Joint Modeling of Immune Reconstitution Post Haploidentical Stem Cell Transplantation in Pediatric Patients With Acute Leukemia Comparing CD34+ Selected to CD3/CD19-Depleted Grafts in a Retrospective Multicenter Study

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Rapid immune reconstitution (IR) following stem cell transplantation (SCT) is essential for a favorable outcome. The optimization of graft composition should not only enable a sufficient IR but also improve graft vs. leukemia/tumor effects, overcome infectious complications and, finally, improve patient survival. Especially in haploidentical SCT, the optimization of graft composition is controversial. Therefore, we analyzed the influence of graft manipulation on IR in 40 patients with acute leukemia in remission. We examined the cell recovery post haploidentical SCT in patients receiving a CD34+ selected or CD3/CD19-depleted graft, considering the applied conditioning regimen. We used joint model analysis for overall survival (OS) and analyzed the dynamics of age-adjusted leukocytes; lymphocytes; monocytes; CD3+, CD3+CD4+, and CD3+CD8+ T cells; natural killer (NK) cells; and B cells over the course of time after SCT. Lymphocytes, NK cells, and B cells expanded more rapidly after SCT with CD34+ selected grafts (P = 0.036, P = 0.002, and P < 0.001, respectively). Contrarily, CD3+CD4+ helper T cells recovered delayed in the CD34 selected group (P = 0.026). Furthermore, reduced intensity conditioning facilitated faster immune recovery of lymphocytes and T cells and their subsets (P < 0.001). However, the immune recovery for NK cells and B cells was comparable for patients who received reduced-intensity or full preparative regimens. Dynamics of all cell types had a significant influence on OS, which did not differ between patients receiving CD34+ selected and those receiving CD3/CD19-depleted grafts. In conclusion, cell reconstitution dynamics showed complex diversity with regard to the graft manufacturing procedure and conditioning regimen.

Keywords: immune reconstitution, allogeneic stem cell transplantation, CD34 selection, CD3/19 depletion, children
INTRODUCTION

The transplantation of allogeneic hematopoietic stem cells [stem cell transplantation (SCT)] provides a curative treatment option for patients suffering from high-risk acute leukemia and other hematological malignancies (1–4). The success of SCT is dependent on the rapid reconstitution of immune-compotent cells, which is influenced by different factors (5–10). Adequate immune recovery may result in the eradication of residual malignant cells (11) and can protect the patient from infectious complications (12, 13). Allogeneic SCT is mostly performed from an HLA-matched related or unrelated donor. For patients who lack a suitable donor, a haploidentical SCT and umbilical cord blood SCT (14) serve as an alternative. Haploidentical SCT (haplo-SCT) carries an increased risk of both graft vs. host disease (GvHD) and graft rejection compared to HLA-matched SCT but shows a decreased relapse rate (2, 15–17). GvHD is mainly induced by donor T cells (18, 19). Therefore, T cells need to be depleted prior to the infusion of the haploidentical graft. Different strategies for the purification of haploidentical grafts are available, such as the positive selection of CD34+ stem cells leading to indirect T-cell depletion (2, 15, 17) and the direct depletion of CD3+ T cells or TCR alpha/beta T cells, both in combination with CD19+ B-cell removal (20–23). CD34+-selected grafts contain highly purified stem cells and can be grafted in the absence of GvHD prophylaxis (24). CD3/CD19 or TCR alpha/beta-depleted grafts contain hematopoietic progenitors, natural killer (NK) cells, and antigen-presenting cells, probably leading to an enhanced graft vs. leukemia effect (16, 20, 23).

To date, studies about cellular immune reconstitution (IR) in pediatric patients post haplo-SCT are mainly done as single center studies and have long-term monitoring intervals, leading to small and heterogeneous patient cohorts. Therefore, in a retrospective multicenter study (Frankfurt, Ulm, Wuerzburg), we examined the IR post haplo-SCT in a relatively homogeneous group of 40 pediatric patients suffering from acute leukemia. We analyzed and compared the regeneration of various leukocyte subpopulations from children receiving CD34+-selected and CD3/CD19-depleted grafts. Our study considered age-adjusted leukocytes subpopulations as well as the individual progress of IR of each patient. In this respect, we were able to set up a joint model for IR, which allowed modeling of the continuous recovery process over time post SCT.

PATIENTS AND METHODS

Patients

In this retrospective study, we included 40 pediatric patients suffering from high-risk leukemia transplanted in complete remission (CR) with HLA-haploidentical peripheral blood (PB) stem cells between 1997 and 2012. Informed consent was obtained from all patients/parents, and the retrospective study was approved by the local ethic committee (Frankfurt, D Ethical No: 499/16). Inclusion criteria for patient recruitment in the three centers were as follows: (1) patients with acute lymphoblastic (ALL) or acute myeloblastic leukemia (AML), (2) a PBSC graft from a haploidentical donor processed with either positive CD34+ immunomagnetic selection (PBSCCD34Sel) or CD3/CD19 depletion (PBSCCD3/CD19dep) and (3) in case of multiple transplantation, inclusion criteria requires a >6-month interval between the last and penultimate transplant. Only immune monitoring after the last transplantation was considered in these cases.

Depending on the underlying disease, either myeloablative conditioning regimens (MAC) based on total body irradiation (12 Gy TBI) or busulphan, or a reduced-intensity conditioning regimen (RIC) with fludarabine, thioepa, and melphalan were used. The positive selection of CD34+ PB stem cells with immunomagnetic microbeads and the direct depletion of T and B cells with-coated microbeads under good manufacturing practice (GMP) were performed with-coated microbeads using a CliniMACS (Miltenyi Biotec, Bergisch-Gladbach, Germany) device under GMP as we described previously (23, 25). For prophylaxis of GvHD, OKT3 or anti-thymocyte globulin was given. Posttransplantation, the GvHD prophylaxis consisted of mycophenolate mofetil in cases of patients receiving PBSCCD3/ CD19dep grafts, while for patients transplanted with PBSCCD34Sel stem cells, no GvHD prophylaxis was given. The grade of acute GvHD was defined according to published consensus criteria (26). Engraftment of neutrophils was defined as the first of 3 days with a neutrophil count >500 cells/μl.

Flow Cytometric Analyses for Monitoring of IR

In all three centers, PB samples were collected regularly post haplo-SCT (>10 times/year) using EDTA tubes and analyzed the same day to monitor IR. The absolute numbers of leukocytes, CD14+ monocytes, lymphocytes, CD3+ T cells, CD3+CD4+ helper T cells, CD3+CD8+ cytotoxic T cells, CD3+CD56 NK cells and CD19+ B cells were determined by flow cytometry in a lysis-no-wash procedure as we described previously (8, 9, 25, 27). Briefly, two tubes with 100 μl of PB were labeled with tetraCHROME (Frankfurt) or manually pipetted (Wuerzburg, Ulm) with combinational monoclonal antibody reagents for CD45/ CD4/CD8/CD3 and CD45/CD56/CD19/CD3 conjugated with fluorescein isothiocyanate, phycoerythrin (PE), phycoerythrin Texas red (ECD), phycoerythrin-cyanine 5.1 (PC5), PerCp, and APC, respectively. The accredited tetraCHROME antibodies included anti-CD45 (clone: B3821F4A), anti-CD4 (clone: SFCI12TD4D11), anti-CD8 (clone: SFCI21Thy2D3), anti-CD3 (clone: UCHT1), anti-CD56 (clone: N901) and anti-CD19 (clone: J3-119). Monocytes (clone: RMO52) were detected by adding phycoerythrin-cyanine 7 (PC7) labeled anti-CD14 monoclonal antibodies to the first tube or by a separate CD45/CD14 analysis. All reagents are purchased from Beckman Coulter® Immunotech (Marseille, France) or BD/Pharmigen (Heidelberg). The samples were measured with an FC500™ (Coulter) or a FACSCalibur/Canto-II (BD) flow cytometer. Absolute cell counts were calculated from the percentage values using a dual-platform approach.

For assessment of quality control, flow-setTM fluorospheres or similar products were used to set up the photomultiplier tube values weekly. Fluorescence overlap was compensated for, and both the flow cytometer optical alignment and the fluidic
stability were tested daily as we described previously. Control cells (i.e., Immuno-Trol™) were used for verification (27). During the study, the values of all controls remained within the designated limits.

**Statistical Analysis**

Sequential measurements of eight leukocyte subpopulations after haplo-SCT were used to compare the dynamics of IR between patient cohorts receiving PBSC<sub>CD34 sel</sub> or PBSC<sub>CD3/CD19 dep</sub> grafts in relation to outcome. We characterized the IR dynamics as a continuous process over time and took the strong age-dependency of IR into account (27–35) by normalizing each quantification value with the corresponding age-specific expected mean value (see Table S1 in Supplementary Material). Furthermore, the regression models used normalized levels of the leukocyte subpopulations after log-transformation to obtain an approximately Gaussian distribution for the residuals.

Then, each of the eight leukocyte subpopulations and patient survival were analyzed with a joint model for longitudinal and time-to-event data (36). Thereby, the whole recovery process over time post SCT was assessed. As immune recovery after SCT is not linear in time, the longitudinal submodel was a linear mixed-effect model using B-splines of the third order with two inner knots (37). The time-to-event outcome was overall survival (OS), which was assessed with a Cox proportional hazard submodel (38). Furthermore, the Kaplan-Meier method, standard Cox proportional hazard regression and the log-rank test were used for analyzing OS.

In addition, patient characteristics are described by median and range. Fisher’s exact test, the Wilcoxon-Mann-Whitney test and the Kruskal-Wallis test were used to compare characteristics between the two groups.

All tests were two sided, and \( P < 0.05 \) was considered significant. Statistics analysis used the R 3.2.5 (R Foundation for Statistical Computing, Vienna, Austria) with the JM package for model fitting (39) and ‘BIAS for Windows’ v11.02 (Epsilon-Verlag, Frankfurt, Germany).

**RESULTS**

The study cohort included 40 patients, with 11 patients receiving PBSC<sub>CD34 sel</sub> and 29 patients receiving PBSC<sub>CD3/CD19 dep</sub> in CR at transplantation time. Table 1 and Figure 1 give detailed information about both the patients and the transplant characteristics.

**Dynamics of Immune Recovery**

Our joint modeling approach primarily compared two graft manipulations (PBSC<sub>CD34 sel</sub> vs. PBSC<sub>CD3/CD19 dep</sub>) considering the conditioning regimen (MAC vs. RIC).

We fitted a joint model for OS and each of the following leukocyte subsets: overall leukocytes, lymphocytes, CD14<sup>+</sup> monocytes, CD3<sup>+</sup> T cells, CD3<sup>+</sup>CD4<sup>+</sup> helper T cells, CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T cells, CD3<sup>+</sup>CD56<sup>+</sup> NK cells, and CD19<sup>+</sup> B cells, as shown in Figure 2. Such modeling allowed a comparison of leukocyte subset recovery after PBSC<sub>CD34 sel</sub> and PBSC<sub>CD3/CD19 dep</sub> transplantsations. Tables 2 and 3 present the respective immune recovery prognosis values of our models showing the predicted percent values (% of

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**TABLE 1 | Patient’s characteristics.**

| CD34 sel | CD3/CD19 dep | \( P \) |
|----------|-------------|------|
| n = 11   | n = 29      |      |
| SCT time period | August 1997–January 2005 | April 2005–March 2012 |
| IR follow-up, median (range) (months) | 5.7 (2.3–160.3) | 8 (1.4–138) |
| Survival follow-up, median (range) (months) | 149 (72–160.3) | 101 (44–138) |
| Patient-related factors | | |
| Age at SCT, median (range) years | 7.5 (3.5–23) | 10.6 (1.3–26) | 0.437 |
| Sex (male/female) | 8/3 | 20/9 | 1 |
| BMI at SCT, median (range) kg/m² | 17.9 | 17.3 | 0.802 |
| Time from diagnosis to SCT, median (range) years | (14.3–20.2) | (11.9–28.7) |
| Disease-related factors | | |
| Diagnosis | | 0.158 |
| AML | 2 | 13 |
| M0/M1/M2/M4/M5/M6/M7 | 0/0/1/0/0/0/0/0 | 2/2/3/1/2/1 |
| No data | 2 |
| ALL | 9 | 16 |
| T-ALL/BCP-ALL/ | 3/6/0 | 7/7/2 |
| Biphen-ALL | | |
| Status at SCT | | |
| CR1/CR2/ ≥ CR3 | 2/5/4 | 8/12/9 | 0.820 |
| Time from diagnosis to SCT, median (range) years | 2.4 (0.7–4.2) | 1.3 (0.3–5.7) | 0.417 |
| Donor-related factors | | |
| Age, median (range) years | 38 (28–42) | 37 (24–51) | 0.628 |
| Sex (male/female) | 9/2 | 16/13 | 0.158 |
| Patient–donor sex | | 0.547 |
| Female–female | 1 | 5 |
| Male–male | 7 | 12 |
| Male–female | 1 | 8 |
| Female–male | 2 | 4 |
| ABO compatibility | | |
| Compatible | 1 | 20 |
| Incompatible | 3 | 7 |
| No data | 7 | 2 |
| CMV status (recipient–donor) | | |
| Positive–positive | 2 | 11 |
| Negative–positive | – | 9 |
| Positive–negative | – | 1 |
| Negative–Negative | 2 | 4 |
| No data | 7 | 4 |
| Transplant-related factors | | 0.732 |
| SCT-number | 2/5/4 | 8/12/9 |
| Conditioning regimen | | <0.001<sup>a</sup> |
| Standard myeloablative | 9 | 5 |
| TBI-based | 6 | 4 |
| Chemo-based | 3 | 1 |
| Reduced myeloablative | 2 | 24 |
| Flud/TT/Mel | 2 | 24 |
| Serotherapy | | 0.005 |
| Without | – | 2 |
| ATG | 8 | 5 |
| OKT3 | 3 | 21 |
| Graft vs. host disease prophylaxis | | <0.001 |
| Without | 11 | 3 |
| MMF | – | 25 |
| CSA, MTX | – | 1 |
| (Continued) | | |

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(Continued)
Overall, the number of cells did not exhibit significant differences with respect to graft purification. For the overall monocytes, we observed a rapid IR, with approximately 80% of their age norm value already at 30 days after SCT (Figure 2C).

CD3 + T cells, CD3 + CD4 + helper T cells, and the CD3 + CD8 + cytotoxic T cells recovered significantly quicker in the patients receiving the MAC regimen than in those patients receiving MAC regimen (Figures 2D–F, P < 0.001). In the MAC group, the frequency values were nearly 1.5 times higher at each time point compared to the respective kind of manipulation receiving the MAC regimen during the first year post haplo-SCT. No significant differences were detected in the recovery of CD3 + T cells and the CD3 + CD8 + cytotoxic T cells owing to the kind of graft purification, PBSC CD34 sel and PBSC CD3/CD19 dep among patients undergoing the same regimen of conditioning. The CD3 + T cells increased from 1% of the reference values at day 30 to 37% at the end of the first year and from 1 to 31% for PBSC CD34 sel and PBSC CD3/CD19 dep, respectively, with both groups receiving MAC (Table 2).

In the MAC group, the CD3 + T cells increased from 1.7% of the reference values at day 30 to 54% at the end of the first year and from 1 to 45% for PBSC CD34 sel and PBSC CD3/CD19 dep respectively (Table 3).

In contrast, the CD3 + CD4 + helper T cells recovered significantly delayed in patients receiving PBSC CD34 sel grafts than in those transplanted with PBSC CD3/CD19 dep grafts from approximately 6th to 24th months after transplantation for both the MAC and MAC groups (P < 0.01).

In both the MAC and MAC groups, the ratio of CD4 + /CD8 + trended downward because the cytotoxic T cells recovered faster than the helper T cells (Figure 2I).

As expected, in vivo NK expansion post haplo-SCT was very rapid (Figure 2G). No differences in the NK cell recovery were found between the MAC and MAC groups. Interestingly, in the first 4 months, NK cell recovery was faster in patients receiving PBSC CD34 sel grafts than in those transplanted with a PBSC CD3/CD19 dep graft (P = 0.002). Afterward, the in vivo NK cell proliferation was very similar between the two patient groups.

Recovery of CD19 + B cells showed no differences in the IR regarding the conditioning regimen (Figure 2H). With regard to graft manipulation, B-cell recovery was faster in the PBSC CD34 sel patient group than in the PBSC CD3/CD19 dep group (P < 0.001). This difference was very pronounced in the first year after haplo-SCT. At the end of the first year, the B cells in the group PBSC CD34 sel with MAC had already achieved 30% of the age-matched reference values. In contrast, in the PBSC CD3/CD19 dep with MAC group, the B cells had only reached 9% of the age reference values.

**Clinical Outcome and Survival**

The medians (range) OS follow-up time were 149 (72–160.3) and 101 (44–138) months in patients receiving PBSC CD34 sel and PBSC CD3/CD19 dep, respectively.

### Table 1

| Graft's composition | CD34 sel | CD3/CD19 dep | P  |
|---------------------|----------|--------------|----|
|                     | n = 11   | n = 29       |    |
| CD34*, median       | 15.3     | 10.1         | 0.009 |
| (range) × 10³/kg BW | (4.9–36.9)| (4.8–20.7)   |    |
| CD3+, median        | 1.0 (0.4–3) | 8.9 (0–5,000)| <0.001 |
| (range) × 10³/kg BW |          |              |    |

**Impact of Both Conditioning Regimen and Graft on IR of Patients in CR**

The majority of the 29 patients transplanted in CR with a CD3/CD19-depleted graft received reduced intensity conditioning, while the majority of the 11 patients transplanted in CR with a CD34-selected graft underwent full myeloablative conditioning (see Figure 1 for full details). To separate the influence of conditioning regimen and graft manipulation, all four scenarios (CR-MAC PBSC CD34 sel, CR-MAC PBSC CD3/CD19 dep, CR-RIC PBSC CD34 sel, and CR-RIC PBSC CD3/CD19 dep) were compared.

The increase in overall leukocytes was similar in the RIC and MAC patients during the first year (Figure 2A). However, within the same conditioning regimen group, faster leukocyte proliferation was found in patients with PBSC CD34 sel grafts than in patients with PBSC CD3/CD19 dep grafts from the 6 months after SCT. Previously, the leukocyte populations were comparable between the two groups with graft manipulation.

The overall lymphocytes in patients receiving PBSC CD34 sel grafts differed from those with PBSC CD3/CD19 dep grafts, also showing a faster recovery in the early phase until the 6th month after haplo-SCT (Figure 2B, P = 0.036). This was mainly due to a faster reconstitution of NK cells and B cells in patients receiving PBSC CD34 sel vs. PBSC CD3/CD19 dep. Afterward, the course of lymphocyte values was comparable in both groups. Concerning the conditioning regimen, we observed a faster expansion of the overall lymphocytes in the RIC group than in the MAC group. At the end of the first year, the RIC and MAC groups reached approximately 42 and 36% of the normal reference value, respectively.

Monoocyte expansion was similar in RIC and MAC recipients, but the number of cells did not exhibit significant differences with respect to graft purification. For the overall monocytes, we observed a rapid IR, with approximately 80% of their age norm value already at 30 days after SCT (Figure 2C).

CD3 + T cells, CD3 + CD4 + helper T cells, and the CD3 + CD8 + cytotoxic T cells recovered significantly quicker in the patients receiving the RIC regimen than in those patients receiving MAC regimen (Figures 2D–F, P < 0.001). In the RIC group, the frequency values were nearly 1.5 times higher at each time point compared to the respective kind of manipulation receiving the MAC regimen during the first year post haplo-SCT. No significant differences were detected in the recovery of CD3 + T cells and the CD3 + CD8 + cytotoxic T cells owing to the kind of graft purification, PBSC CD34 sel and PBSC CD3/CD19 dep among patients undergoing the same regimen of conditioning. The CD3 + T cells increased from 1% of the reference values at day 30 to 37% at the end of the first year and from 1 to 31% for PBSC CD34 sel and PBSC CD3/CD19 dep, respectively, with both groups receiving MAC (Table 2).

In the MAC group, the CD3 + T cells increased from 1.7% of the reference values at day 30 to 54% at the end of the first year and from 1 to 45% for PBSC CD34 sel and PBSC CD3/CD19 dep respectively (Table 3).

In contrast, the CD3 + CD4 + helper T cells recovered significantly delayed in patients receiving PBSC CD34 sel grafts than in those transplanted with PBSC CD3/CD19 dep grafts from approximately 6th to 24th months after transplantation for both the RIC and MAC regimens (P < 0.01).

In both the RIC and MAC groups, the ratio of CD4 + /CD8 + trended downward because the cytotoxic T cells recovered faster than the helper T cells (Figure 2I).

As expected, in vivo NK expansion post haplo-SCT was very rapid (Figure 2G). No differences in the NK cell recovery were found between the RIC and MAC groups. Interestingly, in the first 4 months, NK cell recovery was faster in patients receiving PBSC CD34 sel grafts than in those transplanted with a PBSC CD3/CD19 dep graft (P = 0.002). Afterward, the in vivo NK cell proliferation was very similar between the patient groups.

Recovery of CD19 + B cells showed no differences in the IR regarding the conditioning regimen (Figure 2H). With regard to graft manipulation, B-cell recovery was faster in the PBSC CD34 sel patient group than in the PBSC CD3/CD19 dep group (P < 0.001). This difference was very pronounced in the first year after haplo-SCT. At the end of the first year, the B cells in the group PBSC CD34 sel with RIC had already achieved 30% of the age-matched reference values. In contrast, in the PBSC CD3/CD19 dep with MAC group, the B cells had only reached 9% of the age reference values.

**Clinical Outcome and Survival**

The medians (range) OS follow-up time were 149 (72–160.3) and 101 (44–138) months in patients receiving PBSC CD34 sel and PBSC CD3/CD19 dep, respectively.
The incidence of aGvHD grades I and II was 45% (5/11) in patients with PBSCCD34sel grafts and 48% (14/29) in patients with PBSCCD3/CD19dep grafts. The incidence of aGvHD grades III and IV was 18% (2/11) and 14% (4/29) in the cohorts with PBSCCD34sel vs. PBSCCD3/CD19dep grafts, respectively. The incidence of aGvHD did not significantly differ between both groups. Table 4 presents details of the clinical outcome.

Overall, 21/40 patients died. The main cause of death was relapse (n = 13), followed by non-relapse mortality (NRM, n = 8). The Kaplan–Meier curves for OS were compared between the different groups (Figure 3A). The log rank test was not significant for differences between the PBSCCD34sel and the PBSCCD3/CD19dep groups (Table S3 in Supplementary Material). Likewise, there were no significant differences in the causes of death between the PBSCCD34sel and the PBSCCD3/CD19dep patient groups.

Using standard Cox regression, we analyzed the association between transplant-associated factors and the probability of OS without considering the evolution of IR after haplo-SCT. In the univariable analysis, age, gender, diagnosis, TBI (yes/no), conditioning regimen, graft purification, graft composition, and engraftment day were not associated with OS. In the multivariable analysis, considering graft purification, conditioning regimen, graft composition, and the univariable analysis, age, gender, diagnosis, TBI (yes/no), OS without considering the evolution of IR after haplo-SCT. In this respect, it has to be noted that direct comparisons of IR after transplantation with PBSCCD34sel compared with PBSCCD3/CD19dep grafts have to be handled with caution because patients receiving the latter normally do not need immunosuppressive therapy. However, the most important factor for T-cell and T subsets recovery was the conditioning regimen. For patients in CR receiving either PBSCCD34sel or PBSCCD3/CD19dep grafts, IR was much faster after the RIC regimen than after the MAC regimen. However, it has to be mentioned that the main limitations of our study are its retrospective nature and the small number of patients, although it is a multicenter study.

Comparison of our results with other haploidentical pediatric studies in the literature showed that most of the other studies about IR were using very heterogeneous patient groups (43–45) with only some of them treated with haplo-SCT (2, 46–52) or analyzed IR including patients with PR/NR (45, 53). Other investigations are not comparable with our study due to the administration of fixed addback (54, 55), or reported about other malignant (56) or nonmalignant diseases (57).

A high dose of CD34 cells is required in the haploidentical setting for a successful transplant and therefore rapid and sustained engraftment (58). Despite the differences in the CD34 doses between the CD34 selected group and the CD3/CD19-depleted

**DISCUSSION**

To date, there are ongoing discussions about optimization of graft composition in haplo-SCT in order to reach a sufficient IR with an additional goal to improve the GVL/GVT effect and, finally, the survival of the patients. Various factors affecting IR after SCT, especially haplo-SCT (5–10), have been identified, such as age, diagnosis, stage of disease, remission status at transplantation, conditioning regimen, HLA-typing, and graft manipulation (6, 40–42).

Investigation of IR post SCT, especially haplo-SCT, has been done in single-center studies, mostly in heterogeneous patient groups using univariate analyses. Here, we compared the dynamics of IR in patients receiving PBSCCD34sel vs. PBSCCD3/CD19dep when used for haplo-SCT in a homogeneous pediatric patient group with acute leukemia from a retrospective multicenter study. To investigate the complexity of various factors on IR, we were able to develop joint models for longitudinal and time-to-event data. To our knowledge, this is the first time that the influence of multivariable clinical factors, including RIC, MAC, and age-related dependence of leukocytes and their subpopulations, has been evaluated in detail in haplo-SCT from pediatric patients suffering leukemia.

Surprisingly, IR for T cells did not differ between patients receiving PBSCCD34sel or PBSCCD3/CD19dep grafts with the exception of CD4+ helper T cells, which showed much slower recovery after transplantation using a PBSCCD34sel graft. NK and B cells had a faster reconstitution after transfusion of the PBSCCD34sel graft. In this respect, it has to be noted that direct comparisons of IR after transplantation with PBSCCD34sel compared with PBSCCD3/CD19dep grafts have to be handled with caution because patients receiving the latter normally do not need immunosuppressive therapy. However, the most important factor for T-cell and T subsets recovery was the conditioning regimen. For patients in CR receiving either PBSCCD34sel or PBSCCD3/CD19dep grafts, IR was much faster after the RIC regimen than after the MAC regimen. However, it has to be mentioned that the main limitations of our study are its retrospective nature and the small number of patients, although it is a multicenter study.

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A high dose of CD34 cells is required in the haploidentical setting for a successful transplant and therefore rapid and sustained engraftment (58). Despite the differences in the CD34 doses between the CD34 selected group and the CD3/CD19-depleted
group, no differences in the neutrophil engraftment or aGVHD was found.

In contrast to our findings that the IR of the CD3+ T cells and CD8+ cytotoxic T cells was comparable between recipients receiving haplo-PBSC_{CD3/CD19dep} and haplo-PBSC_{CD34sel} if both groups received the same conditioning regimen, other researchers reported slower expansion of T cells, CD4+, and CD8+ cells in recipients receiving mismatch/haplo-PBSC_{CD34sel} than in...
| Cells | Day 30 | Day 60 | Day 90 | Day 180 | Day 365 |
|-------|--------|--------|--------|--------|--------|
|       | CD34sel | CD3/C19dep | CD34sel | CD3/C19dep | CD34sel | CD3/C19dep | CD34sel | CD3/C19dep | CD34sel | CD3/C19dep |
| Leukocytes | 34.22 (25.94–45.15) | 39.98 (34.91–45.79) | 52.49 (38.82–70.98) | 47.05 (41.29–53.60) | 64.26 (47.11–87.64) | 50.23 (44.15–57.14) | 66.80 (53.09–84.04) | 49.21 (43.78–56.31) | 75.69 (61.47–93.19) | 56.14 (49.47–63.72) |
| Lymphocytes | 15.69 (12.39–19.86) | 12.75 (10.80–15.04) | 35.30 (28.66–42.23) | 21.93 (18.68–25.75) | 42.23 (34.98–50.99) | 25.75 (22.13–29.98) | 31.67 (27.21–36.85) | 25.05 (21.72–28.90) | 36.43 (31.11–42.67) | 36.18 (31.36–41.74) |
| Monocytes | 64.81 (48.21–87.14) | 86.29 (72.61–102.56) | 68.52 (53.69–87.44) | 71.44 (60.32–84.61) | 69.99 (53.85–88.39) | 62.25 (52.62–73.64) | 64.91 (54.22–77.71) | 51.04 (44.01–59.19) | 72.76 (60.36–87.71) | 54.14 (46.34–63.26) |
| CD3* | 1.18 (0.82–1.69) | 1.15 (0.91–1.45) | 5.23 (3.76–7.30) | 4.90 (3.91–6.15) | 9.13 (6.98–11.94) | 7.98 (6.54–9.73) | 21.05 (16.80–26.38) | 16.35 (13.48–19.82) | 36.98 (29.83–45.85) | 30.94 (25.31–37.81) |
| CD4* | 0.91 (0.72–1.16) | 1.32 (0.94–1.98) | 3.22 (1.90–2.85) | 3.22 (2.46–4.21) | 3.10 (2.59–3.72) | 3.63 (3.65–4.44) | 5.15 (4.29–6.18) | 6.18–9.54 | 9.27 | 14.08 |
| CD8* | 2.24 (1.82–2.76) | 1.52 (1.0–2.33) | 7.13 (6.17–8.24) | 7.22 (5.49–9.52) | 11.91 (10.47–13.16) | 15.85 (12.80–19.63) | 22.13 (18.57–25.01) | 26.30 | 51.33 | 37.96 |
| CD3*CD56* | 88.93 (68.93–115.23) | 65.10 (53.59–79.07) | 145.31 (114.59–184.27) | 78.72 (65.0–94.34) | 85.19 (72.35–100.31) | 58.80 (49.56–69.77) | 47.80 (40.37–66.60) | 44.25 (37.23–52.59) | 47.66 (41.79–54.35) | 46.51 |
| CD19+ B | 2.82 (1.86–4.26) | 1.71 (1.40–2.09) | 10.57 (7.12–15.70) | 9.09 (4.23–6.13) | 21.46 (14.45–31.89) | 8.59 (7.10–10.40) | 24.52 (18.55–32.40) | 7.30 (6.16–8.65) | 25.49 | 9.35 |

The table shows the predicted percent values (% of the norm values) derived from our joint model with 95% confidence interval at +30, +60, +90, +120, and +365 days after transplantation. The values were obtained for children in complete remission (CR) at time point of haplo-SCT. The percent values represent the fraction with respect to the predicted age-matched norm values by Huenecke et al. (27). See also Table S2A in Supplementary Material for a representation of absolute numbers for predicted values for a 10-year-old child.
recipients receiving haplo-PBSC_{CD3/CD19dep} (41, 49). A possible reason for this discrepancy may be that the mentioned studies did not differentiate between the influences of the graft and the conditioning on IR. The majority of patients received PBSC_{CD34sel} combined with the MAC regimen or PBSC_{CD3/CD19dep} combined with the RIC regimen. When we compared the IR between the PBSC_{CD34sel} vs. PBSC_{CD3/CD19dep}, without considering conditioning, our results were consistent with these previous findings (data not shown).

In our study, there was no difference in the CD3^+CD4^+ helper T-cell recovery between patients receiving depleted or selected grafts until the sixth month after haplo-SCT. Therefore, it seems unlikely that better expansion of helper T cells at late time points is explained by residual T cells in the CD3/CD19-depleted graft. However, after the sixth month, the CD3^+CD4^+ helper T cells recovered significantly faster in patients receiving CD3/CD19-depleted grafts than in those receiving CD34-selected grafts, regardless of the type of conditioning. This could also be the effect of other therapeutic agents applied combined with the CD3/CD19-depleted graft.

It is well known that RIC facilitates rapid reconstitution of T cells and its subsets in PBSC transplantation. However, the RIC method is often not strong enough to eradicate the malignant cells, and it is associated with a higher incidence of mixed chimerism (59), higher relapse, and mortality risk (60). After SCT, non-remission rates found in studies with patients who received haplo-PBSC_{CD3/CD19dep} combined with an RIC regimen were 50.2% (61), 68% (45), and 57% (53), but note that 31 of 61 adult patients did not achieve CR in the first study, 22 of 26 analyzed childhood patients in the second study suffered from malignancies, and 43%...
of patients from the third study had active disease at the time of transplantation.

Surprisingly, we observed higher expansion of NK cells in the early phase of SCT in patients receiving a PBSCCD34sel graft than in those patients who received a PBSCCD3/CD19dep graft, despite the fact that PBSCCD3/CD19dep grafts contain considerably higher numbers of NK cells. Furthermore, we did not find any differences between the immune recoveries of NK cells when comparing positive selection or negative depletion with conditioning preparative treatments MAC vs. RIC. Our results about NK expansion in the PBSCCD34sel combined with MAC are in conformity with the study from Lang et al. (50). In that trial ($n$ = 29; 62%) and those patients receiving mismatch-PBSCCD34sel grafts with MAC in pediatric patients. They found that only at day +14 was there a significant difference advantageous for PBSCCD3/CD19dep recipients (45). However, the HLA-disparity and related donors were different between the PBSCCD34sel groups in our and the mentioned study, and the latter included heterogeneous diseases with 64% cases of acute leukemia in the mismatch-PBSCCD34sel group and only 42% cases of acute leukemia in the haplo-PBSCCD3/CD19dep group. Additionally, Pfeiffer reported a rate of 42% with active diseases (PR/NR) prior to SCT in the CD3/CD19-depleted group, while our study included patients in CR only.

Significantly higher NK cell proliferation in patients receiving PBSCCD3/CD19dep grafts than in patients undergoing PBSCCD34sel (both groups combined with RIC) was presented by Gonzalez-Vicent et al. (51). However, in this investigation, 74% of patients with PBSCCD34sel had a matched donor, while all patients with PBSCCD3/CD19dep received a transplant from mismatched donors.

Alternatively, unmanipulated blood and marrow transplantation is another feasible option in haploidentical transplantations. Marek et al. (62) examined lymphoid recovery of haploidentical in vivo ($n$ = 19) vs. in vitro T-cell depletion ($n$ = 53, 89% haploidentical donors, 81% acute leukemia, 76% children). The in vitro T-cell depletion was mostly PBSCCD34sel (94%) with the MAC regimen (78%). They reported faster IR not only of the NK cells but also of all lymphocyte subpopulations in patients receiving grafts with in vitro T-cell depletion than observed in our results. Nevertheless, they used a MAC regimen based on cyclophosphamide + TBI instead of VP16 + TBI as in our study.

There are no studies comparing B-cell recovery in pediatric patients who received RIC vs. MAC regimens after haplo-SCT. However, in ALL childhood patients, the chemotherapy strongly affects B cells (63). In adult studies, there is no consensus about this point (7, 64–67).

Concerning the graft manipulation technique, similar to our results, Bettege et al. (20) reported faster B-cell expansion in adult patients receiving haplo-PBSCCD34sel than in those who received haplo-PBSCCD3/CD19dep grafts. The influence of serotherapy on the IR especially on the B cells is reported in several studies (68–70). In our study, the size of the cohort did not allow the inclusion of this factor in our model.

Booth et al. reported the unpublished observations from Lawton and Darbyshire (71). They found no significant difference between the CD34 selection compared to CD3/CD19 depletion in terms of engraftment, IR, viral infections, or non-relapse mortality.

**CONCLUSION**

We analyzed a homogeneous pediatric group suffering from high-risk acute leukemia in CR, who underwent in vitro haplo-PBSC in a retrospective study. A limitation of our study is the small number of patients, although it is a multicenter study.

Comparing transplantation with CD34-positive selected and CD3/CD19-depleted grafts patients did not show marked differences in the IR, while differences were stronger between the conditioning regimens. For further optimization of the treatment of acute childhood leukemia, conditioning regimen, graft purification, and their interplay should always be considered, particularly for novel techniques of graft manipulation and engineering, such as TCR alpha/beta depletion and in vivo T-cell depletion, as well as genetically modified T-cell products.

**ETHICS STATEMENT**

Informed consent was obtained from all patients/parents, and the retrospective study was approved by the respective local ethic committee (Frankfurt EK 499/16, 50/07), Würzburg (EK 133/04) and has been included in part in the registered studies for both, transplantation with CD3/CD19 depleted grafts (EudraCT-No.: 2006-000393-76) as well as the ALL-SCT study (register No. 3352).

**AUTHOR CONTRIBUTIONS**

Study design and wrote the manuscript: UK, EH, and ES-M. Provided clinical data: ME, AS, and PB. Analyzed the data: RE, MB, and SH. Coordinated the research: UK and EH. Organized and data collection: ES-M, MS, and MB. Performed statistical analyses: ES-M. Revised the manuscript: UK, EH, SH, MB, ME, and PB. Supervised the research: EH, UK, and TK. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01841/full#supplementary-material.
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