Biol Blood Marrow Transplant 23 (2017) 87–95

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Clinical Research: Pediatric

Pre-Emptive Immunotherapy for Clearance of Molecular Disease in Childhood Acute Lymphoblastic Leukemia after Transplantation

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Article history:
Received 20 June 2016
Accepted 7 October 2016

Key Words:
Allogeneic hematopoietic stem cell transplantation
Chimerism
Minimal residual disease
Pre-emptive immunotherapy

A B S T R A C T

Monitoring of minimal residual disease (MRD) or chimerism may help guide pre-emptive immunotherapy (IT) with a view to preventing relapse in childhood acute lymphoblastic leukemia (ALL) after transplantation. Patients with ALL who consecutively underwent transplantation in Frankfurt/Main, Germany between January 1, 2005 and July 1, 2014 were included in this retrospective study. Chimerism monitoring was performed in all, and MRD assessment was performed in 58 of 89 patients. IT was guided in 19 of 24 patients with mixed chimerism (MC) and MRD and by MRD only in another 4 patients with complete chimerism (CC). The 3-year probabilities of event-free survival (EFS) were .69 ± .06 for the cohort without IT and .69 ± .10 for IT patients. Incidences of relapse (CIR) and treatment-related mortality (CITRM) were equally distributed between both cohorts (without IT: 3-year CIR, .21 ± .05, 3-year CITRM, .10 ± .04; IT patients: 3-year CIR, .18 ± .09, 3-year CITRM .13 ± .07). Accordingly, 3-year EFS and 3-year CIR were similar in CC and MC patients with IT, whereas MC patients without IT experienced relapse. IT was neither associated with an enhanced immune recovery nor an increased risk for acute graft-versus-host disease. Relapse prevention by IT in patients at risk may lead to the same favorable outcome as found in CC and MRD-negative-patients. This underlines the importance of excellent MRD and chimerism monitoring after transplantation as the basis for IT to improve survival in childhood ALL.

INTRODUCTION

In recent years, several techniques have been developed to assess treatment response and risk stratification in patients with acute lymphoblastic leukemia (ALL) [1-7]. These efforts have led to the concept of minimal residual disease (MRD) assessment [8]. Beginning in the late 1990s, the clinical importance of MRD in childhood ALL could retrospectively be confirmed [1,2,6]. Thereafter, several studies were based mainly on clone-specific Ig and TCR gene rearrangements as real-time quantitative PCR targets, as well as on quantitative flow cytometry analysis for monitoring MRD in bone marrow samples [9-14].

Moreover, a special situation occurs in patients receiving allogeneic hematopoietic stem cell transplantation (HSCT) for treatment of ALL, where impending relapse can be assessed by chimerism analysis in both peripheral blood and bone marrow. Recurrent recipient signals, ie, the detection of mixed chimerism (MC) after transplantation in addition to the detection of MRD, strongly predict the risk for relapse in children with ALL after allogeneic HSCT [15-20].

Several approaches have been used to treat leukemia relapse after HSCT, such as reinduction chemotherapy, reapplied transplantation, and immunotherapy (IT), including the

Financial disclosure: See Acknowledgments on page 94.

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http://dx.doi.org/10.1016/j.bbmt.2016.10.006
1083-8791/

discontinuation of immunosuppression (IS) or donor lymphocyte infusion (DLI) [21,22]. IT is intended to induce graft-versus-leukemia reaction, but IT patients are also at risk of developing graft-versus-host disease (GVHD) or marrow aplasia. Particularly in the case of DLI, the efficacy and the risk of side effects depend on the type of leukemia and the dose of infused T lymphocytes, respectively [22-26]. Furthermore, because the response is generally limited in cases of florid relapse of ALL, IT is increasingly applied pre-emptively at the time of detectable MRD or MC [15]. Using serial MRD or chimerism analysis enables the detection of impending relapse in advance to allow pre-emptive IT [27,28].

Here, we retrospectively report our experience of pre-emptive IT guided by consistent MRD and chimerism monitoring for the detection of impending relapse in childhood ALL after transplantation. Outcomes of patients at risk for relapse with or without IT were compared with outcomes of patients not identified as being at risk for relapse after transplantation. Our follow-up analyses included molecular responses and the relation to outcome, as well as the impact on immune reconstitution and toxicity.

METHODS

Patients

Consecutive children older than 1 year of age were included in our study if they had received their first allogeneic HSCT for ALL in Frankfurt/Main, Germany, between January 1, 2005, and July 1, 2014 after written informed consent for retrospective data analysis. Written informed consent for retrospective studies was given at the time of transplantation. The institutional ethics committee approved this best practice approach at the center (number 529/15).

Molecular Target Identification and MRD Analysis

MRD analysis was performed in patients if a diagnostic sample of their leukemia cells was available. This analysis was possible in 58 of 89 (65%) patients because several patients were referred from abroad without a diagnostic sample taken at diagnosis or relapse. Cell sample isolation and identification of the markers for MRD evaluation have been previously reported [29,30]. Real-time quantitative PCR analysis was performed and interpreted according to the guidelines developed within the European Study Group for MRD detection in ALL (ESG-MRD-ALL) [3].

Risk Group Definition and Stratification According to MRD

If MRD levels differed between 2 MRD markers, the highest MRD level was chosen for MRD assessment, provided that the markers had a sensitivity of at least 10^{-4}. EuroMRD guidelines were used to reduce the risk for false-negative and false-positive results.

Chimerism Analysis

Chimerism determination started at the time of leucocyte recovery after transplantation. A previously described semi-quantitative PCR approach, based on the amplification of short-tandem-repeat (STR) markers, was used for chimerism analyses [15]. The sensitivity of our assay for detecting autologous cells was 1%. In this regard, singleplex STR-PCR approaches were run to avoid interference of the baseline, as observed frequently in multiplex PCR. Fragment analyses of fluorescence-labeled PCR products was performed by capillary electrophoreses using a 3100-avant device (Applied Biosystems, Darmstadt, Germany) and peak detection cut-off was defined as >50 relative fluorescence units (RFUs). For valid detection of 1% minority genotype, the majority peaks must exceed 5000 RFUs for STR-heterozygous or 2500 RFUs for STR-homozygous recipients.

In addition, if a patient showed 1% of autologous (recipient) cells in a peripheral blood or bone marrow sample, another sample was taken and assessed within 1 week. Patients with confirmed 1% autologous cells in 2 consecutive samples and patients with >1% of autologous cells in a single sample after transplantation were considered as having mixed chimeras.

Flow Cytometry

Flow cytometric analyses for immune monitoring were performed monthly as previously described [31]. In brief, immune reconstitution monitoring of $CD3^+CD56^-$ T cells, $CD3^-CD56^+$ natural killer (NK) cells, and $CD3^+CD56^+$ T-NK cells was performed in patients with or without IT treatment. As childhood blood values strongly depend on age, each patient's longitudinally determined measurement was calculated from its corresponded age-matched norm published by Huenecke et al. [32]. The age-adjusted values allowed for the comparison among the cell counts from patients of different ages.

Stratification

Serial and semi-quantitative analyses of post-transplantation hematopoietic chimerism in peripheral blood started at the time of leucocyte engraftment and were performed weekly until day 200 and monthly thereafter. For analyses of both hematopoietic chimerism and MRD, bone marrow was assessed at days 30 ± 15, 60 ± 15, 90 ± 15, 180 ± 30, and 365 ± 30 and also at 18 months $\pm$ 30 days after transplantation. Bone marrow punctures were not performed routinely at later time points, but they were performed in case of suspected relapse or other situations.

Patients with detectable MRD or MC (confirmed 1% autologous (recipient) signals in 2 consecutive samples or >1% of autologous signals in a single sample after transplantation) and with no or only mild signs of acute (aGVHD) (grade 1 aGVHD) were immediately offered pre-emptive IT. Regardless of the MRD status before transplantation and of the donor type or stem cell source, IT included discontinuation or tapering of IS for patients still receiving IS in the early post-transplantation period or administration of DLI as frontline therapy in patients without IS. DLI was also applied in patients without responses after stop of IS. The interval between both IT options as well as between respective DLI doses was 3 to 4 weeks. The recommended starting dose of DLI was 1×10^6 T cells/kg in cases of HLA-matched related donors, $.5 \times 10^6$ T cells/kg in cases of HLA-matched unrelated donors, and $.1 \times 10^6$ T cells/kg in cases of HLA-haploidentical donors. With pre-emptive IT, chimerism monitoring was performed weekly in peripheral blood samples and monthly in parallel with MRD monitoring in bone marrow samples. In case of persistence of MRD or MC 3 to 4 weeks after DLI, prudent dose escalation was considered for subsequent DLIs. In the matched transplantation setting, a doubling of the $CD3^+$ T cell numbers infused was contemplable, if no additional signs of GVHD had appeared.

Pre-emptive IT was stopped if MC converted to complete donor chimerism (CC) or in cases of clearance of MRD. Furthermore, pre-emptive IT was not applied if patients experienced aGVHD exceeding grade 1.

Statistical Analysis

The median follow-up time for all patients was obtained using the reversed Kaplan-Meier estimator. Fisher's exact test or the Wilcoxon-Mann-Whitney test were used to compare the patients' categorical data. Kaplan-Meier estimates were performed to predict the overall survival and event-free survival (EFS) probabilities. The log-rank test was used for comparisons. EFS was defined as survival without relapse and treatment-related mortality (TRM). TRM was defined as death in complete remission (CR) without pervious relapse. Cumulative incidence (CI) curves were calculated for the incidence of relapse (CIR) and TRM (CITRM) considering TRM as a competing risk for relapse. Gray's test was used for comparisons of CIs.

Cox regressions were performed to identify associations with patient or transplantation characteristics and MC (if applicable) and EFS, as well as CIR. Only factors that attained significance in the univariable regression were included in the multivariable analysis.

Mixed-effect regression with the linear spline model was fitted for the longitudinal analysis of each immune cell subpopulation after transplantation. Previously, the absolute cell values of T and NK cells were age adjusted and logarithmically transformed [32]. T-NK cells were only logarithmically transformed because of a lack of pediatric norm values. Furthermore, patients were classified into groups according the IT and GVHD grade.

Statistical tests were 2-sided with a significance level of 5% and 95% confidence interval. Data analysis was performed using the R software for statistical computing, Version 3.1.3. (R: A Language and Environment for Statistical Computing, http://www.R-project.org/). The survival package (Therneau, http://CRAN.R-project.org/package=survival), cmprsk package (Gray, http://CRAN.R-project.org/package=cmprsk), and nlme package (Pinheiro et al., http://CRAN.R-project.org/package=nlme) were used.

RESULTS

Patients

From January 1, 2005 to July 1, 2014, 89 consecutive patients with ALL received their first allogeneic HSCT in Frankfurt/Main, Germany.

Patients' ages ranged from 2.2 to 26.0 years (Table 1). Precursor B cell ALL was diagnosed in 63 patients, T cell ALL in 20 patients, and biphenotypic/bilinear ALL in 6 patients. Transplantation was performed as recommended by the respective treatment protocols (Cooperative study group for childhood acute lymphoblastic leukaemia or Associazione Italiana

Table 1
Patient Characteristics, n = 89

Characteristic	n	(%)	IT		*P*
			No (n = 66;73%)	Yes (n = 23,21%)	
Gender					.625
Female	36	40%	28 (42%)	8 (35%)	
Male	53	60%	38 (58%)	15 (65%)	
Age at HSCT					
Median (range), yr	11.5 (2.2-26)		10.5 (2.2-26)	12 (3.5-18.8)	
Chromosome					.469
BCR/ABL+	16	18%	11 (17%)	5 (22%)	
Hypodiploid	3	2%	3 (5%)	0	
Normal	60	68%	46 (70%)	14 (61%)	
Other	10	12%	6 (9%)	4 (17%)	
Immunophenotype					.798
pB-ALL	63	71%	47 (71%)	16 (70%)	
T ALL	20	22%	14 (21%)	6 (26%)	
Bpheno ALL	6	7%	5 (8%)	1 (4%)	
Donor					.023
MSD	18	20%	9 (14%)	9 (39%)	
MUD	61	69%	50 (76%)	11 (48%)	
MMFD	10	11%	7 (11%)	3 (13%)	
Conditioning regimen					.690
Myeloablative	80	90%	60 (91%)	20 (87%)	
RIC	9	10%	6 (9%)	3 (13%)	
TBI					1
Yes	77	87%	57 (86%)	20 (87%)	
No	12	13%	9 (14%)	3 (13%)	
Remission at HSCT					.633
1 CR	47	53%	36 (55%)	11 (48%)	
≥2 CR	42	47%	30 (45%)	12 (52%)	
Stem cell source					.413
BM	64	72%	49 (74%)	15 (65%)	
PB	25	25%	17 (26%)	8 (35%)	
IS					.066
ATG	62	69%	50 (76%)	12 (52%)	
OKT 3	8	9%	6 (9%)	2 (9%)	
Combination	1	1%	1 (2%)	0	
Without serotherapy	18	20%	9 (14%)	9 (39%)	
T cell depletion					1
No	79	89%	59 (89%)	20 (87%)	
Yes	10	11%	7 (11%)	3 (13%)	

pB-ALL indicates precursor B- acute lymphoblastic leukemia; T ALL, T cell ALL; Bpheno ALL, biphenotypic/bilinear ALL; MUD, matched unrelated donor; MMFD, mismatched family donor; RIC, reduced-intensity conditioning; TBI, total body irradiation; BM, bone marrow; PB, peripheral blood; ATG, antithymocyte globulin; OKT3, anti-CD3 antibody.

Ematologia Oncologia Pediatrica - Berlin, Frankfurt, Münster study group) after remission induction treatment. At the time of transplantation, 47 patients were in first CR, 26 patients were in second CR, 15 patients were in third CR, and 1 patient was in fourth CR, but patients were not necessarily MRD-negative at the time of transplantation (for further statistical analyses, patients were grouped into 2 categories: CR1 [n = 47] and ≥CR2 [n = 42]). Eighteen patients received grafts from matched sibling donors (MSD), 61 patients received grafts from matched unrelated donors, and 10 patients received grafts from a haploidentical parent. Patients who received their graft from a matched donor (n = 79) received a fully myeloablative conditioning regimen consisting of total body irradiation (12 Gy) and etoposide, with the youngest patient being 2.2 years of age at the time of transplantation. Patients grafted from a haploidentical donor were prepared with fludarabine, thiotepa, and melphalan. Antithymocyte globulin was used for T cell depletion in the matched unrelated and anti-CD3 antibody or antithymocyte globulin in the haploidentical transplantation setting (Table 1). Patients who were grafted from HLA-identical siblings received cyclosporine A for the prophylaxis of GVHD. Cyclosporine A and methotrexate were used in matched unrelated donor transplantations for prevention of GVHD. Mycophenolate mofetil was given as GVHD prophylaxis in the haploidentical setting.

Chimerism and MRD Monitoring

All patients were fully evaluable for stratification classified by chimerism and/or MRD (Figure 1). Therefore, chimerism analysis was performed in all patients (n = 89), whereas MRD was assessed in 58 of 89 (65%) patients, as initial diagnostic material was not available in 31 of 89 (35%) patients.

The majority of patients (65 of 89 patients, 73%) remained CC at any time point after transplantation, whereas 41 of 58 (70%) patients in whom MRD could be assessed were negative for MRD. Twenty-four of 89 patients (27%) developed MC in post-transplantation follow-up analyses, and 23 of 58 (40%) evaluable patients developed MRD positivity after transplantation. Pretransplantation MRD results were available in 15 of 89 patients. Three of these 15 patients were MRD-negative before transplantation while MRD (ranging between 1×10^{-6} and 6.2×10^{-2}) was detectable in 12 patients. Of these 12 MRD-positive patients before transplantation, 5 became MRD-negative whereas 4 remained MRD-positive after transplantation. Another 3 of these 12 MRD-positive patients before transplantation turned MRD-negative, but they became positive again after transplantation. One of the 3 MRD-negative

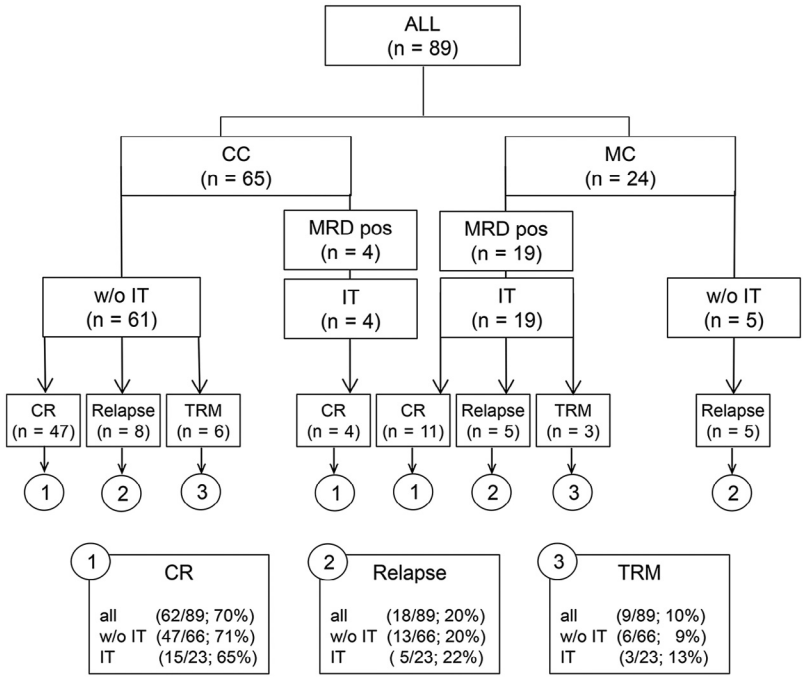


Figure 1. Flowchart of stratification and outcome according to chimerism status, MRD, and immunotherapy. Patients with detectable minimal residual disease (MRD) or mixed chimerism (MC) ≥1% after transplantation (MC of 1% autologous [recipient] signals had to be confirmed by 2 consecutive blood or bone marrow samples taken within 1 week) and with no or only mild signs of acute graft-versus-host disease (aGVHD) were immediately offered pre-emptive immunotherapy (IT). IT included discontinuation or tapering of immunosuppression (IS) for patients still receiving IS in the early post-transplantation period or administration of donor lymphocyte infusion (DLI) as frontline therapy in patients without IS. DLI was also applied in patients without responses after stop of IS. IT was performed until complete molecular remission was restored and was stopped in case of overt relapse or occurrence of aGVHD exceeding grade 1. Stratification of all patients (n = 89) according to chimerism status (CC, n = 65; MC, n = 24), MRD-positivity (but CC, n = 4) and subsequent IT as well as outcome of all patients (CR, n = 62; relapse, n = 18; TRM, n = 9) allocated to particular subgroups with and without IT is shown.

patients before HSCT developed detectable MRD after transplantation.

Overall Outcome

For the total cohort of 89 patients, the 3-year estimates of overall survival and EFS were .77 ± .05 and .69 ± .05, respectively, with a median follow-up of 3.3 years (data not shown). Furthermore, CIR and CITRM were .20 ± .04 and .10 ± .03, respectively, for all patients (data not shown).

Remission status at HSCT showed an association with EFS and CIR. There was a trend towards worse 3-year EFS in patients who underwent transplantation in ≥CR2 versus those who underwent transplantation in CR1 (log-rank test, $P = .068$). The EFS (mean ± SE) at 3 years were 76.2% ± 6.7% and 61.56% ± 7.6% for the CR1 group and ≥CR2 group, respectively. Patients who underwent transplantation in CR1 had a lower relapse incidence compared with patients who underwent transplantation in ≥CR2 ($P = .023$). Relapse incidences (mean ± SE) at 3 years were 12.3 ± 7.2% and 29 ± 5% for the CR1 group and the ≥ CR2 group, respectively. Accordingly, ≥CR2 patients had a worse EFS with a hazard ratio (HR) of 1.96 (95% confidence interval, .89 to 4.32, P=.095) compared with CR1 patients. In addition, patients with ≥CR2 had a HR of 2.99 (95% confidence interval, 1.05 to 8.49, P=.039) considering CIR compared with that of CR1 patients.

With our approach of pre-emptive immune intervention guided by serial chimerism and MRD monitoring, 62 of 89 (70%) patients achieved CR, whereas 18 of 89 (20%) patients experienced relapse and 9 of 89 (10%) patients died from TRM (Figure 1).

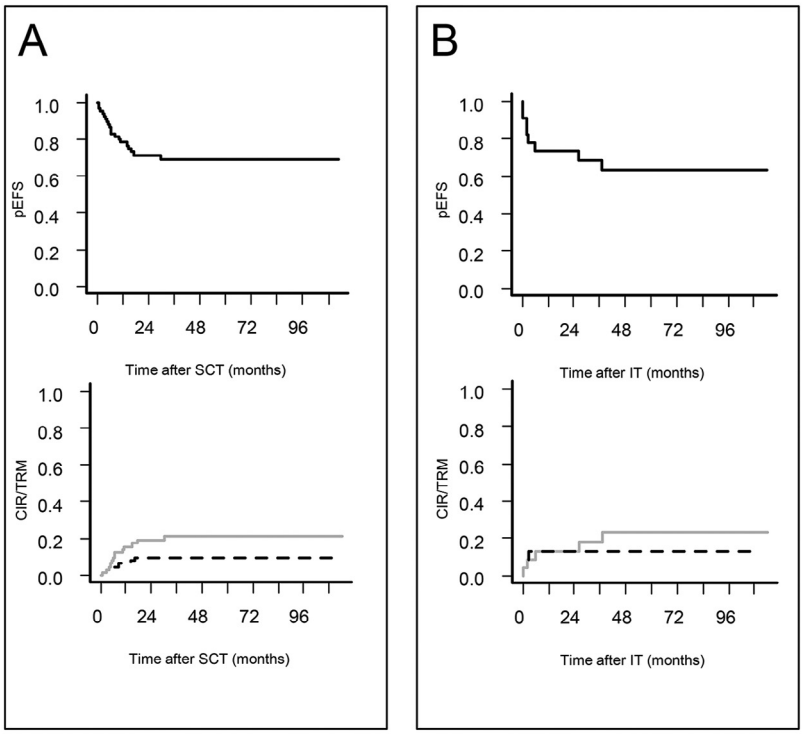
Patients Without Pre-Emptive IT

Immune intervention was not performed in 66 of 89 (74%) patients (Figure 1). Sixty-one of 89 (69%) patients were identified as not being at risk for relapse by serial chimerism and/or MRD monitoring, whereas 5 of 89 (6%) patients at risk were not offered IT intervention. In these patients without IT, 13 (20%) patients relapsed and 6 (9%) patients died from TRM (Figure 1).

Estimates of 3-year EFS of patients without IT were .69 ± .06 (Figure 2A). Furthermore, CIR and CITRM were .21 ± .05 and .10 ± .04, respectively, for patients without immune intervention (Figure 2A).

More detailed analysis showed that evidence of molecular disease was not apparent in 61 of these 66 patients without pre-emptive IT, like mentioned above (Figure 1). Forty-seven patients without evidence of molecular relapse remained in CR. But despite close monitoring, relapse was not detectable in 8 patients. In 3 of these patients, relapses occurred at days +31, +515, and +896 after transplantation; that is, before and beyond close monitoring for relapse. Despite close monitoring, another 2 patients experienced relapse at days +317 and +322 after transplantation. Because of the unavailability of molecular markers for MRD, MRD monitoring was not performed in another 3 patients. These patients relapsed at days +128, +185, and +199.

Another 6 patients in the cohort of patients without immune intervention died from TRM.



	n	Events	3y-predicted
EFS	66	19	0.69 ± 0.06
CIR	66	13	0.21 ± 0.05
TRM	66	6	0.10 ± 0.04

	n	Events	3y-predicted
EFS	23	8	0.69 ± 0.10
CIR	23	5	0.18 ± 0.09
TRM	23	3	0.13 ± 0.07

Figure 2. Estimates of event-free survival (EFS) and cumulative incidence (CI) of relapse (CIR) and treatment-related toxicity (TRM). (A) Probability of EFS, CIR, and CITRM for all patients without IT (n = 66) at 3 years after stem cell transplantation (SCT): EFS, events, n = 19; .69 ± .06; CIR, events, n = 13; .21 ± .05; CITRM, events, n = 6; .10 ± .04. (B) Probability of EFS, CIR, and CITRM for patients who received IT (n = 23) at 3 years after IT: EFS, events, n = 8; .69 ± .10; CIR, events, n = 5; .18 ± .09; CITRM, events, n = 3; .13 ± .07.

In contrast, because of the rapid progression followed by the overt relapse of their disease, which was censored as an event in our analysis, pre-emptive IT was not initiated in 5 patients despite evidence of MC (Figure 1).

Patients with Pre-Emptive Immune Intervention

Pre-emptive IT was performed in 23 of 89 (26%) of patients and was guided based on chimerism and MRD analysis in 19 patients and by MRD detection only in another 4 patients (Figure 1). Altogether, in 15 of 23 (65%) patients with IT, CR was achieved, whereas 5 (22%) patients relapsed and 3 (13%) died from TRM (Figure 1).

Estimates of 3-year EFS, CIR, and CITRM of IT patients were .69 ± 010, .18 ± .09, and .13 ± .07, respectively (Figure 2B).

More detailed analysis showed that all 4 patients who received IT based on MRD only achieved MRD negativity; no patient in this group relapsed or died from TRM (Figure 1). In 11 of 19 MC patients with IT, CR was achieved, whereas 5 patients relapsed and 3 died from TRM (Figure 1) (1 in the context of cumulative transplantation-related toxicity developed impairment of liver and renal function followed by cerebral edema, 1 patient died from multiorgan failure caused by a disseminated adenovirus infection, 1 patient died from multiorgan failure after a transplantation-associated microangiopathy and hemolytic uremic syndrome triggered by an Epstein-Barr virus reactivation). Of note, TRM in these patients was not related to immune intervention.

There was no difference in outcome of IT patients who either received withdrawal of IS or DLI (data not shown). In 9 patients, pre-emptive IT consisted of discontinuation of IS, 11 patients were given DLIs, and another 3 patients received both, discontinuation of IS and DLI. Discontinuation of IS was performed at a median of 45 days after transplantation, while DLI was given at a median of 150 days after transplantation, with a minimum of 44 days after transplantation, ie, in some extent before T cell engraftment. Eleven of 14 patients with DLIs received a single dose, 1 patient received 3 doses, 1 patient received 7 doses, and 2 patients received 9 doses of DLI. The cell dose administered was based on the number and potential severity of HLA mismatches between donor and recipient. In patients with 1 dose of DLI, median doses of T cells per kilogram body weight infused were 1×10^6 (mean, $1.0 \pm 0 \times 10^6$, n = 3) in cases of MSD, 5×10^5 (range, 1-10 $\times 10^5$; mean, $4.40 \pm 1.66 \times 10^5$, n = 5) in cases of matched unrelated donors, and 1×10^5 (range, .5-1 $\times 10^5$; mean, $.83 \pm 0.17 \times 10^5$, n = 3) in the haploidentical setting. In case of persistence of MRD or MC, DLIs with escalating numbers of $CD3^+$ T cells were infused, provided that no additional signs of GVHD had appeared. One patient who was given 3 doses of DLI received 1.0, 1.3, and 3.5×10^6 T cells/kg

Table 2
Univariate and Multivariate Analysis of Clinical/Molecular Characteristics Associated with Mixed Chimerism

Univariable Cox Regression				Multivariable Cox Regression		
n = 89, events = 24				n = 87, events = 23, missing = 2		
	HR	95% CI	*P*	HR	95% CI	*P*
Donor						
MUD	1					
MSD	5.33	2.21 to 12.88	.002	5.44	2.25 to 13.18	.0002
Status at SCT						
CR1	1					
≥CR2	2.36	.99 to 5.56	.05	2.51	1.05 to 6.01	.039
Age at HSCT						
≤10 years	1					
>10 years	1.9	.76 to 4.8	.171			

Gender, T cell depletion, transplanted source, conditioning regimen, recipient/donor cytomegalovirus status, and TBI were not associated with mixed chimerism. HR indicates hazard ratio; CI, confidence interval.

in the matched unrelated setting. One patient with 7 infusions was given $.075 \times 10^6$ T cells/kg once and $.1 \times 10^6$ T cells/kg another 6 times in the haploidentical setting. One of the 2 patients with 9 DLIs received T cell doses of 1×10^6 T cells/kg once and 5×10^6 T cells/kg another 8 times from a MSD, respectively. The other patient with 9 DLIs, which were applied in the matched unrelated setting, was given doses of 1×10^6 and 5×10^6 T cells/kg once, followed by 7 doses of 1×10^7 T cells/kg.

GVHD

aGVHD occurred in 49 of 89 (55%) patients but was not increasingly apparent in patients with IT (13 of 23; 56%). Grade 1 or 2 aGVHD was observed in 32 of 66 (48%) patients without IT and in 8 of 23 (35%) patients with IT. Grade 3 or 4 aGVHD occurred in 7 of 66 (11%) patients without IT and in 2 of 23 (9%) patients with IT. There was no correlation of aGVHD and EFS (data not shown). Furthermore, 8 of 89 (9%) patients developed chronic GVHD (cGVHD), of whom 2 (9%) patients had received IT. One of these 8 cGVHD patients relapsed.

Univariate and Multivariable Cox Regression Analyses

We investigated the association between MC and clinical/molecular characteristics. Gender, T cell depletion, transplant source, conditioning regimen, recipient cytomegalovirus, donor cytomegalovirus, and total body irradiation were not associated with MC.

In contrast, MSD and remission status (≥CR2) at the time of HSCT were associated with higher probability of developing MC after transplantation (Table 2).

Immune Reconstitution

From January 2005 to January 2015, immune regeneration of T cells, NK cells, and T-NK cells was monitored in 88 of 89 patients. Altogether, 2740 data points were obtained, with a median of 33 (range, 1 to 73) T cell values, 26 (range, 1 to 64) NK cell values, and 24 (range, 1 to 64) T-NK cell values per patient. The results from patients with IT (total, 186; median, 6.5; range, 1 to 19 per patient) were compared with the results from patients without IT (total, 2513; median, 29; range, 1 to 73 per patient). Values were age dependent and were therefore transformed for analyses [32].

Immune reconstitution of T, NK, and T-NK cells was not significantly different among patients with and without IT (T cells, $P = .542$; NK cells, $P = .053$; T-NK cells, $P = .348$; Figure 3). The regeneration of NK cells was slightly faster among patients without IT. However, IT was not associated with an increased risk for aGVHD.

DISCUSSION

Studies exploring the importance of post-transplantation monitoring for residual disease have been based on the detection of MC, flow cytometry, PCR (using clone-specific Ig or TCR sequences), and real-time quantitative PCR detection of clonal Ig/TCR rearrangements [16,33-39]. Consistently, all studies demonstrated that any evidence of MRD after transplantation represents a substantial risk for relapse. MRD monitoring using antigen receptor gene rearrangements offers a highly sensitive tool for the detection of impending relapse compared with the less sensitive chimerism analysis. Chimerism analysis in general detects persisting or reappearing recipient cells—ie, surviving leukemia blasts, surviving host hematopoiesis, or both. Hence, detection of residual host hematopoietic cells may not definitely identify patients at risk for relapse. But when performed sequentially, chimerism analysis may lead toward the alloreactive (decreasing recipient signals) or tolerance induction potential of the graft (eg, stable or increasing recipient signals) and, as a consequence, serves more as a prognostic factor than an indirect molecular marker for MRD. Additional MRD monitoring after allogeneic HSCT can highlight patients with increased risk for subsequent relapse. Hence, when assessed sequentially, both methods in combination may be a useful means of assessing the efficacy of HSCT and of identifying patients at risk for relapse which might open a window for pre-emptive IT [15,19,40-46]. Here, the first therapeutic option may be the reinforcement of the graft-versus-leukemia effect without administering additional toxicity.

In this retrospective study, we analyzed the impact of pre-emptive IT based on post-transplantation chimerism or lineage-specific MRD detection in a larger cohort of ALL patients who received their first allogeneic HSCT at our center. In our cohort, 28 patients out of 89 patients showed evidence of molecular relapse and, therefore, fulfilled the criterion for pre-emptive IT intervention.

Over the last decade, there have been many attempts to target ALL relapses. Hereby, treatment with unmanipulated donor lymphocytes is not effective in treating overt relapse. However, pre-emptive IT such as the tapering of IS and DLI has been reported to be successful especially in case of molecular relapse [15,47]. However, unspecific IT approaches may be associated with an increased risk for developing aGVHD.

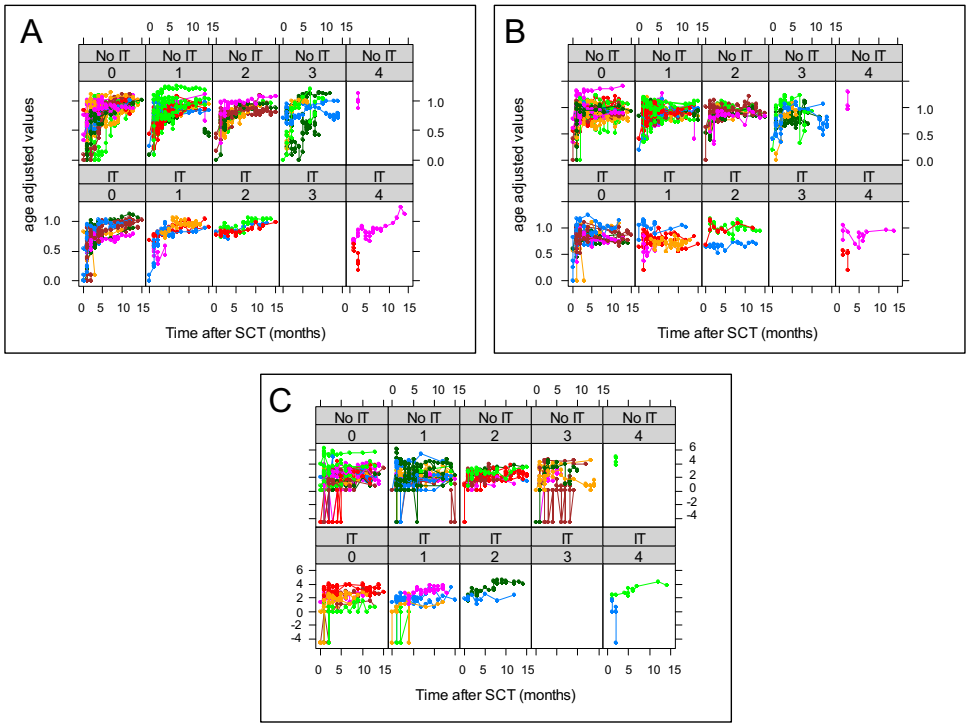


Figure 3. Level of T, NK, and T-NK cells according to aGVHD and IT. IT may have increased levels of immune cells and may have raised risk for developing aGVHD. Figure 3 shows the individual (age-adjusted) values of (A) T, (B) NK, and (C) T-NK cells of patients with and without IT related to the grade of aGVHD (0 = no aGVHD, 1-4 = grade 1 to 4 aGVHD). There is a nonsignificant trend toward lower levels of NK cells in patients with IT and severe aGVHD. We did not observe such differences in the number of T cells in patients with and without IT in correlation with aGVHD.

Overall aGVHD occurred in 39 of 66 (59%) patients without intervention and in 10 of 23 (43%) patients with tapering of IS or DLI, 32 of 66 (48%) patients without IT and 8 of 23 (35%) patients with IT developed grade 1 or 2 aGVHD. Only 7 of 66 (11%) patients in the study cohort without intervention and only 2 of 23 (9%) patients of the IT group experienced grade 3 or 4 aGVHD. DLIs led to grade 1 aGVHD in 1 case, whereas aGVHD developed in another 9 patients after cessation of IS. Hence, our data showed that unspecific DLI intervention is feasible and is not associated with an increased risk for developing aGVHD. In addition, TRM in our cohort with preemptive immune interventions was not associated with increased risk for aGVHD or cGVHD and, therefore, was not related to IT. The risk for GVHD in our cohort may have been minimized by small initial T cell doses according to donor type and prudent dose escalation provided that no or only mild signs of aGVHD (≤grade 1) were apparent at the time of IT intervention. In contrast, the risk for aGVHD was previously reported to be substantial, especially with unmanipulated DLI approaches [23,24]. Further T cell approaches with minimized risk for GVHD may include limiting T cell doses, selective depletion of alloreactive T cells, insertion of suicide genes or chimeric antigen receptors, or T cell activation [28,48-53].

Minimized risk for GVHD may not translate to a lack of efficacy, although several studies reported that GVHD appeared to be correlated with response to DLI [23,24]. In our cohort, despite the minimal risk for GVHD, the intervention did affect EFS in the MRD and MC groups. In 15 of 23 (65%) IT patients, molecular remission was restored, whereas 13 (56%) and 8 (35%) of 23 patients with IT showed no or only transient grade 1 or 2 aGVHD, respectively. Although DLIs were effective in treating relapsed chronic myeloid leukemia, similar effects were not reported in the treatment of ALL by other groups [22,23,25,54-56]. In contrast, several reports described that activated T cells do recognize and lyse ALL cells despite the rapid growth characteristics of ALL cells [57-59]. Balduzzi demonstrated that the timing and level of MRD after transplantation are predictive of subsequent relapse, which may identify patients at risk and may open a window for IT intervention [44]. However, all patients in this study with post-transplantation MRD $\geq 1 \times 10^{-3}$ relapsed, regardless of IS discontinuation or DLI. The improvement in efficacy in our study may, in part, be related to intensive chimerism in addition to MRD monitoring, as IT intervention may only be protective when the load of residual leukemia is limited [60], with immediate and consistent IT intervention until chimerism converted to full donor chimerism and the parallel clearance of MRD, if applicable. Monitoring of chimerism in peripheral blood is easier than that for MRD in the bone marrow, such as in our study, as diagnostic samples to establish the leukemia markers were not available [61]. For this purpose, chimerism monitoring has recently been standardized [62]. In comparison with historical control outcome data with an 3-year EFS of 37% for patients with increasing MC and immune intervention our data with immediate and consistent IT intervention confirmed a superior 3-year EFS of 69%. Of the 46 patients with increasing MC in the historical control group, 31 received IT showing a significantly higher 3-year EFS estimate (37%) than the 15 patients who did not receive IT (0%). Overall 3-year EFS of the historical control group with CC, decreasing ,or low-level chimerism was 66% compared to a 3-year EFS estimate of 69% of the actual study cohort without IT [15]. However, in the current study, patients were

treated on the basis of any MC (1% recipient signals on 2 occasions or >1% recipient signals in a single sample) rather than increasing MC. With this approach, patients without a definite risk may have received immune intervention. However, the trigger for IT intervention as well as the way how preemptive IT was implemented in this study (low applied T cells doses, time interval between interventions, prudent dose-escalating strategies, and close monitoring for GVHD) did not result in increased toxicity. Taken together, the close monitoring for MC and MRD, the trigger for IT, and the use of preemptive immunotherapy as a means of preventing relapse after transplantation in childhood ALL may have improved outcome in our cohort. In contrast, with the recent approach relapse was still not identified in 8 of 89 (9%) patients. However, 3 of these 8 patients had no MRD monitoring and in another 3 patients relapses occurred before and beyond close monitoring for residual disease. Despite serial chimerism and MRD monitoring, 2 patients relapsed at days +317 and +322 after transplantation, suggesting that MRD monitoring was not close enough between days +180 and +356 after transplantation or was hampered by the rapid recurrence of the disease. Furthermore, in patients without available MRD markers, analysis of chimerism subpopulations or flow cytometry might be considered.

Pre-emptive IT did not significantly influence the immune reconstitution of T, T-NK, and NK cells in our analysis. In our cohort, aGVHD was not significantly increased among patients with IT, whereas most patients with impending relapse were rescued by pre-emptive IT.

In conclusion, in our cohort about one-third of childhood ALL patients received immune intervention guided by chimerism and MRD monitoring after transplantation, which, despite the risk for relapse, may have influenced the excellent overall outcome of our cohort. Our results confirm that the analysis of chimerism and MRD enables the prediction of impending relapse in the majority of children transplanted for ALL and that pre-emptive IT can safely be performed to clearly improve outcome in these high-risk patients, which was comparable to the outcome of patients with CC and MRD negativity after transplantation.

ACKNOWLEDGMENTS

The authors thank the LOEWE Center for Cell and Gene Therapy Frankfurt, Hessian Ministry of Higher Education, Research and the Arts, reference number: III L 4- 518/17.004 (2013).

Financial disclosure: This work was supported by Hessian Ministry of Higher Education, Research and the Arts, funding reference number: III L 4- 518/17.004 (2013).

Conflict of interest statement: The authors declare no conflicts of interest.

Authorship statement: E.R., M.M., E.S.-M., H.K., T.K., M.D., J.F., S.H., C.C., M.B., A.W., S.B., A.J., J.S., G.B., T.K., and P.B. collected, analyzed, and interpreted the data. E.R., M.M., E.S.-M., and P.B. wrote the manuscript. E.R., T.K., M.D., J.F., A.W., S.B., A.J., J.S., T.K., and P.B. enrolled and cared for the patients. E.R., M.M, and E.S.-M. contributed equally.

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