proportions of IFN-γ producing NK cells after HSCT were associated with an increased incidence of acute GVHD in humans (79). IFN-γ boosts the recognition of CD8 T cells for target cells and promotes the differentiation of CD4 T cells toward a T-helper type 1 (Th1) phenotype (80), which plays an important role in the pathophysiology of GVHD (81). Importantly, the cytokines (including type I IFN, interleukin-2, interleukin-18, and interleukin-15) secreted by dendritic cells, macrophages, monocytes can further promote NK-cell cytolysis and IFN-γ

secretion (82). In short, NK cells can suppress GVHD by killing APCs, while NK cells can exacerbate GVHD due to enhanced NK-cell cytolysis or cytokine secretion facilitated by APCs. Further investigations are essential to elucidate the regulatory interactions between NK cells and other immune cells including T cells, monocytes, and neutrophils during aGVHD.

There are several intriguing perspectives that merit further studies. In patients with aGVHD, the differentiation bias of HSPCs toward monocytes occurs even prior to day 21 post-transplantation. Exploring the fundamental mechanisms behind the hematopoietic differentiation bias of HSPCs is anticipated to uncover potential targets for counteracting the abnormal accumulation of monocytes. In addition, there is uncertainty regarding the microenvironment in patients with aGVHD during the early hematopoietic reconstruction. The altered niche associated with aberrant monocyte accumulation could offer additional perspectives on the underlying pathogenic mechanisms. In addition, the regulatory role of immunosuppressive cells like PreNeus in the development of aGVHD remains under further investigation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GSE224714 (GEO) and HRA001359 (GSA; https://ngdc.cncb.ac.cn/gsa-human/browse/HRA001359).

Ethics statement

The studies involving humans were approved by the ethics committee of Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

HS: Conceptualization, Investigation, Validation, Visualization, Writing – original draft, Resources. LW: Data curation, Formal analysis, Visualization, Writing – original draft. XZ: Formal analysis, Visualization, Writing – original draft. YYH: Investigation, Writing – original draft. PD: Validation, Writing – original draft. AP: Resources, Writing – original draft. YZ: Resources, Writing – original draft. YWH: Validation, Writing – original draft. SM: Resources, Writing – original draft. EJ: Resources, Writing – original draft. FD: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft. TC: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft. SH: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1433091/full#supplementary-material

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Umbilical cord blood stem cells as third-party adjuvant infusions in human leukocyte antigen antibody-positive patients undergoing haploidentical hematopoietic stem cell transplantation

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Introduction: Graft failure (GF) or poor graft function (PGF) remain critical obstacles in haploidentical hematopoietic stem cell transplantation (haplo-HSCT), especially in recipients with HLA antibodies. Here, we performed a retrospective cohort study to investigate the efficacy and safety of the use of unrelated umbilical cord blood stem cells (UCBs) as a third-party adjuvant infusion in patients with HLA-antibodies undergoing haplo-HSCT.

Methods: A total of 90 patients were divided into three groups: 17 patients in Group A (with positive HLA antibodies and who received UCB infusion), 36 patients in Group B (with positive HLA antibodies without UCB infusion), and 37 patients in Group C (without HLA antibody or UCB infusion).

Results: The median age of patients included in Groups A, B, and C was 43 (IQR, 27 - 49.5), 33 (IQR, 20 - 48.75), and 30 (IQR, 18 - 46.5) years, respectively. All but one patient in Group B achieved granulocyte recovery within 28 days after transplantation. The median time to granulocyte engraftment were all 12 days for patients in Groups A, B, and C, respectively. All the patients in Group A achieved 100% donor chimerism without UCB engraftment. There were no significant differences in granulocyte or platelet engraftment time between the three groups. There were 1, 5, and 0 patients in Groups A, B, and C, respectively, who developed PGF. The cumulative incidence rates for any grade of acute graft-versus-host disease (aGVHD) were comparable among the three groups. Patients in Group B presented a greater incidence of cGVHD than did those in Group A ($P = 0.002$) and Group C ($P = 0.006$). Patients in Group A presented more limited and milder cGVHD than those in Group C ($P < 0.0001$). The 1-year relapse-free survival (RFS) was 70.6% (95% CI, 0.47 - 0.87), 55.6% (95% CI, 0.40 - 0.70), and 77.9% (95% CI, 0.63 - 0.89) in Groups A, B, and C, respectively.

Discussion: Our results indicated that patients who were positive for HLA antibodies were at a greater risk of developing GF/PGF. Co-infusion with UCBs was safe and improved engraftment, cGVHD, and improved the 1-year RFS to some extent.

KEYWORDS

unrelated umbilical cord blood, haploidentical hematopoietic stem cell transplantation, graft failure, poor graft function, graft-versus-host disease, relapse-free survival

1 Introduction

With the progression of haploidentical hematopoietic stem cell transplantation (haplo-HSCT) technology, almost every patient with malignant hematopoietic diseases can find a donor receiving allogeneic-HSCT and achieve long overall survival (OS). However, the therapeutic benefits and wider application of haplo-HSCT are limited by graft-versus-host disease (GVHD), the latter remains a major obstacle to long-term survival for this population. Furthermore, rejection remains a critical reason for graft failure (GF) in the haplo-HSCT setting. The incidence of rejection was <3% for matched human leukocyte antigen (HLA)-identical sibling donors (MSDs) or matched unrelated donors (MUDs), and these data increased to >10% for haplo-HSCT. Furthermore, the incidence of poor graft function (PGF) with complete donor chimerism is also greater in the haplo-HSCT setting (1). Both GF and PGF often result in an increased incidence of transplant-related mortality (TRM) and inferior OS.

Previous studies have suggested that rejection is mainly related to donor-specific antibody (DSA), severe acute GVHD (aGVHD), HLA mismatching, stem cell number, etc. (2). DSA is the most important risk factor for rejection, and a previous study confirmed that DSA is the only risk factor for GF (3). In haploidentical donor selection, due to the presence of multiple donor-recipient mismatches, anti-HLA antibody screening must be performed in the recipient to detect the presence of DSA. In 2018, the European Society for Blood and Marrow Transplantation (EBMT) recommended DSA testing in all haploidentical donor transplant recipients and suggested an MFI > 1,000 as DSA positivity (4). If multiple donors are available, DSA-positive donors should be avoided. Furthermore, donors who have the same allele as patients with DSA (MFI ≥ 10,000) should be excluded. For patients with HLA antibodies but not DSAs, transplantation can be conducted as scheduled. However, these patients also have a higher GF rate than those without HLA antibodies, especially in haplo-HSCT (5). To date, there is no consensus on whether HLA antibodies but not DSAs should be managed before transplantation, and there is no ideal strategy for eradicating or decreasing HLA antibodies in this population (6).

Umbilical cord blood cells (UCBs) are characterized by abundant stem cell sources and low immunogenicity. UCB has a greater number of natural killer (NK) cells and a greater proportion of immature T cells (7). UCBs have previously been shown to contain a distinct regulatory T-cell (Treg) subset that exists at a relatively high frequency compared to that in peripheral blood (PB). Tregs and other components of UCB may act as immunomodulators to reduce immune rejection and regulate the hematopoietic microenvironment (7). Previous studies reported satisfactory results using UCBs as third-party stem cells in haplo- or MUD transplantations (8, 9). Lyu et al. confirmed a superior outcome of haplo-HSCT combined with third-party UCBs compared with MUD transplantation in 66 patients with a high risk of hematopoietic neoplasm relapse (10). Here, we hypothesized that third-party UCBs could offer some advantages for patients with HLA antibodies and decrease the incidence of GF/PGF.

In this retrospective, single-center, controlled study, we aimed to investigate the efficacy and safety of unrelated UCBs as third-party adjuvant infusions in patients with HLA antibodies receiving haplo-HSCT. The primary objectives were the incidence of GF/PGF and the transplant-related mortality (TRM) within 100 days posttransplantation. The secondary objectives included the following: incidence of aGVHD grades II-IV, chronic GVHD (cGVHD), relapse-free survival (RFS), GVHD- and relapse-free survival (GRFS), and overall survival (OS).

2 Material and methods

2.1 Patients and controls

A total of 90 eligible participants with hematological diseases who underwent haplo-HSCT from May 2017 to December 2022 in the Department of Hematology of Shandong Provincial Hospital Affiliated to Shandong First Medical University were enrolled in this study. The inclusion criteria for patients were haplo-HSCT with weakly positive/positive HLA antibodies (MFI >500) but not DSAs. Age- and sex-matched patients who underwent haplo-HSCT without any HLA antibodies were chosen concurrently as controls. The exclusion criterion was haplo-HSCT with positive DSAs (MFI>1,000). HLA antibodies from each patient were routinely tested before transplantation. In this retrospective study, participants were assigned to three groups in a ratio of 1:2:2. Group

A (experimental group: patients with weakly positive/positive HLA antibodies who received UCB infusion), Group B (positive control group: patients with weakly positive/positive HLA antibodies who did not receive UCB infusion), Group C (blank control group: patients who did not receive HLA antibody or UCB infusion). All protocols and consent forms were obtained from the patients or their guardians and approved by the Human Subjects Review Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University. This project is registered by the Shandong Data Protection Agency and approved by the Joint Ethics Committee of Jinan. UCBs were obtained from the Shandong Umbilical Cord Blood Bank. All the study procedures were conducted in compliance with the Declaration of Helsinki. The consort flow chart of this study is shown in Figure 1.

2.2 Conditioning and prophylaxis for aGVHD

Patients included in this study received myeloablative conditioning (MAC), which included antithymocyte globulin (ATG) at a total of 10 mg/kg administered on days -5 to -2 before the transplant. The conditioning regimens used in this study are summarized in Table 1. The calcineurin inhibitor (CCI) combined with mycophenolate mofetil (MMF) and short-term methotrexate (MTX, 15 mg/kg on days +1, 10 mg/kg on days +3, +5, and +11) were administered for aGVHD prophylaxis. Glucocorticoid (GC)-based treatment was given when Grade II or higher aGVHD developed. For patients who developed steroid-resistant/refractory aGVHD (SR-aGVHD), second-line strategies, including ruxolitinib, anti-CD25 monoclonal antibody, and mesenchymal stem cell (MSC) infusion, were considered. For patients with cGVHD, GC combined with CCIs was the most common strategy used in our study.

Acyclovir, fluconazole/posaconazole, and cotrimoxazole were administered prophylactically against herpes viruses, fungi, and pneumocystis pneumonia, respectively. Granulocyte colony-stimulating factor (G-CSF) was administered subcutaneously at a dose of 5 - 10 mg/kg from day +6 after stem cell transfusion and was discontinued until neutrophil engraftment. All blood products were irradiated, and leukocytes were depleted.

2.3 Transplant products

All patients received peripheral blood stem cells (PBSCs) from their haplo-donors. For patients in Group A, single unrelated UCBs were infused four hours before haplo-donor stem cells. The following criteria for cord blood unit selection were applied: 1) HLA matching was preferred at > 4/6 loci (HLA-A, HLA-B, and HLA-DRB1 loci serological matching) between donors and recipients. 2) The total nucleated cells (TNCs) were not less than

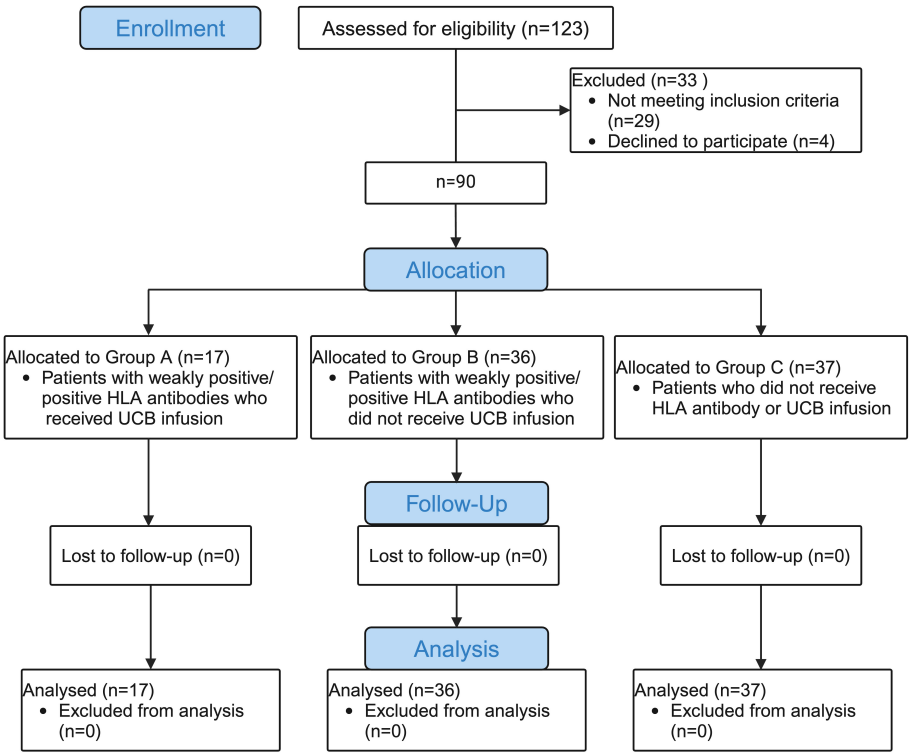

FIGURE 1
The consort flow chart of the study.

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TABLE 1 Demographic and clinical characteristics of patients and donors.

	No. (N = 90)	%
Donor		
Median age at mobilization, yr (IQR)	33.5(23.75 - 47)	–
Male	64	71.1%
Female	26	28.9%
Patient		
Median age at transplant, yr (IQR)	33(19 - 48)	–
Male	51	56.7%
Female	39	43.3%
Diagnosis		
AML, MDS	43	47.8%
ALL, MPAL	27	30.0%
CMML, CML	16	17.8%
SAA and other benign diseases	4	4.4%
Remission status at transplant		
CR1	59	65.6%
≥CR2	16	17.8%
NR	11	12.2%
SAA and other benign diseases	4	4.4%
Conditioning regimen		
IDA+BU/FLU	21	23.3%
BU/FLU	12	13.3%
BU/CY	6	6.7%
mBU/CY	4	4.4%
VP16+BU/CY	20	22.2%
TBI/CY	3	3.3%
FLU/CY	3	3.3%
Others	21	23.3%
Donor–patient gender matching		
Male-Female	28	31.1%
Male-Male	36	40.0%
Female-Male	15	16.7%
Female-Female	11	12.2%
Donor–patient relationship		
Patient-child	40	44.4%
Child-patient	34	37.8%
Sibling	16	17.8%
HLA match		
>5/10	15	16.7%
5/10	75	83.3%
Donor–patient blood type		
Match	62	68.9%
Mismatch	28	31.1%
Cellularity in haplo-grafts, median (IQR)		
Mononuclear cells, 10^8/kg	10.46(8.16 - 13.26)	
CD34+ cells, 10^6/kg	3.94(2.69 - 4.95)	

AML, acute myelocytic leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphocytic leukemia; MPAL, mixed phenotype acute leukemia; CMML, chronic myelomonocytic leukemia; CML, chronic myelogenous leukemia; SAA, severe aplastic anemia; CR, complete remission; NR, non-remission; IDA, idarubicin; BU, busulfan; FLU, fludarabin; CY, cyclophosphamide; mBU/CY, modified BU/CY; VP16, etoposide; TBI, total body irradiation. IQR, interquartile range.

1×10^7/kg of the recipient's body weight after thawing. 3) Blood type-matched cord blood was preferred at an equal level of HLA-type matching. The matching landscape of the haplotype donor and cord blood is shown in Table 2.

2.4 Definitions and patient management

The first day of absolute neutrophil count (ANC) > 0.5×10^9/L for 3 consecutive days was defined as neutrophil engraftment. The first day when the platelet count was > 20×10^9/L without platelet transfusion support for 7 consecutive days was defined as platelet engraftment. Primary GF was defined as failure to achieve neutrophil engraftment within the first 28 days posttransplantation and lack of evidence of donor-type implantation. Poor graft function (PGF) was defined as the presence of multilineage cytopenias in the presence of 100% donor chimerism. The diagnostic and grading criteria for acute and chronic GVHD were determined according to the EBMT-NIH-CIBMTR Working Group Position Statement on Criteria and Guidelines for the Evaluation of Graft-versus-Host Disease (11). Serum levels of cytomegalovirus (CMV)- and Epstein–Barr virus (EBV)-DNA were monitored twice weekly during the first 30 days posttransplantation and every month thereafter. Donor chimerism was evaluated every month in the first year and every 3 months until 3 years posttransplantation.

2.5 Data collection

The occurrence of granulocyte and platelet engraftment time, GVHD, CMV/EBV reactivation, hemorrhagic cystitis (HC), TRM within 100 days posttransplantation, 1-year relapse-free survival (RFS), 1-year GVHD- and relapse-free survival (GRFS), and 1-year overall survival (OS) between the three groups were analyzed in this study.

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TABLE 2 The cellularity of the stem cells and the engraftment time of neutrophil and platelet.

	Group A (n = 17)	Group B (n = 36)	Group C (n = 37)
HLA match (Cord blood)			
5/10	3 (17.6%)	–	–
>5/10	14 (82.4%)	–	–
Donor–patient blood type (Cord blood)			
Match	15 (88.2%)	–	–
Mismatch	2 (11.8%)	–	–
Cord blood TNC, 10^8/kg			
Median (IQR)	15.16 (12.45 – 18.23)	–	–
Cord blood CD34+, 10^6/kg			
Median (IQR)	4.63 (3.75 – 5.55)	–	–
Cell compositions in haplo-grafts, median (IQR)			
MNC, 10^8/kg	11.03 (9.43 – 17.35)	9.95 (7.70 – 11.99)	10.80 (8.20 – 13.69)
CD34+ cells, 10^6/kg	3.58 (2.58 – 4.81)	4.01 (2.61 – 6.50)	4.13 (2.85 – 4.87)
Granulocyte recovery at +28 days, n (%)			
Median (IQR)	12 (10.5 – 15)	12 (11 – 16)	12 (11 – 14)
Platelet recovery at +28 days, n (%)			
Median (IQR)	14 (12 – 16)	13 (12 – 17)	13 (12 – 17.75)

HLA, human leukocyte antigen; TNC, total nucleated cells; MNC, mononuclear cell; IQR, interquartile range.

2.6 Statistical analysis

SPSS 26.0 software (SPSS, Chicago, IL) and GraphPad Prism software (La Jolla, CA) were used to conduct the statistical analyses and construct the figures. All quantitative values are expressed as the mean (range) or median (IQR, interquartile range). The normality of the continuous variables was assessed by the Kolmogorov-Smirnov test (K-S test). The Kruskal-Wallis test and analysis of variance (ANOVA) were used for comparisons among the three groups. A nonparametric test (Mann-Whitney U test) was used to compare the nonnormally distributed continuous variables between the two groups. The chi-squared test and Fisher's exact probability test were performed to analyze categorical variables, including the incidence and grade of aGVHD, cGVHD, incidence of CMV, EBV reactivation, and HC, among the different groups. The TRM, RFS, GRFS, and OS were estimated using the Kaplan-Meier product limit estimation method, and differences in subgroups were assessed by the log-rank test. Throughout, two-sided P values < 0.05 were obtained via t-tests, and 95% confidence intervals (95% CIs) that included unity were considered to indicate statistical significance.

3 Results

3.1 Patient characteristics

A total of 90 patients were included in this study (17 in Group A, 36 in Group B, and 37 in Group C). The median age of patients included in Groups A, B, and C was 43 (IQR, 27 - 49.5), 33 (IQR, 20 - 48.75), and 30 (IQR, 18 - 46.5) years, respectively. These participants were aged 8 to 65 years, and the median age of the patients at transplantation was 33 years. The clinical characteristics of the patients and the paired donors are listed in Table 1. The median follow-up time was 26.5 (range, 1 - 79 months), and the last follow-up time point was December 2023.

3.2 HLA antibody distribution

The prevalence of antibodies to HLA according to different cutoff values of mean fluorescence intensity in Groups A and B are shown in Figures 2A, B. For patients with HLA antibodies, the three most frequent alleles were HLA-B76, HLA-DP1, and HLA-DR4. The first three highest MFI values were 23,768, 22,741, and 22,428, which corresponded to the HLA loci HLA-B13, HLA-B61, and HLA-B60, respectively. One patient in Group B who developed GF presented with a weakly positive HLA-A02 (MFI 1294) antibody.

3.3 Transplant products and engraftment

For all 90 participants, the median numbers of reinfused haplograft mononuclear cells (MNCs) and CD34+ cells were 10.46 (IQR, 8.16 - 13.26) × 10^8/kg and 3.94 (IQR, 2.69 - 4.95) × 10^6/kg, respectively. The median MNCs and CD34+ cells for the three groups were compared, and there was no significant difference among them.

No obvious adverse effects were observed during the process of UCB infusion. For Group A, the median cellularity of the third-party UCB units was 15.16 (IQR, 12.45 - 18.23) × 10^8/kg for TNCs and 4.63 (IQR, 3.75 - 5.55) × 10^6/kg for CD34+ cells. The median number of MNCs in the UCBs was 11.03 (IQR, 9.43 - 17.35) × 10^8/kg, and the median number of CD34+ cells was 3.58 (IQR, 2.58 - 4.81) × 10^6/kg.

All the patients in Group A achieved 100% donor chimerism without UCB engraftment. There were no significant differences in granulocyte or platelet engraftment time between Group B and the other two groups.

The median time to granulocyte engraftment was 12 (IQR, 10.5 - 15), 12 (IQR, 11 - 16), and 12 (IQR, 11 - 14) days for patients in Groups A, B, and C, respectively. The median time to platelet recovery was 14 (IQR, 12 - 16), 13 (IQR, 12 - 17), and 13 (IQR, 12 - 17.75) days in Groups A, B, and C, respectively. The cellularity of the stem cells and the graft times of the granulocytes and platelets in the three groups are shown in Table 2.

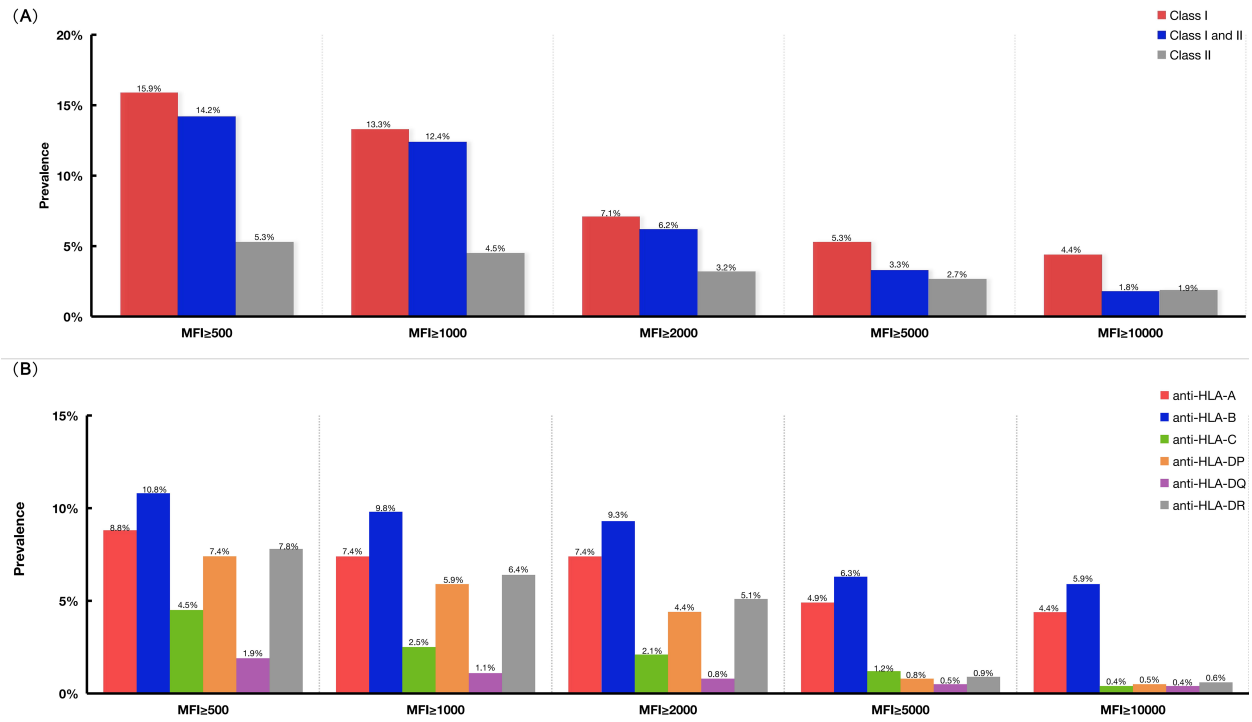

FIGURE 2
The prevalence of HLA antibodies according to different cut-off values of MFI in Groups A and B. **(A)** The prevalence of HLA antibodies in Group A and Group B patients according to different MFI cut-off values. **(B)** The prevalence of HLA antibodies specific for antigens coded by different HLA loci, such as HLA-A, -B, -C, -DP, -DQ, -DR. HLA, human leukocyte antigen; MFI, mean fluorescence intensity.

3.4 Incidence of GF or PGF

All but one patient in Group B achieved granulocyte recovery within 28 days after transplantation. This patient died of multiple organ dysfunction syndrome (MODS) at 50 days posttransplantation.

There were 1, 5, and 0 patients in Groups A, B, and C, respectively, who developed PGF. In Group A, the patient who developed PGF received a child-parent donation with a matched blood type. The HLA-antibody MFI of this patient was 18,649.3, which corresponded to the HLA locus of HLA-B13. The number of MNCs and CD34+ cells from his haplo-donor group were 11.89×10^8/kg and 4.7×10^6/kg, respectively. This patient eventually died from a serious infection. According to Group B, 4 males and 1 female developed PGF. There were 3 parent-child and 2 child-parent donor-patient relationships. Three donor-recipient blood types were matched, and 2 donor-recipient blood types were not matched. The mean numbers of MNCs and CD34+ cells in these five patients were 9.90 (range, 7.01 - 15.2) $\times 10^8$/kg and 4.75 (range, 2.33 - 8.52) $\times 10^6$/kg, respectively. All five patients died, two died of severe infection, two died of respiratory and circulatory failure, and one died of posttransplant lymphoproliferative disease (PTLD).

3.5 The incidence of aGVHD and cGVHD

In all 90 patients, the cumulative incidence rates for Grade I-II aGVHD and Grade III-IV aGVHD were 75.5% (37/90) and 24.5% (12/90), respectively. Eight patients (1 patient in Group A, 3 patients in Group B, and 4 patients in Group C) developed grade IV intestinal aGVHD, three of whom developed SR-aGVHD, and all of whom responded to second-line anti-aGVHD therapy. There were no significant differences in the incidence or grades of aGVHD among the patients in the three groups (Figures 3A, B). The median time points of aGVHD occurrence were 17.5 (range, 8 - 31), 21 (range, 10 - 45), and 20 (range, 11 - 31) days after transplant for Groups A, B, and C, respectively. The clinical characteristics and grades of aGVHD in the three groups of patients are shown in Table 3. The incidence of cGVHD in 84 patients who survived to +100 days was evaluated. The cumulative incidence rate of cGVHD was 20.2% (17/84). There were 2, 10, and 5 patients who developed cGVHD in Groups A, B, and C, respectively. There was no significant difference in the incidence of cGVHD between Group A and Group C. However, patients in Group B presented a greater incidence of cGVHD than did patients in the other two groups (Group B vs. Group A: 31.3% vs. 12.5%, P = 0.002; Group B vs. Group C: 31.3% vs. 13.9%, P = 0.006) (Figure 3C). Patients in Group A presented with limited cGVHD, which was relatively mild compared with that in Group B and Group C (0% vs. 40%, P < 0.0001, Figure 3D). There was no statistically significant difference in the degree of cGVHD organ involvement between patients in Group B and those in Group C.

At the end of follow-up, the median cGVHD durations of the three groups were 7 (range, 5 - 9), 7 (range, 3 - 23), and 8 (range, 3 - 12) months, respectively. The distribution of characteristics of patients with cGVHD in the three groups is shown in Table 3. The organs most frequently involved in cGVHD were the oral

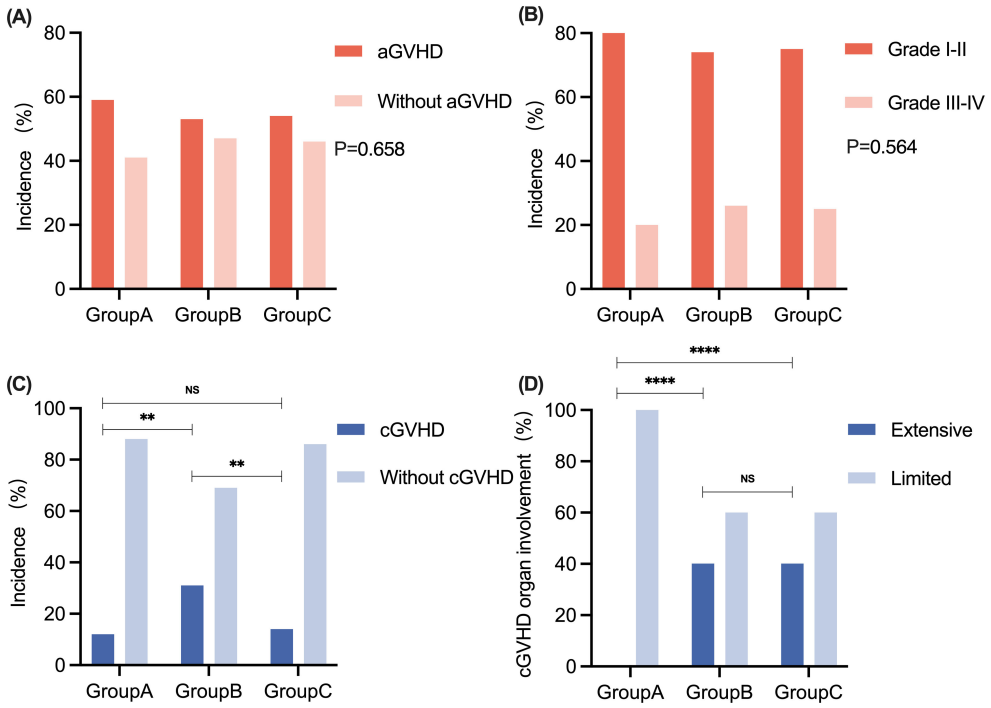

FIGURE 3
The comparison of aGVHD and cGVHD among patients in Groups A, B, and C. **(A)** The incidence of aGVHD in Groups A, B, and C. **(B)** The grades of aGVHD in Groups A, B, and C. **(C)** The incidence of cGVHD in Groups A, B, and C. **(D)** The degree of cGVHD organ involvement in Groups A, B, and C. No significant difference was observed in the incidence and grading of aGVHD between patients in Groups A, B, and C, respectively. **P < 0.01. ****P < 0.0001. aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; NS, no significance.

TABLE 3 The clinical characteristics of patients with aGVHD/cGVHD.

	Group A	Group B	Group C	P-value
Occurrence of aGVHD, n (%)	10 (58.8)	19 (52.8)	20 (54.1)	0.658
Median time of aGVHD occurrence, days (range)	17.5 (8 – 31)	21 (10 – 45)	20 (11 – 31)	0.553
Mean age, yr (range)	37.60 (16 – 63)	29.42 (9 – 65)	30.95 (14 – 56)	0.110
Gender				0.222
Male, n (%)	6 (60.0)	10 (52.6)	13 (65.0)	–
Female, n (%)	4 (40.0)	9 (47.4)	7 (35.0)	–
Primary disease, n (%)				0.482
AML, MDS	5 (50.0)	8 (42.1)	8 (40.0)	–
ALL, MPAL	3 (30.0)	6 (31.6)	9 (45.0)	–
CMML, CML	1 (10.0)	3 (15.8)	2 (10.0)	–
SAA and other benign diseases	1 (10.0)	2 (10.5)	1 (5.0)	–
Pre-transplantation status, n (%)				0.240
CR1	7 (70.0)	11 (57.9)	12 (60.0)	–
≥CR2	3 (30.0)	2 (10.5)	5 (25.0)	–
NR or other	0 (100.0)	6 (31.6)	3 (15.0)	–
Grades of aGVHD, n (%)				0.564
I	3 (30.0)	7 (36.8)	7 (35.0)	–

(Continued)

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TABLE 3 Continued

	Group A	Group B	Group C	P-value
II	5 (50.0)	7 (36.8)	8 (40.0)	–
III-IV	2 (20.0)	5 (26.3)	5 (25.0)	–
aGVHD organ involvement, n (%)				0.051
Skin	6 (60.0)	9 (47.4)	8 (40.0)	–
Liver	1 (10.0)	0 (0.0)	0 (0.0)	–
Gastrointestinal tract	1 (10.0)	3 (15.8)	4 (20.0)	–
Two or more organs involved	2 (20.0)	7 (36.8)	8 (40.0)	–
Occurrence of cGVHD, n (%)	2 (12.5)	10 (31.3)	5 (13.9)	<0.001
Median time of cGVHD occurrence, months (range)	7 (5 – 9)	7 (3 – 23)	8 (3 – 12)	0.830
Early aGVHD before cGVHD, n (%)				0.048
Yes	2 (100.0)	5 (50.0)	3 (60.0)	–
No	0 (0.0)	5 (50.0)	2 (40.0)	–
cGVHD Organ Involvement, n (%)				<0.0001
Lung	0 (0.0)	1 (10.0)	0 (0.0)	–
Skin, joint, and connective tissue	0 (0.0)	0 (0.0)	2 (40.0)	–
Liver	0 (0.0)	3 (30.0)	1 (20.0)	–
Oral cavity	2 (100.0)	2 (20.0)	0 (0.0)	–
Two or more organs involved	0 (0.0)	4 (40.0)	2 (40.0)	–

AML, acute myelocytic leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphocytic leukemia; MPAL, mixed phenotype acute leukemia; CMML, chronic myelomonocytic leukemia; CML, chronic myelogenous leukemia; SAA, severe aplastic anemia; CR, complete remission; NR, non-remission; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease.

cavity (4/17, 23.5%) and liver (4/17, 23.5%). One patient in Group B developed bronchiolitis obliterans syndrome (BOS) and lived with poor quality of life at the last follow-up (Supplementary Table S1).

3.6 The incidence of CMV and EBV reactivation

The differences in the reactivation of CMV and EBV among the three groups are presented in Table 4. The total incidences of CMV and EBV viremia were 47.8% (43/90) and 72.2% (65/90), respectively. As shown in Figure 4A, there were no significant differences in CMV reactivation among the three groups in the first 100 days after transplantation. In terms of EBV reactivation, patients in Group A presented with less EBV reactivation than did those in Group B (52.9% vs. 70.3%, P = 0.020) and Group C (52.9% vs. 83.3%, P < 0.001). Patients in Group B had a greater incidence of EBV reactivation than did those in Group C (83.3% vs. 70.3%, P = 0.045) in the first 100 days posttransplantation (Figure 4A). As shown in Figure 4B, when the data were analyzed by month, the rate of CMV reactivation was greater in Group A than in Group B at 1 month after transplantation (41.2% vs. 22.2%, P = 0.006), and there was no significant difference between Group C and any of the other two groups. There was no statistically significant difference in the percentage of patients who experienced CMV reactivation in the 2nd month after transplantation among the groups. At 3 months posttransplantation, no CMV reactivation occurred in Group A patients, and the rate of CMV reactivation was greater in Group B patients than in the other two groups (Group B vs. Group A 19.4% vs. 0.0%, P < 0.001; Group B vs. Group C 19.4% vs. 5.4%, P = 0.004).

When we analyzed the data by month, there was no significant difference in the rate of EBV reactivation between patients in Group A and those in Group C at 1 month posttransplantation (Figure 4C). However, the rate of EBV reactivation in Group B patients was greater than that in Groups A (47.2% vs. 29.4%, P = 0.013) and C (47.2% vs. 24.3%, P = 0.001). At 2 months posttransplantation, the rate of EBV reactivation in Group A patients was lower than that in Group B patients (23.5% vs. 58.3%, P < 0.001) and Group C patients (23.5% vs. 62.2%, P < 0.001). However, there was no significant difference between Groups B and C. At 3 months after transplantation, the percentage of patients who were positive for EBV reactivation was lower in Group A than in Group B (23.5% vs. 50.0%, P < 0.001) and Group C (23.5% vs. 37.8%, P = 0.046). Similarly, there was no significant difference in the rate of EBV reactivation between patients in Groups B and C.

In addition, a total of 4 patients developed PTLD posttransplantation (1 in Group A, 2 in Group B, and 1 in Group C). The patient in Group C has survived to date, and the patient in Group A died of hemophagocytic lymphohistiocytosis (HLH). Two patients in Group B died of PTLD complicated with severe infection secondary to hematopoietic failure.

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TABLE 4 The reactivation of CMV and EBV between the three groups.

	Group A	Group B	Group C	P-value
CMV reactivation within 100 days				0.443
Yes	9(52.9)	16(44.4)	18(48.6)	–
No	8(47.1)	20(55.6)	19(51.4)	–
CMV reactivation by month				
1st-month	7(41.2)	8(22.2)	13(35.1)	0.014
2nd-month	5(29.4)	10(27.8)	8(21.6)	0.478
3rd-month	0(0.0)	7(19.4)	2(5.4)	<0.0001
EBV reactivation within 100 days				<0.0001
Yes	9(52.9)	30(83.3)	26(70.3)	–
No	8(47.1)	6(16.7)	11(29.7)	–
EBV reactivation by month				
1st-month	5(29.4)	17(47.2)	9(24.3)	0.001
2nd-month	4(23.5)	21(58.3)	23(63.9)	<0.0001
3rd-month	4(23.5)	18(50.0)	14(37.8)	<0.001

CMV, cytomegalovirus; EBV, Epstein-Barr virus.

3.7 Other complications posttransplantation

The mean incidence of HC in the first 100 days post-transplantation in Groups A, B, and C transplantation was 29.4% (5/17), 30.6% (11/36), and 27.03% (10/37), respectively. There was no significant difference in HC incidence among the three groups (all P values > 0.05).

Forty-three (47.8%) patients developed lung infections, and 38 (42.2%) patients suffered multiple organ infections (abdominal, sinus infections, etc.). There were no significant differences in the number of infectious organs or pathogens among the three groups.

3.8 Survival analysis

The TRM within 100 days after transplantation was 5.9%, 11.1%, and 2.7% in Groups A, B, and C, respectively. The comparison of TRM within 100 days after transplantation did not significantly differ among the three groups (Figures 5A–C).

The 1-year RFS rates were 70.6%, 55.6%, and 77.9% in Groups A, B, and C, respectively. The 1-year RFS of patients in Group A was not significantly different from that of patients in the other two groups (Figures 5D, E). However, the 1-year RFS of patients in Group C was greater than that of patients in Group B [HR = 0.41 (0.18 - 0.92), P = 0.031] (Figure 5F).

The 1-year GRFS for the three groups were 23.5%, 16.7%, and 29.7%, respectively. As shown in Figures 5G and 5H. Compared with Groups B and C, the 1-year GRFS was not statistically significant in patients in Group A. The 1-year GFRS was higher in patients in Group C than in Group B [HR = 0.66 (0.39 - 1.12), P = 0.035] (Figure 5I).

After a median follow-up period of 26.5 (range, 1 - 79) months, 61 patients survived. The 1-year OS rates of patients in Groups A, B, and C were 70.6%, 58.3%, and 83.8%, respectively. As shown in Figures 5J and 5K, there was no significant difference in the 1-year OS between patients in Group A and those in the other two groups. However, the 1-year OS of patients in Group C was greater than that of patients in Group B [HR = 0.35 (0.15 - 0.83, P = 0.017] (Figure 5L).

4 Discussion

In the present study, we investigated the efficacy and safety of unrelated UCBs as third-party adjuvant infusions in patients with HLA antibodies receiving haplo-HSCT. Preexisting or *de novo* HLA antibodies can be derived from blood transfusion, pregnancy, history of allogeneic transplantation, self-peptides, tumor antigens, CMV, influenza virus infection, bacterial infection, etc. (12, 13) There has been no consensus on the necessary and ideal strategy for eradicating or decreasing HLA antibodies before transplantation. However, former studies indicated that patients with HLA antibodies but not DSA are also at a higher risk of developing GF/PGF.

As alternative stem cells, UCBs are characterized by abundant stem cell sources and low immunogenicity (14, 15). UCBs are easy to achieve and have a high matching success rate. Lyu et al. confirmed that haplo-HSCT co-infused with third-party cord blood induced immune tolerance and modulated allogeneic reactions in patients who underwent mismatched HSCT (10). The incidence of grade II-IV aGVHD in their cohort was 14.3%, which was lower than that in previous reports of 28% (16). Cheng et al.

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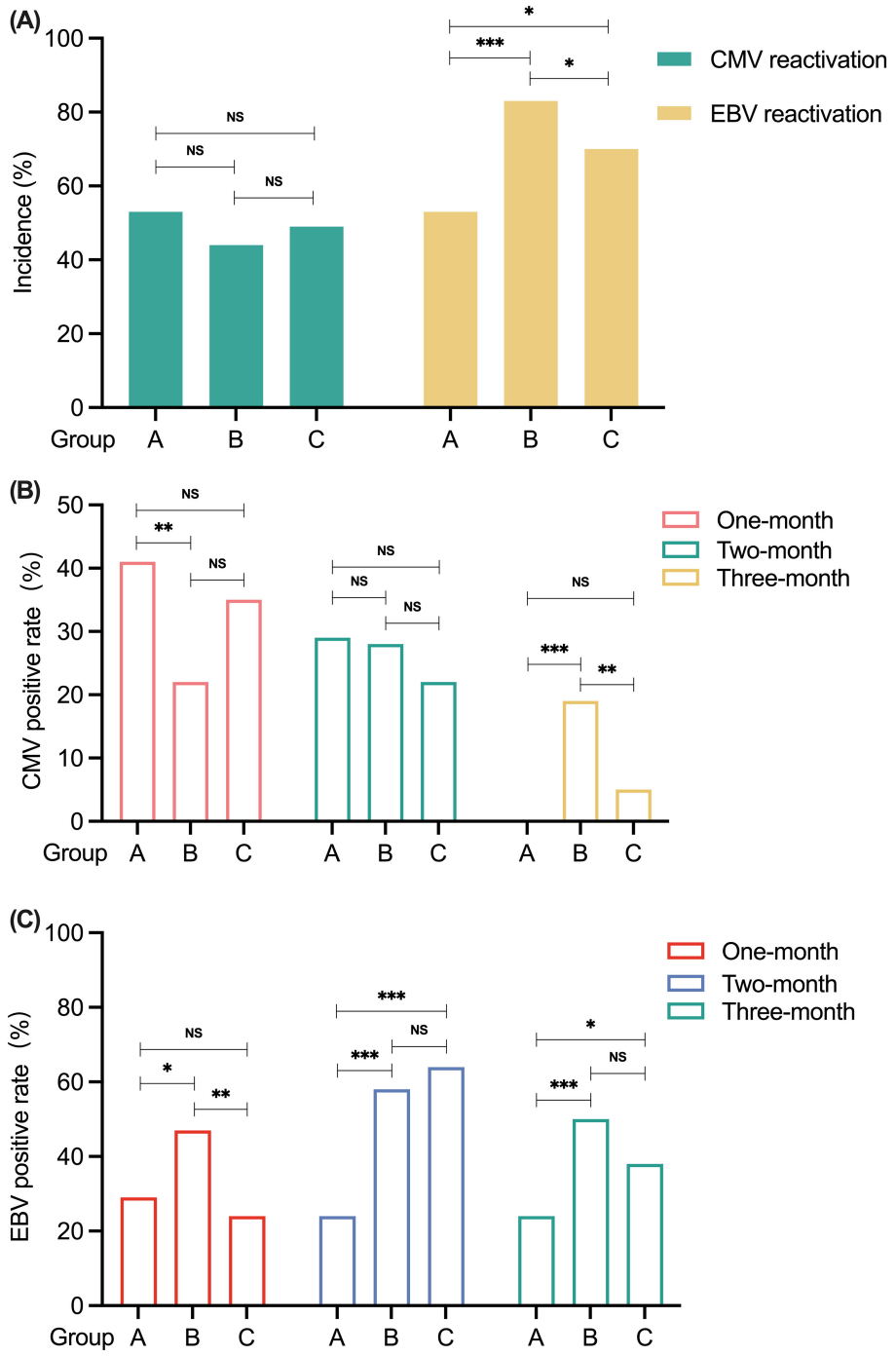

FIGURE 4
The comparison of CMV and EBV reactivation among patients in Groups A, B, and C. **(A)** The incidence of CMV and EBV reactivation within 100 days after transplantation in Groups A, B and C. **(B)** CMV reactivation in Groups A, B, and C by month post-transplantation. **(C)** EBV reactivation in Groups A, B, and C by month post-transplantation. *P < 0.05. **P < 0.01. ***P < 0.001. NS, no significance. CMV, cytomegalovirus; EBV, Epstein-Barr virus.

confirmed a similar outcome with MSD-PBSCT when they infused cord blood stem cells with PBSCT in haplo-setting (8). The feasibility and efficacy of UCBs as third-party adjuvant infusions in previous studies may be related to low immunogenicity and immunomodulation. Therefore, we hypothesized that third-party UCBs could offer some advantages for patients with HLA antibodies at a high risk of GF/PGF.

In the present study, the incidence of GF/PGF in Group B (5 cases) was greater than that in Group A (1 case) and Group C (0 case), respectively, indicating that UCB transfusion could benefit the engraftment of haplo-donor stem cells in patients with HLA antibodies. The potential reasons for this improvement might include the following aspects. First, compared with BM/PB cells, UCB cells might have more robust differentiation and proliferation

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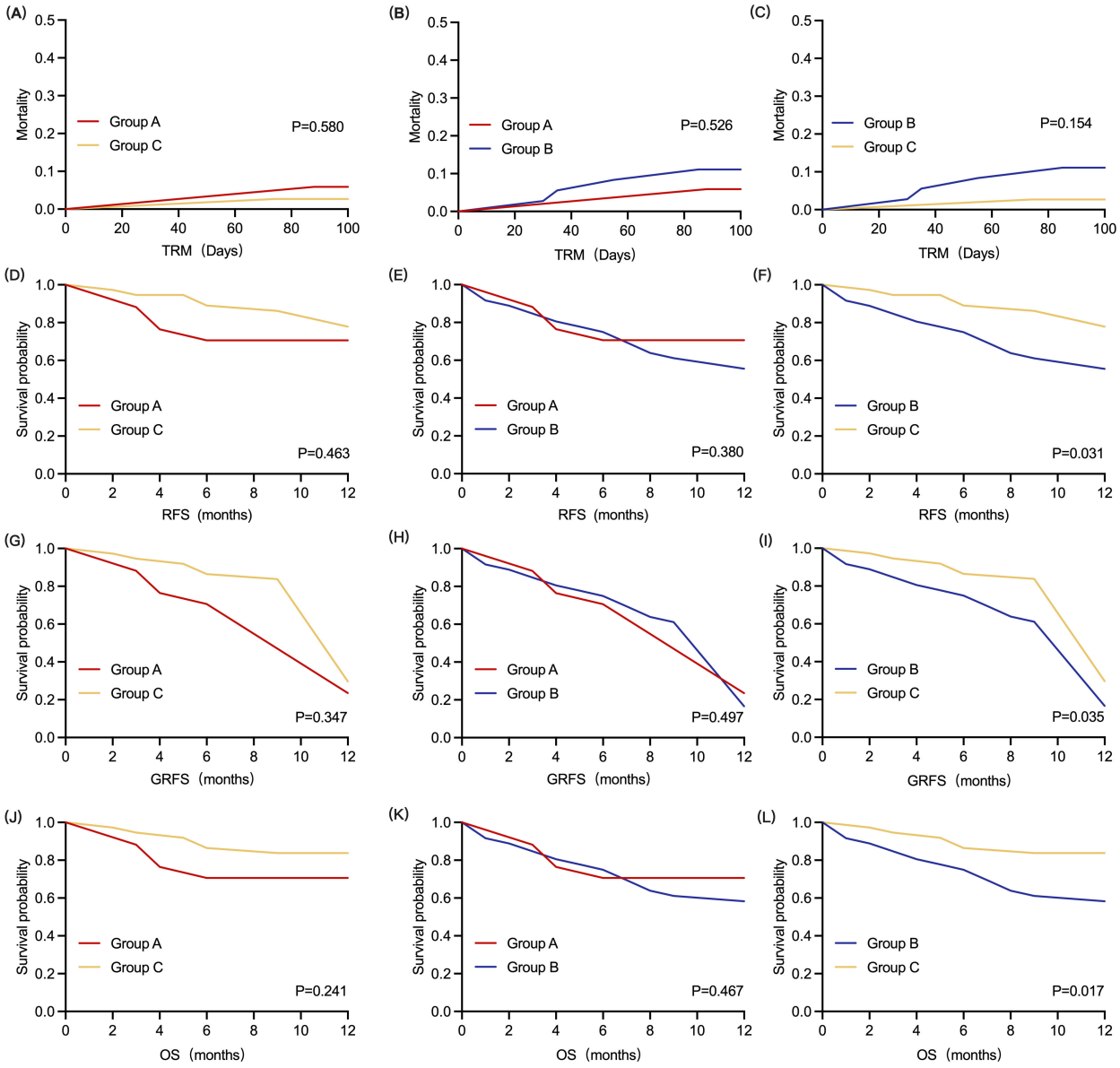

FIGURE 5
Kaplan-Meier analysis of all patients (Groups A, B, and C). **(A–C)** TRM within 100 days for all patients. **(D–F)** 1-year RFS for all patients. **(G–I)** 1-year GRFS for all patients. **(J–L)** 1-year OS for all patients. Significant differences in 1-year RFS, 1-year GRFS and 1-year OS were observed between patients in Group B and Group C, respectively ($P < 0.05$, log-rank test, for each cohort). TRM, transplant-related mortality; RFS, relapse-free survival; GRFS, GVHD- and relapse-free survival; OS, overall survival.

capabilities. Cairo et al. demonstrated an 80-fold increase in stem cell factor (SCF) and G-CSF or GM-CSF after a 14-day expansion of UCB versus adult BM using IL-11 (17). Moreover, compared with adult BM, UCB has been shown to have increased serial *in vitro* replating efficiency (18) and increased culture lifespan with increased progenitor cell production (19, 20). Second, the phenotype and constitution of the precursor cells in UCB are different from those in BM/PB cells. Hao et al. found that the primitive cell population that expresses the CD34 antigen is fourfold more prevalent than in BM or peripheral blood progenitor cells (PBPCs). This subpopulation of UCB has a greater *in vitro* cloning efficiency than the same population isolated from adult BM (21). Third, other components, such as Thy-1 antigen (CD90), expressed on UCB progenitors might have synergistic effects on precursor cell differentiation (22). Thy-1 is thought to assist in hematopoietic cell development. These data support that UCB has more primitive immature colony-forming cells (CFCs) than adult BM/PB and that these hematopoietic progenitors are capable of long-term repopulation (23). Last, it has been shown that stimulated NKT cells can facilitate hematopoietic reconstitution through dual immunoregulatory functions by secreting IL-10, IL-4, and TGF-β through the direct cell-cell contact pathway or cytokine pathway to induce immune tolerance and improve immune survival (7). Overall, due to their robust differentiation and proliferation capabilities, different phenotypes and constitutions of precursor cells, and ability to

stimulate NKT cells, UCBs might facilitate haplo-donor stem cell homing and differentiation after transplantation, thus overcoming the adverse effects of HLA-antibodies on engraftment.

In our study, the cumulative incidence rates for grades I-II aGVHD, grades III–IV aGVHD, and cGVHD were 41.1% (37/90), 13.3% (12/90), and 18.9% (17/90) respectively, which is similar to that in previously reported studies (16, 24, 25).

We also observed a slight increase in Grade IV intestinal aGVHD in Groups B (3 patients) and C (4 patients) compared with Group A (1 patient). The exact mechanism by which UCBs improve aGVHD efficacy is not yet fully understood. The reasons may include the following aspects. First, in contrast to the complexity of T cells in the PB, T cells are largely naïve in UCB (26). Tregs are a subset of CD4+ T cells that are known to limit inflammatory reactions, and they could be considered for prophylaxis and treatment of severe and refractory GVHD (27). UCB contains a significant number of CD4+CD25+ Tregs and mesenchymal stem cells (MSCs), which have immune regulatory mechanisms that can regulate the hematopoietic microenvironment and therefore play important roles in the prevention of GVHD. Clinical-grade expansion of Tregs from UCB has been performed successfully (28) *in vitro*. Second, UCB is a rich source of NK cells. Janelle A Olson et al. investigated the impact of donor NK cells on donor alloreactive T cells in GVHD induction in an animal model. Donor T cells exhibited less proliferation, lower CD25 expression, and decreased interferon-gamma (IFN-gamma) production in the presence of donor NK cells. Interestingly, the GVL effect was retained in the presence of donor NK cells (29). Third, type II NKT cells can secrete IL-4 to induce Th2-type immune responses, both of which together contribute to alleviating GVHD (30). Pillai et al. reported that CD4-CD8-NKT cells, a subtype of NKT cells, were able to kill T cells and APCs expressing CD1d molecules, thereby inhibiting immune cell proliferation recognized by specific antigens and alleviating aGVHD (31). Larger samples are needed to validate the efficacy of UCB co-infusion in mitigating aGVHD.

In the present study, it is worth noting that patients in Group B presented a greater incidence of cGVHD than did patients in the other two groups. Patients in Group A presented more limited cGVHD than did patients in Group C. The pathophysiology of cGVHD is characterized mainly by impaired immune tolerance, and alloreactive donor-derived T and B cells are involved in this process (32). During cGVHD, regulatory cell populations, including Tregs, regulatory B cells (Bregs), regulatory NK cells, invariant NKT cells, and regulatory type 1 T cells, are impaired or reduced in frequency or number, resulting in the continuous release of inflammatory factors and ultimately pathogenic immunoglobulin deposition in various organs (33, 34). The history and severity of aGVHD are the strongest predictors among the known risk factors for cGVHD (26). The abundance of Tregs, Bregs, and NK cells in UCB might benefit the immune reconstitution, and alleviate aGVHD, thus improving cGVHD in patients who received a co-infusion of UCBs. In the future, more investigations performed both *in vivo* and *in vitro* are needed to confirm our findings.

In the present study, we did not find a significant difference in the TRM within 100 days after transplantation among the three groups. The 1-year RFS and 1-year GRFS of patients in Group A were not significantly different from those in the other two groups. However, if patients with HLA-antibodies did not receive UCB transfusion, their 1-year RFS was obviously lower than that of patients in Group C. These findings indicated that the presence of HLA antibodies was an adverse factor for relapse and that the co-infusion of UCBs can reverse this adverse effect to some extent. Yang et al. confirmed that the addition of UCBs could improve the prognosis of patients receiving haplo-HSCT and enhance the GVL effect without increasing the incidence of GVHD (35). This might be interpreted that faster engraftment and immune reconstitution in Group A may contribute to a GVL effect. In our previous perception, recipients who develop PGF will be at a greater risk of relapse than recipients who achieve good hematopoietic reconstitution. The GVL effect is considerably dependent on the amount and function of NK cells, especially allogenic mismatched NK cells. The effects of GVL can also be initiated by antigen-specific T cells and activated dendritic cells (DCs) of leukemic origin (36). The concurrent increase in NKT cell numbers and activities, the promoted host DC activation, subsequent CD8-dependent GVL effects, and increased generation of Tregs can all contribute to the preservation of the GVL and the prevention of CD4-dependent GVHD. Compared with NK cells derived from PB, cord blood-derived NK cells are younger, proliferate more, and have greater efficacy in targeting and killing malignant cells (19). Many NK progenitor cell populations can be found in UCB, and these populations are usually not present in PB (37). These include the CD34-CD133-CD7-CD45+lin- population, which can differentiate into NK cells after culture with IL-15 and stromal cells. Furthermore, CD34+CD7+ and CD34-CD7+ progenitor cells were also more abundant in UCB, and these cells were also able to develop into NK cells (13). Furthermore, when aGVHD is initiated, excessive cytokines can activate CTLs and NK cells and directly exert an immune response. Combined with our experimental results, co-infusion with UCBs in a haplo-HSCT setting might utilize this 'shortcoming' of increased risk of aGVHD and favor NK cells to facilitate the GVL effect. Therefore, the co-infusion of UCBs with haplo-stem cells might provide a certain number of progenitor cells and derived NK cells, and the latter will play an important role in anti-infection and anti-leukemia effects.Furthermore, there was no significant difference in the 1-year OS of patients in Group A compared with the other two groups. A larger sample size and longer follow-up are anticipated to draw more exact conclusions about the long-term survival of the participants in this study. Overall, co-infusion of UCBs might alleviate the relapse rate to some extent by potentially maintaining the GVL effect. The abundant NK cells and the cross-talk effect of cytokines might be involved in this complex process.

Our results indicated that the total incidences of CMV and EBV viremia were 47.8% and 72.2%, respectively, which is consistent with previous studies in developing countries. There were no significant differences in CMV reactivation among the three groups in the first 100 days after transplantation. Patients in Group A presented with less EBV reactivation than did those in Group B and Group C in the first 100 days posttransplantation. This may be due to the faster neutrophil reconstitution in Group A than

in Group B. Furthermore, NK cells derived from UCBs might also be an important cause of less virus activation. NKT cells can activate the immune system involved in resisting viral and bacterial infection (38). The anti-infective effect of NKT cells is achieved by direct recognition of CD1d-presenting bacterially derived lipid antigens or by the development of responses to self-lipid antigens presented by APCs and infectious agents when interacting with pathogen-associated molecules (29). Therefore, the anti-infection ability of NKT cells can be used to effectively reduce the infection rate, improve the survival rate, and solve the problem of viral and fungal infection in the first few months after HSCT. Kotsianidis et al. (39) reported that NKT cells secrete hematopoietic-related cytokines, such as granulocyte giant cell colony-stimulating factor (GM-CSF) and IL-3, which are involved in regulating stable hematopoietic processes through CD1d recognition of hematopoietic precursor cells.

In addition, a total of 4 patients developed PTLD posttransplantation (1 in Group A, 2 in Group B, and 1 in Group C). EBV activation is the most important risk factor for developing PTLD. Patients in Group A might benefit from a reduced incidence of EBV activation due to cotransfusion with UCBs. In the future, a larger sample size should be investigated to determine why patients in Group A presented lower EBV activation than those in Group C.

In our study, a 4/6 match for HLA markers was the minimum criterion for choosing a UCB. As recommended by the EBMT, the minimum amount for UCB transplantation after thawing is 2.0 - 2.5 $\times 10^7$/kg for TNC and 1.0 - 1.2 $\times 10^5$/kg for CD34+ cells (40). Koen van Besien et al. proposed that a cell dose of > 3 $\times 10^6$ total cord blood units is the minimum threshold cell dose for haplo-cord engraftment. They also determined that a dose lower than 1 $\times 10^7$/kg has a high risk of rejection of the UCB graft (41). In our study, the median doses of third-party UCBs to the TNC and CD34+ cells were 15.16 $\times 10^7$/kg and 4.63 $\times 10^5$/kg, respectively. All the patients who achieved successful engraftment had haploid-stem cell engraftment without cord blood engraftment, which may be related to factors such as the number of haploid-stem cells being far greater than the number of cord blood hematopoietic stem cells. Furthermore, there might be both intrinsic cord blood factors and recipient factors that can lead to cord blood not engrafting. The exact mechanisms underlying the lack of UCB engraftment should be investigated in more in-depth studies. Our results indicated that the use of UCBs as a complement to haplo-HSCT is a feasible and safe strategy without concern about UCB engraftment.

Notably, our study has several limitations. First, the sample size was small, and the observation time was short. The clinical outcomes relating to survival and disease control seem to be preliminary. It would be better to classify the patients into subgroups according to their titers of HLA antibodies and analyze the differences between these groups. Second, as a retrospective cohort study, certain imbalanced features may exist in our study such as the unbalance in the number of cases between groups, although we made adjustments in the multivariate analysis. Finally, it is necessary to monitor HLA antibody titers after transplantation to find more evidence of the benefit of UCB.

5 Conclusions

In the present study, UCBs administered to patients who were positive for HLA antibodies as a third-party adjunctive infusion were safe and improved the engraftment, the grade and number of organs involved in cGVHD, decreased the incidence of EBV reactivation, and improved the 1-year RFS to some extent. Patients who are positive for HLA antibodies without UCB infusion are at a greater risk of developing GF/PGF and a greater risk for relapse. The patients who were negative for HLA antibodies still had better 1-year RFS, GRFS, and OS than those who were positive for HLA antibodies regardless of the UCB infusion. In the future, we will also expand the sample size to further validate our results.

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 humans were approved by Human Subjects Review Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YW: Investigation, Methodology, Writing – original draft, Conceptualization. YZ: Data curation, Investigation, Writing – review & editing. XF: Project administration, Writing – review & editing. DY: Supervision, Writing – review & editing. MD: Data curation, Writing – review & editing. KL: Data curation, Writing – review & editing. HQ: Supervision, Writing – review & editing. NW: Supervision, Writing – review & editing. XL: Writing – review & editing. PL: Writing – review & editing. CZ: Writing – review & editing. HX: Supervision, Writing – review & editing. YJ: Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1459699/full#supplementary-material

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Metachronous spinal cord involvement B cell and subcutaneous tissue involvement NK/T cell lymphoid proliferations and lymphomas arising in post-transplantation mimicking general NK/T cell lymphoma: a case report and review of the literature

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Lymphoid proliferations and lymphomas arising in post-transplantation are potentially life-threatening complications after solid organ transplant (SOT) and hematopoietic stem cell transplant (HSCT). The lymphoid proliferations and lymphomas arising in post-transplantation originating from different cell lineages in the same patient are highly unusual. Herein, we delineate a case of isolated spinal cord involvement with B cell lymphoid proliferations and lymphomas arising in post-transplantation at 11 months post-transplantation, which was successfully treated with chemotherapy and intrathecal injection. Six months later, the patient again developed lymphoma arising in post-transplantation, presenting with predominant subcutaneous tissue involvement deriving from EBV-positive NK/T cells, and received four courses of chemotherapy. Ultimately, she achieved complete remission (CR). The report further contributes to our new insights into the unusual clinical presentations of lymphoid proliferations and lymphomas arising in post-transplantation.

KEYWORDS

transplantation, Nk/T cell lymphoma, lymphoid proliferations and lymphomas associated with immune deficiency/dysregulation, spinal cord, subcutaneous

Introduction

Lymphoid proliferations and lymphomas arising in post-transplantation used to be termed post-transplant lympho proliferative disorders, a heterogeneous group of lymphoid and plasmacytic proliferations, which are categorized as "Lymphoid proliferations and lymphomas associated with immune deficiency/dysregulation (IDD)" in the 5th edition of the World Health Organization Classification of hematolymphoid tumors (1). It encompasses a spectrum of disorders ranging from indolent reactive lesions to malignant and aggressive diseases (2). For patients with lymphomas arising in post-transplantation, failure to receive timely and appropriate treatment will result in a 3-year overall survival rate of 20% (3). In comparison, the 3-year overall survival rate significantly improves to 60% when patients receive prompt diagnosis and appropriate management (4). The manifestation of lymphoid proliferations and lymphomas arising in post-transplantation is nonspecific, including fever, night sweats, fatigue, loss of appetite, lymphadenopathy, and enlarging masses, and some patients are asymptomatic, which poses challenges for early diagnosis. Approximately 90-95% of lymphoid proliferations and lymphomas arising in post-transplantation display B cell lineage derivation (5), with a high incidence of extranodal involvement, including frequently the gastrointestinal tract, lung, and bone marrow (6). In contrast, NK/T cell lymphoid proliferations and lymphomas arising in post-transplantation are uncommon. Here, we report a rare case of isolated spinal cord involvement with B cell lymphoid proliferations and lymphomas arising in post-transplantation at 11 months post-transplantation. Six months later, the patient again developed lymphoma arising in post-transplantation, presenting with predominant subcutaneous tissue involvement deriving from EBV-positive NK/T cells.

Case presentation

A 29-year-old woman presented to an outside hospital with a prolonged fever (>38.5°C). Although receiving antibiotic treatment with meropenem (500mg q8h for 7 days) combined with dexamethasone (5mg qd for 2 days) and Tylenol (0.65g q8h for 7 days), her body temperature was repeatedly elevated. The in-patient examination indicated that sCD25(18964pg/ml), hypertriglyceridemia (3.28mmol/l), hypofibrinogen(0.81g/l), hepatosplenomegaly and hemophagocytosis in the bone marrow. She presented with EBV DNA positivity of plasma and Peripheral Blood Mononuclear Cells (PBMCs), accompanied by bilateral lymph node enlargement in the neck and inguinal areas, and was diagnosed with EBV-HLH according to HLH-2004 diagnostic criteria in November 2019. Subsequently, she was initially treated with the DEP chemotherapy regimen, which consisted of etoposide (110mg day1), doxorubicin hydrochloride liposomes (40mg day1), methylprednisolone (80mg, day1 to 3, 30mg, day4 to 7, 10mg, day8 to 10, and 4mg, day11 to 14) on November 28, 2019 and achieved CR after two cycles of induction therapy according to efficacy evaluation criteria of the HLH (7).

On December 20, 2019, the patient was admitted to our hospital for HSCT. She had no other medical history or family history of primary immunodeficiency. On physical examination, the patient was afebrile, with normal vital signs. The neck, axilla, and groin ultrasound detected no lymph node enlargement. On the functional examination of NK cells and cytotoxic T lymphocytes (CTL), the expression of associated proteins, such as ΔCD107a, perforin, and Granzyme, is normal. Whole exome sequencing (WES) did not also detect any significant pathogenic variant.

On February 20, 2020, she received allogeneic HSCT from her father following a conditioning regimen including busulfan, cyclophosphamide, etoposide, and anti-thymocyte globulin(ATG). Prior to HSCT treatment, the serologic workup of the recipient was positive for EBV and negative for CMV, whereas the donor was serologically negative for EBV and CMV. Cyclosporin A (CsA, 50mg intravenously daily) and mycophenolate mofetil (MMF, 500mg orally daily) were used as prophylaxis against graft versus host disease (GVHD). Grade II hyperacute GVHD of the gastrointestinal tract occurred 4 days after HSCT and was in remission after short course of methotrexate (24mg/day, +4 day, 16mg/day, +6day, and 18mg/day, +9 day), methylprednisolone (40mg qd), cyclosporinA (CsA, 100mg intravenously twice daily), and mycophenolate mofetil (MMF, 500mg orally twice daily) therapy. At 3 months after transplantation, the immunosuppressive therapy regimen was adjusted, consisting of CsA (50mg orally twice daily initially, dosage adjusted according to drug concentration), methylprednisolone (8mg once daily) and MMF (500mg orally twice daily). The immunosuppressive treatment was gradually reduced and discontinued 1 year after transplantation. The patient had been maintaining complete donor chimerism since 20 days after transplantation.

At 11 months post-transplantation, the patient was admitted to our hospital with posterior neck pain and limb numbness for 2 months. Magnetic resonance imaging (MRI) of the spine revealed diffuse swelling and increased signal intensity of the spinal cord extending from cervical 2 to thoracic 3. DNA copy numbers of EBV-DNA in both plasma, PBMC and cerebrospinal fluid (CSF) measured by real-time qPCR were positive. The sorting of EBV-infected peripheral blood cells revealed a predominance of B lymphocytes. However, bone marrow and CSF cytology demonstrated no abnormal cells. We eliminated other etiologies such as ischemic myelopathy, compressive myelopathy, autoimmune/infectious/parainfectious myelitis and metabolic/toxic myelopathy by a comprehensive analysis of clinical and MRI findings. Eventually, the patient was clinically diagnosed with EBV-positive lymphoid proliferations and lymphomas arising in post-transplantation with spinal cord involvement according to National Comprehensive Cancer Network (NCCN) guidelines. Consequently, the patient received four courses of chemotherapy treatment that incorporated Rituximab, Reduction in immunosuppression (RIS) combined with intrathecal injection of methotrexate (MTX) and dexamethasone. Her symptoms and spinal cord swelling gradually remitted and nearly completely disappeared (Figure 1).

In July 2021, 17 months after the transplantation, the patient reported a superficial mass in the left elbow joint with mild pain. The ultrasonography revealed a 2cm*2cm mass in the medial aspect of the left elbow joint. The biopsy was delayed due to the novel coronavirus epidemic. Twenty-one months after transplantation,

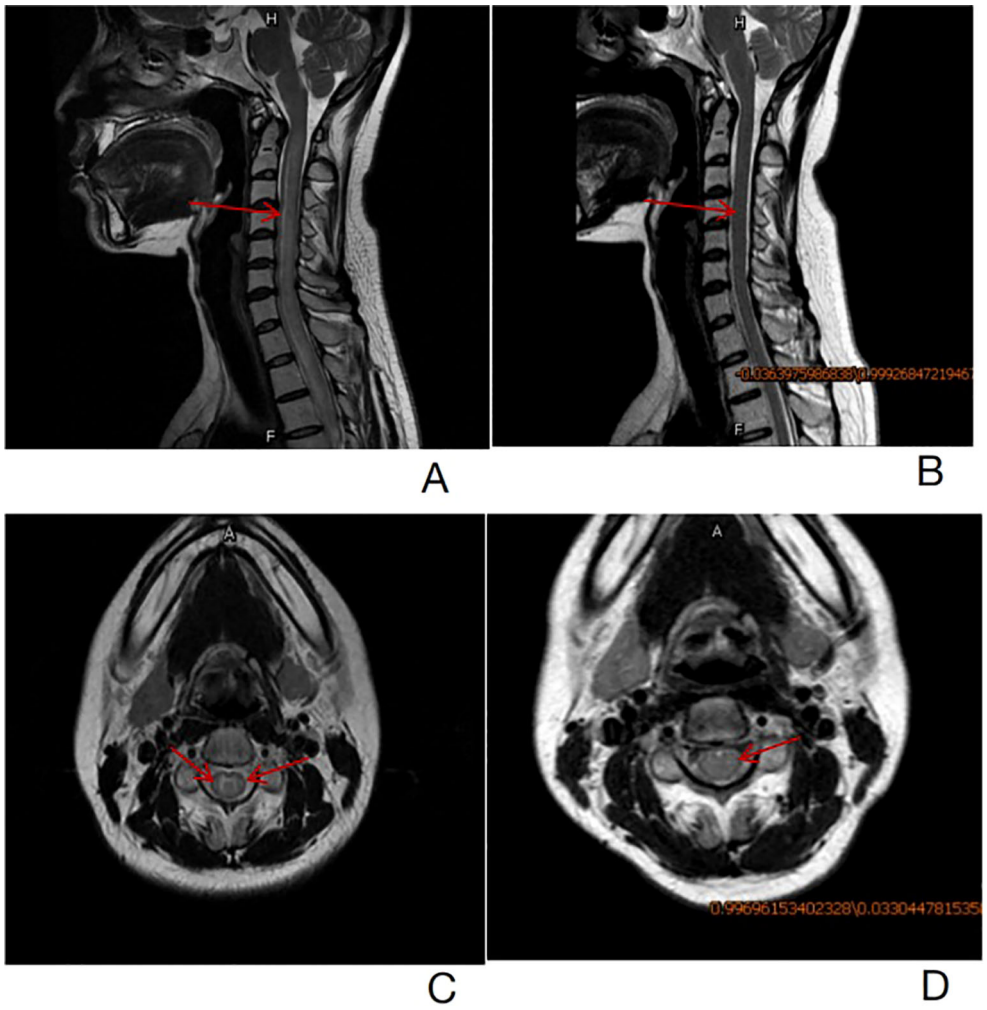

FIGURE 1
(A)-T2 weighted sagittal images revealed diffuse swelling and increased spinal cord signal intensity from cervical 2 to thoracic 3 before treatment (the red arrow). **(B)**- T2 weighted sagittal images showed that spinal cord swelling and abnormal strengthening signals from cervical 2 to thoracic 3 were significantly remitted after treatment (the red arrow). **(C)**- T2 weighted axial images revealed hyper-intense signal in the spinal cord more in the white matter region before treatment (the red arrow). **(D)**- T2 weighted axial images revealed hyper-intense signal in the white matter of the spinal cord return to normal (the red arrow).

the patient presented a 4.6*2cm subcutaneous mass on the right upper extremity. Puncture biopsies of bilateral upper extremity masses were performed, and similar characteristics were demonstrated. Pathological examination revealed that the mass was surrounded by the infiltration of abundant lymphocytes and heterogeneous cells, accompanied by granuloma formation and plentiful cellular necrosis. The immunohistochemical examination demonstrated the tumor cells expressed CD20, CD3, CD4, CD8, CD56, Ki67, Gr B, TIA-1, and EBNA2. They were also positive for EBV-encoded RNA (EBER) (Figure 2). EBV DNA was positive in PBMC at a low level and negative in plasma. A Positron Emission Tomography/computed tomography (PET/CT) indicated increased uptake of Fluoro-2-deoxy-D-glucose (FDG) in masses in both upper limbs, multiple lymph nodes, liver, spleen, truncal bones, colorectum, and multiple subcutaneous nodules. Consequently, the patient was treated with four courses of chemotherapy with L-GDP (L-Asparaginasum, Gemcitabine, Dexamethasone, Cisplatin) plus PD-1 inhibitor and was routinely evaluated by PET CT imaging at the end of treatment. The metabolic activity and volume of masses in both upper limbs and enlarged lymph nodes significantly decreased (with a Deauville score of 1-3), and no other abnormal lesions were revealed. CR was confirmed according to the Lugano efficacy evaluation criteria (8).

Discussion

Lymphoid proliferations and lymphomas arising in post-transplantation display a bimodal distribution with an increase in incidence within one year of transplant and then another peak, which occurs around five years after transplant. Early-onset lymphoid proliferations and lymphomas arising in post-transplantation are mainly derived from polyclonal or monoclonal polymorphic B-cell proliferations, which occur within one year of transplantation and are frequently associated with EBV (9). In this case, the patient presented diffuse swelling and increased signal intensity of the spinal cord 11

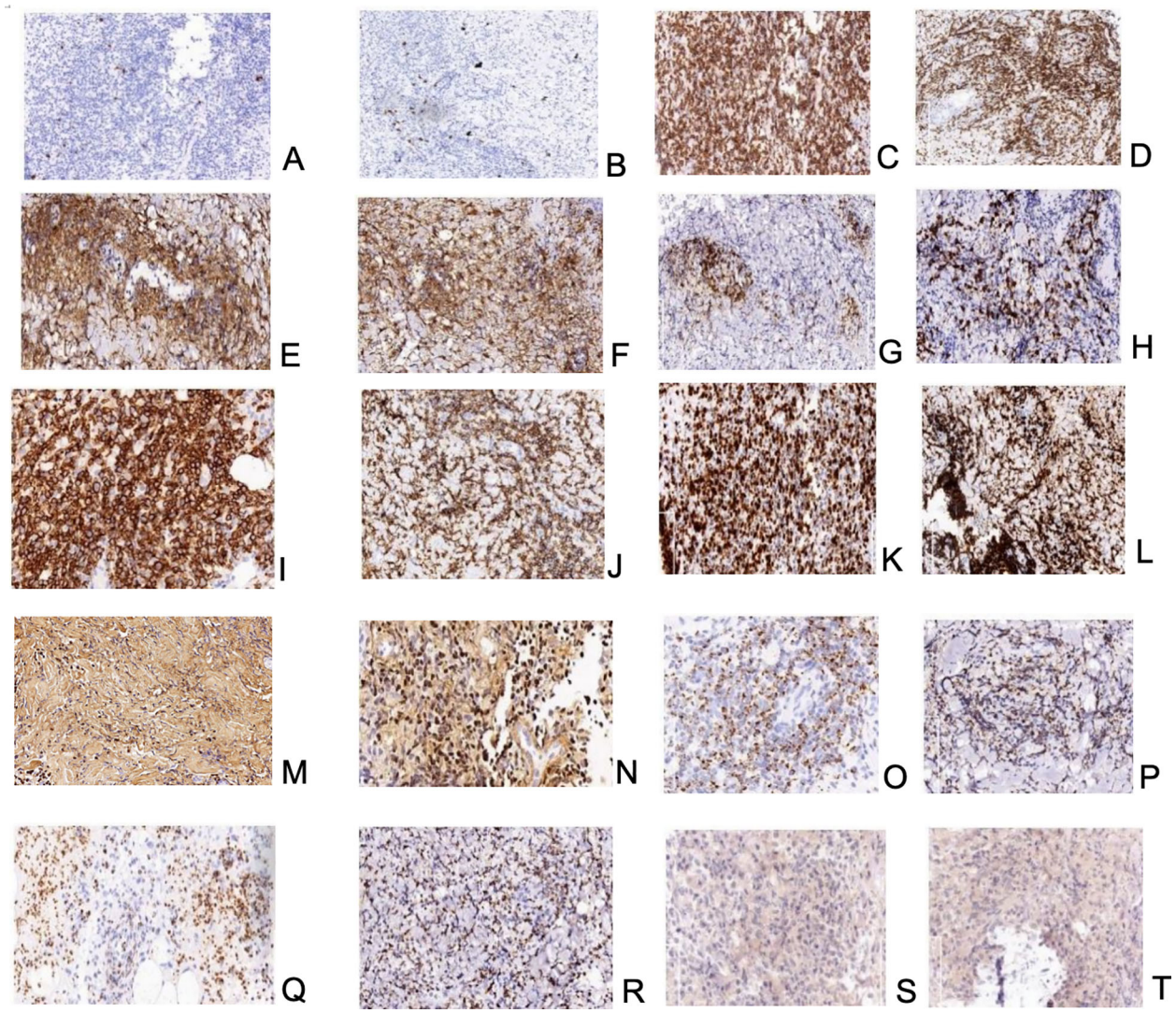

FIGURE 2
(A, B)-neoplastic NK/T-cells-positive reaction for CD20; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(C, D)**–neoplastic NK/T-cells-positive reaction for CD3; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(E, F)**–neoplastic NK/T-cells-positive reaction for CD4; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(G, H)**–neoplastic NK/T-cells-positive reaction for CD8; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(I, J)**–neoplastic NK/T-cells-positive reaction for CD56; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(K, L)**– neoplastic NK/T-cells; high proliferative index – almost all cells showed expression of Ki67; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(M, N)**–neoplastic NK/T-cells-positive reaction for EBER; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(O, P)**–neoplastic NK/T-cells-positive reaction for Granzyme B; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(Q, R)**–neoplastic NK/T-cells-positive reaction for TIA-1; biopsy of mass on the left upper extremity and the right upper extremity respectively; **(S, T)**–neoplastic NK/T-cells-positive reaction for EBNA2; biopsy of mass on the left upper extremity and the right upper extremity respectively.

months after transplantation and was clinically diagnosed with EBV-positive central nervous system (CNS) lymphoid proliferations and lymphomas arising in post-transplantation (10). Historically, only a minority of published cases of lymphoid proliferations and lymphomas arising in post-transplantation with neurological symptoms have been reported as case reports (11–13). Among them, involvement of the internal structure of the CNS occurs in approximately 5-30% of patients with lymphoid proliferations and lymphomas arising in post-transplantation (14). They often present with multiple supratentorial lesions in the periventricular regions (14). However, Beukelaar et al. reported that a rare lymphoid proliferations and lymphomas arising in post-transplantation case occurred in the ventral side of the spinal cord after HSCT (15). Another uncommon case of intraspinal lymphoid proliferations and lymphomas arising in post-transplantation involvement was reported in a pediatric patient who underwent renal transplantation (16). Our patient is a rare case of lymphoid proliferations and lymphomas arising in post-transplantation involving the spinal cord.

Due to the complexity of obtaining specimens from the CNS, multiple postoperative complications, and the rapid progression of CNS lymphoid proliferations and lymphomas arising in post-transplantation, most patients did not receive a histologically confirmed diagnosis or appropriate therapy during their lifetime. They commonly passed away within a year of receiving a transplant,

with autopsy results ultimately confirming the diagnosis. According to previous reports, a combination of clinical presentation, imaging studies such as MRI, and the detection of EBV DNA in CSF can aid in the clinical diagnosis of CNS lymphoid proliferations and lymphomas arising in post-transplantation. Early initiation of treatment after clinical diagnosis of CNS lymphoid proliferations and lymphomas arising in post-transplantation can significantly improve the survival of patients, with an overall survival rate of up to a median of 47 months (17). However, standard prophylactic or therapeutic protocols for CNS lymphoid proliferations and lymphomas arising in post-transplantation are still under investigation (18). Current regimens for treating CNS lymphoid proliferations and lymphomas arising in post-transplantation include monotherapy with Rituximab (19), intrathecal injection of methotrexate (MTX) (20), high-dose MTX intravenous treatment (6), RIS combined with Rituximab and whole brain radiation therapy (WBRT) (21), and EBV-specific cytotoxic T lymphocytes (CTL) (22), all of which have demonstrated promising efficacy.

At 17 and 21 months post-transplantation, the patient presented with a mass on the bilateral upper extremities, along with enlarged multiple lymph nodes. However, the tissue biopsy was insufficient to confirm whether it was general NK/T cell lymphoma or NK/T cell lymphoma arising in post-transplantation. Misdiagnosis often occurs due to the similar pathological features shared by NK/T cell lymphoma arising in post-transplantation and common NK/T cell lymphoma (23). Early differentiation is especially crucial between NK/T cell lymphoma arising in post-transplantation and NK/T cell lymphoma, as it allows for the initiation of treatment. Notably, our patient had presented with generalized lymphadenopathy prior to treatment initiation. However, upon admission to our hospital, the enlarged lymph nodes had disappeared following chemotherapy with the DEP regimen, and no lymph node biopsy was performed for a definitive diagnosis. Therefore, it is reasonable to suspect the presence of occult lymphoma at the initial diagnosis, with a recurrence of occult lymphoma. However, NK/T cell lymphoma is predominantly extranodal (24), and patients with nodal involvement typically progress rapidly (25). The majority of patients have chromosomal abnormalities, such as del (6), del (8), and del (13), as well as frequent gene mutations, such as JAK3, STAT3, and STAT5b (26), which are not consistent with our patient's clinical presentation at the time of initial treatment. Additionally, in NK/T cell lymphoma, increases in circulating EBV DNA are usually found due to viral DNA release from apoptosis of proliferating tumor cells. However, in this case, EBV DNA was positive in PBMCs at a low level and negative in plasma. On the other hand, multiple risk factors that increase the likelihood of developing lymphoid proliferations and lymphomas arising in post-transplantation have been elucidated, including the use of ATG prior to transplantation and immunosuppression following HSCT, elderly donor (51 years), difference of EBV serological status between donor and recipient, and haplo-identical HSCT (27). Pathology indicated that the tumor cells predominantly exhibited an EBV latency type III (LMP1-positive, EBNA2-positive, EBER-positive), mainly expressed in immunodeficient patients (28). Wang S.H. et al. reported a patient who underwent HSCT for cutaneous NK/T cell lymphoma and developed hepatosplenomegaly and cervical lymphadenopathy two months after transplantation. The patient was ultimately diagnosed with EBV-positive lymphoma arising in post-transplantation, although recurrence of NK/T cell lymphoma was suspected initially (29). Even though the pathological presentations of lymphoid proliferations and lymphomas arising in post-transplantation with cutaneous involvement are commonly characterized by polymorphic or monomorphic B cell and plasma cell subtypes (30–32), the NK/T cell lymphoid proliferations and lymphomas arising in post-transplantation primarily manifesting as subcutaneous nodules have also been reported (9, 33, 34). The majority of them are usually present late after transplantation and are EBV-negative (27). Nonetheless, approximately 15% of NK/T cell lymphoid proliferations and lymphomas arising in post-transplantation occur in the early post-transplant stage (5), and about 40% of these patients are EBV-positive. Based on the evidence presented, the final diagnosis of the subcutaneous mass was established as NK/T cell lymphoma arising in post-transplantation.

RIS has been the cornerstone of first-line treatment for lymphoma arising in post-transplantation (35), and it is often used in combination with chemotherapy, radiotherapy, surgery, adoptive T-cell therapy, and antiviral and immunological agents. The combined treatment has become the mainstream for NK/T cell lymphoma arising in post-transplantation, and the 5-year survival rate has risen to 60% (34). NK/T cell lymphoma is an aggressive disease with a poor response to therapy and a high risk of replase, resulting in a poor long-term prognosis. The overall 5-year survival rate is approximately 10 to 40%, with the median survival being 15 months (36–38). Patients with extracutaneous involvement show shorter median survival (39). On the contrary, as of the last follow-up in March 2023, our patient maintained CR without any evidence of disease recurrence. The satisfactory treatment efficacy of the patient further supported the diagnosis of NK/T cell lymphoma arising in post-transplantation.

EBV infection status is a significant factor associated with the development of lymphoid proliferations and lymphomas arising in post-transplantation (40). Unlike post-transplant B cell lymphoid proliferations and lymphomas arising in post-transplantation, the role of EBV in EBV-positive NK/T cell lymphoid proliferations and lymphomas arising in post-transplantation is still unclear. Magro et al. suggest that regulatory T cells can undergo tumorigenic transformation under conditions of immunosuppression. EBV-infected B cells, serving as a continuous antigenic stimulus, may induce an excessive immune response in T cells, leading to the development of EBV-positive NK/T cell lymphoid proliferations and lymphomas arising in post-transplantation (41). The incidence of lymphoid proliferations and lymphomas arising in post-transplantation has significantly increased over the last two decades due to various factors, including an increasing number of HSCT, older donors and recipients, the use of novel immunosuppressive agents, and the introduction of unrelated donors (42). Despite

significant improvements in supportive strategies following HSCT in recent years, many problems still need to be better controlled. Monitoring EBV DNA allows for early recognition of impending lymphoid proliferations and lymphomas arising in post-transplantation, thus providing a basis for timely treatment initiation (43). Notably, only 30% of case reports showed positive results for EBV DNA in the CSF of patients with CNS lymphoid proliferations and lymphomas arising in post-transplantation (44); for post-transplant patients who present with CNS symptoms, peripheral blood EBV DNA monitoring does not meet clinical needs, making combined imaging examinations necessary for monitoring CNS lymphoid proliferations and lymphomas arising in post-transplantation. Furthermore, there needs to be more standardization across institutions in the detection methods and the sample types used for EBV DNA surveillance. The management of lymphoid proliferations and lymphomas arising in post-transplantation also needs a common consensus around the EBV DNA threshold for preemptive therapy. The standard RIS regimens that allow for the elimination of lymphoid proliferations and lymphomas arising in post-transplantation while maintaining the level of immunosuppression to prevent graft rejection and GVHD have yet to be elucidated. Additionally, the optimal dosage of Rituximab in first-line treatment regimens warrants further investigation (45).

Conclusion

Conclusively, lymphoid proliferations and lymphomas arising in post-transplantation with predominantly intraspinal involvement is a rare disorder that is difficult to diagnose definitively and has a dismal clinical prognosis. This case report serves as a reminder to clinicians to maintain a high index of suspicion for lymphoid proliferations and lymphomas arising in post-transplantation with spinal cord involvement when neurological complications arise after HSCT. It also highlights the importance of long-term imaging and CSF monitoring in post-transplant patients. Early diagnosis is crucial for disease management and improvement in prognosis due to the differences in pathomechanisms and prognosis between NK/T cell lymphoma arising in post-transplantation and general NK/T cell lymphoma. This article further explains the current treatment modalities and clinical shortcomings of lymphoid proliferations and lymphomas arising in post-transplantation, providing more references to enhance the knowledge of clinicians and pathologists on the disease and pointing out the direction for future exploration.

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) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YZ: Formal analysis, Writing – original draft. LH: Formal analysis, Writing – review & editing. HZ: Data curation, Writing – review & editing. SY: Data curation, Writing – review & editing. JH: Data curation, Writing – review & editing. JG: Methodology, Writing – review & editing. YW: Methodology, Writing – review & editing.

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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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Cord blood T regulatory cells synergize with ruxolitinib to improve GVHD outcomes

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Background: Adoptive therapy with umbilical cord blood (UCB) T-regulatory (Treg) cells can prevent graft vs. host disease (GVHD). We hypothesize that UCB Tregs can treat GVHD and synergize with ruxolitinib, Jak2 inhibitor, to improve outcomes.
Methods: UCB Treg potency and efficacy was examined using cell suppression assay and xenogeneic GVHD model, respectively. Ruxolitinib was fed continuously in presence or absence of CellTraceViolet tagged UCB Tregs on days +4, +7, +11, +18. Mice were followed for survival, GVHD score, hematology parameters and inflammation.
Results: Addition of ruxolitinib to UCB Tregs exerted synergistic suppressor function *in vitro* and improved persistence of UCB Tregs *in vivo*. Lower GVHD score, improved survival, increased hemoglobin level and platelet count, decreased inflammatory cytokines and decrease in $CD3^+$ T cell lung infiltrate was observed in UCB Tregs+ruxolitinib recipients.
Conclusion: UCB Treg+Ruxolitinib combination improves outcomes in xenogeneic GVHD and should be explored in a clinical setting.

KEYWORDS
GVHD, T regulatory cell, allogeneic, cord blood, ruxolitinib

Introduction

Graft vs. host disease (GVHD) is a fatal complication of allogeneic stem cell transplantation, driven by donor derived T cell proliferation and characterized by excessive inflammation which can lead to widespread tissue injury and wasting phenomenon (1). $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) are a subset of T cells that regulate immune function and resolve unwanted inflammation (2). Tregs have been extensively studied for prevention and treatment of GVHD (3–5), with promising clinical results (6–11).

We have previously shown that Tregs derived from umbilical cord blood (UCB) co-express $CD45RA^+CD45RO^+$ (12) that allow for sustained *in vivo* proliferation of the injected cells; as well as retain their suppressor function in presence of dexamethasone (12, 13). Additionally, treatment with multiple injections of UCB Tregs can reduce burden of inflammation without interfering in the anti-tumor activity of CD19 CART cells in a xenogeneic lymphoma model (14). Recently, Kadia et al., showed that a single infusion of CK0801 Tregs can lead to durable independence from blood and platelet transfusion in patients with bone marrow failure (15). In a randomized

placebo control trial, multiple infusions of CK0802 Tregs led to improvement in survival in patients with COVID associated acute respiratory distress syndrome (13). Combination of donor derived Tregs and ruxolitinib, a Jak2 inhibitor currently approved agent for steroid refractory GVHD (16), has been shown to exert synergistic effect to improve GVHD outcomes without interference in graft vs. leukemia effect (17). We hypothesize that addition of UCB Tregs to ruxolitinib can improve results in GVHD.

Methods

UCB Treg cell generation and function

UCB derived Tregs were generated as described previously (12, 18) and/or provided by Cellenkos® Inc. (Houston, TX, USA). Cell characterization was performed as described in Supplementary Material. Cell suppression assay was performed as described previously (14).

In vivo GVHD model

Animal procedures were performed according to an approved protocol by The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee. Xenogeneic GVHD model was established as described previously (4). CellTraceViolet (CTV) dye labeled UCB Tregs (1×10^7 cells) were injected on days +3, +10, +17, and +24. Mice received 1 mg ruxolitinib daily by oral gavage for 14 consecutive days. Mice were monitored and weighed twice weekly. GVHD score was calculated as described previously (Supplementary Table S1) (19). Peripheral Blood (PB) from mice was collected weekly and at euthanasia, for flow analysis. Euthanasia was performed according to institutional guidelines. Room air in the mice chamber was gradually replaced by 100% CO_2, from a compressed gas cylinder at a flow rate that displaced 30%–70% of the chamber volume per minute until the concentration of CO_2 reached 100%. Upon achieving this concentration, the mice remained in the chamber for at least, an additional three minutes to ensure effective euthanasia. Plasma was analyzed for inflammatory cytokines using Human Cytokine 42-plex Discovery Assay Kit (Eve Technologies, Calgary, Canada). Organs of xenografts were harvested, homogenized, and analyzed as described previously (14).

Statistical analysis

All statistical analyses were done with GraphPad Prism 9 software (San Diego, CA, US). Data are presented as mean ± SEM. *P*-values were calculated using 2-tailed *t*-test with 95% confidence interval, one-way ANOVA, or two-way ANOVA for evaluation of statistical significance compared with the untreated controls. $P < 0.05$ was considered statistically significant.

Results and discussion

UCB Tregs can treat GVHD without affecting GVL

Ex vivo expanded UCB Tregs express $CD4^+CD25^+CD127^{lo}$ $FOXP3^{hi}Helios^{hi}$ phenotype (Supplementary Figure S1) and have been shown to retain their function in presence of dexamethasone (12). In an established haploidentical murine GVHD model, donor Tregs injection on day +11 led to improvement in survival and decrease in GVHD score (20). In an exploratory study, two out of five patients suffering from chronic GVHD, who received donor derived, *ex vivo* expanded, Treg cells at a median of 35 months after their allogeneic hematopoietic cell transplantation showed improvement in their condition which allowed decrease in their steroid intake and the other three patients had stable disease (21). To evaluate whether UCB Tregs can treat established GVHD in a completely mismatched setting, we injected multiple of UCB Tregs, in a fixed dose of 10 million cells, in a xenogeneic GVHD model (Supplementary Figure S2). As shown in Figure 1A, UCB Treg cell treatment led to improvement in survival; preservation of weight (Figure 1B) and decrease in GVHD score (Figure 1C). A major co ncern exists in regard to impact of any GVHD treatment modality on possibly interfering in the donor T cell mediated graft vs. leukemia (GVL) effect (22). To evaluate the impact of UCB Tregs on GVL, 3×10^6 GFP labeled HL60 leukemia cells were injected through tail vein into sublethal irradiated non-SCID gamma null (NSG) mice to establish acute leukemia. On day +1, mice were injected with 3×10^6 $CD4^+25^-$ Tcon cells in presence or absence of 3×10^6 UCB Tregs injected on day +4 (Supplementary Figure S2). No evidence of leukemia was detected in Tcon and UCB Tregs + Tcon recipients by day +28 by non-invasive bioluminescence imaging (Figures 1D,E). However, all Tcon recipients succumbed to GVHD without evidence of leukemia by day +35 (Figure 1D). Whereas highest GVHD score was observed in Tcon recipients, addition of UCB Tregs reduced it significantly over 35 days ($p < 0.0001$; Figure 1F). While body weight was comparable between Tcon and Tcon + UCB Treg recipients (Figure 1G), a significantly superior survival was observed in the latter arm ($p < 0.001$; Figure 1H). Our findings support that UCB Tregs can treat xenogeneic GVHD without compromising GVL effect. Similar uncoupling effect has been reported in a xenogeneic lymphoma model, where UCB Tregs dampened systemic inflammation witho ut interfering in the on-target anti-tumor effect of CD19 CART cells (14). Long term follow up of multiple clinical studies examining Treg cells in GVHD, also do not report any detrimental effect of Tregs on the risk of leukemia relapse (6–10, 23). Thus, adoptive therapy with UCB Tregs presents itself as a viable strategy for treatment of GVHD.

UCB Tregs synergize with ruxolitinib to improve GVHD outcomes

Next, we proceeded to examine whether UCB Tregs can be added to ruxolitinib, an approved agent for treatment of steroid

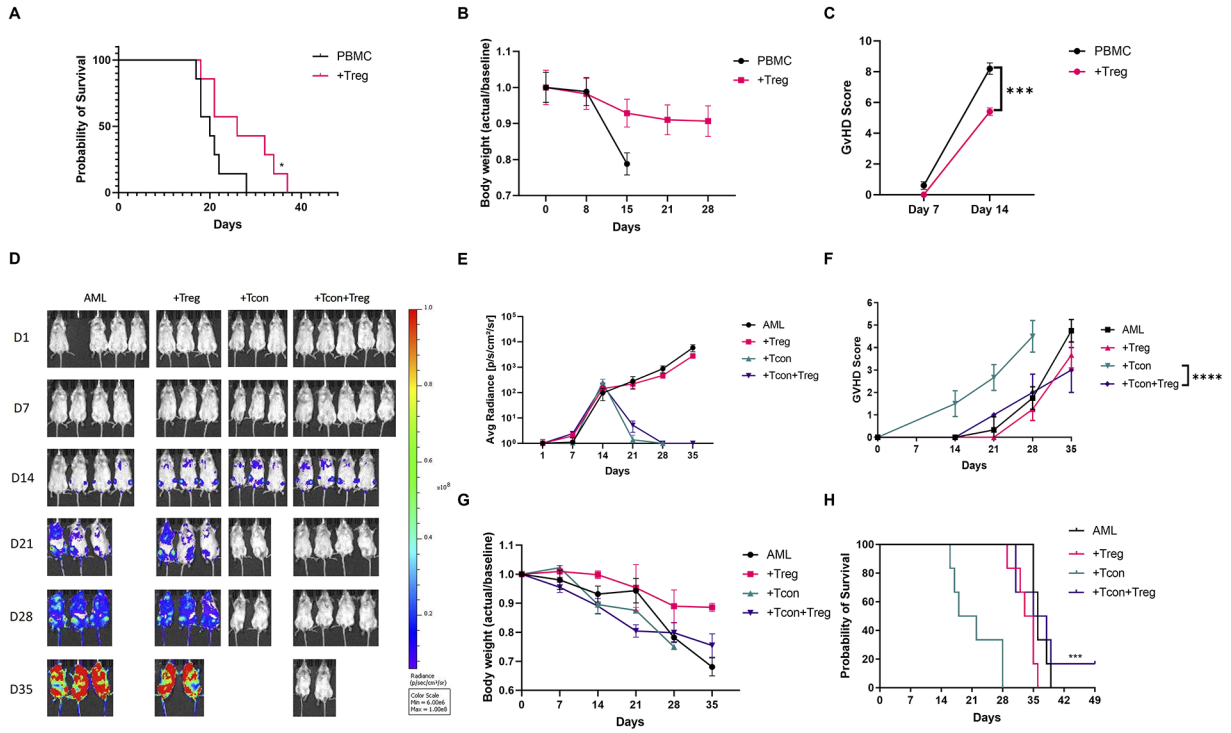

FIGURE 1
UCB Tregs can treat GVHD and preserve GVL. Xenogeneic GVHD model using female Rag2-γc- mice, that underwent sublethal irradiation on day-1 and received tail vein injection of 10^7 human peripheral blood mononuclear cells (PBMCs) on Day 0. In the treatment arm, 10^7 UCB Treg cells were injected through tail vein on days +4, +11, +18, and +25. $N = 6$ each arm. **(A)** UCB Tregs improve survival compared to control. **(B)** UCB Tregs maintain body weight compared to continued weight loss in control arm. **(C)** UCB Tregs significantly improve GVHD scores, from days 7 and 14 compared to control groups. Graft vs. Leukemia (GVL) xenogeneic model was set up using female Rag2-γc- mice, that underwent sublethal irradiation, followed by 3×10^6 GFP-labeled HL60 AML cells in presence or absence of by 3×10^6 UCB Tregs on Day 0, followed by tail vein injection of 3×10^6 Tcon cells. Treatment groups were divided into: Arm 1: HL60 alone (AML); Arm 2: HL60 + UCB Tregs (+Treg); Arm 3: HL60+Tcon (+Tcon); Arm 4: HL60+UCB Tregs+Tcon (+Tcon+Treg), $N = 6$ each arm. **(D)** UCB Tregs do not increase tumor burden in GVL model. HL60 tumor burden in mice was evaluated by Non-invasive bioluminescence imaging (BLI) with the IVIS Lumina X5 Imaging System. Ventral images are shown for each day of capture. Non-invasive BLI showed clear evidence of disease progression in control arm, AML and +Treg. No evidence of disease in +Tcon and +Tcon+Tregs recipients on day 21 and day 28. **(E)** Quantitative analysis of the BLI measurements (photons/sec/cm^2/sr) showed no differences in the +Tcon and +Tcon+Tregs recipients by day 35. **(F)** UCB Tregs improve GVHD score in GVL model. Significant improvement in GVHD scores in +Treg+Tcon arm compared to +Tcon arm alone. Scores were evaluated bi-weekly, with statistical analysis conducted using data from day 28 to compare the different arms. **(G)** UCB Tregs maintain weight in GVL model. No differences observed between +Tcon vs. +Tcon+UCB Treg arm. Weight measured twice weekly, and results presented as fold changes compared to baseline. **(H)** UCB Tregs do not compromise survival in GVL. Over a 49 day follow up, +Tcon+UCB Treg arm shows survival benefit. Addition of UCB Tregs to AML did not have any significant impact on tumor burden. Error bars represent SEM. Statistical differences compared with control were quantified by one-way ANOVA or paired student's *t*-test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ns, not significant; SEM, standard error of means.

refractory GVHD (16). In a recent study of 35 patients with aplastic anemia, prophylatic administration of ruxolitinib significantly lowered incidence of moderate to severe acute GVHD (17.1% vs. 48.6%) (24). However, since ruxolitinib can suppress both T cells (25) and Tregs (26) and is accompanied by hematologic toxicity including anemia and thrombocytopenia (16) that can be dose limiting in GVHD treatment, we examined whether the addition of UCB Tregs can mitigate such a side effect. We analyzed the proliferation of CTV labeled $CD4^+25^-$ Tcon cells cultured with different ratios of UCB Tregs in presence or absence of ruxolitinib. A slight, but significant improvement in the cell suppression was observed with the addition of ruxolitinib at UCB Treg:Tcon ratio of 2:1 and 1:1 ($p < 0.01$; Figure 2A). Similar synergistic effect of 0.1 um ruxolitinib on cell suppression function of human Treg cells has been reported at 120 h of co-culture, where superior survival was observed in recipients of ruxolitinib and Tregs compared to either agent alone (17). To understand whether a comparable effect will be recapitulated in a xenogeneic GVHD model, ruxolitinib and UCB Treg treatment combination was examined (Supplementary Figure S2). Improvement of survival was observed in the UCB Tregs + ruxolitinib recipients compared to all other arms (Figure 2B), which was accompanied with preservation of body weight (Figure 2C) and lowering of GVHD score (Figure 2D). When gated on live cells, while the circulating human $CD45^+$ cells in the peripheral blood increased over time, a significantly lower level was observed in the UCB Tregs + ruxolitinib recipients when compared to UCB Tregs alone recipients at day +14 ($p < 0.001$; Figure 2E). This synergistic effect might be due to engagement of complimentary pathways since the effects of ruxolitinib on Tregs are immune-context dependent (27).

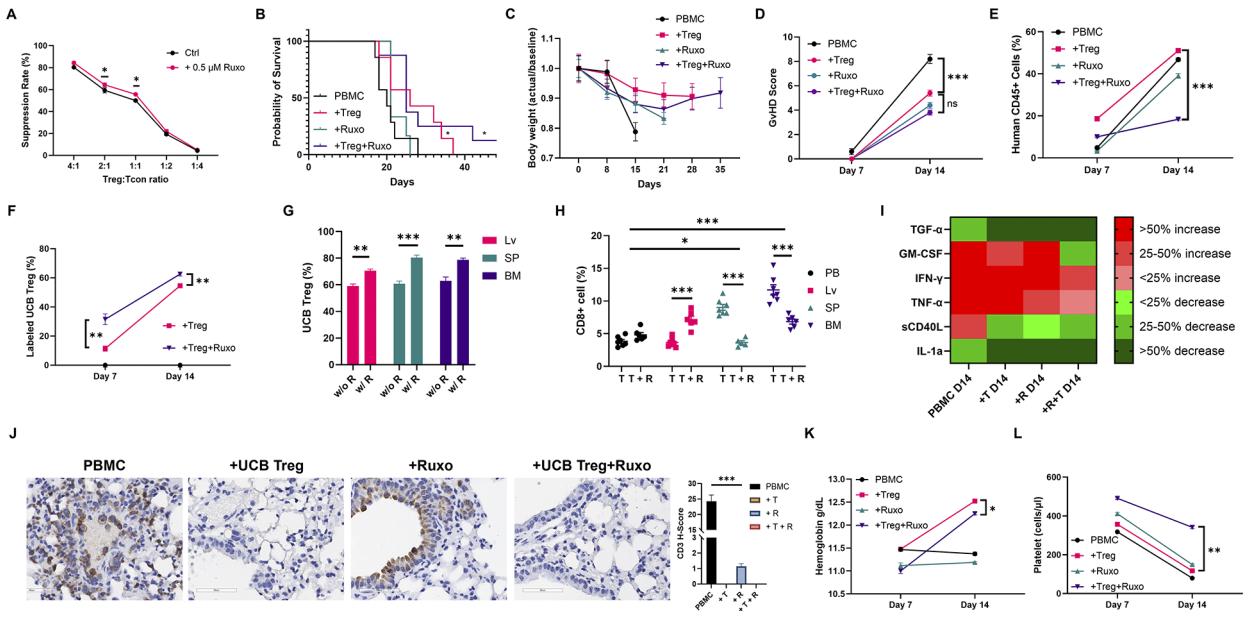

FIGURE 2
UCB tregs synergize with ruxolitinib to improve GVHD outcomes. **(A)** Ruxolitinib improves UCB Treg suppression. UCB Tregs co-cultured with CTV-labeled Tcon ($CD4^+CD25^-$) cells at different ratios of 4:1, 2:1, 1:1, 1:2, and 1:4 in the presence of CD3/28 beads. Proliferation of CTV-labeled Tcons was assessed by the LSR Fortessa Cell Analyzer after 96 h of culture. Percentage suppression was calculated using the following formula: 100% × (1–percentage of proliferating CTV-diluting Tcons in the presence of UCB Tregs at a different ratio/percentage of proliferating CTV-diluting Tcons when cultured alone). Ruxolitinib (0.5 μM) led to improvement in UCB Treg suppression at 2:1 and 1:1 Treg: Tcon ratio. Impact of addition of ruxolitinib at different UCB Tregs: Tcon ratios was: 4:1 (84.0% vs. 80.2%); 2:1 (64.1% vs. 59.3%); 1:1 (55.4% vs. 49.1%); 1:2 (22.4% vs. 19.1%); 1:4 (5.4% vs. 4.1%), respectively. Xenogeneic GVHD model was established using female Rag2-γc- mice, that received tail vein injection of 10^7 human PBMCs on Day 0. In the treatment arm, mice received 1 mg of Ruxolitinib (45 mg/kg) daily orally from day −1 until day 14. Additionally, 10^7 UCB Tregs were injected by tail vein on days +4, +11, +18, and +25. Arm 1: control (PBMC); Arm 2: PBMC+UCB Tregs (+Treg); Arm 3: PBMC +ruxolitinib (+Ruxo); Arm 4: PBMC+UCB Tregs+ruxolitinib (+Treg+Ruxo). $N = 6$ each arm. **(B)** UCB Tregs improve survival in xenogeneic GVHD. When compared to control arm, PBMC, significant improvement in survival was seen in +Treg and +Treg+Ruxo arms, at a follow up of 42 days. **(C)** UCB Tregs maintain body weight in xenogeneic GVHD. Weights were recorded twice a week, and the data are presented as fold changes over successive days relative to the baseline. **(D)** UCB Tregs lower GVHD score. When compared to control arm, +Treg, +Ruxo and +Treg+Ruxo led to significantly lower GVHD score at day 14. The scoring was performed bi-weekly. **(E)** UCB Tregs synergize with ruxolitinib to decrease circulating human $CD45^+$ T cells (gated on live cells) at day 14 in xenogeneic GVHD. **(F)** Ruxolitinib increases UCB Tregs persistence. Significantly higher circulating CTV labeled Tregs on day 7 ($p < 0.01$) and day 14 ($p < 0.001$) in +Treg+Ruxo vs. +Treg arm, respectively. **(G)** Ruxolitinib increases UCB Tregs tissue persistence. Flow cytometric analysis of harvested organ cell suspensions at euthanasia on day 14 showed higher level of CTV labeled Tregs in UCB Tregs+ruxolitinib (w R) recipients compared to UCB Tregs recipients (w/o R), across different lymphoid sites, including spleen (SP), liver (LV) and bone marrow (BM). **(H)** Ruxolitinib addition to UCB Tregs leads to decrease in $CD8^+$ T cells in spleen and bone marrow in xenogeneic GVHD at day 14. *Y*-axis shows $CD8^+$ T cells gated on human $CD45^+$ cells. T = UCB Tregs; T + R = UCB Tregs + ruxolitinib. Compared to peripheral blood (PB), proportion of $CD8^+$ cells was significantly higher in the spleen (SP) ($p < 0.05$) and bone marrow (BM) $p < 0.001$. Addition of ruxolitinib led to a significant decrease of $CD8^+$ cells in spleen and bone marrow and an increase in liver ($p < 0.001$). **(I)** UCB Tregs and ruxolitinib decrease systemic inflammation in xenogeneic GVHD. On day 7 and 14, plasma cytokines level was quantified and analyzed. Cytokines at day 14 were normalized to day 7. The categorical heatmap shows the percentage changes in cytokine levels at day 14 compared to day 7 for TGF-α, GM-CSF, IFN-γ, TNF-α, sCD40l, and IL-1a. Changes are color-coded: shades of green indicate decreases (less than 25%, 25%-50%, more than 50%) and shades of red indicate increases (less than 25%, 25%-50%, more than 50%). **(J)**. Histopathologic examinations of lung at 40× magnification, shows tissue destruction and high $CD3^+$ staining in the control PBMC arm. Tissue architecture is somewhat preserved in PBMC +ruxolitinib arm, with high concentration of $CD3^+$ staining in the alveolar lining as well as in the parenchyma. Complete resolution of $CD3^+$ infiltrate as well as tissue architecture preservation is seen in UCB Treg recipients with or without ruxolitinib. Quantification analysis of the H-score for human CD3 positivity (right panel). The H-score was defined by the percentage of strongly positive stain × 3 + moderately positive stain × 2 + weakly positive stain × 1. A final value of 0–300 was also calculated at 40× magnification using the software HALO (v3.5-3,577.140). A $p < 0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(K)**, UCB Tregs improve hemoglobin in ruxolitinib recipients. On day 14, ruxolitinib recipients show lower hemoglobin levels compared to UCB Tregs recipients. Addition of UCB Tregs to ruxolitinib increases day 14 hemoglobin level. **(L)** UCB Tregs improve platelet decrease in ruxolitinib recipients. Compared to day 7, decrease in platelet counts observed in all arms on day 14. UCB Tregs + ruxolitinib recipients preserved their platelet count. The statistical differences were quantified by a one-way ANOVA or student's *t*-test. Error bars represent SEM ($n = 7$); statistical differences compared with the control arm were quantified by one-way or two-way ANOVA using GraphPad Prism software: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

To study their *in vivo* distribution pattern, UCB Tregs, were labeled with CTV, a low-cytotoxicity intracellular dye, that is detectable for at least seven days post-labeling. As shown in Supplementary Figure S3, when gated on human lymphocytes, nearly all the $CD4^+25^+127^{lo}$ Treg cells comprised of CTV-labeled cells. On day +14, CTV labelled UCB Tregs percentage was significantly higher in the UCB Tregs + ruxolitinib vs. UCB Tregs alone recipients in peripheral blood (PB) (Figure 2F), and in liver, spleen and bone marrow (Figure 2G). When gated on human $CD45^+$ cells, $CD8^+$ T cells percentages decreased in spleen and bone marrow (Figure 2H). Cytotoxic $CD8^+$ T cells play a pivotal role in the pathogenesis of acute GVHD because they directly attack

nonmalignant host tissues through effector molecules (28), and therefore, their decrease at the level of the target tissue in the UCB Tregs recipients, alone and in combination with ruxolitinib, highlights an important mechanism deployed by UCB Tregs to resolve GVHD. Although a relative increase in $CD8^+$ T cells was observed by flow cytometry in the liver, such an increase was not reflected on the immunohistology studies (Supplementary Figure S5).

To further examine whether addition of UCB Tregs has an impact on tissue infiltration with $CD3^+$ lymphocytes, IHC staining was performed and quantified using H-score (12). In xenogeneic GVHD model, lung has been identified as a target organ for immune mediated tissue destruction (29). IHC section of lung histologic section showed a decrease in $CD3^+$ staining in ruxolitinib recipients, with a complete resolution of $CD3^+$ infiltrate in UCB Tregs and in UCB Tregs + ruxolitinib recipients. Quantification of the $CD3^+$ cell infiltrate using H-score mirrored the histology findings, which shows that UCB Tregs decrease the T-cell mediated tissue damage in GVHD. Histology examination of spleen and liver is shown in Supplementary Figure S5. Furthermore, a corresponding decrease in the inflammatory cytokines, including GM-CSF, IFNγ, TNFα, sCD40l, IL-1a, observed in the treatment arms at day +14 (Figure 2I), underscores the multiple mechanisms at play for dampening GVHD (30).

Since ruxolitinib is associated with grade 3 and/or 4 thrombocytopenia in steroid refractory GVHD (16), we sought to examine whether UCB Tregs has an impact on this drug toxicity. As shown in Figure 2L, on day 14, platelet count decrease was lesser in UCB Tregs + ruxolitinib recipients when compared to ruxolitinib alone or UCB Tregs alone recipients. Furthermore, an improvement in the hemoglobin levels was observed in the UCB Tregs + ruxolitinib recipients when compared to ruxolitinib alone recipients (Figure 2K). In addition to a possible direct protective effect on the bone marrow, the improvement in cytopenias might be attributed to the relief from IFNγ mediated bone marrow suppression which is reversed by the UCB Tregs (31).

We conclude that UCB Tregs synergize with ruxolitinib to treat xenogeneic GVHD through multiple mechanisms and lead to improve outcomes. This combination should be examined in a clinical setting.

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 humans were approved by University of Texas MD Anderson IRB committee. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from cord blood bank of MD Anderson Cancer Center. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. The animal study was approved by University of Texas at MD Anderson IACUC committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KZ: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. HM: Conceptualization, Data curation, Formal Analysis, Methodology, Writing – review & editing. MH: Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. M-AL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. TS: Funding acquisition, Project administration, Resources, Writing – review & editing. CF: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. SP: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

SP is scientific founder of Cellenkos and has equity, is on board, has patents and royalties and funding support from Cellenkos Inc. TS is employed by Cellenkos and has equity.

The remaining 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/frtra.2024.1448650/full#supplementary-material

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