SUPPLEMENTARY APPENDIX

Intentional donor lymphocyte-induced limited acute graft-*versus*-host disease is essential for long-term survival of relapsed acute myeloid leukemia after allogeneic stem cell transplantation

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 doi:10.3324/haematol.2013.089565
Manuscript received on April 7, 2013. Manuscript accepted on November 8, 2013.
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Supplementary methods section

Between January 2000 and January 2010, 44 patients with relapsed AML after alloSCT were treated. The patients were categorized according to their disease characteristics prior to alloSCT as intermediate, poor-risk, or very poor-risk AML. Intermediate risk (int-risk) AML was defined as <60 years old, white blood cell count (WBC) $<100x10^9$/L at diagnosis, cytogenetically normal (except for -X, -Y), and transplanted in first complete remission (CR1). Poor-risk AML was defined as ≥60 years old and/or WBC $>100x10^9$/L at diagnosis, and/or transplanted in second complete remission (CR2) and/or not cytogenetically normal, favorable (t(15;17), t(8;21), or inv(16)) or very poor (monosomal karyotype), and/or FLT3/ITD positive. In addition, acute erythroid leukemia, acute megakaryocytic leukemia and mixed-phenotype acute leukemia were also considered poor-risk. AML with a monosomal karyotype (MK) was defined as very poor-risk.

Analysis of the data was approved by Leiden University Medical Center Research Ethics Committee. Informed consent was obtained prior to data collection. Data was analyzed as of November 2012.

Transplantation protocol

Patients had been transplanted with a myeloablative (MAC) or a reduced-intensity conditioning (RIC) regimen. The MAC regimen consisted of cyclophosphamide 60 mg/kg i.v. for 2 days, and TBI 9 Gy (n=26) or busulphan (Busilvex©) 3.2 mg/kg i.v. for 4 days (n=1). Patients transplanted with an unrelated donor also received alemtuzumab 15 mg i.v. for 2 days, and cyclosporine 3 mg/kg as GvHD-prophylaxis from day -1 until day 60 in the absence of GvHD. The RIC regimen consisted of fludarabine 30 mg/m2 i.v. or 50 mg/m2 orally for 6 days, busulphan 3.2 mg/kg i.v. for 2 days and either alemtuzumab 15 mg i.v. for 2 days (n=10) or horse-derived anti-T lymphocyte globulin (Lymphoglobulin©) 10 mg/kg/day i.v. for 4 days (n=8). After RIC, patients did not receive additional GvHD-prophylaxis. In both MAC and RIC transplanted patients in vitro T-cell depletion (TCD) of the stem cell product was performed by incubation with alemtuzumab (20 mg) for 30 min at room temperature under continuous agitation (n=41), or by $CD34^+$-cell MACS-sorting (n=3).(1-4) Fully matched donors were defined as 10 out of 10 HLA-matched (HLA-A, -B, -C, -DR and -DQ).

From 2002, all patients with AML or MDS were eligible for prophylactic DLI at 6 months after alloSCT when the patient showed mixed-chimerism in the absence of GvHD requiring systemic immunosuppression. From 2007, all patients with high-risk MDS (IPSS >1), poor-risk AML and MK AML were candidate for low dose prophylactic DLI at 3 months after alloSCT, if overall grade 2 (or higher) acute GvHD was absent at that time point.

Relapse

Relapse after alloSCT was defined as an increase of blasts in the bone marrow (BM) to 5% or more by morphology, and/or by the presence of more than 1% blasts in peripheral blood (PB), and/or by the reappearance of molecular and/or cytogenetic markers.

Smoldering relapse was defined as a low percentage of blasts by morphology (<10% blasts in BM and <5% blasts in PB) during a prolonged interval of at least 4 weeks evaluated by repeated bone marrow aspiration. Other relapses were defined as high tumor burden relapse. Remission status after DLI was assessed by bone marrow aspiration at 6 week, at 12 weeks after DLI, and at every 3 months hereafter. Second relapse was defined as first relapse after DLI.

Treatment strategies for relapse

Patients with a poor performance status, defined as WHO performance status 3 or higher, and/or with severe GvHD requiring systemic immuno-suppression at the time of relapse were unlikely to benefit from an intensive DLI-based strategy and received palliative treatment only.

Patients with high tumor burden relapse received salvage re-induction therapy prior to infusion of donor lymphocytes. In case of a response to re-induction therapy, defined as absence of circulating leukemic blasts, unmanipulated DLI collected from the original donor was administered at 3 weeks after start of re-induction therapy (during the neutropenic phase). In case of a smoldering relapse, patients received DLI without prior cytoreductive treatment.

DLI-dosing depended on the time point after alloSCT, administration of pre-emptive DLI prior to relapse and donor type. Median DLI-dose of patients transplanted with a (10 out of 10 HLA-matched) sibling donor was $5.0x10^6$ $CD3^+$-cells/kg (range $1.0\text{-}100x10^6$ CD3+cells/kg) and $2.5x10^6$ $CD3^+$-cells/kg (range $1.5\text{-}7.8x10^6$ CD3+cells/kg) for patients transplanted with an unrelated donor (or a HLA-mismatched related donor), respectively.

The occurrence and impact of GvHD on outcome after relapse was evaluated in all patients receiving DLI for relapse. Assessment and grading of GvHD was performed using modified Glucksberg and Shulman criteria.(5, 6). If no acute GvHD was observed within 3 weeks after DLI, interferon-α was administered subcutaneously in a daily dose of 3 million units until GvHD occurred. Escalating doses of DLI were administered in case of failure to develop acute GvHD at 3 months after start of treatment. Finally, grade 2-4 acute GvHD and severe chronic GvHD were treated with prednisone 1-2 mg/kg/day. Ciclosporine or mofetil mycophenolate were added if prednisone was administered for more than 2 weeks.

Statistical analysis

For different analyses, time was measured since relapse, initiation of re-induction therapy or infusion of donor lymphocytes. Non-relapse mortality (NRM) was defined as death after DLI while in continuous complete remission. Relapse-free survival was defined as survival after DLI without second relapse. Probabilities of overall survival with associated 95% confidence intervals (95% CI) were calculated by the Kaplan-Meier method; differences between groups were compared using long-rank test. The cumulative incidence of acute GvHD after DLI was calculated using R2.15.0 software, library 'cmprsk' (http://www.r-project.org/foundation).

To investigate the impact of a rapid immune response associated with acute GvHD on long-term survival and disease recurrence after DLI, extended Cox regression models as proposed by Andersen&Gill for overall survival, relapse-free survival and relapse incidence from starting point DLI were constructed (see online supplementary method section for details).(7) These models included onset of acute GvHD after infusion of donor lymphocytes as a time-dependent variable, separating between grade 1-3 and grade 4. The small number of events prevented the inclusion of other relevant predictors, for which reason the estimated Hazard Ratios should be interpreted as averages over different patient characteristics. Impact of acute GvHD on survival was also studied by landmark analysis (see supplementary Figure 1).

All P-values of <0.05 were considered significant. Statistical software used was SPSS, PASW Statistics 20, release 20.0.0 (2011).

References of the supplementary method section

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Supplementary Table 1. Severity and distribution pattern of GvHD after DLI according to administration of interferon-α.

	Interferon-α	No interferon-α
Acute GvHD	(n=15)	(n=12)
None	1	5
Grade 1	3	1
Grade 2	1	1
Grade 3	2	3
Grade 4	4	2
Localization acute GvHD	(n=10)	(n=7)
Skin only	5	1
Intestines only	0	1
Skin and liver	5	3
Skin, liver and intestines	0	2
Chronic GvHD	(n=15)	(n=12)
None	8	2
Limited	1	1
Extensive	4	2
Not applicable (Follow-up ≤ 3 months)	2	7

Supplementary Figure 1. Landmark analysis of the impact of the presence of acute GvHD at 3 months after DLI on overall survival. Kaplan meier curves starting at 3 months after infusion of donor lymphocytes showing overall survival according to maximum grading of acute GvHD before that time point: 10 patients without signs of GvHD (dashed line), 8 patients with grade 1-3 acute GvHD (solid line) and 2 patients with grade 4 acute GvHD (dotted line).