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Effects of Rapid Donor T-lymphocyte Engraftment on Disease Control and Graft *versus* Host Disease in Persons Undergoing Reduced-Intensity Conditioning Allotransplantation for Advanced CLL

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Abstract

Eradication of minimal residual disease (MRD) after allotransplantation in persons with chronic lymphocytic leukemia (CLL) is associated with lower rates of relapse. Rapid engraftment of donor lymphocyte elements may contribute to MRD control but it remains unclear if this strategy will benefit patients. Here we report incidence of MRD eradication and graft *versus* host disease (GVHD) in persons with rapid versus later donor T-lymphocyte engraftment after lymphodepleting chemotherapy and reduced intensity conditioning (RIC) allotransplantation. Twenty-seven subjects received lymphodepleting chemotherapy to facilitate donor engraftment followed by fludarabine and cyclophosphamide RIC and a blood cell allograft. MRD was monitored by multicolor flow cytometry post transplantation. Complete donor T-lymphoid (TLC) and myeloid (MC) chimerism were achieved in 25 subjects at a median of 28 days (range 14–60 days) and 21 days (range 14–180 days). Achieving complete donor TLC by day 14 versus day 28 correlated with occurrence of grade-2 acute GVHD (90% [95% confidence interval (CI), 78–100%] versus 35% [95% CI, 16–54%], $P=0.014$) and better control of minimal residual disease in the bone marrow at day 100, median 0% (range, 0–0.1%) versus 8.5% (range, 0–92%; $P=0.016$). Among 11 persons with early donor TLC none had progressive disease (PD) and 7 died of treatment related mortality (TRM). In persons with later development of TLC 8 of 16 had PD and 2 died of TRM. Time to donor myeloid chimerism had no impact on outcomes. Rapid establishment of donor TLC

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results in more complete eradication of early MRD but greater incidence of acute GvHD and TRM in persons with CLL undergoing RIC allotransplantation.

Keywords

Chronic lymphocytic leukemia; allogeneic transplantation; chimerism; reduced intensity conditioning

Introduction

Reduced-intensity conditioning allotransplants are effective in persons with advanced chronic lymphocytic leukemia (CLL). (1–5) This is due to a well described graft-*versus*-leukemia (GvL) reaction that contributes to disease control. (6–8) Despite this, relapse after allotransplantation occurs in 30–40% of persons with CLL and is a major cause of death in this population. (1, 9) Minimal residual disease monitoring has emerged as a technique capable of studying the kinetics of CLL response after allotransplantation and may offer insight into the biology of relapse. (8, 10) These techniques are well validated and can predict individuals with CLL at higher risk for clinical relapse both pre and post allotransplantation. (8, 10–12) Clearance of MRD after transplantation is associated with improved disease-free and overall survival. (5) Thus, MRD monitoring post allotransplantation in CLL offers a unique opportunity to study the disease kinetics quantitatively in the setting of a presumed GvL reaction.

Donor chimerism analysis is common in the context of RIC allotransplantation, where both recipient and donor hematopoietic elements form blood cells and the anti-tumor effect is dependent largely on donor immune function. Most studies support that conversion to full donor chimerism either precedes or is associated with initiation of acute GvHD. (13–16) Retrospective studies support a graft *versus* CLL effect that may be initiated with withdrawal of immune suppression or donor lymphocyte infusion. (8, 10) Furthermore, the CLL3X study conducted by the German CLL Study Group suggests that hampering donor alloimmunity with *in vivo* allograft T-cell depletion leads to increased rates of relapse post transplantation. (5) Thus, the association between donor chimerism, immune function and disease control in CLL suggests that development of rapid donor chimerism could lead to a greater degree of early disease control. Whether therapeutic manipulation of the allotransplant to promote donor alloreactivity is beneficial in persons with CLL is unclear. Given these concerns, it is important to quantitatively measure the effects of donor alloimmune function on disease control and GvHD in order to facilitate further study of relapse prevention.

We have previously demonstrated in clinical and murine studies that adequate suppression of host-versus-graft immunity (rejection) promotes rapid and sustained engraftment of donor hematopoietic elements after a RIC allotransplant. (17–19) In the context of CLL, rapid engraftment of donor T-lymphocytes is associated with improved control of MRD in persons undergoing RIC allotransplantation. (7) We used a pretransplant regimen of etoposide, doxorubicin, vincristine, cyclophosphamide, fludarabine and prednisone with or without rituximab (EPOCH-F±R) to deplete recipient lymphocytes in persons subsequently receiving a RIC allotransplant. We hypothesized that serial administration of EPOCH-F±R would facilitate rapid donor lymphocyte engraftment via depletion of host alloimmunity and result in improved outcomes in persons with hematologic malignancies. (20) We demonstrated that this approach results in good disease control and transplantation outcomes in persons with hematologic malignancies including CLL. (21–22) In the current study we describe the relationship between donor engraftment kinetics, disease control using bone

marrow MRD-flow, and acute GVHD from 27 persons with CLL treated with with EPOCH-F±R and RIC allotransplantation. We hypothesized that individuals with more rapid establishment of full donor TLC would eradicate CLL from the bone marrow to a greater extent but would suffer from a greater degree of GVHD.

Methods

Subjects

Subjects with CLL receiving a RIC allotransplant at the National Cancer Institute (NCI) 1999 to 2010 are included in the analysis. Persons with CLL who had progressed after a fludarabine based regimen and at least one other prior regimen were eligible. Subjects participated with informed consent in the following clinical trials: NCT00410631, NCT00074490, NCT00020592, and NCT00438958. All trials were conducted according the Declaration of Helsinki and approved and monitored by the institutional review board of the National Institutes of Health. Diagnosis of CLL, response and progression were based on the 1996 NCI-Working Group (WG) criteria. (23) Acute and chronic GVHD were diagnosed and graded as described. (24)

The median follow-up of surviving patients is 32 months (range 13 to 99 m). Clinical and laboratory features of the 27 subjects at study-entry are summarized in the Table. Median age was 56 years (range, 37–71 years); 21 were male. Fifteen subjects had high-risk CLL by modified Rai staging and 12 persons had higher risk cytogenetic abnormalities (either deletion of 11q or 17p). (25) The median percent of bone marrow involvement with CLL at enrollment was 45% (range 0–95%). Four had less than stable disease (SD), 9 had SD, and 14 responded (13 PR, 1 CR) to chemotherapy given immediately before study-entry. Rai stage 0–1 at enrollment was the only prognostic clinical variable associated with survival ($P = 0.0006$). Age ($P = 0.54$), time from diagnosis to transplant ($P = 0.66$), donor source (URD versus MRD, $P = 0.61$), Sorror comorbidity index score ($P = 0.38$), and lymph node size >5 cm ($P = 0.80$) were not associated with prolonged survival.

Laboratory Studies

Cytogenetic abnormalities were studied by chromosome analyses and/or fluorescent *in situ* hybridization (FISH) using probe sets for standard cytogenetic abnormalities. (25) Computed tomography (CT) scans were performed at study-entry, after completing EPOCH-F±R, and at day 28, 60, 100, 180, 270 and 360 post-transplant and then yearly or at investigator discretion. Toxicities were graded according to the NCI Clinical Toxicity Criteria versions 2.0 or 3.0. Lymphocyte subsets were quantified at study-entry, at day 17 of each EPOCH-F±R cycle and on the day of transplant. Chimerism analyses were done using the variable number tandem repeats–polymerase chain reaction method in a CLIA–certified laboratory at the Blood Center of Southeastern Wisconsin or at the NCI Department of Pathology. Chimerism was determined at day 14, 28, 56, 100, and 365 post transplantation on blood mononuclear cells enriched for myeloid (CD15⁺ or CD33⁺) or T-lymphoid (CD3⁺) subsets. Myeloid or lymphoid cell subset enrichment was by positive selection using magnetic beads (Miltenyi, Inc., Auburn, CA) or a rosette technique (Stem Cell Technologies, Inc., Vancouver, Canada) as per the manufacturer guidelines. Complete donor chimerism was declared when 95% of analyzed cells were of donor origin.

Flow Cytometric Immunophenotyping

Minimal residual disease burden was quantitated on the bone marrow 28, 60, 100, 180, 270, and 360 days post transplantation as follows: Specimens were stained within 12 h of collection with a panel of antibodies. Erythrocytes were lysed by incubating with lysing solution (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 10 min at room temperature

(maintained at 21–23°C) at a ratio of 1:9 (volume of sample: volume of lysing solution). Specimens were then washed with phosphate buffered saline (PBS) to remove cytophilic antibodies before determining cell number. Specimens were stained for 30 min at room temperature (maintained at 21–23°C) with a cocktail of four to 8 antibodies (antibody concentrations used per manufacturer's recommendations) according to Clinical Laboratory Standards Institute (CLSI) document H43-A recommendations.(26–27) Panels included antibodies against kappa, lambda, CD3, CD4, CD5, CD8, CD11c, CD14, CD16, CD19, CD20, CD22, CD45, and CD56 prior to September 2009 when CD43, CD79b and CD81 were added. All cells were fixed in 1.0% paraformaldehyde post staining and stored at 4°C for up to 12 h before acquisition. Specimens were acquired with 6-parameter 4-color FC on the FACSCalibur (BD Biosciences) using CellQuest Pro software prior to September 2009, and from September 2009 onward, with 10-parameter 8-color FC on the FACSCanto (BD Biosciences) using DIVA software (sensitivity of fluorescent detectors monitored using standard beads according to the manufacturer's recommendations). At least 5,000 lymphocytes were acquired per tube, and 1,000,000 total events were acquired in cases of minimal residual disease. Data (collected in list mode) were analyzed with FCS Express (De Novo Software).

For analysis, cell populations were gated according to characteristic forward and side scatter properties, in conjunction with antigen back-gating. In addition, analysis gates were drawn on CD19 positive B-cells. CD19 and CD5 positive B-cells were evaluated for the typical CLL immunophenotype (i.e. CD20 dim, CD22 dim, CD45 dim, CD79b dim, CD43 bright and CD81 dim) and light chain restriction. Normal lymphoid cells within specimens were used as internal positive and negative controls (e.g. T-cells served as negative controls for B-cell directed antibodies). Bright expression was defined as higher than normal B-cells while dim expression was defined as lower than that observed in normal B-cells, in concordance with the 1997 U.S- Canadian consensus guidelines.(28) Sensitivity of detection was validated by serial dilutions to be 10^{-4} .

Therapy

Subjects with $0.2 \times 10^9/\text{L}$ CD3+CD4+ lymphocytes received EPOCH-F±R chemotherapy every 21 days until CD3+CD4+ lymphocytes were $<0.2 \times 10^9/\text{L}$, had toxicity grade-3 or received a maximum of 3 cycles of EPOCH-F±R. (20–21) In 25 patients EPOCH-F±R consisted of doxorubicin 10 mg/m²/day, vincristine 0.4 mg/m²/day, etoposide 50 mg/m²/day continuous IV infusion days 1–4, fludarabine 25 mg/m²/day IV days 1–4, cyclophosphamide 750 mg/m² IV on day 5, rituximab 375 mg/m² IV on day 1 (n=22), and prednisone 60 mg/m²/day PO days 1–5. Two patients were treated with reduced doses of cyclophosphamide (600 mg/m²), prednisone (60 mg/m²/day days 1–4), fludarabine (25 mg/m²/day days 1–3), and were administered etoposide, vincristine, and doxorubicin as above only on days 1–3. Subjects with stable disease advanced to a RIC allotransplant. Pre transplantation conditioning was with fludarabine, 30 mg/m²/day and cyclophosphamide, 1.2 g/m²/day (N=25) or 0.3 g/m²/day (N=2) on days -6, -5, -4, and -3. Donors were an HLA-matched relative (N=17), an HLA-matched unrelated person (N=8) or a one HLA-antigen mismatched unrelated donor (N=2). Grafts were filgrastim mobilized blood cells. Mean CD34+ and CD3+ doses were $7.7 \times 10^6/\text{kg}$ (range, $3.3\text{--}17.8 \times 10^6/\text{kg}$) and $3.0 \times 10^8/\text{kg}$ (range, $0.9\text{--}9.9 \times 10^8/\text{kg}$). Subjects received filgrastim until granulocytes were $>0.5 \times 10^9/\text{L}$. GVHD prevention was cyclosporine or tacrolimus based. Three subjects also received alemtuzumab.

Statistics

Actuarial analyses of survival were performed using the Kaplan-Meier method. Survival times were censored if the subject was alive at last follow-up. Cumulative incidence curves

of progression and TRM were constructed in a competing risk framework. (29) Analyses of progression according to whether complete lymphoid or myeloid chimerism was attained by day 14 or 28+ were also performed. Statistical significance of the correlation between two dichotomous variables was determined by Fisher exact-test. An exact Wilcoxon rank-sum test was used to determine statistical significance of the difference in continuous parameters between groups. All p-values are two-sided and unadjusted for multiple comparisons. Only comparisons where $p < 0.01$ are considered statistically significant because of the number of comparisons performed p-values between 0.01 and < 0.05 are referred to as trends.

Results

Response and Adverse Effects to EPOCH-F±R

No subject had $< 0.2 \times 10^9/L$ CD3+CD4+ lymphocytes at study-entry. Subjects received a median of 2 cycles of EPOCH-F±R (range, 1–3). Thirteen responded to treatment (1 CR and 12 PRs). The remaining 14 patients had SD. There was no difference in the number of responding patients in the early (6/11 responded) versus later (7/16 responded) full donor TLC groups ($P=0.70$). Incidence of grade-3 non-hematologic adverse events was 37% (95% CI, 18–55%) including infection ($N=7$) and neutropenic fever ($N=6$). Grade-4 non-hematologic adverse events included cerebro-vascular accident ($N=1$) and pulmonary embolism ($N=1$). There were 8 episodes of grade-4 thrombocytopenia and 17 episodes of grade-4 neutropenia. Effect of EPOCH-F±R on lymphocyte subsets is illustrated in Figure 1. Median CD4+ lymphocyte level before EPOCH-F±R was $0.44 \times 10^9/L$ (range, 0.008–1.8) compared to $0.97 \times 10^9/L$ (range, 0.007–0.55; $P=0.0002$) after therapy.

Engraftment, Graft versus Host Disease, and Minimal Residual Disease Kinetics

The median days from transplantation to a granulocyte count $1.0 \times 10^9/L$ was 10 days (range, 8–13 days). Complete donor lymphoid and myeloid chimerism were achieved in 25 subjects at a median of 28 days (range, 14–60 days) and 21 days (range, 14–180 days). The three individuals in this cohort that did not develop full donor T-lymphocyte chimerism died rapidly of disease progression ($N=1$) or infection ($N=2$). We then analyzed the effects of engraftment before versus during or after the median. Achieving complete donor lymphoid chimerism by day 14 versus day 28 correlated with occurrence of grade-2 acute (90% [95% CI, 78–100%] versus 35% [95% CI, 16–54%], $P=0.014$) but not chronic GVHD (50% [95% CI, 30–70%] versus 41% [95% CI, 32–60%], $P=0.71$). There was no correlation between interval to complete donor myeloid chimerism and acute or chronic GVHD. Fourteen patients developed grade 2+ acute GVHD and 12 patients developed chronic GVHD. Patients with acute GVHD were not more likely to have negative MRD ($P=0.7$). Six of the 8 patients surviving in remission have chronic GVHD.

Earlier achievement of complete donor lymphoid chimerism correlated with fewer bone marrow CLL cells at day 100 (median 0% [range, 0–0.1%] versus 8.5% [range, 0–92%]; $P=0.016$; Figure 2). There was no association of early engraftment with survival ($P=0.077$). Sixteen patients had MRD flow performed at 180 days post transplant. Twelve patients were MRD negative at this time and of these patients only one had PD.

Transplantation Outcomes

The best response post transplantation was a CR in 14 subjects, PR in 5, SD in 7 and PD in 1. Eight of the 14 subjects achieving CR are alive in remission. Four died of treatment-related mortality (TRM) and 2 relapsed. TRM at 1 and 2 years was 21% (95% CI, 5–37%) and 30% (95% CI, 12–58%). Fourteen subjects had no evidence of CLL posttransplant, all of whom achieved complete donor lymphoid chimerism by day 14 posttransplant. Among 11 persons with early establishment of donor TLC none had progressive disease (PD) and 7

died of treatment related mortality (TRM) (Figure 3). In persons with later development of TLC 8 of 16 had PD and 2 died of TRM. All TRMs resulted from infectious causes. Only one infectious death occurred outside the setting of immune suppression for GVHD. There was no correlation between early myeloid chimerism and CLL progression. Cumulative incidence of progression at 3 years was 30% (95% CI, 12–58%). Three year PFS is 56% (95% CI, 32–75%) and 3 year survival is 57% (95% CI, 32–76%). Median PFS was 3.8 years and survival was 6 years (Figure 4).

Donor Cell Infusions

Six subjects received donor lymphocyte infusions postransplant, 3 for progressive disease, 2 for less than a complete donor lymphoid or myeloid chimerism and 1 for residual CLL detected by flow cytometry.

Discussion

In the current study we report the effects of donor engraftment on MRD eradication and GVHD in persons with CLL. The underlying transplantation program is designed to promote rapid engraftment of donor hematopoietic elements via host lymphodepletion with EPOCH-F±R chemotherapy. We have previously demonstrated in persons with non-Hodgkin lymphoma that this approach results in high rates of early complete donor TLC and resembles the engraftment kinetics seen in conventional myeloablative transplants.(17, 20) Here we examined the effects of this regimen in persons with CLL. We sought to compare alloreactivity between individuals within the group with rapid versus later conversion to full donor TLC in terms of GVHD and GVL using MRD-flow.

Multiple studies have demonstrated that prolonged mixed chimerism or slow engraftment results in increased rates of relapse. (2, 7, 30–31) Early reports of the association between mixed chimerism and relapse are complicated by lack of lineage specific analysis. More recent publications describe a relationship between the development of full donor T-lymphocyte specific chimerism with relapse-free survival suggesting that this test is at least a partial surrogate for the degree of GVL. (31–32) The mechanism underlying the relationship between mixed chimerism and relapse is unclear but may be related to induction of donor-recipient tolerance. (33–34) Few patients in this study developed stable mixed chimerism, thus, we could not evaluate between these two groups. A more common clinical scenario is the patient with delayed engraftment, thus, we evaluated the rate of conversion to full donor TLC. This analysis is supported by previous evidence that demonstrates a relationship between rapid engraftment and disease control in persons with CLL. (7) The outcomes in this cohort confirm this association but also define rapid engraftment as a risk factor for acute GVHD. No benefit in survival was seen in persons with rapid conversion to donor TLC. These findings would suggest that methods designed to promote rapid donor engraftment may not result in improved survival without improved prophylaxis of acute GVHD. Furthermore, monitoring of early MRD and donor TLC may be a useful tool to determine which persons are at high risk for relapse or acute GVHD. These data could be useful in design of future clinical trials of preemptive therapy for either of these disorders.

The use of MRD-flow after allogeneic transplantation has gained wider acceptance in clinical practice; however, questions remain regarding the utility of this method in predicting outcomes. Several previous reports have described the relationship between MRD response and clinical events in persons with CLL. Ritgen and colleagues demonstrated only 1 relapse in 12 persons with a negative MRD study after measures to promote alloreactivity including withdrawal of immune suppression or DLI compared to 3 relapses in 4 persons with positive MRD. (10) In this study both MRD-flow and MRD-PCR were used with similar results in terms of disease burden. A causative effect of promotion of GVL with MRD control was also

noted by Farina and colleagues using a nested PCR based method for MRD detection. (8) These reports suggest that MRD is a useful tool to monitor the GvL reaction post transplantation. MRD monitoring may also provide prognostic information. Several studies have noted a relationship between MRD negativity remotely after allotransplantation and EFS and OS. (3, 5, 35) Our results support this phenomenon: Only 1 patient of 12 with a negative MRD-flow study at 180 days post transplantation developed progressive disease. Similarly, no patient progressed among 11 patients with rapid conversion to fully donor TLC. This analysis is complicated by high rate of acute GvHD and TRM in this population—some patients may have expired before developing relapse. Limited numbers of persons included in this study is also a major limitation to broader application of these findings. Nevertheless, the similarity of our data to the broader CLL experience supports our application of MRD-flow and chimerism analysis in predicting outcomes.

In summary, these data support the notion that both a possible graft-*versus*-leukemia effect and rapid complete donor lymphoid chimerism are important in early control of CLL post transplantation. Furthermore, testing of lineage specific donor chimerism after transplantation may be of benefit in CLL. Efforts to improve donor engraftment without improved control of GvHD may not improve survival due to increased TRM. These findings may be important for developing future strategies for improving CLL control after allotransplantation. Further trials including larger numbers of persons are needed to confirm these results.

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Figure 1. Bar plot showing paired 25th, median, and 75th percentiles of selected lymphocyte subsets at study-entry (shaded) and after (open) EPOCH-F±R.

Figure 2.

Degree of bone marrow involvement of CLL in subjects with day 14 (solid line) versus day 28 (dashed line) development of complete CD4+ T-lymphocyte donor chimerism. Bone marrow CLL cells were assessed by multi-color flow cytometry at study-entry (E) and days +28, +60, +100, +180 post transplantation.

Figure 3. Probability of progression of subjects with day 14 (dashed line) *versus* day 28 (solid line) development of complete CD4+ T-lymphocyte donor chimerism.

Figure 4.
Overall survival.

Table 1

Table Patient characteristics at enrollment

Age (y)	56 (37–71)
Male	21
Sorrow comorbidity score	
0–1	15
2–3	9
4–5	3
Number prior lines of therapy	2 (2–4)
Prior alemtuzumab	5
Fludarabine refractory	6
Unresponsive to prior chemotherapy	13
Modified Rai stage	
Low	1
Intermediate	11
High	15
Cytogenetics	
Normal	8
Del (13q)	2
Ts (12)	3
Del (11q)	7
Del (17p)	5
Unknown	2
Median bone marrow CLL involvement	45% (0–95%)
Lymph node > 5 cm	6
Splenomegaly	7
DLBCL transformation	2
Mo from diagnosis to allotransplant	58 (17–205)