

## ORIGINAL ARTICLE

# Immune recovery in children undergoing allogeneic stem cell transplantation: absolute CD8<sup>+</sup>CD3<sup>+</sup> count reconstitution is associated with survival

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To evaluate the correlation between kinetics of immune reconstitution and survival, we prospectively evaluated lymphocyte subsets in 32 paediatric patients undergoing allogeneic stem cell transplantation (SCT) for haematological malignancies. Four-colour flow cytometric analysis was performed at short intervals with a median follow-up of 4 years post SCT. A total of 50% of patients reached age-matched 5th percentile of natural killer, cytotoxic T, B and helper T cells 4, 9, 20 and 28 weeks after SCT, respectively, which increased to more than 80% within 1 year after SCT. Transplantation of peripheral blood stem cells (PBSC) seemed to elicit the fastest reconstitution of CD3<sup>+</sup>, CD4<sup>+</sup>CD3<sup>+</sup>, CD8<sup>+</sup>CD3<sup>+</sup> and naïve T cells compared to bone marrow (BM) or CD34-selected PBSC, which did not differ. Most importantly, we observed a significantly higher number of survivors among patients whose CD8<sup>+</sup>CD3<sup>+</sup> absolute counts rose above the 5th percentile of age-matched normal levels during the first year post SCT compared to patients who never reached these levels (19/25 vs 0/7,  $P < 0.001$ ). This was still present in both subgroups, BM- and CD34-selected grafts ( $P = 0.03, 0.02$ ). These results from a small patient sample underline the importance of particular lymphocyte subsets for the outcome of children undergoing SCT. A larger study with detailed subset analysis is underway.

*Bone Marrow Transplantation* (2007) 39, 269–278.  
doi:10.1038/sj.bmt.1705584

**Keywords:** allogeneic transplantation; haematopoietic reconstitution; children

## Introduction

Allogeneic haematopoietic stem cell transplantation (SCT) is a recognized treatment for a subgroup of patients with haematological malignancies. Post SCT, prolonged lymphocytic immune deficiency is related to infectious morbidity<sup>1–3</sup> and an increased risk of relapse,<sup>4</sup> both associated with increased mortality. A number of factors influence the haematopoietic reconstitution after SCT, such as the graft source,<sup>5</sup> the immunosuppressive therapy and the age of the patients.<sup>6</sup> SCT with full peripheral blood stem cell (PBSC) grafts results in an earlier immune reconstitution compared to bone marrow (BM) grafts, whereas CD34-selected (PBSC-CD34<sup>+</sup>) grafts elicit a haematopoietic recovery similar to BM.<sup>5,7</sup> The differences are attributed to the number of lymphocytes transfused with the grafts. PBSC contain approximately one log more lymphocytes compared to BM.<sup>7</sup> Data on the incidence of infections after SCT with regard to the stem cell source are conflicting. Storek *et al.*<sup>8</sup> reported a higher incidence of infections after BM transplantation (BMT) than after PBSC transplantation (PBSC) in adults, whereas Behringer and co-workers<sup>5</sup> did not observe differences between PBSC, PBSC-CD34<sup>+</sup> transplantation and BMT in spite of the extremely low amount of T cells infused in the case of PBSC-CD34<sup>+</sup>. Lymphocytic immunodeficiency after adult BMT is prolonged and more profound than after childhood BMT,<sup>9</sup> associated with a significantly increased risk of life-threatening infections, which in part may be owing to delayed reconstitution of the T-cell receptor repertoire.<sup>10</sup> T cells may be regenerated through two different mechanisms. One is thymus-dependent and plays a major role in children, leading to an early broad T-cell receptor repertoire. The other, predominant mechanism in case of adults whose thymus is involuted seems to be the expansion of peripheral T cells infused with the graft.<sup>10</sup>

As most studies on immune reconstitution after SCT were performed in adults, data in paediatric patients were limited. Moreover, to date, lymphocyte subsets were assessed in monthly or even longer intervals,<sup>3,5–9</sup> which does not allow recognition of transient changes in lymphocyte subsets. Therefore, we measured the

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Received 8 September 2006; revised 27 November 2006; accepted 18 December 2006

reconstitution of natural killer (NK)-, B- and T-cell subsets as well as that of naïve, memory and activated cells in children after SCT at short intervals in a pilot study. To this end, we compared the absolute values with age-matched normal values from the literature<sup>11</sup> and analysed the impact of reaching the 5th or 50th percentile for various lymphocyte subgroups as well as the impact of the stem cell source. The data were analysed with regard to predictive-ness for survival.

## Patients and methods

### Patients

Immune reconstitution was prospectively measured in 37 paediatric patients undergoing SCT. Thirty-two patients who survived for at least 6 months after SCT and who were monitored at regular intervals were included in the analysis. Two patients were excluded because of early transplant related death; three patients were lost for follow-up. Diagnoses of the analysed patients included high-risk acute lymphoblastic leukaemia (ALL) in first complete remission (CR) ( $n=5$ ), ALL in second CR ( $n=10$ ) or third CR ( $n=2$ ) and ALL not in remission (NR) ( $n=1$ ), AML in first ( $n=2$ ) or second CR ( $n=5$ ) and AML NR ( $n=2$ ), CLL ( $n=1$ ), juvenile myelomonocytic leukaemia/myelodysplastic syndrome ( $n=2$ ) and refractory cytopenia ( $n=2$ ). The conditioning regimens varied according to the underlying disease (Table 1). Informed consent was obtained for all patients. For the evaluation of immune reconstitution the patients were divided into age groups as follows, because absolute numbers of lymphocyte subsets in healthy children differ among these groups:<sup>11</sup> 2–5 years ( $n=10$ ), 5–10 years ( $n=7$ ), 10–17 years ( $n=14$ ) and one young adult.

### Stem cell source and graft manipulation

Grafts were obtained from matched related donors ( $n=11$ ), mismatched family donors ( $n=6$ ) and from matched unrelated donors ( $n=15$ ). Graft types included unmanipulated BM ( $n=14$ ), unmanipulated PBSC ( $n=5$ ) and highly purified PBSC-CD34<sup>+</sup> ( $n=13$ ). CD34<sup>+</sup> cells were immunomagnetically selected and processed observing GMP as described previously.<sup>12</sup> Briefly, the PBSC were washed for platelet reduction and incubated with human immunoglobulin to reduce unspecific binding, then labelled with the CD34 CliniMacs reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany). After incubation and washing, the CD34<sup>+</sup> cells were purified using the automated CliniMacs device.

### Flow cytometric analysis

During the first 3 months after SCT, peripheral blood (PB) samples with ethylenediaminetetraacetic acid were obtained at weekly intervals, then bi-weekly for another 3 months, then monthly up to 1 year and finally each 3 months for up to at least 2 years post SCT. Four-colour flow cytometric analyses of the lymphocyte subsets in the PB was performed on a Coulter Epics XL (Coulter, Krefeld, Germany). Briefly, 100–500  $\mu$ l of PB containing  $4 \times 10^4$ –

$1 \times 10^6$  white blood cells (WBC) were labelled for 20 min with each of the 45/4/8/3 or 45/56/19/3 tetraCHROME reagents or four different antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, phycoerythrin-Texas red (ECD) and phycoerythrin-cyanine 5.1. The samples were further processed with a lyse-no wash procedure, and then measured within 2 h. The samples were analysed for the content of CD19<sup>+</sup> B cells, CD3<sup>+</sup> T cells, CD4<sup>+</sup>CD3<sup>+</sup> T helper cells, CD8<sup>+</sup>CD3<sup>+</sup> cytotoxic T cells and CD56<sup>+</sup>CD3<sup>-</sup> NK cells. At every other time point, flow cytometric analyses were extended to the determination of naïve cells (CD45RA<sup>+</sup>CD4<sup>+</sup>CD3<sup>+</sup>, CD45RA<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup>CD28<sup>+</sup>), memory cells (CD45RO<sup>+</sup>CD4<sup>+</sup>CD3<sup>+</sup>, CD45RO<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup>), activated cells (CD8<sup>+</sup>CD3<sup>+</sup>CD69<sup>+</sup>, CD8<sup>+</sup>CD3<sup>+</sup>HLA-DR<sup>+</sup>, CD4<sup>+</sup>CD3<sup>+</sup>CD69<sup>+</sup>, CD4<sup>+</sup>CD3<sup>+</sup>HLA-DR<sup>+</sup>) and NK cell subsets (CD56<sup>+</sup>158a<sup>+</sup>CD3<sup>-</sup>, CD56<sup>+</sup>158b<sup>+</sup>CD3<sup>-</sup>); as well as CD14<sup>+</sup> monocytes. Additionally, appropriate isotypic negative controls were set up. All antibodies were obtained from Coulter Immunotech (Marseilles, France).

The cytometer was calibrated at weekly intervals. Daily, the settings were validated using ImmunTrol control cells (Coulter Immunotech, Marseilles, France) and the tetraCHROME reagents. The tetraCHROME automatic gating algorithms (Beckman Coulter, Krefeld, Germany) for samples with normal or low lymphocyte contents were employed to measure the major lymphocyte subsets, which use gating on CD45<sup>high</sup>, FSC and SSC<sup>low</sup> cells to define lymphocytes.<sup>13</sup> All analyses were double-checked for correct setting of regions. The percentage of lymphocytes determined in these analyses was used to calculate the absolute lymphocyte count in a dual-platform approach. The percentages of activated, naïve and memory subsets of CD4<sup>+</sup> or CD8<sup>+</sup> T cells and of NK cell subsets were evaluated using standardized gates. They were converted to absolute numbers corrected by the percentages of these major populations in the tetraCHROME analyses. Leucocyte counts were measured with the CellDyn 3500 (Abbott, Chicago, IL, USA) as part of on-time WBC analyses.

### Statistics

As normal values in childhood vary considerably with age, absolute numbers of NK cells, B cells, T cells and their CD4<sup>+</sup> and CD8<sup>+</sup> subsets were related to age-specific reference values generated by Comans-Bitter *et al.*<sup>11</sup> in a study with 429 healthy children. For end points of analyses, we chose the first time patients crossed their age-matched 5th and 50th percentile. Using this first transition time, we examined the distribution of patients' immune reconstitution time of lymphocyte subpopulations as measured. Kaplan–Meier estimates were used to determine the distribution of the censored patients' cellular immune reconstitution time. For testing the homogeneity of these survival functions across strata, we employed the log-rank<sup>14</sup> and the Wilcoxon test using censored data linear rank statistics based on exponential scores.<sup>15</sup> Additionally, we used these rank test to analyse the age-related immune reconstitution for significant differences between the kinds of graft (BM, PBSC, PBSC-CD34<sup>+</sup>). *P*-values <0.05 were regarded significant.

**Table 1** Patients' and grafts' characteristics

No.	Sex age (years)	Diagnosis status	Conditioning regimen	Donor	Stem cell source	CD34 <sup>+</sup> selection	CD34 <sup>+</sup> /kg body weight	CD3 <sup>+</sup> /kg body weight	DLI	Steroide application	Current state, months post transplant, grade of GvHD
1	m, 7	AML, CR2	Bu, Cy	MFD	BM	–	9.1 × 10 <sup>6</sup>	3.7 × 10 <sup>7</sup>	–	–	cCR, + 39 months
2	m, 14	ALL, CR1	TBI, VP16, Cy	MFD	BM	–	3.9 × 10 <sup>6</sup>	2.1 × 10 <sup>7</sup>	–	+	GvHD III, died + 7 months
3	m, 4	JMML, NR	Bu, Cy, Mel, ATG	MFD	BM	–	15.1 × 10 <sup>6</sup>	3.0 × 10 <sup>7</sup>	+	–	Relapse, died + 26 months
4	f, 12	ALL, CR2	TBI, VP16	MFD	BM	–	4.4 × 10 <sup>6</sup>	1.0 × 10 <sup>7</sup>	–	–	cCR, + 59 months
5	m, 12 <sup>1</sup> / <sub>2</sub>	ALL, CR3	TBI, VP16, Cy, ATG	MUD	BM	–	2.9 × 10 <sup>6</sup>	2.7 × 10 <sup>7</sup>	–	+	cCR, + 46 months
6	f, 6	ALL, CR1	TBI, VP16, Cy	MFD	BM	–	8.3 × 10 <sup>6</sup>	2.5 × 10 <sup>7</sup>	+	–	Relapse, died, + 21 months
7	m, 17	AML, CR2	Bu, Cy, Mel, ATG	MUD	BM	–	5.6 × 10 <sup>6</sup>	4.3 × 10 <sup>7</sup>	–	+	cCR, + 34 months
8	m, 17	MDS	Bu, Cy, Mel, ATG	MFD	BM	–	2.2 × 10 <sup>6</sup>	3.7 × 10 <sup>7</sup>	–	+	cCR, + 25 months
9	m, 2 <sup>1</sup> / <sub>2</sub>	proB-ALL, CR2	TBI, VP16	MFD	BM	–	10.5 × 10 <sup>6</sup>	3.8 × 10 <sup>7</sup>	–	–	cCR, + 24 months
10	m, 16	AML, CR1	Bu, Cy	MFD	BM	–	1.8 × 10 <sup>6</sup>	1.4 × 10 <sup>7</sup>	+	–	Relapse, died + 6 months
11	f, 17	AML, CR2	Bu, Cy, Mel, ATG	MUD	BM	–	5.2 × 10 <sup>6</sup>	2.6 × 10 <sup>7</sup>	–	+	Relapsed, GvHD II, died + 6 months
12	f, 13	ALL, CR1	TBI, VP16, ATG	MUD	BM	–	5.0 × 10 <sup>6</sup>	3.3 × 10 <sup>7</sup>	–	+	cCR, GvHD I, + 18 months
13	m, 4	RC	Bu, Cy	MFD	BM	–	2.7 × 10 <sup>6</sup>	2.9 × 10 <sup>7</sup>	+	–	cCR, + 14 months
14	f, 10	AML, NR	Bu, Cy, Mel, ATG	MUD	BM	–	6.9 × 10 <sup>6</sup>	5.8 × 10 <sup>7</sup>	–	+	cCR, + 12 months
15	m, 17	ALL, CR2	TBI, VP16, Cy	MFD	PBSC	–	7.8 × 10 <sup>6</sup>	3.4 × 10 <sup>8</sup>	–	–	Relapse, died + 10 months
16	f, 3	RC	Bu, Cy, Mel, RATG	MFD	PBSC	–	7.3 × 10 <sup>6</sup>	1.2 × 10 <sup>9</sup>	–	+	cCR, GvHD III, + 68 months
17	f, 12	CLL, CR1	Flu, Bu, RATG	MUD	PBSC	–	3.3 × 10 <sup>6</sup>	5.5 × 10 <sup>8</sup>	–	–	cCR, + 57 months
18	m, 6	ALL, CR2	TBI, VP16, Cy, ATG	MUD	PBSC	–	9.5 × 10 <sup>6</sup>	5.7 × 10 <sup>8</sup>	–	+	Infection, died + 11 months
19	m, 10	AML, CR2	Bu, Cy, Thio, RATG	MUD	PBSC	–	6.6 × 10 <sup>6</sup>	1.3 × 10 <sup>9</sup>	–	+	cCR, GvHD I, + 68 months
20	m, 6	AML, NR	Topo, Cy, Ara-C	MUD	PBSC	+	9.8 × 10 <sup>6</sup>	3.1 × 10 <sup>5</sup>	+	+	Relapse, GvHD IV, died + 6 months
21	m, 3 <sup>1</sup> / <sub>2</sub>	ALL, CR2	TBI, VP16, Cy, ATG	MUD	PBSC	+	16.2 × 10 <sup>6</sup>	6.6 × 10 <sup>3</sup>	–	+	cCR, + 67 months
22	f, 5	ALL, CR2	TBI, VP16, Cy, RATG	MUD	PBSC	+	12.4 × 10 <sup>6</sup>	9.3 × 10 <sup>3</sup>	–	–	Infection, died + 29 months
23	m, 11	ALL, CR1	TBI, VP16, Cy, RATG	MUD	PBSC	+	7.2 × 10 <sup>6</sup>	9.3 × 10 <sup>3</sup>	+	+	Relapse, died + 22 months
24	m, 15	AML, CR1	Bu, Cy, Thio, ATG	MUD	PBSC	+	14.4 × 10 <sup>6</sup>	9.4 × 10 <sup>3</sup>	–	–	cCR, + 54 months
25	m, 4 <sup>1</sup> / <sub>2</sub>	AML, CR2	Bu, Cy, Mel, RATG	MUD	PBSC	+	31.9 × 10 <sup>6</sup>	1.4 × 10 <sup>4</sup>	–	–	cCR, + 50 months
26	m, 5 <sup>1</sup> / <sub>2</sub>	ALL, CR2	TBI, VP16, Cy, ATG	MUD	PBSC	+	13.4 × 10 <sup>6</sup>	2.1 × 10 <sup>4</sup>	–	+	Relapse, died + 9 months
27	m, 7	ALL, CR2	TBI, Flu, VP16, RATG	MMFD	PBSC	+	16.4 × 10 <sup>6</sup>	1.6 × 10 <sup>4</sup>	–	+	cCR, + 56 months
28	m, 4	ALL, CR2	TBI, Flu, VP16, RATG	MMFD	PBSC	+	15.4 × 10 <sup>6</sup>	1.6 × 10 <sup>4</sup>	–	+	GvHD IV, died + 6.5 months
29	m, 3 <sup>1</sup> / <sub>2</sub>	ALL, CR1	TBI, Flu, VP16, RATG	MMFD	PBSC	+	36.9 × 10 <sup>6</sup>	2.4 × 10 <sup>4</sup>	+	+	cCR, GvHD III, + 48 months
30	f, 12 <sup>1</sup> / <sub>2</sub>	ALL, NR	TBI, Flu, VP16, RATG	MMFD	PBSC	+	20.1 × 10 <sup>6</sup>	2.0 × 10 <sup>4</sup>	–	+	TRD, died + 7 months
31	f, 9	ALL, CR2	Flu, Thio, Mel, OKT3	MMFD	PBSC	+	12.7 × 10 <sup>6</sup>	7.1 × 10 <sup>3</sup>	+	+	cCR, GvHD III/IV, + 13 months
32	m, 23	ALL, CR3	Flu, Thio, Mel, OKT3	MMFD	PBSC	+	10.3 × 10 <sup>6</sup>	4.4 × 10 <sup>3</sup>	–	+	cCR, + 12 months

Abbreviations: ATG=anti thymocyte globulin; Bu=busulphan; cCR=continuous complete remission; CR=complete remission; Cy=cyclophosphamide; DLI=donor lymphocyte infusion; Flu=fludarabine; MFD=matched family donor; MMFD=mismatched unrelated donor; MUD=matched unrelated donor; NR=not in remission, RATG=highly purified rabbit ATG<sup>14</sup>; RC=refractory cytopenia; TBI=total body irradiation; Thio=thiotepa; Topo=Topotecan; TRD=transplant-related death.

Finally, we determined the impact of age-related lymphocyte subset reconstitution on survival after transplantation. Indicators were the time for a lymphocyte subset to reach its age-matched 5th or 50th percentile with the condition that the values stayed on or above this level for more than 1 month ('run') during the first year post SCT. We examined two-way contingency tables for these indicators. Owing to the small sample size, Fisher's exact test was used to detect associations between the variables. Additionally, the indicators defined above served to calculate the relationship between lymphocyte subset reconstitution and survival in an age-dependent manner in log-rank analyses. Kaplan–Meier estimates were employed for the survival curves. For testing the equality homogeneity of survival functions across strata we used the log-rank and the Wilcoxon test with censored data linear rank statistics.

Statistical analyses were performed with the SAS software Version 8, graphics were generated with GraphPad Prism version 4-00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

## Results

*Characteristics of transplant groups and clinical follow-up*  
CD34 purification of PBSC resulted in 3 or 4 log depletion of T cells (median  $1.4 \times 10^4$ , range  $0.4\text{--}31 \times 10^4$  CD3<sup>+</sup>/kg) compared to unmanipulated BM (median  $2.9 \times 10^7$ , range  $1\text{--}5.8 \times 10^7$  CD3<sup>+</sup>/kg) or PBSC (median  $5.7 \times 10^8$ , range  $3.4\text{--}13.4 \times 10^8$  CD3<sup>+</sup>/kg). The number of transplanted CD34<sup>+</sup> stem cells was 2–3 times higher for PBSC-CD34<sup>+</sup> (median  $14.4 \times 10^6$ , range  $7\text{--}36.9 \times 10^6$  CD34<sup>+</sup>/kg) compared to unmanipulated PBSC or BM grafts (median  $7.3 \times 10^6$  and  $5.0 \times 10^6$  CD34<sup>+</sup>/kg).

Successful engraftment was observed in all children. Overall, 19 of 32 children survived (59%) among these seven of 13 (54%) were receiving PBSC-CD34<sup>+</sup>, nine of 14 (64%) were receiving BM and three of five (60%) were receiving unmanipulated PBSC. Overall disease status and donor source were more heterogeneous in the PBSC-CD34<sup>+</sup> group compared to the groups with unselected grafts (Table 1). The median follow-up of survivors was 4 years (range, 12–68 months). Causes of death were relapse of the underlying malignancy ( $n=8$ ) or transplant-related mortality including severe infections ( $n=3$ ) and severe graft-versus-host disease (GvHD) ( $n=2$ ).

### *Immune reconstitution of the main lymphocyte subsets with regard to age-matched 5th and 50th percentile*

CD56<sup>+</sup>CD3<sup>-</sup> NK cells were the first lymphoid cells to emerge following SCT. Fifty per cent of all patients reached age-matched 5th percentile of CD56<sup>+</sup>CD3<sup>-</sup> NK, cytotoxic CD8<sup>+</sup>CD3<sup>+</sup> T, CD19<sup>+</sup> B and CD4<sup>+</sup>CD3<sup>+</sup> helper T cells 4, 9, 20 and 28 weeks after SCT, respectively. This increased up to 100% of the patients for NK cells and up to 80–90% of the patients for cytotoxic T, helper T and B cells within the first year of SCT. The higher level of age-matched 50th percentile was reached by 50% of all patients for CD56<sup>+</sup>CD3<sup>-</sup> NK, cytotoxic T and B cells 7, 19 and 36

weeks after SCT, respectively. In contrast, 28% of all patients reached the age-matched 50th percentile for helper T cells within the first year of SCT.

### *Impact of BM, PBSC or PBSC-CD34<sup>+</sup> grafts*

Significant differences between the types of graft with regard to the lymphocyte subsets were found (Figure 1). PBSC seemed to elicit the fastest reconstitution of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells with regard to the 5th percentile, an observation that was reverted for B cells, which recovered faster after BMT (Figure 1a–e). No significant differences were found between BMT and PBSC-CD34<sup>+</sup> transplantation for either the 5th or the 50th percentile (Figure 1a–k).

*CD3<sup>+</sup> T-lymphocytes:* Overall T cells reconstituted significantly faster to 5th percentile levels after PBSC compared to BMT or PBSC-CD34<sup>+</sup> transplantation, whereas no differences were found between BMT or PBSC-CD34<sup>+</sup> transplantations. This finding continues to be even for the 50th percentile take (Table 2).

*CD8<sup>+</sup> CD3<sup>+</sup> cytotoxic T cells:* Like the overall T cells, the evaluation of cytotoxic T cells showed a significant delay in the 5th percentile reach after BMT or PBSC-CD34<sup>+</sup> transplantation compared to PBSC. The differences were still present for 50th percentile takes comparing PBSC and PBSC-CD34<sup>+</sup> transplantations.

*CD4<sup>+</sup> CD3<sup>+</sup> helper T cells:* The 5th percentile reconstitution of helper T cells was the fastest after PBSC with significant differences compared to PBSC-CD34<sup>+</sup> and BMT, whereas no differences were found for the 50th percentile takes.

*CD56<sup>+</sup> CD3<sup>-</sup> NK cells:* NK cells reconstituted fast and efficiently with all graft types without significant differences between the stem cell sources, except the 50th percentile comparing PBSC and BMT.

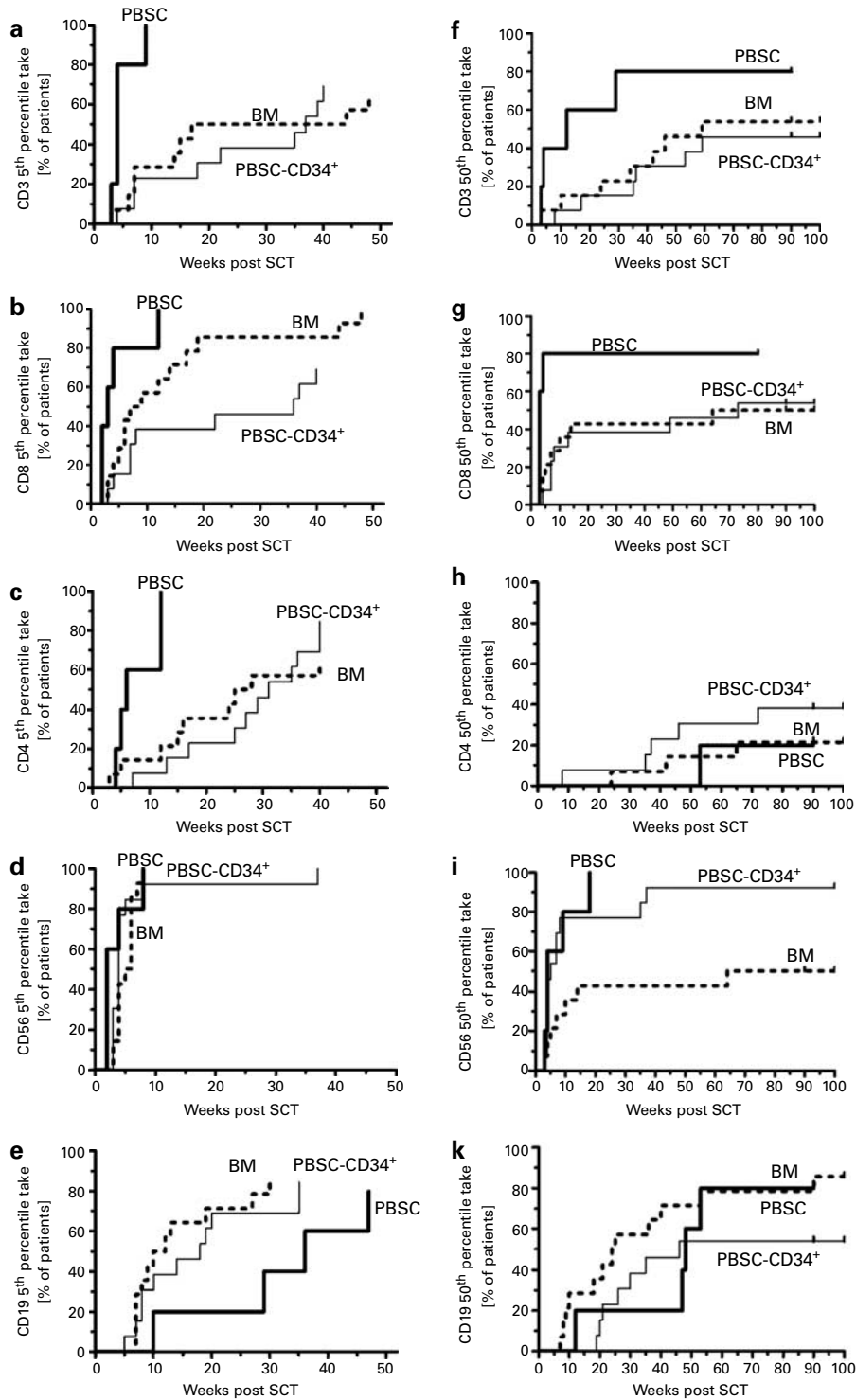
*CD19<sup>+</sup> B-lymphocytes:* B-cell reconstitution with regard to the 5th and 50th percentile take was significantly faster following transplantation with BM compared to unmanipulated PBSC, whereas no differences were found between the other groups for either percentile.

The detailed log-rank analysis results for the different lymphocyte subsets are listed in Table 2.

### *Engraftment of CD45 isotypes and NK cell subsets*

During the first month post SCT, median absolute numbers of both naïve cytotoxic T cells (CD8<sup>+</sup>CD3<sup>+</sup>CD45RA<sup>+</sup>CD28<sup>+</sup>) and naïve helper T cells (CD4<sup>+</sup>CD3<sup>+</sup>CD45RA<sup>+</sup>) were lower in patients with CD34-selected grafts compared to PBSC or BM (Figure 2a and b). From the second month onwards, these differences gradually decreased. From the fourth or fifth month onwards naïve T cells fairly increased in all patients. Whereas the median of patients reached levels similar to those before transplantation between 8 and 12 months after SCT, there was a wide range in naïve T cells among patients with BM and PBSC-CD34<sup>+</sup> grafts. In some patients, after BMT, both cytotoxic and helper naïve T cells remained low even at 1 year post SCT.

The absolute numbers of cytotoxic T cells with a memory phenotype were markedly higher 1 month post SCT compared to the naïve T cells with median levels of >100



**Figure 1** Empirical distribution of the time needed to reach the 5th percentile (a–e) and 50th percentile (f–k) of different lymphocyte subsets using unmanipulated PBSC and BM or PBSC-CD34<sup>+</sup> as stem cell source.

CD8<sup>+</sup>CD3<sup>+</sup> CD45RO<sup>+</sup> cells/ $\mu$ l in all three transplant groups. The PB pool of these cells gradually expanded 3–5-fold over the first 8–10 months (data not shown). In the first month post SCT, the absolute number of memory T helper cells was lower in patients receiving PBSC-CD34<sup>+</sup> (median, 90/ $\mu$ l CD4<sup>+</sup>CD3<sup>+</sup> CD45RO<sup>+</sup> cells) compared

to patients transplanted with BM or PBSC (median, >200/ $\mu$ l CD4<sup>+</sup>CD3<sup>+</sup> CD45RO<sup>+</sup> cells). In all patients, memory helper T cells also increased 3–5-fold during the first 7–10 months (data not shown).

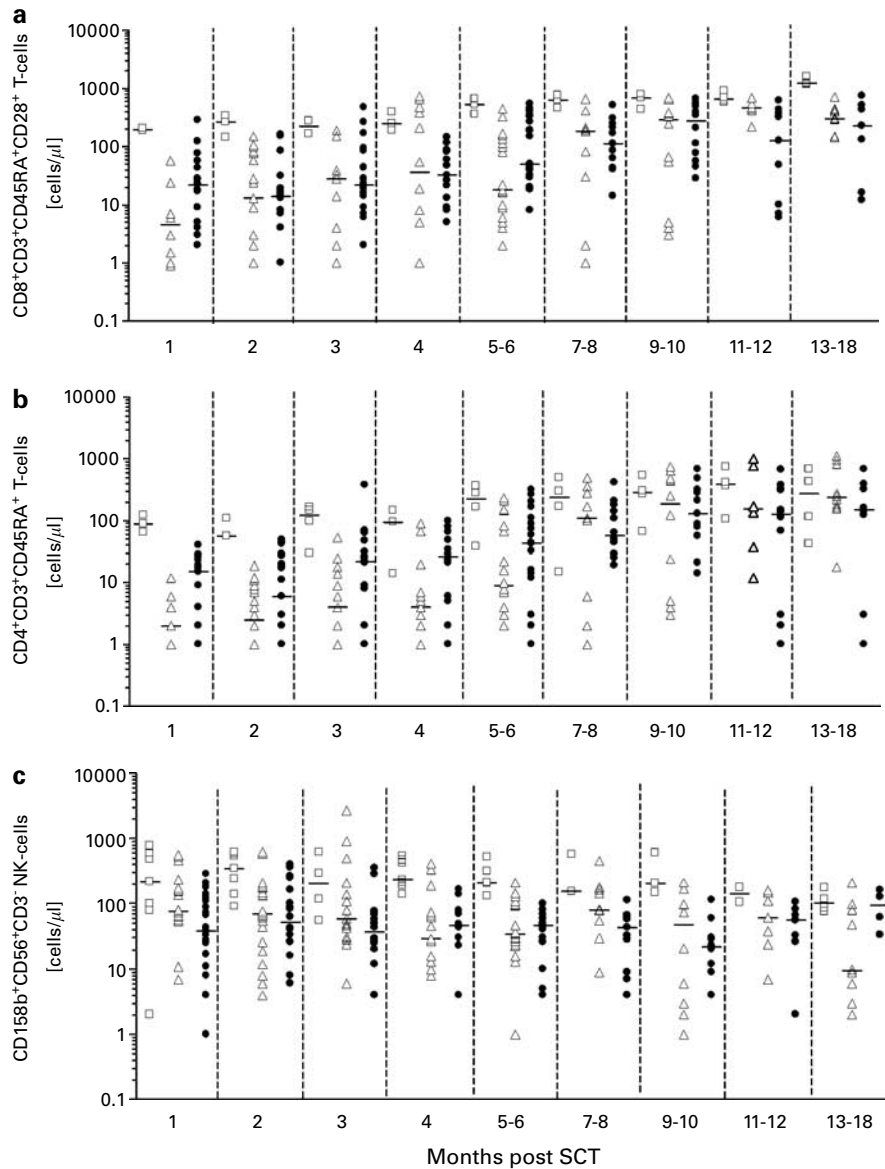
NK cell subsets and their corresponding killer cell inhibitor receptors (KIRs) are of interest in the context of

**Table 2** Comparative empirical distribution of the time-lags for 5th and 50th percentile of lymphocyte subsets with regard to stem cell source

Lymphocyte subsets	5th percentile			50th percentile		
	<i>PBSC vs PBSC-CD34<sup>+</sup></i>	<i>PBSC vs BM</i>	<i>BM vs PBSC-CD34<sup>+</sup></i>	<i>PBSC vs PBSC-CD34<sup>+</sup></i>	<i>PBSC vs BM</i>	<i>BM vs PBSC-CD34<sup>+</sup></i>
	P-value	P-value	P-value	P-value	P-value	P-value
CD3 <sup>+</sup>	0.0006	0.0009	NS	0.005	0.008	NS
CD8 <sup>+</sup> CD3 <sup>+</sup>	0.003	0.03	NS	0.02	NS	NS
CD4 <sup>+</sup> CD3 <sup>+</sup>	<0.0001	0.001	NS	NS	NS	NS
CD56 <sup>+</sup> CD3 <sup>-</sup>	NS	NS	NS	NS	0.02	NS
CD19 <sup>+</sup>	NS	0.02	NS	NS	0.03	NS

Abbreviations: BM = bone marrow; NS = nonsignificant; PBSC = peripheral blood stem cells.

Log-rank analyses of the immune reconstitution of different lymphocyte subsets with regard to stem cell source. *P*-values >0.05 were regarded not significant.

**Figure 2** Haematopoietic reconstitution of naïve T-cell subtypes and of the NK-cell subtype CD56<sup>+</sup>CD3<sup>-</sup>158b<sup>+</sup> using different stem cell sources. PBSC (open squares), PBSC-CD34<sup>+</sup> (open triangle) or BM (closed circles), means (lines).

GvHD and graft-versus-leukaemia/lymphoma.<sup>16,17</sup> Reconstitution of the NK cell subtype CD158b<sup>+</sup>CD56<sup>+</sup>CD3<sup>-</sup>, which is associated with KIR HLA-C group II, is exemplarily given in Figure 2c. Similar to the overall NK cells, cells expressing the CD158b<sup>+</sup>CD56<sup>+</sup>CD3<sup>-</sup> phenotype expanded rapidly during the first month post PBSC or PBSC-CD34<sup>+</sup> transplantation with a high absolute plateau for up to month 3, which decreased during the next 12 months.

*Reconstitution of lymphocyte subsets in relation to survival*  
We investigated the association of the immune reconstitution of lymphocyte subsets during the first year post SCT with survival. Cell counts rising above the 5th and 50th percentile, and cell counts above the levels lasting for more than 1 month ('runs') were analyzed with relation to survival.

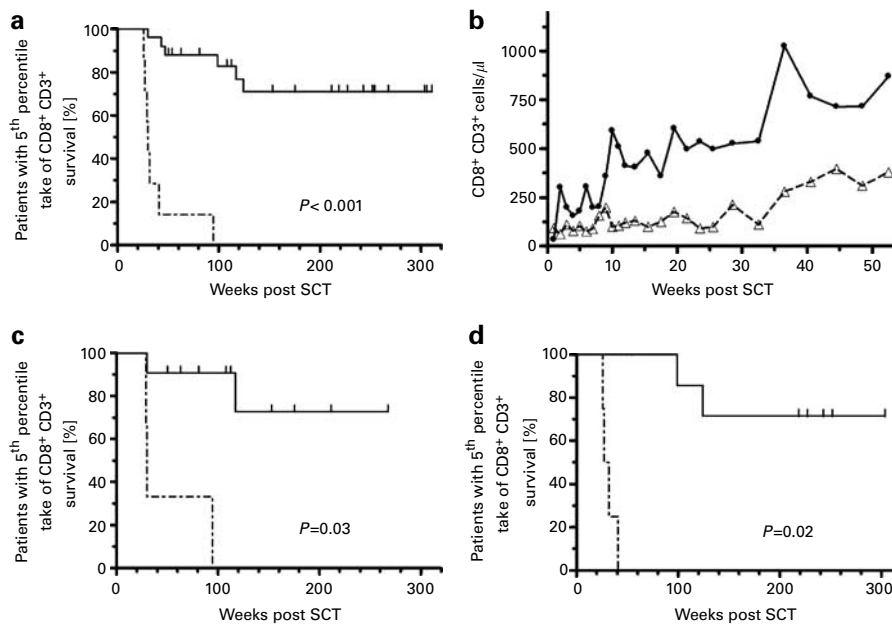
We found a significantly higher number of long-term survivors (>12 months) among those patients in whom CD8<sup>+</sup>CD3<sup>+</sup> absolute counts rose above the 5th (Figure 3a) and the 50th percentile of age-matched normal levels (19 survivors out of 25 patients and 17 survivors out of 23 patients, respectively) compared to patients who never reached these levels (0 survivor out of seven patients and two survivors out of nine patients, respectively) with  $P < 0.001$  and  $P = 0.01$  (two-tailed Fisher's exact test). Similarly, there was a significant difference in CD3<sup>+</sup> absolute counts regarding the 5th and the 50th percentiles ( $P = 0.01$  and  $0.02$ ). Most importantly, transplant type seemed not to be a discriminator for delayed T-cell reconstitution and inferior outcome. In both subgroups, BMT and PBSC-CD34<sup>+</sup> transplantations, we found a significant difference among those patients in whom

CD8<sup>+</sup>CD3<sup>+</sup> absolute counts rose above the 5th (Figure 3c and d; Table 3) of age-matched normal levels compared to patients who never reached these levels ( $P = 0.03$  and  $P = 0.02$ ; two-tailed Fisher's exact test).

No major differences were found in different parameters between patients remaining below age-related 5th percentile (group I) and those with a fast immune reconstitution (group II). In both groups, matched family donor, matched unrelated donor and mismatched family donor were used as stem cell donors (Table 3). Donor lymphocyte infusion (DLI) and steroids were used in 43 and 71% of the patients in group I compared to 20 and 60% of the patients in group II. Development of GvHD was 43% in group I and 24% in group II, but GvHD was related to grade III/IV in group I, only and it split one half to grade I/II and the other half to grade III/IV in group II. Among those three patients with a slow immune reconstitution receiving a BMT, there was one patient with a severe GvHD grade III who did not receive a DLI. Among the four patients with a slow immune reconstitution, who received a PBSC-CD34<sup>+</sup> transplantation, two patients developed a severe GvHD grade III/IV, one after DLI and the other patient after haploidentical SCT without DLI.

An association to survival after SCT regarding CD19<sup>+</sup> B-cell counts was seen that was significant for the 50th percentile, only ( $P = 0.003$ ). Because the absolute values of the phi- and contingency coefficient are rather high (>97%), in all cases where Fisher's test indicates significance, a strong association can be assumed. Kaplan-Meier estimates of reconstitution-dependent survival were generated for the indicators found (Figure 3).

The lymphocyte subsets CD4<sup>+</sup>CD3<sup>+</sup> and CD56<sup>+</sup>CD3<sup>-</sup> were not found to discriminate between a proposed



**Figure 3** Kaplan-Meier estimates of overall survival in relation to immune reconstitution of cytotoxic T cells (a, c, d) and CD8<sup>+</sup>CD3<sup>+</sup> immune reconstitution of good-risk and low-risk patients (b). Patients with a fast immune reconstitution of cytotoxic T cells after SCT (b, solid line), rising above the age-related 5th percentile of CD8<sup>+</sup>CD3<sup>+</sup> cells, had a significant better survival (a, c, d solid line) compared to patients who remained below the age-related 5th percentile of CD8<sup>+</sup>CD3<sup>+</sup> cells (dotted line). The difference was statistically significant in the overall patient cohort ( $n = 32$ ) receiving various kinds of stem cell grafts (a) as well as in the more homogeneous patient subgroups, receiving BMT (c,  $n = 14$ ) or PBSC-CD34<sup>+</sup> grafts (d,  $n = 13$ ) only.

**Table 3** characteristics of the high- and poor-risk groups

	<i>Patients remaining below age-related 5th percentile of CD8<sup>+</sup>CD3<sup>+</sup> cells/<math>\mu</math>l, group I</i>	<i>Patients exceeding age-related 5th percentile of CD8<sup>+</sup>CD3<sup>+</sup> cells/<math>\mu</math>l, group II</i>
Survivors/all patients	0/7	19/25
<i>Cause of death</i>		
Relapse	4	4
Severe infection or GvHD	3	2
<i>Survivors related to the stem cell source</i>		
CD34-selected PBSC	0/4	7/9
BM unmanipulated	0/3	9/11
PBSC unmanipulated	0	3/5
<i>Survivors related to the kind of donor</i>		
Matched family donor	0/3	6/8
Matched unrelated donor	0/2	9/13
Mismatched family donor	0/2	4/4
<i>Survivors related to the diagnosis</i>		
ALL (CR3 and NR)	0/1	2/2
ALL (CR1 and CR2)	0/4	7/11
AML, MDS, JMML, CLL, RC	0/2	10/12
<i>Number of patients receiving or developing</i>		
DLI	3/7	5/25
Steroids	5/7	15/25
GvHD grade I/II	0/7	3/25
GvHD grade III/IV	3/7	3/25

Abbreviations: BM = bone marrow; CR = complete remission; DLI = donor lymphocyte infusion; GvHD = graft-versus-host disease; NR = not in remission; PBSC = peripheral blood stem cells.

high risk and a low risk group. The cell subsets CD158a<sup>+</sup>CD56<sup>+</sup>CD3<sup>-</sup>, CD158b<sup>+</sup>CD56<sup>+</sup>CD3<sup>-</sup>, CD8<sup>+</sup>CD3<sup>+</sup>CD69<sup>+</sup>, CD8<sup>+</sup>CD3<sup>+</sup>HLA-DR<sup>+</sup>, CD4<sup>+</sup>CD3<sup>+</sup>HLA-DR<sup>+</sup> and CD4<sup>+</sup>CD3<sup>+</sup>HLA-DR<sup>+</sup> were found to show a trend toward correlation with survival that was not significant.

## Discussion

Among the factors known to influence immune reconstitution are the stem cell source, the amount of CD34<sup>+</sup> cells and the content of CD3<sup>+</sup> cells in the graft,<sup>3,5,6,18–21</sup> the cytomegalovirus status before and after SCT,<sup>6,8,22</sup> the additional application of donor lymphocyte infusion,<sup>9,23,24</sup> the relationship and disparity between donor and recipient,<sup>23,25,26</sup> the age of the patients,<sup>9,21,22</sup> and the development of GvHD.<sup>18,22,27</sup>

The influence of the stem cell source on the speed of immunological recovery has been controversial. Kalwak *et al.*<sup>26</sup> saw a faster T cell reconstitution after non-T-cell depleted compared to T-cell depleted allogeneic SCT, associated with a lower risk of relapse and infection, but a higher rate of GvHD. In contrast, Behringer and co-workers<sup>5</sup> described a similar T lymphocyte reconstitution in adult patients after SCT using unmanipulated BM or PBSC and CD34 selected PBSC. In our study in children, PBSC

seemed to elicit the fastest reconstitution of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells with regard to the age-related 5th percentile followed by BMT and PBSC-CD34<sup>+</sup> transplantation, which did not differ.

Adult patients show a very slow immune reconstitution reaching normal values 2–3 years post transplantation, finally.<sup>28</sup> This is due to the thymus, which rapidly involutes after puberty.<sup>10,18</sup> In contrast, in children immune reconstitution, cell function and T-cell receptor repertoire recover much faster within the first 1–1½ years post SCT.<sup>3,6,19,22,29</sup> This is associated with a decreased risk of life-threatening opportunistic infections compared to adult patients.<sup>9</sup> We and others could show for children, that NK cell counts recover rapidly within 1 month post-transplantation, followed by cytotoxic CD8<sup>+</sup>CD3<sup>+</sup> T cells 2–3 months and CD19<sup>+</sup> B-cell 3–5 months post SCT. Initially, most of the cytotoxic T cells are memory CD8<sup>+</sup>CD3<sup>+</sup>CD45RO<sup>+</sup>, whereas the naïve CD8<sup>+</sup>CD3<sup>+</sup>CD28<sup>+</sup>CD45RA<sup>+</sup> T cells regenerate in the second ½ year post SCT. CD4<sup>+</sup>CD3<sup>+</sup> T helper cells emerge very slowly reaching normal values 6–10 months post SCT.<sup>3,6,30,31</sup> Although in our study >80% of the children reached age-related 5th percentile values of the main lymphocyte subsets within the first year post SCT, the remaining children showed still very low levels of the different T-lymphocyte subsets 1 year post SCT associated with a high risk of morbidity and mortality. Similar to the CD56<sup>+</sup>CD3<sup>-</sup> NK-cell reconstitution, we could show an early high peak/plateau of NK subsets expressing different KIRs. There is evidence that these cells play an important role in minimal residual disease (MRD) control in the HLA-mismatch setting.<sup>17</sup> We and others could show that NK cells can be used as adoptive immunotherapy after haploidentical SCT.<sup>32,33</sup> In addition, high levels of NK cells may be protective against the development of GvHD.<sup>34</sup>

Immune reconstitution studies, to date, have not measured lymphocyte subsets in short intervals. The majority of these studies have been performed in adult patients using monthly or quarter-year determinations. Therefore, transient but possibly influential changes may not have been recognized with additional impact on statistical analysis. A relation between a delayed immune reconstitution and a relapse in an infection of the patient has been suspected regularly; however, no strong statistical correlation has been found. In our study, 5th and 50th percentiles of healthy children were used as reference values because these are studied in the literature.<sup>11</sup> However, to date, the minimum cell number of immune subpopulations necessary for adequate infection or relapse control is not known.

Our data of 32 paediatric patients presented here using short monitoring intervals provide preliminary evidence that patients with slow recovery of cytotoxic T cells have a high risk of relapse or life-threatening infections. We found a significant difference compared to those patients reaching or exceeding the reference value between patients who did not reach the absolute count of age-related 5th CD8<sup>+</sup>CD3<sup>+</sup> percentile taken during the first ½ or even during the whole first year post SCT, leading to death in all cases.<sup>11</sup> This was independent of the graft source and could be shown for our heterogenous overall patient cohort, as

well as in the more homogenous patient subgroups, receiving BMT or PBSC-CD34<sup>+</sup> grafts only. In the overall and the patient subgroups, a CD8<sup>+</sup>CD3<sup>+</sup> take was associated with more than 70% chance of survival. This hints at a high-risk and a low-risk group of patients that can be discriminated. This discrimination might be possible as early as 2 and 5 months post SCT, respectively, when 50 and 70% of the patients reached the 5th percentile. In addition, patients with a low immune reconstitution do not differ dramatically from patients with a fast immune reconstitution regarding DLI or steroid application and GvHD development. Although our results are limited by the relatively small sample size of the groups, our data indicate a minimum number of cytotoxic T cells needs be reached within a certain period of SCT.

Novitzky *et al.*<sup>35</sup> described similar to our paediatric patients, in 42 adult patients, that treatment failure was associated with low total CD8<sup>+</sup> cells, when tested at 6 months post SCT. The high importance of CD8<sup>+</sup> cells after SCT is confirmed by clinical reports where a higher relapse rate was observed in patients with lower CD8<sup>+</sup> and NK cells, whereas blood levels of the CD4<sup>+</sup> populations were not associated with outcome.<sup>23</sup> Similarly, we did not find a significant correlation of CD4<sup>+</sup> reconstitution and survival. Supportive evidence for the importance of cytotoxic T cells is also given by an animal study where CD8<sup>+</sup> cells were found to be effective in eliminating leukaemic cells.<sup>36</sup>

To conclude, our results indicate that the reconstitution of CD8<sup>+</sup>CD3<sup>+</sup> cells to as low level as 5th percentile may be a prognostic marker for outcome after SCT. Such a marker might be a valuable tool to the clinicians apart from MRD or chimerism analyses. These two latter only indicate impending failure when known therapeutic measures do not work satisfactorily any more. In contrast, detection of age-related CD8<sup>+</sup>CD3<sup>+</sup> reconstitution may assist as an early predictor for potentially fatal complications in addition to clinical observations. Our work was intended as a pilot investigation for a larger study with the long-term goal of identifying indicative parameters for a reasonable tailored monitoring system to recognize the need of immunotherapeutic intervention before the occurrence of relapse or life-threatening infections.

## Acknowledgements

This project was supported by 'Frankfurter Stiftung für Krebskranke Kinder', 'Paul und Ursula Klein-Stiftung', 'Alfred und Angelika Gutermuth-Stiftung' and 'Messer-Stiftung'. We acknowledge the excellent technical support of Andrea Brinkmann, Stephanie Grohal, Sibylle Wehner, Regine Quaritsch and Rabiä el Kalaäoui.

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