High preharvest donor Foxp3 mRNA level predicts late relapse of acute lymphoblastic leukaemia after haematopoietic stem cell transplantation

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Abstract

Objectives: The curative effect of allogeneic haematopoietic stem cell transplantation (HSCT) for acute leukaemia is due in part to the donor T cell–mediated graft-versus-leukaemia immune reaction (GvL). Several studies have suggested that donor CD25+CD4+Foxp3+regulator T cells (Tregs) may decrease graft-versus-host disease (GvHD) without abrogating GVL. This notion may need modification in acute lymphoblastic leukaemia (ALL).

Methods: Foxp3 mRNA level was measured by qPCR in preharvest donor blood CD4+ T cells. The study comprised 45 patients with ALL in 1st or 2nd CR who received myeloablative HSCT using T-replete bone marrow grafts.

Results: Relapse occurred in 17 patients median 363 days after HSCT. The relapse risk was estimated by Cox univariate and multivariate proportional hazard regression. The proportionality assumption was met by analysing the preharvest donor Foxp3 mRNA level as a time-dependent covariate. Early relapse was not modified by the Foxp3 mRNA level. However, a higher Foxp3 mRNA level was associated with a significantly increased relapse risk after day 363 after transplantation, compatible with inhibition of GvL. In contrast, a higher preharvest donor CD4+ T-cell concentration was associated with reduced relapse risk.

Conclusion: A higher preharvest donor Foxp3 mRNA level may be predictive of late ALL relapse after HSCT.

Keywords

acute lymphoblastic leukaemia, Foxp3, haematopoietic stem cell transplantation, regulatory T cells
The curative effect of allogeneic T-cell replete myeloablative haematopoietic stem cell transplantation (HSCT) for acute leukaemia is due in part to the donor T-cell–mediated graft-versus-leukaemia reaction (GvL). However, despite pretransplant conditioning and GvL, leukaemia relapse remains a major cause of treatment failure. CD25+CD4+Foxp3+regulatory T cells (Tregs) maintain peripheral immunological tolerance by inhibiting auto- and alloreactive T-cell activation and function. Expression of the transcription factor Foxp3 is essential for the suppressive function of Tregs. In murine HSCT models, adoptive transfer of donor Tregs has been reported to abrogate T-cell–mediated graft-versus-host disease (GvHD) associated mortality and yet preserve GvL. A similar reduced incidence of acute GvHD without increased leukaemia relapse rate has been observed in patients with acute leukaemia after myeloablative conditioning followed by HSCT with T-cell replete peripheral blood stem cell (PBSC) allografts containing higher Treg concentrations. These patients received post-transplant GvHD prophylaxis with cyclosporine. Studies in man after HSCT with PBSC or umbilical cord cells enriched with effector (ie non-regulatory) T cells and immuno-selected or ex vivo expanded donor Tregs have supported the notion that Tregs may inhibit GvHD without abrogating GvL. A single study using bone marrow stem cells (BMSC) rather than PBSC showed no effect on acute GvHD associated with high Tregs in the graft. This study did not report relapse rate.

We asked the question, whether the level of Foxp3 messenger RNA (mRNA) assessed by quantitative PCR (qPCR) in peripheral blood CD4+ T cells from the donor prior to stem cell harvest was predictive of acute leukaemia relapse after HSCT. This analysis included children and adults with ALL in 1st or 2nd complete remission (CR) who received T-cell replete bone marrow allografts after myeloablative conditioning.

2 | PATIENTS AND METHODS

The study was approved by the Committees on Health Research Ethics for the Capital Region of Denmark (H-4-2013-188) and the Data Protection Agency (30-1168). Informed written consent was obtained according to The Declaration of Helsinki.

2.1 | Patients

The study comprised 45 patients who received allogeneic myeloablative HSCT with T-cell replete bone marrow at a single centre during 1998-2006 due to ALL in 1st CR (n = 25) or 2nd CR (n = 20). The median patient age at HSCT was 16.7 years, range 4-52 years. Further selection criteria were restricted to known preharvest donor CD4+ and CD8+ T-cell blood concentrations, cytogenetics tested at diagnosis, preharvest frozen donor blood cells available for retrospective Foxp3 analysis, and no previous allogeneic transplantation.

Significance statements

1. This study elucidates aspects of T-cell–mediated graft-versus-leukaemia reaction (GvL) after haematopoietic stem cell transplantation for acute lymphoblastic leukaemia (ALL).

2. A high concentration of Foxp3 mRNA, a molecular marker of regulatory T cells, in predonation donor blood is associated with an increased risk of late ALL relapse after transplantation suggesting that regulatory T cells from donor may inhibit GvL in ALL.

3. This observation may become relevant for donor selection and for elimination of potential harmful cells in the donor stem cell graft or leukocyte products before infusion to the patient.

Definition of CR was based on morphological examination by microscopy and included normalisation of neutrophil granulocytes and thrombocytes. Patients with high-risk leukaemia, based on cytogenetics and time interval to achieve CR after onset of chemotherapy were preferentially transplanted in 1st rather than in 2nd CR.

2.2 | Donor selection

Donor was an HLA identical sibling (n = 11) or an alternative HLA compatible donor, comprising other related (n = 1) or unrelated donors (n = 33), Table 1. Twenty alternative donor-recipient pairs were typed for HLA-ABC, -DRB1 and -DQB1 by high resolution and 14 alternative donor-recipient pairs were typed by low resolution for HLA-ABC and by high resolution for DRB1 and DQB1. Eleven alternative donor-recipient pairs had at least one documented allele or antigen mismatch, counting only GvHD directed mismatch.

2.3 | Transplant procedures

All patients received myeloablative conditioning with fractionated TBI, 12 Gy with lung shielding to 9 Gy, combined with cyclophosphamide iv (60 mg/kg/d for two days), n = 19, or with etoposide iv (60 mg/kg once), n = 24. Instead of irradiation, two patients received weight and age-adjusted busulfan, 16 mg/kg orally or 12.8 mg/kg iv, combined with cyclophosphamide (n = 1) or etoposide (n = 1).

Antithymocyte globulin (ATG) (ATGAM, Ujohib, 20 mg/kg/d or Thymoglobulin, Mercierx, 2.5 mg/kg/d) was given for three days during conditioning to 28 of 34 recipients with an alternative donor to prevent graft rejection. GvHD prophylaxis consisted of cyclosporine day –1 to day 180, mean target whole blood valley concentration (excluding metabolites) 300 μg/L, with or without 3 or 4 doses of methotrexate (MTX) iv day +1 to +11. Prednisolone 2 mg/kg/d was initial treatment for moderate-severe acute or extensive chronic
GvHD. Short courses of granulocyte colony-stimulating-factor (G-CSF) were given to 30 patients to accelerate granulocyte recovery, starting median 23.5 days after transplantation (range 11-100 days). Leukaemia relapse was defined morphologically. Acute and chronic GvHD were diagnosed according to published criteria.28,29

2.3.1 | The graft

Donor BMSC were harvested by bone marrow aspiration and given iv at day zero. No ex vivo T-cell depletion was employed. Median dose was $3.18 \times 10^8$ nucleated cells/kg recipient weight.

2.4 | Blood lymphocytes and Foxp3 mRNA qPCR analysis

Peripheral blood samples were obtained 2-3 weeks prior to bone marrow harvest. CD3+ CD4+ and CD3+ CD8+ T cells were assessed by a single platform lyse-no-wash procedure using Becton-Dickinson TRUcount beads and BD TriteSTM monoclonal antibodies. Mononuclear cells were obtained by Lymphoprep® gradient separation and stored in liquid nitrogen. After thawing, CD4+ T cells were isolated using magnetic beads (Dynabeads, Life Technologies). Flow cytometry after CD4+ T-cell isolation showed a purity of >97% CD3+ CD4+ T cells with contamination of <2% CD3+ CD8+ T cells and <1% CD14+ CD4dim monocytes. Foxp3 mRNA including full length and splice forms was analysed in duplicate by qPCR in purified CD4+ T cells using CD4 mRNA as a population-specific internal reference. The resulting ΔCT value, which denotes the PCR cycle threshold (CT) for the target mRNA minus the cycle threshold (CT) for CD4 mRNA, provided an estimate of the amount of target mRNA copies relative to reference mRNA copies.30 The number of CD4 mRNA copies has been found to be proportional to the number of CD4+ T cells, independent of T-cell activation.31 The ratio Foxp3 mRNA copies per CD4 mRNA copy therefore provides an estimate of the number of Foxp3 mRNA copies relative to the number of CD4+ T cells. This estimate was expressed in a non-log scale and multiplied by the CD4+ T-cell concentration in donor blood yielding

### TABLE 1 (Continued)

| No | ATG | ATG | Total | P |
|----|-----|-----|-------|---|
| Donor ≤ 34.0 y | 9 | 14 | 23 |   |
| Donor > 34.0 y | 8 | 14 | 22 |   |
| Total | 17 | 28 | 45 | 1.00 |
| Recipient ≤ 16.7 y | 9 | 14 | 23 |   |
| Recipient > 16.7 y | 8 | 14 | 22 |   |
| Total | 17 | 28 | 45 | 1.00 |
| Donor CD4 T cells ≤ 0.87 10^9/l | 6 | 17 | 23 |   |
| Donor CD4 T cells > 0.87 10^9/l | 11 | 11 | 22 |   |
| Total | 17 | 28 | 45 | 1.00 |
| HLA identical sibling donor | 11 | 0 | 11 |   |
| HLA match alternative donor | 4 | 19 | 23 |   |
| HLA mismatch alternative donor | 2 | 9 | 11 |   |
| Total | 17 | 28 | 45 | <.0001 |
| Donor CMV negative | 8 | 19 | 27 |   |
| Donor CMV positive | 9 | 9 | 18 |   |
| Total | 17 | 28 | 45 | .29 |
| Recipient CMV negative | 5 | 10 | 15 |   |
| Recipient CMV positive | 12 | 18 | 30 |   |
| Total | 17 | 28 | 45 | .91 |
| Female donor to male recipient | 2 | 4 | 6 |   |
| Other combinations | 15 | 24 | 39 |   |
| Total | 17 | 28 | 45 | 1.00 |
| Stage at HSCT: 1st CR | 9 | 16 | 25 |   |
| Stage at HSCT: 2nd CR | 8 | 12 | 20 |   |
| Total | 17 | 28 | 45 | 1.00 |
| High-risk cytogenetics | 4 | 10 | 14 |   |
| Not high-risk cytogenetics | 13 | 18 | 31 |   |
| Total | 17 | 28 | 45 | .60 |
| Time to latest CR > median 32 d | 10 | 13 | 23 |   |
| Time to latest CR ≤ median 32 d | 7 | 15 | 22 |   |
| Total | 17 | 28 | 45 | .62 |
| Busulfan | 0 | 2 | 2 |   |
| Total body irradiation | 17 | 26 | 43 |   |
| Total | 17 | 28 | 45 | .70 |
| Cyclophosphamide | 5 | 15 | 20 |   |

Abbreviations: ATG, Antithymocyte globulin; CMV, cytomegalovirus; CR, complete remission; HSCT, Haematopoietic stem cell transplantation.
Foxp3 mRNA copies in arbitrary units per volume of blood, in the following denoted the Foxp3 mRNA level. CD25 and CTLA-4 mRNA expression were assessed by analogous procedures. Thus, "donor CD4+ T cell concentration" and "donor Foxp3 mRNA level" refer to preharvest peripheral blood values.

2.5 | Statistical analysis

Correlations between continuous covariates were tested using the Pearson correlation coefficient r. Risk factors for relapse were identified by univariate and multivariate Cox proportional hazard regression analysis and tested by Wald test, unless otherwise stated.32,33 For the analysis of relapse, patients were censored at last follow-up, if they died in continuous CR after HSCT, or if they received a second infusion of cells from the same donor. Treatment failure was defined as relapse or death in continuous CR after HSCT, whatever is first, using last follow-up or a second cell infusion from the same donor as censoring. Disease-free survival is equal to the absence of treatment failure. CD4+ T-cell concentration and Foxp3 mRNA level were analysed as continuous covariates unless otherwise stated. The proportionality assumption was tested by time-dependent covariates using the log-likelihood χ²-test. P < .050 indicated lack of proportionality. Post-transplant events, including GvHD, were tested as time-dependent covariates. Probability of disease-free survival was analysed by Kaplan-Meier product-limit estimates using treatment failure as the event and last follow-up or a second infusion of cells from the same donor as censoring. The Mantel-Cox log-rank test was used to test for significance. Cumulative relapse incidence was analysed treating death in continuous CR after HSCT as a competing event. Differences between cumulative incidences were tested according to Fine and Gray.34,35 All P values were two-tailed. P < .050 was considered significant.

3 | RESULTS

Patient, donor and procedure-related characteristics are shown in Table 1 and Table S1. Relapse occurred in 17 of 45 patients median 363 days (range 108 -1876 days) after HSCT. Twelve of 25 patients in 1st CR and five of 20 patients in 2nd CR experienced relapse after HSCT. Patients transplanted in 1st CR had marginally more often high-risk cytogenetics at diagnosis compared with patients in 2nd CR (P = .078) (Table S1). Moderate acute GvHD and chronic extensive GvHD were diagnosed in 16 and 6 patients, respectively. Five patients died in continuous post-transplant CR median 133 days after HSCT (range 20 - 1357 days). One patient died with a respiratory syncytial virus infection, two patients died with acute GvHD, two patients died with acute GvHD combined with cytomegalovirus (CMV) infection, and one patient died due to a secondary neoplasm. No graft rejection occurred. One patient received additional stem cells from the same donor 121 days after HSCT due to pancytopenia without evidence of relapse or rejection. This patient was censored at the time of infusion according to the protocol. The median observation time for disease-free survivors was 3143 days (range 121-4879 days).

In purified CD4+ T cells, the expression of Foxp3 mRNA was strongly correlated with the expression of CD25 mRNA (r = .79, P < .001, Figure 1A) and CTLA-4 mRNA (r = .74, P < .001, Figure 1B), consistent with the Treg phenotype.5,36-38 There was a weak correlation between the donor Foxp3 mRNA level and total donor CD4+ T-cell concentration (r = .25, P = .004, 1C). Figure 1D illustrates the distribution of Foxp3 mRNA -ΔCT values. Figure 1E illustrates the corresponding -ΔCT values expressed as Foxp3 mRNA copy levels in arbitrary units per volume of blood.

3.1 | Cox proportional hazard regression analysis of relapse

Preharvest donor Foxp3 mRNA level and donor CD4+ T-cell concentration were analysed as continuous variables. The Cox analysis requires that the effect of each covariate be proportional with time since HSCT. However, the effect of donor Foxp3 mRNA level on the incidence of relapse did not meet the proportionality assumption. This was documented in univariate as well as in multivariate analysis by testing the effect of inclusion of donor Foxp3 mRNA as a time-dependent covariate in the Cox models, using the chi-square test for significance (P<.042 and .043, respectively). The lack of proportionality indicated that the effect associated with higher donor Foxp3 level on relapse differed significantly between early and late relapse. Proportionality was obtained when early and late relapse were analysed separately (≤363 days: P<.050; >363 days: P>.85), using dichotomy at the median relapse time. All other risk factors tested met the proportionality assumption without dichotomy.

3.2 | Univariate analysis of relapse

When early and late relapse were analysed separately, donor Foxp3 mRNA level had no influence on risk of early relapse (hazard rate (HR) 0.60, P = .38, Table 2). However, a higher donor Foxp3 mRNA level was associated with a significantly increased risk of late relapse (HR 2.86, P = .0039, Table 2). In contrast, in the overall analysis of relapse, a higher donor CD4+ T-cell concentration was associated with a significantly reduced relapse risk (HR = 0.092, P = .014, Table 2).

3.3 | Multivariate analysis of relapse

The results are presented in Table 3. To meet the proportionality assumption, donor Foxp3 mRNA level was analysed in a single Cox model as a time-dependent covariate characterised by disparate hazard rates early and late after HSCT, using dichotomy at the median time of relapse. No significant effect of higher donor Foxp3 mRNA level on early relapse was seen (HR = 1.24, P = .71, Table 3).
In contrast, a higher donor Foxp3 mRNA level was associated with a significantly increased risk of late relapse (HR = 4.26, P = .00043, Table 3). Multivariate analysis confirmed that higher donor CD4+ T-cell concentration was associated with an overall decreased relapse risk (HR = 0.0061, P = .00032). Relapse occurred in nine of 28 patients who received ATG and in eight of 17 patients who did not receive ATG. We observed a significant statistical interaction between donor CD4+ T cells and ATG (P<.027), indicating that the effect associated with donor CD4+ T cells was significantly different in patients who received ATG during the pretransplant conditioning compared with patients who did not. This is illustrated in Table 3 which shows the effect of donor CD4+ T-cell concentration after stratification for ATG administration. In patients who did not receive ATG, a higher donor CD4+ T-cell concentration was associated with

**FIGURE 1** Data description. A-C. Each dot represents one donor. Data are from 136 normal donors, median age = 34 years. Foxp3 mRNA expression correlates strongly with the expression of CD25 mRNA (A, r = 0.79, P < .001) and CTLA-4 mRNA (B, r = 0.74, P < .001) in purified CD4+ T cells from preharvest donor blood. Values were obtained by qPCR using CD4 mRNA level as the internal reference. *r* denotes the Pearson correlation. P is the probability that the correlation is zero. Results are presented as minus ΔCT. ΔCT denotes the PCR cycle threshold (CT) for the target mRNA minus the cycle threshold (CT) for the internal reference. C. Weak correlations were found between preharvest donor Foxp3 mRNA level and preharvest donor CD4+ T-cell concentration (r = 0.25, P = 0.004). D and E. Data are from the present patient series (n = 45). Black circles denote patients who had relapse after HSCT (n = 17). White circles denote patients who were censored in CR after HSCT (n = 28). Horizontal dashed lines denote the median Y value for all patients. Unbroken lines denote median Y value pertaining to each subgroup. D, illustrates the distribution of Foxp3 mRNA -ΔCT values. The figure presents separate columns for patients who had relapse or were censored in CR (n = 17). E, presents separate columns for patients who had relapse or were censored in CR, either early (≤363 d) or late (>363 d) after HSCT [Colour figure can be viewed at wileyonlinelibrary.com]
a reduced risk of relapse (HR 0.0002, P = .00044) This effect was not significant in patients who received ATG (P = .12). No significant interaction was observed between donor CD4 and donor-recipient HLA mismatch or alternative donor. Furthermore, no statistically significant interaction between donor Foxp3 mRNA level and ATG was observed (Pχ2 = .34). There were too few patients to allow estimation of the relapse risk after splitting according to whether they received ATG.

The model presented in Table 3 included adjustment for the effect of leukaemia-associated risk factors known to be associated with increased relapse risk after HSCT, including stage at HSCT, high-risk cytogenetics at diagnosis,19,20 and time from onset of chemotherapy to achievement of CR,21 as well as the use of ATG during the pre-HSCT conditioning. Results of pretransplant measurable residual disease (MRD) were not available and could not be obtained retrospectively. Other factors tested and found to be insignificant included graft nucleated cell dose, donor and recipient age and pretransplant CMV status, alternative donor, documented donor-recipient HLA mismatch, administration of MTX or G-CSF, and moderate-severe acute or extensive chronic GvHD.

### 3.4 Cumulative relapse incidence

For this analysis, preharvest donor Foxp3 mRNA level and preharvest CD4+ T-cell concentration were analysed as categorical variables, that is high versus low, using dichotomy at the median. Figure 2A,B illustrate the effect of donor Foxp3 mRNA level on the cumulative incidence of early and late relapse after HSCT, respectively. No effect on early relapse was observed (Figure 2A, P = .61).
TABLE 3 Multivariate Cox analysis of relapse

| Variable                          | E : n  | Hazard rate [95% CI] | P Wald |
|----------------------------------|--------|----------------------|--------|
| Donor Foxp3 mRNA level, early relapse | 9:45   | 1.24 [0.39-4.01]     | .71    |
| Donor Foxp3 mRNA level, late relapse | 8:31   | 4.26 [1.87-9.67]     | .00043 |
| Donor CD4+ T cells 10^9/L, ATG = 0 | 8:17   | 0.0002 [-0.0001-0.025] | .00044 |
| Donor CD4+ T cells 10^9/L, ATG = 1 | 9:28   | 0.070 [0.0023-2.11]  | .12    |

Note: Donor Foxp3 mRNA level and donor CD4+ T-cell concentration were assessed in donor blood obtained prior to harvest, expressed per litre blood, and analysed as continuous variables. To obtain proportionality, donor Foxp3 mRNA level was included into the model as a time-dependent covariate. A significant interaction was found between donor CD4+ T cells and conditioning with ATG. ATG = 0: conditioning not including ATG. ATG = 1: conditioning including ATG. The results were adjusted for the effect of stage, high-risk cytogenetics, time to achieve CR, and ATG during conditioning. Other factors tested and found to be insignificant included graft nucleated cell dose, donor and recipient age and pretransplant CMV status, alternative donor, documented donor-recipient HLA mismatch, administration of Methotrexate or Granulocyte colony-forming factor, and moderate-severe acute or extensive chronic Graft-versus-host disease.

Abbreviations: ATG, Antithymocyte globulin; CI, confidence interval; E, n: number of patients with post-transplant relapse: number of patients in the group.

However, high donor Foxp3 mRNA level was associated with a four-fold increased cumulative incidence of late relapse compared with low donor Foxp3 mRNA level (Figure 2B, P = .046). These results are compatible with the results of univariate and multivariate Cox proportional hazard regression (Table 2 and 3, respectively). In contrast to the effect of high donor Foxp3 mRNA level, a higher donor CD4+ T-cell concentration was associated with a reduced cumulative incidence of relapse compared with lower donor CD4+ T-cell concentration. However, this donor CD4+ T-cell-associated effect appeared to be restricted to patients who did not receive ATG during conditioning (Figure 2C,D), compatible with the results of the multivariate analysis (Table 3).

3.5 | GvHD and treatment failure

By univariate Cox proportional hazard regression analysis of acute GvHD, there was a non-significant trend of decreased risk of acute GvHD associated with higher donor Foxp3 mRNA level, analysed as a continuous variable, (P = .096, Table 2). The number of cases with chronic GvHD (n = 6) was too low to justify analysis. Treatment failure was defined as relapse (n = 17) or death in continuous remission after HSCT (n = 5). Donor Foxp3 mRNA level and donor CD4+ T-cell concentration were analysed as continuous variables. Multivariate models included recipient age and CMV immune state, conditioning with ATG and total number of nucleated cells in the graft. Test for proportionality showed that the effect of donor Foxp3 mRNA level failed to meet the proportionality assumption with respect to treatment failure (P^2 = .021). Early and late univariate (Table 2) and multivariate (Table 4) analysis showed an increased risk of late treatment failure associated with higher preharvest donor Foxp3 mRNA (multivariate analysis, HR 2.31, P = .017, Table 4). In contrast, higher donor CD4+ T-cell concentration was associated with an overall decreased risk of treatment failure (HR = 0.17, P = .028). This effect was significant when the conditioning did not include ATG (multivariate analysis, HR = 0.014, P = .012). No significant effect of donor CD4+ concentration was observed when ATG was included in the conditioning. Recipient age above median was a marginally significant risk factor (not shown).

Kaplan-Meier estimates of the probability of disease-free survival confirmed that high preharvest donor Foxp3 mRNA level was associated with a significantly decreased probability of late disease-free survival (>363 days, P = .025, Figure 2F), which is equivalent with the increased risk of late treatment failure shown in Tables 2 and 4. No similar effect was observed during the first 363 days after HSCT (P = .75, Figure 2E).

4 | DISCUSSION

Patients with high-risk ALL were transplanted in 1st rather than in 2nd CR (Table S1). This preference was defined by treatment protocols. As a result of this risk-adapted strategy, the effect of leukaemia-associated risk factors and stage per se on post-HSCT relapse could not be estimated. However, the bias introduced by this strategy did not compromise the retrospective analysis of the risk associated with donor Foxp3 mRNA level and CD4+ T-cell concentration, since these values were unknown at the time of HSCT. More importantly, we were unable to adjust for the adverse effect associated with pretransplant presence of MRD, since this information was not available at the time of analysis and could not be obtained retrospectively. However, since Foxp3 expression is a donor associated risk factor, and MRD is associated with the recipient, and none of them were involved in the procedure of donor-recipient matching, MRD cannot be a confounder for the observed association between Foxp3 mRNA and post-HSCT relapse. Yet, the absence of MRD data increases the degree of statistical uncertainty associated with analysis of donor Foxp3 mRNA and CD4+ T cells.

With this precaution, this study strongly suggested that a higher preharvest donor Foxp3 mRNA level was associated with an increased risk of late, but not early, relapse of ALL in patients who received allogeneic myeloablative HSCT with T-replete bone marrow grafts and received GvHD prophylaxis including cyclosporine with or without MTX. Given that a patient at the end of one year was alive and relapse-free, the risk of subsequent relapse was significantly increased when the preharvest donor blood Foxp3 mRNA level was above median.

In contrast, a higher preharvest donor Foxp3 mRNA level was not associated with an increase of early relapse. The absence of an early effect may be due to the administration of cyclosporine, which
was given day −1 – +180 as prophylaxis against GvHD. Treg expansion and suppressor function are highly dependent on an exogenous supply of interleukine-2 (IL-2), mainly produced by activated effector T cells. Cyclosporine is a strong inhibitor of IL-2 production and may consequently abrogate Treg function. When Treg function is abrogated, donor effector T cells may mediate GvL independently of the donor preharvest Treg level. We hypothesise that when cyclosporine prophylaxis is discontinued, increasing IL-2 release may result in expansion of Tregs which may inhibit GvL, and consequently increase the risk of late relapse. It follows that it is only in the absence of cyclosporine that an effect of higher donor Treg concentration may be observed.

This mechanism does not explain why a higher dose of prophylactic cyclosporine may be associated with an increased risk of leukaemia relapse after HSCT. The precise explanation for this requires further investigation.

The association between higher donor Foxp3 mRNA expression and late post-transplant relapse of ALL has not previously been reported. Previous studies have suggested that grafts containing a high Treg concentration may not abrogate GvL. These studies measured Treg concentration within the graft by flow cytometry and comprised AML as well as ALL patients who received PBSC grafts. Several factors may account for this discrepancy. First, assessment by qPCR of Foxp3 mRNA level may not be equivalent.
with flow cytometric assessment of CD4+ T cells isolated from preharvest donor blood was associated with an increased risk of late relapse in patients with ALL who received myeloablative conditioning followed by allogeneic HSCT using bone marrow grafts, suggesting that Tregs may inhibit GvL. In contrast, a higher preharvest donor CD4+ T-cell concentration was associated with a reduced relapse risk, which was abrogated in patients who received ATG during the conditioning.

**4.1 | Conclusion**

A higher level of expression of Foxp3 mRNA in purified CD4+ T cells isolated from preharvest donor blood was associated with an increased risk of late relapse in patients with ALL who received myeloablative conditioning followed by allogeneic HSCT using bone marrow grafts, suggesting that Tregs may inhibit GvL. In contrast, a higher preharvest donor CD4+ T-cell concentration was associated with a reduced relapse risk, which was abrogated in patients who received ATG during the conditioning.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

Data that support the findings of this study are available from the corresponding author upon request. Data pertaining to specific patients are not publicly available.
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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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