ADVANCES IN PEDIATRIC HEMATOPOIETIC CELL THERAPIES AND TRANSPLANTATION

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PUBLISHED IN: Frontiers in Pediatrics and Frontiers in Oncology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88974-531-9
DOI 10.3389/978-2-88974-531-9

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ADVANCES IN PEDIATRIC HEMATOPOIETIC CELL THERAPIES AND TRANSPLANTATION

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Dr Patrick Hanley, a Topic Editor for this collection, is a co-founder and serves on the board of directors of Mana Therapeutics, a private biotech company focused on cellular therapy for AML. All other Topic Editors declare no competing interests with regards to the Research Topic.

Citation: Katsanis, E., Hanley, P. J., Simpson, R. J., eds. (2022). Advances in Pediatric Hematopoietic Cell Therapies and Transplantation. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-531-9

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frontiers in Pediatrics

EDITORIAL
published: 28 January 2022
doi: 10.3389/fped.2022.847288

Editorial: Advances in Pediatric Hematopoietic Cell Therapies and Transplantation

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Keywords: transplant, hematopoietic, cell therapeutic, leukemia, haploidentical hematopoietic stem cell transplantation

Editorial on the Research Topic

Advances in Pediatric Hematopoietic Cell Therapies and Transplantation

Hematopoietic cell transplantation (HCT) offers curative treatment for numerous malignant and non-malignant disorders and has evolved into a relatively safe procedure with reduced transplant related mortality. There have been notable advances in HCT in recent years. Undoubtedly one of the most significant has been the re-emergence of haploidentical (haplo) HCT which has rapidly evolved into a comparable alternative to unrelated donor transplantation (1, 2). However, haplo-HCT has not been as widely adopted in pediatrics in part due to the option of umbilical cord blood (UCB) transplantation that younger patients may have (3). T-cell replete haplo-HCT with post-transplant cyclophosphamide (PT-CY) is the most frequently applied platform, while αβ T-cell and $CD19^+$ B-cell depletion is utilized in some pediatric European centers (4). In the accompanied collection of publications, Sano et al. report their experience in 19 pediatric patients with relapsed/refractory acute lymphoblastic leukemia receiving T-cell replete peripheral blood stem cell (PBSC) haplo-transplantation following total body irradiation (TBI) or busulfan (BU) based myeloablative conditioning (MAC) in 89% of patients. Thymoglobulin, tacrolimus, methotrexate and prednisolone were used as graft-versus-host disease (GvHD) prophylaxis. Not unexpectedly and partially due to the high CD3 cell dose infused, the incidence of grade II-IV and III-IV acute and chronic GvHD was unacceptably high, 100, 50, and 67%, respectively. Nonetheless, they demonstrated an encouraging 3-year overall survival of 75% in patients in complete remission (CR) and 45% in those not in remission at the time of transplantation, whilst acknowledging that their results should be interpreted with caution due to their small numbers. Katsanis et al. updated their pediatric haplo-BMT experience of 21 patients with hematologic malignancies (89% in CR) using TBI or BU based MAC followed by T-replete haplo-BMT with PT-CY or PT-CY/bendamustine (5). Grade II-IV and III-IV acute and chronic GvHD were manageable at 30, 15, and 18%, respectively. With a median follow-up >2 years, their outcomes were excellent with OS of 84%, progression free survival (PFS) 74% and graft-versus-host disease relapse-free survival (GRFS) of 50%. Gupta and Wagner review the current status of UCB transplantation for malignant and non-malignant disorders. They also discuss strategies to enhance hematopoietic recovery as well as alternative uses of UCB such as for the generation of virus-specific T cells, T regulatory cells for prevention of immune reactivity, and NK cell therapy for hematologic malignancies.

Supportive care pre-, during and post-HCT is of critical importance in reducing adverse drug effects, organ toxicity, infectious complications and ultimately non-relapse mortality. The next series of articles address some of these important issues. A report by Ifversen et al. from

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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: *01 January 2022*
Accepted: *07 January 2022*
Published: *28 January 2022*

Citation:
Katsanis E, Hanley PJ and Simpson RJ (2022) Editorial: Advances in Pediatric Hematopoietic Cell Therapies and Transplantation. Front. Pediatr. 10:847288. doi: 10.3389/fped.2022.847288

the Supportive Care of the Pediatric Diseases Working Party (PDWP) of the European Society for Blood and Marrow Transplantation (EBMT) provide an updated and comprehensive set of recommendations of infection prevention for children and young adults. They introduce consensus guidelines on environmental protective measures, microbial prophylaxis, post discharge precautions, which are especially important in the COVID-19 era, and vaccinations. Boelens et al. provide an overview of the association between specific conditioning agents and immune reconstitution (IR) post-HCT. Given the correlation between IR and outcome, they discuss a rationale for selection of a more standardized parameter set to monitor immune recovery that could be further studied and applied more widely. Additionally, Winger et al. address an important and often overlooked aspect of HCT which is the reconciliation of medications in the pre-HCT period to avoid harmful drug-drug interactions or overlapping toxicities with conditioning agents. As part of their report, they provide a very practical and extensive appendix with timelines for discontinuation or modification of common drugs prior to initiation of conditioning.

Relapse remains the primary cause of failure following HCT. Strategies to enhance graft-versus-leukemia (GVL) without increasing GvHD have been a major focus of research for decades. Summers et al. discuss the use of minor H antigens as T cell targets for augmenting GVL. Most minor H antigens are expressed ubiquitously, including on epithelial tissues, and as such can be recognized by donor T cells resulting in GvHD. This report focuses on donor-derived T cell responses against minor H antigens with hematopoietic-restricted expression. Following HCT, these hematopoietic-restricted minor H antigens present on residual recipient malignant hematopoietic cells can serve as tumor-specific antigens for donor T cells and thus be targets for selective GVL. This novel approach may hold promise for preventing relapse if given with the stem cell graft or as cell therapy for treating minimal residual disease (MRD) post-HCT. Wang et al. discuss the importance of monitoring MRD over the long treatment course of pediatric acute lymphoblastic leukemia (ALL). They followed over one thousand ALL patients documenting MRD re-emergence in ∼15%. Three fourths of patients with MRD re-emergence continued to receive maintenance chemotherapy while 25% underwent mostly haplo-HCT. The HCT group had significantly better outcomes than the patients continuing with chemotherapy.

The final two papers in this series are disease specific. Krishnamurti discusses the complex topic of HCT for patients with sickle cell disease (SCD). He addresses the importance of careful patient selection considering the intricate compromise between the possibility of ameliorating SCD manifestations and early or late complications of HCT. Moreover, careful selection of donor, and choice of conditioning and GvHD prophylaxis regimens are examined as well as pre-HCT evaluation and post-HCT management of the unique complications encountered in patients with SCD. Ussowicz et al. performed a retrospective analysis of patients with high-risk neuroblastoma treated with MIBG I [131] therapy most of who then received high-dose chemotherapy with stem cell rescue. Their results suggest that MIBG I [131] may have utility pre-HCT and may warrant further study.

This collection of original and review articles addresses new developments in this exciting and very diverse field. Despite ongoing challenges, much hope exists in developing more effective and safer approaches to treat both malignant and none malignant disorders with cell therapies and transplantation.

AUTHOR CONTRIBUTIONS

All authors listed contributed to the editorial and approved the submitted version.

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Conflict of Interest: PH is a co-founder and on the board of directors of Mana Therapeutics, is on the scientific advisory board of Cellevolve, and on an advisory board of Maxcyte.

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.

frontiers in Pediatrics

REVIEW
published: 03 June 2020
doi: 10.3389/fped.2020.00284

Minor Histocompatibility Antigen-Specific T Cells

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Minor Histocompatibility (H) antigens are major histocompatibility complex (MHC)/Human Leukocyte Antigen (HLA)-bound peptides that differ between allogeneic hematopoietic stem cell transplantation (HCT) recipients and their donors as a result of genetic polymorphisms. Some minor H antigens can be used as therapeutic T cell targets to augment the graft-vs.-leukemia (GVL) effect in order to prevent or manage leukemia relapse after HCT. Graft engineering and post-HCT immunotherapies are being developed to optimize delivery of T cells specific for selected minor H antigens. These strategies have the potential to reduce relapse risk and thereby permit implementation of HCT approaches that are associated with less toxicity and fewer late effects, which is particularly important in the growing and developing pediatric patient. Most minor H antigens are expressed ubiquitously, including on epithelial tissues, and can be recognized by donor T cells following HCT, leading to graft-vs.-host disease (GVHD) as well as GVL. However, those minor H antigens that are expressed predominantly on hematopoietic cells can be targeted for selective GVL. Once full donor hematopoietic chimerism is achieved after HCT, hematopoietic-restricted minor H antigens are present only on residual recipient malignant hematopoietic cells, and these minor H antigens serve as tumor-specific antigens for donor T cells. Minor H antigen-specific T cells that are delivered as part of the donor hematopoietic stem cell graft at the time of HCT contribute to relapse prevention. However, in some cases the minor H antigen-specific T cells delivered with the graft may be quantitatively insufficient or become functionally impaired over time, leading to leukemia relapse. Following HCT, adoptive T cell immunotherapy can be used to treat or prevent relapse by delivering large numbers of donor T cells targeting hematopoietic-restricted minor H antigens. In this review, we discuss minor H antigens as T cell targets for augmenting the GVL effect in engineered HCT grafts and for post-HCT immunotherapy. We will highlight the importance of these developments for pediatric HCT.

Keywords: minor histocompatibility antigen, T cell immunotherapy, hematopoietic stem cell transplantation, leukemia, pediatric, graft-vs.-leukemia, graft engineering, polymorphism

OPEN ACCESS

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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: *15 April 2020*
Accepted: *06 May 2020*
Published: *03 June 2020*

Citation:
Summers C, Sheth VS and Bleakley M (2020) Minor Histocompatibility Antigen-Specific T Cells. Front. Pediatr. 8:284. doi: 10.3389/fped.2020.00284

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HCT) is widely employed in the management of very high-risk leukemia in children, adolescents, and young adults (1, 2), because HCT as consolidation therapy is generally associated with a reduced relapse risk compared to chemotherapy alone (3, 4). Although HCT reduces the risk, relapse remains the major cause of death following HCT for leukemia (5). Reported post-HCT relapse rates are variable, with rates of 10–30% for patients transplanted with leukemia in minimal residual disease (MRD) negative remission, 20–70% for those in remission but with MRD, and 50–90% for those in relapse (6, 7). Long-term survival following relapse after HCT is infrequent (8–10).

HCT involves two important elements that confer protection from relapse: first, "conditioning" incorporating chemotherapy and/or radiotherapy as the pre-HCT preparative regimen, and second, donor lymphocytes in the hematopoietic cell graft as cellular therapy. Donor T cells respond to non-donor antigens on recipient cells, including minor histocompatibility (H) antigens, and thereby lead to a graft-vs.-leukemia (GVL) effect when those antigens are presented on leukemic cells. Minor Histocompatibility (H) antigens are Human Leukocyte Antigen (HLA)-presented peptides derived from normal self-proteins that differ in amino acid sequence between donor and recipient due to genetic polymorphisms outside of the chromosome 6 HLA loci (11). The GVL effect and intensive conditioning both substantially contribute to relapse prevention in myeloablative HCT, while the GVL effect is particularly critical with use of less intensive, "non-myeloablative" and "reduced intensity" HCT preparative regimens.

The efficacy of HCT for pediatric leukemia currently depends highly on both the GVL effect and on delivery of myeloablative chemo(radio)therapy in the pre-HCT conditioning regimen. Intense myeloablative conditioning, particularly involving total body irradiation (TBI), causes serious short- and long-term adverse effects, including growth and neurocognitive impairments in pediatric patients (12–16). Thus, there is a critical need to advance HCT strategies that allow reduced conditioning intensity and associated toxicity, while mitigating relapse risk. Furthermore, avoiding severe and chronic graft-vs.-host disease (GVHD) in children is highly desirable due to the morbidity, mortality, disability, and social handicaps, and late effects associated with chronic GVHD (12, 15, 17).

In this review, we discuss minor H antigens as T cell targets for augmenting the GVL effect in engineered HCT grafts and for post-HCT immunotherapy. We will highlight the importance of these developments for pediatric HCT.

MINOR HISTOCOMPATIBILITY ANTIGENS

The GVL effect in allogeneic HLA-matched related or unrelated donor HCT is largely attributed to T cell responses to recipient minor H antigens. Specifically, when a HCT recipient has a homozygous or heterozygous polymorphism that encodes a minor H antigen and their HLA-matched donor is homozygous for the "negative" allele, the donor may have T cells in their repertoire that recognize the minor H antigen peptide/HLA complex on the recipient's leukemia cell surface, and those cells may eliminate any residual leukemia after HCT (**Figure 1**).

From the perspective of the donor T cells, minor H antigens are foreign antigens and consequently donor T cells specific for minor H antigens are not subject to self-tolerance mechanisms, allowing for highly avid minor H antigen-specific T cell responses. Most known minor H antigens arise from single nucleotide substitutions in the coding sequences of homologous donor and recipient genes, which change peptide-HLA binding or T cell receptor (TCR) recognition of the peptide-HLA complex. There are at least 660,000,000 single nucleotide polymorphisms (SNPs) and insertion-deletion polymorphisms (indels) in the human genome (18) although less than 1% of SNPs are non-synonymous limiting the number of potential minor H antigens (19). Moreover, only non-synonymous SNPs that give rise to recipient donor mismatches in the graft-vs.-host direction (recipient homozygous positive or heterozygous, donor homozygous negative for the immunogenic allelic variant) are relevant to the GVL effect. T cell recognition of most minor H antigens is unidirectional, mostly due to the lack of T cell recognition of the allelic variant peptide despite cell surface presentation with HLA molecules (20). Alternatively, the corresponding donor peptide may not be generated (21–25), transported by antigen processing machinery (26), escape proteasomal degradation (27), or stably bind to MHC molecules (28–31).

MINOR H ANTIGENS AND SELECTIVE GVL

HCT outcome data demonstrate the potency of the GVL effect, with reduced relapse rates noted in patient cohorts that develop acute and/or chronic GVHD following allogeneic HCT (32–34). However, the GVL effect is apparently separable from GVHD; reduced relapse rates are still observed in patients who underwent allogeneic HCT and did not develop clinically significant GVHD, as compared to relapse rates in syngeneic HCT recipients (33).

Most minor H antigens are expressed ubiquitously, including on epithelial tissues. Recognition of such minor H antigens by donor T cells following HCT can potentially lead to GVHD as well as GVL (35, 36). However, those minor H antigens that are expressed predominantly on hematopoietic cells can be targets of a selective GVL effect (37, 38) (**Figure 2**). Once full donor normal hematopoietic chimerism is achieved after HCT, only residual or recurrent recipient malignant hematopoietic cells will present hematopoietic-restricted minor H antigens, and these minor H antigens serve as tumor-specific antigens for donor T cells (**Figure 3**). Over 100 fully-characterized or candidate human minor H antigens have been identified (22, 25, 28, 39–52). Of these, an important minority are expressed predominantly or exclusively on hematopoietic cells and are of particular interest as targets for GVL-augmenting therapeutic T cells delivered with or following HCT (**Table 1**). Examples, of well-characterized minor

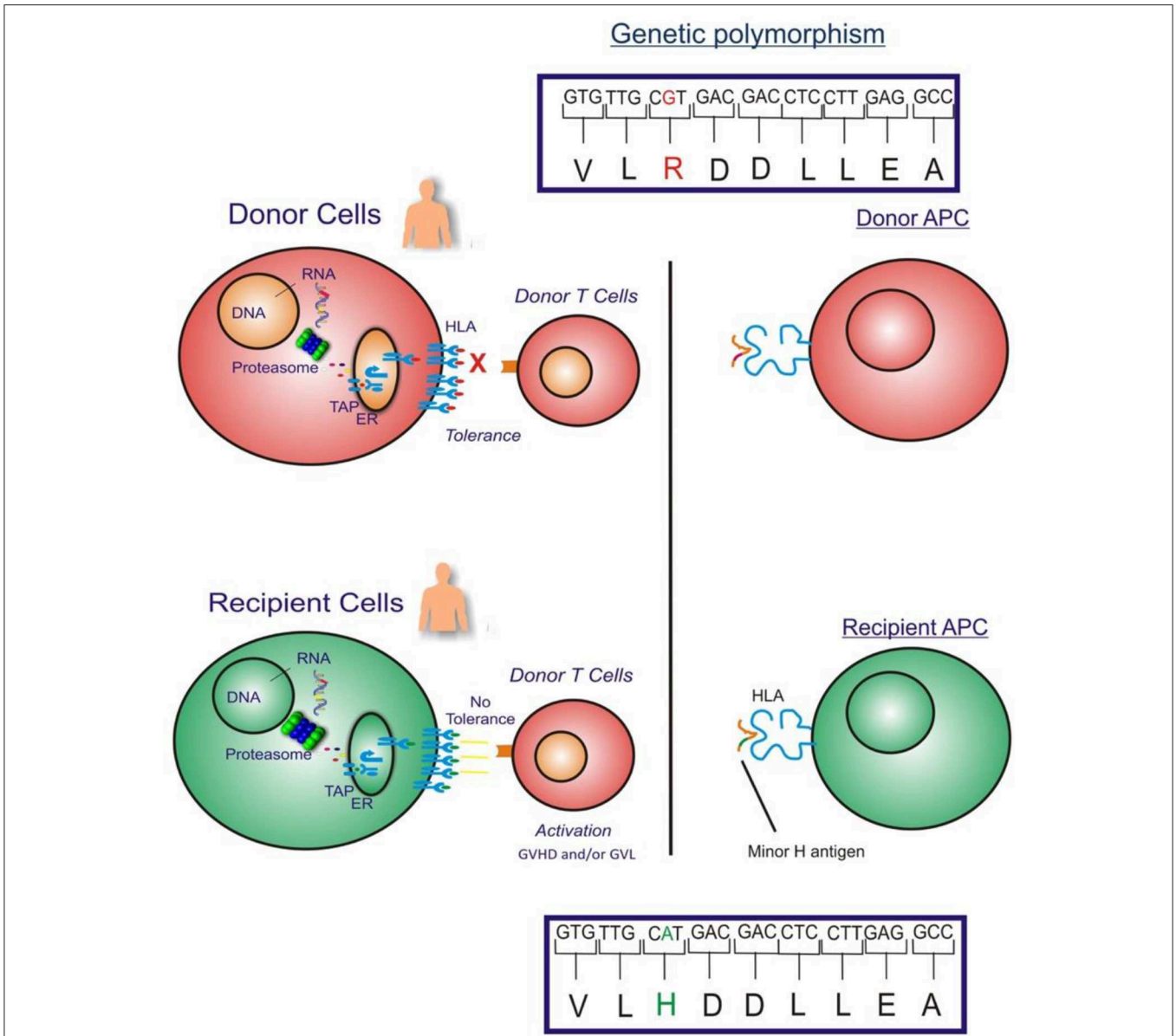

FIGURE 1 | The figure illustrates the GVHD and/or GVL effect in allogeneic HLA-matched donor HCT which is largely attributed to donor T cell responses to recipient cell minor H antigens. When an HCT recipient has a homozygous or heterozygous polymorphism that encodes a minor H antigen and their donor is homozygous negative for the allele, the donor may have T cells in their repertoire that can target the minor H antigen peptide/HLA complex on the recipient's leukemia cell surface leading to an elimination of any residual leukemia following HCT.

H antigens of high therapeutic interest include HA-1, ACC-1, ACC-2, and LRH1, described below.

There is direct evidence for the anti-leukemic activity of minor H antigen-specific T cells. In humans, donor-derived $CD4^+$ and $CD8^+$ T cells that have been activated and expanded *in vivo* following recognition of minor H antigens on recipient cells can be isolated and grown *in vitro* and evaluated for anti-leukemic activity (38). Additionally, minor H antigen-specific T cells can be generated by primary *in vitro* stimulation (53). Minor H antigen-specific $CD8^+$ T cell clones can inhibit acute myelogenous leukemia (AML) colony growth and lyse primary AML and acute lymphoblastic leukemia (ALL) cells *in vitro* (38, 53–55). Furthermore, minor H antigen-targeting T cells prevent the engraftment of AML in immunodeficient murine models, supporting the hypothesis that early leukemic progenitors are targets of these cells (56).

In vivo anti-leukemic efficacy of minor H antigen-specific T cells has also been demonstrated in murine models of HCT and GVL. Perreault and colleagues demonstrated that adoptive transfer of T cells specific for a single immunodominant murine minor H antigen (B6dom1, also known as $H7^a$) can eradicate

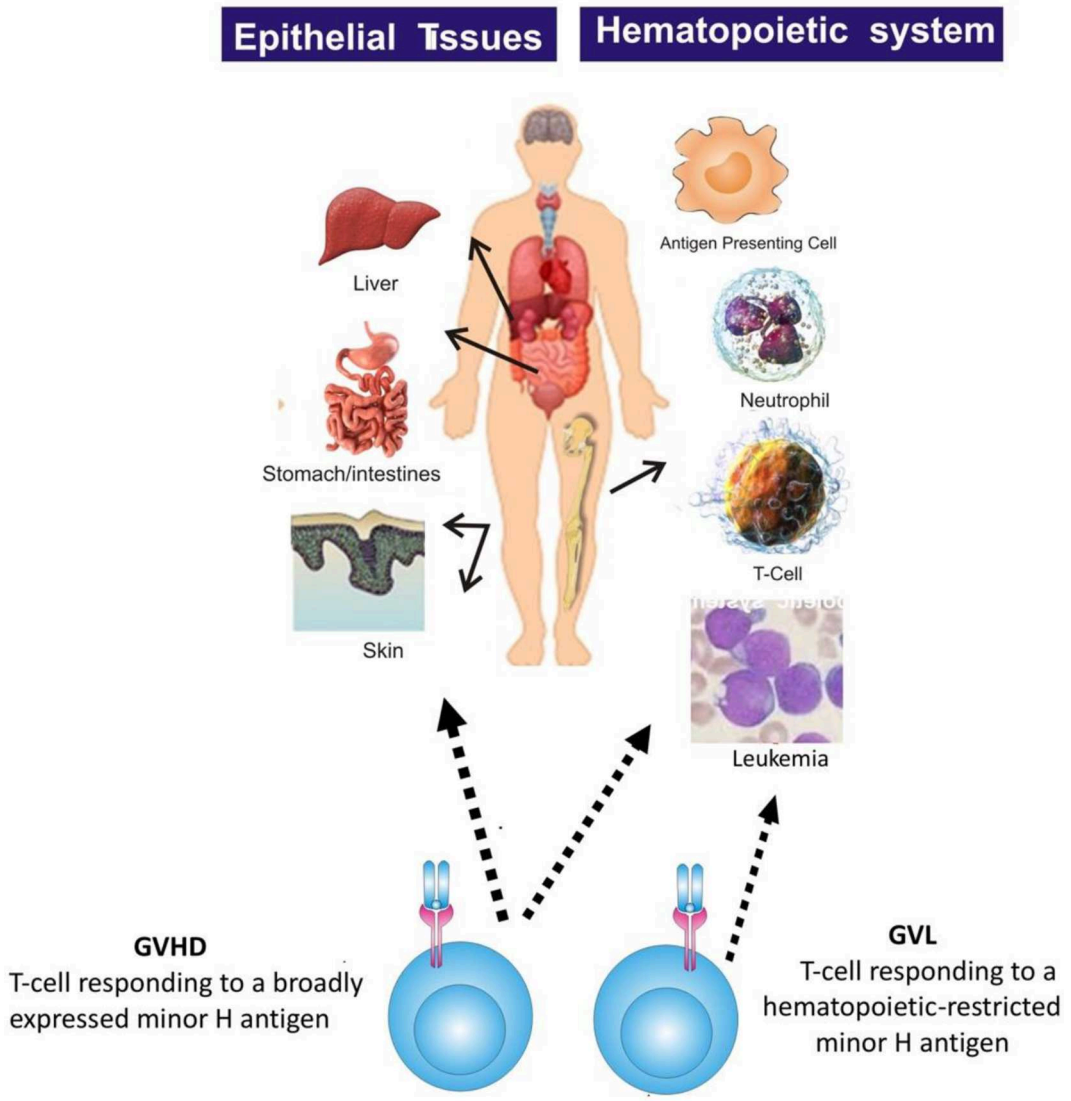

FIGURE 2 | The figure illustrates that most minor H antigens are ubiquitously expressed on epithelial tissues and hematopoietic cells. Donor T cell recognition of such antigens leads to GVHD as well as GVL. However, minor H antigens expressed predominantly on hematopoietic cells can be targets of a selective GVL effect.

leukemia and has anti-cancer activity in solid tumor models (57–60). Shlomchik and colleagues demonstrated antigen-specific memory T cell (T_M)-mediated GVL against chronic phase and blast crisis chronic myeloid leukemia (CML) when they transferred $CD8^+$ T_M from murine donors vaccinated against the H60 minor H antigen (61). In both the Perreault and Shlomchik studies, little to no GVHD was observed when the transferred T cells were specific for a single minor H antigen, even if expression of the minor H antigen was not restricted to the hematopoietic system. However, the anti-tumor efficacy was improved if the minor H antigen was not ubiquitously expressed (57, 59–61). Better efficacy of T cells specific for minor H antigens with hematopoietic-restricted vs. ubiquitous expression can be explained by less activation-induced cell death and T cell exhaustion, and better expansion of T cells targeting hematopoietic-restricted minor H antigens (61).

Focusing the T cell response on a limited number of minor H antigens may favor GVL over GVHD. In mice, leukemia was eradicated following adoptive transfer of $CD8^+$ T cells specific for a single broadly-expressed minor H antigen (B6dom/H7a), without the development of GVHD. However, GVHD occurred if B6dom-specific T cells from vaccinated donors were delivered with naïve T (T_N) cells specific for other minor H antigens (57). Earlier experiments by Korngold and colleagues, using numerous combinations of congenic mouse strains, also did not reveal GVHD in any experiment where donors and recipients were incompatible for single minor H antigens (62). Research by the Falkenburg group using human cells also demonstrated that the magnitude and diversity of the immune response influences

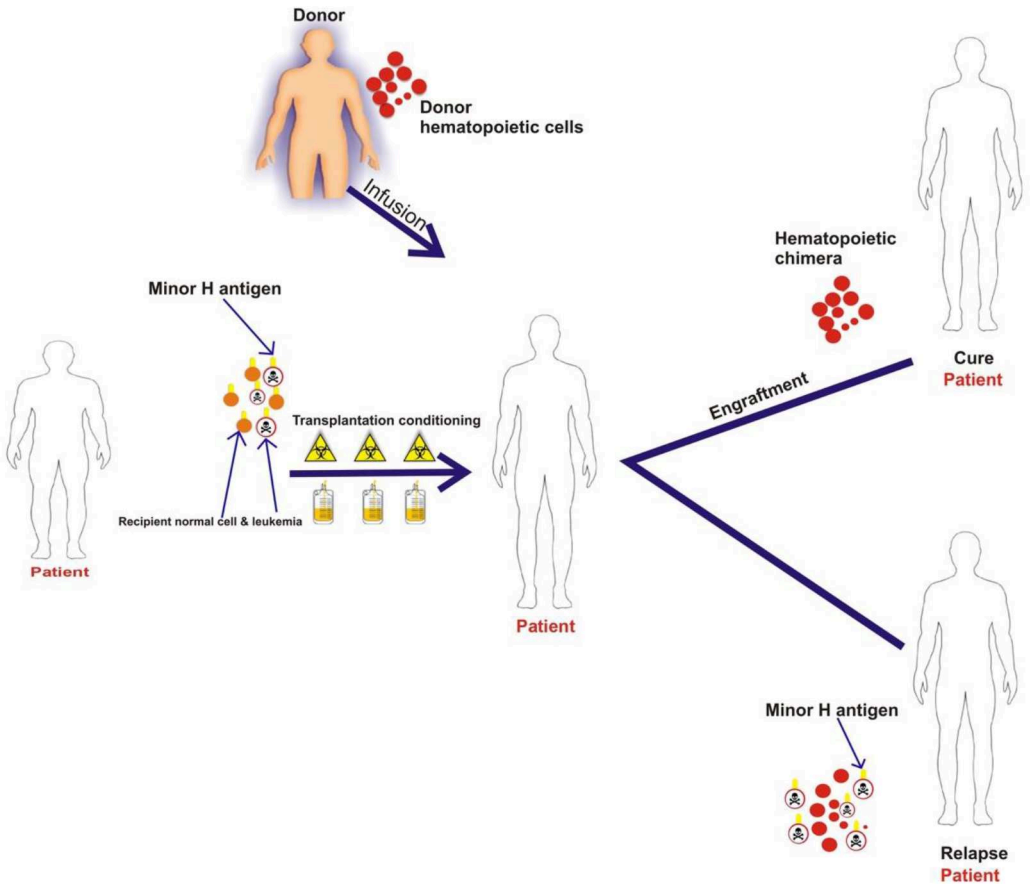

FIGURE 3 | The schematic illustrates that patient normal hematopoietic and leukemia cells with minor H antigens are eliminated by the transplant conditioning regimen and replaced with donor hematopoietic cells. Once full donor normal hematopoietic cell chimerism is achieved only recipient malignant hematopoietic cells present hematopoietic-restricted minor H antigens if disease persists or recurs following HCT. These minor H antigens serve as tumor-specific antigens for donor T cells.

the balance between GVHD and GVL (51). They characterized alloreactive $CD8^+$ T cell responses in recipients of T cell-depleted (TCD) HLA-matched HCT who achieved a clinical complete response and/or full donor chimerism after donor lymphocyte infusion (DLI). Minor H antigen-specific T cell frequency and diversity was lower in patients with who cleared residual leukemia but did not develop GVHD (i.e., selective GVL) compared to those who developed GVHD and although, patients who developed selective GVL had predominantly hematopoietic-restricted minor H antigen-specific T cells, some did also have T cells that recognized more broadly expressed minor H antigens.

Together, these studies imply that minor H antigens targeted with T cells to augment GVL may not need to be absolutely hematopoietic-restricted from a safety perspective, particularly if the T cell infusion occurs beyond the pro-inflammatory period immediately post-HCT and if a limited number of minor H antigens are targeted. However, targeting minor H antigens that are predominantly expressed on hematopoietic tissue may still be safer, and may be more effective as T cells specific for broadly-expressed antigens tend to become exhausted and dysfunctional (60, 61).

MINOR H ANTIGEN DISCOVERY

A major challenge for developing minor H antigen-targeting, GVL-augmenting strategies is to create therapeutics for *all* patients who may benefit from them, which will require the identification of multiple minor H antigens presented by various HLA types. Techniques used for minor H antigen discovery have been reviewed and include forward and reverse immunology approaches. Forward immunology approaches involve several combinations of different component methods of isolation of activated T cells from HCT recipients, primary *in vitro* stimulation of normal donor T cells, haplotype mapped (HapMap) cell line screening, genetic linkage analysis, genome-wide association studies and cDNA library screening. Combinations of *in silico* analysis, mass spectrometry, HLA/multimer and functional T cell screening have been used in reverse immunology approaches (63, 64).

As there are numerous non-synonymous SNPs (ns-SNPs) with a variant allele frequency between 0.1 and 0.9 across the human genome and a significant minority are encoded by genes that are predominantly expressed in hematopoietic cells, it is anticipated that there remain many minor H antigens that will

TABLE 1 | Selected hematopoietic-restricted minor H antigens of interest for therapeutic targeting.

Minor H antigen (reference)	Gene/Chromosome	HLA allele	Heme/non-heme expression	Polymorphism	Immunogenic peptide Epitope (underlined)	Genotype-phenotype frequencies (%)	Estimated disparity MSD (%)	Estimated disparity MUD (%)
HA-1(28, 39)	HMHA1/19p13.3	A*02:01 A*02:06	1–2 logs higher in heme.	rs1801284	VL[**H/R**] DDLLEA	H/H = 13 H/R = 45.8 R/R = 41.2	6.4 +A206 < 1	11.6 +A206 <1
LRH-1(22)	P2X5/17p13.3 (frameshift mutation)	B*07:02	1.5–2.0 logs higher in heme	rs3215407	TP**NQRQNVC**	+/+ = 4 +/– = 50 –/– = 46	4.9	7.5
LB-EBI3-1(40)	EBI3/ 19p13.3	B*07:02	2 logs higher in heme	rs4740	RPRARYY[**I/V**] QV	I/I = 10.6 I/V38.1 V/V = 51.3	3.7	7.5
HB-1(41–43)	HMHB1/ 5q31.3	B*4402 B*4403	B cell	rs161557	EEKRGSL[**Y/H**] VW	Y/Y = 5.2 H/Y = 41.2 H/H = 53.7	3.9 (Y) 1.2 (H)	6.8 (Y) 1.3 (H)
ACC-2 (44)	BCL2A1/ 15q24.3	B*44:03	1–2 logs higher in heme.	rs3826007	KEFED[**D/G**] IINW	D/D = 6.4 D/G = 38.1 G/G = 55.5	3.6	6.7
ACC-1 (44, 45)	BCL2A1/ 15q24.3	A*24:02	1–2 logs higher in heme.	rs1138357	DYLQ[**Y/C**] VLQI	Y/Y = 6.7 Y/C = 39.5 C/C = 53.5	2.8 (Y) <1 (C)	5.2 (Y) <1 (C)
ACC-6 (46)	HMSD/ 18q21.3	B*4402 B*4403	Leukemia. Not normal hematopoietic	rs9945924	**MEIFIEVFSHF**	V/V = 10 V/wt = 23 wt/wt = 66.7	2.3	5.9
HA-2 (47)	MYO1G/ 7p13	A*02:01	1–2 logs higher in heme	rs61739531	YIGEVLVS[**V/M**]	V/V = 57 V/M = 38 MM = 6	1.8	2.5
HA-1/B60 (48)	HMHA1/ 19p13.3	B*40:01	1–2 logs higher in heme	rs1801284	KECVL[**H/R**] DDL	H/H = 13 H/R = 46 R/R = 42	1.4	2
LB-ITGB2-1 (25)	ITGB2/ 21q22.3 (transcript variant)	B*15:01	1–2 log higher in heme	rs760462	**GQAGFFPSPF**	+/+- = 5 +/– = 31 –/– = 63	1	2

Minor H antigens were selected for inclusion in the table based on (a) having a predominantly hematopoietic gene expression pattern; (b) there being published functional T cell data that clearly demonstrates recognition of hematopoietic cells that endogenously present the minor H antigen(not only peptide-pulsed target cell recognition or tetramer binding); and (c) a reasonably common HLA-restricting allele and a balanced minor H antigen genotype/phenotype distribution such that >1% of the HCT recipients would have the correct HLA and minor H antigen genotype and a donor with the alternative minor H antigen allele.

be suitable targets for therapeutic T cell yet to be discovered. An *in silico* analysis was performed by Lansford et al. to predict minor H antigens. Analysis of recurrent SNPs among 101 HLA-matched HCT recipient donor pairs resulted in the identification of 102 peptides with desirable properties for public, leukemia-associated minor H antigens, specifically with: (a) predicted high binding affinity to a common HLA molecule; (b) RNA expression in AML, but not in GVHD target organs; and (c) optimal allele frequencies to give rise to common minor H antigen mismatches and therefore feasible T cell targeting (52). A proportion of these candidate minor H antigens would be expected to be naturally processed and presented on HLA molecules in leukemic cells, and to elicit a T cell response.

In a second example of the potential for additional minor H antigen discovery, Granados et al. focused on identifying HLA-A*02:01 or -B*44:03-restricted polymorphic peptides using a mass spectrometry-based proteogenomic approach and cells from 13 volunteer donors and found thousands of candidate minor H antigens (50). Of the nearly 6,773 candidate minor H antigens generated by ns-SNPs, the authors identified 100 relatively common candidates with a minor allele frequency of >0.05, including a set of 39 putative minor H antigens with RNA expression at least two times higher in bone marrow cells than in skin cells. Two of the 39 were tested and induced a T cell response *in vitro*. A proportion of the 39 candidate minor H antigens with predominantly hematopoietic expression would be expected to be both adequately hematopoietically-restricted for therapeutic targeting and immunogenic to have therapeutic utility.

EXAMPLES OF HLA CLASS I MINOR H ANTIGENS WITH POTENTIAL FOR THERAPEUTIC TARGETING

HA-1

HA-1 is the most comprehensively investigated human minor H antigen and is selectively expressed on normal and malignant hematopoietic cells, including AML, myelodysplastic syndromes (MDS), B lineage ALL, and T lineage ALL. The HA-1 peptide epitope is presented on the cell surface in association with HLA-A*02:01 and is recognized by HA-1-specific T cells.

The HA-1^H peptide (VL**H**DDLLEA) is encoded by a nucleotide sequence spanning a single nucleotide polymorphism (RS_1801284) within the *HMHA1* gene (28). Individuals with a rs1801284 A/A or A/G genotype express the histidine variant (HA-1^H, also referred to as HA-1) and are considered HA-1 "positive." Conversely, HA-1 "negative" people with the G/G genotype express only the arginine allelic variant (HA-1^R, VL**R**DDLLEA), and may have T cells in their repertoire that can respond to HA-1^H. Both HA-1^H and HA-1^R undergo similar intracellular processing from the HMHA1 protein, with appropriate proteasomal cleavage and transporter associated with antigen processing (TAP) (29, 30). However, the presence of an arginine at position three of the peptide reduces the affinity of VL**R**DDLLEA binding to HLA-A*02:01, relative to VL**H**DDLLEA (29, 30). HA-1^H-specific T cells do not recognize VL**R**DDLLEA at peptide concentrations that are naturally presented on cells. Therefore, HA-1-specific T cells can be employed following HCT to selectively target residual leukemic cells from a HA-1 positive patient, without damaging normal hematopoietic cells of HA-1 negative donor origin.

Approximately 50% of the population presents HLA-A*02:01, and HA-1 allelic variants are phenotypically balanced in the population (rs1801284 A/A 16%, A/G 36%, G/G 48%; HA-1 positive 52%, HA-1 negative 48%). This means that ∼10–15% of the HCT population will express both HA-1^H and the HLA-A*02:01-restricting allele and also have a suitably mismatched HA-1 negative or HLA-A*02:01 negative donor, making HA-1 a relatively feasible minor H antigen target for T cell immunotherapy.

Multiple publications have documented that *HMHA1* gene expression is very low to absent in non-hematopoietic cells (65–67), and that HA-1 genotypically-positive hematopoietic cells but not non-hematopoietic cells are recognized by HA-1 -specific T cells *in vitro* (37). Furthermore, HA-1-specific T cells induce little or no tissue damage when co-cultured with HA-1 positive skin biopsy specimens (68). Following HCT from an HA-1 negative donor to an HLA-A*02:01 HA-1 positive recipient, HA-1-specific T cells can be identified in approximately one-third of patients (69). One study reported a temporal relationship between the presence of HA-1-specific T cells and GHVD early post-HCT (70), but HA-1-specific T cells have been identified in patients without GVHD following DLI therapy (51, 71–73). Additionally, associations between HA-1 donor-recipient genotypic disparity and GVHD have been observed in some (74–77) but not all (78–81) HCT studies. One explanation for this apparent inconsistency is that patient hematopoietic cells remain in the tissues for several months before being replaced by donor-derived cells (72). Thus, HA-1-specific T cells generated by *in vivo* priming following HCT may contribute to GVHD early post-HCT but are less likely to do so in the context of DLI or delayed HA-1 targeted T cell immunotherapy.

Circumstantial evidence suggests that HA-1 can serve as a therapeutic target in the context of HCT and unmanipulated DLI. Specifically, there have been several reports in which patients with hematological malignancies responded DLI to treat recurrent disease following HCT and coincident emergence of HA-1-specific T cells *in vivo* was documented using peptide/HLA tetramer analysis and/or isolation of the HA-1-specific T cells from the peripheral blood (29, 51, 71–73). In some of these reports HA-1-specific T cell clones isolated from the patients were further evaluated and demonstrated specific killing of HLA-A*02 positive$^+$ HA-1^H -pulsed target cells and primary leukemic cells (71–73). There have also been reports of HA-1 positive patients with multiple myeloma who were treated with dendritic cell vaccines loaded with minor H antigen peptides and adjuvant followed by DLI. Several patients developed a detectable HA-1-specific T cell responses, without adverse effects and two achieved disease control for 6–7 months (82, 83). Together, these observations suggest HA-1 will be a safe and effective target for T cell immunotherapy. HA-1-directed T cell immunotherapy is currently in development, as discussed below.

BCL2A1/ACC-1 and ACC-2

BCL2A1 is a member of the Bcl-2 family of anti-apoptotic genes. Two minor H antigens, ACC-1^Y and ACC-2^D, result from distinct nucleotide polymorphisms in the *BCL2A1* gene and are presented by HLA-A*24:02 and HLA-B*44:03, respectively (44). *BCL2A1* is frequently highly expressed in hematologic malignancies and may contribute to the malignant phenotype, making ACC-1 and ACC-2 attractive targets for T cell immunotherapy (84, 85).

The polymorphisms, rs1138357 and rs3826007, lead to single amino acid substitutions in exon 1 of *BCL2A1*, creating the immunogenic HLA-A*24:02-restricted ACC-1^Y [DYLQ**Y**VLQI, rs1138357 AA (6%) or AG (39%)] and B*44:03-restricted ACC-2^D [KEFED**D**IINW, rs3826007 AA (4%) or AG (50%)] (44). ACC-1-specific T cells can be generated from HLA-A*24:02 positive ACC-1 "homozygous negative" donors (rs1138357, GG encoding DYLQ**C**VLQI) and distinguish the single amino acid difference (tyrosine vs. cysteine in ACC-1). Similarly, ACC-2-specific T cells from homozygous negative donors (rs3826007, GG encoding KEFED**G**IINW) can distinguish the aspartic acid from the glycine.

ACC-1 and ACC-2 are feasible targets for T cell immunotherapy. Based on the prevalence of the HLA-A*24:02 and HLA-B*44:03 restricting alleles and the frequency of distribution of immunogenic and non-immunogenic variants of ACC-1 and ACC-2, the calculated estimate of finding a polymorphism-discrepant, matched related and unrelated donor is 2.8 and 5.2% for ACC-1, and 3.6 and 6.7% for ACC-2, respectively (86).

There has been controversy regarding how selectively *BCL2A1* is expressed in hematopoietic cells. Gene expression analysis by Northern blot (44), quantitative polymerase chain reaction (67), and database microarray (http://biogps.gnf.org) suggests hematopoietic-restricted expression. Although the Goulmy group showed that *BCL2A1* expression could be upregulated in non-hematopoietic cells (mesenchymal stromal cells) by simultaneous exposure to interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), but not to IFNγ alone (87), Akatsuka's research group subsequently demonstrated comparable up-regulation of *BCL2A1* and *HMHA1* after administration of IFNγ and TNFα (67). In any case, the clinical relevance of upregulation of minor H antigen-encoding genes

after exposure to high doses of cytokines *in vitro* has not been established. ACC-1^Y and ACC-2^D genotypically positive non-hematopoietic cells are not recognized in cellular assays without exogenous cytokines (44, 67, 87). Moreover, an analysis of HCT outcomes in 320 patients expressing HLA-A*24:02 did not show an increase in GVHD in recipients with an immunogenic *BCL2A1* allele and an antigen negative donor (88). Together these data suggest that ACC-1^Y is likely to be a safe target.

ACC-1- and ACC-2-specific T cells lysed primary leukemic cells *in vitro* after isolation from HCT recipients (44). In a subsequent study, direct tetramer analysis was used to identify ACC-1-specific T cells in the bone marrow and peripheral blood in a patient who received an HLA-matched, ACC-1-disparate HCT for CML 14 months earlier (89). The bone marrow ACC-1-specific T cells proliferated in response to ACC-1 peptide stimulation and demonstrated cytotoxicity against a cell line endogenously presenting ACC-1, demonstrating the presence of functional ACC-1 specific T cells that may contribute to protection against post-HCT relapse. Gene expression data suggests that BCL2A1 is not expressed at high levels in all leukemia cells. Although high-level expression is not necessarily required to render leukemia susceptible to lysis by high-avidity minor H antigen-specific T cells, functional assays demonstrating lysis of numerous ACC-1 and ACC-1 genetically positive leukemia will be necessary before ACC-1 and 2 directed T cell immunotherapy is advanced to the clinic.

P2X5/P2RX5/LRH-1

The *P2RX5* gene is a member of the P2X purinergic ATP-gated non-selective cation channel protein group and has been recognized as promoting cancer growth and aggression (90–93). A frameshift polymorphism (rs3215407) in *P2RX5* leads to major differences in the *P2RX5*-transcribed sequence, including the immunogenic HLA-B*07:02-restricted LRH-1 minor H antigen TPNQRQNVC (22). On the basis of the frequency of the HLA-B*07:02 restricting allele and 46% prevalence of the homozygous cytosine deletion, the calculated estimate of finding discrepant matched related and unrelated donors is 4.9 and 7.5%, respectively (86). If HCT donors have the rs3215407 polymorphic sequence with a homozygous deletion of a cytosine at position 732, they may generate T cells that recognize the TPNQRQNVC minor H antigen generated in individuals without the cytosine deletion.

The *P2RX5* gene is expressed in normal lymphocytes, B and T lineage ALL, a range of lymphoma and multiple myeloma cases, the $CD34^+$ fractions of CML and AML, and possibly at low levels in brain and skeletal muscle, but there is minimal expression in GVHD target tissues (intestine, liver, lung, skin) (22). LRH-1 genotypically-positive fibroblasts, a representative non-hematopoietic tissue, are not recognized in cellular assays (22).

LRH-1-specific T cells kill ALL $CD34^+$, AML $CD34^+$, CML $CD34^+$, and multiple myeloma $CD138^+$ cells *in vitro* (22, 94, 95). Moreover, LRH-1-specific T cells have been detected in the peripheral blood of patients responding to DLI (22, 95, 96). Dolstra's group studied seven HLA-B*07:02^+ LRH-1 positive patients who received HCT and DLI from a HLA-B*07:02 positive LRH-1 negative donor, and detected LRH-1-specific T responses coinciding with declines in detectable leukemia in three of the seven patients, including two patients with CML and one with AML (22, 95). Gene expression data indicates that LRH-1 is not expressed at high levels in all leukemia blasts or progenitors, although progenitors with relatively low levels of LRH-1 expression can be inhibited by LRH-1-specific T cells in functional assays (22, 95). Further functional assays demonstrating activity of LRH-1-specific T cells against multiple LRH-1 genetically positive leukemia cell targets *in vitro*, and ideally in patient-derived murine xenograft models, are required before translation of LRH-1-specific T cell immunotherapy to the clinic.

HLA CLASS II MINOR H ANTIGENS

The primary focus of minor H antigen discovery has been on HLA class I-restricted minor H antigens as targets for $CD8^+$ T cells. However, class II-restricted minor H antigens are also of interest particularly given that HLA class II molecules are generally expressed at relatively low levels on non-hematopoietic cells under non-inflammatory conditions. As such, class II-restricted minor H antigens may be more likely to induce a selective GVL response even if the gene encoding the minor H antigen is relatively broadly expressed. In a recent study of $CD4^+$ enriched DLI from HLA-identical sibling donors, GVL reactivity without GVHD was associated with $CD4^+$ T cells targeting HLA class II-restricted minor H antigens, some of which were associated with genes expressed in non-hematopoietic cells (97). However, HLA class II gene expression is often downregulated on leukemic cells after HCT (98, 99), which implies that while class II-restricted minor H antigen-specific T cells may make a major contribution to GVL after HCT and drive the HLA class II downregulation, class II-restricted minor H antigens may not be optimal targets for T cell immunotherapy to treat post-HCT relapse.

T CELL IMMUNOTHERAPY TARGETING MINOR H ANTIGENS (FIGURE 4)

Minor H antigens have several advantages as targets for therapeutic T cells aimed to prevent or manage relapse. First, minor H antigens arise from germline variants and are therefore likely to be expressed, at least initially, in all leukemia cells in an individual, unlike neoantigens that are often subclonal, permitting escape from neoantigen-specific T cells. Second, minor H antigens are foreign to donor T cells, like neoantigens and unlike overexpressed non-polymorphic leukemia-associated antigens. High-affinity minor H antigen-specific TCRs can be relatively readily found in the repertoire of normal donors and exploited as therapeutics (100). Lastly, as described above,

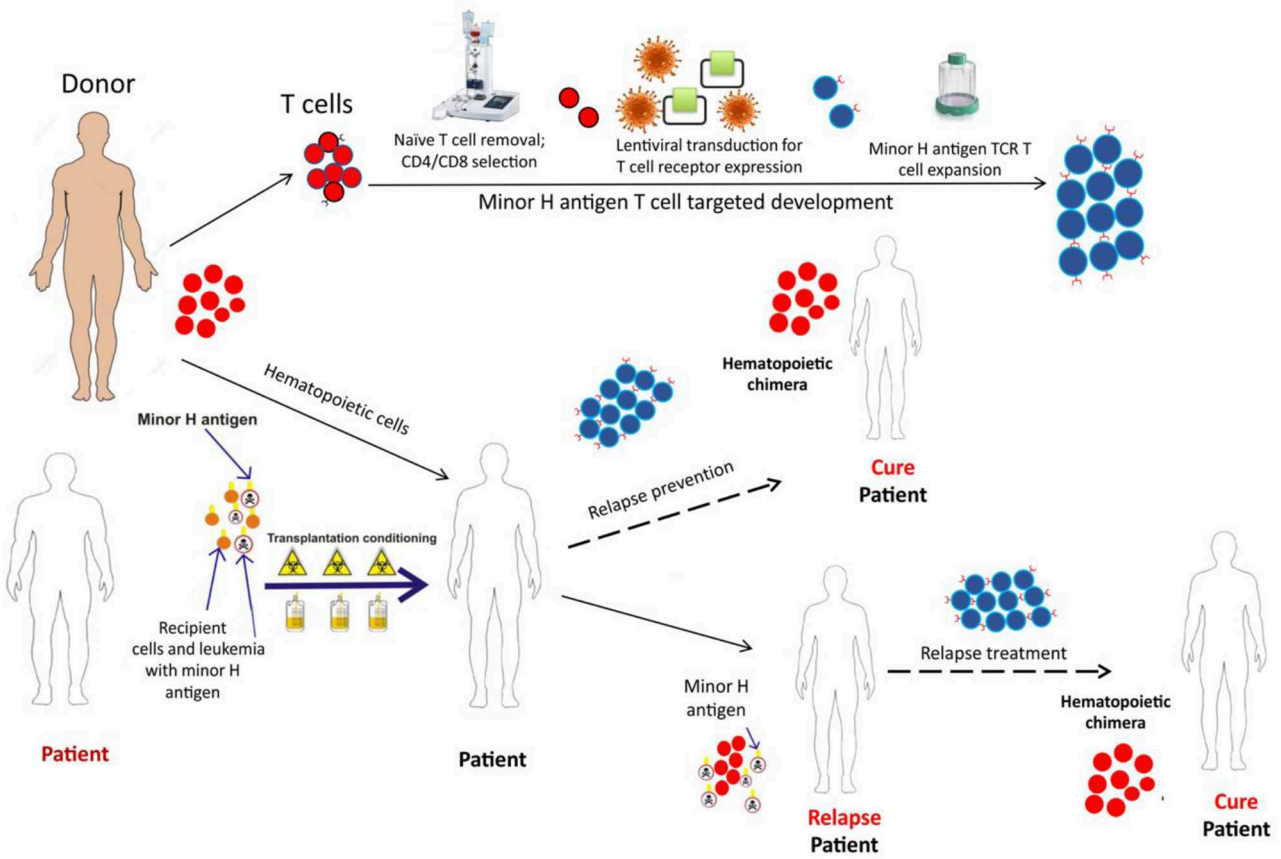

FIGURE 4 | The schematic depicts the development and potential therapeutic use of donor derived minor H antigen targeting T cell therapy. A patient, with the hematopoietically-restricted minor H antigen target, would undergo allogeneic HCT. The donor, lacking the minor H target, would donate hematopoietic cells for the HCT graft and T cells for the immunotherapy product. The T cells would undergo depletion of the naïve T cell fraction (to reduce GVHD risk) and then undergo modification using a lentiviral construct containing a potent TCR targeting the minor H antigen of interest. Following this modification, the cell product would be expanded to develop a therapeutically active cell dose which could be infused soon after the HCT as a relapse prevention strategy or following relapse detection to treat post-HCT relapse.

minor H antigen expression is inherently specific to HCT-recipient cells, and therefore specific to recipient leukemia after myeloablative HCT. This inherent specificity avoids many of the challenges for chimeric antigen receptor T cells (CAR-T) that target cell surface antigens that are shared with normal recipient or donor hematopoietic cells, leading to problems with prolonged marrow aplasia in the case of AML CAR-T, for example.

Immunotherapy employing T cells genetically modified with transgenic TCR alpha and beta chains is a promising strategy for treating hematologic malignancies and solid tumors (101, 102). Genetic TCR transfer into a selected T cell subset facilitates relatively rapid production of a T cell immunotherapy product with high potential for expansion, function and persistence after infusion into the patient. We developed T cell immunotherapy employing donor T_M cells transduced with a lentiviral vector encoding a TCR specific for HA-1 and are currently evaluating this novel therapeutic in a phase I clinical trial for the treatment of post-HCT MRD or relapse (NCT03326921) (100). The cell product incorporates multiple elements designed to optimize efficacy and safety: (1) a high-avidity HA-1 specific TCR with potent anti-leukemic activity; (2) a $CD8^+$ co-receptor to promote function of the class I-restricted TCR in $CD4^+$ T cells; (3) an inducible caspase 9 safety switch that can be triggered by the dimerizer AP1903/rimiducid in the event of unexpected side effects; (4) a CD34-CD20 epitope to facilitate selection and tracking of the engineered T cells; and (5) predominantly central memory T cells to promote persistence after infusion and to avoid infusing GVHD-inducing T_N cells (100, 103, 104). This is the currently the only minor H antigen targeting clinical trial enrolling pediatric patients that we are aware of. Another HA-1 TCR T cell immunotherapy trial (MDG1021) will open in Europe this year, employing a HA-1 TCR T cell product developed in Leiden (105, 106).

Of note, while current clinical trials targeting minor H antigens are evaluating the safety profile of minor H antigen-specific T cells as treatment for post-HCT leukemia recurrence,

a longer-term goal is to deliver hematopoietic-restricted minor H antigen-specific T cells soon after the HCT graft to augment GVL and prevent relapse.

GRAFT ENGINEERING TO AUGMENT THE T CELL RESPONSE TO SELECTED MINOR H ANTIGENS

The term "graft engineering" refers to manipulation of the composition of cells collected from an allogenic HCT donor prior to infusion into the patient. To date, graft engineering has primarily involved selections and/or depletions of particular cell subsets for the purpose of mitigating the risk of GVHD. However, more complex manipulations can be considered with emerging technology, including enrichment for rare antigen-specific T cells, genetic modification of cells with CAR-T or TCR-T, and/or knockdown of genes in order to protect certain cells or augment their function. Here we will discuss recent progress in graft engineering to create an improved platform for delivery of antigen-specific T cells, and to develop strategies for enhanced delivery of leukemia-associated minor H antigen-specific T cells.

Minimizing the risk of serious GVHD is a critical first step to enabling the effective delivery and function of hematopoietic-restricted, minor H antigen-specific T cells, since the management of GVHD requires pharmacologic immunosuppression that is not conducive to T cell expansion and function. Non-selective removal of T cells from the donor graft can be achieved through pre-infusion physical depletion of T cells or enrichment of $CD34^+$ stem cells using immunomagnetic beads, or by *in vivo* depletion using T cell antibody-directed therapy. Non-selective T cell depletion strategies have led to reduced GVHD rates but also increased infection risk due to prolonged immune reconstitution (107–111). Selective T cell depletion strategies, including $CD45RA^+$ T_N depletion and αβ TCR T cell depletion, are aimed at reducing GVHD but retaining lymphocytes with activity against pathogens and malignant cells and are being evaluated in children and adults.

T_N-Depletion

Mature αβ TCR T cells can be divided into categories based on differentiation: naïve, T memory stem cells, central memory, effector memory, and effectors (112). Naïve T cells (T_N) which are antigen inexperienced, include cells that can react to minor H antigens expressed on epithelial tissues following recipient infusion, resulting in GVHD. Murine modeling using MHC matched and mismatched mice have demonstrated that antigen experienced T_M cells cause less or no GVHD compared to T_N (113–121). They do, however, show appropriate antigen-specific T cell responses and beneficial GVL (114, 120). Additionally, *in vitro* studies found that human minor H antigen-specific T cells were more prevalent among T_N than T_M cells (53). Bleakley et al. developed a technique to engineer $CD34^+$ cell-enriched, T_N-depleted peripheral blood stem cells (PBSC) for HCT (122). Initial published experience has demonstrated remarkably low levels of chronic GVHD without increases in relapse or infection rates following HCT of T_N-depleted HLA-matched related donor PBSC in patients with leukemia (104). Additional unpublished data shows comparable results in a larger cohort of pediatric and adult recipients of T_N-depleted PBSC from HLA-matched related and unrelated donors. This approach is currently being further studied in randomized prospective trials comparing the T_N-depleted PBSC to conventional bone marrow stem cells for pediatric patients (NCT03779854) and to alternative strategies for GVHD reduction in adults (NCT03970096).

αβ TCR T Cell Depletion (αβ-TCD)

HCT strategies that deplete the donor stem cell graft of all αβ TCR T cells to remove alloreactive T cells and CD19 B cells to avoid EBV post-transplant lymphoproliferative disorder, while retaining γδ TCR T cells and NK cells, are also being investigated. HCT with αβ-TCD grafts shows promise as a platform to reduce GVHD, especially in pediatric patients (123–128). γδ TCR T cells and NK cells both have activity against pathogens and malignant cells, so their retention in the graft may protect patients against infection and relapse, respectively (128–134). In long-term analysis of 98 pediatric patients with leukemia, the outcomes of patients who received haploidentical αβ-TCD grafts were encouraging with 3-year DFS, relapse, severe acute and extensive chronic GVHD rates of 62, 29, 0, and 1%, respectively (126, 127). Moreover, in a large retrospective analysis comparing outcomes of unmanipulated HLA matched or mismatched unrelated donor HCT (MUD; MMURD) with haploidentical αβ-TCD HCT, 5-year chronic GVHD free, relapse free survival (GRFS) was superior in the haploidentical αβ-TCD compared to the MMURD (135).

Assuming continued success in reducing GVHD by graft engineering, the next challenge is to augment HCT grafts for enhanced anti-leukemic activity, including but not limited to enriching for hematopoietic-restricted minor H antigen-specific T cells. The primary aim of this approach would be to overcome quantitative deficiencies in minor H antigen-specific T cells in the donor HCT graft. Minor H antigen-specific T cells will be numerically deficient in grafts that have been depleted of T_N or of all αβ TCR T cells. Additionally, minor H antigen-specific T cells are rare in the donor T cell repertoire even in the absence of any T cell depletion (53) and may not consistently adequately expand, migrate to the bone marrow, and persist with durable anti-leukemic activity after HCT. Antigen-specific T cells are effective at controlling and eliminating cancer cells only when the T cells are present in sufficient numbers relative to the cancer cells. Therefore, particularly for patients with residual disease at the time of HCT, increasing the number of hematopoietic-restricted minor H antigen-specific T cells delivered with the graft should facilitate the GVL effect. Delayed sequential infusion of antigen-specific T cells after infusion of the HCT graft (i.e., a "split graft") may also be considered to allow the pro-inflammatory state resulting from chemo/radiotherapy conditioning to abate and the recipient tissue-resident normal hematopoietic cells to be largely replaced by cells of donor origin, in order to mitigate the risk of inducing GVHD. Delayed sequential T cell infusions should also circumvent the antigen-induced T cell death that may occur when hematopoietic-restricted minor H antigen-specific T cells

encounter residual recipient hematopoietic cells immediately post-HCT, improving persistence. Multiple infusions of cells will also avoid the functional limitation of the donor T cells that results from progressive expression of inhibitory molecules over time (99, 136).

Current hurdles to augmenting HCT grafts for hematopoietic-restricted minor H antigen-specific T cells include the relatively limited number of suitable target minor H antigens that have been discovered and characterized (**Table 1**) and technical issues largely related to inadequate options for clinical-grade sorting of rare cells. Streptamer technology is being evaluated for isolating antigen-specific T cells (137–140). The technology is based on the direct labeling of $CD8^+$ T cells with HLA I-Streptamers composed of peptide-loaded HLA I-Strep-tag fusion proteins reversibly multimerized on magnetically labeled Strep-tactin. After separation, the HLA I-Streptamers can be dissociated from the positively selected cells by the addition of D-Biotin, allowing for rapid enrichment of unlabeled antigen-specific T cells under GMP conditions. However, in a clinical trial of Streptamer-enriched multi-antigen-specific T cells to prevent complications early after T cell-depleted HCT, neither tumor associated antigen or minor H antigen-specific T cells could be confirmed in the majority of antigen-specific T cell products or after HCT, although EBV and CMV-specific T cells were readily detected in the products and sometimes after HCT (140). The greater success in isolating virus-specific T cells compared to minor H antigen- or other tumor-associated antigen-specific T cells, may be due to the relatively high frequencies of virus-specific T cells in the repertoire of normal viral antigen-experienced donors.

Next-generation high-speed cell sorting technology may solve the challenge of isolating very rare cells from donor cell collections. For example, a novel cell sorting technology called OrcaSort™ is being developed by OrcaBio. OrcaSort™ uses fluorescent markers for identification of target cells and high-speed laser pulses to sort cells in fully-enclosed, sterile, disposable cassettes (141). The technology will first be evaluated in clinical trials that require sorting of multiple cell populations for GVHD reduction (NCT03802695, NCT01660607) but could also be adapted to positive selection of antigen-specific T cells.

An alternative strategy for enriching selected minor H antigen-specific T cells in donor cell collections is to first vaccinate the HCT donor against minor H antigens to generate a memory T cell response and increase the frequency of the minor H antigen-specific T cells (61). Shlomchik et al. demonstrated that donor vaccination with recipient minor H antigens, and subsequent transplantation of donor T memory T cells, transferred leukemia- and pathogen-specific immunity in murine bone marrow transplantation (BMT) recipients (61). The transferred memory T cells expanded after BMT and augmented GVL. This approach could be translated to humans by vaccinating donors months before HCT or intended post-HCT infusion and then specifically selecting the minor H antigen T cells using a immunomagnetic bead-based technique, such as Streptamer selection (137–140) or the Miltenyi Biotec CliniMACs Cytokine Capture System (142, 143). Alternatively, after donor vaccination with hematopoietic-restricted minor H antigens, donor T cells could be collected and depleted of naïve T cells to avoid GVHD (104), and the minor H antigen-specific T cell enriched memory T cells could be delivered with or following the stem cell graft. Minor H antigen vaccination of donors is likely to be safe, given that clinical trials of vaccination of HCT recipients against minor H antigens have been completed without excess toxicity (82, 83). Donor vaccination could also be employed to improve the efficacy of DLI to prevent or treat post-HCT relapse by producing a product enriched for particular minor H antigen-specific T_M cells. The DLI product could be further engineered by depletion of T_N.

HEMATOPOIETIC CELL TRANSPLANTATION FOR PEDIATRIC LEUKEMIA IN THE CURRENT ERA AND FUTURE

Allogeneic HCT is the current standard of care for pediatric patients with very high-risk hematologic malignancies (1, 2). The overall success of HCT as a treatment for pediatric leukemia has improved, with reduced non-relapse mortality rates (144) and leukemia-free survival rates in the range of 60–80% (145, 146) in the current era. However, conditioning regimens and GVHD have long lasting adverse effects, particularly for the pediatric population who undergo significant neurologic and physical development. Long-term effects of HCT, particularly of conditioning and especially related to TBI, include growth disturbance, hormone deficiencies, cataracts, seizures, cerebrovascular events, dyslipidemia, and secondary malignancies (12–16). GVHD can also lead to long-lasting morbidity involving multiple organs, most commonly the skin, gut, and liver, but also lungs, mouth, eyes and joints in the chronic setting (15, 17).

The mortality and morbidity of HCT may be improved by graft engineering and adjunctive T cell immunotherapy to reduce GVHD and to augment GVL. Because T cell immunotherapy has the potential to prevent relapse, it may permit de-escalation of conditioning intensity, which will be critically important for the youngest HCT recipients in whom myeloablative HCT can be associated with devastating neurodevelopmental complications (13). In infants, leukemia is often refractory to chemotherapy and relapse occurs frequently (147). HCT is indicated for infants with ALL in first remission with the highest risk of relapse, and for those who achieve a second remission after relapse. Unfortunately, the youngest infants with ALL generally have the highest risk of relapse with chemotherapy alone, but also the greatest risk of late effects of HCT conditioning. Because of this combination of high risk from both disease and complications of therapy, there is an urgent need to develop reduced toxicity HCT strategies for infants, supplemented by add-back of minor H antigen-specific T cells and/or post-HCT T cell immunotherapy to prevent relapse.

The presence of detectable disease at the time of HCT is consistently and strongly associated with an increased risk of relapse post-HCT (6, 7). As such, other important recent developments that enable reduction of conditioning intensity

include sensitive techniques for detecting measurable residual disease (MRD) and therapies for reducing MRD prior to HCT. These advances are particularly important when one contemplates HCT strategies that rely on the T cell-dependent GVL effect, which is relatively delayed compared to the immediate anti-leukemic effect of intensive conditioning. MRD detection has moved from morphologic evaluation to the use of flow cytometry or PCR, which have sensitivities of 0.1% for AML and 0.01% for ALL (148, 149). Technologies to detect even lower levels of disease have been developed and the clinical implications are being studied. Next-generation sequencing (NGS) measuring immunoglobulin heavy chain (variable, diversity and joining) have been developed for ALL; patients who are NGS negative prior to HCT have been shown to have a reduced risk of relapse (150). NGS is also currently being evaluated for AML (151, 152) and may prove useful for risk stratification and perhaps monitoring, but is likely to be complex (153). Highly sensitive MRD evaluation in the pre-HCT period may be used to guide which pediatric patients can undergo HCT with reduced intensity/toxicity conditioning without an excessive risk of relapse, and which patients do have a high risk of relapse and may benefit from the addition of novel relapse prophylaxis, such as minor H antigen-specific T cells. In cases where MRD is detected pre-HCT, new targeted strategies, particularly CAR-T cell immunotherapy and bispecific T cell engagers, can also produce deep MRD negative remissions prior to HCT and thereby may improve post-HCT prognosis (154–158).

Significant success has been achieved using CAR-T cell immunotherapy targeting lineage-specific antigens to treat pediatric and adult patients with acute leukemia. This is highlighted by the efficacy of some CAR-T cell products targeting CD19, an antigen expressed on normal and leukemic B cells in some acute leukemias and lymphomas (154, 156). Patients who have received effective CD19 CAR-T cell therapy develop B cell aplasia and hypogammaglobulinemia as a result. Given hypogammaglobulinemia can be supported with intravenous immunoglobulin administration, and patients do not have significant infectious complications (159), B cell aplasia is not a major barrier for CD19 CAR-T cell immunotherapy. Targeting myeloid-lineage antigens, such as CD33 or CD123, is likely to be more problematic due to the risk of marrow suppression or aplasia placing patients at risk for severe associated complications including infection (160, 161). Minor H antigen-targeted therapies are not suitable for use in patients who have not received allogeneic HCT and therefore have a recipient hematopoietic system. However, following HCT the presence of a normal donor hematopoietic system lacking the minor H antigen target limits the therapeutic targets to neoplastic cells or residual recipient normal cells that are no longer necessary, and should protect the recipient from marrow suppression, B cell aplasia, and other hematopoietic complications. Additionally, many CAR constructs incorporate non-human components, frequently using murine derived svFc, which can be immunogenic leading to a rejection response, whereas the TCR in minor H antigen-specific T cells and TCR-T cell products are of human origin and are inherently less immunogenic favoring *in vivo* persistence (162). Lastly, although minor H antigen-targeted therapy is restricted to patients with the restricting HLA-allele and appropriate recipient-donor mismatch for the polymorphism, minor H antigen-targeted therapy is not limited to one leukemia subtype, in contrast to most CAR-T cell immunotherapy.

Ultimately, superior survival with reduced morbidity after pediatric HCT could be achieved by a combination of (a) therapies to achieve remission without any detectable disease, potentially including CAR-T cell therapy or other immunotherapy (b) a reduced-intensity/toxicity conditioning regimen, (c) a HCT graft selectively depleted of GVHD-inducing T cells and augmented with T cells specific for a limited number of hematopoietic restricted minor H antigens, and (d) post-HCT minor H antigen-targeted T cell immunotherapy. Although this vision has not yet been reached, and minor H antigen-targeted T cell therapies are currently in early phase clinical trials without proven benefit for pediatric or other patients, steady progress is being made in the fields of antigen discovery, graft engineering and genetically-modified T cell therapy. We anticipate that these advances will continue and come together to benefit children with very high-risk leukemia.

CONCLUSIONS

Donor-derived T cell responses to minor H antigens with hematopoietic-restricted expression are a key component of effective GVL. Targeting leukemia-associated minor H antigens with T cells in hematopoietic grafts and/or with post-HCT T cell immunotherapy are potentially low-toxicity, high-efficacy prophylactic and therapeutic strategies for pediatric patients. Pre-clinical studies and clinical trials of T cell immunotherapies to enhance the response to hematopoietic-restricted minor H antigens are underway. These advancing technologies should enable a reduction in HCT conditioning intensity without sacrificing protection from post-HCT relapse, thereby mitigating dangerous late effects of HCT for pediatric patients with leukemia.

AUTHOR CONTRIBUTIONS

CS, VS, and MB reviewed the literature, wrote, and edited the manuscript.

FUNDING

This work was supported by National Cancer Institute (NCI CA018029-42) Alex's Lemonade Stand Foundation, and by a Stand Up To Cancer Innovative Research Grant, Grant Number SU2C-AACR-IRG 14-17. Stand Up To Cancer is a division of the Entertainment Industry Foundation. Research grants are administered by the American Association for Cancer Research, the scientific partner of SU2C.

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Conflict of Interest: MB is a Founder and Scientific Advisory Board member of HighPassBio, a Scientific Advisory Board member of Orca Bio, and has also received compensation from Miltenyi Biotec for presentations at conferences and corporate symposia.

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.

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 09 June 2020
doi: 10.3389/fped.2020.00282

T-Cell Replete Myeloablative Haploidentical Bone Marrow Transplantation Is an Effective Option for Pediatric and Young Adult Patients With High-Risk Hematologic Malignancies

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OPEN ACCESS

Edited by:
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Jennifer Whangbo,
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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: 26 February 2020
Accepted: 05 May 2020
Published: 09 June 2020

Citation:
Katsanis E, Sapp LN, Reid SC, Reddivalla N and Stea B (2020) T-Cell Replete Myeloablative Haploidentical Bone Marrow Transplantation Is an Effective Option for Pediatric and Young Adult Patients With High-Risk Hematologic Malignancies. Front. Pediatr. 8:282. doi: 10.3389/fped.2020.00282

Twenty-one pediatric and young adult patients (1.1–24.7 years) with hematologic malignancies underwent myeloablative T-cell replete haploidentical bone marrow transplant (haplo-BMT) between October 2015 to December 2019. Fifty-seven percent of the patients were ethnic or racial minorities. Thirteen patients had B-cell precursor acute lymphoblastic leukemia (B-ALL) with 10 receiving 1,200 cGy fractionated total body irradiation with fludarabine while the remaining 11 patients had targeted dose-busulfan, fludarabine, melphalan conditioning. Graft-vs.-host disease (GvHD) prophylaxis consisted of post-transplant cyclophosphamide (15 patients) or cyclophosphamide and bendamustine (six patients), with all patients receiving tacrolimus and mycophenolate mofetil. Twelve patients were in first or second remission at time of transplant with five in >2nd remission and four with measurable disease. Three patients had failed prior transplants and three CAR-T cell therapies. Only one patient developed primary graft failure but engrafted promptly after a second conditioned T-replete peripheral blood stem cell transplant from the same donor. An absolute neutrophil count of 0.5 $\times$ 10^9/L was achieved at a median time of 16 days post-BMT while platelet engraftment occurred at a median of 30 days. The cumulative incidence of grades III to IV acute GvHD and chronic GvHD was 15.2 and 18.1%, respectively. With a median follow-up of 25.1 months the relapse rate is 17.6% with an overall survival of 84.0% and a progression-free survival of 74.3%. The chronic graft-vs.-host-free relapse-free survival (CRFS) is 58.5% while acute and chronic graft-vs.-host-free relapse-free survival (GRFS) is 50.1%. Myeloablative conditioned T-replete haploidentical BMT is a viable alternative to matched unrelated transplantation for children and young adults with high-risk hematologic malignancies.

Keywords: myeloablative, T-replete, haploidentical BMT, pediatric, GvHD

INTRODUCTION

There has been a global resurgence in the use of haploidentical hematopoietic cell transplantation (haplo-HCT). One of the factors that has contributed to this increase has been the ability to use T-replete stem cell grafts whether bone marrow (BM) or peripheral blood (PBSC) followed by post-transplant cyclophosphamide (PT-CY) (1–5). While there have been countless adult reports using haplo-HCT with PT-CY for hematologic malignancies, application of this approach in pediatric patients has lagged behind. Potential contributing factors may have included the comfort level and familiarity of using matched unrelated donor (MUD) or umbilical cord blood (UCB) by pediatric transplant physicians, the absence of haplo-HCT as a transplant option in many cooperative group trials, and the relative lack of pediatric haplo-HCT publications demonstrating comparable efficacy to MUD transplantation. Moreover, the most widely published conditioning regimen used with PT-CY has been a reduced intensity conditioning (RIC) consisting of low dose cyclophosphamide, fludarabine and 200 cGy total body irradiation (TBI) developed at Johns Hopkins, which is not ideal for high-risk pediatric acute leukemias as relapses have been reported in more than half of patients (**Table 1**) (8).

Only a handful of pediatric studies using T-cell replete grafts with myeloablative conditioning (MAC) followed by PT-CY have been published (**Table 1**). An Italian group described their pediatric haploidentical bone marrow transplantation (haplo-BMT) experience from five centers, with 14 patients (43%) receiving MAC and the remaining RIC, all followed by PT-CY (6). A team from India reported on the use of PT-CY following a chemotherapy based MAC regimen and unmanipulated PBSC transplant in 20 pediatric leukemia patients in India (7). More recently, a South Korean group reported on 22 pediatric patients with hematologic malignancies also undergoing PBSC transplant using a chemotherapy-based MAC and PT-CY (10). We have previously published our initial experience with MAC haplo-BMT using TBI-fludarabine primarily for B-cell precursor acute lymphoblastic leukemia (B-ALL) and busulfan, fludarabine, melphalan for the other hematologic malignancies (9). Herein, we update our results with MAC T-cell replete haplo-BMT, making this the largest single institution pediatric report in North America. Our experience confirms that T-cell replete haplo-BMT utilizing MAC is a safe and effective HCT modality for pediatric and young adults with hematologic malignancies and should be readily offered to HCT candidates without a matched sibling donor.

METHODS

Patients

Twenty-one pediatric and young adult patients (1.1–24.7 years) with hematologic malignancies underwent myeloablative T-cell replete haplo-BMT on the pediatric hematopoietic cell therapy and transplant (HCTT) service at Banner University Medical Center from October 2015 to December 2019. University of Arizona institutional review board approval was obtained to review and report our findings. Eligible patients for haplo-BMT were those who had no matched related donor or a readily available MUD, met organ criteria allowing for MAC and had no evidence of active untreated infection.

Conditioning Regimens

Ten patients were conditioned with fractionated TBI of 200 cGy BID given on days −8, −7, and −6 (1,200 cGy total dose with lungs shielded to 900 cGy by custom cerrobend blocking), followed by fludarabine (FLU) 30 mg/m^2 on days −5, −4, −3, and −2 (2, 9). Eleven patients received busulfan (BU) at 0.8 mg/kg IV every 6 h for a total of 12 doses (days −8 to −6), targeting an average area under the curve (AUC) of 1,000–1,100 μMol/min for the duration of the course. BU pharmacokinetics of the first dose were performed at the Seattle Cancer Care Alliance laboratory. The seventh and remaining doses were modified to achieve the average exposure of 1,000–1,100 μMol/min. BU was followed by FLU 30 mg/m^2 on days −5, −4, −3, and −2 and melphalan (MEL) 100 mg/m^2 on day −2 (9, 12).

Graft-vs.-Host Disease (GvHD) Prophylaxis

Fifteen patients received PT-CY 50 mg/kg on days +3 and +4. Six patients that were part of an IRB–approved phase I single institution clinical trial through the University of Arizona Cancer Center (NCT02996773) received PT-CY 50 mg/kg on days +3 and post-transplant bendamustine (PT-BEN) with or without PT-CY on day +4. One patient (cohort 1) received 40 mg/kg PT-CY on day +4, immediately followed by PT-BEN 20 mg/m^2. Two patients (cohort 2) were treated PT-CY 20 mg/kg followed by PT-BEN 60 mg/m^2 while three patients (cohort 3) received only PT-BEN 90 mg/m^2 on day +4. All patients were started on mycophenolate mofetil on day +5 until day +28 and Tacrolimus from day +5. In the absence of GvHD, tacrolimus was weaned starting day +70 to +90 and discontinued by day +120 to 180. GvHD was graded according to the consensus criteria for grading acute and chronic GVHD (13, 14).

Supportive Care

Antifungal prophylaxis with voriconazole was administered in all patients. Patients received i.v. pentamidine for Pneumocystis jirovecii and acyclovir for herpes simplex and varicella virus prophylaxis. Bi-weekly polymerase chain reaction (PCR) monitoring for cytomegalovirus (CMV) and weekly for adenovirus, Epstein-Barr virus (EBV) and human herpes virus-6 (HHV-6) were performed until discharge from hospital and subsequently at least every other week during first 100 days. All patients were transplanted in HEPA filtered rooms on a HEPA filtered unit and encouraged to walk laps on the unit daily.

Donor Selection

Donors were first degree relatives who were HLA-haploidentical based on high-resolution typing at HLA-A, -B, -Cw, -DRB1, and -DQB1. Fourteen of the donors were five of 10 antigen matches, five were six of 10 and two 7/10 (**Table 2**). None of the patients had anti-donor HLA antibodies. There were eight major, and four minor ABO incompatibilities that required donor red blood cell

TABLE 1 | Pediatric T-cell replete haploidentical hematopoietic cell transplant studies with PT-CY in patients with hematologic malignancies.

	n	Single or multi-institution	Age	Disease	Disease status %	Regimen %	Graft	GvHD prophy	Engraft %	aGvHD III-IV %	cGvHD %	NRM %	Relapse %	OS %	PFS %	F/U mo
Berger et al. (6)	33	M-#5 Italy	1–21	ALL 45% AML 21% OL 3% MDS 12% CML 3% HD-NHL 5%	24 CR1 24% 30 CR2 30% 15 >CR2 15% NR 30%	RIC 57% CY-FLU-200cGy MAC 43% BU-FLU-TT 1,200cGy-FLU	BM	PT-CY Tacro MMF	97	3	4	9	24	72 @1yr	61 @1 yr	12
Jaiswal et al. (7)	20	S India	2–20	ALL 35% AML 65%	NR 100%	MAC BU-FLU-MEL	PBSC	PT-CY CSA MMF	100	20	5	20	26	64 @2yr	59 @2 yr	22
Klein et al. (8)	40	S Baltimore MD, US	1–25	ALL 23% AML 23% MDS 13% AUL 2% CML 2% HD-NHL 38%	CR 43 % NR 57%	RIC CY-FLU-200cGy	BM	PT-CY Tacro MMF	94	13	24 7*	13	52	56 @1yr	43 @1 yr	20
Katsanis et al. (9)	13	S Tucson AZ, US	4–26	ALL 54% AML 15% AUL 7% CML 7% HD-NHL 15%	CR1 23% CR2 46% >CR2 8% NR 23% 2nd BMT 15% failed CAR-T 7%	MAC BU-FLU-MEL 1,200cGy-FLU	BM	PT-CY PT-CY/BEN Tacro MMF	100	0	29 19*	0	0	100 @1yr	100 @1 yr	15
Hong et al. (10)	22	S South Korea	1–20	ALL 50% AML 32% AUL 14% HD 14%	CR1 73% >CR2 27%	MAC BU-FLU-CY	PBSC	PT-CY Tacro MMF	100	6	9*	0	21	82 @2yr	78 @2 yr	26
Symons et al. (11) Abstract	32	M-#9 US-Canada	1–23	ALL 41% AML 41% AUL 3% MDS 15%	CR1 47% CR2 34% 2nd BMT 3%	MAC BU-CY 1,200cGy-CY	BM	PT-CY Tacro MMF	84	0	4*	0	32	77 @1yr	68 @1 yr	39
Katsanis Current	21	S Tucson AZ, US	1–24	ALL 62% AML 14% AUL 5% CML 5% HD-NHL 14%	CR1 19% CR2 38% >CR2 24% NR 19% 2nd BMT 14% failed CAR-T 14%	MAC BU-FLU-MEL 1,200cGy-FLU	BM	PT-CY PT-CY/BEN Tacro MMF	95	15	18 12*	9	17	91 @1yr 84 @2 yr	86 @1 yr 74 @2 yr	25

*n, number; GvHD, graft-vs.-host disease; a, acute; c, chronic; NRM, non-relapse mortality; OS, overall survival; PFS, progression-free survival; F/U, follow-up; M, multi-institutional; #, number of institutions; S, single institution; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; OL, other leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; HD, Hodgkin's disease; NHL, non-Hodgkin lymphoma; AUL, acute undifferentiated leukemia; CR, complete remission; NR, not in remission; PT-CY, post-transplant cyclophosphamide; PT-CY/BEN, post-transplant cyclophosphamide/bendamustine; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; *, extensive cGvHD.*

TABLE 2 | Patient, disease and transplant characteristics.

Age, median yr, (range)	16.8 (1.1–24.7)
Male gender, *n* (%)	14 (66.7)
Race/Ethnicity, *n* (%)	
White Hispanic	10 (47.6)
Native American	1 (4.8)
African American	1 (4.8)
White	9 (42.9)
Diagnosis, *n* (%)	
B-ALL	13 (61.9)
AML	3 (14.3)
AUL	1 (4.8)
CML	1 (4.8)
NHL	2 (9.5)
HD	1 (4.8)
Pretransplant Status, *n* (%)	
CR1	4 (19)
CR2	8 (38.1)
>CR2	5 (23.8)
Other	4 (19)
Prior BMT	3 (14.3)
Failed prior CAR-T	3 (14.3)
Disease risk index, *n* (%)	
Low	1 (4.8)
Intermediate	19 (90.4)
High	1 (4.8)
Lansky/Karnofsky, median (range)	90 (50–100)
HCT Comorbidity index, median (range)	0 (0–7)
Conditioning, *n* (%)	
TBI-FLU	10 (47.6)
BU-FLU-MEL	11 (52.4)
GvHD prophylaxis	
PT-CY, Tacro, MMF	15 (71.4)
PT-CY/BEN, Tacro, MMF	6 (28.6)
Graft composition median (range)	
CD34+ $\times 10^6$/kg	4.05 (1.5–7.5)
RBC incompatibility, *n* (%)	
None	9 (42.9)
Major	8 (38.1)
Minor	4 (19.0)
Donor age, median yr, (range)	34.7 (16.3–47.7)
Donors of male recipients, *n* (%)	
Mother	6 (42.9)
Father	4 (28.6)
Brother	4 (28.6)
Donors of female recipients, *n* (%)	
Mother	2 (28.6)
Father	2 (28.6)
Sister	2 (28.6)
Brother	1 (14.3)
Donor Match, *n* (%)	
5/10	14 (66.7)
6/10	5 (23.8)
7/10	2 (9.5)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AUL, acute undifferentiated leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma; HD, Hodgkin's dsease; CR, complete remission; TBI, total body irradiation; FLU, fludarabine; BU, busulfan; MEL, melphalan; CY, cyclophosphamide; BEN, bendamustine; Tacro, Tacrolimus; MMF, mycophenolate mofetil.

(RBC) reduction using Hespan® (6% hetastarch in 0.9% sodium chloride injection) for RBC sedimentation or plasma reduction, respectively (15).

Engraftment and Donor Chimerism Monitoring

Granulocyte-colony stimulating factor (G-CSF) was started on day +5 at 5 μg/kg/day until an absolute neutrophil count (ANC) of 2.5 × 10^9/L was achieved for three consecutive days. Day of myeloid engraftment was defined as the first of three consecutive days with an ANC of 0.5 × 10^9/L. Day of platelet engraftment was considered the first of three consecutive days with platelet counts of >20 × 10^9/L with no platelet transfusions administered in the previous 7 days. Donor chimerism was evaluated on days +28, +100, +180, and +365 by short tandem repeats (STRs) on peripheral blood or bone marrow. Engraftment testing was performed using labeled primers to PCR-amplify STR polymorphic DNA markers followed by capillary electrophoresis to distinguish between the DNA contributed by the recipient vs. the donor, and estimate the percentage of the contribution. The Promega GenePrint 24 System which includes 24 polymorphic markers was used (Promega Corporation, Madison, WI) (16).

Statistical Analysis

Time to event endpoints were estimated using the Kaplan-Meier method. The association between the number of $CD34^+$ × 10^6/kg cells infused with time to neutrophil or platelet engraftment was estimated using linear regression analysis.

RESULTS

Patient, Disease, and Transplant Characteristics

Twenty-one patients with hematologic malignancies underwent T-replete haplo-BMT following myeloablative conditioning. The clinical characteristics of the patients are outlined in **Table 2**. The median age at transplant was 16.8 years (1.1–24.7 years). Two thirds of patients were male. Ethnic and/or racial minorities constituted 57% of all patients, the majority of whom (48%) were Hispanic. Sixty-two percent of the patients had B-ALL. All 13 patients with B-ALL were minimal residual disease (MRD) negative by flow cytometry prior to initiation of conditioning. Only one patient was in first complete remission (CR1), who had failed two chemotherapy induction regimens and achieved CR1 only after blinatumomab, while seven were in CR2 and five in third or greater remission. One B-ALL patient had haplo-BMT following a prior MUD BMT, one following failure of chimeric antigen receptor (CAR)-T therapy and one following relapse after unrelated cord blood transplant and failure of multiple CAR-T cell therapies (17). Cytogenetics were unfavorable in nine of the 13 patients with B-ALL including two with 9:22 translocation, two with 11q23.3 translocation, three with intrachromosomal amplification of chromosome 21 (iAMP21) with RUNX1 amplification and two with complex abnormalities. Two patients had *de novo* acute myeloid leukemia (AML) one of which had 11q23/MLL-rearranged AML, another patient developed a secondary AML eight months following

completion of chemotherapy for osteogenic sarcoma and also was t(9,11),11q23 positive (12) and one had acute undifferentiated leukemia. All of these patients were in morphologic remission, three in CR1 and one AML in CR2. One patient had chronic myelogenous leukemia (CML) in chronic phase and had failed tyrosine kinase inhibitor therapy, having developed T315I kinase domain mutation. The other three patients had relapsed/refractory Hodgkin's (HD) or non-Hodgkin lymphoma (NHL). One patient with HD was in partial remission following an autologous PBSC transplant. One patient with refractory diffuse large B-cell lymphoma (DLBCL) had never achieved complete remission having failed multiple lines of therapy including CAR-T cells and was in partial remission at the time of transplant as was a patient with anaplastic large cell lymphoma.

Ten of the B-ALL (77%) patients were conditioned with fractionated TBI followed by fludarabine (TBI-FLU) while the other three (one infant with B-ALL, one who had a prior MUD BMT with TBI, and one with poor performance status) received BU-FLU-MEL. All other patients with non-lymphoid leukemias and those with lymphomas received BU-FLU-MEL.

Engraftment and Chimerism

Mothers were donors in 38%, fathers in 29% and siblings in 33% of transplants (**Table 2**). The median age of the donors was 34.7 years (range 16.3–47.7). The median number of $CD34^+$ cells infused was 4.05×10^6/kg (range 1.5–7.5). There was only one patient with B-ALL conditioned with BU-FLU-MEL and given 4.8×10^6/kg $CD34^+$ cells that developed primary graft failure but engrafted 11 days after a second conditioned PBSC transplant from the same donor. ANC of 0.5 x 10^9/L was achieved at a median time of 16 days post-BMT (**Figure 1A**) while platelet engraftment occurred at a median of 30 days (**Figure 1B**). We found no correlation between the number of $CD34^+ \times 10^6$/kg infused and time to neutrophil ($p = 0.86$, $R^2 = 0.002$) or platelet engraftment ($p = 0.39$, and $R^2 = 0.04$). Mean absolute lymphocyte counts (ALC) doubled over time from 1.2×10^9/L at 3 months to 2.4×10^9/L at 1-year post haplo-BMT (**Figure 1C**). All patients, including the one receiving a second haplo-HCT, had complete donor chimerism on their day +28 bone marrows and as did all those that were re-assessed by peripheral blood chimerisms on days +100, +180, and +365.

Graft-vs.-Host Disease

The cumulative incidence of grades II to IV and III to IV acute GvHD (aGvHD) was 30.3 and 15.2% (**Figure 2A**). The three patients with grade III aGvHD all had stage III lower GI GvHD and responded to steroids. The cumulative incidence of chronic GvHD (cGvHD) and extensive cGvHD was 18.1 and 11.8%, respectively (**Figure 2B**). Of the two patients with extensive cGvHD one had GI, skin, and joint involvement while the other had GI, skin and liver. A third patient had cGvHD limited to his oral mucosa. All patients responded to steroid therapy and are all off immunosuppression with resolution of their cGvHD symptoms.

Infections

All patients undergoing haplo-BMT have three lines, usually a double lumen broviac or double lumen PICC line and a port-a-cath. In the first 100 days post-BMT there were 14 blood cultures drawn from at least one lumen that grew Gram-positive bacteria in 12 patients (57%). All these occurred between day +0 and day +24 (**Table 3**). In 10 of the 12 patients (83%) the gram+ infections arose before neutrophil engraftment. All patients responded promptly to appropriate antibiotic therapy except for one who died from overwhelming methicillin resistant staphylococcus aureus (MRSA) sepsis which developed on day +10. Of note is that this patient had required prolonged ventilatory support for MRSA infection following reinduction chemotherapy months prior to her BMT. There were three Gram-negative line infections/bacteremias in two patients. One patient died from septic shock after developing recurrent bacteremia with multi-drug resistant Enterobacter cloacae. There were no fungal infections documented in any of our patients undergoing haplo-BMT.

CMV reactivation was not uncommon after haplo-BMT. All of our 21 patients were at risk (seropositive recipient and/or seropositive donor) with seven developing CMV viremia (33%) (**Table 4**). The median time to peak CMV viremia was day +40 post haplo-BMT (**Figure 3**) with viral loads

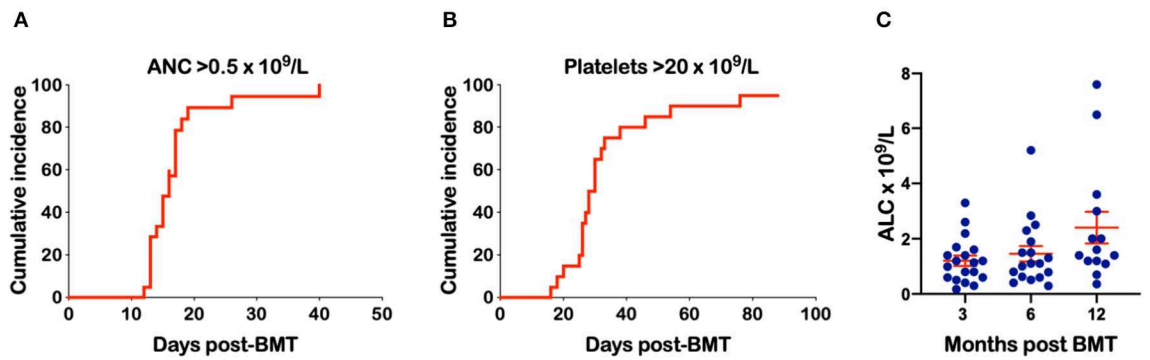

FIGURE 1 | Engraftment of neutrophils and platelets and absolute lymphocyte count (ALC) over time. **(A)** Time to an ANC of 0.5×10^9/L. **(B)** Time to a platelet count of 20×10^9/L **(C)** ALC at 3, 6, and 12 months following haplo-BMT.

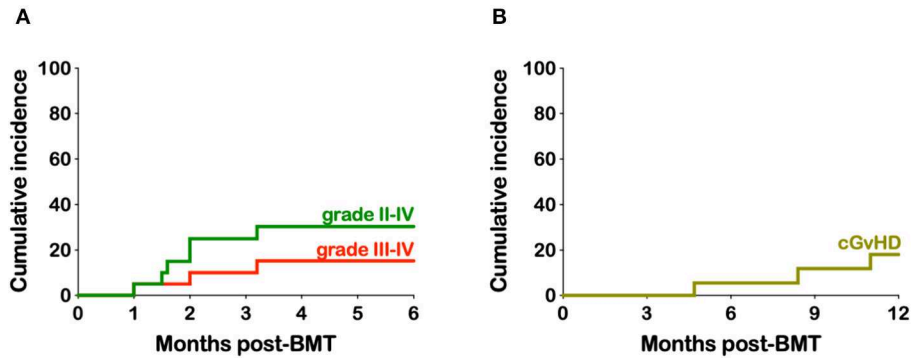

FIGURE 2 | Kaplan-Meier estimates of acute and chronic- graft-vs.-host disease. **(A)** Cumulative incidence of grades II-IV and III-IV aGvHD. **(B)** Cumulative incidence of cGvHD.

TABLE 3 | Central line infections/Bacteremias and fungal infections.

First 100 days post BMT *n* (%)*	
Gram (+)	**14 (57.1)**
Coagulase negative staphylococcus	5
Staphylococcus aureus	1
Streptococcus	3
Enterococcus	2
Other gram (+) bacteria	3
Gram (–)	**3 (9.5)**
Klebsiella	1
Enterobacter	2
Fungal	**0**

**Total of 14 gram (+) positive cultures in 12 patients, total of 3 gram (–) positive cultures in two patients. CMV, cytomegalovirus.*

TABLE 4 | CMV viremia and BK viruria.

First 100 days post BMT *n* (%)	
CMV status	
R+/D+	6/14 (42.9)
R+/D–	1/6 (16.7)
R–/D+	0/1 (0)
Total at risk	**7/21 (33.3)**
R–/D–	0/0 (0)
BK virus	
BK viruria >7.5 × 10^8	4/21 (19)

R, recipient; D, donor; CMV, cytomegalovirus.

peaking between 700 and 11,500 IU/ml. All patients responded to ganciclovir/valganciclovir, generally requiring 3–4 weeks of anti-viral therapy. BK viruria (>7.5 × 10^8 viral copies/ml) was detected in four patients (19%) with symptoms of BK hemorrhagic cystitis consisting of dysuria, frequency and microscopic or macroscopic hematuria (**Table 4**). A patient that developed end stage renal failure requiring dialysis also had significant BK viremia of 7500 viral copies/ml and was treated with cidofovir. None of our patients had clinically significant reactivation of EBV, HHV-6 or adenovirus warranting therapeutic intervention.

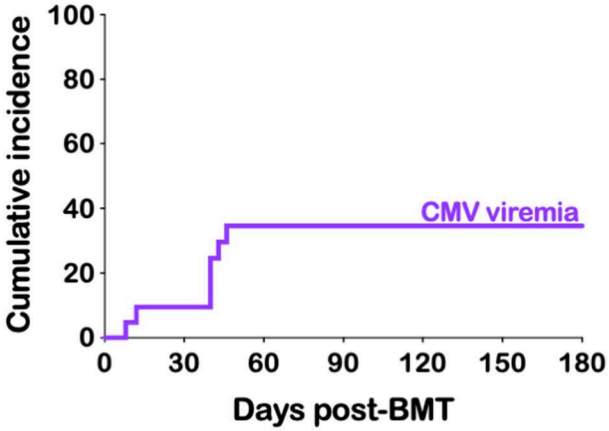

FIGURE 3 | CMV viremia. Cumulative incidence of CMV viremia.

Transplant Related Toxicities

Three patients (14.3%) required admission to the intensive care unit (ICU) within their first 100 days post-BMT (**Table 5**). Two of these patients required mechanical ventilation. One died MRSA sepsis as noted above (**Figure 4A**). The second patient who needed mechanical ventilation had developed significant ascites from veno-occlusive disease (VOD) compromising his respiratory effort and requiring CRRT for hepato-renal syndrome. This patient with infant leukemia had a short-lived response to CAR-T cell therapy and achieved a CR3 following inotuzumab ozogamicin known to predispose to VOD. As noted above, he eventually succumbed to septic shock following recurrent bacteremia with multi-drug resistant Enterobacter cloacae and was one of two patients contributing to a non-relapse mortality (NRM) of 9.5%. The third patient admitted to ICU did so for CRRT after developing renal failure from BK nephritis (as noted above) and cidofovir treatment and after prolonged therapy with liposomal amphotericin B prior to his haplo-BMT. He was also our sole patient that had failed engraftment and received a second PBSC transplant. He remains on renal dialysis.

Survival

Only three patients have relapsed, all which were heavily treated relapsed/refractory young adults (24, 23, and 24 years). One with HD, had failed autologous PBSC transplantation and was only in partial remission prior to haplo-BMT, another had refractory diffuse large B cell lymphoma (DLBCL) never having achieved a CR despite multiple lines of therapy, including CAR-T cells, and the third patient had B-ALL since she was 12 years of age with complex cytogenetics and had at least six prior relapses including one after an unrelated BMT. The cumulative incidence of relapse is 17.6% (**Figure 4B**). With a median follow-up of 25.1 months (range 4.6–52.9) the overall survival (OS) is 84%, with a progression-free survival (PFS) at 74.3% (**Figure 4C**). Taking into consideration both relapse and grade III-IV acute or chronic GvHD requiring treatment, the GvHD-free relapse-free survival (GRFS) at 2 years stands at 50.1% while the cGvHD-free, relapse-free survival (CRFS) at 58.5% (**Figure 4C**).

DISCUSSION

The preferred donor for allogeneic HCT is an HLA-matched sibling. However, <30% of patients will have a matched sibling donor (MSD), a probability that continues to decline in developed countries due to decreasing birth rates. Notably, the likelihood of having an MSD is estimated to be only 22% for the U.S. pediatric population (0–19 years) and is even lower in younger patients (1–5 years) at 17% (18). Traditionally, seeking a matched unrelated donor (MUD) is considered the second-best alternative after an MSD. While national and international hematopoietic cell registries have diversified and expanded in an attempt to increase access to unrelated donors, finding a MUD has continued to be a challenge for minority populations. Younger pediatric patients who do not have an MSD may have the option of receiving umbilical cord blood transplant (UCB) in place of a MUD. As UCB units are cryopreserved, they are readily available. The low numbers of T cells in UCB allows for mismatched units to be utilized, thereby expanding the donor pool for younger pediatric patients. However, disadvantages of UCB include low numbers of hematopoietic stem cells, which are associated with slow engraftment, and the high cost of cord blood unit acquisition. The current trend in the U.S. and especially Europe now favors the use of haplo-HCT over UCB transplants, particularly for malignant diseases (19, 20).

The benefits of haploidentical over unrelated donor (URD) HCT are numerous, with arguably the most notable being that haplo-HCT extends donor availability to nearly all patients. With more than half of our patients being ethnic and/or racial minorities (**Table 2**), our program relies heavily on haploidentical familial donors as an unrelated donor is secured in <40% of our patients (21). In fact, since the initiation of our pediatric haplo-BMT program in October 2015 we have performed 3-fold more haplo-BMTs than URD HCTs for hematologic malignancies. Haplo-BMT offers additional advantages by circumventing the delays and costs associated with unrelated donor searches and hematopoietic stem cell procurement. Haplo-HCT, therefore, can expedite transplantation in time-sensitive circumstances such as pediatric acute leukemias potentially preventing relapses. Moreover, haploidentical familial donors, especially parents, which were donors in two-thirds of our patients, are eager to donate and readily available not only for the initial harvest but also potential additional collections of bone marrow, PBSCs or donor leukocyte infusions (DLI), if needed.

Early attempts at T-cell depleted haplo-HCT in the 1980s proved to be challenging for various reasons, including a high incidence of graft rejection and delayed immune reconstitution

TABLE 5 | Transplant related toxicity.

First 100 days post BMT *n* (%)	
Admission to ICU	3 (14.3)
Mechanical ventilation	2 (9.5)
CRRT/dialysis	2 (9.5)
VOD/SOS	1 (4.8)
TRM	2 (9.5)

BMT, bone marrow transplant; ICU, intensive care unit; CRRT, continuous renal replacement therapy; VOD/SOS, nonocclusive disease/sinusoidal obstruction syndrome; TRM, transplant related mortality.

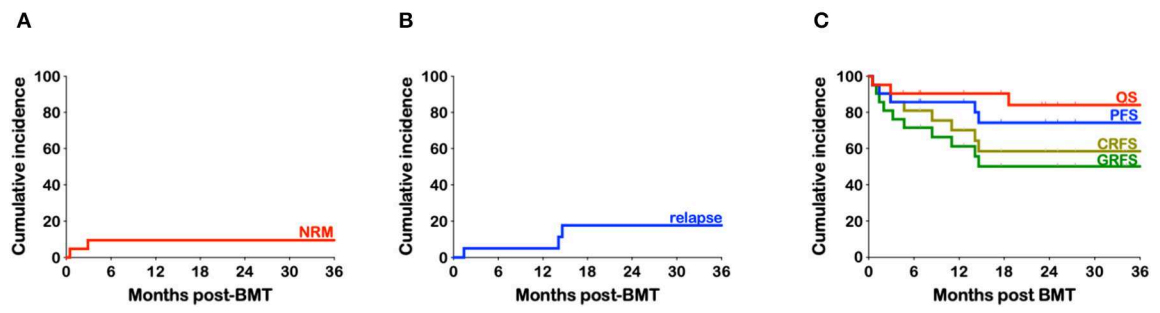

FIGURE 4 | Kaplan-Meier estimates of non-relapse mortality, relapse, and survival. **(A)** Cumulative incidence of non-relapse mortality (NRM) **(B)** Cumulative incidence of relapse. **(C)** Cumulative incidence of overall survival (OS), progression-free survival (PFS), chronic graft-vs.-host-free relapse-free survival (CRFS) and grade III-IV aGvHD-free, cGvHD-free, relapse-free survival (GRFS).

leading to infections and relapse (22, 23). After decades of research a more refined graft engineering approach to T-cell depletion used primarily in Europe consists of haplo-HCT with αβ T and B cell depleted grafts. This transplant methodology allows for the transfer of $CD34^+$ stem cells, without GvHD inducing αβ T cells, but with inclusion of and NK and γδ T-cells, both of which are capable of eliciting anti-leukemic and anti-pathogenic effects. A group from Germany reported their results with this approach in pediatric patients with mostly advanced hematologic malignancies (24). A team from Italy more recently added their experience in acute leukemia pediatric patients in complete remission (25). Patients received MAC which was TBI based in the majority of cases. Primary graft failure was low, there was an absence of grade III-IV aGvHD, with only 5% cGvHD. With a median follow-up of 46 months OS, PFS and relapse were 72, 71, and 24% respectively. Comparison of their haplo-HCT outcomes was performed to their acute leukemia patients in CR that received MSD or MUD HCT during the same time period, with all three populations having comparable disease characteristics. A lower incidence of grade III-IV aGvHD and cGvHD was observed in haplo-HCT patients with no significant difference in PFS amongst the three transplant groups.

Fifty-seven years ago, it was found that a single dose of CY, if given between the first and fourth day following implantation of a skin graft from a haploidentical donor, it was able to prolong the survival of the allograft (26). In the setting of T-cell replete hematopoietic grafts, GvHD prevention becomes of utmost importance, given that the graft contains all of the immune cells necessary to attack the immunocompromised host. Therefore, pre- and especially post-transplant immunosuppression are essential. The use of PT-CY originated at Johns Hopkins University in experimental murine models of HCT and was successfully translated clinically (27). PT-CY effectively targets rapidly dividing alloreactive donor T-cells responsible for GvHD while not affecting quiescent hematopoietic stem cells which express high levels of aldehyde dehydrogenase (28, 29). Moreover, this approach is simple as it circumvents the need to *ex-vivo* manipulate stem cell grafts and thus can be applied by almost any center performing allogeneic HCT.

There have been no randomized trials comparing haploidentical to MUD HCT. Such trials are difficult to conduct given the diverse disease conditions, conditioning regimens, donor characteristics, stem cell sources and GvHD prophylaxis utilized. However, countless adult haplo-HCT trials for hematologic malignancies have been conducted generally reporting comparable outcomes to concurrently reported MUD HCT (2, 3, 5, 30). Many of these contemporaneous studies have indicated that haplo-HCT may be associated with less acute and chronic GvHD with no differences in NRM, relapse and OS compared to MUD-HCT. Similarly, there have been no randomized studies directly comparing mismatched unrelated donor (MMUD) and haplo-HCT, but most reports indicate that outcomes with MMUD-HCT are inferior to haplo-HCT (31–36). This has led many centers, including ours, to favor the selection of a haploidentical donor over a MMUD. There has been however, a Phase III, randomized trial of RIC comparing double unrelated umbilical cord blood (dUCB) to haplo-BMT (BMT-CTN 1101) the results of which have not been published yet.

Despite the numerous adult reports there have only being a limited number of pediatric studies utilizing PT-CY in patients with hematologic malignancies. As shown in **Table 1**, the two largest studies used primarily or exclusively RIC (6, 8). While it is difficult to compare outcomes from these reports given the generally advanced stages of disease including >CR2 and, in many cases, refractory disease, PFS trended lower in RIC patients compared to MAC underscoring the consideration of more intensive regimens for better disease control. Two Asian studies have reported on the use of chemotherapy based MAC followed by T-cell replete PBSC transplant and PT-CY (7, 10). There were no graft failures, severe acute and chronic GvHD were low and improved PFS correlated with CR status. A recent conference abstract from nine US and Canadian institutions using myeloablative BU-CY or TBI-CY, surprisingly reported high graft failure and relapse rates at 1-year despite their patients being in CR at time of BMT (**Table 1**) (11).

In our updated report of 21 pediatric and young adult patients we have continued to observe excellent results with our MAC regimens. For patients with B-ALL we primarily use a TBI-based regimen previously described in adult patients by the Genoa group (2). For non-ALL patients we have been applying a chemotherapy-based MAC regimen comprised of BU-FLU-MEL. Coincidently, our BU-FLU-MEL regimen resembles that reported by Jaiswal et al. with regards to busulfan, but ours has 4 days of FLU rather than five which is infused following BU rather than concurrently, and includes a lower dose of MEL (100 mg/m^2 instead of 140) (7, 9, 12). While the majority of our patients received PT-CY, six patients were part of our phase I study which is progressively substituting PT-BEN for PT-CY. This trial is based on our murine investigations demonstrating that PT-BEN has advantages in preserving GvL effects over PT-CY (37). While all six patients received PT-CY on day +3, three patients had partial substitution and three complete substitution of PT-CY with PT-BEN on day +4. A report of our preliminary trial findings is in press (38).

Our outcomes remain excellent, with donor engraftment occurring in >95% of patients. We observed a high incidence of mostly Gram positive bacterial infections but no documented fungal infections. The incidence of CMV viremias and BK virurias was similar to other haplo-HCT reports (1, 4, 39–44). The cumulative incidence of grades III-IV aGvHD and cGvHD were low at 15.2 and 18.1%, respectively, with all patients responding to steroid therapy. With a median follow-up of 25.1 months the OS and PFS at 2 years is 84 and 74.3% which compares favorably to the published pediatric haplo-HCT studies (6–8, 10, 11, 24, 25). As noted above the only three patients relapsing after haplo-BMT all had advanced disease with two not in remission at time of BMT, two had failed previous transplants and one had not responded to CAR-T cell therapy. CRFS, which is a composite end point of survival without cGvHD requiring systemic therapy or relapse and GRFS, the endpoint of grade III-IV aGVHD or systemic therapy–requiring cGVHD or relapse are 58.5 and 50.1%, respectively at 2-years.

Previous, pediatric T-cell replete pediatric haplo-HCTs have not reported on this composite end point. However, Locatelli's group compared outcomes in a younger pediatric population (<18 years) with acute leukemia in remission receiving haplo-HCT with αβ T and B cell depleted grafts, MUD or MMUD and reported 5-year PFS of 62, 65, and 55% and CRFS of 58, 67, and 34% (45) which parallels our 2-year haplo-BMT PFS of 74.3%, and CRFS of 58.5%. Moreover, our GRFS and CRFS with haplo-BMT appear superior when compared to a recent CIBMTR analysis of over 1,600 pediatric acute leukemia patients receiving either a UCB or a MMUD transplanted in CR that found GRFS of 33 and 22% and CRFS of 38 and 27%, respectively (46). We realize that our numbers are small, but to provide a contemporaneous comparison between haploidentical and MUD transplantation, we analyzed the outcomes of our patients undergoing MUD transplantation for hematologic malignancies performed on our unit during the same period. The 2-year OS, PFS, CRFS and GRFS of our patients receiving MUD transplantation is 71.5, 71.5, 42.9, and 42.9% compared to 84, 74.3, 50.1, 58.5% in those receiving haplo-BM.

In summary, the use of haplo-HCT has advanced faster in adult HCT programs compared to pediatric and in Europe and Asia compared to North America. Various options exist with respect to the choice of conditioning regimen, graft manipulation and GvHD prophylaxis. The introduction of PT-CY has transformed haplo-HCT into a practical procedure easily applicable in every transplant center. Our experience continues to support the application of MAC T-cell replete haplo-BMT as a safe and effective alternative to MUD HCT.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Subjects Protection Program The University of Arizona. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EK reviewed and analyzed the data and wrote the manuscript. He also designed and is PI of the clinical trial. LS collected and reviewed data and edited the manuscript. SR, NR, and BS reviewed the data and edited the manuscript. All authors contributed in the treatment of the patients.

ACKNOWLEDGMENTS

The authors wish to thank the inpatient and outpatient nursing and other staff on the pediatric HCTT unit at Banner University Medical Center in Tucson for their outstanding care of our patients. This work was supported in part by pilot research funding, from the University of Arizona Cancer Center Support Grant P30 CA023074, the Leukemia and Lymphoma Society Translational Research Program, Hyundai Hope on Wheels, Tee up for Tots, Angel Charity for Children and PANDA.

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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.

frontiers in Pediatrics

REVIEW
published: 21 August 2020
doi: 10.3389/fped.2020.00454

Immune Monitoring After Allogeneic Hematopoietic Cell Transplantation: Toward Practical Guidelines and Standardization

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Hematopoietic cell transplantation (HCT) is often a last resort, but potentially curative treatment option for children suffering from hematological malignancies and a variety of non-malignant disorders, such as bone marrow failure, inborn metabolic disease or immune deficiencies. Although efficacy and safety of the HCT procedure has increased significantly over the last decades, the majority of the patients still suffer from severe acute toxicity, viral reactivation, acute or chronic graft-versus-host disease (GvHD) and/or, in case of malignant disease, relapses. Factors influencing HCT outcomes are numerous and versatile. For example, there is variation in the selected graft sources, type of infused cell subsets, cell doses, and the protocols used for conditioning, as well as immune suppression and treatment of adverse events. Moreover, recent pharmacokinetic studies show that medications used in the conditioning regimen (e.g., busulphan, fludarabine, anti-thymocyte globulin) should be dosed patient-specific to achieve optimal exposure in every individual patient. Due to this multitude of variables and site-specific policies/preferences, harmonization between HCT centers is still difficult to achieve. Literature shows that adequate immune recovery post-HCT limits both relapse and non-relapse mortality (death due to viral reactivations and GvHD). Monitoring immune parameters post-HCT may facilitate a timely prediction of outcome. The use of standardized assays to measure immune parameters would facilitate a fast comparison between different strategies tested in different centers or between different clinical trials. We here discuss immune cell markers that may contribute to clinical decision making and may be worth to standardize in multicenter collaborations for future trials.

Keywords: immune monitoring, immune reconstitution, hematopoietic (Stem) cell transplantation (HCT), cellular therapies, harmonization

OPEN ACCESS

Edited by:
Emmanuel Katsanis, University of Arizona, United States

Reviewed by:
Leo D. Wang, City of Hope National Medical Center, United States
Geoff D. E. Cuvelier, CancerCare Manitoba, Canada

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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: *05 March 2020*
Accepted: *30 June 2020*
Published: *21 August 2020*

Citation:
Boelens JJ, Hosszu KK and Nierkens S (2020) Immune Monitoring After Allogeneic Hematopoietic Cell Transplantation: Toward Practical Guidelines and Standardization. Front. Pediatr. 8:454. doi: 10.3389/fped.2020.00454

INTRODUCTION

The probability of long-term survival after hematopoietic cell transplantation (HCT) has steadily improved in the last decade with advances in treatments (1, 2). However, long term survival after HCT is still hampered by adverse events, such as graft-versus-host disease (GvHD), infections and relapse of the underlying disease (3–6). Delayed immune reconstitution plays a central role in most

of these events (7, 8), suggesting that strategies that increase immune recovery are of great interest to increase survival chances post-HCT (9).

Among the graft-related variables that can influence immune reconstitution are the graft source and composition, degree of HLA-match and the cell dose (10–12). Transplantation with matched related bone marrow (BM) or peripheral blood (PB) (stem) cells is considered the standard for allo-HCT, but also matched unrelated donors (MUD) (mis)matched unrelated cord blood (CB), a haplo-identical or a mismatched family donor can be considered as alternative graft sources. The HCT-source and its manipulation, such as CD34+ positive selection (13) or other *ex vivo* T-cell depletion (14) and *in vivo* T cell depletion with serotherapy [e.g., anti-thymocyte globulin (ATG) or Alemtuzumab] may be important factors defining the probability of T-cell immune reconstitution (IR). Moreover, grafts are selected based on HLA-matching criteria, where the degree of HLA-match is, in general, stricter for BM/PB than for CB grafts, and may depend on the indication. Also, the use of grafts from younger donors is preferred by centers because it associates with better survival chances, due to lower non-relapse mortality (15–17). The graft's cell dose is considered, particularly in CB transplants, because units with low numbers of CD34+ cells were associated with inferior outcomes (18). A retrospective European Society for Blood and Marrow Transplantation (EBMT) analysis by Czerw et al. showed that T cell numbers in the HCT graft are highly variable (range; 50–885 × 10e6/kg) and positively correlate with an increased incidence of grade III-IV acute (a) GvHD (19). Others found that higher numbers of γδT cells (20) in the graft associate with favorable immune reconstitution and superior clinical outcome (21). However, whether the combination of graft source, match grade, cell dose and graft composition will result in optimal immune recovery post-HCT is impossible to deduce from these data. To get more insight into the contribution of different parameters to outcome, we need to identify the best combination of markers that associate with clinical outcome. A reliable predictor for outcomes, across the variety of transplant platforms (e.g., T replete and T deplete), seems to be CD4+ T cell counts above 50/μL within 100 days after transplant (22–24).

A decisive factor that may even overrule the graft-related effects, is the type and timing of the conditioning regimen used. Pharmaco-kinetic and -dynamic studies show strong correlations between post-transplant recovery of immune cells and the timing and dose of conditioning agents [e.g., fludarabine (Flu), busulfan (Bu), and ATG] (25, 26). These data point toward personalized dosing strategies to achieve an optimal exposure in every individual patient (27–29).

Due to the multitude of variables described above and the site-specific policies/preferences, a precise understanding of how these variables can be influenced to optimize outcomes of HCT is difficult to achieve. While donor-selection in cell transplantation has been increasingly standardized over the last decades (30, 31), harmonization of the conditioning regimen is still lacking and the highly variable pharmacokinetics of drugs used in the conditioning regimen and their dramatic effects on outcome are still not widely considered. In the first part of this review, we will provide an overview of the association between different conditioning regimens, outcomes and IR post-HCT. Given the strong relationship between immune recovery and outcome (32, 33), monitoring immune parameters post-HCT may serve as a relatively fast predictor for outcomes (including survival, relapse, and non-relapse mortality) and accelerate comparisons between different strategies in different centers and between different clinical trials. The use of standardized assays across laboratories will be imperative for this purpose. In the second part, we discuss a rationale for selection of a minimal parameter set to monitor immune recovery that could be considered for standardization.

CONDITIONING REGIMENS, IMMUNE RECONSTITUTION, AND OUTCOMES: TOWARD INDIVIDUALIZED DOSING

We recently showed that the pharmacokinetics of rabbit ATG (rATG) is highly dependent on absolute lymphocyte count (ALC) as a representation for the receptor-load (26). In adults, the ALC before rATG-dosing was the only predictor for rATG clearance, while in pediatric patients a weight of <40 kg also influenced clearance. In **Table 1** the suggested rATG (Thymoglobulin) exposure targets for pediatric and adult patients are listed. It is important to realize that the ligands recognized by rATG and rATLG (anti-T-lymphocyte globulin; Neovii Biotech, Rapperswil, Switzerland) are not identical, so the target exposures for rATG do not apply for rATLG (26, 35, 36). A nomogram for ATLG should still be made, as its effects are likely similar to those of rATG. Soiffer et al. showed that ATLG was associated with inferior chronic-GvHD-free, leukemia-free survival in a *post-hoc* analysis of a randomized controlled trial with three different regimens (25). This study nicely illustrates that it is essential to understand the effects of all agents on immune reconstitution and the interrelationships between the agents that may significantly alter the outcomes. In this trial, patients receiving total body irradiation (TBI) + cyclophosphamide (Cy) had lower "absolute lymphocyte counts" on the day of rATLG dosing compared to patients receiving a chemo (Bu-Cy or Bu-Flu)-based regimen or patients that received Cy first, before receiving TBI (usually because of practical issues). This resulted in a slower clearance of rATLG, delayed immune reconstitution and subsequently higher transplantation related mortality. Other forms of serotherapy, such as Alemtuzumab, as well as equine-ATG (less frequently used), can have highly variable PK and a significant influence on IR and survival chances.

A prospective validation of a new dosing nomogram for rATG in pediatric patients (PARACHUTE trial) proved to be effective to expedite immune reconstitution (primary endpoint) and survival (secondary endpoint) (34). This study confirms our previous results showing that a timely recovery of CD4 T cells (>50 CD4+ T cells/μL at two consecutive measurements within 100 days post-HCT) is the strongest predictor of leukemia-free survival, non-relapse mortality, as well as overall survival (23, 35, 37, 38). We previously showed that adequate CD4 recovery is associated with a lower chance for relapse in acute myeloid leukemia (AML) (22) and with higher survival

TABLE 1 | Suggested novel ATG (thymoglobulin) dosing nomograms based on PKPD modeling for (non-)myelo-ablative settings in pediatrics and adults [For pediatrics based in PARACHUTE clinical trial; (34)].

Setting	Dosing on	Target AUC after HCT (AU*d/mL) and donor source	Starting day	References
Pediatrics; myelo-ablative	Weight ALC Cell source	<20 for cord blood <50 for bone marrow (T-Replete)	−9	(26, 34)
Adults: non-myelo-ablative	ALC	60–90 for peripherally mobilized stem cells (T-Replete)	−9	(35)

Absolute lymphocyte count (ALC). Area under the curve (AUC).

chances for patients with adenoviral reactivation (24). Recently this association was also found to be a significant predictor in a predominantly T cell depleted cohort of pediatric and adult patients (23, 26, 39). Moreover, a recent multicenter trial also showed the association of CD4 T cell recovery with survival chances in patients developing GvHD (23). Interestingly, the CD4 IR is preceded by increasing cell numbers of the myeloid lineage and could even be retraced to the proliferative potential of myeloid cells in BM and CB grafts (38). This makes CD4+ T >50/μL a reliable biomarker for clinical decision making, e.g., patients who fail to reconstitute CD4 T cells may, for instance, be eligible for prophylactic therapy or for pre-emptive treatment upon the first positive virus measurement. Alternatively, patients with adequate CD4 T cell numbers should be monitored carefully, but would not receive treatment, as there is a reasonable chance that the reconstituting immune system will clear the pathogen by itself. Of course, these observations should be further studied in the context of a clinical trial, and follow-up studies and validations are needed to confirm whether predictions can be further improved.

In a recent retrospective cohort analysis (including > 650 pediatric and young adult patients), the cumulative exposure to Bu significantly influenced clinical outcomes (40). The optimal Bu-exposure (80–100 mg*h/L) was associated with the highest survival chances and lowest toxicity and was independent of the indication, chemo-combination (Bu + Flu, Cy + Bu, or Bu + Cy and Melphalan), age and donor source. The method of Bu-exposure estimation may differ between centers and could be responsible for a large variation in the reported estimated exposures (40). This emphasizes the value of standardizing the entire process of sample logistics up to data reporting, in order to be able to compare different treatment strategies.

More recently, Flu-exposure (given prior to transplant) was also found to influence survival by negatively affecting IR in patients who were over-exposed (41, 42). This is an interesting observation, given that the exposure in blood was only before transplant, and it is important to note that the PK in the organs in experimental models can be different, as discussed by the authors. These studies show that the pharmacokinetic variation between individuals is high and that these differences in exposure can have significant impact on outcomes, including survival. It is still daily practice that a variety of conditioning regimens are used, which can complicate comparisons of HCT outcomes across different centers and even within trials as illustrated by Soiffer et al. (25). Also, post-transplant Cy is a frequently used (and a simple and cheap) transplant platform to cross the HLA barrier in allogeneic HCT and induce a state of immunologic tolerance (43, 44). While its simplicity makes it an attractive approach, there is not much data available about immune reconstitution in this transplant setting, which would be of interest to study in more detail to identify predictors for failure or success.

Together, these data suggest that the recovery of (CD4+) T cells may be an easy-to-obtain biological marker and a potential biomarker/predictor to monitor treatment success in population studies. Additional work is needed to identify further biomarkers with clinical predictive value. A biological predictor for clinical outcomes, even before clinical signs will be visible, may be valuable to anticipate graft-versus host responses. It may also facilitate stratification of patient subgroups for treatment interventions after HCT, e.g., prophylactic antiviral therapy, or the use of checkpoint inhibition, to ensure adequate treatment for responsive patients, while predicting non-responders or patients with a high probability of developing life-threatening side effects who may be directly eligible for other treatments (45). As different institutions have their own policies on sample collection and monitoring, it is of crucial importance to set up multicenter validation trials to establish standardized protocols for sampling, handling logistics, measurements and data processing, to reduce result variability and allow for more accurate data comparison.

MONITORING IMMUNE CELL RECONSTITUTION

Multicolor flow cytometry is the technology by default in many accredited transplant laboratories for immune cell reconstitution monitoring. It enables the analysis of a large number of parameters simultaneously, in a short time and for a reasonable cost. Many centers monitor the recovery of the common leukocyte subsets based on CD45 (leukocytes), CD3, CD4, CD8 (T cells), CD19 (B cells), αβTCR, γδTCR and CD16/CD56 (NK cells), and may analyze the maturation of T cells from naïve to effector/memory cells to discriminate lymphopenic proliferation and the recovery of thymic output. There are, however, no stringent guidelines for clinical decisions based on quantification of cell subsets post-HCT.

Many effector and regulatory cell types have been linked to the anti-cancer immune responses (46). Different subsets of T cells play crucial roles in controlling disease progression, and effectors like CD8+ and CD4+ T cells have been associated with direct anti-tumor activity and a favorable prognosis, particularly when those T cells express memory and activation markers (47–50). NK cells were shown to mediate tumor regression in AML patients, eliminate graft rejection and protect patients against GvHD (51, 52). The presence of mature antigen-presenting dendritic cells (DCs) has been correlated with improved survival (53–56). T regulatory (Treg) cells down-modulate T-cell activation

through the production of immunosuppressive cytokines (TGFβ, IL-10), as well as through surface receptors (CTLA4), and can drastically impact both the anti-tumor immune response as well as the control of GvHD (57–59). Myeloid suppressive cells (60–62) and regulatory B cells (63–65) also play a potential role in attenuating the immune response and controlling effector cells and signaling mechanisms. Moreover, the presence of certain biomarkers may be predictive for the functioning of effector mechanisms; e.g., sorafenib-related IL-15 production causes an increase in CD8+CD107a+IFN-γ+ T cells with features of longevity and eliminates leukemia in secondary recipients, indicating that sorafenib after HCT might be more effective through induction of IL-15-mediated metabolic reprogramming of leukemia-reactive T cells (66). Others showed that the presence of peripheral blood DCs in high frequencies relates to clinical response to high-dose IL-2 (67). This data suggests that DCs may be instrumental for endogenous- and immunotherapy-induced immunity against cancer.

In summary, data from multiple studies suggest that monitoring immune cell subsets may hold predictive value for outcome, development of adverse effects, or may be used for patient stratification for certain treatment modalities. Multicenter validation studies are required using standardized protocols for sampling, handling logistics, measurements and data processing. Diagnostic laboratories in transplant centers have standardized protocols to diagnose and measure minimal residual disease burden in patients with hematological malignancies. Most centers provide validated assays for CD34 cells and immune monitoring of leukocyte subsets post-HCT in a standardized way. It thus seems a small step to collect and collectively report these data in international databases (e.g., EBMT), and to relate biological markers, such as CD4 T cell reconstitution, to outcome parameters and to the treatment procedures.

CYTOKINE PROFILING

Profiling soluble markers in blood may be of value to assess the status of the immune system before, during and after immunotherapeutic interventions for infections or GvHD, or to provide additional insight into the therapeutic mechanisms of action. Blood markers should be regarded as surrogate markers and may not always reflect local responses in affected tissues, such as skin or gut in GvHD. As this topic has been reviewed recently (9, 68), we here just describe two scores that may be close to multicenter validation studies and emphasize the standardization of techniques. Previous studies characterized biomarkers for aGvHD-related mortality post-HCT; a biomarker score using ST2, TNFR1, and Reg3a to guide risk-adapted therapy at aGvHD onset irrespective of the conditioning regimen intensity (69, 70). Another formula, including the markers lactate dehydrogenase, creatinine and thrombocytes, termed the Endothelial Activation and Stress Index (EASIX), was also reported useful for prognosis of survival chances in patients suffering from aGvHD after HCT with reduced intensity conditioning (71). These scores should still be prospectively validated in the different HCT settings, preferentially in coordinated multicenter trials before they can be implemented in patient care.

The technology to acquire the parameters for EASIX may be more standardized in and between different centers than those for analyzing proteins, such as ST-2. Many technologies are available e.g., antibody-based ELISA's or multiplex platforms, liquid chromatography—mass spectrometry (LC-MS), electro-chemiluminescence (72), but concentrations may differ depending on the methods used. The latter is problematic for implementation of mathematical scores. Standardization is needed for the procedures of sample processing and isolation, selection of tubes and duration of storage/cryopreservation. Protein levels can differ considerably between serum and plasma samples (due to release of platelet-associated molecules into serum), even between the type of anticoagulant used, and are prone to changes due to variations in time (from sampling to processing and time of storage) and/or temperature (73).

IN SUMMARY

Survival after HCT depends on many factors, including a balanced and timely immune reconstitution in the first 3 months post-HCT. Delayed immune reconstitution is associated with a lack of disease control, viral infections, and seems to increase the chances of immune dysregulation, resulting

TABLE 2 | Suggested harmonized panels.

	Cell type	Standard panels	Advanced monitoring suggestions
Cell Phenotyping of Graft Composition and IR	αβT γδT Treg B NK/NKT DC/mono	αβTCR, CD45RO/RA, CD3, CD4, CD8, CD27 γδTCR, CD45RO/RA, CD3, CD27 CD45, CD3, CD4, CD25, CD127, FoxP3 CD45, CD19, CD38, CD27, IgM/G/D, CD21 CD45, CD3, CD56, CD16 CD11c, HLA-DR, CD14, CD16, CD1c, CD141, CD303	• Intracellular cytokines after PMA/ionomycin stimulation • Specific TCR by multimer approach. • TCRγδ
Secretome		-	MultiPlex panel (e.g., IL-7, IL-15, ST2, TNF-a, IL-6, HGF, IL-2R, IL-8, GM-CSF, etc.)
Cell function		-	• NK cell lysis • T cell proliferation after stimulation with antigens and mitogens • B cell maturation

General parameters that could be included in harmonized immune monitoring protocols across most studies/centers, and advanced parameters that may be of great value in specific studies that can only be performed in specialized immunology labs or analyzed in a central laboratory.

in higher non-relapse mortality rates. Currently, only a few HCT programs consider therapeutic drug monitoring of agents used in the conditioning (a main factor influencing immune recovery) or apply frequent systematic monitoring of immune cell reconstitution to predict unwanted events.

Based on current knowledge, recommendations can be made with regards to personalized drug dosing and application of a standardized minimal panel for immune monitoring to generate fast responsive surrogate markers for efficacy or the development of unwanted effects. It may be valuable to register the details of the treatment modality, i.e., drug doses and if possible, drug exposure, graft composition, and standardized immune reconstitution parameters to the Center for International Blood and Marrow Transplant Research (CIBMTR) and EBMT databases. This could also initiate the establishment of consensus guidelines in clinical trials on monitoring and reporting a minimal set of parameters, which can be extended to add-on trial-specific parameters (**Table 2**). Efforts to harmonize HCT protocols/platforms aiming to create the optimal "immune milieu" to exert the most optimal effector mechanism may have more impact on survival chances than many novel maintenance therapies (35, 74). Moreover, it will provide a more straightforward comparison between different treatment modalities, due to better prediction of the immune milieu.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: JB has received consulting fees from Race Oncology, Takeda, Omeros, Advances Clinical, Bluebird Bio, Bluerock, Sanofi (all not related to research described in this review).

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.

frontiers in Pediatrics

REVIEW
published: 02 October 2020
doi: 10.3389/fped.2020.570282

Umbilical Cord Blood Transplants: Current Status and Evolving Therapies

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Hematopoietic cell transplants using stem cells from umbilical cord blood are used worldwide for the treatment of malignant and non-malignant disorders. Transplant procedures from this stem cell source have shown promising outcomes in successfully treating various hematologic, immunologic, malignant, and inherited metabolic disorders. Rapid availability of these stem cells is an important advantage over other unrelated donor transplants, especially in situations where waiting can adversely affect the prognosis. The umbilical cord blood is rich in CD34+ stem cells, though with a limited cell dose and usually takes longer to engraft. Limitations around this have been addressed by *in vivo* and *ex vivo* expansion techniques as well as enhanced engraftment kinetics. Development of adoptive immunotherapy using other components of umbilical cord blood such as regulatory T cells, virus-specific T cells, and natural killer cells has further transformed the field and enhanced the utility of umbilical cord blood unit.

Keywords: umbical cord stem cells (UCSC), cord expansion, immune effector function, cord selection, alternative use of UCB

OPEN ACCESS

Edited by:
Richard J. Simpson, University of Arizona, United States

Reviewed by:
Pietro Merli, Bambino Gesù Children Hospital (IRCCS), Italy
Atsushi Manabe, Hokkaido University, Japan

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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: *07 June 2020*
Accepted: *26 August 2020*
Published: *02 October 2020*

Citation:
Gupta AO and Wagner JE (2020) Umbilical Cord Blood Transplants: Current Status and Evolving Therapies. Front. Pediatr. 8:570282. doi: 10.3389/fped.2020.570282

INTRODUCTION

Umbilical cord blood (UCB) stem cells have been successfully used for hematopoietic cell transplant (HCT) since the first report in 1989 (1). Over 40,000 UCB transplants have since been performed worldwide for a wide variety of malignant and non-malignant disorders (2, 3). The treatment outcomes of UCB transplant are comparable to those of related or unrelated bone marrow (BM) or peripheral blood (PB) used as graft source in hematologic malignancies (4, 5). In children with inherited metabolic disorders, outcomes are comparable for non-carrier-matched sibling BM and fully matched UCB (6, 7) and have been used in majority of transplants in this patient population (8). In other disorders such as primary immunodeficiency disorders, bone marrow failure syndromes, and hemoglobinopathies such as sickle cell disease and thalassemia, UCB transplant outcomes continue to improve (9–13). The establishment of cord blood banks have enabled safe storage and rapid availability of UCB stem cells for timely transplants for these disorders.

Cord blood banking was first established in 1993, and now about 5 million cord blood units are banked worldwide. About 800,000 of these are in public banks, and over 4 million UCB units are stored in private or family banks (3). The biologic properties of UCB units can be safely cryopreserved for more than 20 years under appropriate conditions, with efficient recovery of functional hematopoietic stem cell (HSC) and hematopoietic progenitor cells (HPCs) (14). The availability of these UCB banks has led to faster procurement of unrelated donor cord blood stem

cells, significantly reducing the median search time from 3 to 4 months for bone marrow and peripheral blood stem cells to as early as 2 weeks for UCB stem cells (15). This is an important attribute which plays a role in choosing the appropriate donor HSC source for disorders where timing and flexibility are particularly critical such as high-risk malignancies and rapidly progressive inherited metabolic disorders. To maintain the optimum quality of these cord blood units, various organizations such as the National Marrow Donor Program and NetCord and Foundation for the Accreditation of the Cellular Therapy have established regulatory guidelines for the collection, processing, and storage of these units (16).

HEMATOPOIETIC PROPERTIES

UCB is a rich source of primitive HSCs and progenitor cells. Though quantitatively limited, the proportion of highly proliferative HSCs is greater in UCB as compared to BM or PB allografts and has an enhanced capacity for homing and hematopoietic reconstitution (17, 18). These UCB cells also have higher capability for self-renewal, proliferation, and expansion under optimal conditions (19, 20). Higher expression of CD34+ antigen on their cell surface along with longer telomere length is an advantage. This provides a unique alternative to the invasive extraction of BM HSC and exposure to mobilizing agents prior to the collection of HSC in PB. As the collection of UCB takes nothing away from the neonate or the mother, safety is another major advantage.

Immune Properties

With limited immunological memory in the neonate and high frequency of regulatory T cells that play an important role in maternal–fetal tolerance, these attributes likely play a critical role in the tolerability of human leukocyte antigen (HLA) mismatch in the recipients of UCB. However, studies also demonstrate that neonatal immune cells produce immune responses similar to that of adults in some aspects but not in others (21). Transplacentally acquired maternal antibodies play an important role in the initial neonatal defense against micro-organisms, followed by rapid maturation of T cells. Development of the maturation of neonatal T cells is facilitated by the cytokine milieu, with interleukin (IL)-2 mediating the differentiation of these naïve T cells into Tregs. These forkhead box p3 (Foxp3) + Tregs are present in higher proportion in neonatal lymph nodes as well as in umbilical cord blood (22, 23) and play an important role in immune tolerance in a developing fetus as well as in HLA-mismatched immune milieu.

Impact of HLA Mismatch in Recipients of UCBT

The composition of cord blood from a relatively naïve immune system bestows the advantage of a lower frequency of alloreactive T cells in the graft. This results in a significantly lower incidence of graft vs. host disease (GVHD) in UCB recipients as compared to other graft sources (24). Because of these properties, less restrictive HLA matching criteria (HLA-A, HLA-B, and DRB1) are used for donor selection, thereby expanding the availability of UCB stem cells. However, previous studies have shown the impact of closely HLA-matched UCB units and cell dose on engraftment and risk of graft failure (25, 26). The role of total nucleated cell (TNC) dose and HLA matching was investigated by Barker et al. in 1,061 UCBT recipients which showed best transplant-related outcome and engraftment with 6/6 HLA-matched cord irrespective of TNC, while the effect of TNC was important in more mismatched cord recipients (27). The importance of enhanced matching, including HLA-C locus, was subsequently investigated by the combined Eurocord and Center for International Blood and Marrow Transplant Research (CIBMTR) study, analyzing the outcomes of 803 UCBT recipients in patients with hematologic malignancies (28). Significantly higher day 28 neutrophil recovery was noted in UCBT matched at HLA-A, HLA-B, HLA-C, and-DRB1 (8/8) compared to those mismatched at three or more HLA loci. More studies have since reported similar outcomes and the importance of allele-level HLA matching in UCBT recipients (29–31).

STRATEGIES TO ENHANCE THE SUCCESSFUL USE OF UCB

UCB has been widely accepted as a useful HSC source; however, delayed hematopoietic recovery and consequent increases in initial hospital costs, concerns about delayed immunological recovery, and even the challenge of knowing how to select the best UCB unit for transplantation have been important barriers. This review will summarize the state of the art.

UCB Unit Selection

The greater availability of high-quality and high-cell-content UCB units has resulted in increasingly improved engraftment and survival outcomes after UCBT. However, unit selection is often considered to be a major barrier to its use because multiple characteristics must be considered simultaneously. Several reports have previously outlined country-specific selection guidelines (32–37). This review provides a simplified step-by-step unit selection guide with additional principles for the selection of a cord blood unit (CBU) (**Table 1**). The guiding principle for CBU selection includes acceptable quality, adequate cell dose, and optimum high-resolution HLA matching. While not every transplant center experienced with UCB will use exactly the same selection criteria, the principles are uniform, with all centers recognizing the importance of finding the unit with a highly viable CD34 cell dose and HLA match at four of eight HLA antigens when possible, recognizing the importance of allele level typing at HLA-A, HLA-B, HLA-C, and DRB1.

STRATEGIES FOR EARLY HEMATOPOIETIC RECOVERY

In vivo Expansion

The use of double UCB resulting in *in vivo* expansion of stem cells and thereby improving engraftment was first shown by Barker et al. (38). Since then, other strategies for *in vivo* UCB expansion have been used, such as haplo-cord transplants where a small dose of haploidentical stem cells is used for early engraftment and

TABLE 1 | Umbilical cord blood unit search algorithm.

NMDP search algorithm	Step 1	Identify all CBUs that are HLA matched at 4/8 (considering A, B, C, and DRB1 at allele level based on haplogic) with a TNC dose $>1.5 \times 10^7$/kg filtering out those with a known CD34 dose $<1.0 \times 10^5$/kg)
	Step 2	List best to worst HLA match
	Step 3	Within each HLA match category list highest to lowest by NC dose
	Step 4	Provide information on CD34 dose, ABO types, race/ethnicity and any missing identity or history information
Center specific filters	Step 5	Center's will be able to adjust filters and how units are sorted: (a) Restrict or relax HLA match (e.g., permit 3/8 or eliminate < 5/8) (b) Relax CBU age (e.g., include older units >10 years) (c) Restrict or relax eligible CBBs (e.g., include those that are not FACT accredited, or located out of country) (d) Relax RBC replete status (e) Eliminate HLA antigens (based on recipient anti-HLA antibodies) (f) Change sort and simply list units based on highest to lowest cell dose and not group by HLA match
Additional principals		(1) RBC replete units are not recommended as these have been associated with more adverse events including life-threatening infusion reactions (2) Consider cryovolume for units expected to undergo post-thaw dilution (3) Focus on units with attached segments for confirmatory typing (if not available, consider rapid HLA screen if possible at time of unit thaw and prior to infusion) (4) Perform minimum of 8 high-resolution (HLA-A, HLA-B, HLA-C, and HLA-DRB1) for both patient and CB unit (5) While balancing CD34 cell dose and HLA matching, the greater the HLA mismatch, the higher cell dose is needed for a successful outcome

CBU, cord blood unit; CBT, Cord blood transplant; HLA, human leukocyte antigen; TNC, total nucleated cells; CBBs, cord blood banks; RBC, red blood cell.

TABLE 2 | Clinical trials for *ex-vivo* cord blood stem cell expansion.

Study	Expansion technique	Model	Number of Patients	Duration of culture	Median CD34 cell dose after expansion ($\times 10^6$)/kg	Median CD34 expansion (folds)	Neutrophil engraftment (in days, range)	Platelet engraftment (in days, range)
Delaney et al. (39)	Notch ligand delta 1	Double UCBT	10	16 days	6.1 (0.9–13.6)*	164 (41–471)	16 (7–34)	NA
de Lima et al. (40)	Copper chelation (TEPA)	Double UCBT	8	21 days	9.4 (0.39–247.7)	161 (2–620)	30 (16–46)	48 (35–105)
Stiff et al. (41)	Copper chelation (TEPA)	Double UCBT	101	21 days	1.028 (0.137–939.6)	77	21 (18.4–23.5)	54 (43.3–61.9)
Horwitz et al. (42)	Nicotinamide	Double UCBT	11	21 days	3.5 (0.9-18.3)	72 (16–86)	13 (7–26)	33 (26–49)
Horwitz et al. (43)	Nicotinamide	Single UCBT	36	21 ± 2 days	6.3 (1.4–14.9)	33	11.5 (9–14)	34 (32–42)
Wagner et al. (44)	StemRegenin 1	Double UCBT	17	21 days	17.5 (1.4–48.3)	248 (66–446)	15 (6–30)	49 (28–136)
de Lima et al. (45)	MSC co-culture	Double UCBT	31	7 days	0.95 (1.6–9.34)	30	15 (9–42)	42 (15–62)
Cohen et al. (46)	UM 171	Double UCBT/ Single UCBT	27 (4 received double UCBT)	7 days	2.87 (1.91–3.96)	28 (19–35)	18 (12.5–20)	42 (35–47)

**Reported mean.*
TEPA, tetraethylenepentamine; UCBT, Umbilical cord blood transplant; MSC, mesenchymal stem cell.

UCB expansion in the optimal cytokine milieu (47). However, with these *in vivo* expansion techniques, there is a concern of higher risk of graft-versus-host disease (GvHD) and prolonged mixed chimerism, though studies have shown otherwise (48, 49).

Double Cord Blood Transplants

The use of two umbilical cord blood units *in vivo* has shown promising outcomes and has helped obviate some of the limitations of single UCB HCTs in older children and adults. The two UCB units can be either infused unmanipulated or one unit can be infused unmanipulated and the other one selected for HSCs and HPCs. In majority of patients, one unit predominates either by rejection by the other unit or having a competitive advantage with higher CD34+ stem cells (50). Overall HCT outcomes with double UCBT (dUCT) have been comparable to matched-related and unrelated outcomes (51).

Haplo-Cord Transplants

This approach combines infusion of a smaller dose, but well-matched, of UCB unit together with mobilized PB CD34+ cells from a haploidentical donor. In this setting, early engraftment and hematopoietic recovery is achieved by usually the haploidentical donor, which then subsequently yields sustained and durable engraftment to the UCB unit. Outcomes

of this approach using both myeloablative and reduced-intensity conditioning regimens have shown reduced infectious and immunologic complications with good outcomes (49, 52). This approach can be useful for adults with limited matched unrelated donor and UCB availability where a haploidentical donor is available, allowing the use of a single UCB.

UCB HSPC Expansion

Various approaches have been used to expand HSCs and HPCs from UCB with the use of feeder stromal cells, epigenetic modifiers, and small molecules. Over the past decade, UCB expansion has been successfully achieved, demonstrating rapid recoveries of neutrophils and platelets, better rates of engraftment, and fewer days in the hospital as surrogate for reducing costs.

Ex vivo Expansion Culture

Several *ex vivo* expansion strategies used over the last few years show promising results today (**Table 2**). Irrespective of the technique, there is a robust increase in CD34+ stem cells and their progenitors, leading to much faster neutrophil recovery and myeloid engraftment after infusion as compared to historical controls (39–44). A regimen containing different cytokines or small molecules is used to exponentially enhance the stem cell population, thereby influencing the kinetics of engraftment and reducing the duration of severe neutropenia.

In early clinical trials with *ex vivo* expanded UCB, investigators often first evaluated the engraftment potential of the expansion product in the context of double UCBT, where one unit was expanded and one was infused without prior manipulation. The notch signaling pathway has an important regulatory role in hematopoietic differentiation as well as in the proliferation of HSCs and HPCs (53). Using engineered notch ligand delta 1, the expanded UCB unit resulted in rapid neutrophil recovery, but long-term engraftment was most often from the unmanipulated UCB unit (39). This approach demonstrated safety and feasibility as well as early neutrophil recovery [16 days; range (r) 7–34 days] as compared to the median time of 26 days (r, 16–48 days; $p = 0.002$) in historical controls with double UCBT without expansion (38).

Co-culture with mesenchymal stem cells (MSCs) to provide the necessary factors for HSC expansion was explored as an *ex vivo* expansion strategy (45). It used an initial co-culture with MSCs for 7 days, followed by culture with cytokines. This trial enrolled 31 adults who underwent dUCBT, one with expanded cord and another with an unmanipulated cord. About 30-fold higher CD34+ cell dose was noted in the expanded unit. This study also reported earlier neutrophil and platelet recovery as compared to the CIBMTR data for dUCBT. The expanded cord lasted for about a year, when entire donor chimerism was noted from the unmanipulated cord. This technique has been further refined by the addition of fucosylation during UCB expansion (clinical trial NCT03096782) to investigate further improvement in hematopoietic recovery.

While promising, the results of these two studies suggested that these culture methods preferentially expanded primitive progenitors at the expense of HSC, providing only a transient wave of hematopoietic recovery. However, in the absence of T cells after expansion culture, the expanded product is at an immunological disadvantage in the setting of dUCBT, where one unit actively rejects the other (54). Based on this observation, transient engraftment after expansion culture may have been due to the absence of T cells rather than the loss of HSC. Therefore, subsequent expansion trials re-cryopreserved the CD34-depleted fraction after the CD34-enriched population was placed in expansion cultures.

Copper chelation technique using tetraethylenepentamine (TEPA) was investigated, with pre-clinical evidence demonstrating the prevention of stem cell differentiation in an *in vitro* culture (55). The UCB graft is derived from a single unit, where a fraction of the UCB unit undergoes a 21-days expansion culture in the presence of TEPA, followed by infusion of expanded and unmanipulated fractions on transplant day. The phase I/II clinical trial enrolling 10 patients confirmed safety and feasibility and showed neutrophil engraftment at day 30 (r, 16–46) and platelet engraftment at day 48 (r, 35–105 days) (40). A larger international multi-center trial was then conducted and reported results from 101 patients, which showed a median nucleated cell expansion of about 400-fold, with CD34 expansion of 77-fold (41). The 100-days survival was superior to dUCBT in the contemporary period. Neutrophil and platelet engraftments were significantly earlier than the comparison group (21 vs. 28 days and 54 vs. 105 days, respectively).

More recent trials evaluated small molecules, including vitamin B derivatives, aryl hydrocarbon receptor antagonists (AHRa), and pyrimidoindole derivatives that impeded HSC differentiation in cultures containing stimulatory cytokines, like SCF, Flt-3L, and thrombopoietin, but also infused the unit's T cells, in contrast to prior trials. Nicotinamide, a vitamin B derivative, inhibits differentiation, thereby enhancing the expansion of HSC and HPCs expanded in *ex vivo* cultures with stimulatory hematopoietic cytokines. In the initial phase I trial, 11 patients were enrolled. Of the 11 patients, the median time to neutrophil and platelet recovery was 13 and 33 days, respectively, faster than the controls (25 days, $p < 0.001$ and 37 days, $p = 0.085$). However, it is most notable that sustained myeloid engraftment from the NiCord-derived unit was observed in eight patients (42). Subsequently, a phase II study was completed using NiCord as a stand-alone graft in 36 patients (median age, 44 years), with high-risk hematologic malignancies treated with myeloablative conditioning. The results were compared to those of 146 patients who received standard UCB transplantation, with data reported to the CIBMTR. In the recipients of NiCord, the cumulative incidence of neutrophil engraftment was 94% at day 42, and the median time to neutrophil recovery was 11.5 days (95% CI, 9–14) vs. 21 days (95% CI, 20–23) for patients who received standard transplant ($p < 0.001$). Similarly, the median time to platelet recovery was 34 days (95% CI, 32–42) with NiCord vs. 46 days (95% CI, 42–50) with standard UCB ($P < 0.001$). The unadjusted probability of overall survival after 2 years was 51% (95% CI, 33–67), and the 2-years disease-free survival was 43% (95% CI, 24–60) (43).

Boitano et al. reported the first use of an AHRa, StemRegenin 1 (SR1), for purified CD34+ expansion when cultured in media

with SCF, Flt3L, TPO, and IL6, resulting in about a thousand-fold expansion (56). In the initial phase I/II clinical trial, 18 patients were treated with the lower dose unit placed in expansion culture and the larger dose unit which was unmanipulated. All the patients demonstrated sustained engraftment. In the 12 that had unit predominance with the expanded unit, the median time to recovery was 10.5 days, in contrast to 23 days in those engrafted with the unmanipulated unit (44). With sustained engraftment in the 12 patients recovering with the expanded product, the subsequent study evaluated the safety and the efficacy of the expanded product as a stand-alone graft. In addition, because of the marked expansion with the AHRa, lower-dose UCB units containing a cell dose of 1×10^7 nucleated cells, rather than 3×10^7 nucleated cells, per kilogram of recipient body weight were considered, potentially increasing the chance of better HLA-matched units for adults. An interim analysis demonstrated CD34+ cell expansion of 421-fold (r, 219–1,476), with the patients receiving a median of CD34+ cell dose of 2.6×10^7/kg (r, 0.9–13.5 $\times$ 10^7/kg) and CD34+CD90+ cell dose of 1.3×10^6/kg (r, 0.5–7.0 $\times$ 10^6/kg). Neutrophil recovery occurred in 100% of patients at a median of 13 days (*r*, 8–31) vs. 25 days in prior recipients of unmodified CB ($p < 0.01$). Similarly, platelet recovery and red blood cell transfusion independence occurred in 100% at a median of 36 days (r, 30–56) and 48 days (r, 13–196), respectively. Time to neutrophil and platelet recovery strongly correlated with CD34+CD90+ dose, and there had been no transplant-related mortality reported so far (57).

The pyrimidoindole derivative, UM171, was evaluated as another strategy for UCB HSC expansion which enhances the self-renewal potential of human long-term repopulating HSCs independently of AHR suppression (58). The clinical phases 1 and 2 trial using this compound was conducted in two parts (46). Part 1 enrolled four patients who received dUCBT—one with unmanipulated UCB unit and the other expanded with UM171—until the patients showed UM171 UCB unit-derived engraftment. In part 2 of this study, 22 patients received single UM171 expanded UCBT with a dose de-escalation design. The minimal UCB unit that achieved prompt engraftment as a single UM171-expanded UCBT was 0.52×10^5 CD34+ cells. The median time to neutrophil (ANC > 500/μl) and platelet recovery was 18 (r, 12.5–20 days) and 42 days (interquartile range, 35–47), respectively, with no incidence of graft failure.

Augmenting Homing

Based on preclinical models, it is clear that relatively few HSCs make it to the hematopoietic niche. Therefore, investigators explored ways that might augment homing and engraftment as an alternative to expansion culture. The first studies evaluated direct intra-bone marrow injection (IBMI) of UCB stem cells (59). This phase I/II study enrolled 32 adult patients with acute leukemia. The median time to neutrophil and platelet recovery was 23 and 36 days, respectively, and early sustained donor-derived engraftment was noted among all patients. There was no incidence of grade III and IV acute GVHD on this study. In a subsequent study by Brunstein et al., a dUCBT platform was used for the IBMI of one of the UCB units, while the other was given intravenously (60). Ten adult patients were enrolled on this trial, and the median time to neutrophil and platelet recovery was 21 and 69 days, respectively. In nine out 10 patients that engrafted, four engrafted with IBMI UCB unit. The trial demonstrated the safety of the procedure, but the technique offered no advantage over the traditional intravenous route.

Alternatively, agents like the dimethylated form of prostaglandin E2 (dmPGE2) and fucosylation have been used to augment the homing of HSCs (61). In the earlier approach, dmPGE2 was used to augment the homing of stem cells by increasing the number of stem cells that reach the bone marrow niche. This was considered to deliver a greater number of stem cells to the target site without the need for *in vivo* or *ex vivo* expansion (61). A phase I safety and efficacy trial was conducted, evaluating this concept using co-transplantation of a dmPGE2-treated UCB with an unmanipulated cord in patients with hematologic malignancies (62). The trial initially enrolled nine patients, with median time to neutrophil and platelet engraftment at 24 and 72.5 days, respectively. Two of seven patients undergoing engraftment demonstrated prolonged hematopoiesis from the $dmPGE_2$-UCB units. Given the lack of accelerated engraftment in the initial trial, dmPGE2 was optimized in the subsequent trial with a modulation protocol, and 12 additional patients were enrolled. The median time to neutrophil engraftment was 17.5 days (r, 14–31 days) compared to 21 days for the historical cohort ($p = 0.045$). The median time to platelet engraftment was 43 days (r, 26–60 days), and 10 of 12 patients had early and sustained engraftment of the $dmPGE_2$-UCB unit.

Another approach was evaluated by exploring the role of complement 3a (C3a), which attaches to the HSCs and improves homing by its immunomodulatory properties, including stromal-derived factor I (SDF I)-mediated homing (63). Based on pre-clinical data, the phase I study was conducted in adults receiving non-myeloablative conditioning in a dUCT model. Engraftment was noted in two-thirds of the patients from the non-manipulated cord, thus failing to show earlier homing and engraftment with this technique (64). Another group investigated the effect on homing with inhibition of dipeptidyl peptidase (DPP)-4, which is a peptide cleavage protein that truncates the chemotaxis factor, SDF-1-alpha. A pre-clinical investigation in mice demonstrated that deletion or inhibition of DPP-4 enhanced engraftment of human CD34+UCB cells in mouse marrow (65, 66). In the subsequent clinical trial, an oral inhibitor of DDP-4, sitagliptin, was used to enhance the engraftment of single-unit UCB transplants in adults with high-risk hematological malignancies (67). In this feasibility trial, 24 patients received sitagliptin on days 1 and 2 at a dose of 600 mg daily and engrafted at a median of 21 days (r, 13–50). Though sitagliptin was well-tolerated, a significant reduction in area under the curve was noted. After dose optimization, 600 mg every 12 h administered on days −1 to +2, another 15 adult patients were treated, and all engrafted by day 30, with 12 (80%) engrafting by day 21 (68). The median time to neutrophil engraftment was 19 days (r, 12–30).

Slower homing and engraftment with UCB relative to bone marrow HSC had also been attributed to poor binding to adhesion molecules P- and E-selectins present on bone marrow

endothelial cells (69). Pre-clinical models showed that both endogenous as well as *ex vivo* fucosylation of UCB HSCs increased the affinity for these adhesion molecules, resulting in earlier engraftment (70, 71). In the phase-I clinical trial using the dUCT model, one unit underwent fucosylation using fucosyltransferase-I enzyme, while the other unit was infused unmanipulated. Significantly faster neutrophil (17 vs. 26 days; $p < 0.05$) and platelet engraftment (36 vs. 46 days; $p < 0.05$) was noted from both units compared to the historical controls, suggesting that endogenous fucosylation benefited the engraftment of the unmanipulated cord as well (72).

Alternative Uses of UCB

Regulatory T Cells for Prevention of Immune Reactivity

GVHD has been one of the most serious complications for patients undergoing allogeneic HCT. The complex interaction between donor immune cells and residual host immunity results in extensive tissue damage requiring prolonged immune suppression, thereby increasing the risk of infection. Different strategies targeting *in vivo* or *ex vivo* T cell depletion have been shown to reduce the risk of GVHD, but these T cells also assist with engraftment and hasten immunologic recovery.

UCB has unique immunologic properties as it unites the maternofetal hematologic and immune environment and is one of the best examples of immune tolerance. This led to the identification of immunomodulatory cells in the UCB which dampen the pro-inflammatory immune response of the activated T cells. These T cells, also known as regulatory T cells (Tregs), are CD4+CD25+Foxp3+ and can proliferate in the presence of IL-2 (73). These Tregs have since shown to play an important role in autoimmune diseases (74, 75) as well as in regulating systemic pro-inflammatory response such as GVHD (76, 77). Edinger et al. demonstrated the role of CD4+CD25+Tregs in inhibiting the GVHD while preserving the graft-versus-tumor effect in mice with leukemia and lymphoma (78). Pre-clinical studies investigating the UCB components demonstrated the role of Tregs within the UCB, responsible for maternal–fetal tolerance (79, 80). These UCB-derived Tregs can be successfully isolated and expanded *ex vivo* to about 100-fold using anti-CD3/28 monoclonal antibody (mAb) along with supplemental IL-2 (81).

Early clinical trials investigating the safety profile of UCB-derived Treg infusion in adults with malignant disorders showed an encouraging safety profile and reduced grades II–IV acute GVHD rates (43 vs. 61%; $p = 0.05$) when compared to the historical cohort (82). Much greater *in vitro* expansion of isolated Tregs was obtained using the artificial antigen-presenting cells (aAPCs) to about 1,250-fold in 2.5–3 weeks compared to anti-CD3/CD28 mAb beads (83). These expanded UCB Tregs using modified aAPCs expressing OX40 and 4-1BBL had significantly better survival without loss of suppressor potency. A second dose expansion trial with Tregs showed a significant reduction of grades II–IV acute GVHD (45 vs. 9%; $p = 0.05$) compared to the historical controls, with no dose-related infusional toxicity or adverse reactions (84).

Virus-Specific T Cells

Viral infections after HCT result in substantial morbidity and mortality. Cytomegalovirus, Epstein–Barr virus, and adenovirus constitute the majority of significant viremias after HCT. Transplant-related variables that contribute to the risk include the underlying disease, donor and graft source, preparative regimen, and the degree of T cell depletion (*in vivo* or *ex vivo*) (85, 86). Current pharmacologic antiviral prophylaxis and treatment are limited in efficacy and are toxic to various organs (87, 88). The use of adoptive virus-specific T cell therapy was initially reported in 1992 by Ridell et al. (89) and has since developed and is increasingly used in HCT patients at high risk of organ toxicities or who have failed in conventional therapies. Readily available "off-the-shelf" third party is now available, targeting multiple viral infections (90–94), which is the major advantage these UCB-derived virus-specific T cells offer over other sources. However, the inability to do T cell expansion after UCBT and the naivety of UCB-derived T cells with limited priming resulting in a blunted immune response (95, 96) continue to be the main challenges.

UCB-Derived NK Cells

Natural killer (NK) cells are innate lymphocytes characterized by CD16+/CD56+ surface proteins and play an important role in identifying non-self-antigens without preemptive exposure. These are not antigen specific and are capable of identifying cells with reduced major histocompatibility complex expression as well. Their response is regulated through a balance between activating and inhibitory signals derived from surface receptors on the cell membrane that engage with related ligands on target cells (97, 98). The inhibitory killer cell immunoglobulin-like and NKG2A family of receptors recognize self HLA class I antigens, while the activating receptors include the natural cytotoxicity receptors NKp46, NKp30, NKp44, CD16, NKG2D, NKG2C, DNAX accessory molecule-1, and 2B4 recognize viral proteins as well as antibodies on target cells (99–101). NK cells also express checkpoint inhibitory receptors which further play an important role in antitumor activity and prevent disease relapse after HCT. In addition to their cytotoxic effect on target cells, their interaction with other immune cells, like dendritic cells and T cells, further potentiates the overall immune response.

NK cells are the first ones to reconstitute after HCT, providing an important immunologic barrier to invading pathogens in this critical period and especially more so after UCBT compared to other donor sources (102). UCB-derived NK cells are noted to express higher inhibitory receptors and fewer activating receptors in the early post-transplant period. Though these NK cells have high proliferative capacity, they are immature in function and have relatively less cytotoxic potential (103–105). Despite these issues, NK cells continue to play an important role in early post-HCT period, and their role in facilitating engraftment has been demonstrated in murine models (106, 107). This supports the premise of using *ex vivo*-expanded NK cells after UCB infusion to enable early engraftment and reduce the duration of neutropenia. Another important feature of NK cells in the post-HCT period is their strong potential for antitumor activity. The role of NK cell alloreactivity and kir-mismatch in antitumor activity and preventing acute myeloid leukemia relapse was first

demonstrated by Ruggeri et al. (108). The interaction of NK cells with other immune cells and the direct cytotoxic effect on tumor cells play an important role in reducing tumor relapse, which has since been shown by other studies (109, 110). This again supports the role of early NK recovery and infusion of expanded NK cells after UCBT.

A major challenge with UCB-derived NK cells is the limited number of available cells in a cord blood unit. Isolation and *ex vivo* expansion using an optimal cytokine cocktail (IL-2- or IL-7-mediated) thus become crucial to increase the cell dose (111–113). The expansion techniques under good manufacturing practices are now well-established, providing an important tool for adoptive immunotherapy against hematologic malignancies (114, 115) as well as solid tumors (116, 117). NK-cell-based immunotherapy continues to be a developing area with ongoing research on enhancing NK cell function by the increased expression of activating receptors or blocking inhibitory receptors (118), overcoming tumor resistance by blocking inhibitory signals and better understanding of the NK cell–tumor interactions to develop targeted immunomodulation.

In the recent phase I/II study by Liu et al., 11 patients with CD19+ hematologic malignancies (relapsed or refractory) were administered HLA-mismatched anti-CD19 CAR-NK cells derived from UCB (119). These HLA-mismatched NK cells were transduced using a retroviral vector expressing genes that encode anti-CD19 CAR, IL-15, and inducible caspase 9 as a safety switch, expanded *ex vivo*, and infused at different doses after lymphodepleting chemotherapy. There was no increase in the levels of inflammatory cytokines post-infusion and no incidence of cytokine release syndrome, neurotoxicity, or GVHD. Eight patients tolerated the infusion, with seven demonstrating complete and rapid response within 30 days of infusion. The infused CAR-NK cells expanded and persisted at low levels for at least 12 months, which was possibly supported by IL-15 in the construct as well as the lymphodepleting chemotherapy. These preliminary results from this study paves the way forward for the development of off-the-shelf NK-CAR products using UCB.

Other UCB-Derived Products

Induced pluripotent stem cells (iPSCs) can be derived from the UCB unit and now have been investigated in the development of multiple regenerative therapies including the skin, cartilage, neurodegenerative and spinal cord injuries, ocular degenerative diseases, and musculoskeletal disorders among many (120, 121). As regenerative therapies continue to develop, these UCB-derived iPSCs will continue to play a major role with their unique properties and easy availability. The role of UCB in immune modulation and regeneration is also

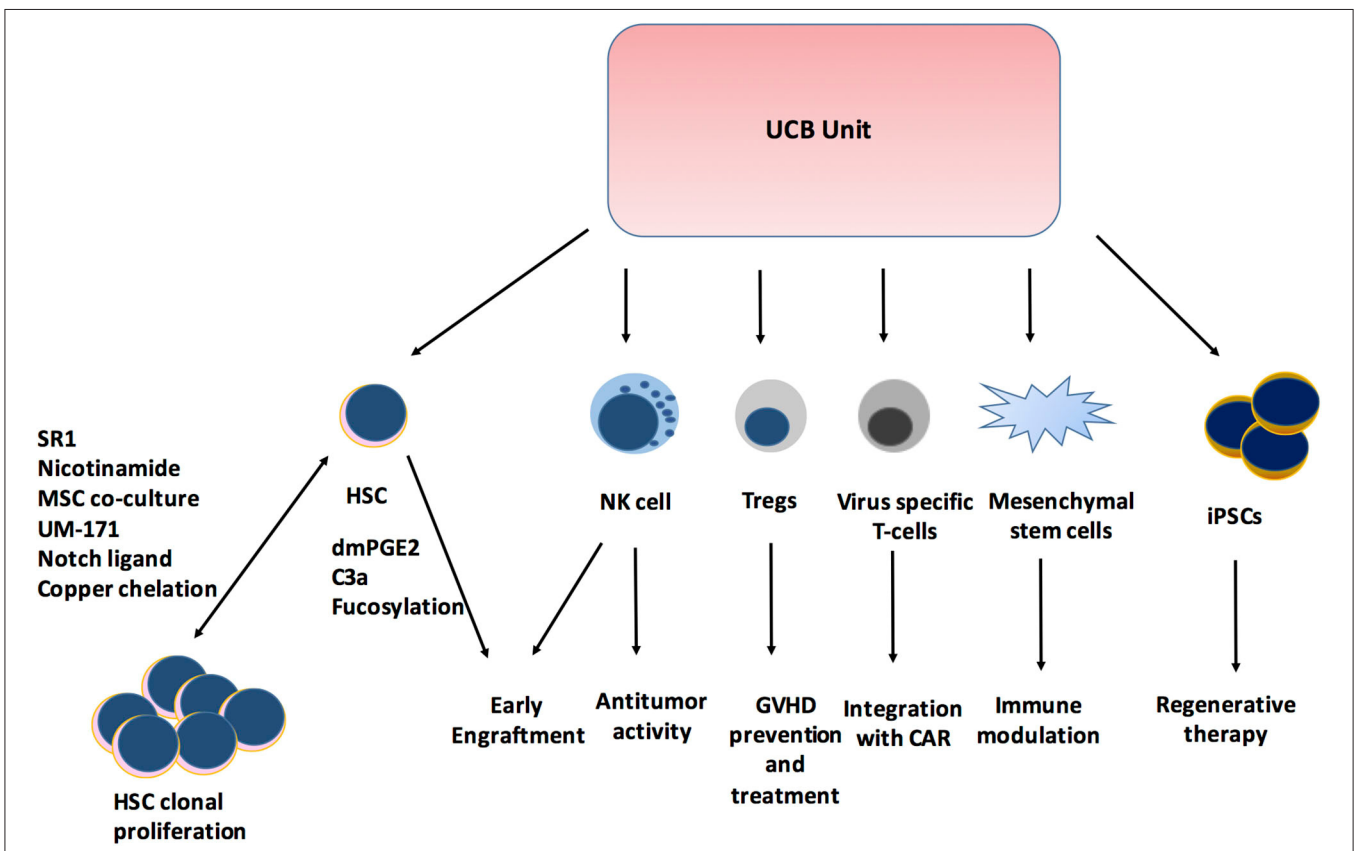

FIGURE 1 | Expanding role of Cord blood unit. SR1, StemReginin1; HSC, hematopoietic stem cell; dmPGE2, dimethyl prostaglandin E2; C3a, Complement 3a; NK cell, Natural killer cell; Tregs, Regulatory T cells; GVHD, Graft versus host disease; CAR, Chimeric antigen receptor; iPSCs, induced Pleuripotent stem cells.

being explored in several non-hematologic and non-malignant diseases. Currently, autologous or allogeneic UCB units are being investigated under ongoing clinical trials for hypoplastic left heart (NCT01856049 and NCT01883076), acute ischemic stroke (NCT03004976), cerebral palsy (NCT01072370), and hypoxic neurologic injuries (NCT03526588).

Future Directions

The use of UCBT has evolved from being limited to HCT for pediatric patients to expanding the role in adoptive cellular therapy (**Figure 1**). Remarkable research in the field has helped overcome the limitations of UCB with stem cell expansion and significantly improved hematopoietic and immunologic recovery, which has led to a shorter duration of hospitalization and hence lesser healthcare resource utilization. The outcomes of UCBT continue to improve with these newer modalities. In this new era of UCB-derived cells, the role of *in vivo* and *ex vivo* UCB expansion, tolerant Tregs, expansion of multi-virus-specific T cells, NK cells, and iPSCs will be crucial. The expanding use of UCB-derived immunotherapy will play an important role beyond HCT and continue to improve disease outcomes.

AUTHOR CONTRIBUTIONS

AG performed literature review and drafting of manuscript. JW provided critical review of manuscript, contributed further literature, and finalized the manuscript. All authors contributed significantly for preparation of this manuscript.

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

frontiers in Pediatrics

REVIEW
published: 05 January 2021
doi: 10.3389/fped.2020.551170

Hematopoietic Cell Transplantation for Sickle Cell Disease

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Sickle cell disease (SCD) is a severe autosomal recessively inherited disorder of the red blood cell characterized by erythrocyte deformation caused by the polymerization of the abnormal hemoglobin, which leads to erythrocyte deformation and triggers downstream pathological changes. These include abnormal rheology, vaso-occlusion, ischemic tissue damage, and hemolysis-associated endothelial dysfunction. These acute and chronic physiologic disturbances contribute to morbidity, organ dysfunction, and diminished survival. Hematopoietic cell transplantation (HCT) from HLA-matched or unrelated donors or haploidentical related donors or genetically modified autologous hematopoietic progenitor cells is performed with the intent of cure or long-term amelioration of disease manifestations. Excellent outcomes have been observed following HLA-identical matched related donor HCT. The majority of SCD patients do not have an available HLA-identical sibling donor. Increasingly, however, they have the option of undergoing HCT from unrelated HLA matched or related haploidentical donors. The preliminary results of transplantation of autologous hematopoietic progenitor cells genetically modified by adding a non-sickling gene or by genomic editing to increase expression of fetal hemoglobin are encouraging. These approaches are being evaluated in early-phase clinical trials. In performing HCT in patients with SCD, careful consideration must be given to patient and donor selection, conditioning and graft-vs.-host disease regimen, and pre-HCT evaluation and management during and after HCT. Sociodemographic factors may also impact awareness of and access to HCT. Further, there is a substantial decisional dilemma in HCT with complex tradeoffs between the possibility of amelioration of disease manifestations and early or late complications of HCT. The performance of HCT for SCD requires careful multidisciplinary collaboration and shared decision making between the physician and informed patients and caregivers.

Keywords: transplantation, hematopoietic stem cell, sickle cell disease, sickle cell anemia, gene editing, clinical trial, curative treatment

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: *12 April 2020*
Accepted: *16 November 2020*
Published: *05 January 2021*

Citation:
Krishnamurti L (2021) Hematopoietic Cell Transplantation for Sickle Cell Disease. Front. Pediatr. 8:551170. doi: 10.3389/fped.2020.551170

INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessively inherited disorder of the red blood cell (RBC) (1) characterized by the polymerization of the abnormal hemoglobin. Polymerization of the hemoglobin leads to erythrocyte deformation triggering downstream pathological changes, including abnormal rheology, vaso-occlusion mediated by abnormal adhesivity, ischemic tissue damage, and hemolysis-associated endothelial function. These acute and chronic physiologic disturbances contribute to the morbidity, organ dysfunction, impairment of health-related quality of life (QoL) (2), and diminished survival (3, 4). SCD is a major public health problem in the

United States affecting more than 100,000 individuals (5), predominantly African Americans, and is associated with substantial healthcare costs. Hydroxyurea, L-glutamine (6), voxelotor (7), and crizanlizumab (8) offer the possibility of the modification and amelioration of the disorder. Patients have to take these medications indefinitely, and access to care, health disparities, and other sociodemographic factors may influence uptake, usage, and effectiveness of long-term disease modification therapy. Hematopoietic cell transplantation (HCT) results in excellent outcomes in excellent overall survival (OS) and SCD-free survival (DFS) following matched related donor (MRD) HCT (9–13). There is increasing application of HCT from matched siblings, or from alternate donors (14–17) using myeloablative, reduced intensity, or non-myeloablative (NMA) conditioning regimens (15, 18–21). More than 65% of HCTs for SCD procedures reported from 2008 to 2017 were performed after 2013 (22). While the majority of HCTs performed have been from an MRD, recently, there is increasing use of alternative donor HCT (22). There are more than 100,000 individuals in the United States with SCD (5). There are many millions of patients worldwide, with more than 300,000 newborns with SCD born each year (23, 24). Therefore, we anticipate that increasing numbers of patients worldwide will consider the option of HCT for SCD. Further, the transplantation of autologous hematopoietic cells modified by gene addition or genomic editing offers the possibility of expanding the application of HCT for SCD (25–27).

INDICATIONS FOR HCT

HCT for SCD has been typically reserved for patients with severe complications of SCD, such as stroke or who were considered to be at risk of long-term disease-related complications (**Table 1**). In the initial clinical trials of HCT for SCD, the most common indication for HCT was stroke in 57% of patients and frequent vaso-occlusive pain crises (VOCs) in 23% of patients (9). In contrast, in recent studies, recurrent episodes of pain exacerbation requiring healthcare utilization have been reported as the most frequent indication for HCT (13, 18, 28). That patients are increasingly seeking HCT because of the impact of recurrent acute and possibly chronic pain on their QoL suggests that HCT is moving from a lifesaving treatment to one intended to improve the QoL. The number of hospitalizations or emergency department (ED) visits for SCD-associated pain is a poor surrogate measure of the total burden of pain. Patients manage most of their pain at home; hence, a hospital or ED visit represents a small fraction of the patients' pain experience (29). The duration of daily pain and the presence of disability provide a better measure of the impact of pain on the lives of individuals with SCD and is beginning to be used as an eligibility criterion for HCT for SCD (30, 31).

No randomized controlled trials have compared HCT with non-transplant treatment therapies such as hydroxyurea; however, favorable long-term survival has been reported on hydroxyurea. The efficacy of other disease-modifying therapies must be weighed against the fact that they need to be continued indefinitely and that morbidity, QoL, and the risk of premature death worsen with age in SCD patients. An ongoing trial (*NCT02766465*, BMT CTN 1503) compares the outcomes of patients with severe disease and an available donor who undergo HCT and those who do not have a donor and continue observation on standard clinical care.

TABLE 1 | Disease severity criteria for consideration of HCT for SCD adapted from Walters et al. (9).

Stroke or CNS event lasting >24 h
ACS with recurrent hospitalizations or previous exchange transfusion
Recurrent VOC (≥2/year for several years) or recurrent priapism
Impaired neuropsychological function and abnormal brain MRI
Stage I or II sickle lung disease
Sickle nephropathy (moderate or severe proteinuria or GFR 30–50% of predicted)
Bilateral proliferative retinopathy and major visual impairment in at least one eye
Osteonecrosis of multiple joints
Red cell alloimmunization (≥2 antibodies) during long-term transfusion therapy

An international expert panel recommended the consideration of HCT in young patients preferably at preschool age with symptomatic SCD who have an available HLA-identical sibling donor (32). The panel recommended using bone marrow and umbilical cord blood (UCB) from HLA-identical sibling donors as the preferred stem cell sources. They also suggested that HCT from an alternate donor source be restricted to those with severe disease, preferably in a clinical trial and at a center experienced in the performance of the procedure.

DECISION MAKING FOR HCT FOR SCD

Recipient Considerations

Recipient factors in the consideration or decision making for HCT and used as eligibility criteria in clinical trials have typically included the severity of the phenotype, including QoL, adequacy of organ function, response to current disease-modifying therapies, and the imminence of future complications (**Table 1**). In initial reports of HCT, patients had undergone the procedure most commonly because of a history of stroke (9), although more recently, recurrent VOC requiring medical care is reported as the most common indication for HCT (12).

The best OS and event-free survival (EFS) are reported in children younger than 5 years with an increased risk of complications with each year of increasing age (12, 14, 33). SCD-related organ damage progresses with age. SCD-related damage to the kidneys (34) and to the spleen (35) commences in infancy. Silent cerebral infarction is observed in nearly one-quarter of patients under 6 years of age (36). Silent cerebral infarcts are associated with cognitive deficits and poor school performance (37). High rates of OS and EFS in adult patients undergoing MRD HCT (13, 18) have expanded the applicability of HCT in this age group. Thus, HCT from an HLA-identical

sibling donor may be considered for young children without the severe disease phenotype because of excellent outcomes and the potential for the patient to have a normal childhood and avoid disease progression and complications of SCD in later life. However, these benefits must be carefully weighed against the risk of late effects such as infertility and subsequent malignancy.

Donor Considerations

The availability and use of an HLA matched donor are the primary donor factor that impacts outcomes. Stem cell source and donor age are important secondary considerations. The majority of sibling donors have sickle cell trait (HbAS) (11), but a heterozygous carrier state in the donor does not appear to impact outcomes. HCT using UCB from an HLA-identical sibling is associated with excellent outcomes. An acceptable total nucleated cell (TNC) dose for UCB is at least 3×10^7 TNC/kg recipient weight (38). However, in one case series in which a median dose of 3.9×10^7 TNC/kg (range, 1.5–14 $\times$ 10^7 TNC/kg) was administered, no impact was observed of cell dose administered on engraftment or DFS (39). If the cell dose is low with TNC 1×10^7/kg to 3×10^7/kg, a combination of UCB and bone marrow may be infused (10). Donor weight exceeding 10 kg and age exceeding 1 year are generally considered safe for collecting an adequate cell dose. If more than one HLA-identical–related donor is available, additional factors to consider in donor selection include donor size/age, ABO typing, and cytomegalovirus (CMV) serostatus. Donor age may impact the risk of chronic graft-vs.-host disease (GVHD) risk in malignant disease (40, 41) and may similarly impact HCT recipients with SCD. ABO major or minor mismatch may add to the risk of graft stem cell loss (42). Major ABO mismatch should be avoided as it may be associated with delayed RBC engraftment (43), decreased OS (44), and pure red cell aplasia (45). Donor and recipient should be matched for CMV serostatus whenever possible to minimize risks of CMV disease.

Decision-Making by Patient, Families, and Caregivers

HCT for SCD poses a decisional dilemma involving tradeoffs between the possibility of cure, amelioration of symptoms on the one hand and the risk of acute toxicities and late effects on the other hand (46). Advances in care have greatly improved outcomes of SCD, but there is a lack of data comparing outcomes of HCT with that of standard clinical care. HCT has curative intent but is associated with treatment-related morbidity and mortality risk. The perceived severity of disease and availability of a suitable donor influence the decision-making by SCD patients, their caregivers (46), and their physicians (47). The decision is also influenced by the availability of family support, resources, and BMT-related education and awareness (46). A quarter of SCD patients and caregivers are unwilling to accept *any* risk of GVHD or mortality (48). Seventy-two percent of parents of a child with SCD were willing to accept a $\geq$5% risk of mortality, whereas 57% are willing to accept a risk of $\geq$10% of GVHD (48). Interestingly, following a successful HCT, SCD patients and caregivers do not report decisional regret (46). In that study, even the few patients who were currently dealing with chronic GVHD indicated that although they are dealing with frequent clinic visits and receiving treatments, they were glad to be rid of SCD and its complications.

The Physician Perspective on Decision Making

Physicians may be influenced by their past experiences, outcomes of previous patients undergoing HCT, their perception of the severity of disease manifestation in the individual, and a variety of patient and institutional characteristics in the decision to refer a patient for consideration of HCT (47). We performed a qualitative study of physician perceptions and approach to decision making regarding disease-modifying therapies in SCD (47). We identified two different narratives in the physician's approach to patient decision making. In the collaborative approach, the physician co-opts the patient in a joint examination of all available treatment options to jointly arrive at a treatment choice. In the proponent approach, on the other hand, the physician strongly advocates a particular treatment and educating patients/families to convince them to accept that treatment. The ethics of this decision-making have been extensively reviewed (49, 50). As the decision for HCT for SCD is complex, shared decision making about HCT must involve the cooperation of the informed and empowered patient and caregivers, the primary hematologist, and the transplant physician.

PRE-HCT RECIPIENT EVALUATIONS

Eligibility for HCT is examined based on disease severity, the adequacy of organ function, and for any SCD-related or treatment-related complications that may impact the HCT course or outcome.

Before HCT, patients are evaluated for organ damage. SCD-associated neurologic damage is assessed by brain magnetic resonance imaging (MRI)/magnetic resonance angiography. Patients with severe cerebral vasculopathy or moyamoya disease may be considered for encephaloduroarteriosynangiosis (51). Cerebral blood flow velocity determined by transcranial Doppler velocity (age <16 years) and neurocognitive function may stabilize or improve posttransplant (52) and may be evaluated pre-HCT. Transfusional hemosiderosis and its effect are examined by the quantification of the liver and cardiac iron. The presence and severity of liver fibrosis are evaluated by magnetic resonance or ultrasound elastography and by liver biopsy as needed. As patients are at risk of renal dysfunction (34, 53), glomerular filtration rate (GFR), urine specific gravity, and albumin-to-creatinine ratio are evaluated pre-HCT (54). The splenic function is impaired and hence is evaluated pre-HCT with liver–spleen nuclear medicine scan (35) or RBC pit count. These studies are repeated 1 year post-HCT to determine the recovery of splenic function (55). The presence of donor-directed HLA antibodies in high titers may predict an increased risk of graft rejection. Donor-directed HLA antibodies can be reduced by desensitization strategies (56, 57), and patients with SCD have subsequently successfully undergone haploidentical HCT

(56). Patients are evaluated by transfusion medicine specialists to assist in assessing the alloimmunization status or special needs for packed RBCs and formulate a plan for the management of RBC transfusion before conditioning and in the peritransplant period (58).

Patients are typically evaluated by social workers, psychologists, and child life specialists and counseled regarding preparation for the anticipated stressors associated with HCT. Patients are encouraged to consult reproductive endocrinologists and receive counseling regarding their options for clinical or research procedures for fertility preservation.

HCT CONDITIONING REGIMEN

Myeloablative conditioning (MAC) has been used most often in MRD HCT (12). The regimen used in the early transplant series consisted of a myeloablative combination of busulfan and cyclophosphamide (9). More recent reports describe the use of reduced-intensity conditioning (RIC) (10, 12, 14, 15, 59, 60). The most common RIC approach has been the substitution of cyclophosphamide with fludarabine in combination with another agent (12), typically an alkylator such as busulfan or melphalan (10). Reduced-toxicity conditioning regimen (61, 62) has also been reported (13, 63, 64), including reduced doses of busulfan in combination with fludarabine with or without cyclophosphamide (63) or substitution of busulfan with treosulfan and the addition of thiotepa (64). NMA conditioning with TBI 200 cGy and fludarabine (65, 66) was associated with poor long-term engraftment. Recently, NMA strategies combining TBI (300 cGy) with alemtuzumab have been associated with high OS and EFS in adults (18, 67) and children (20, 68).

GVHD PROPHYLAXIS

T-cell depletion *in vivo*, using antithymocyte globulin (ATG; 70.6%) or alemtuzumab (11.5%), has been used in most patients undergoing MRD HCT (12). Bernaudin et al. demonstrated that the use of ATG was associated with the decrease of graft failure rate from 22.6% to 3% (11). ATG use is, however, uncommon in MRD UCB transplant (39). T-cell depletion *in vivo*, with alemtuzumab, has been administered in MRD HCT (10, 18) and URD UCB transplant (15, 59). T-cell depletion *in vivo*, with posttransplant cyclophosphamide with ATG or alemtuzumab (16, 19, 21, 56, 60), has been used for haploidentical HCT for SCD. The use of *ex vivo* T-cell depletion, with $CD34^+$ selection (69), CD3/CD19 depletion (70), or T-cell receptor (TCR) α/β and CD19 depletion (71), has also been reported in haploidentical HCT for SCD. *Ex vivo* TCR α/β and CD19 depletion have been associated with a reduction in risk of GVHD, but may, however, be complicated by delayed immune reconstitution, infection, and graft failure (71, 72). Calcineurin inhibitors (CNIs) are the most commonly used GVHD prophylaxis and may be combined with methotrexate or mycophenolate mofetil (12). Locatelli et al. observed decreased DFS following MRD UCB transplant with MTX (39). MMF is substituted in this setting (15, 59). The addition of abatacept, a selective inhibitor of T-cell costimulation, to the GVHD prophylaxis regimen has the potential to decrease the GVHD and thus improve the safety profile and applicability of HCT to SCD (73).

CELL DOSE CONSIDERATIONS

Cell dose predicts engraftment, with the rate of graft failure decreasing to 5% from 10% when TNC was $\geq 2.5 \times 10^8$/kg (74). In unrelated donor transplantation, increased mortality was predicted by a peripheral blood mononuclear cell dose of TNC $<2 \times 10^8$/kg or a bone marrow graft nucleated cell dose $<5 \times 10^8$/kg, respectively (75). In URD UCB, TNC $>5 \times 10^7$/kg increased engraftment and DFS (76). The generally recommended target cell dose is 4×10^8 to 5×10^8 TNC/kg for bone marrow, and 4×10^7 to 5×10^7 TNC/kg for UCB (prethaw) grafts.

ENGRAFTMENT FOLLOWING TRANSPLANT

Factors predicting graft failure include the degree of HLA mismatch, the titers of donor-directed HLA antibodies if present, the intensity of conditioning, and the presence of active infection at the time of engraftment (74).

Lineage-specific chimerism in the lymphoid lineage[CD3] and myeloid lineage[CD15 or CD33], as hemoglobin level, and HbS% are typically used to evaluate the degree of engraftment and donor-derived hematopoiesis. Whole-blood chimerism of 11% to 74% may be associated with stable donor-derived erythropoiesis (77–79). Lineage-specific chimerism may provide additional information, although red cell chimerism assays are still under evaluation in research studies (80, 81). Myeloid chimerism of 20–25% (13, 78, 79) may best predict stable donor-derived erythropoiesis. An HbS >50% suggests the likelihood of impending autologous recovery. In the case of mixed or declining donor chimerism or increasing HbS percentage, more frequent assessments may be necessary. The role of donor lymphocyte infusions in improving donor chimerism is unknown and, in any case, associated with a significant risk of GVHD.

The majority of patients rejecting the allograft reconstitute autologous hematopoiesis (11, 78) even in the case of alternative donor HCT (13–16, 78). If marrow aplasia occurs or if prolonged cytopenia is observed, a salvage HCT may be required urgently (13). In patients with autologous reconstitution, following graft failure, a second HCT may be considered after at least 6 months following the first HCT procedure.

PREVENTION AND MANAGEMENT OF COMPLICATIONS DURING HCT

SCD patients are uniquely susceptible to certain neurologic, cardiovascular, pulmonary, hepatobiliary, renal, and infection risks in the immediate peri-HCT period and the long term (82).

Neurologic

Neurologic complications include seizures and hemorrhagic stroke, which were observed in 30% of patients of the initial group of patients who underwent MRD HCT (83). The following risk factors for neurologic complications in HCT for SCD were identified: (i) stroke particularly in patients with a history of prior stroke; (ii) hypertension due to neurologic and renal dysfunction exacerbated by nephrotoxic medications such as CNIs and hypertension due to prednisone; (iii) hemorrhagic stroke with concurrent thrombocytopenia in patients with preexisting cerebral vasculopathy exacerbated by concurrent postchemotherapy thrombocytopenia (11), and (iv) a high incidence of posterior reversible encephalopathy syndrome (PRES) (22–34%) (14, 84), with the risk of PRES in SCD patients (85) exacerbated during HCT (84). Patients who developed PRES following MRD HCT had decreased OS and DFS (84). BMT CTN 0601, a multicenter trial of URD BMT, reported a high incidence of PRES (14). Of note, this study included the use of prednisone through day +28 as part of GVHD prophylaxis.

Thus, precautions instituted to decrease neurologic complications include (i) drug prophylaxis to prevent seizures starting prior to conditioning, especially if busulfan is used and continued for the duration of CNI administration, and (ii) strict control of hypertension. Particular caution must be exercised in the monitoring and management of hypertension when patients with SCD are receiving both prednisone and CNI post-HCT because both drugs are likely to cause hypertension. Blood pressures (BPs) in SCD are lower compared to age-matched peers (86). As such, the BP control regimen should take into account these lower BP parameters, as well as the baseline BP of the patient (iii) magnesium supplementation to prevent hypomagnesemia (87, 88) and (iv) platelet transfusions to maintain platelets >50,000/μL and RBC transfusion to maintain hemoglobin 9–11 g/dL.

Cardiovascular and Pulmonary

Individuals with SCD run lower BP compared to individuals matched for age, sex, and race (86). BP above the 50th percentile for age may be associated with an increased risk of stroke (86, 89). Thus, the prevention of neurologic complications such as seizures or PRES requires careful monitoring and strict control of BP (9, 83), with a target BP within 10% of the median for age and sex, for SCD patients (86).

A combination of echocardiographic tricuspid regurgitant jet (TRJ) velocity >3.0 m/s, and Brain Natriuretic Peptide BNP >160 pg/mL is a strong predictor of premature mortality in adult SCD patients (90, 91). Successful HCT may be associated with the improvement of TRJ velocity (13).

Infections

SCD patients undergo autoinfarction of the spleen with impaired splenic dysfunction in most patients by age 3 years (35, 92), thus increasing the risk of pneumococcal sepsis mostly with non-vaccine serotypes (93). Pediatric SCD patients may recover splenic function post-HCT, but older patients and those with extensive chronic GVHD are at risk of poor post-HCT splenic recovery (55). While pneumococcal infections are rare following HCT for SCD (55), deaths from pneumococcal sepsis have been reported post HCT for SCD. As such, pneumococcal prophylaxis during HCT, monitoring of splenic function recovery post-HCT, and timely reimmunization starting with conjugated pneumococcal vaccines 6 months posttransplant (94) is recommended.

Management of Iron Overload

Patients may have transfusional hemosiderosis pre-HCT and may have received several transfusions of packed red blood cells PRBCS with the HCT. As such, residual iron overload is measured by serum ferritin and MRI at 1 year post-HCT by which time patients are typically off immunosuppression and are transfusion independent. Removal of excess body iron stores must be instituted after HCT and continued with close follow-up monitoring until the resolution of iron overload is demonstrated. Transfusional hemosiderosis may be treated with oral iron chelation, by monthly phlebotomy, or a combination thereof (95–98). The method adopted for reducing iron overload should be dictated by the degree of iron overload and the preferences and circumstances of the individual patient. Individuals with cardiac iron overload must undergo follow-up MRI evaluation of cardiac iron overload post-HCT.

Renal

Prior to HCT, serum blood urea nitrogen/creatinine and GFR or 24-h creatinine clearance are obtained to determine the adequacy of renal function and are followed yearly for at least 2 years post-HCT. Patients are evaluated for SCD-associated proteinuria pre-HCT and followed for recovery post-HCT (82). Patients who are on prolonged course of CNIs are at high risk of renal dysfunction. Patients with SCD may have prior complement-mediated vascular injury due to their underlying primary hemolytic disease and then again from additional stressors during the transplantation process leading to progressive endothelial injury and end-organ dysfunction (99). This may place them at a greater risk of transplant-associated thrombotic microangiopathy (TA-TMA) and subsequent chronic kidney disease. Patients must be monitored for HCT TA-TMA, and interventions such as control of hypertension, consideration of alternatives to CNIs, and the introduction of eculizumab therapy may be considered prior to overt clinical manifestations (100).

OUTCOMES FOLLOWING HCT

The first HCT for SCD was performed in a child who developed acute myeloid leukemia, thus curing both diseases (101). Since the first multicenter clinical trial (9), HCT for SCD has been rapidly expanding in its applicability. The majority of patients reported are children who have undergone HCT from HLA-identical–related donors (9, 10, 12, 13, 18, 39, 67, 77, 81, 102, 103) Gluckman et al. reported in a joint Eurocord-CIBMTR registry–based study an OS of 92.9% and EFS of 91.4% in 1,000 children who underwent HCT for SCD (12). On multivariate analysis, survival was found to decline with increasing recipient age and was to be higher in patients undergoing HCT after the year 2006. Previously, most patients undergoing HCT received MAC. In more recent series, reduced toxicity/intensity (10, 13, 81)

and NMA (18, 20, 67, 103) conditioning regimens have been demonstrated to be safe and effective in ameliorating SCD clinical manifestations (10, 13, 81). Following HCT, the transcranial Doppler velocity returns to normal; there is a stabilization of organ function, stabilization of full-scale IQ, improved health-related QoL (HRQoL), decreased prescriptions of opioid pain medications, and improvement in splenic function (11, 13, 14, 52, 55, 104–107). Adult patients followed in the 2nd year after a successful HCT for SCD, as compared to pre-HCT, and as compared to a group of SCD patients who had not undergone HCT, had significantly lower rates of healthcare utilization and healthcare costs (108).

Following a successful HCT pain eventually resolves in the large majority of SCD patients. Pain, however, may persist for a prolonged period, especially in older patients, those with higher pain burden, with more anxiety, or more use of long-acting opioids prior to HCT (109). While pain intensity may remain unchanged at 1 year in 40% (109), HRQoL may improve, with decreased pain interference and improved physical function (13). Thus, patients with a history of chronic pain with high pain burden and pain medication use pre-HCT and require close follow-up and multidisciplinary rehabilitation post-HCT.

PREDICTORS OF OUTCOMES

The age at the performance of HCT is the strongest predictor of EFS in HLA-identical sibling donor HCT (12, 33, 104). Brazauskas et al. described a simple risk score to guide patients with SCD and hematologists who are considering HCT as a curative treatment relative to other available contemporary treatments (110). Patients 12 years or younger with MRD were at the lowest risk with a 3-year EFS of 92% (score, 0). Patients 13 years or older with an MRD or those 12 years or younger with an HLA-matched unrelated donor were at intermediate risk (3-year EFS, 87%; score, 1). All other groups, including patients of any age with a haploidentical relative or HLA-mismatched unrelated donor and patients 13 years or older with an HLA-matched unrelated donor, were high risk (3-year EFS, 57%; score, 2 or 3).

CURRENT RESEARCH GAPS

To date, a small proportion of SCD with severe clinical manifestations has undergone HCT. In one survey of SCD programs, only 8% of patients with severe manifestations who would have met eligibility for participation in a trial of HCT for SCD underwent a transplant. The lack of an available MRD is the primary barrier to the consideration of HCT (111). The likelihood of an African American finding a suitable 8 of 8 antigen HLA-matched donor is as low as 19%, with <5% finding a potential matched (6/6) unrelated UCB donor (112, 113). BMT CTN 0601 results revealed an unacceptably high rate of chronic GVHD following URD BMT (13, 14). Conversely, the rates of graft failure following unrelated UCB transplants were found to be unacceptably high (59, 76), but modifications in conditioning may result in improved rates of engraftment (15). The expansion of UCB units *ex vivo* has the potential to improve the applicability of this cell source for HCT for SCD (114). Increasing the cell dose would enable the use of an otherwise suitably HLA-matched UCB, which may have had a cell dose below the threshold of acceptability. HCT from related haploidentical donors can expand the donor pool, thus expanding the applicability of SCD. Early reports suggested that while the safety of the procedure was acceptable, the rates of stable engraftment were low (16). Refinements in conditioning regimen by either the addition of thiotepa or by increasing the dose of TBI have demonstrated improvement in EFS rates (16, 17, 21, 56, 107). Despite improvements in outcomes of MRD and the development of multiple options for alternate donor HCT, concerns for serious short-term complications such as GVHD, as well as long-term risks specifically of infertility or subsequent malignancy, remain a substantial barrier to the acceptability of HCT. Further, awareness of these options remains poor, and access to quality care remains challenging. Health disparities, even in high-resource countries, and the lack of health infrastructure in low-resource countries severely limit the general applicability of HCT for SCD. Thus, there is a need for progress on multiple fronts before HCT can become a standard therapy that is applicable and acceptable to a sizable proportion of patients with SCD.

POTENTIAL DEVELOPMENTS IN THE FIELD

SCD, the first molecular disease (115), has long been the target for the development of gene therapy for producing long-term amelioration of the disease. Gene addition and genomic editing are two approaches to gene therapy. Gene addition involves the addition of a non-sickling beta-globin gene into the genome of the hematopoietic stem cell. Genomic editing involves the use of molecular techniques to reverse the silencing of the fetal gamma globin gene with a view to increase the expression of non-sickling fetal hemoglobin (27). Early results of the addition of a non-sickling beta-globin gene using a lentiviral vector have been encouraging (116). Multiple studies of genomic editing aimed at fetal hemoglobin induction through targeting BCL11a are ongoing (117–121). Chemotherapy-based conditioning and the resultant bone marrow aplasia, mucositis, and late effects such as infertility remain a barrier to the acceptability of HCT and gene therapy. Non-genotoxic conditioning based on antibody targeting hematopoietic stem cells has the potential to reduce the toxicity of HCT and gene therapy (122–124). The development of novel GVHD prophylaxis such as ruxolitinib, a selective Janus kinase (JAK1 and JAK2) inhibitor (125, 126), and the use of biomarker panels to predict outcomes and direct therapy of GVHD (127) may further improve the safety of HCT and expand its applicability for SCD.

SUMMARY AND CONCLUSIONS

Excellent outcomes in MRD HCT, improvements in the conditioning regimen, novel methods of GVHD prophylaxis, and

the ability to safely use alternative donors have all contributed to the applicability of HCT for SCD. Encouraging early results in the clinical trials of the feasibility and safety of gene therapy suggest that this approach may further increase the applicability of HCT (116). Hence, the discussion about available curative options should be integrated with the comprehensive care of SCD patients. In young children with symptomatic SCD with an available HLA-identical–related donor, careful consideration should be given to proceeding to HCT, even in the absence of severe SCD-associated comorbidities. HCT from alternative donors is typically undertaken only in patients with severe symptoms, causing or likely to cause organ damage, and undertaken in the context of clinical trials. Patients undergoing these therapies require care and counseling regarding psychosocial aspects, including the importance of adherence to medications, fluid intake, and precautions to prevent infections. The study of long-term outcomes following HCT for SCD (128, 129) through long-term follow-up registries is key to determining long-term efficacy and late effects of HCT (82). The transplantation of autologous gene-modified HPCs is not associated with GVHD and eliminates the need for a matched allogeneic donor. Questions remain, however, regarding the level of non-sickling hemoglobin required for long-term disease amelioration and organ function. Ongoing studies will also address the durability of gene-modified cell engraftment, as well as the long-term risk, if any, of insertional mutagenesis (116, 130, 131). Currently, cost and availability are major barriers to the application of gene therapies (132).

Further research must focus on how to make these treatments generally available and at a reasonable price point. Studies of incremental cost-effectiveness must account for the individual and societal impact of chronic illness, associated utilization of healthcare, and the loss of educational opportunity and suitable employment. There is a need for ongoing clinical and translation research, clinical trials, and long-term follow-up studies, to explore the enormous potential of HCT in the treatment of SCD.

AUTHOR CONTRIBUTIONS

This study was conceived, written, and edited by LK.

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Conflict of Interest: The 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.

The reviewer NS declared a shared affiliation, though no other collaboration, with the author LK.

frontiers in Oncology

ORIGINAL RESEARCH
published: 05 February 2021
doi: 10.3389/fonc.2020.596677

Re-Emergence of Minimal Residual Disease Detected by Flow Cytometry Predicts an Adverse Outcome in Pediatric Acute Lymphoblastic Leukemia

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Purpose: While the role of minimal residual disease (MRD) assessment and the significance of achieving an MRD-negative status during treatment have been evaluated in previous studies, there is limited evidence on the significance of MRD re-emergence without morphological relapse in acute lymphoblastic leukemia (ALL). We sought to determine the clinical significance of MRD re-emergence in pediatric ALL patients.

Methods: Between 2005 and 2017, this study recruited 1126 consecutive patients newly diagnosed with ALL. Flow cytometry was performed to monitor MRD occurrence during treatment.

Results: Of 1030 patients with MRD-negative results, 150 (14.6%) showed MRD re-emergence while still on morphological complete remission (CR). Patients with white blood cell counts of ≥50 × 10^9/L (p = 0.033) and MRD levels of ≥0.1% on day 33 (p = 0.012) tended to experience MRD re-emergence. The median re-emergent MRD level was 0.12% (range, 0.01–10.00%), and the median time to MRD re-emergence was 11 months (range, <1–52 months). Eighty-five (56.6%) patients subsequently developed relapse after a median of 4.1 months from detection of MRD re-emergence. The median re-emergent MRD level was significantly higher in the relapsed cohort than in the cohort with persistent CR (1.05% vs. 0.48%, p = 0.005). Of the 150 patients, 113 continued to receive chemotherapy and 37 underwent transplantation. The transplantation group demonstrated a significantly higher 2-year overall survival (88.7 ± 5.3% vs. 46.3 ± 4.8%, p < 0.001) and cumulative incidence of relapse (23.3 ± 7.4% vs. 64.0 ± 4.6%, p < 0.001) than the chemotherapy group.

Conclusions: MRD re-emergence during treatment was associated with an adverse outcome in pediatric ALL patients. Transplantation could result in a significant survival advantage for these patients.

Keywords: acute lymphoblastic leukemia, pediatric, minimal residual disease, re-emergence, hematopoietic cell transplantation (HSCT)

OPEN ACCESS

Edited by:
Emmanuel Katsanis, University of Arizona, United States

Reviewed by:
Holly Pariury, University of Arizona Medical Center, United States
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Aron Flagg, Yale University, United States

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Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *20 August 2020*
Accepted: *21 December 2020*
Published: *05 February 2021*

Citation:
Wang Y, Xue Y-J, Jia Y-P, Zuo Y-X, Lu A-D and Zhang L-P (2021) Re-Emergence of Minimal Residual Disease Detected by Flow Cytometry Predicts an Adverse Outcome in Pediatric Acute Lymphoblastic Leukemia. Front. Oncol. 10:596677. doi: 10.3389/fonc.2020.596677

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most prevalent hematological malignancy in children (1). Advances in our understanding of the clinical features, immunobiological characteristics, and cytogenetic alterations associated with ALL have led to better risk stratification and risk-directed treatment of ALL patients (2, 3). In pediatric ALL, minimal residual disease (MRD) levels reflect the efficacy of chemotherapy and have shown to be the most powerful prognostic factor. While the role of MRD assessment and the significance of achieving an MRD-negative status at the end of induction and consolidation therapy have been evaluated in previous studies, there is limited evidence on the significance of MRD re-emergence without morphological relapse in ALL, in the context of sequential MRD monitoring. Our previous study showed that MRD re-emergence was an adverse prognostic factor in children at high risk of ALL (4). Pui et al. (5) and Pemmaraju et al. (6) have also reported that MRD re-emergence is associated with a poor outcome in ALL.

Flow cytometry (FCM) was explored as a less labor-intensive, less expensive, and faster MRD technique than polymerase chain reaction (PCR)-based methods and has been used extensively in pediatric ALL patients (7). Since 2005, we have monitored MRD sequentially using FCM at our institution. This study therefore aimed to determine the significance of MRD re-emergence in pediatric ALL patients after achieving an MRD-negative status.

MATERIALS AND METHODS

Patients

Between January 2005 and December 2017, this trial recruited consecutive patients aged 0 to 18 years who were newly diagnosed with ALL. Patients with mature B-cell leukemia were excluded. The study was approved by the Ethics Committee of Peking University People's Hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents or guardians of the patients. The BOSHI Network Database (https://www.boshicloud.com), an online platform for clinical patient information management and data analysis, was used to retrieve and monitor patient data.

Diagnosis, Minimal Residual Disease Measurement, and Risk Classification

ALL was diagnosed based on morphological, immunophenotypic, and cytogenetic evaluation using standard techniques (8, 9). Fusion transcripts of t(12;21)/*ETV6-RUNX1*, t(1;19)/*TCF3-PBX1*, t(9;22)/*BCR-ABL1*, and 11q23/*KMT2A* rearrangement (*KMT2A-r*) were measured using PCR and/or fluorescence *in situ* hybridization, as previously described (10, 11).

MRD was measured using FCM, with a sensitivity of 0.01% (12, 13). The MRD monitoring schedule was planned in advance, and the scheduled time points for induction therapy were on day 15 and day 33. Patients were classified as either M1 (blast cells, <5%), M2 (5–25%), or M3 (≥25%) based on morphological evaluation. After induction therapy, MRD measurements were performed every 2–3 months during consolidation chemotherapy and every 6 months during maintenance chemotherapy (4). More frequent MRD monitoring was performed for some patients, depending on their conditions.

For initial risk stratification, we referred to the National Cancer Institute (NCI) risk group criteria (14) and cytogenetic subtypes, while the final assessment was based on treatment response and MRD levels during and after induction therapy (15) (**Supplementary Figure 1**). Standard-risk (SR) patients with an M3 marrow status on day 15 or MRD measurements of 0.01–0.99% on day 33 were upstaged to intermediate risk (IR), whereas IR patients with an M3 marrow status on day 15 were assigned high risk (HR). Patients who did not achieve complete remission (CR) upon completion of induction therapy or had MRD levels of ≥1% on day 33 or ≥0.1% on week 12 were also upstaged to HR.

Definition

CR was defined as a percentage of leukemic blasts of <5% in the bone marrow (BM) sample reviewed at the time of peripheral blood count recovery, the absence of circulating peripheral blasts, and the absence of extramedullary disease. Relapse was defined as the presence of leukemic blasts in any extramedullary location, or in the BM at a level of ≥5%. Moreover, MRD re-emergence was defined as at least two consecutive detectable recurrences of MRD (sensitivity for positive value, ≥0.01%), despite the persistence of morphological CR. The level for MRD positivity was based on first MRD re-emergence level. After the first MRD re-emergence, a second MRD test was scheduled within the next two weeks.

Treatment

All patients underwent a modified version of the ALL-Berlin-Frankfurt-Munster (BFM) protocol described previously (4). Briefly, the patients underwent induction therapy, including vincristine, idarubicin, cyclophosphamide, prednisone/dexamethasone, and L-asparaginase (COIPL), followed by consolidation therapy with one to two cycles of re-induction and maintenance therapy (**Supplementary Figure 1** and **Supplementary Table 1**). The consolidation chemotherapy regimen included high-dose methotrexate (HDMTX) (targeted steady-state concentration of 16 μM/L for SR patients and 24 μM/L for IR/HR patients), high-dose cytarabine (HDAra-C) (cytarabine for SR patients and cytarabine + idarubicin for IR/HR patients), and ifosfamide (IFO) (only for HR patients), which were given alternately. Re-induction comprised 1 course of COIPL for SR patients and 2 courses of COIPL for IR/HR patients. Maintenance therapy included daily mercaptopurine and weekly methotrexate. Re-induction was administered every 6 months during the consolidation chemotherapy. The scheduled consolidation chemotherapy comprised 9 rounds of HDMTX and 2 rounds of HDAra-C for SR patients, 11 rounds of HDMTX and 2 rounds of HDAra-C for IR patients, and 13 rounds of HDMTX and 3 rounds of HDAra-C for HR patients. Since 2010, for patients diagnosed with *BCR-ABL1* ALL (days 8–15 of induction), imatinib mesylate was initiated at a dose of 260 to

340 mg/m^2/day. The total doses of idarubicin and L-asparaginase were 80 mg/m^2 and 200 000 units/m^2 for SR patients, and 100 mg/m^2 and 300,000 units/m^2 for IR/HR patients, respectively.

All patients regularly received triple intrathecal therapy to prevent central nervous system (CNS) leukemia. The total number of intrathecal therapies administered ranged from 16 in SR patients to 23 in IR/HR patients. Patients presenting with CNS leukemia received twice-weekly intrathecal chemotherapy until normalization of cerebrospinal fluid levels, after which they received weekly CNS therapy for four more doses. The total duration of treatment was 3 years for SR patients and 3.5 years for IR/HR patients. Patients in the HR group who achieved CR were offered the option of undergoing allogeneic hematopoietic cell transplantation (allo-HSCT). The transplant conditioning regimens were administered as previously described (4).

Statistical Analysis

The outcome data used in the analysis were last updated on April 15, 2020. Overall survival (OS) was defined as the time between the date of diagnosis and the date of death due to any reason or the date of last contact. Event-free survival (EFS) was defined as the time between the date of diagnosis and the date of an event (e.g., relapse, second malignancy, death due to any reason) or the date of the last follow-up. The Kaplan-Meier method was used to estimate the survival rates, and log-rank tests were used to compare their differences. Multivariate analyses were performed using a Cox proportional hazards model. The cumulative incidence of relapse (CIR) for competing events was constructed using the Kalbfleisch-Prentice method. The $OS_{MRD\text{-}r}$ and $CIR_{MRD\text{-}r}$ were evaluated from the time of MRD re-emergence. Fisher's exact test was used to compare differences between categorical variables among the groups. Logistic regression was used to evaluate factors affecting the re-emergence of MRD. R software version 4.0.1 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS version 26.0 (SPSS Inc., Chicago, IL) were used for statistical analyses.

RESULTS

Patient Characteristics and Treatment Outcomes

There were 1126 patients with newly diagnosed ALL during the study period in our center. Among them, 25 (2.2%) did not complete the induction treatment and lost contact, while 50 (4.4%) who were on CR without serious toxicities gave up treatment because of financial difficulties, of whom 40 were in the early intensification phase and 10 were in the consolidation phase. Ultimately, 1051 patients were enrolled in the study. At a median follow-up of 60.6 months (range, 0.8–184.5 months), the estimated 5-year OS, EFS, and CIR in the 1051 patients were 84.0 ± 1.0%, 79.0 ± 1.0%, and 17.8 ± 1.2%, respectively.

Multivariate predictors of outcome in pediatric ALL are presented in **Table 1**. In the multivariate analysis, the re-emergence of MRD during treatment was the most powerful prognostic factor for OS ($p < 0.001$, hazard ratio = 6.135), EFS ($p < 0.001$, hazard ratio = 5.848), and CIR ($p < 0.001$, hazard ratio = 7.476). The 5-year OS, EFS, and CIR for patients with re-emergent MRD were 49.8 ± 4.3%, 38.4 ± 4.2%, and 60.2 ± 4.3%, respectively. In patients with persistently MRD-negative results, the corresponding values were significantly better, at 91.7 ± 1.0%, 88.5 ± 1.1%, and 9.2 ± 1.0% ($p < 0.001$) (**Figures 1A–C**).

Re-Emergence of Minimal Residual Disease

Among the 1051 patients, 8 died during induction therapy and 13 maintained a persistently MRD-positive status until relapse. Finally, 1030 patients achieved an MRD-negative status on BM examination. Of these patients, 150 (14.6%) ultimately developed re-emergent MRD while still on morphological CR and were the focus of this analysis. **Figure 2** depicts the study flowchart for patient disposition. Further, we analyzed the characteristics of patients with persistently MRD-negative results and re-emergent MRD (**Table 2**), and found that those with white blood cell (WBC) counts of ≥50 × 10^9/L (hazard ratio, 1.609; 95%

TABLE 1 | Factors associated with outcomes in multivariate analysis in the whole group (N = 1,051).

Variable	OS Multivariate (*p*) HR (95% CI)	EFS Multivariate (*p*) HR (95% CI)	CIR Multivariate (*p*) HR (95% CI)
Age (1–10 years)	0.032 0.709 (0.518–0.971)	0.019 0.715 (0.540–0.945)	0.054 0.728 (0.527–1.005)
WBC < 50 × 10^9/L	0.431 0.861 (0.593–1.250)	0.313 0.841 (0.624–1.177)	0.190 0.791 (0.556–1.125)
Immunophenotype(T)	<0.001 2.017 (1.365–2.982)	0.008 1.637 (1.135–2.361)	0.100 1.409 (0.935–2.123)
Day 33 MRD ≥ 0.1%	0.164 1.353 (0.884–2.070)	0.016 1.599 (1.093–2.339)	0.150 1.372 (0.894–2.106)
Week 12 MRD ≥ 0.01%	0.589 1.148 (0.696–1.893)	0.452 1.190 (0.756–1.873)	0.230 1.358 (0.8221–2.248)
Re-emergent MRD	<0.001 6.135 (4.367–8.621)	<0.001 5.848 (4.329–7.874)	<0.001 7.476 (5.405–10.309)
Risk group (high-risk)	0.001 1.795 (1.279–2.518)	0.028 1.392 (1.037–1.868)	0.120 1.289 (0.939–1.770)

MRD, minimal residual disease; WBC, white blood count; HR, hazards ratio; CI, confidence interval; EFS, event-free survival; OS, overall survival; CIR, cumulative incidence of relapse.

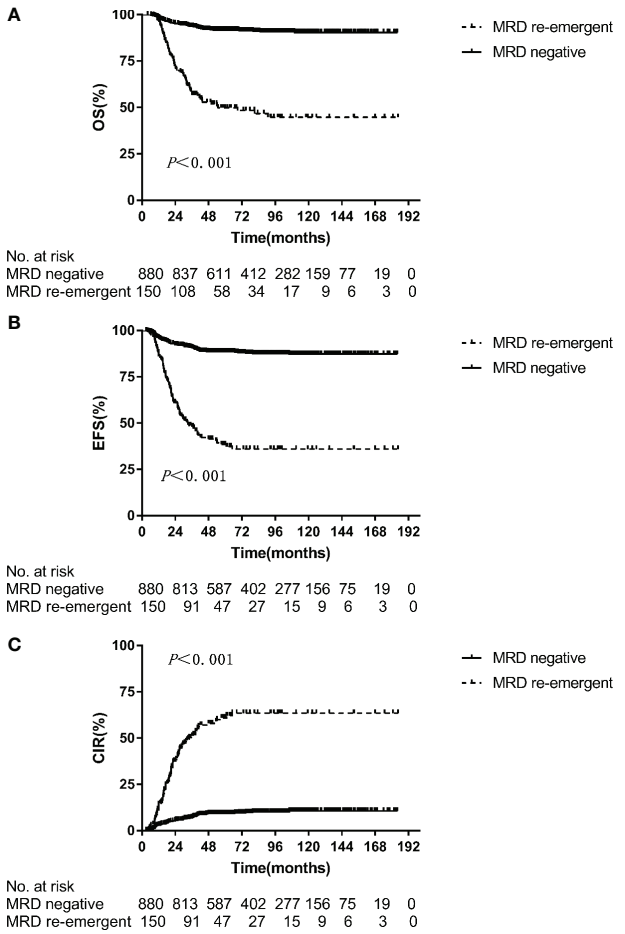

FIGURE 1 | Overall survival **(A)**, event-free survival **(B)**, cumulative incidence of relapse **(C)** for MRD negative and MRD re-emergent group.

confidence interval [CI], 1.034–2.488; $p = 0.033$) and MRD levels of ≥0.1% on day 33 (hazard ratio, 1.908; 95% CI, 1.145–3.145; $p = 0.012$) tended to have re-emergent MRD.

The overall median level for MRD positivity in the 150 patients was 0.12% (range, 0.01–10.00%). The median duration from MRD negativity to MRD re-emergence was 11 months (range, <1–52 months). Eighty-five (56.6%) patients subsequently developed relapse (78 patients with BM leukemia, 4 with BM + CNS leukemia, 2 with BM + testicular leukemia, and 1 with leukemia in other extramedullary sites) after a median of 4.1 months (range, <1–47.4 months) from the detection of MRD re-emergence. Among the 150 patients with re-emergent MRD, the median level for the first MRD-positive result was significantly higher in the relapsed cohort than in the cohort with persistent CR (1.05% vs. 0.48%, $p = 0.005$) (**Figure 3A**). To further investigate the predictive role of re-emergent MRD in relapse, we performed a receiver operating characteristic (ROC) curve analysis of the first re-emergent MRD level and the actual development of relapse. It turned out that the area under the ROC curve (AUC) was 0.631 (95% CI, 0.540–0.708; $p = 0.004$). Further, we investigated the diagnostic accuracy using different MRD levels as cutoff points. The optimal cutoff point to predict relapse was 0.15%, with a sensitivity and specificity of 61.65% and 71.69%, respectively. The median duration from MRD negativity to MRD re-emergence tended to be shorter for patients who experienced a subsequent relapse, although the differences did not reach statistical significance (11.9 vs. 15.0 months, $p = 0.068$) (**Figure 3B**).

When the $OS_{MRD\text{-}r}$ was evaluated from the time of MRD re-emergence to the last follow-up, its median value was 20.6 months in the 150 patients. To determine if the time of MRD re-emergence had an effect on the outcome of ALL, patients were divided into two groups: <12 months and ≥12 months from MRD negativity to MRD re-emergence. No statistically significant differences in 2-year $OS_{MRD\text{-}r}$ (60.6 ± 6.2% vs. 54.0 ± 5.5%, $p = 0.745$) and 2-year $CIR_{MRD\text{-}r}$ (49.7 ± 6.4% vs. 56.8 ± 5.5%, $p = 0.582$) were found between the two groups. Regarding treatments prior to morphological relapse among these 150 patients, 113 patients continued to receive maintenance chemotherapy according to the specified treatment protocol and 37 underwent allo-HSCT (30 from haploidentical donors, 5 from HLA-identical sibling donors, and 2 from matched unrelated donors). Moreover, the HSCT group showed significantly better 2-year $OS_{MRD\text{-}r}$ (88.7 ± 5.3% vs. 46.3 ± 4.8%, $p < 0.001$) and 2-year $CIR_{MRD\text{-}r}$ (23.3 ± 7.4% vs. 64.0 ± 4.6%, $p < 0.001$) than the chemotherapy group (**Figures 4A, B**).

DISCUSSION

With a median follow-up of 60.6 months, this single-institution trial showed that the 5-year OS and EFS of the 1051 pediatric patients with ALL were 84.0 ± 1.0% and 79.0 ± 1.0%, which are comparable to the results of other studies (15–18). Multiple studies have established MRD detection as an independent prognostic factor for ALL and have demonstrated that achievement of an MRD-negative status could lead to better clinical outcomes (3, 19, 20). In our study, we applied FCM for sequential post-remission MRD measurement and found that patients with higher end-induction MRD levels (≥0.1%) and positive levels of MRD on week 12 (≥0.01%) exhibited a worse EFS and OS. However, in the multivariate analysis of the whole cohort, MRD at any particular time point did not show a strong prognostic significance. We calculated that there may be two reasons for this result. First, the risk stratification of the patients in this study was adjusted based on the MRD level at end-induction and week 12. Therefore, risk stratification-oriented treatment may affect the results. Second, MRD may not show a strong prognostic significance on the context of MRD-guided therapy and MRD alone was not sufficient to fully predict outcomes. The significance of MRD on treatment outcomes varied depending on leukemia subtypes and measurement time, such as different genotypes. Meanwhile, re-emergent MRD during treatment was the most powerful adverse prognostic indicator, even after adjusting for other risk factors. The 5-year OS (91.7 ± 1.0% vs. 49.8 ± 4.3%, $p < 0.001$) and EFS (88.5 ± 1.1% vs. 38.4 ± 4.2%, $p < 0.001$) were significantly better

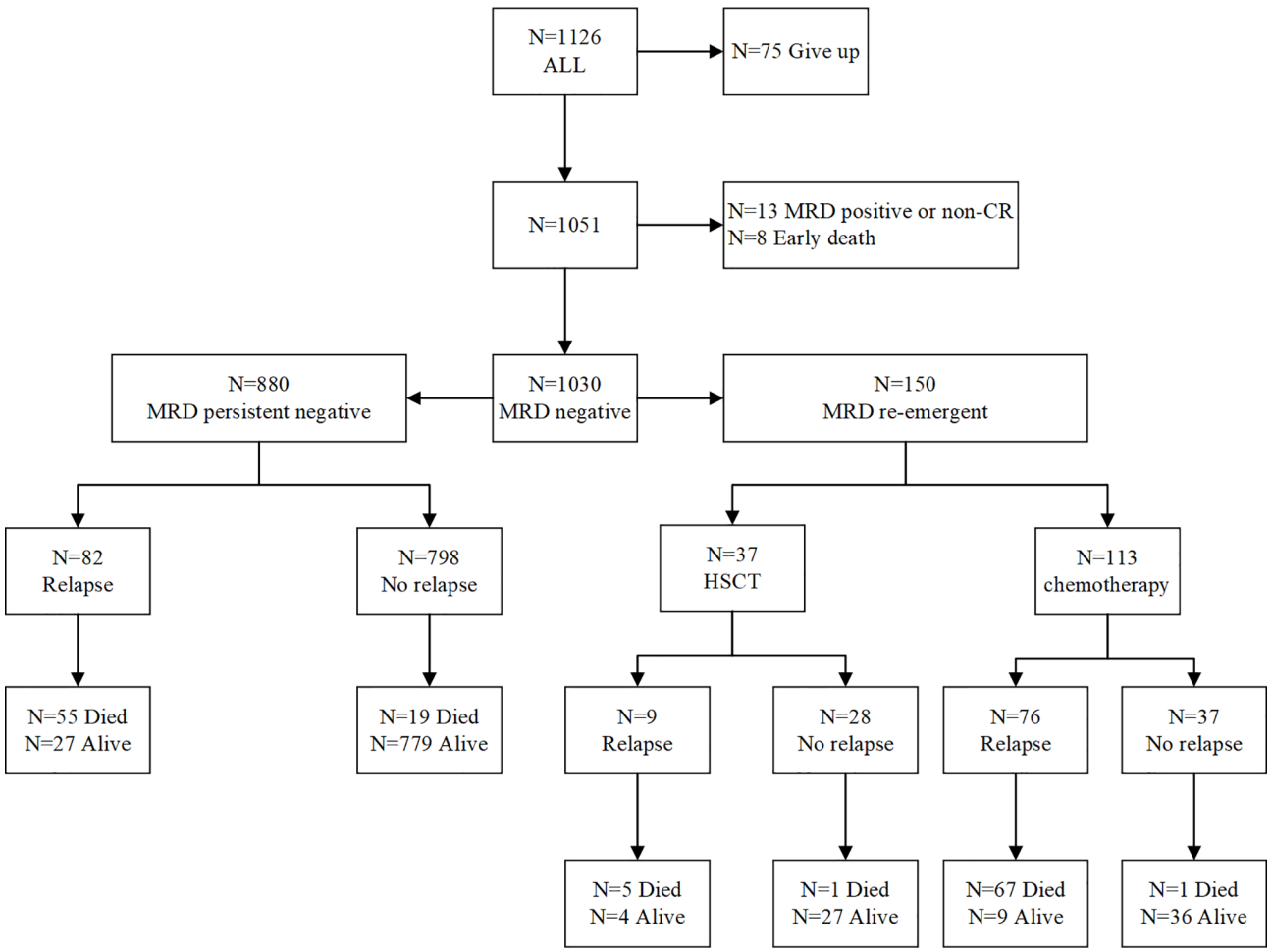

FIGURE 2 | Patient disposition: study flowchart.

in the persistently MRD-negative group. This obvious survival gap strongly confirmed the poor prognostic significance of MRD re-emergence in ALL.

In this study, 14.6% (150/1030) of pediatric ALL patients experienced MRD re-emergence while still on morphological CR. Patients with a high leukemia burden (WBC ≥ 50×10^9/L) and a poor response to early treatment (MRD levels ≥ 0.1% on day 33) were prone to MRD re-emergence, which indicates the need for further strengthening MRD monitoring in these patients. Several previous investigations demonstrated the clinical potential and prognostic value of FCM- or PCR-based MRD quantification in the post-remission setting, producing lead times from clinical relapse of 3.6 to 4.1 months (6, 21). In our analysis, 85 (56.6%) patients subsequently developed relapse after a median of 4.1 months from the detection of re-emergent MRD, and this finding was consistent with those of previous studies. Additionally, a strong correlation was observed between re-emergent MRD levels and clinical relapse, suggesting that a higher re-emergent MRD level (cutoff, 0.15%) may signify an impending relapse. It was worth mentioning that a total of five patients had a re-emergence of MRD > 2%, but never developed a morphologic relapse. One of the patients had a large deletion of IKZF gene. He started taking tyrosine kinase inhibitors after MRD recurrence, and continued to survive disease-free. As of the last follow-up date, he had been followed up for 61.6 months. The other four patients all chose further transplantation rescue treatment after MRD recurrence, and all of them survived disease-free.

As re-emergent MRD can reliably predict clinical relapse, we should monitor MRD sequentially to expand the time window for a more effective preemptive treatment against a potential relapse (22, 23). In this retrospective study, patients with re-emergent MRD were given the choice between HSCT or chemotherapy according to their preference. The results showed that the HSCT group had a significantly higher survival advantage than the chemotherapy group. Re-emergent MRD may be a group of residual leukemia cells that are out of the detection range of FCM and resistant to chemotherapy (3, 20). Although the intensification of chemotherapy may not fully eliminate re-emergent MRD, the strong graft-versus-leukemia

TABLE 2 | Characteristics of patients with MRD-negative (n=880) and MRD-re-emergent (n=150).

Variables	MRD-re-emergent		MRD-negative		*p*
	N	%	N	%	
Sex					0.997
Male	91	60.6	534	60.6	
Female	59	39.3	346	39.3	
Age(years)					0.362
<1	2	1.3	9	1.0	
1–10	93	62.0	598	67.9	
≥10	55	36.3	273	31.0	
Initial WBC (10^9/L)					0.001
<50	102	68.0	705	80.1	
≥50	48	32.0	175	19.8	
Immunophenotype					0.029
Precursor B	122	81.3	773	87.8	
T	28	18.6	107	12.1	
Molecular subtype					0.538
TCF3-PBX1	9	6.0	54	6.1	
BCR-ABL1	9	6.0	62	7.0	
ETV6-RUNX1	13	8.6	143	16.2	
KMT2A-r	4	2.6	27	3.0	
Hyper-diploidy>50					0.830
Yes	23	15.3	129	14.6	
No	127	84.6	751	85.4	
Day 33 remission					0.014
Yes	144	96.0	871	98.9	
No	6	4.0	9	1.0	
Day 33 MRD					<0.001
<0.01%	81	54.0	651	73.9	
0.01%–0.1%	17	11.3	81	9.2	
0.1%–1%	29	19.3	73	8.2	
≥1%	21	14.0	63	7.1	
Week 12 MRD					<0.001
<0.01%	126	84.0	829	94.2	
0.01%–0.1%	10	6.6	22	2.5	
≥0.1%	14	9.3	29	3.2	
Risk group					<0.001
SR	20	13.3	301	34.2	
IR	88	58.6	417	47.3	
HR	42	28.0	162	18.4	

MRD, minimal residual disease; WBC, white blood count; SR, standard risk; IR, intermediate risk; HR, high risk.

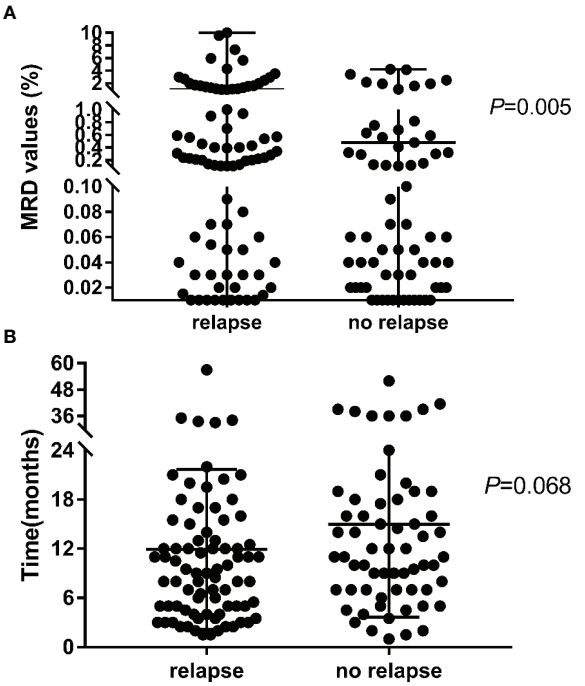

FIGURE 3 | The MRD value **(A)** and duration time **(B)** for the first MRD re-emergent in the subsequent relapse and no relapse cohort.

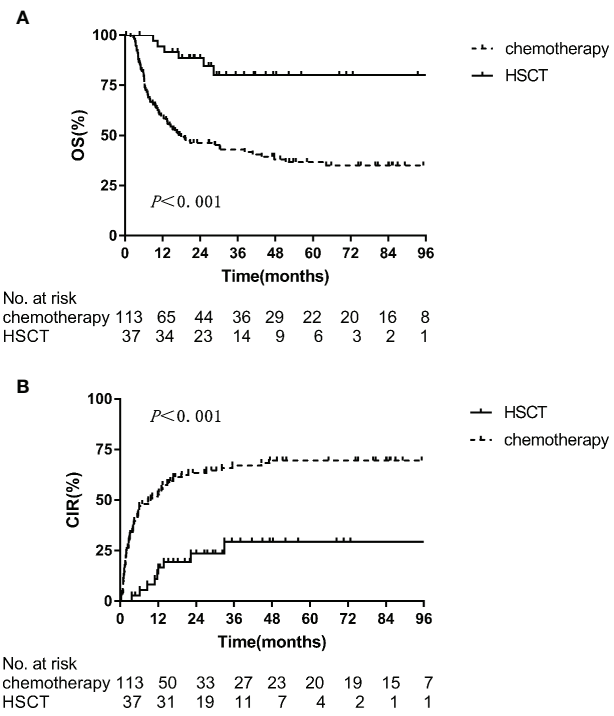

FIGURE 4 | Kaplan-Meier estimates of 2-year outcomes in the HSCT and chemotherapy arms in MRD re-emergent group. **(A)** OS, **(B)** CIR.

effect of HSCT may help (24). However, the outcome of the HSCT group in this study was unsatisfactory, highlighting the urgent need for novel, less toxic strategies and enrollment of subjects in clinical trials specifically designed for ALL patients with MRD persistence or re-emergence.

This study has a few limitations. It is a retrospective single-center study without predetermined enrollment criteria. Limited by the availability of donors and patients' preference of whether to undergo HSCT, we were unable to define the indications of transplantation in advance. Furthermore, technical constraints such as low tumor burden, immunophenotypic shifts, and clonal selection may have contributed to a decreased sensitivity of measurements, leading to more false-negative results, as suggested by the occurrence of relapse in patients with negative FCM-MRD findings (25). Recent studies have described a highly sensitive next-generation sequencing platform to monitor MRD and have observed the conversion

of an MRD-negative status to a positive one as early as 25.6 weeks prior to clinical relapse (21). Besides, patients in this study did not undergo a unified treatment escalation after the recurrence of MRD, because although multiple studies have confirmed the poor prognosis of MRD re-emergence, the current international standards have not yet reached a consensus on the treatment of risk escalation after the recurrence of MRD. However, we believe that the findings of this study will provide more powerful evidence to support the future treatment options for patients with re-emergent MRD.

In conclusion, this study revealed that MRD re-emergence at any time after induction and consolidation therapy was associated with relapse in pediatric ALL patients. We also found that patients with re-emergent MRD could benefit from HSCT, reflecting the necessity of sequential MRD monitoring for better risk stratification and earlier preemptive therapies against impending relapse, thus potentially improving outcome for pediatric B-ALL. Prospective studies on sequential MRD monitoring coupled with less toxic strategies such as chimeric antigen receptor T cell therapy designed to eradicate MRD are warranted to address the unmet medical needs of pediatric ALL patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Material preparation and data collection were performed by Y-PJ, Y-XZ, and A-DL. Data analysis was performed and the first draft of the manuscript was written by YW and Y-JX, and they contributed equally to this work. L-PZ designed the research and was the chief person in charge of the manuscript. All authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Foundation of 2018 Beijing Key Clinical Specialty Construction Project-Pediatrics (2199000726).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.596677/full#supplementary-material

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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.

frontiers in Oncology

ORIGINAL RESEARCH
published: 12 April 2021
doi: 10.3389/fonc.2021.647361

Factors Modifying Outcome After MIBG Therapy in Children With Neuroblastoma—A National Retrospective Study

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Edited by:
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Monica Davini, University of Arizona, United States

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Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Oncology

Received: *29 December 2020*
Accepted: *16 March 2021*
Published: *12 April 2021*

Citation:
Ussowicz M, Wieczorek A, Dłużniewska A, Pieczonka A, Dębski R, Drabko K, Goździk J, Balwierz W, Handkiewicz-Junak D and Wachowiak J (2021) Factors Modifying Outcome After MIBG Therapy in Children With Neuroblastoma—A National Retrospective Study. Front. Oncol. 11:647361. doi: 10.3389/fonc.2021.647361

Background: Neuroblastoma is the most common pediatric extracranial tumor with varied prognoses, but the survival of treated refractory or relapsing patients remains poor.

Objective: This analysis presents the outcomes of children with neuroblastoma undergoing MIBG therapy in Poland in 2006-2019.

Study Design: A retrospective cohort of 55 patients with refractory or relapsed neuroblastoma treated with I-131 MIBG in Poland in 2006-2019 was analyzed. The endpoints were overall survival (OS), event-free survival (EFS), cumulative incidence (CI) of second cancers and CI of hypothyroidism. Survival curves were estimated using the Kaplan-Meier method and compared between the cohorts by the log-rank test. Cox modeling was adopted to estimate hazard ratios for OS and EFS, considering factors with $P < 0.2$.

Results: Fifty-five patients with a median age of 78.4 months (range 18-193) with neuroblastoma underwent one or more (4 patients) courses of MIBG I-131 therapy. Fifteen patients were not administered chemotherapy, 3 children received standard-dose chemotherapy, and 37 patients were administered high-dose chemotherapy (HDCT) (busulfan-melphalan in 24 and treosulfan-based in 12 patients). Forty-six patients underwent stem cell transplantation, with autologous (35 patients), haploidentical (6), allogeneic (4), and syngeneic grafts (1). The median time from first MIBG therapy to SCT was 22 days. Children with relapsing tumors had inferior OS compared to those with primary resistant disease (21.2% vs 58.7%, p=0.0045). Survival was better in patients without MYCN gene amplification. MIBG therapy was never curative, except in patients further treated with HDCT with stem cell rescue irrespective of the donor type.

31 patients were referred for immune therapy after MIBG therapy, and the 5-year OS in this group was superior to the untreated children (55.2% vs 32.7%, p=0.003), but the difference in the 5-year EFS was not significant (25.6% vs 32.9%, p=ns). In 3 patients, a second malignancy was diagnosed. In 19.6% of treated children, hypothyroidism was diagnosed within 5 years after MIBG therapy.

Conclusion: MIBG therapy can be incorporated into the therapeutic strategy of relapsed or resistant neuroblastoma patients as preconditioning with HDCT rather than stand-alone therapy. Follow-up is required due to the incidence of thyroid failure and risk of second cancers.

Keywords: MIBG, high-dose chemotherapy, pediatric, hematopoietic stem cell transplant, treosulfan-based conditioning, busulfan and melphalan

INTRODUCTION

Neuroblastoma, which is derived from sympathoadrenal progenitor cells within the neural crest, is the most common extracranial tumor in children and shows hallmarks of neural tissue, such as the production of catecholamines and the expression of neurotrophin receptors (1). The outcome correlates with biological and clinical factors such as age, tumor stage, histology, and genetic profile, with MYCN protooncogene amplification being a prominent unfavorable risk factor (2). The prognosis varies, ranging from spontaneous regression to fatal outcomes despite aggressive therapy; in particular, in refractory or relapsing disease, survival remains very poor, with a 10-year survival probability below 15% (3). The search for therapeutics to improve the prognosis of this group of patients is warranted, but no significant break-through has been achieved in chemotherapy for the last 20 years. Because high-risk neuroblastoma patients benefit from consolidation megatherapy, many attempts have been made to improve the efficacy of this treatment. The idea of megatherapy is based on the administration of high-dose chemotherapy (HDCT) aimed at eradicating the primary malignancy, and bone marrow damage manifesting as myeloablation or myelosuppression must be subsequently managed by hematopoietic stem cell transplantation from autologous (autoSCT) sources or allogeneic (alloSCT) or haploidentical (haploSCT) donors. The results of chemotherapy and HDCT in neuroblastoma still leave a room for improvement that can be achieved by exploiting biological characteristics of malignant cells. Neural differentiation features can be used for targeted therapy in neuroblastoma and improve the clinical outcome. Ninety percent of neuroblastomas express NET, a 12-domain transmembrane protein encoded by the SLC6A2 gene with high affinity and specificity for norepinephrine and its analogs, which enables uptake and targeted therapy with I-131 radiolabeled metaiodobenzylguanidine (MIBG) (4). The indications of I-131 MIBG therapy include treatment-resistant neuroblastoma, unresectable or metastatic pheochromocytoma and paraganglioma, unresectable or metastatic carcinoid tumors, and unresectable or metastatic medullary thyroid cancer (5). The primary toxicity of I-131 MIBG therapy is hematologic, with stem cell rescue typically required with doses ≥ 15 mCi/kg (6). Based on the results of the study by Matthay et al., the MIBG dose given in megatherapy is usually 12 mCi/kg, which corresponds to a total whole body dose of 2.74 to 5.2 Gy absorbed by a patient (7, 8). The role of MIBG therapy in neuroblastoma has been confirmed, but its place in the therapeutic strategies remains unclear, and different approaches have been studied over the years. This analysis presents the results of children with neuroblastoma undergoing MIBG therapy in Poland in 2006-2019.

PATIENTS AND METHODS

A retrospective cohort of 55 patients with refractory or relapsed neuroblastoma treated with I-131 MIBG in Poland in 2006-2019 was analyzed. The patient characteristics and treatment stratifications are presented in **Table 1**.

The prerequirement for therapy was an avid MIBG disease within 3 months prior to therapy and unfavorable prognostic factors such as relapse or primary chemoresistance. The stage at initial diagnosis (in refractory patients) or at relapse (in relapsed children) and response at subsequent follow-up visits were assessed by a review of CT scans, bone marrow biopsies and MIBG scans and evaluated according to the INRC criteria of response (9, 10). The patients were grouped according to the response to first-line chemotherapy as patients with primary refractory (or persistent disease) or as relapsed patients who had disease recurrence at any time prior to study enrollment. The group of primary refractory patients consisted of 27 children not achieving complete remission (CR) after first-line chemotherapy, that corresponded to PR (partial response) in 21 patients, SD (stable disease) in 1 child, MR (mixed response), or PD (progressive disease) in 5 patients according to the INRC criteria. Among the 22 relapsing patients, 18 suffered from 1 relapse and 4 from multiple relapses. Autologous peripheral blood stem cells (PBSCs) were collected if bone marrow evaluation showed complete remission. The target CD34+ cell dose yield was 3×10^6/kg of the recipient's weight per autoSCT. Patients not eligible to the autologous PBSC apheresis due to poor mobilization were qualified for allogeneic stem cell

TABLE 1 | Patient characteristics.

Category		Value
Total number	Patients	55
	MIBG therapies	59
Sex	Male	32
	Female	23
Age at therapy in months	Median	78.4
	Range	18.3-193.2
MYCN status	Amplified	10
	Non-amplified	26
	Unknown	19
Disease stage	Stage 3	2
	Stage 4	53
Disease status	Relapsed	22
	Resistant	27
	Unknown	6
Interval from diagnosis to MIBG therapy (in months)	Median	15.6
	Range	5.8-133
MIBG-activity in mCi	Median	300
	Range	100-500
MIBG-activity in mCi/kg	Median	17.85
	Range	6.25-26.6
Post-MIBG Curie score	Median	4
	Range	0-30
Post-MIBG Curie score	Standard risk ≤2	25
	High risk >2	28
Extraosseus	Yes	16
MIBG uptake	No	35
Post-MIBG chemotherapy	None	15
	Standard dose	3
	HDCT	37
High dose chemotherapy	Busulfan-melfalan	24
	Treosulfan-melfalan-thiotepa	9
	Treosulfan-cyclophosphamide	3
	Fludarabine-melphalan-thiotepa	1
Incidence of SOS/VOD after MIBG therapy	Yes	5
	No	50
Interval from MIBG therapy to SCT	Median	22
	Range	13-103
Stem cell transplantation	None	11
	Autologous	37
	Allogeneic, matched sibling donor	2
	Allogeneic, matched unrelated donor	2
	Allogeneic, haploidentical donor	6
	syngeneic	1

HDCT, high-dose chemotherapy; MIBG, meta-iodobenzylguanidine; SOS/VOD, sinusoidal obstruction syndrome/veno-occlusive disease.

transplantation from related or unrelated donor. Four children referred for palliative care were scheduled for low-dose MIBG therapy and not referred for post-therapy stem cell rescue. The decisions on palliative MIBG therapy were reached in patients with advanced and progressive malignancy on individual basis and with consent of patients' families. The chart showing the grouping of treated patients is presented in **Figure 1**.

The administration of MIBG was performed at the central facility of Maria Sklodowska Curie Memorial Cancer Center and Institute of Oncology in Gliwice. The MIBG dose given in palliative treatment (without available stem cell rescue) was 8-12 mCi/kg and in megatherapy (patients with option of any SCT) - usually 18 mCi/kg. The dose of 131I-MIBG was adjusted to the upper limit of radioisotope use in the treating center (500 mCi). The patients received a median activity of 300 (range 100-500) mCi of MIBG. Median time from diagnosis to MIBG therapy was 15.6 months (range 5.8-133). All patients remained in radiation protective isolation for up to seven days after MIBG administration. Thyroid protection consisted daily of 1% solution of potassium iodide at dose of 2 mg/kg starting 72 hours before MIBG administration until 120 hour post therapy. Four days after MIBG injection the post therapy MIBG whole body scan was performed, and assessed with the Curie score (11).

After discharge from MIBG therapy, the patients were referred to stem cell transplantation units and treated with chemotherapy. The HDCT protocols consisted of busulfan or treosulfan backbone with additional alkylating agents. BuMel HDCT consisted of busulfan administered in 16 doses from day −7 to day −3 before autoSCT (at a cumulative dose of 16 mg/kg) and after 2009 intravenously (cumulative dose according to weight range: < 9 kg: 16 mg/kg; 9-16 kg: 19.2 mg/kg; 16-23 kg: 17.6 mg/kg; 23-34 kg: 15.2 mg/kg; >34 kg: 12.8 mg/kg), and melphalan was administered on day −1 in a single intravenous dose of 140 mg/m^2 or in children below 12 kg at a dose of –4 mg/kg. The TreoMelTT HDCT protocol consisted of treosulfan (14 g/m2/day for 3 days), melphalan (70 mg/m2/day for 2 days), and thiotepa (2 × 5 mg/kg BW for 1 day). One of the transplant centers used a stand-alone MIBG-megatherapy in a series of patients with or without stem cell rescue as a consolidation. The parents gave their written informed consent for the treatment and analysis of clinical data. Ethical approval was waived by the local Ethics Committee of Wroclaw Medical University in view of the retrospective nature of the study, and all procedures being performed were part of routine care.

Statistical Analysis

The endpoints were overall survival (OS), defined as the time from MIBG therapy to death or the last report from patients with no event, and event-free survival (EFS), defined as the time from MIBG therapy to progression, relapse, second malignancy or death. Survival curves were estimated using the Kaplan-Meier method and compared between the cohorts by the log-rank test. Cox modeling was adopted to estimate hazard ratios for OS and EFS, considering factors with $P < 0.2$. Statistical analysis and data formatting for presentation were performed with the computer software GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and STATISTICA 13.3 (TIBCO Software Inc. 2017, STATISTICA, version 13, Dell, OK, USA).

RESULTS

Fifty-five patients with a median age of 78.4 months (range 18-193) with neuroblastoma underwent one or more (4 patients) courses of MIBG I-131 therapy. Among 15 patients without chemotherapy, 6 required SCT due to prolonged cytopenia, and 1 died due to fulminant disease progression. Standard dose

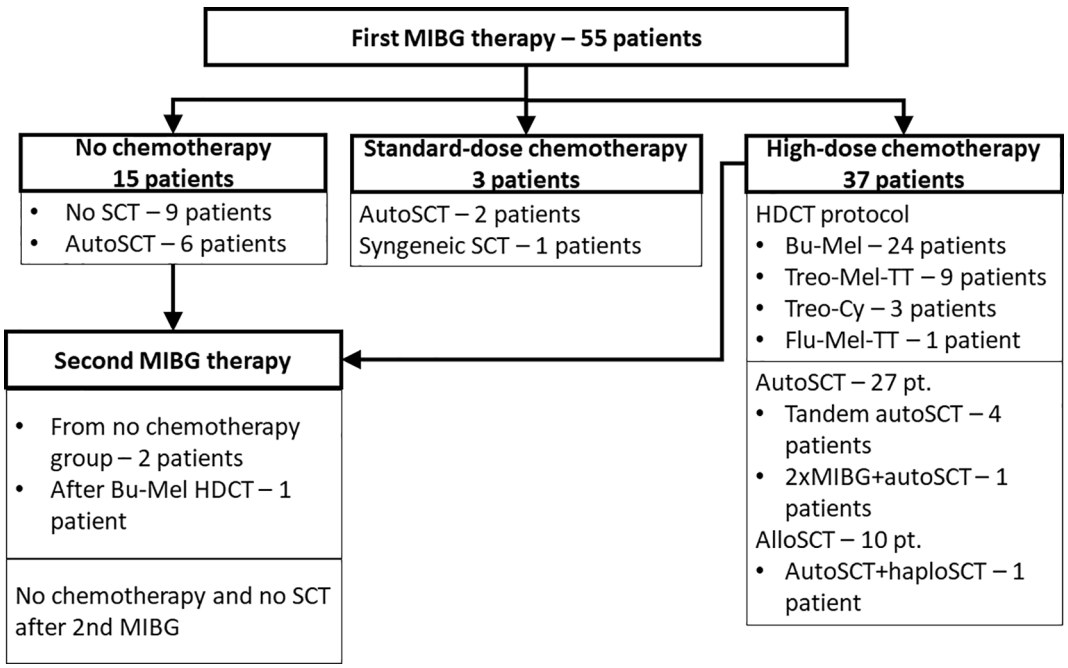

FIGURE 1 | Distribution of patients treated with MIBG therapy.

chemotherapy was given in 3 patients: VP-Carbo/etoposide-carboplatin in 1, and CADO/cyclophosphamide, doxorubicin and vincristine in 2 children. In 37 patients, HDCT was performed (BuMel in 24 patients, treosulfan-based therapy in 12 patients, and other in 1 patient). The grafting material was autologous in 35 children, haploidentical from the parent in 6 children, allogeneic in 4 children, and syngeneic in one child. The median time from first MIBG therapy to SCT was 22 days (range 13-103). Detailed survival results are presented in **Table 2**. In the whole group, the probability of 5-year OS was 38%, and the probability of 5-year EFS was 25.2% (**Figure 2A**). Patients with relapsing disease had inferior survival compared with those with primary resistant disease (5-year OS 15.2% vs 58.7%, p=0.001) (**Figure 2B**). The MYCN status affected survival, which was superior in patients without amplification (5-year OS 58.6% and 5-year EFS 39.1%, **Figures 2C, D**). MIBG therapy was never curative (5-year OS and 5-year EFS 0%), except in patients further treated with HDCT with stem cell rescue irrespective of their donor type (p<0.001) (**Figures 3A, B**). The HDCT protocol was based on busulfan or treosulfan in almost all patients, and the 5-year OS and 5-year EFS were not different between the groups. Sinusoidal obstruction syndrome/veno-occlusive disease (SOS/VOD) was diagnosed in 5 of 24 patients (20.8%) in the BuMel HDCT group, but not after any other chemotherapy protocols.

In the analyzed group, 10 patients were transplanted from allogeneic donors due to a lack of sufficient stem cell harvest or upfront strategy for haploidentical transplantation in relapsing tumors. HDCT resulted in better outcomes than non-HDCT, and the results of autologous SCT were similar to those of non-autologous grafts (**Figure 3C**). However, among different types of donors, haploidentical transplantations showed a trend toward worse survival, and no long-term survivors were observed after haploSCT (**Figure 3D**).

In 53 patients a post-therapy MIBG whole-body scan was performed, and in 40 cases the uptake was detected, with 28 patients showing the Curie score above 2 and in 17 the extraosseus foci were observed. In 13 patients, the post-therapy scan did not reveal MIBG uptake, and 9 of these patients showed subsequent relapse and 7 died of disease.

Among 55 patients, 31 were referred for immune therapy after MIBG therapy, and the 5-year OS in this group was superior to the untreated children (55.2% vs 32.7%, p=0.003, **Figure 3E**), but the difference in the 5-year EFS was not statistically significant (25.6% vs 32.9%, p=ns, **Figure 3F**).

In the Cox multivariate analysis, autoSCT and post-MIBG immune therapy emerged as the only factors significantly associated with improved OS (hazard ratio 0.58, 95% CL 0.17-1.93; P=0.04; and hazard ratio 0.08, 95% CL 0.0-0.72; P=0.02). The other factors were not significantly associated with either OS or EFS.

Adverse Effects and Second Malignancies

In 19.6% of treated children, hypothyroidism was diagnosed within 5 years of MIBG therapy (**Figure 4**). In 3 patients, a second malignancy was diagnosed, which corresponded to a 5-year cumulative incidence of 10.9%. In a girl coded as UPN 26, diffuse large B-cell lymphoma of the brain was diagnosed, and the child died as a consequence of it. In patients UPN 32 and UPN 33, myelodysplastic syndrome with excess blasts (MDS-EB)

TABLE 2 | Survival after MIBG therapy.

		Number of patients	5 year OS	log rank p	5 year EFS	log rank p
All patients		55	38%	n/a	25.2%	n/a
Sex	Male	32	51.9%	ns	31.3%	ns
	Female	23	29%		19.7%	
Disease status	Resistant	27	58.7%	p=0.0045	45%	p=0.0003
at MIBG therapy	Relapsed	22	21.2%		11.4%	
	Not specified	6	0%		0%	
MYCN status	Amplified	10	26.7%	p=0.02	26.7%	p=0.0036
	Not amplified	26	58.6%		39.1%	
	Unknown	19	19.8%		6.3%	
Post-MIBG	≤2	25	42.2%	ns	32.1%	ns
Curie score	>2	28	36.7%		25.7%	
Extraosseus	Yes	17	41.7%	ns	30%	ns
MIBG uptake	No	36	36.8%		27.4%	
Chemotherapy	No	15	0%	p<0.0001	0%	p<0.001
	Standard	3	0%		0%	
	HDCT	37	53.8%		37.1%	
HDCT	Busulfan	24	43.9%	ns	31%	ns
	Treosulfan	12	66.7%		50%	
SCT	No	9	0%	p=0.03	0%	ns
	Auto	35	47.1%		31.2%	
	Allo/haplo/syn	11	43.6%		27.3%	
Anti-GD2	No	20	32.7%	p=0.003	32.9%	ns
immunotherapy	Yes, after MIBG	31	55.2%		25.6%	
	Yes, prior to MIBG	4	0		0	

Allo, allogeneic; auto, autologous; HDCT, high-dose chemotherapy; n/a, not applicable; ns, not significant; SCT, stem cell transplantation; syn, syngeneic.

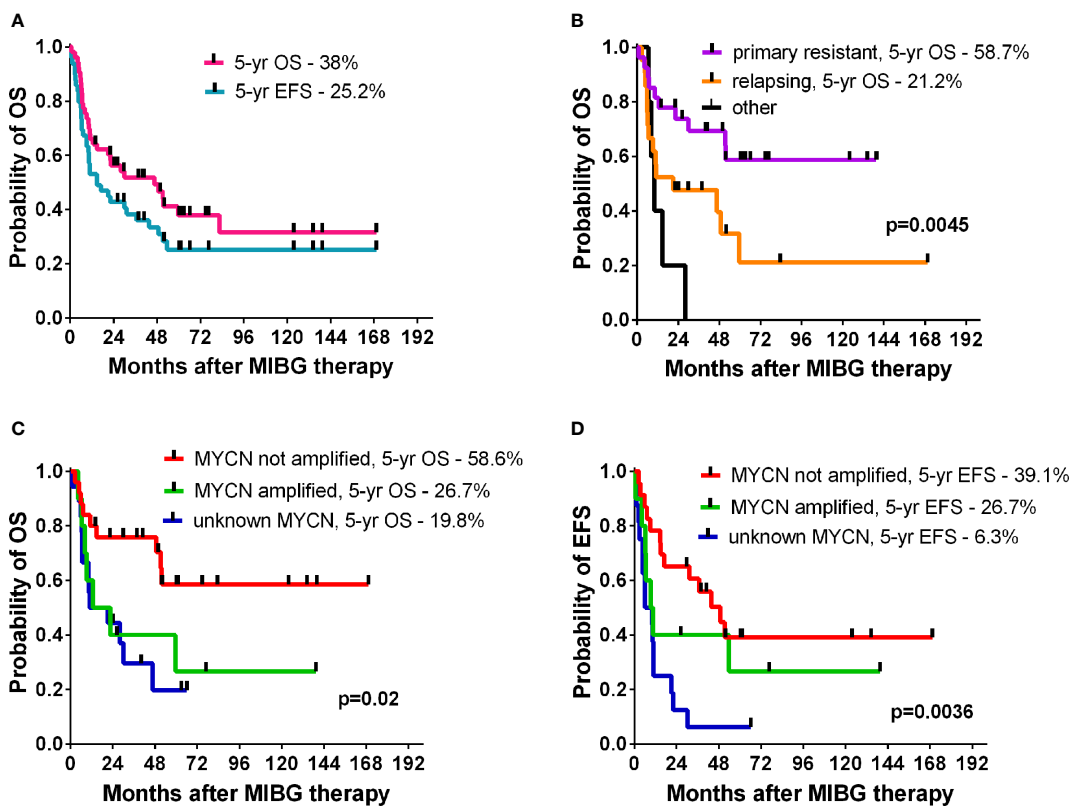

FIGURE 2 | The OS and EFS probabilities of patients treated with MIBG therapy **(A)**, OS in relapsing or resistant disease **(B)**, impact of MYCN status on OS **(C)** and EFS **(D)**.

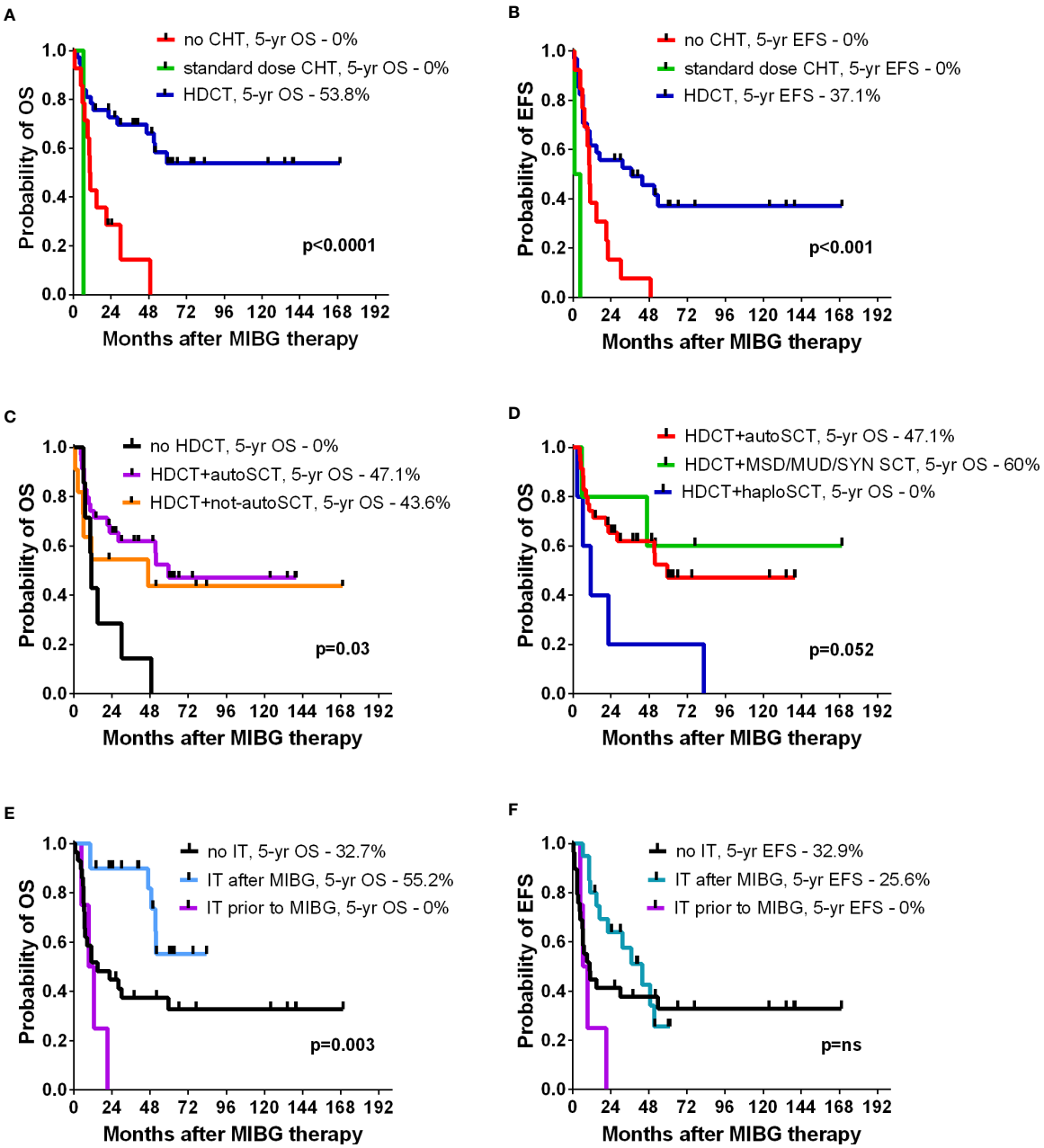

FIGURE 3 | The effect of chemotherapy on OS **(A)** and EFS **(B)**. OS in patients with or without HDCT **(C)**, and impact of different donor types on OS **(D)**. The effect of immune therapy (IT) on OS **(E)**, and EFS **(F)**.

was diagnosed. Both patients with MDS-EB were transplanted from matched donors and are alive and well.

DISCUSSION

I-131 MIBG is one of the successful examples of theranostic radiopharmaceuticals but requires specialized facilities with appropriate personnel, radiation safety equipment, procedures available for waste handling and disposal, the handling of contamination, monitoring personnel for accidental contamination and the controlling contamination spread, which are limiting factors of the widespread use of this therapy (12, 13). Despite the high number of neuroblastoma patients treated with MIBG-megatherapy, the mean tumor response rate in 25 studies was estimated at 32%, but the meta-analysis by Wilson et al. showed high heterogeneity of analyzed studies and a lack of randomized controlled trials (14). The 38% 5-year OS in

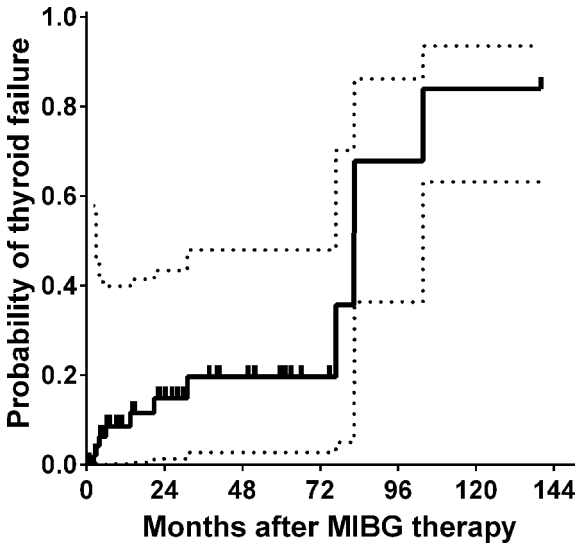

FIGURE 4 | Probability of thyroid failure after MIBG therapy.

our whole study group combined the better outcome (58.7%) of patients with primary resistant disease and the worse outcome (15.2%) of patients with relapsing disease, which is consistent with that in other studies. Our study was limited by the small number and heterogeneously treated group of patients over a long period of time in different centers, but worse outcomes in relapsing patients were also observed in other studies. According to the study by Zhou et al. who reviewed the retrospective cohort of 218 patients treated with (131)I-MIBG between 1996 and 2014, the probability of 24-month OS was 47.0%, and for refractory patients was significantly higher at 65.3%, compared to 38.7% for relapsed patients ($p<0.001$) (15). In our group the respective 24-month OS results were 56.2% (in the whole group), 73.7% in refractory, and 38.5% in relapsing patients, which suggests similar early outcome despite different treatment protocols, but deteriorating survival after 5 years emphasizes incomplete and impermanent responses.

Matthay et al. reported that stand-alone MIBG therapy showed hematological toxicities (thrombocytopenia and/or neutropenia), resulting in HSCT in 33% of treated children (6). The response rate was significantly higher for patients with disease limited either to bone and bone marrow or to soft tissue (compared with patients with both) for those with fewer than three prior treatment regimens and for patients older than 12 years; OS was 49% at 1 year and 29% at 2 years, but EFS was only 18% at 1 year (6). The influence of skeletal or extraskeletal disease was not confirmed in our study, and the post-therapy whole body scan result was not associated with differences in survival. This effect might be a consequence of maturation of neuroblastoma cells in the persisting skeletal MIBG avid lesions, but no surviving patients in our study underwent the bone biopsy.

In our study, eleven of 15 children treated with stand-alone MIBG-therapy were not treated with intention of palliative care, and all of them died. Long-term survivors were noted only in the arm combining MIBG therapy with HDCT, which suggests, that the survival benefit is unlikely in stand-alone MIBG treatment. However, it must be noted that the HDCT protocol choice and time of administration require prospective study.

The combination of MIBG therapy with HDCT has raised the issue of therapy tolerance and excessive toxicities on many occasions, and different approaches have been suggested. Among posttransplant risks for neuroblastoma patients, the incidence of SOS/VOD is high. In our study, SOS/VOD incidence of 20.8% was similar to the 22% observed in the patients treated with BuMel HDCT alone (16). In a study by Yanik et al., the combination of MIBG megatherapy administered on day -21, with CEM given days -7 to -4, and SCT given on day 0 was evaluated (17). The study showed that the SOS/VOD incidence was 12%, which is similar to the 9% reported for the CEM protocol alone (16). A study by Miano showed an increased risk of toxicities induced by the addition of MIBG therapy 7 days prior to BuMel (11 patients) or BuMel with thiotepa (4 patients) HDCT but showed the feasibility of such a combination in children with neuroblastoma (18). A recent study by Giardino et al. showed that MIBG therapy administered shortly before BuMel HDCT was well tolerated, and the SOS/VOD incidence was 7.1% (19). The cumulative incidence of hypothyroidism was 31.1% in this study, the three-year and five-year rates of the cumulative risk of progression/relapse were 64% and 73%, respectively, and MYCN amplification emerged as the only risk factor significantly associated with OS (HR, 3.58; $P = 0.041$). The concern of toxicities resulted in the strategy of delayed HDCT after MIBG therapy and was studied by French et al., who administered MIBG therapy with first autoSCT and 6-8 weeks later administered BuMel with second autoSCT (20). The straightforward combination (MIBG therapy-HDCT-1st autoSCT) in our study was used in 32/37 patients, and due to the low number of tandem transplantations (MIBG therapy-1st autoSCT-HDCT-2nd autoSCT), a detailed analysis of this group was not performed. The strategy of combining MIBG therapy with BuMel as tandem transplantation is a safer approach, as demonstrated by the French group, although it requires double the amount of collected stem cells (21). The strategy of tandem transplantation reduces the chemotherapy dose intensity, which may be associated with worse efficacy, as MIBG stand-alone therapy effectiveness is less potent than combination with HDCT. In addition, some reports have suggested that the toxicities after combination therapy are manageable and do not significantly differ from megachemotherapy alone. Preconditioning with MIBG is feasible with treosulfan-melphalan-thiotepa HDCT, and the 2-week interval between both therapies was not associated with excessive toxicities (22). The combination of MIBG preconditioning with megachemotherapy can reduce the need for a high stem cell yield in pretreated patients, which reduces the delay due to ineffective stem cell mobilizations. Another strategy for patients with inadequate autologous stem cell harvest is the choice of allogeneic donor, but this option is not actively encouraged today

due to limited evidence for graft versus tumor effects against neuroblastoma and the availability of plerixafor, which improves the autologous stem cell yield (23, 24). Indeed, no benefit of haploSCT was observed in our study despite the promising results reported by Illhardt et al. (25).

According to the SIOPEN study group, the recommended HDCT protocol in neuroblastoma is BuMel (16). Recently, treosulfan has been viewed as a promising option, especially because of the data supporting the *in vitro* activity of treosulfan in neuroblastoma lines (26). In our study, 12 children were treated with treosulfan-based HDCT protocols and achieved solid survival rates (5-year OS – 66% and 5-year EFS – 50%). This observation can support the planning of treosulfan studies in neuroblastoma patients who are not eligible for busulfan-based HDCT.

The post MIBG therapy whole body scan in high-risk neuroblastoma patients may reveal hidden mIBG-avid lesions, which are under the level of detection by diagnostic 123I-MIBG images (27–29). The fact, that in 13 patients the MIBG uptake was absent can be explained by a time interval between the qualification and administration of the therapy, during which patients received chemotherapy. In the analyzed group, skeletal or extraskeletal uptake and Curie scoring were not associated with different survival, but it can be explained by the fact, that Curie score has been validated to assess the postinduction response in neuroblastoma patients, and not in the megatherapy settings (30).

Immune therapy with dinutuximab or dinutuximab beta is associated with the most important recent treatment improvement in neuroblastoma, but it has not been studied in patients undergoing the MIBG therapy (31, 32). Through 2014 immunotherapy with dinutuximab beta was not available as a therapeutic option for Polish children and those who received it did so abroad within the LTI (Long-Term Infusion study, EudraCT 2009-018077-31). In children referred for immune therapy an overall survival advantage was observed, but not the probability of 5 year EFS. Interestingly, four of 15 patients not receiving the chemotherapy were treated with immune therapy after MIBG (and 3 - before), and all of them died. The impact of immunotherapy after MIBG therapy should be studied in a prospective trial in a more homogenous cohort, and from our data no further conclusions can be drawn.

The prevention and early diagnosis of long-term sequelae are now important issues in pediatric oncology. Thyroid protection is an issue that warrants attention in patients treated with MIBG megatherapy. Clement et al. studied long-term survivors after MIBG therapy and showed that at a median follow-up of 9.0 years, the incidence of thyroid disorder was 50% (12/24 patients); among them, 5 were diagnosed with a thyroid nodule, and 1 patient was subsequently diagnosed with differentiated thyroid carcinoma (33). At 5 years after MIBG therapy, we observed a cumulative incidence of thyroid failure of 19%, and among the long-term survivors, the incidence could increase to over 80%, but the number of observed patients was very low. The risk of second malignancies also needs to be considered in patients after MIBG therapy. The review of 644 patients treated with MIBG therapy performed by Huibregtse et al. showed a second malignant neoplasm cumulative incidence of 7.6% and 14.3% at 5 and 10 years from the first therapy, with myelodysplastic syndromes/therapy-related acute myeloid leukemia being most common (10/19 patients), followed by solid tumors (inflammatory myofibroblastic tumors, bone and soft tissue sarcomas, and thyroid cancers) (34). In our group, 3 patients developed second malignancies, but it is not possible to associate the incidence of MDS with either MIBG therapy or HDCT because the patients were treated with intensive chemotherapy before referral for HDCT. In the third case, the diagnosis of lymphoma was associated with T-cell–depleted haploidentical transplantation and subsequent EBV replication.

CONCLUSION

Prospective, randomized controlled trials are needed to optimize therapeutic strategies incorporating MIBG therapy in patients with neuroblastoma. Currently, 2 ongoing studies are focusing on MIBG, the upfront Children Oncology Group NCT03126916 study and SIOPEN VERITAS study (NCT03165292). MIBG therapy can be incorporated into the therapeutic strategy of relapsed or resistant neuroblastoma patients, but our analysis suggests that the advantage is achieved by using MIBG therapy as preconditioning with HDCT rather than stand-alone therapy.

Due to the lack of systematic publications on the effects of immune therapy in patients undergoing MIBG therapy, such analysis is necessary in uniform cohorts of treated children.

According to our observations, MIBG therapy patients require endocrinological follow-up due to the incidence of thyroid gland failure and the risk of second cancers.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: available on request. Requests to access these datasets should be directed to ussowicz@o2.pl.

ETHICS STATEMENT

The parents gave their written informed consent for the treatment and analysis of clinical data. Ethical approval was waived by the local Ethics Committee of Wroclaw Medical University in view of the retrospective nature of the study, and all procedures being performed were part of routine care.

AUTHOR CONTRIBUTIONS

Concept, data collection, analysis, writing, and final draft: MU. Data collection, patient care, and manuscript acceptance: AW, AD, AP, RD, KD, JG, WB, DH-J, and JW. All authors contributed to the article and approved the submitted version.

FUNDING

Source of support- Wroclaw Medical University statutory grant ST.C200.18.013 and SUB.C200.21.058.

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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.

frontiers in Pediatrics

PERSPECTIVE
published: 19 July 2021
doi: 10.3389/fped.2021.713091

A Guidance for Concomitant Drug Reconciliation Prior to Allogeneic Hematopoietic Cell Transplantation in Children and Young Adults

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OPEN ACCESS

Edited by:
Emmanuel Katsanis, University of Arizona, United States

Reviewed by:
Pooja Khandelwal, University of Cincinnati College of Medicine, United States
Alix Eden Seif, Children's Hospital of Philadelphia, United States

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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: 21 May 2021
Accepted: 23 June 2021
Published: 19 July 2021

Citation:
Apsel Winger B, Long SE, Brooks J, Gupta AO, Dvorak CC and Long-Boyle JR (2021) A Guidance for Concomitant Drug Reconciliation Prior to Allogeneic Hematopoietic Cell Transplantation in Children and Young Adults. Front. Pediatr. 9:713091. doi: 10.3389/fped.2021.713091

Pediatric diseases treated by allogeneic hematopoietic stem cell transplantation (alloHCT) are complex and associated with significant comorbidities and medication requirements that can complicate the transplant process. It is critical to reconcile pre-transplant concomitant medications (pcon-meds) in the weeks prior to alloHCT and to consider the potential for pcon-meds to cause harmful drug-drug interactions (DDIs) or overlapping toxicities with conditioning agents. In this perspective, we describe a systematic process to review pcon-meds and determine the drug modifications needed to avoid DDIs with conditioning regimens. We provide an extensive appendix with timelines for discontinuation or modification of common pcon-meds that patients are taking when presenting to the HCT medical team. The timelines are based on the pharmacokinetic (PK) properties of both the pcon-meds and the planned conditioning medications, as well as anticipated DDIs. They also account for the ages seen at pediatric transplant centers (0–30 years old). Common scenarios, such as when pcon-med discontinuation is not an option, are discussed. Since alloHCT patients are often dependent upon psychiatric medications with problematic DDIs, a table of alternative, non-interacting psychiatric medications is also presented. The appendix provides details regarding how to adjust pcon-meds prior to the start of chemotherapy for children and young adults undergoing alloHCT, however patient-specific circumstances always need to be taken into account. Careful attentiveness to pcon-meds at the time the decision is made to pursue transplant will result in more consistent HCT outcomes, with lower toxicity and increased efficacy of conditioning agents.

Keywords: drug–drug interactions, pharmacokinetics, pediatric, hematopoietic cell transplantation, concomitant medication, chemotherapy

INTRODUCTION

Indications for allogeneic hematopoietic cell transplantation (alloHCT) in children and young adults range from malignant disorders such as high-risk leukemia to nonmalignant conditions such as primary immunodeficiencies, hemoglobinopathies, and inherited metabolic disorders (1). Pediatric diseases treated by alloHCT are complex and are often associated with significant comorbidities and medication requirements that can further complicate the transplant process (2, 3). Therefore, it is critical to reconcile the medications that patients are taking when they first present to the HCT medical team, referred to as pre-transplant concomitant medications (pcon-meds), in the weeks leading up to alloHCT. The medication reconciliation process evaluates the potential for pcon-meds to cause harmful drug-drug interactions (DDIs) or exacerbate toxicities with conditioning agents. DDIs can either increase or decrease the exposure of various conditioning agents, and thereby negatively impact outcomes by contributing to severe drug-related toxicities, graft rejection, and disease relapse.

Pcon-meds vary widely in their potential to cause harmful DDIs with alloHCT conditioning. The detailed mechanisms by which DDIs result in altered pharmacokinetics (PK) and pharmacodynamics (PD) are well described elsewhere in the literature (4). The goals for this perspective are: (a) to provide an overview of ideal medication changes prior to the start of conditioning, and, (b) to provide a framework for assessing the potential impact of pcon-meds on HCT conditioning agents. As an extensive resource, **Appendix 1** lists common pcon-meds, provides recommendations for the optimal timing for discontinuation of pcon-meds in relation to the start of conditioning chemotherapy, and also provides potential medication alternatives for commonly used psychiatric medications that have problematic DDIs.

BACKGROUND

Impact of Pcon-Meds on Cytochrome P450 (CYP450) Enzymes

Most clinical trials evaluating new therapies enroll a homogeneous population with strict eligibility criteria for limiting DDIs. Thus, the potential effects of pcon-meds are rarely evaluated formally in a real-world setting. Instead, the potential for a pcon-med to alter the PK or PD of a conditioning agent is often extrapolated based on the known impact of similar drugs "in-class" or based on individual case reports. For example, the potential for pcon-meds to alter drug metabolism outside the setting of alloHCT is often well-understood and thus applied to how these medications may impact conditioning agents used during alloHCT. Pcon-meds most commonly alter the metabolism of conditioning agents via induction or inhibition of cytochrome p450s or via interference with drug transporters (**Figure 1**) (4). This can have a profound effect on the exposure and efficacy of conditioning agents. If metabolism is induced, the conditioning agent can be less effective; if metabolism is inhibited, the conditioning agent can have increased toxicity. Aside from busulfan, therapeutic drug monitoring is not routine for conditioning agents, therefore drug exposure cannot be tracked in real-time and the dose of the conditioning agent cannot be adjusted to counteract the impact of interfering pcon-meds. Therefore, it is critical to be aware of the potential impact of pcon-meds on metabolism with enough time to make medication adjustments prior to alloHCT conditioning and avoid DDIs whenever possible. When the clinical scenario makes it impossible to avoid a DDI, awareness of the potential for a DDI can direct appropriate clinical monitoring for toxicity.

The enzymatic system responsible for the majority of phase one metabolism leading to elimination of both endogenous substrates and xenobiotics is the hepatic cytochrome P450 (CYP450) system (5). There are multiple mechanisms by which CYP450 enzymes can be modulated by medications (4). The mechanisms include direct competitive inhibition at the CYP450 active site, induction of CYP450 protein synthesis, and disruption of CYP450 transcription, translation and/or post-translational processing (6). The mechanism of CYP450 dysregulation dictates the time it will take for the enzyme to recover, and is complicated by the fact that different CYP450 isoforms have different half-lives (6). In addition, CYP450 synthesis is a zero order process (meaning not dependent on the concentration of CYP450 enzyme in the body), while CYP450 degradation is a first order process (meaning it is dependent on the concentration of enzyme in the body) (6–8). This is an important difference because it means that synthesis and degradation of CYP450 enzymes often do not occur directly in parallel, and that if the system is disrupted, time is required to re-establish a steady-state level of enzymatic expression.

For pcon-meds that are competitive inhibitors of CYP450 function, the elimination half-life of the drug determines the amount of time that the CYP is inhibited, leading to variable, drug-specific durations in DDI effect (9). Based on basic PK principles, it is well-known that discontinuing competitive CYP inhibitors 5–7 elimination half-lives prior to the start of conditioning will ensure the majority of medication is cleared from systemic circulation. In contrast, CYP450 recovery after discontinuation of enzyme inducers requires the liver to re-establish normal cellular enzyme levels (9). This process takes longer because additional enzyme that has accumulated needs to be degraded. The limited data available suggest that waiting 14 days after stopping a CYP450 inducer should result in ∼90% enzyme recovery (9). This concept is not specifically addressed in the appendix, but should be considered by clinicians.

MATERIALS AND METHODS

This section describes a systematic process of reviewing pcon-meds and determining what drug modifications are recommended to avoid DDIs with conditioning agents. The process is initiated at the time the decision is made to pursue alloHCT in the weeks to months leading up to pre-transplant conditioning. All medications are reviewed and confirmed for the dose, dose frequency, indication, and potential for DDIs with the anticipated alloHCT conditioning agents. Pcon-meds with well-established or highly suspected DDIs are discontinued whenever

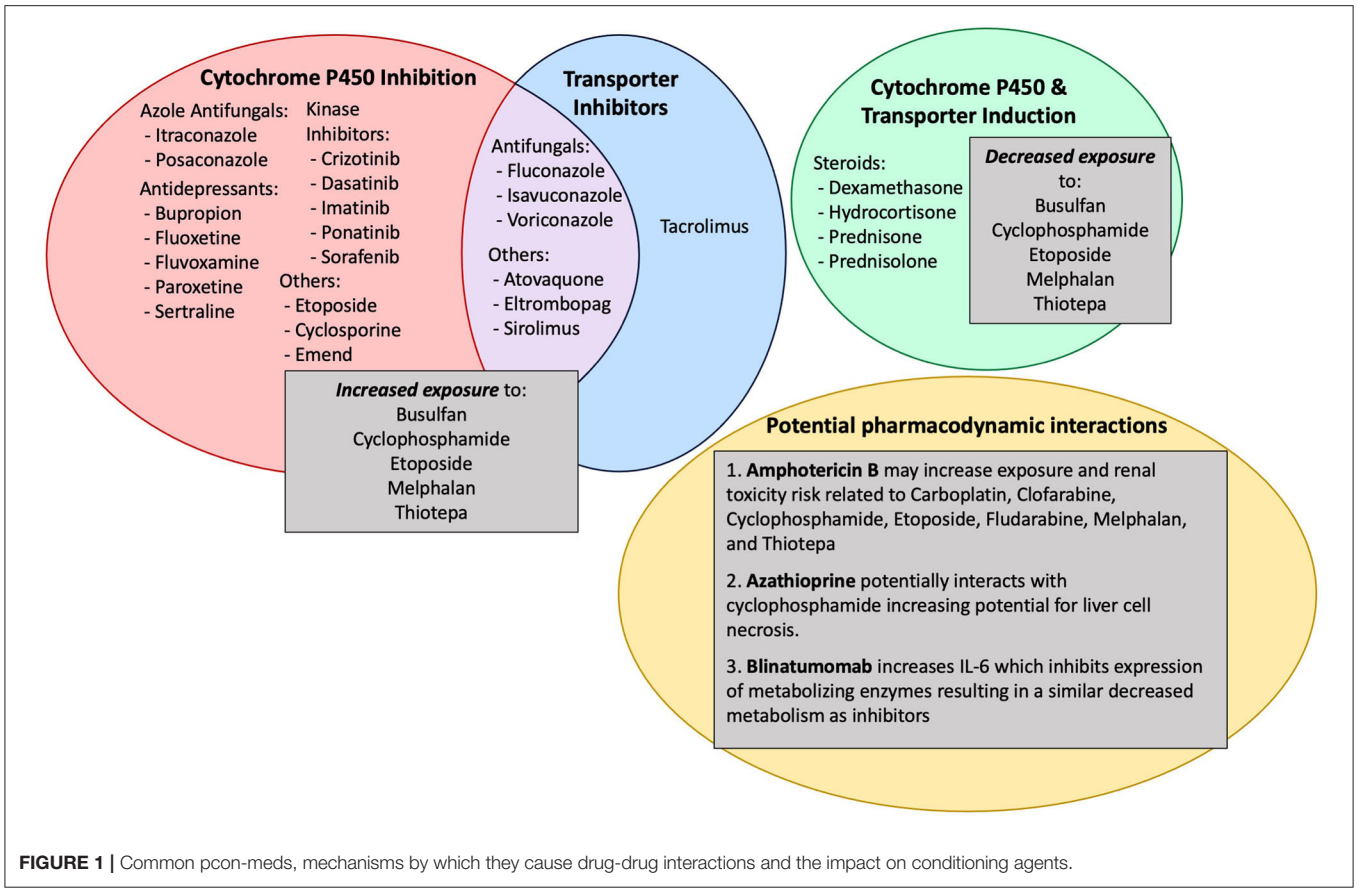

FIGURE 1 | Common pcon-meds, mechanisms by which they cause drug-drug interactions and the impact on conditioning agents.

possible based on the patients' disease state and medical needs. **Appendix Table 1A**, a tool developed by the authors to streamline the medication review process, has specific guidelines for discontinuation of commonly used pcon-meds based on DDIs with conditioning agents used in alloHCT (specifically: busulfan, carboplatin, clofarabine, cyclophosphamide, etoposide, fludarabine, melphalan, and thiotepa). The pcon-meds in the table were selected based on input from pharmacists and physicians at UCSF Benioff Children's Hospital and the University of Minnesota Masonic Children's Hospital. In the table, the "Standard Stop Time" was determined by the 5–7 half-lives necessary to clear an offending agent and the impact of the pcon-med on metabolism of the conditioning agents. If not clinically feasible for the patient to stop the medication within the "Standard Stop Time", a "Minimum Stop Time" is included. In **Appendix Table 1A**, the "Minimal Stop Time" is the time in days required to limit or minimize the most significant/harmful DDIs and was determined based on the drug clearance of the offending agent and the DDIs present. The timeline for stopping a medication is always rounded up or down in days to maximize patient comprehension and compliance prior to admission. Additionally, the timing for discontinuation of medications does not include the administration of serotherapy prior to the start of cytotoxic chemotherapy given the limited evidence for DDIs with monoclonal antibodies (10).

RESULTS AND DISCUSSION

When Discontinuation Is Not an Option

Discontinuation of a pcon-med may not be possible for a variety of reasons, including risk of toxicities associated with rapid drug discontinuation, risk of inflammation which may contribute to graft rejection, and risk of relapse of the underlying disease. Certain drugs, such as antidepressants, anticonvulsants and steroids, need to be tapered or converted to alternate agents rather than abruptly stopped to limit symptoms of drug withdrawal (11, 12). For antidepressants and anticonvulsants, coordination with the prescribing provider to develop an appropriate plan for tapering the drug and/or transitioning to another agent is critical for establishing a clear discontinuation timeline, maximizing patient compliance and safety, and ensuring the drug is discontinued by the start of conditioning. For corticosteroids, several forms of steroids (e.g., prednisone and dexamethasone) can lead to enzyme induction which will decrease the activation of cyclophosphamide and thiotepa and can increase the clearance of busulfan (2). Given this effect can be dose-dependent, an attempt to lower the total daily dose of steroids is routinely considered prior to HCT, and conversion of other steroids to hydrocortisone is recommended at least a week prior to start of conditioning, unless methylprednisolone is planned for GVHD prophylaxis. However, for patients with severe, refractory hemophagocytic

lymphohistiocytosis (HLH) whose disease is uncontrollable off steroids, a steroid taper prior to HCT may not be possible. For such patients, acceptance of the DDIs described above may be required in order to maintain the patient in a clinical condition acceptable for HCT. In this scenario, implementation of a rapid steroid taper can generally commence after the start of immunoablative conditioning and serotherapy. One final example is a patient with chemo-refractory, CD19-negative acute lymphoblastic leukemia who only achieves remission with inotuzumab, a drug ideally discontinued 3 months prior to transplant due to the risk of veno-occlusive disease. In this scenario, discontinuing inotuzumab closer to transplant (e.g., 2–3 weeks) may be the only way to maintain sufficient disease control to get to definitive therapy. Therefore, some centers would accept the risk of organ toxicity over the risk of leukemia relapse. There are many other examples of situations in which the ideal discontinuation timeline is not an option. In these situations the use of busulfan as the primary alkylator is an attractive option since both model-based dosing and therapeutic drug monitoring of busulfan can overcome drug interactions between busulfan and pcon-meds, enabling optimal exposure.

Considerations for Drug Formulation

Absorption can vary during the post-transplant period secondary to mucositis or intestinal graft verses host disease. Therefore, transition off oral medications is important to consider pre-transplant. Particularly following myeloablative conditioning, patients are routinely unable to swallow pills, which is a challenge for medications only available in pill form. Extended-release formulations do not allow for splitting or crushing and may not be available in an IV form. For example, as discussed above, many alloHCT patients are dependent upon antidepressants that are only available in pill form. If possible, under the guidance of the prescribing provider, tapering and discontinuation of the patient's current antidepressant should be performed in the weeks prior to alloHCT (**Appendix Table 2A**).

Antimicrobials

Several of the "azoles" are well-known for their role in DDIs. Itraconazole, voriconazole and posaconazole are considered significant inhibitors of CYP450 enzymes and have interactions with several of the conditioning agents used for pediatric HCT, as well as interactions with many other pcon-meds (2, 13, 14). Studies have shown that azoles, for example fluconazole verses itraconazole, differ in their impact on metabolic enzymes (15). To minimize any impact, discontinuing all azoles except for fluconazole at least 7 days prior to the start of conditioning is recommended. Echinocandins (e.g., caspofungin) and/or amphotericin B may be used as alternatives for patients who require anti-fungal therapy during conditioning, though amphotericin B must be used carefully in patients with renal impairment. In contrast to anti-fungal medications, anti-viral medications and antibiotics used for pneumocystis (PCP) prophylaxis (trimethoprim-sulfamethoxazole, dapsone) have few DDIs. These agents can be safely continued through conditioning if needed.

Anti-neoplastic Medications

Targeted therapies, especially tyrosine kinase inhibitors (TKIs), are well-known for having problematic DDIs (16). Based on established PK profiles most require discontinuation over a week prior to the start of conditioning. Other anti-neoplastic agents commonly used for leukemia control prior to transplant (e.g. azacytidine, cytarabine, 6-mercaptopurine (6MP), 6-thioguanine (6TG), vincristine) have minimal DDIs. Although these agents pose little to no risk for altered PK of conditioning agents, stopping a week prior to conditioning may be considered because of concerns for overlapping drug-related toxicity (e.g., hepatic toxicity) that may occur with combination conditioning. Other chemotherapies, such as cytarabine and etoposide do not have the same concerning overlapping toxicities and can be discontinued 24–48 h prior to conditioning.

For intrathecal (IT) therapies, it is recommended to stop IT cytarabine at least 7 days prior to conditioning and IT methotrexate 14 days prior to conditioning. This conservative approach is based on limited formal drug studies evaluating the effects of IT drug administration and DDIs (17) and a theoretical concern that IT chemotherapy could make patients more susceptible to central nervous system (CNS) toxicity by disrupting the blood-brain barrier.

Interactions Between Drugs and Total Body Irradiation

Drug interactions with TBI are not well defined, however there is some data evaluating changes in the permeability of the blood brain barrier following total body irradiation (TBI) (18, 19). The data available from rodent models demonstrates an increase in the CNS permeability to both endogenous and exogenous substances following both moderate and low doses of TBI. The CNS permeability is most significant at 24–48 h post-TBI (18, 19). Given the sparse data and limited understanding of the potential risk, limiting certain pcon-meds with known neurologic toxicity (e.g., IT chemotherapy), regardless of the conditioning regimen, in the days prior to TBI is recommended.

Additionally, TBI has been linked to increased risk of veno-occlusive disease (VOD), particularly when used in combination with an alkylator, in which case the risk of VOD is dependent on the dose of the alkylator (20, 21). Thus, for all patients receiving TBI, it is important to limit hepatotoxic pcon-meds (e.g., fluoxetine, paroxetine, and isoniazid) and discontinue such medications prior to transplant whenever possible.

Other Considerations

In addition to analyzing DDIs between pcon-meds and conditioning agents, clinicians should also review patient related risk factors when recommending alternative medications. These include special attention to patient allergies, and patient use of illicit substances, herbal medications, essential oils and/or nutritional supplements. Special attention should be made for very young pediatric patients (<1 year), patients that weigh <10 kg and/or obese patients. Adjustments may also be necessary for patients with renal dysfunction, hepatic dysfunction or cardiac dysfunction (e.g., prolonged QTc). When recommending

supportive medications, clinicians should also consider patient-specific preferences. These recommendations are outside of the scope of the paper, however still important to address with both the patient and the HCT medical team prior to starting conditioning.

CONCLUSION

The tables included in the **Appendix** of this manuscript provide detailed guidance regarding how to adjust and discontinue pcon-meds for pediatric patients prior to conditioning for alloHCT. However, as outlined above, the considerations that we have incorporated into this table are extensive and detailed, and the table is a guideline that requires provider interpretation and clinical expertise individualized to each patient. As outlined in this perspective, careful attentiveness to pcon-meds at the time the decision is made to pursue transplant will result in more consistent HCT outcomes, with lower toxicity and/or increased efficacy of conditioning agents.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

BAW, SL, JB, AG, CD, and JLB conceived of the manuscript, selected the drugs to include in the main table (**Supplementary Table 1A**), manuscript and generated the table. All authors contributed to the article and approved the submitted version.

FUNDING

JLB received funding for this study from an anonymous donor. BAW is grateful for support from the Frank A. Campini Foundation.

ACKNOWLEDGMENTS

We would like to acknowledge our patients and their families.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.713091/full#supplementary-material

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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.

frontiers in Pediatrics

ORIGINAL RESEARCH
published: 29 July 2021
doi: 10.3389/fped.2021.705179

Supportive Care During Pediatric Hematopoietic Stem Cell Transplantation: Prevention of Infections. A Report From Workshops on Supportive Care of the Paediatric Diseases Working Party (PDWP) of the European Society for Blood and Marrow Transplantation (EBMT)

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Specialty section:
This article was submitted to Pediatric Oncology, a section of the journal Frontiers in Pediatrics

Received: 04 May 2021
Accepted: 25 June 2021
Published: 29 July 2021

Citation:
Ifversen M, Meisel R, Sedlacek P, Kalwak K, Sisinni L, Hutt D, Lehrnbecher T, Balduzzi A, Diesch T, Jarisch A, Güngör T, Stein J, Yaniv I, Bonig H, Kuhlen M, Ansari M, Nava T, Dalle J-H, Diaz-de-Heredia C, Trigoso E, Falkenberg U, Hartmann M, Deiana M, Canesi M, Broggi C, Bertaina A, Gibson B, Krivan G, Vettenranta K, Matic T, Buechner J, Lawitschka A, Peters C, Yesilipek A, Yalçin K, Lucchini G, Bakhtiar S, Turkiewicz D, Niinimäki R, Wachowiak J, Cesaro S, Dalissier A, Corbacioglu S, Willasch AM and Bader P (2021) Supportive Care During Pediatric Hematopoietic Stem Cell Transplantation: Prevention of Infections. A Report From Workshops on Supportive Care of the Paediatric Diseases Working Party (PDWP) of the European Society for Blood and Marrow Transplantation (EBMT). Front. Pediatr. 9:705179. doi: 10.3389/fped.2021.705179

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Specific protocols define eligibility, conditioning, donor selection, graft composition and prophylaxis of graft vs. host disease for children and young adults undergoing hematopoietic stem cell transplant (HSCT). However, international protocols rarely, if ever, detail supportive care, including pharmaceutical infection prophylaxis, physical protection with face masks and cohort isolation or food restrictions. Supportive care suffers from a lack of scientific evidence and implementation of practices in the transplant centers brings extensive restrictions to the child's and family's daily life after HSCT. Therefore, the Board of the Pediatric Diseases Working Party (PDWP) of the European Society for Blood and Marrow Transplantation (EBMT) held a series of dedicated workshops since 2017 with the aim of initiating the production of a set of minimal recommendations. The present paper describes the consensus reached within the field of infection prophylaxis.

Keywords: infection precaution, allogeneic hematological stem cell transplantation, children, antibiotic prophylactic therapy, vaccination

INTRODUCTION

Infectious complications are a major cause of morbidity and mortality in children undergoing hematopoietic stem cell transplantation (HSCT). In the early history of HSCT many patients died due to reactivated viruses of the herpes family and *de novo* infections especially by fungi and respiratory viruses. Modern HSCT programs therefore rely heavily on efficient infection prevention and early treatment of infections to continue the significant improvement in transplant related mortality (TRM) experienced during the past decades.

Measures to avoid exposure to infectious pathogens include amongst others cohort isolation, use of physical barriers, pharmaceutical prophylaxis, food restriction and vaccinations. In order to help the families and health care workers on how to manage infection risk, local supportive care guidelines are issued in most HSCT centers. However, such guidelines are often based on local preferences rather than on evidence-based studies due to difficulties of carrying out controlled studies in this field. Yet, comprehensive international guidelines were published in 2009 for non-pharmacologic infection prevention during HSCT. The guidelines included the use of HEPA filtered rooms, isolation precautions, restriction of certain food items and crowd isolation (1, 2). Recently, such general recommendations were reviewed in the 2019 handbook issued by the European Society for Blood and Marrow Transplantation (EBMT) (3).

In order to provide an updated and comprehensive set of recommendations directed specifically for children and young adults, the Board of the Pediatric Diseases Working Party (PDWP) of the EBMT decided to review the current evidence within infection prevention in children, during the work on supportive care in pediatric HSCT.

The work was based on repeated focused meetings of the board of PDWP and experts within the fields of pediatric HSCT: Different topics on supportive care were each prepared and finalized by a sub-committee within a total of three meetings held between October 2017 and November 2018 as detailed in the initial paper by Nava et al. (4). Briefly, prior to the initial meeting experts from each sub-committee reviewed the literature and presented during the first meeting a list of issues to be addressed and a draft proposal for the structure and major content of a consensus statement. Based on thorough discussions within the sub-committee and then the entire group, the major objectives/topics and most substantial pieces of evidence but also uncertainties were identified and formed the basis for discussing a more detailed consensus recommendation during the second

meeting. During the third meeting the final recommendations were agreed upon within each sub-committee and in consensus of all workshop attendants.

The current publication is the 3rd volume following these supportive care workshops and presents the consensus of infection prevention in pediatric HSCT recipients with a special focus on an updated vaccination program and guidelines for restrictions following discharge until full de-isolation. Following re-evaluation with two further virtual meetings in April 2020 and February 2021, the manuscript also includes considerations regarding Coronavirus disease 2019 (COVID-19).

PROTECTIVE MEASURES AT THE HSCT WARD

In general, it is recommended to reduce all risks of community acquired infections. The level of inpatient isolation varies between centers due to local logistics and priorities but also due to lack of evidence of specific actions. We here provide the updated recommendations based on international guidelines (1, 2, 5–8) and on the practice in the highly specialized pediatric EBMT centers involved in the workshops. Recently, specific guidelines for SARS-CoV-2 prevention and control have been worked out by scientific societies, including the EBMT (9).

Rooms

Allo-HSCT recipients should be treated in a highly shielded environment with single patient rooms, preferentially with HEPA filters for prevention of airborne fungal infections, especially *Aspergillus* (1). Filters should be replaced regularly according to the manufacturer's recommendations, especially in centers with ongoing construction work (10, 11). There should be at least 12 air exchanges per hour, keeping a consistent positive air pressure of at least 2.5 Pa between the patient room and hallway (1, 12). In case of a recipient affected with disease transmissible by droplets (i.e., COVID-19, measles), a switch to negative pressure might be considered, in order to protect the ward.

Barrier Precautions

When entering and leaving the room and before and after patient contact, hand hygiene including alcohol-based hand rubs/hand washing with soap (plain or antimicrobial) and water is absolutely essential (13, 14). In the absence of visible soiling of hands or potential contact with spore-forming organisms (e.g., *Clostridium difficile*), alcohol-based hand rub can be used. Rings, bracelets, artificial nails and adhesive bandages should be avoided. Personal protective equipment (gloves, masks, and gowns) should be worn during procedures generating splashes of body fluids or causing soiling of clothes. Additionally, when indicated on the basis of coexisting conditions, patients should be placed on airborne, droplet or contact precautions (7). Toys including games, videos, mobile phones, and tablet computers should be wiped with certified cleansing material before being brought into the room and thereafter at least once weekly (15–17). Plants, dried or fresh flowers are prohibited. Each patient should have designated examination tools and routine examination equipment should not be transferred from room to room.

Health Care Workers and Visitors

All HCWs should be vaccinated according to national guidelines including annual influenza vaccination (18). A HCW with known or suspected transmissible infection should have no direct contact with the patients or other HCW and if possible, should be temporarily referred to back-office tasks, or in the case of COVID19 pandemic stay home according to guidelines (19). Visitors should be restricted to as few as possible and any showing signs of infection should be excluded from the transplant unit and from direct contact with HSCT recipients or candidates undergoing conditioning therapy until all symptoms have resolved (1). No absolute minimum age for visitors can be recommended, some centers accept visitors older than 12 years old including siblings, whereas other centers accept even younger visitors. All visitors, including children must be able to follow strict hand hygiene and isolation precautions. Furthermore, during the COVID-19 pandemic, strict adherence to masks by staff and visitors must be followed. Visitor restrictions to transplant units often imply one parent only following negative SARS-CoV-2 testing. The visit of the second parent is rarely allowed, except in case of end-of-life situations.

Given the suboptimal immunogenicity in HSCT patients, family members and healthcare professionals involved in the care of these populations should be vaccinated during influenza seasons, prior to and at least 12 months after HSCT, till patients are able to get and respond efficiently to vaccination. Furthermore, measles and chicken pox status of the family should be reviewed.

DIETARY RESTRICTION DURING ADMISSION

Enteral nutrition is generally encouraged in order to preserve the natural microbiota, thus reducing the risk of graft vs. host disease (GvHD) and possibly the speed of platelet recovery (20). Conditioning-associated nausea and mucositis generally leads to a reduction in the appetite and oral food intake during HSCT, especially during the neutropenic phase (4). Traditionally, certain dietary items have been widely restricted to reduce the risk of introducing harmful food-borne microorganisms to the HSCT patient ("neutropenic diet"), which is increasingly questioned. A retrospective study analyzed infectious complications in 726 consecutive adult HSCT recipients, 25% receiving an allogeneic transplant (21). A neutropenic diet was provided to 363 patients who underwent HSCT between 2004 and 2006, whereas 363 patients undergoing HSCT after 2006 did not receive a neutropenic diet. The only significant difference between the groups was a higher number of microbiologically documented infections after resolution of neutropenia in patients receiving neutropenic diet. A small prospective and randomized pilot study compared a total of 46 adult patients receiving neutropenic diet or a diet without restrictions from day 1 of conditioning until engraftment and did not find differences in infections (22), which corroborates the results of a meta-analysis including 1,116 patients, with 772 (69.1%) having undergone HSCT (23). Unfortunately, data of the effect of a neutropenic diet in pediatric HSCT is lacking to date, but, superiority of a strict neutropenic

TABLE 1 | Handling of food items during allogeneic hematopoietic cell transplantation.

Steps	Handling and preparing food items	Selecting the lower risk option of food items	
		Low risk	**High risk**
Clean	Wash hands and surfaces often. Rinse fruits and vegetables, and rub firm-skin fruits and vegetables under running tap water.	Washed fresh vegetables including lettuce/salads; cooked vegetables	Unwashed fresh vegetables including lettuce/salads
Separate	Separate raw meat, poultry, seafood, and eggs from other foods to avoid cross-contamination (e.g., in the refrigerator, using different cutting boards for raw foods and ready-to-eat food).		
Cook	Cook to safe temperatures, consider using a food thermometer to measure the internal temperature (e.g., beef, lamb, pork, veal and fish to at least 63°C, ground meat to at least 70°C, eggs until yolks and whites are firm). Reheat hot dogs and luncheon meats until steaming hot or 75°C.	Sufficiently cooked meat, poultry, seafood and eggs; canned fish and seafood; pasteurized milk, milk products, egg and egg products	Raw or undercooked meat, poultry, seafood; unpasteurized (raw) milk and milk products Hot dogs and luncheon meats that have not been reheated
Chill	Refrigerate promptly and follow cold storage charts for refrigerator (below 4°C) and freezer (<-16°C). Never thaw food at room temperature.		

diet was not demonstrated in either adults or pediatric oncology patients receiving chemotherapy (24–27).

There is considerable variation in practices in dietary restrictions of specific food items between HSCT-centers (28, 29). However, the growing body of evidence of the lack of benefit of a neutropenic diet has led to an increasing number of cancer centers replacing the strict neutropenic diet with safe food handling guidelines (30, 31). In this regard, the US Department of Agriculture/FDA continues to recommend the avoidance of uncooked ground meat or unpasteurized milk and milk products, whilst categorizing other foods such as fresh vegetables and salad as lower risk food permitted for cancer patients provided certain conditions of food handling and preparation are strictly adhered to (**Table 1**). Four essential steps ("clean, separate, cook and chill") are highlighted, and detailed recommendations regarding washing hands and surfaces ("clean"), how to prevent cross-contamination from one food product to another ("separate"), how to cook different food items to safe temperatures ("cook") and how to refrigerate properly ("chill") are given.

In conclusion, replacing the strict neutropenic diet in HSCT recipients with a more palatable diet should not result in an increased risk of infection and would improve the quality of life and further result in an increase in oral intake of calories and protein, helping to prevent undesirable weight loss. However, this consensus statement of European pediatric HSCT-centers needs to be adapted by each center according to regional and local circumstances.

PHARMACEUTICAL MICROBIAL PROPHYLAXIS DURING AND AFTER HSCT

Exposure to infectious pathogens is unavoidable and necessitates antimicrobial prophylaxis during and after HSCT in all patients. The duration of prophylaxis may depend more on the degree of immune reconstitution in any individual patient rather than any specific time from HSCT. Current evidence has been reviewed and the PDWPs recommendations for the antimicrobial prophylaxis are summarized in **Table 2**. Local circumstances may modify these recommendations.

Pre-assessment

Thorough pre-HSCT assessment including virus specific antibody titers, syphilis and toxoplasmosis is a pre-requisite for the risk management of either reactivation or *de novo* infection and can guide prophylactic treatment pre- and post-discharge. Patients testing IgG-seronegative should in general remain on prophylactic treatment to avoid *de novo* infection prior to HSCT. Interferon-gamma-release assay (IGRA) rather than a tuberculin skin test, is recommended if tuberculosis is clinically suspected. Occasionally in non-acute HSCT, the time from initial referral of the child until HSCT may allow for considering targeted antimicrobial therapy or (re)- immunization and/or immunoglobulin prior to transplant. Pre-transplant serology may not be relevant for anamnestic IgG in those who were recently exposed to products containing plasma (fresh frozen plasma, platelet concentrates, granulocyte transfusions, …) or received intravenous immunoglobulin infusion.

Antibacterial Prophylaxis

Systemic antibacterial prophylaxis and selective gut decontamination is no longer recommended during the neutropenic period (32–35). Instead, immediate administration of intravenous antibiotic treatment when infection is suspected is mandatory. Empiric antibiotic treatment should be adapted to local resistance patterns, patients' colonization status and cover gram-negative aerobic bacteria (*Enterobacteriaceae* and Pseudomonas aeruginosa) and gram-positive cocci (streptococci, Staphylococcus aureus and *Enterococci*). A strict implementation of the guidelines for treatment of febrile neutropenia is mandatory (35–37).

Late infection prevention (>100 days posttransplant), targeting mainly encapsulated bacteria (*Streptococcus pneumoniae* and *Haemophilus influenzae*), should include penicillin or macrolide antibiotics during immunosuppressive treatment for GvHD (38, 39), immunoglobulin

TABLE 2 | Antimicrobial prophylaxis in children undergoing allogeneic hematopoietic cell transplantation.

Phase	Antibacterial	Antiviral	Pnemocystis/toxoplasmosis	Antifungal	IVIG
Conditioning	NGR	NGR	NGR	NGR	NGR
Pre-engraftment	NGR	aciclovir	NGR	L-AmB, azoles or echinocandins	NGR
Post-engraftment w/o a/c GvHD	NGR	aciclovir	SMX/TMP	NGR	NGR
Post-engraftment with a/c GvHD	PNC	aciclovir	SMX/TMP	azoles or echinocandins	NGR

NGR, not generally recommended; PNC, penicillin; SMX/TMP, sulfamethoxazole/trimethoprim; L-AmB, intravenous liposomal amphotericin B; IVIG, intravenous immunoglobulin. If IgG levels below 400 mg/dL IVIG could be given. a/c GvHD, acute or chronic graft vs host disease.

replacement therapy (i.v. or s.c.) in patients with severe hypogammaglobulinemia (serum IgG level $<$ 400 mg/dL) and vaccinations (see later).

Antiviral Prophylaxis

The risk of HSV disease after allo-HSCT in the absence of prophylaxis is ∼80%, especially during the first neutropenic period after HSCT or associated with stomatitis. Prophylaxis with aciclovir (ACV), at a standard dose of 2 × 20 mg/kg from day +1 is recommended in all HSV seropositive patients until neutrophil engraftment or mucosal recovery (3, 40). However, the emergence of drug resistance to ACV may hamper the efficacy of HSV prophylaxis, thus ACV-resistant HSV isolates has been reported in up to 30% among allogeneic bone marrow transplant patients (41).

Following discharge, it is recommended that VZV-seropositive patients receive ACV (2 × 20 mg/kg or 2 × 800 mg in adolescents) or valaciclovir (2 × 500 mg in adolescents) until day +365. There may be an increased risk of delayed VZV-reactivation after discontinuing ACV (rebound) and optimal ACV dose and duration of the prophylaxis is still to be defined (40, 42). Post-exposure prophylaxis with anti-VZV-immunoglobulins (within 96 h) and ACV/valaciclovir is recommended for seronegative patients exposed to VZV. Prophylaxis should be started as soon as possible and continued until 21 days after exposure (43). Primary VZV-infection after allo-HSCT is associated with a high mortality and should be avoided by isolation measures or vaccination (2). Cytomegalovirus (CMV) is a well-known cause of disease occurring after allogeneic HSCT. The manifestations of CMV range from asymptomatic infection, defined as active CMV replication in the blood in the absence of clinical and organ manifestations to CMV disease, characterized by CMV infection with clinical symptoms and/or organ function abnormalities. Fever, cytopenia, pneumonia, gastrointestinal involvement and retinitis are the most frequent presentations. CMV prophylaxis or pre-emptive therapy has changed the natural history of the disease, reducing the risk of CMV disease, CMV-associated death and transplant-related mortality.

Prevention of CMV disease is a prerequisite in allo-HSCT and includes preference of CMV-matched donor/recipient pairs and prevention of virus exposure by discontinuation of breast feeding by CMV-positive mothers (44), physical protection measures (isolation) and use of leukodepleted and/or gamma-irradiated blood products. A preemptive strategy based on weekly monitoring of CMV replication is strongly recommended using foscarnet before and ganciclovir after stable neutrophil engraftment in the presence of rising CMV-loads (45). CMV-monitoring should continue until acceptable immune recovery and/or tapering of immunosuppressive therapy (IST). CMV prophylaxis with letermovir is recommended in adult CMV-positive HSCT recipients (45) and may be an option for seropositive children in an off-label setting (46).

Prevention of EBV-disease following HSCT relies on prevention of exposure (isolation) and preemptive strategies in EBV-seropositive patients (47, 48). Post-transplant lymphoproliferative disease (PTLD) is the most frequent EBV-associated transplant related disease. For timely diagnosis, it is recommended to weekly monitor by quantitative PCR until immune recovery. In case of rapidly increasing viral loads, preemptive treatment with Rituximab is recommended (2, 49). Rituximab-induced B cell depletion has a prophylactic role in controlling EBV-DNAemia after HSCT. Rituximab targets CD20+ B cells which proliferate under the viral trigger, thus removing the EBV reservoir. However, it is not clear if rituximab improves survival, beyond reducing EBV DNAemia. Therefore, the latest ECIL-6 guidelines only recommend prophylactic rituximab for patients at very high risk of developing PTLD, with surveillance of hypogammaglobulinemia and prompt administration of intravenous immunoglobulins to limit rituximab-related complications (12). EBV-specific cytotoxic T lymphocytes (CTLs) showed promising results but remain limited to second-line settings. Antiviral agents are ineffective *in vivo* since their target is not expressed by latent B cells (50).

Primary infections or reactivations of other respiratory tract viruses (RSV, parainfluenza, influenza, and adenovirus) are associated with severe illness and should be monitored closely. Prevention of disease relies on preventing exposure before and after HSCT, see below. HHV-6, parvovirus B19 and the polyomaviruses (BK and JC) are associated with specific clinical entities in allo-HSCT recipients. However, no established prophylaxis exists for these and no recommendations can be made currently (51).

Antifungal Prophylaxis

Invasive fungal infections (IFI) remain a challenge due to substantial morbidity and mortality, despite the availability of new antifungal drugs such as broad spectrum triazoles or echinocandins. Primary antifungal prophylaxis is strongly recommended irrespective of primary diagnosis during the

neutropenic phase and until immune reconstitution and in situations with augmented immunosuppression due to GvHD, which is in line with the updated guidelines according to the Eighth Conference on Infections in Leukemia (ECIL-8) (35). We recommend practice according to the ECIL-8 guidelines to include fluconazole (only if the institutional incidence of invasive mold infections is low) or voriconazole (therapeutic drug monitoring (TDM) strongly recommended), posaconazole (not approved for children, TDM recommended); itraconazole (not approved for children), liposomal amphotericin B (off-label) or micafungin. Other options against pulmonary mold infection may include aerosolized liposomal amphotericin B or caspofungin (off-label indication). Secondary prophylaxis is highly recommended in patients with IFI prior to allo-HSCT.

Monitoring of *Aspergillus* using serum galactomannan levels is feasible, yet the negative predictive value is relatively high and other molds remain undetected (37). According to ECIL-8 guidelines galactomannan monitoring is valuable in children not receiving mold-active prophylaxis, but experts discourage its value in those receiving mold-active prophylaxis (35).

The choice of antifungal drug during and after HSCT should be determined by local circumstances and resistance patterns, organ toxicity (i.e., nephrotoxicity) and drug-drug interactions (e.g., azoles with calcineurin inhibitors) (52). During conditioning, azoles are not recommended whereas L-Amb (off-label) may be preferred until discharge, though nephrotoxicity may limit its use. Triazoles incl. posaconazole have numerous drug interactions including interaction with calcineurin inhibitors that must be considered, and therapeutic drug monitoring is mandatory (53). At the time of writing, posaconazole is still not licensed for recipients <18 years in the EU.

Prophylaxis Against Toxoplasmosis

Toxoplasmosis is a widely distributed zoonosis produced by the parasite *T. gondii* and continues to be a major challenge in the management of pediatric allogeneic HSCT recipients (54). The incidence of toxoplasmosis varies widely due to variable seroprevalence among patient populations, but a majority of patients (up to 3/4) with post HSCT toxoplasmosis disease were IgG-positive prior to HSCT, thus indicating that reactivation plays a major role rather than *de novo* infection (55, 56). Most cases of *Toxoplasma* are observed between 2 and 6 months after transplant with a risk persisting across the first year of transplant and sometimes even later. Early cases occurring during the first 4 weeks of transplant are very rare but have a high mortality rate. Prevention of disease by pre-HSCT assessment of sero-positivity and either prophylaxis with trimethoprim/sulfamethoxazole or preemptive strategy with weekly PCR-monitoring is recommended in seropositive patients starting immediately after transplant until 6 months post HSCT (2, 56).

Prophylaxis Against Pneumocystis Pneumonia

Prior to routine *Pneumocystis jirovecii* pneumonia (PJP) prophylaxis, the cumulative incidence of PJP after allo-HSCT was estimated at 9–16%. Early mortality rates are high, and prophylaxis is strongly recommended after engraftment until at least day +180. Trimethoprim/sulfamethoxazole given 2–3 times weekly is the drug of choice for the primary prophylaxis of PJP in adults and children and should be given during the entire period at risk. All other drugs, including pentamidine, atovaquone and dapsone, are considered second-line alternatives when trimethoprim/sulfamethoxazole is poorly tolerated or contraindicated and are preferred over inhaled pentamidine (57). PJP prophylaxis is relevant in patients with delayed immune reconstitution (CD4 T-cell $< 200/\mu L$) or patients receiving IST for GvHD (58).

Prophylaxis Against COVID-19

The Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), continues to expand worldwide, since it was declared a pandemic by the WHO in March 2020 (59). One year later, 116 million confirmed cases and 2.6 million deaths worldwide have been reported to WHO [World Health Organization Coronavirus (COVID-19) Dashboard https://covid19.who.int]. A cancer diagnosis in adults doubles the risk of COVID-19-associated intensive care unit admission and death, compared with the general population (60, 61). Data regarding COVID-19 in transplanted pediatric patients are very scarce. Despite pediatric cancer patients mostly presenting with a mild or asymptomatic course, the risk of severe COVID-19 may be higher compared with the general pediatric population (62, 63). The Center for International Blood and Marrow Transplant Research (CIBMTR) recently reported a 68% survival 1 month after COVID-19 diagnosis in 318 transplant recipients of whom only 29 of were children, adolescents or young adults. The mortality risk was 2.7 higher in case the infection occurred in the first 12 months after transplant (61). Organ toxicities induced by chemo/radiotherapy, especially in the lungs, and risks of additional infections, due to pancytopenia and immunosuppression, are likely to contribute to exacerbate the COVID-19 course, even though an ultimately protective role of immunosuppression cannot be ruled out (61).

National and local guidelines, policies and procedures should be followed, as the COVID-19 situation varies amongst regions. Yet, the EBMT provides guidelines on COVID precautions in the EBMT-setting, including considerations in pediatric HSCT (9). These guidelines are continuously updated on the EBMT website (https://www.ebmt.org/sites/default/files/2021-02/EBMT%20COVID-19%20guidelines%20v.%2015.02%202021-02-18.pdf). The section dealing with prevention policies confirms case tracking and isolation of SARS-CoV-2 infected individuals, hand hygiene, masks and social distancing as the main prevention strategy. Furthermore, patients and donors should be tested upon admission and/or before the conditioning regimen starts. Visits in the outpatient setting should be limited to patients who are in real need, whereas long-term patients may be followed by telemedicine. The risks associated with postponing scheduled appointments should be balanced (59).

TABLE 3 | Risk grouping following discharge from allogeneic hematopoietic cell transplantation in childhood or adolescence.

Risk assessment at time of discharge, day 100, day 180		
Low risk	**Int risk**	**High risk**
No GvHD	No or controlled GvHD	Active/uncontrolled GvHD
No IST	Low IST	Multimodal IST
No infections	Recurrent infections	Viral reactivations
CD4 > 200	CD4 100-200	CD4 < 100
$N > 0.5 \times 10^9$/l	$N \leq 0.5 \times 10^9$/l	$N \leq 0.5 \times 10^9$/l

GvHD, graft vs. host disease; IST, immune suppressive therapy; CD4, CD positive T-lymphocytes; N, neutrophil count.

Transplant deferral during the pandemic may be considered in non-urgent patients, as hemoglobinopathies, even though a careful evaluation of the risk-benefit balance is recommended.

In case of recipient SARS-CoV-2 positivity, transplantation should be delayed up to two consecutive negative results, even though persistence of virus positivity for several weeks or months in severely immunocompromised patients is not uncommonly reported (61). Donation from a SARS-CoV-2 positive donor should also be deferred. Graft cryopreservation is recommended during the peaks of the pandemic, due to travel restrictions which may prolong its delivery time. The impact of cryopreservation on primary and long-term engraftment is still to be assessed, but many centers prefer mobilized peripheral blood stem cell to ensure good counts, also after thawing, in order not to jeopardize engraftment (60).

POST DISCHARGE PRECAUTIONS

On discharge after allo-HSCT, most patients are still severely immune compromised. Local logistical and geographical factors determine whether patients are discharged to their home or to a local patient hotel or alike. In either case, individual precautions to avoid community acquired infections during immune reconstitution, must be taken. The considerable risk of morbidity or mortality due to acquired infection should be minimized by avoiding microbial exposure. However, no studies support specific approaches of how to protect the child or young adult from community acquired infections. Instead, a number of observations have historically led to recommendations regarding social distancing, food intake and exposure to indoor or outdoor microbes (1, 7, 64–66). The scope of the precautions is to reduce the risk of exposure to especially RSV, *Influenza A, B and C, Parainfluenza, Coronavirus, Norovirus, Rota virus*, *Adenovirus, Salmonella Enteritidis*, *Toxoplasma and Aspergillus* species and should be comprehensive in children due to high rates of airborne infections amongst other children below 6–8 years.

As a part of the consensus workshops, a survey demonstrated that most centers recommend avoiding crowds during the first year post HSCT by not attending school or kindergarten and by avoiding public transportation etc., but the timing of return to normal activities differs widely.

Therefore, in order to provide expert based guidelines on these complex scenarios, we developed a schematic algorithm during the three workshops recommending a set of minimal precautions that should be implemented based on risk stratification of the individual transplanted child. During the workshops we constructed the algorithm defined by three different risk groups, defined as low, intermediate or high risk. The risk grouping is based on immune recovery (CD4 positive T-cells and granulocytes), presence of GvHD, level of IST and infection rate (recurrent viral re-activation, recurrent airway infections), see **Table 3**. According to this, low risk patients are GvHD-free, off IST, do not experience recurrent infections and CD4 counts are >200/μL. High risk group patients have uncontrolled GvHD, receive multimodal IST, have viral reactivations or CD4 counts are <100/μL, whereas intermediate risk group are in between. Patients may move between risk groups in the event of occurrence/recurrence of GvHD or frequent infections.

This risk stratification can then be used to guide patients and their families to a risk adapted set of precautions listed in **Figure 1**. We suggest that such risk classification is done at three different timepoints post HSCT: timepoint 1 at initial discharge post-HSCT, timepoint 2 approximately day +100 and timepoint 3 at approximately day +180. At each timepoints, a patient should be stratified according to the risk group (**Table 3**), which then indicate the set of precautions that should be kept.

The precautionary measures agreed upon by the experts during the workshops include food restrictions, social distancing and behavior at home and outdoors. Importantly though, local (geographical) specific circumstances and outbreaks in infection should be considered and food restrictions may be adjusted accordingly. In principle, the more people in a crowd the higher the risk for transfer of airborne microbes. Thus, childcare centers, indoor waterparks, malls, cinemas and public transportation are regarded as high risk, whereas small family gatherings or small restaurants are regarded intermediate risk. Individual circumstances should be considered when relaxing restrictions as a return to a more normal life may improve the general well-being of the child.

Specific counseling of the family could according to the work shops include:

If possible, patients should not return to a home with high mold and re-housing should be considered until the problem is fixed. Sharing the bedroom with sick siblings should be avoided if possible. Furthermore, the acquisition of new pets and shifting a cat litter box should be avoided in the early post HSCT period.

Outdoor precautions include avoiding bathing in pools and playing in sandboxes. Bathing in the ocean is considered safe, provided the patient has no indwelling catheters.

VACCINATIONS

Children undergoing HSCT lose immunity to vaccine-preventable diseases and are exposed to various pathogens upon reintegration into social life with potentially life-threatening pneumococcal infections playing a prominent role. Thus, early and comprehensive re-immunization is important post-HSCT and should recognize stepwise recovery of the immune system after HSCT and potential impact of IST for

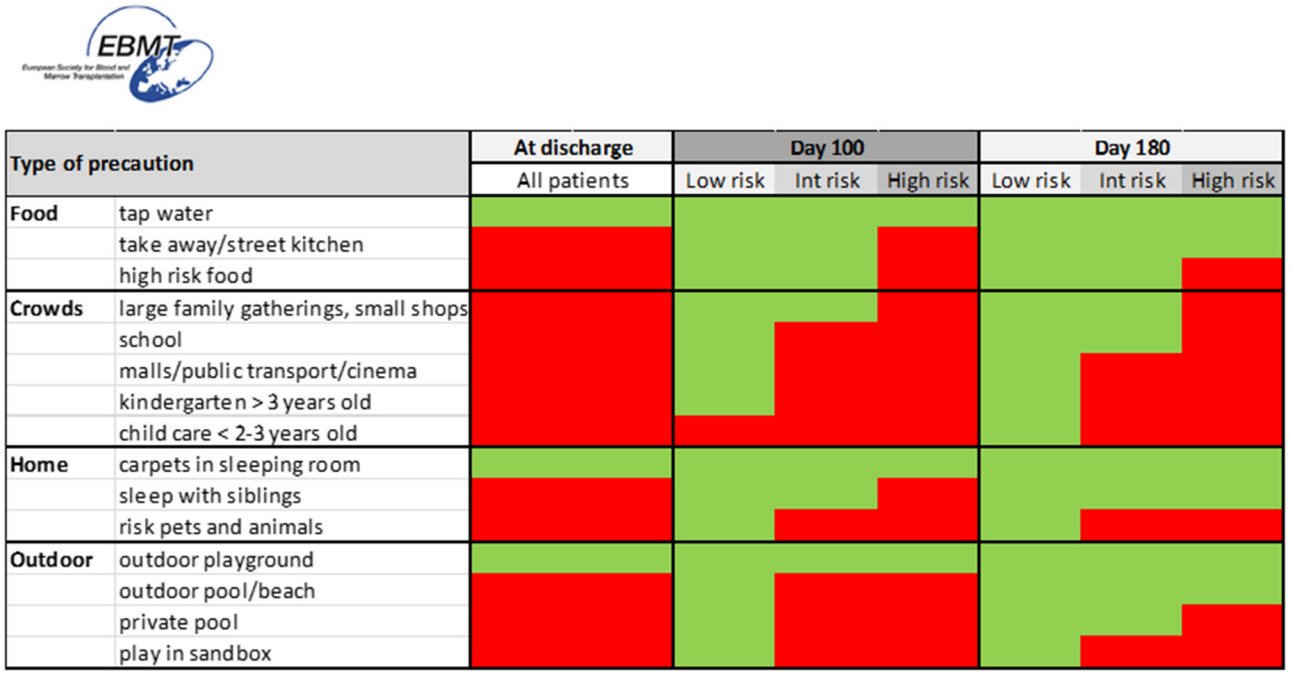

FIGURE 1 | Schematic algorithm for protective measures following discharge from allogeneic hematopoietic cell transplantation in childhood or adolescence. Red color indicates not recommended, green color indicates no restrictions. High risk foods: unpasteurized milk; undercooked egg; blue cheese; honey; raw and undercooked fish, meat and seafood; selected nuts; dried spices from uncontrolled shops/markets; unwashed or unpeeled fresh fruit, water from private wells. Nursery: 0–3 years. Kindergarten: 3–5/6 years. High risk pets: turtles (yersinia), shifting cat trays (toxoplasma), visit stables (aspergillus), acquire new pets. Private pool: if not at bacterial infection. Tap water: local outbreaks may moderate.

prophylaxis/treatment of GvHD that impacts immune recovery. Considerations on the timing of re-vaccination post-HSCT must balance the clinical demand for swift protection and the risk of immunization failure if vaccination is given too early. In the setting of this background an expert group of pediatric infectious disease and transplant physicians identified the specific clinical demand and reviewed currently available evidence focussing on the pediatric age group and generated the consensus recommendation detailed in **Figure 2**.

General Principles for Immunization Post-HSCT in Children

Based on data from a retrospective analysis of revaccination of pediatric HSCT recipients from Great-Britain (67), the prospective IKAST trial on vaccination of children after HSCT (68) and a trial on 13-valent pneumococcal conjugate vaccination in HSCT recipients (69) the following recommendations are made (**Table 4**):

- Use a fixed starting time point for re-vaccination with the newborn DTaP/IPV/HBV/Hib combination vaccine and the 13-valent pneumococcal conjugate (PCV13) vaccine six months post-HSCT [if leukocyte engraftment and platelets $\geq$ 50 $\times$ 10(9)/l] and immunize irrespective of donor/graft type, GvHD, IST and/or measures of immune recovery.
- Use combination vaccine DTaP/IPV/HBV/Hib irrespective of chronologic age.
- Optional/conditional vaccinations (highlighted in yellow in **Figure 2**) should not interfere with evidence-based immunizations (DTaP/IPV/HBV/Hib, PCV13) starting at 6 months. Optional/conditional vaccinations preferably start 12 months post-HSCT.
- Immunization with non-live vaccines is safe during IVIG replacement as there is no specific risk besides non-response. Check titres 3 months after stopping IVIG.
- Start vaccination with live vaccines (MMR-V) not earlier than 24 months post-HSCT and restrict to immunocompetent patients without GvHD and IST $\geq$ 3 months and off IVIG substitution.
- Consider checking antibody concentrations prior and 1 month after primary series in patients with GvHD, IST, IVIG treatment and/or delayed immune reconstitution.

Additional note: Single-center experience indicates that providing non-live vaccines earlier than 6 months post-HSCT may be feasible in children with very swift immune recovery. However, there are no published data on this policy and limited induction of immunologic memory and duration of protection must carefully be weighed against potential earlier protection.

Specific Recommendation for Selected Vaccines

Influenza

High risk for life-threatening influenza-virus infection post-HSCT mandates annual immunization with inactivated influenza

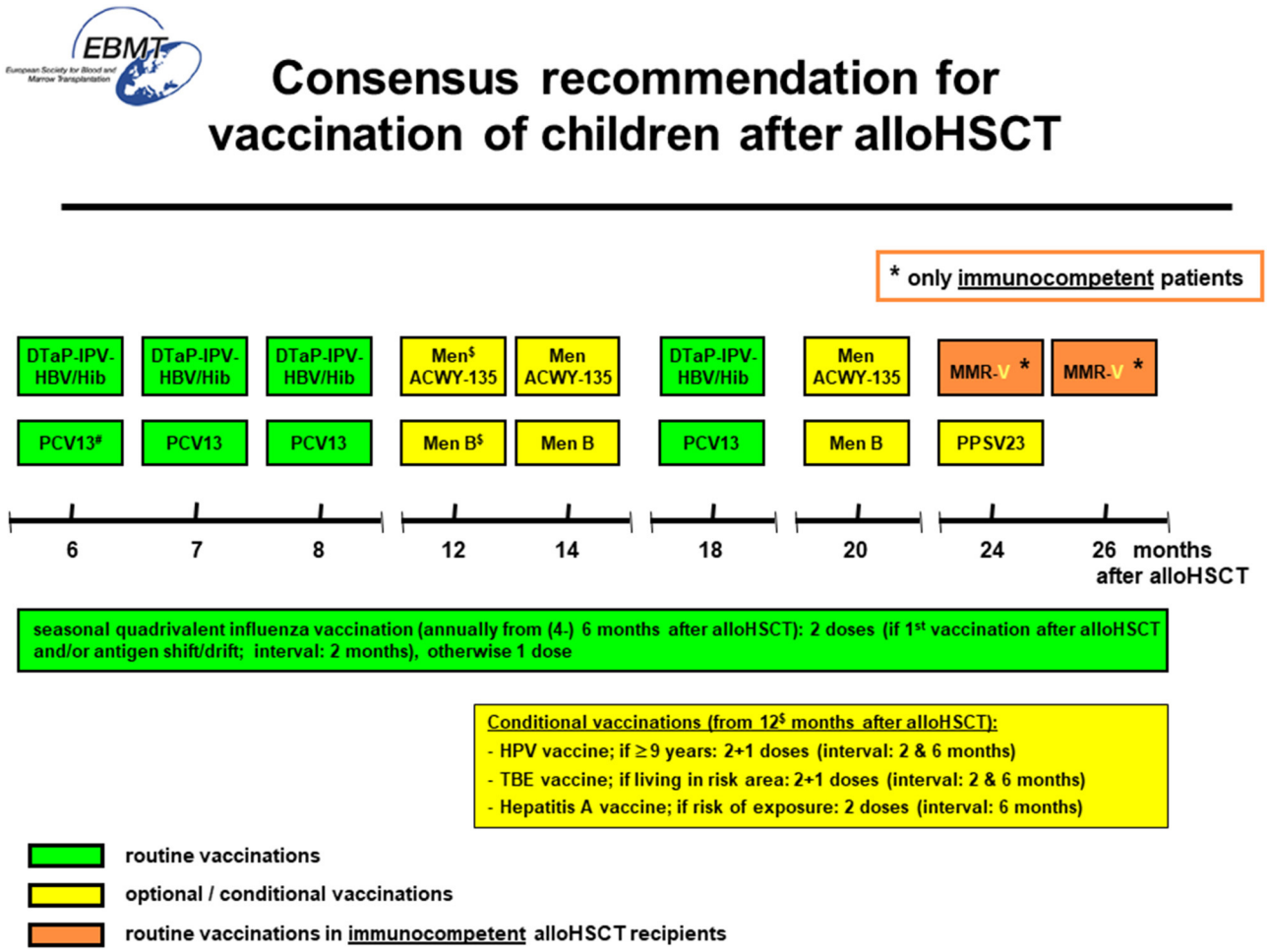

FIGURE 2 | Recommended and optional/conditional vaccinations after allogeneic hematopoietic cell transplantation in childhood. DTaP-IPV-HBV/Hib, hexavalent diphtheria, tetanus, acellular pertussis, inactivated poliovirus, hepatitis B, *Haemophilus influenzae type B* vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; Men ACWY-135, quadrivalent meningococcal conjugate vaccine; Men B, recombinant meningococcal type B vaccine; PPSV23, 23-valent pneumococcal polysaccharide vaccine; MMR-V, live attenuated measles, mumps, rubella, varicella-zoster-virus vaccine; HPV, human-papilloma-virus vaccine; TBE, tick-born-encephalitis vaccine; HAV, hepatitis A vaccine. # start of vaccination at 3 months post-HSCT possible after individual risk-benefit analysis (please refer to text). $ start of vaccination at 6 months post-HCT possible after individual risk-benefit analysis (please refer to text). * only immunocompetent patients post-HSCT, if ≥ 3 months without immunosuppressive therapy and ≥ 3 months without active cGvHD.

vaccines comprising quadrivalent strain coverage. Two doses should be given for first influenza vaccination post-HSCT and after antigenic shift/drift. Live influenza vaccine is contra-indicated post-HSCT. Starting influenza vaccination at 4 months after transplantation may be considered in cases of influenza pandemic or upcoming season.

Pneumococcus

PCV13 comprises the majority of pneumococcal serotypes detected in invasive pneumococcal disease post-HSCT. Administration of the 23-valent pneumococcal polysaccharide vaccine (PPSV23) at 24 months may broaden protection. However, sparse data on the immunogenicity of PPV23 with regard to serotypes reaching beyond PCV13 result in an optional recommendation for PPSV23 post-HSCT.

Meningococcus

No data exist on the specific risk for invasive meningococcal disease post-HSCT but immunecompromised patients represent candidates for meningococcal vaccination. Clinical relevant protection requires vaccination with both A/C/W/Y135 conjugate and recombinant MenB vaccines. Only few disappointing data are available for A/C/W/Y135 conjugate and no data with MenB vaccination post-HSCT resulting in an optional recommendation for meningococcal vaccination starting at 12 months post-HSCT.

Human Papilloma Virus

Profound risk of HPV-associated squamous cell carcinoma exists post-HSCT and most European countries recommend universal HPV vaccination. Consequently, all adolescent transplant

TABLE 4 | Recommendations on specific vaccines following allogeneic hematopoietic cell transplantation in childhood.

Vaccine	Start*	No of doses	Schedule**	Specific notes
Routine vaccinations				
Hexavalent diphtheria, tetanus, acellular pertussis, inactivated poliovirus, Hepatitis B, *Haemophilus influenzae type B* vaccine (DTaP-IPV-HBV/*Hib*)	6	4	0-1-2-12	
13-valent pneumococcal conjugate vaccine (PCV13)	6	4	0-1-2-12	PCV13 comprises most serotypes in invasive pneumococcal disease post-HSCT
Quadrivalent, inactivated influenza virus vaccine (comprehensive current seasonal strain coverage)	(4-)6	1-2	0-2	For first vaccination post-HSCT or after substantial antigenic shift/drift two doses should be given. Yearly revaccination is recommended. Early start at 4 months post-HSCT is possible in case of pandemia or up-coming influenza season. Live influenza vaccine is contra-indicated in HSCT recipients.
Live, attenuated measles- mumps-rubella virus vaccine (MMR)	24	2	0-2	*If immunocompetent (≥ 3 months without GvHD/immunosuppression)*
Conditional vaccinations				
Human papilloma virus vaccine (HPV)	12	3	0-2-8	Substantial risk for HPV-related cancer post-HSCT. Vaccination of both boys and girls *if age ≥ 9years*
Hepatitis A vaccine (HAV)	12	2	0-6	*If risk of exposure*
Tick-borne encephalitis vaccine (TBE)	12	3	0-2-8	*If living in endemic region*
Optional vaccinations				
Live, attenuated varicella-zoster-virus vaccine (in combination with MMR; MMR-V)	24	2	0—2	*If immunocompetent (≥ 3 months without GvHD or immunosuppression.* VZV-reactivations frequently occur before 24 months and are thus beyond effect of the live vaccine which can only be administered from 24 months post-HSCT.
Quadrivalent meningococcal type A,C,W,Y-135 conjugate vaccine (Men ACWY-135)	12	3	0-2-8	No data on specific risk of invasive meningococcal disease post-HSCT. Few and disappointing data for tetra-valent conjugate vaccine post-HSCT.
Recombinant meningococcal type B vaccine (Men B)	12	3	0-2-8	No reported experience with MenB vaccination after HSCT.
23-valent pneumococcal polysaccharide vaccine (PPSV23)	24	1	n/a	PPSV23 may broaden serotype protection. Sparse data on the immunogenicity of PPSV23 with regard to the serotypes beyond PCV13.

**Months from HSCT.*
***Months from start of vaccination.*

recipients should receive HPV vaccination starting from 12 months post-HSCT.

Varicella-Zoster-Virus

High incidence of VZV reactivations with substantial morbidity exists in the first 2 years post-HSCT. Only few case-series report on the use of the live-attenuated VZV vaccine in pediatric HSCT recipients. Immunization can only be instituted at 2 years after HSCT coming too late to prevent the major burden of VZV reactivation. These considerations lead to an optional recommendation for immunization with live-attenuated VZV vaccine in immunocompetent children at least 24 months post-HSCT. Vaccination of family members and household contacts is urgently recommended. If a post-vaccination rash develops the vaccinated should avoid contact with HSCT recipients who may receive aciclovir prophylaxis. Non-live VZV vaccines have recently been investigated in immunocompromised hosts. An inactivated VZV vaccine as well as an adjuvanted VZV subunit vaccine prevented zoster reactivation in adult autologous HSCT recipients. No data are available for either of these vaccines in the post-HSCT setting. Thus, no recommendation can be made for their use in children post-HSCT.

COVID-19

During the COVID-19 pandemic, it seems prudent to administer COVID-19 vaccines—under the condition that they are non-live and non-replicating—to all recipients of allogeneic HSCT, as soon and as far as they are available for use in children and adolescents (of note: the Pfizer/BioNTec COVID-19 vaccine label includes adolescents 12 years or older). Nevertheless, clinical trials are going on to assess safety and efficacy also in younger patients. In analogy with the recommendation for the influenza vaccination, in the current active pandemic situation, the immunization may be started earlier than 6 months after alloHSCT e.g., at 3 months - but it is recommended to do antibody assessments whenever available prior to and 4 weeks after (last) vaccination of the primary series in order to assess immunogenicity as information on this is lacking, in particular in the setting of pediatric alloSCT. This recommendation is in accordance with the current EBMT guideline for COVID-19 vaccination in allogeneic HSCT recipients (https://www.ebmt.

org/sites/default/files/2021-02/COVID%20vaccine%20version%204.03%20with%20table.pdf).

More extensive data on the rationale and risk-benefit assessment for specific vaccines can be found in two international consensus documents on vaccination in HSCT recipients (70, 71).

CONCLUSIVE REMARKS

The emergence and rapid worldwide spread of SARS-COV-2 during early 2020 had substantial impact on general health care and for children and adolescents with the need for allogeneic HSCT it exposed the necessity of strict infection prevention measures. Restrictions regarding hospital resources, donor availability, issues with logistics of stem cell product transport as well as potential consequences of COVID-19 exposure of donors and patients are among the long list of factors potentially impacting provision of care. Nevertheless, the reported incidence of COVID-19 cases among pediatric HSCT recipients is low thus far, which may at least partially reflect the comprehensive measures for social distancing which are implemented as routine standard-of-care in the majority of pediatric HSCT centers. Thus, adherence to the recommendations for preventing infections outlined in this manuscript are considered of particular importance in general but also in the current COVID-19 era.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MI, RM, PS, KK, LS, DH, TL, ABa, and PB wrote the manuscript. PB led the workshops. AJ, TG, JS, IY, HB, MK, MA, TN, J-HD, CD-d-H, ET, UF, MH, MD, MC, CB, ABe, BG, GK, KV, TM, JB, AL, CP, AY, KY, GL, SB, DT, RN, JW, SCe, AD, SCo, and AW participated in workshops and contributed to and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Pediatrics

ORIGINAL RESEARCH
published: 13 October 2021
doi: 10.3389/fped.2021.743294

Effectiveness of T-Cell Replete Haploidentical Hematopoietic Stem Cell Transplantation for Refractory/Relapsed B Cell Acute Lymphoblastic Leukemia in Children and Adolescents

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OPEN ACCESS

Edited by:
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Reviewed by:
Yongsheng Ruan, Southern Medical University, China
Alix Eden Seif, Children's Hospital of Philadelphia, United States

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Specialty section:
This article was submitted to Pediatric Hematology and Hematological Malignancies, a section of the journal Frontiers in Pediatrics

Received: *18 July 2021*
Accepted: *17 September 2021*
Published: *13 October 2021*

Citation:
Sano H, Mochizuki K, Kobayashi S, Ohara Y, Takahashi N, Kudo S, Waragai T, Ikeda K, Ohto H and Kikuta A (2021) Effectiveness of T-Cell Replete Haploidentical Hematopoietic Stem Cell Transplantation for Refractory/Relapsed B Cell Acute Lymphoblastic Leukemia in Children and Adolescents. Front. Pediatr. 9:743294. doi: 10.3389/fped.2021.743294

Background: The prognosis of refractory/relapsed B-cell precursor acute lymphoblastic leukemia (BCP-ALL) remains dismal owing to acquired resistance to chemotherapeutic agents. This study aimed to evaluate the efficacy of T-cell replete HLA haploidentical hematopoietic stem cell transplantation (TCR-haplo-HSCT) for pediatric refractory/relapsed BCP-ALL (RR-BCP-ALL).

Methods: Nineteen pediatric patients with RR-BCP-ALL underwent TCR-haplo-HSCT between 2010 and 2019 at the Fukushima Medical University Hospital. The disease status at TCR-haplo-HSCT included complete remission (CR) in eight patients and non-CR with active disease in 11 patients. Total body irradiation-based, busulfan-based, and reduced-intensity conditioning regimens were employed in 11, 6, and 2 patients, respectively. Low-dose anti-thymocyte globulin (thymoglobulin, 2.5 mg/kg) was used in all patients. Graft-vs.-host disease (GVHD) prophylaxis was administered with tacrolimus, methotrexate, and prednisolone.

Results: All patients received peripheral blood stem cells as the stem cell source. The HLA disparities in graft vs. host directions were 2/8 in one, 3/8 in five, and 4/8 in 13 patients. Among 18 patients who achieved primary engraftment, acute GVHD occurred in all 18 evaluable patients (grade II, 9; grade III, 8; grade IV, 1), and chronic GVHD was observed in 10 out of 15 evaluable patients. Three patients died because of transplant-related mortality. The 3-year overall survival (OS) and leukemia-free survival rates were 57.4 and 42.1%, respectively. Compared to patients older than 10 years in age ($N = 10$), those younger than 10 years in age ($N = 9$) showed an excellent OS rate (3-year OS rate: patients $<$ 10 years old, 100%; patients $>$ 10 years old, 20% [95% confidence interval, 3.1–47.5]; $p = 0.002$).

Conclusions: We suggest that TCR haplo-HSCT with low-dose ATG conditioning has the potential to improve the transplantation outcomes in patients with RR-BCP.

Keywords: acute lymphoblastic leukemia, haploidentical hematopoietic stem cell transplantation, graft-versus-host disease (GVHD), graft versus leukaemia effect, anti-thymocyte globulin (ATG)

INTRODUCTION

In the treatment of pediatric B-cell precursor acute lymphoblastic leukemia (BCP-ALL), patients who fail to achieve complete remission (CR) after relapse, who experience a primary induction failure, or who relapse after hematopoietic stem cell transplantation (HSCT) have extremely poor prognosis (1). Allogeneic HSCT is accepted as the curative treatment option for relapsed or refractory BCP-ALL (RR-BCP-ALL). However, the prognosis of high-risk patients exhibiting early or very early relapse or those positive for minimal residual disease (MRD) is unsatisfactory even if hematological remission is achieved at the time of HSCT (2, 3). In addition, it is extremely difficult to treat HSCT in patients who fail to achieve remission induction after relapse. In recent years, the treatment of RR-BCP-ALL has dramatically changed owing to development of novel therapeutic agents such as blinatumomab, inotuzumab, and CD19 chimeric antigen receptor T (CAR-T) cells (4–6). However, even with the introduction of such novel therapies, some patients fail to achieve remission or have residual MRD; therefore, further development of treatment methods is required.

While HLA haploidentical HSCT (haplo-HSCT) increases the probability of finding a family donor and greatly facilitates donor selection, it is known to be associated with the development of severe graft-vs.-host disease (GVHD) and early transplant-related mortality (TRM) owing to graft rejection (7, 8). Szydlo et al. (9) reported that TRM was significantly higher after haploidentical related or one antigen-mismatched unrelated donor transplants than after HLA-identical sibling transplants. However, unmanipulated haplo-HSCT using post-transplant cyclophosphamide (PTCY) or anti-thymocyte globulin (ATG) has been developed in the last 2 decades. Consequently, TRM due to early post-transplant complications has decreased and the safety of haplo-HSCT has improved. However, the graft-vs.-leukemia (GVL) effect is attenuated in haplo-HSCT using PTCY or high-dose ATG because of suppression of the T-cell-mediated allogeneic immune response. We consider T-cell-replete haplo-HSCT (TCR-haplo-HSCT) using low-dose ATG to be a form of T-cell therapy that has a high degree of efficacy in hematological malignancies based on the allogeneic immune reaction. We had previously reported the outcomes of TCR-haplo-HSCT with low-dose ATG for relapsed or refractory acute pediatric leukemia in 2018 (10), but the outcomes of TCR-haplo-HSCT for BCP-ALL could not be clarified at that time. Thus, in this study, we aimed to evaluate the efficacy of TCR-haplo-HSCT with low-dose ATG for pediatric RR-BCP-ALL by adding new patients to the former cohort.

MATERIALS AND METHODS

Patients

Nineteen patients with RR-BCP-ALL who received unmanipulated haplo-HSCT from a family donor between 2009 and 2019 at Fukushima Medical University Hospital were retrospectively analyzed (**Table 1**). Thirteen of the 19 patients in this study were the same as those in the cohort of our 2018 report (10) and were included here with updated survival information. After adding six new patients, the safety and efficacy of TCR-haplo-HSCT were evaluated for patients with RR-BCP-ALL. We excluded Philadelphia chromosome (Ph)-positive and infant ALL patients from the present analysis. The institutional review board approved the study protocol, and written informed consent was obtained from the patients or their guardians and family donors. Follow-up for all patients was continued through May 2021.

Donor Sources

Donors included fathers ($n = 15$) and mothers ($n = 4$) of the patients. HLA-A, HLA-B, HLA-C, and HLA-DRB1 typing was performed using PCR-Luminex (Luminex Corporation, Austin, TX, USA), based on reverse sequence-specific oligonucleotide (PCR-rSSO) technology (Genosearch HLA, Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). HLA-disparities in both graft-vs.-host and host-vs.-graft directions included two loci mismatches in one patient, three loci mismatches in five patients, and four loci mismatches in 13 patients. Peripheral blood stem cells (PBSCs) were collected with apheresis using COBE Spectra or Spectra Optia (Terumo BCT, Tokyo, Japan) and bone marrow (BM) cells were collected from related donors using standard mobilization protocols. The target amount of CD34-positive and CD3-positive cells was at least 5.0×10^6/kg and $5.0–10.0 \times 10^8$/kg, respectively. Eighteen patients received granulocyte colony stimulating factor (G-CSF)-mobilized PBSCs solely as a stem-cell source, but one patient received BM cells in addition to PBSCs because of low CD34 cell count among the PBSCs.

Conditioning Regimen and GVHD Prophylaxis

Myeloablative conditioning was administered to 17 patients {total body irradiation (TBI) -based for 11 and busulfan (BU)-based for 6 patients}, whereas reduced-intensity conditioning was administered to 2 patients who had organ dysfunction or active infection. For the patients of first transplantation, we employed the regimen consisting of 12 Gy TBI, 1,800 mg/m^2/day (>30 kg) or 60 mg/kg/day (<30 kg) intravenous etoposide (VP-16) and 120 mg/kg/day intravenous cyclophosphamide (CY). Patients who had TBI based regimen during the first transplantation

TABLE 1 | Patient, donor, and graft characteristics.

Pt. No.	Age at HSCT (years)/ Sex	BFM risk at first relapse	Cytogenetics	Disease status at HSCT	Donor	HLA disparity in GVH	Stem cell source	TNC (10^8/kg)	CD34+ cells (10^6/kg)	CD3+ cells (10^8/kg)
1	1.7/F	Very early BM (S4)	*KMT2A-AFF1*	CR2	Father	3/8	PB	32.1	14.2	10.0
2	2.9/M	Early BM (S3)	Hyperdiploid	Active disease	Mother	4/8	PB	18.4	5.7	5.0
3	4.1/M	Very early combined (S4)	*TCF3-HLF*	EM active disease	Father	4/8	PB	21.5	17.8	10.0
4	6.1/F	Relapse after HSCT	*KMT2A-MLLT3*	CR2 after 1st HSCT	Father	4/8	PB	16.5	6.3	5.9
5	6.1/F	Very early BM (S4)		CR2	Father	3/8	PB	32.4	10.3	9.1
6	6.7/M	Very early BM (S4)	*TCF3-PBX1*	CR2	Mother	3/8	PB	13.6	15.1	5.1
7	6.8/M	Early BM (S3)	Hyperdiploid	CR2	Father	4/8	PB	8.5	8.3	1.2
8	8.8/F	Late BM (S2)	*ETV6-RUNX1*	EM active disease	Father	4/8	PB	17.9	12.5	5.6
9	9.7/M	Early BM (S3)	Hyperdiploid	Active disease after 1st HSCT	Father	3/8	PB	21.2	20.2	5.8
10	10.0/F	Early BM (S3)		Active disease	Father	2/8	PB	11.3	11.2	4.4
11	11.5/M	Late BM (S2)	*IKZF1* deletion	Active disease after 1st HSCT	Father	4/8	PB	15.3	10.2	4.7
12	11.9/F	Early BM (S3)		CR2	Father	4/8	PB	15.6	11.5	5.3
13	12.0/M	Late BM (S2)	Hyperdiploid	Active disease after 1st HSCT	Father	4/8	PB	10.8	13.2	4.4
14	12.3/M	Very early BM (S4)		Active disease	Mother	4/8	PB	13.9	6.0	2.5
15	12.5/F	Very early BM (S4)	Hypodiploid	Active disease	Father	4/8	PB	15.2	10.5	3.6
16	12.5/M	PIF		Active disease with PIF	Mother	4/8	PB+BM	13.7	3.7	2.1
17	12.8/M	Early BM (S3)		CR3 after 1st HSCT	Father	4/8	PB	19.8	12.9	8.4
18	13.9/M	Late BM (S2)	Hypodiploid	Active disease after 1st HSCT	Father	4/8 KIR+	PB	14.0	7.4	3.0
19	16.9/M	Very early BM (S4)	*MEF2D-BCL9*	CRi	Father	3/8	PB	10.1	6.3	4.3

Pt.,patient; BM, bone marrow; CR, complete remission; CR2, second complete remission; CR3, third complete remission; CRi, Complete remission with incomplete hematologic recovery; F, female; HSCT, hematopoietic stem cell transplantation; KIR+, killer cell immunoglobulin-like receptor (KIR) ligand mismatch positive; M, male; PB, peripheral blood; TNC, total nucleated cell count.

Berlin-Frankfurt-Münster (BFM) risk stratification for first relapse: very early (<18 months from diagnosis, S4), early (18 months from diagnosis but <6 months after treatment completion, S3), and late (≥6 months after treatment completion, S2).

received the conditioning regimen consisting of intravenous BU 3.2–4.0 mg/kg/day for 4 days, intravenous fludarabine 30 mg/m^2/day for 5 days and intravenous melphalan 70 mg/m^2/day for 2 days. No lung-shielding was performed on patients who underwent TBI. Our GVHD prophylaxis method has been previously reported (11). To prevent GvHD, all patients received ATG (thymoglobulin, 1.25 mg/kg/day; Sanofi, Paris, France) intravenously for 2 consecutive days, from days−2 to−1. GVHD prophylaxis comprised a combination of tacrolimus, methotrexate, and prednisolone for all patients. Tacrolimus was started on day−1 and was continuously administered intravenously. The concentration of tacrolimus in the peripheral blood was adjusted between 7 and 15 mg/ml. 3 or 4 weeks after transplantation, tacrolimus administration was changed to the oral route with the trough level targeted at 5–10 ng/ml. MTX (10 mg/m^2) was administered intravenously on day +1, and doses of 7 mg/m^2 were administered on days +3 and +6 after transplantation. Prednisolone (PSL) was initiated on day 0 with an initial dose of 1 mg/kg/day (Patient No. 1 received 2 mg/kg). When there was no sign of acute GVHD, from day +29, the PSL dose was tapered every 2 weeks and discontinued within 6 months after transplantation. Acute GVHD was graded according to the standard criteria (12). The diagnosis and grading of chronic GVHD followed National Institutes of Health (NIH) criteria (13).

Supportive Care

All patients received prophylaxis with trimethoprim–sulfamethoxazole against *Pneumocystis jirovecii* infection. They received broad-spectrum antibiotics, fluconazole, and acyclovir for bacterial, fungal, and herpes virus infections, respectively. Immunoglobulin (0.2 g/kg/dose, i.v.) was infused weekly until day +100, and then biweekly until 6 months after HSCT. Granulocyte-colony stimulating factor (G-CSF) (5 mg/kg/day) was started on day +1 following stem cell infusion. Cytomegalovirus (CMV) treatment with ganciclovir was initiated when CMV antigenemia was detected in routine weekly examination.

Analysis of Mismatched HLA Loss

Mismatched HLA loss was detected using the PCR-rSSO-Luminex method. The measured data were read using a dedicated software (DNASIS® Call HLA typing software, Hitachi Software Engineering Co., Ltd. Tokyo, Japan).

Statistical Analysis

Overall survival (OS) was defined as the time from TCR-haplo-SCT to death from any cause. Relapse-free survival (RFS) was defined as the time from TCR-haplo-SCT to leukemia relapse or any cause of death. Moderate-severe chronic GVHD/relapse free survival (CGRFS) was defined as the duration from transplantation until death, relapse, development of moderate or severe chronic GVHD that required systemic treatment, and the patients without any of these events at the time of the final follow-up were censored. OS and RFS were calculated using the Kaplan–Meier method (14). The median value of the distribution was used for the age and CD3 cutoff used in the univariate analysis. The cumulative incidences of relapse, TRM and acute GVHD were estimated by analyzing competing risks using Gray's method (15). The Fisher's exact test was used for categorical variables and non-parametric test (Mann-Whitney test) was used for continuous variables. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (16), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patient, Donor, and Graft Characteristics

Patient, donor, and graft characteristics are presented in **Table 1**. The median age of patients at the time of TCR-haplo-HSCT was 10.0 (range: 1.7–16.9) years old. Twelve patients (63%) were male, and seven (37%) were female. Among 19 patients with RR-BCP-ALL, eighteen patients relapsed, and one experienced primary induction failure (PIF). The Berlin-Frankfurt-Münster (BFM) risk classification at first relapse was very early BM or combined (S4) in 7 patients, early BM (S3) in 6 patients, late BM (S2) in 4 patients, and post-HSCT relapse in 1 patient. Thirteen patients who relapsed exhibited cytogenetic aberrations. Of the thirteen, four exhibited hyperdiploidy, two exhibited hypodiploidy, and one each exhibited *KMT2A-MLLT3, TCF3-PBX1, IKZF1 deletion, KMT2A-AFF1, ETV6-RUNX1, and MEF2D-BCL9*. At the time of TCR-haplo-HSCT, eight patients achieved CR, while 11 patients had a non-remission status; of the latter 11 patients, nine patients had BM involvement and two had only extramedullary involvement.

Infused Cell Number and Engraftment

PBSCs were used as the stem-cell source in all patients and were collected from the patients' family (three from mothers and fifteen from fathers). The median number of CD34-positive hematopoietic cells was 10.2×10^6 (range: 3.7–20.2) cells/kg. We used unmanipulated PBSCs to prevent attenuation of the GVL effect and intentionally administered CD3-positive cells; the median dose of CD3 positive cells was 5.0×10^8 (range: 1.2–10.0) cells/kg. Eighteen patients (95%) achieved primary neutrophil engraftment after transplantation. The median time of neutrophil recovery (>500/μL) was 13 (range: 10–16) days and that of platelet recovery ($>2 \times 10^4$/μL) was 23 (range: 7–123) days. One patient who exhibited primary induction failure (Patient No. 8) experienced donor type primary graft failure; because primary graft failure was not based on donor specific antibody (DSA), he underwent a second BMT from the same donor and achieved neutrophil engraftment 16 days after the second BMT. In this study, there were no other patients with positive DSA.

HLA Disparities and GVHD

HLA disparities in the graft-vs.-host direction were 2/8 in one, 3/8 in five, and 4/8 in 13 patients. In Patient No. 18, killer cell immunoglobulin-like receptor (KIR) ligand mismatch was found in HLA-C between the donor and the recipient. Acute GVHD was observed in all 18 evaluable patients; there were nine patients with grade II, eight with grade III, and one with grade IV GVHD. The cumulative incidence of acute GVHD (grade II-IV) was 100% and 70.1% in that of grade III-IV GVHD (**Figure 1**). Chronic GVHD was also observed in 10 (67%) of 15 evaluable patients. With reference to chronic GVHD, five patients developed mild, one developed moderate, and four had severe forms with bronchiolitis obliterans (BO). Three of the four patients who developed BO were under 10 years old, and BU was used in two of the three patients. One of these patients (Patient No.1) avoided TBI because of younger age, and the other (Patient No.4) had a BU based regimen because TBI had been used in the first HSCT.

Complications Within 100 Days After TCR-Haplo-HSCT

Fifteen patients had infectious complications within 100 days of TCR-haplo-HSCT, including CMV antigenemia, Epstein-Barr virus (EBV) reactivation, human herpes virus 6 reactivation, hemolytic cystitis due to BK virus, varicella-zoster virus reactivation, sepsis, and *Aspergillus* infection. Patient No. 14 died because of pulmonary hemorrhage due to invasive aspergillosis on day 23 after TCR-haplo-HSCT, and Patient No. 18 died because of sepsis caused by severe acute GVHD on day 87 after TCR-haplo-HSCT (**Table 2**). Engraftment syndrome (ES) was observed in 5 patients; two of these five patients relapsed, but both survived with CR after the second TCR-haplo-HSCT.

Transplantation-Related Mortality, Relapse, and Outcome

The cumulative incidence of relapse at 3 years was 42.1% (95%CI, 19.5–63.3), and the TRM rate was 15.8% (95%CI, 3.7–35.7) (**Figure 2A**). Five patients died owing to reasons other than leukemia progression; two of these patients died from complications that occurred after the post-HSCT relapse. Therefore, we determined that three patients died from TRM. The causes of TRM were as follows: pulmonary hemorrhage due to invasive aspergillosis, pneumonia due to severe chronic lung GVHD, and sepsis due to severe acute GVHD. Characteristics of patients who relapsed after TCR-haplo-HSCT have been summarized in **Table 3**. Eight patients experienced leukemia relapse; two (Patient No. 2 and 5) of the four patients who received the second TCR-haplo-HSCT survived, and all four patients who did not receive a re-transplantation died due to disease progression. Of the five relapsed patients for whom HLA analysis of blasts was available, three had mismatched HLA loss after TCR-haplo-HSCT and all three died; the two

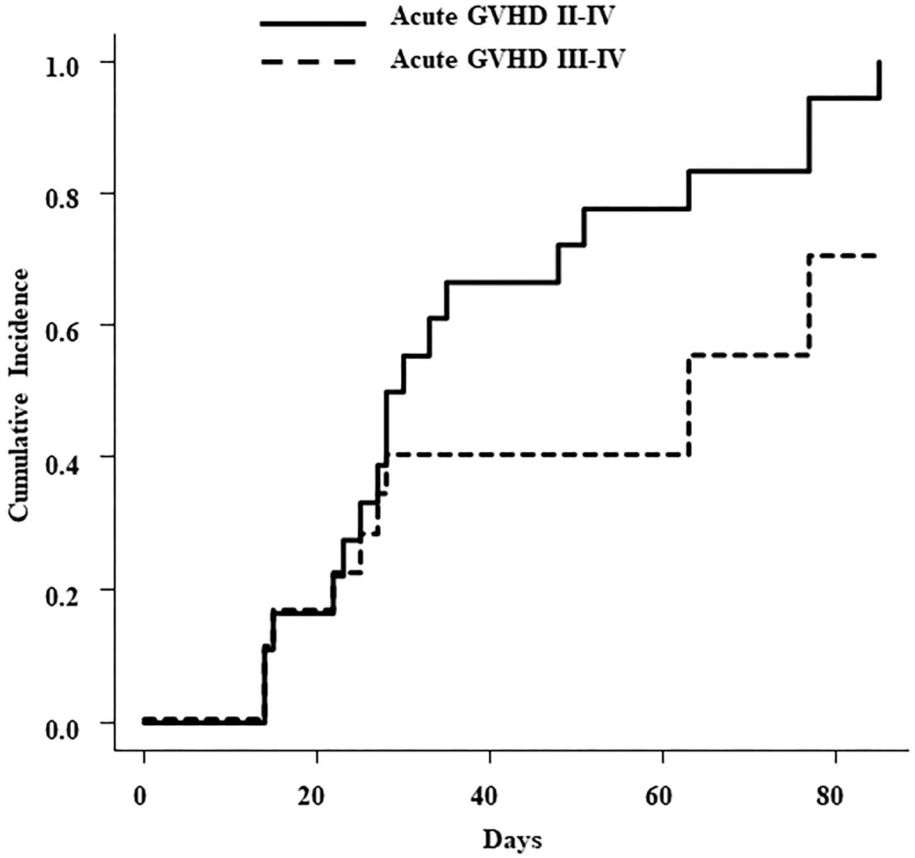

FIGURE 1 | Cumulative incidence of acute GVHD with grade II to IV (solid line) and grade III to IV (dotted line).

patients without mismatched HLA loss were rescued by the second TCR-haplo-HSCT after relapse. Interestingly, Patient No. 4 received TCR-haplo-SCT from his father but relapsed with loss of the maternal HLA haplotype; he then underwent haplo-SCT from his mother and relapsed with loss of the paternal HLA haplotype (17).

Survival

As of the last follow-up date, ten of the 19 patients were still alive after a median follow-up of 2,866 (range: 913–4,190) days. The probability of OS and RFS at 3 years was 57.4% (95%CI, 32.5–76.0) and 42.1% (95%CI, 20.4–62.5), respectively (**Figure 2B**). The probability of 3 years CGRFS was 26.3% (95%CI, 9.6–46.8) (**Figure 2C**). Patients younger than 10 years ($N = 9$) exhibited an excellent overall survival rate compared to patients older than 10 years ($N = 10$) [3-year OS: patients < 10 years old, 100%; patients >10 years old, 20% (95%CI, 3.1–47.5); $p = 0.002$] (**Figure 3A**). However, the disease status at the time of TCR-haplo-HSCT was not associated with the patient's prognosis [3-year OS: CR, 75% (95%CI, 31.5–93.1); NCR, 45.5% (95%CI, 16.7–70.7); $p = 0.285$] (**Figure 3B**). We also examined whether there was a difference in patient characteristics between the patients <10 and those >10 years old (**Table 4**). There was no significant difference between the two groups except for the CD3 dose, which may be confounded by the very small number of cases. Moreover, this result needed to be interpreted carefully because the number of patients in the study cohort was too small to perform multivariate analysis. All three patients who died of TRM were over 10 years in age, and all five patients over 10 years in age who relapsed after TCR-haplo-SCT died; furthermore, all three patients who relapsed with mismatched HLA loss were also over 10 years in age (**Table 3**). Our case series included 13 high-risk patients with a BFM risk classification of very early (S4) and early (S3) for the first relapse; seven of these patients are still alive. Additionally, high infused CD3 dose was also associated with better survival [3 years OS: $>5.0 \times 10^8$/kg ($N = 10$), 88.9% (95%CI, 43.3–98.4); $<5.0 \times 10^8$/kg ($N = 9$), 22.2% (95%CI, 3.4–51.3); $p = 0.0003$]. The CD3 infusion dose itself was correlated with age; therefore, caution should be exercised when considering CD3 infusion dose as an independent prognostic factor. It is important to note that infusing more CD3 does not increase mortality due to complications.

DISCUSSION

Relapse after first-line chemotherapy occurs in 15−20% of pediatric patients with BCP-ALL (18), and survival rates are much lower in cases of BM relapse with a shorter duration

TABLE 2 | T-cell replete haploidentical stem cell transplantation and clinical outcomes.

Patient No.	Conditioning regimen	Engraftment (days)		aGVHD grade and stage (skin, liver, gut)	cGVHD	Complication at <100 days after TCR-haplo-HSCT	Post-HSCT relapse (day+)	Outcomes
		Neutrophil	Platelet					
1	Bu4+Flu+Mel+ATG	10	14	III (2,0,2)	Severe (lung)	ES, EBV	No	Alive with CR
2	TBI(12)+VP16+CY +ATG	15	28	II (3,0,0)	Mild	ES, sepsis (*S. hemolyticus*), HHV6, EBV, PRES	405 (combined)	Alive with CR after 2nd HSCT
3	Bu4+Clo+Flu+ATG	14	123	III (2,0,3)	Mild	ES, CMV, EBV	No	Alive with CR
4	Bu4+Flu+Mel+ATG	11	23	III (2,0,3)	Severe (lung)	BKV-HC, EBV, VZV	No	Alive with CR
5	TBI(12)+VP16+CY +ATG	11	18	II (3,0,1)	Mild	ES	323 (combined)	Alive with CR after 2nd HSCT
6	TBI(12)+VP16+CY +ATG	14	19	III (1,0,3)	Severe (lung)	ES, CMV, aspergillus, atelectasis,	No	Alive with CR
7	TBI(12)+VP16+CY +ATG	11	93	II (3,0,0)	None	CMV	670 (BM)	DOD on day+1986
8	TBI(12)+VP16+CY +ATG	12	19	II (3,0,0)	Mild	None	No	Alive with CR
9	Bu4+Flu+Mel+ATG	15	28	III (3,2,0)	None	BKV-HC, sepsis (*K. pneumoniae*)	No	Alive with CR
10	TBI(12)+VP16+CY +ATG	15	NE	III (3,2,2)	NE	*Candida* sepsis, pancreatitis, aspergillus	67 (BM)	DOD on day+133
11	Bu4+Flu+ATG	13	74	II (3,0,0)	Mild	CMV, EBV, BKV-HC, VZV	105 (BM)	DOD on day+213
12	TBI(12)+VP16+CY +ATG	12	22	II (3,0,0)	None	CMV	No	Alive with CR
13	TBI(12)+VP16+CY +ATG	16	34	II (3,0,0)	Moderate	None	No	Died on day+439 from EB-LPD
14	TBI(12)+VP16+CY +ATG	13	NE	NE	NE	Lung bleeding due to invasive aspergillosis	No	Died on day+23 from aspergillosis
15	Flu+Mel+ATG	11	21	IV (0,4,0)	None	EBV, CMV, BKV-HC, cellulitis, pneumoniae	176 (BM)	Died on day+276 from GF in 2nd HSCT
16	TBI(12)+VP16+CY +ATG	NE	NE	II (3,0,0)	NE	VZV	No	Alive with CR
17	Bu2+Flu+Mel+ATG	10	7	III (2,0,3)	Severe (lung)	EBV, CMV, pancreatitis	115 (CNS)	Died on day+934 from pneumoniae
18	Bu4+Mel+AraC+ATG	13	32	II (3,0,0)	None	None	117 (BM)	DOD on day+549
19	TBI(12)+VP16+CY +ATG	13	NE	III (3,0,3)	NE	Sepsis (*S. oralis*), pancreatitis, HC	No	Died on day+87 from sepsis due to GVHD

aGVHD, acute graft vs. host disease; ATG, anti-thymocyte globulin; BKV, BK virus; BM, bone marrow; Bu2, busulfan for 2 days; Bu4, busulfan for 4 days; cGVHD, chronic GVHD; Clo, clofarabine; CMV, cytomegalovirus; CNS, central nervous system; CR, complete remission; CY, cyclophosphamide; DOD, died of disease; ES, engraftment syndrome; EBV, Epstein-Barr virus; Flu, fludarabine; GF, graft failure; GVHD, graft vs. host disease; HC, hemorrhagic cystitis; LPD, lymphoproliferative disease; Mel, melphalan; NE, not evaluable; PRES, posterior reversible encephalopathy syndrome; TBI, total body irradiation; TBI (12), TBI 12 Gy; TCR-haplo-HSCT, T-cell replete haploidentical hematopoietic stem cell transplantation; VP16, etoposide; VZV, varicella-zoster virus.

from diagnosis than in late and/or extramedullary relapse (19). In the BFM risk classification, very-early relapse is defined as that occurring <18 months from diagnosis, early relapse as that occurring more than 18 months from diagnosis but <6 months from treatment discontinuation, and late relapse as that occurring more than 6 months from treatment discontinuation (20). The 5-year OS rates of early BM (S3) and very-early BM or combined (S4) relapse are very low at 30 and 25%, respectively (21). Patients with high-risk relapses have poor survival rates even after allogeneic HSCT. The pre-HSCT assessment of MRD in patients with high-risk relapse (S3/S4 or third CR) showed that patients with MRD of $<10^{-4}$ leukemic cells had significantly better survival (53% probability of event-free survival [pEFS]) than patients with MRD of over 10^{-4} leukemic cells (30% probability of pEFS) (22). Historically, patients who could not achieve CR were not considered for HSCT because of the

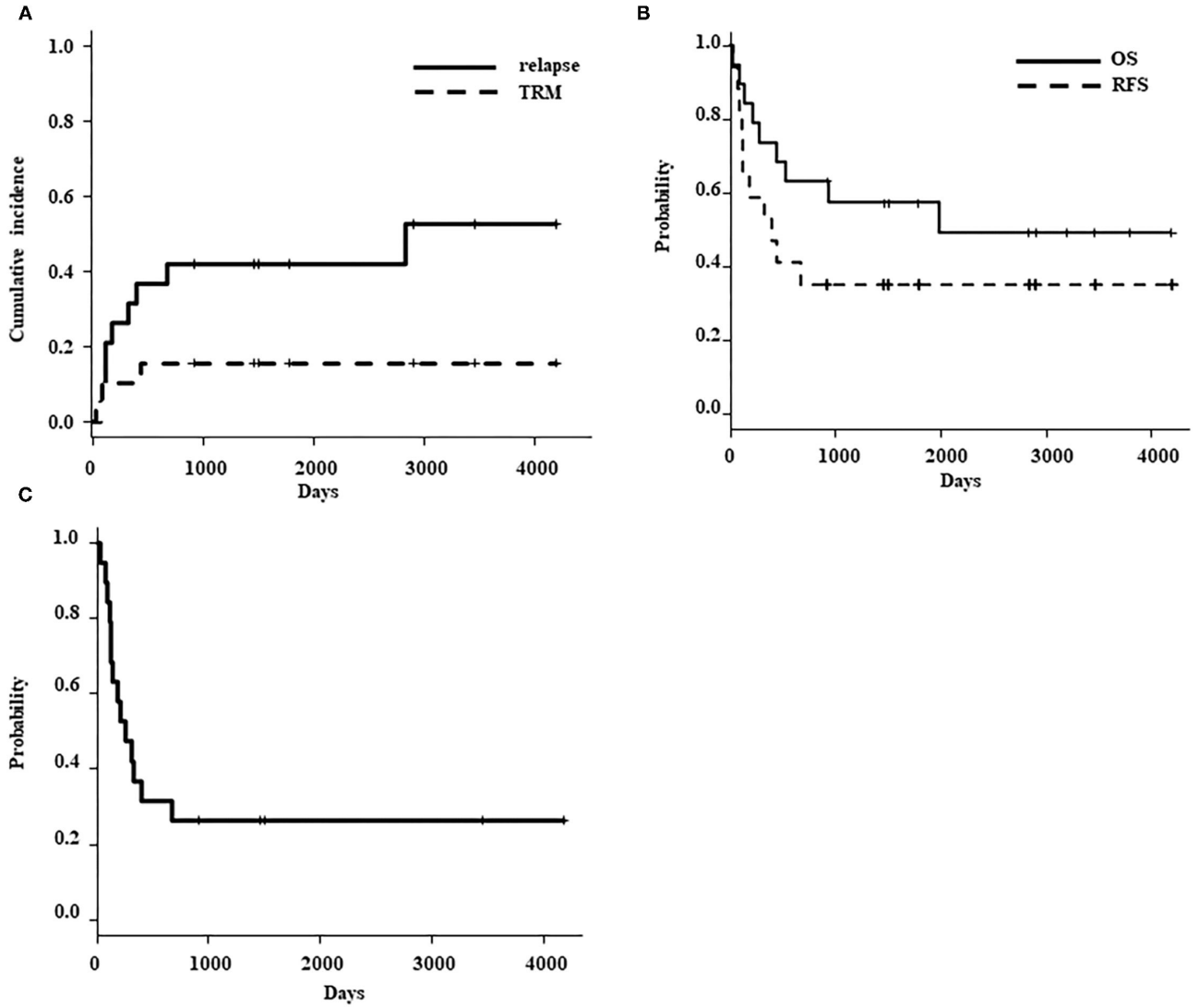

FIGURE 2 | (A) Cumulative incidence of relapse (solid line) and transplant-related mortality (dotted line). **(B)** Probability of overall survival (solid line) and relapse-free survival (dotted line). **(C)** Probability of moderate-severe chronic GVHD/ relapse free survival (CGRFS).

TABLE 3 | Summary of relapsed patients after T-cell replete haploidentical stem cell transplantation.

Patient No.	Age at TCR-haplo-HSCT (years)	Relapse site	Mismatched HLA haplotype loss	2nd TCR-haplo-HSCT (donor)	Outcome
2	2.9	Combined	(-)	YES (uncle)	Alive with CR
5	6.1	Combined	(-)	YES (uncle)	Alive with CR
7	6.8	BM	NE	NO	DOD day+1986
10	10.0	BM	(+)	NO	DOD day+133
11	11.5	BM	NE	NO	DOD day+213
15	12.5	BM	(+)	YES (mother)	Died from graft failure at 2nd HSCT
17	12.8	CNS	NE	NO	Died from pulmonary complication
18	13.9	BM	(+)	YES (mother)	DOD day+549

BM, bone marrow; CNS, central nervous system; NE, not evaluable; CR, complete remission; DOD, died of disease; TCR-haplo-HSCT, T cell replete haploidentical hematopoietic stem cell transplantation.

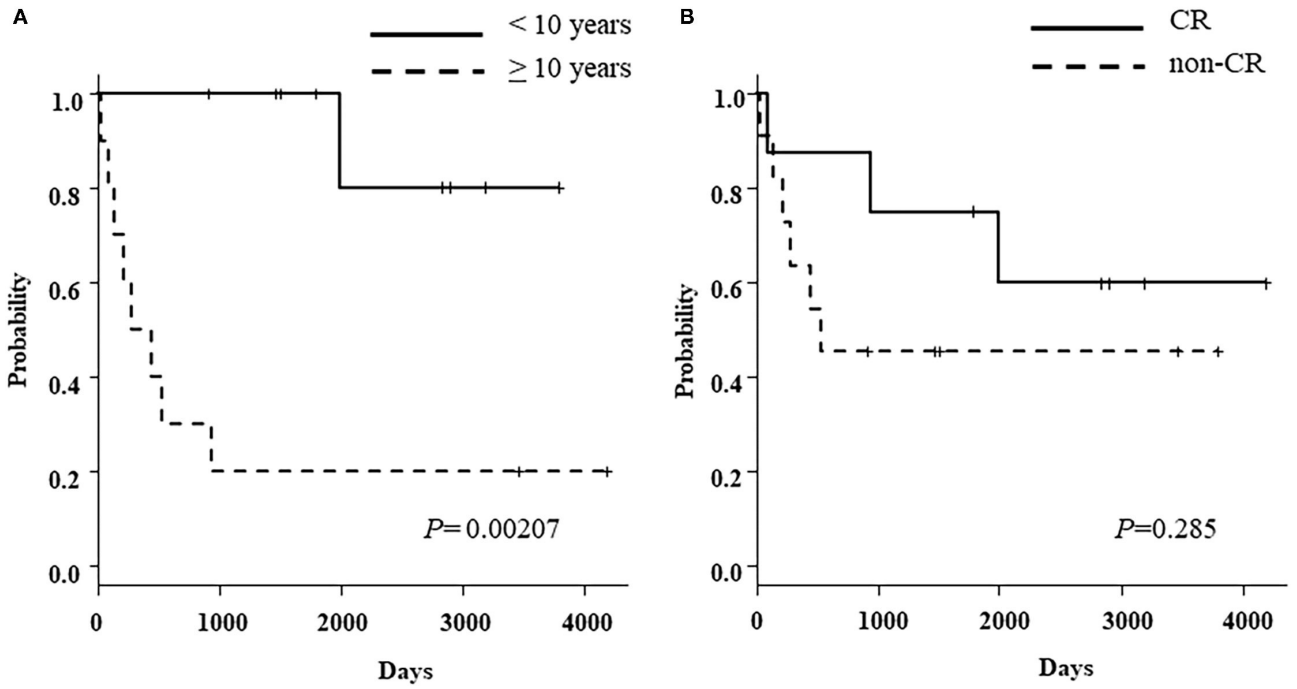

FIGURE 3 | (A) Probability of overall survival according to the age at TCR-haplo-HSCT [<10 years (solid line) vs. more than 10 years (dotted line)] **(B)** Probability of overall survival according to the disease status at TCR-haplo-HSCT [complete remission (solid line) vs. non-complete remission (dotted line)].

TABLE 4 | Comparison of patient characteristics by age.

Variables	<10 years ($n = 9$)	≥10 years ($n = 10$)	*P*-value
Sex			
Male	5	3	0.65
Female	4	7	
Donor			
Father	7	8	1.00
Mother	2	2	
Disease status at TCR-haplo-SCT			
CR	5	8	0.37
Active disease	4	2	
HLA disparity			
2/8	0	1	0.21
3/8	4	1	
4/8	5	8	
CD3 dose			
Median	5.8×10^8/kg	4.35×10^8/kg	0.025
Range	(1.2–10.0)	(2.1–8.4)	
CD34 dose			
Median	12.5×10^6/kg	10.35×10^6/kg	0.22
Range	(5.7–20.2)	(3.7–13.2)	

TCR-haplo-SCT, T cell replete haploidentical hematopoietic stem cell transplantation.

lack of prognostic improvement after treatment. Furthermore, TCR-haplo-HSCT has been shown to be associated with a higher risk of early TRM, owing to complications in severe GVHD arising from allogeneic immune reactions and graft rejection. Therefore, HSCT from HLA-matched donors has traditionally been recommended. In contrast, allogeneic immune reactions induce a strong GVL effect; thus, TCR-haplo-HSCT may be considered as a way to induce GVL in patients with relapsed/refractory leukemia.

The outcome of pediatric BCP-ALL has been gradually improved by the stratification of treatment based on the analysis of MRD and the introduction of new drugs. However, it is difficult to improve the prognosis of patients who have relapsed after HSCT, of patients with early or very early relapse who have high chemo-resistant disease, and of patients with failure to induce remission after treatment with conventional HSCT. In order to treat such cases, we have developed a unique method to maximize the GVL effect of TCR-haplo-HSCT as a cell-mediated immunotherapy for refractory/relapsed acute leukemia including non-CR cases by reducing the amount of ATG, optimizing GVHD prophylaxis, and adding a high-dose of T-cell infusion. We retrospectively analyzed a case series of relapsed and refractory leukemia treated with TCR-haplo-HSCT and reported it in 2018 (10). In the present study, we added six new cases to the 13 previously reported cases and evaluated the efficacy of TCR-haplo-HSCT only in RR-BCP-ALL.

Although the analysis was based on a small number of patients ($N = 19$), the 3-year OS and RFS were 57.4 and 42.1%, respectively, despite including 11 extremely high-risk patients with active disease as non-CR cases. A noteworthy aspect of our study is that the 3-year OS of patients with non-CR ($N = 11$) was 45.5% (95%CI, 16.7–70.7). There were three non-CR

patients under 10 years of age, all of whom are still alive. In our previous report on relapsed/refractory acute leukemia in children, the age at transplantation (<10 years vs. ≥10 years) was considered an independent prognostic factor in multivariate analysis. Similarly, in this study of RR-BCP-ALL, the age at transplantation was a significant prognostic factor. The outcome of haplo-transplantation was excellent in younger children under 10 years of age, with a 3-year OS rate of 100%, compared to a 3-year OS rate of 20% in children over 10 years of age ($p < 0.002$). The absence of TRM and recurrence with loss of mismatched HLA are possible reasons for the better prognosis in children under 10 years of age.

In this study, cumulative incidence of acute GVHD (grade II–IV) was 100% and that of grade III–IV acute GVHD was 70.1%. Of the 18 evaluable patients, grade II–IV acute GVHD developed in the skin of 16 patients, in the gut in 7 patients, and in the liver in 3 patients. Three out of 19 patients died from TRM; thus, the cumulative incidence of TRM was 15.8%. These three patients were over 10 years of age and died from EBV-lymphoproliferative disease, pulmonary hemorrhage due to invasive aspergillosis, or septic shock due to *Stenotrophomonas maltophilia*. The relatively low cumulative incidence of TRM (15.8%) despite the high incidence of GVHD suggests that in these extremely high-risk RR-BCP-ALL patients, some risk of acute GVHD should be tolerated and enhanced immunosuppression should be avoided to maintain the GVL effect. Nevertheless, due to the high risk of GVHD, our TCR-haplo-HSCT should be targeted at high-risk patients who are not eligible for conventional transplantation. Furthermore, since the 5-year leukemia free survival (LFS) of haplo-HSCT with T cell-depleted grafts for BCP-ALL patients who failed to achieve remission was 0% (23), the antitumor effect in TCR-haplo-SCT is clearly based on the allogeneic immune response by cytotoxic T-cells. In 2009, Vago et al. (24) reported that leukemic cells could escape the donor's antileukemic T cells through the loss of the mismatched HLA haplotype after haplo-SCT and that this mismatched HLA loss is caused by acquired uniparental disomy of chromosome 6p. This phenomenon is thought to cause tumor cells to evade attack by alloreactive donor T cells and cause relapse. Mismatched HLA loss after TCR-haplo-HSCT is a clonal evolution based on a high degree of genomic instability; at present, there is no effective way to predict or prevent it. We encountered a rare case (Patient No. 18) of BM relapse with the loss of maternal-derived mismatched HLA haplotype after TCR-haplo-HSCT using PBSCs from his father; after second TCR-haplo-HSCT using PBSCs from his mother, he relapsed again with the loss of paternal-derived mismatched HLA haplotype (17). Such leukemia cells with high genomic instability are likely to develop resistance to TCR-haplo-HSCT, making it difficult to maintain long-term remission. In fact, in this study, all three patients who relapsed owing to mismatched HLA loss died. While donor lymphocyte infusion (DLI) is considered for patients who had relapsed after TCR-haplo-HSCT, it could be dangerous if the presence of mismatched HLA loss is not confirmed. In addition, when a second TCR-haplo-HSCT is conducted, appropriate donor selection is needed after confirming the mismatched HLA loss in leukemic blasts.

The prognosis of patients with post-transplant relapse is dismal (25). A second transplantation for post-transplant relapse of ALL is associated with a high rate of relapse and poor long-term survival of 30% in 2-year OS (26). We conducted TCR-haplo-HSCT again in four out of eight relapsed patients after the first TCR-haplo-HSCT, and two of them are still alive. Of the two patients who died, one patient relapsed owing to loss of mismatched HLA and the other died of graft failure. The two surviving patients were both younger than 10 years of age, and there was no loss of mismatched HLA at the time of relapse after TCR-haplo-HSCT. Since these patients achieved remission with chemotherapy after the post-TCR-haplo-HSCT relapse, it could be suggested that clonal selection by strong allogeneic immunity filtered out and restored chemotherapy sensitivity. Thus, at the time of relapse after TCR-haplo-HSCT, a second TCR-haplo-HSCT may be considered if there is no loss of mismatched HLA and no organ damage in younger children. The limitation of this study is that it is a retrospective study with a small number of patients and was limited to a single center. A multicenter prospective clinical trial is currently underway to validate the efficacy of TCR-haplo-HSCT.

In conclusion, we suggest that our TCR-haplo-HSCT method has the potential to save the lives of RR-BCP-ALL patients with a very poor prognosis and no other treatment options. However, this study was conducted on a small number of patients at a single institution, and the results should be interpreted with caution. In addition, since the incidence of acute GVHD is extremely high, further improvement of the transplantation method needs to be considered. Prospective clinical studies are needed to further clarify the efficacy of this method.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board, Fukushima Medical University. Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin. Written informed consent was obtained from the minor's legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HS and AK conceived and designed the study and collected data from the medical records. HS was responsible for writing the manuscript. HO and KI contributed to this methodology. KM, SKo, and AK contributed to the review of this manuscript. All authors read and approved the final manuscript.

FUNDING

This research was funded by the Department of Pediatric Oncology, Fukushima Medical University Hospital, who supported the open access publication cost.

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