Donor lymphocyte count and thymic activity predict lymphocyte recovery and outcomes after matched-sibling hematopoietic stem cell transplant

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ABSTRACT

Delayed immune recovery is a characteristic feature of allogeneic hematopoietic stem cell transplantation in adult recipients. Although recipient thymic T-cell neogenesis contributes to T-cell regeneration after transplantation, thymic recovery in the transplant recipient decreases with increasing age, and is diminished by intensive preconditioning regimens and graft-*versus*-host disease. In adult recipients, most events that determine transplant success or failure occur during the period when the majority of circulating T cells is derived from the donor's post thymic T-cell repertoire. As a result, the make-up of the donor lymphocyte compartment may strongly influence immune recovery and transplant outcomes. The aim of this study was to examine donor lymphocyte counts in a series of patients undergoing an allogeneic hematopoietic stem cell transplant to identify the potential contribution of donor regulatory and conventional T lymphocyte populations to immune recovery and transplant outcomes. We examined donor lymphocyte subset counts in relation to post-transplant lymphocyte recovery and transplant events in 220 consecutive myeloablative, T-cell-depleted, HLA-identical sibling hematopoietic stem cell transplant recipients with hematologic malignancies. In a multivariate analysis, absolute numbers of donor $CD4^+$ recent thymic emigrants were associated with overall survival (P=0.032). The donors' absolute lymphocyte count and thymic production of regulatory T cells were both associated with extensive chronic graft-*versus*-host disease (P=0.002 and P=0.022, respectively). In conclusion, these results identify donor immune characteristics that are associated with lymphocyte recovery, extensive chronic graft-*versus*-host disease, and survival in the recipient following allogeneic hematopoietic stem cell transplantation. The study reported here was performed using peripheral blood samples drawn from donors and patients enrolled in the ClinicalTrials.gov-registered trials *NCT00001623*, *NCT00001873*, *NCT00353860*, *NCT00066300*, *NCT00079391*, and *NCT00398346*.

Introduction

Immune recovery is frequently delayed after allogeneic hematopoietic stem cell transplantation (HSCT) in adult recipients. Restoration of adaptive T-cell-mediated immune responses and recovery of regulatory T cells (Treg) is required for re-establishment of immune competence after HSCT.[1-4] T-cell regeneration after HSCT is initially derived from the donor's circulating post-thymic lymphocytes, but later regeneration of a naïve T-cell repertoire is achieved through thymic T-cell neogenesis.[5] However, thymic recovery in the transplant recipient decreases with increasing age, and is diminished by intensive preconditioning regimens and graft-*versus*-host disease (GvHD).[6,7] In adult recipients, most events that determine transplant success or failure occur during the period when the majority of circulating T cells is derived from the donor's post-thymic T-cell repertoire.[8,9] Transplanted post-thymic lymphocytes contain a broad repertoire of naïve and memory T cells which include alloreactive T cells responsible for GvHD, and regulatory T cells which can modify GvHD incidence and severity.[10,11] As a result, the make-up of the donor lymphocyte compartment may strongly influence immune recovery in the recipient, and consequently may influence transplant outcomes. Characteristics intrinsic to the donor such as HLA sensitization, viral exposure, the type of killer immunoglobulin-like receptors (KIR) on natural killer cells, and Treg frequencies are already known to influence transplant outcome.[12-16] However, the contribution of mature donor lymphocytes infused at the time of transplantation has not been fully defined.[17] To explore the influence of immunological characteristics of the donor further, we studied donor lymphocyte counts in a series of patients undergoing HSCT to identify the potential contribution of donor regulatory and conventional T lymphocyte populations to immune recovery and transplant outcomes.

Design and Methods

Patients

Between 1997 and 2011, 220 consecutive patients with a hematologic malignancy underwent a myeloablative T-cell-depleted (TCD) HSCT from an HLA-identical sibling on National Heart, Lung and

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Blood Institute institutional review board–approved protocols. Patients and donors provided written informed consent before enrolling in the transplantation protocols. The clinical characteristics of these 220 patients and their respective donors are presented in Table 1.

Transplant approach

All patients received a TCD granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood stem cell transplant. G-CSF was administered to all donors at a targeted dose of 10–12 μg/kg of body weight subcutaneously for 5 consecutive days prior to collection of the mobilized cells. The conditioning regimen consisted of 1200 or 1360 cGy total body irradiation, cyclophosphamide (120 mg/kg over 2 days), with or without fludarabine (125 mg/m^2 over 5 days). T cells were depleted from the stem cell transplant products by selection of CD34$^+$ cells using either the CellPro system (CellPro Inc., Bothel, WA, USA), Isolex system (Nexell Therapeutics Inc., Irvine, CA, USA), or the Miltenyi CliniMacs system (MiltenyiBiotec Inc., Auburn, CA, USA). On the day of HSCT all patients received this enriched CD34$^+$ stem cell product with a target dose of 5x10^6 CD34$^+$ cells/kg, accompanied by a predetermined, protocol-specific dose of 0.2-1x10^5 T cells/kg which was obtained by supplementing the final product with T cells from the original unmanipulated peripheral blood stem cell component. All patients received low-dose cyclosporine A (target plasma level, 100-200 ng/mL), starting on day -4 per protocol. In the absence of significant acute GvHD (grade >1), a donor lymphocyte infusion (1x10^7 CD3$^+$ cells/kg) was administered between day 45 and 100 after transplantation. Standard prophylaxis against infection included fluconazole and trimethoprim/sulfamethoxazole, which were administered for at least 6 months after transplantation, and weekly surveillance was performed for cytomegalovirus antigenemia as described previously.[18,19]

Cell isolation

After written informed consent, and prior to G-CSF mobilization, apheresis was performed in all donors. Peripheral blood samples were collected from patients 30 days and 1 year after HSCT. Peripheral blood mononuclear cells were separated using Ficoll-Hypaque density gradient centrifugation (Organon Teknika, Durham, NC, USA) and subsequently frozen in RPMI 1640 (LifeTechnologies, Gaithersburg, MD, USA) supplemented with 20% heat-inactivated fetal calf serum and 10% dimethyl sulfoxide according to standard protocols. Before use, frozen cells were thawed, washed, and rested overnight in RPMI supplemented with 10% pooled AB serum (Sigma Chemical, St Louis, MO, USA).

Reagents for flow cytometry

The following monoclonal antibodies and fluorescent dyes were used: α-CD27-Cy5-phycoerythrin (Cy5PE; clone 1A4CD27) (from Beckman Coulter); α-HLA-DR peridinin-chlorophyll-protein complex (PerCP) Cy5.5 (clone L243), α-CD4-V500 (clone PRA-T4), α-CD8-allophycocyanin (APC)-H7 or -Cy7APC (clone SK1), α-CD25- Cy5-PE (clone M-A251), α-CD127 Cy7-PE (clone hIL-7R-M21), α-CD16-fluorescein isothiocyanate (FITC; clone 3G8), and α-CD56-PE (clone B159) (all from Becton Dickinson (BD); α-FOXP3-alexa fluor 647 (clone 236A/E7) and α-CD3-eFuor605 (clone OKT3) (from eBioscience); α-CD31-APC (clone 9G11) (from R and D Systems); α-Helios-PE (clone 22F6) and α-TCRγ/δ-APC (clone B1) (from Biolegend); and α-CD31-FITC (clone MBC 78.2), α-CD14-pacific blue (PB; clone TuK4), α-CD19-PB (clone SJ25-C1), α-CD45RA- PE-Texas Red (ECD; clone MEM-56), and the violet dead cell-exclusion dye (ViViD) (all from Invitrogen).

Enumeration of lymphocyte subsets

Absolute lymphocyte counts (ALC) were determined by an automated hematology analyzer in the National Institutes of Health clinical laboratory on donor peripheral blood samples collected at presentation, and on patients' samples 30 days, 100 days, and 1 year after the transplant. In preparation for flow cytometric analysis, cryopreserved samples from both patients and donors were thawed and rested overnight. Cells were stained essentially as previously described.[20,21] Data were acquired on a special order LSR Fortessa (BD) flow cytometer and analyzed using FlowJo (Treestar, version 8.6.6). T-cell subsets were defined as follows: naïve, CD27$^+$CD45RO$^-$; central memory, CD27$^+$CD45RO$^+$; effector memory, CD27$^-$CD45RO$^+$; effector, CD27$^-$CD45RO$^-$; and recent thymic emigrants (RTE), CD45RA$^+$CD31$^+$.[22,23] Treg were defined as CD4$^+$FOXP3$^+$ T cells, and then further subdivided by the Helios marker into induced (Helios$^-$) and natural (Helios$^+$) Treg (Figure 1).[24,25] Finally, the thymic production of nTreg was calculated by comparing the number of nTreg expressing the characteristics of RTE (CD45RA$^+$, CD31$^+$) with the entire proportion of RTE CD4$^+$ T cells (RTE nTreg count/percent CD4$^+$ RTE). B cells were identified as CD19$^+$ lymphocytes, and natural killer cell subsets were identified as CD3$^-$CD56$^+$ lymphocytes.

Definition of clinical terms

As regards relapse risk, patients with acute leukemia in first complete remission, those with myelodysplastic syndrome with an intermediate-I International Prognostic Scoring System score, and patients with chronic myelogenous leukemia in the chronic

Table 1. Characteristics of the donors and patients.

Donors	Patients
Median age 35 years (range, 6 - 68)	35 years (range, 8-68)
Sex Male 109 (49.5%) Female 111 (50.5%)	 Male 111 (50.5%) Female 109 (49.5%)
Race Asian Black Latino White	 39 (17.7%) 19 (8.6%) 113 (51.4%) 49 (22.3%)
Disease Acute lymphoblastic leukemia Acute myelogenous leukemia Chronic myelogenous leukemia Myelodysplastic syndrome Non-Hodgkins leukemia	 42 (19.1%) 67 (30.4%) 67 (30.4%) 30 (13.6%) 14 (6.4%)
Median CD34$^+$ dose	5.58x10^6 cells/kg (range, 2.3-15.9)
Median CD3$^+$ dose	0.5x10^5 cells/kg (range, 0.2-1)
Received donor lymphocyte infusion (between days 45-100)	155 (70%)
Acute graft-*versus*-host disease Grades II-IV Grades III-IV Missing	 65 (31.1%) 25 (12.0%) 11 (5%)
Chronic graft-*versus*-host disease Limited Extensive	 29 (13.2%) 62 (28.2%)
Relapse	73 (33.2%)
Survival	106 (48.2%)
Non-relapse mortality	40 (18.2%)

phase were categorized as standard risk. Patients with more advanced disease (second complete remission or beyond), primary refractory or relapsed disease, and secondary acute myelogenous leukemia were categorized as having a high risk of relapse. Enrollment on protocols began before the National Institutes of Health consensus criteria for chronic GvHD were made available in 2005 and chronic GvHD severity was, therefore, recorded as either limited or extensive. Limited chronic GvHD was defined as localized skin involvement resembling scleroderma with or without liver involvement but no other organ involved. Extensive chronic GvHD was defined as generalized skin or multiple organ involvement.[26] Overall survival was calculated from the interval between the date of transplantation and death, or last follow-up visit. Relapsed disease for acute leukemia and myelodysplastic syndrome was determined from morphological or cytogenetic evidence, either in peripheral blood or in bone marrow. For chronic myelogenous leukemia, relapse was defined by hematologic, cytogenetic or molecular evidence of recurrence. Non-relapse mortality was defined as the time from transplantation until death from an infectious cause, graft failure, GvHD, or any other cause unrelated to disease.

Statistical analysis

Baseline characteristics for the patients were summarized using counts and percents for discrete variables, and means, medians, standard deviations, and ranges for continuous variables. Log transformation was performed on non-normally distributed variables for analysis. Cox proportional hazard models were used to analyze the effects of baseline risk factors on the cumulative incidence of GvHD, relapse, and overall survival. For GvHD, relapse, and overall survival, patients who were alive at the end of the study were treated as censored. Survival was measured to the last contact date or death. For multivariate Cox models, only the risk factors which had significant effects on the corresponding event time were kept in the model. Both continuous and dichotomized covariates were used in survival analysis when a risk factor was a continuous variable. When a dichotomized covariate was used, the median of the continuous variable was used for the threshold value. Effects of the risk factors were evaluated using log-rank tests and two-sided t-tests with a level of statistical significance set at 0.05. Statistical analyses were performed with SPSS 15.0 (IBM SPSS, New York, USA), S-plus 8 statistical package (TIBCO Software Inc., Palo Alto, CA, USA), and Prism 5 (GraphPad Software, San Diego, CA, USA) software.

Results

Patients' characteristics

The patients' characteristics are shown in Table 1. The median age at HSCT for the 111 males and 109 females was 35 years (range, 8 – 68). The indications for HSCT included acute myeloid leukemia (n=67), myelodysplastic syndrome (n=30), acute lymphocytic leukemia (n=42), non-Hodgkin's leukemia (n=14), and chronic myelogenous leukemia (n=67). During the 14 years covered by the consecutive protocols the frequency of chronic myelogenous leukemia decreased and the frequency of high-risk disease increased. Eighty-one patients received 1360 cGy total body irradiation and 120 mg/kg cyclophosphamide, and the remaining patients received total body irradiation (1200 cGy for most, although 11 patients received 400 cGy because they were aged >55 years), 120 mg/kg cyclophosphamide, and 125 mg/m^2 fludarabine for preconditioning. At HSCT patients received a median of 5.58x10^6/kg CD34$^+$ cells (range, 2.3 – 15.9) accompanied by a median CD3$^+$ cell dose of 0.5x10^5 cells/kg (range, 0.2 –1) pre-determined by protocol.

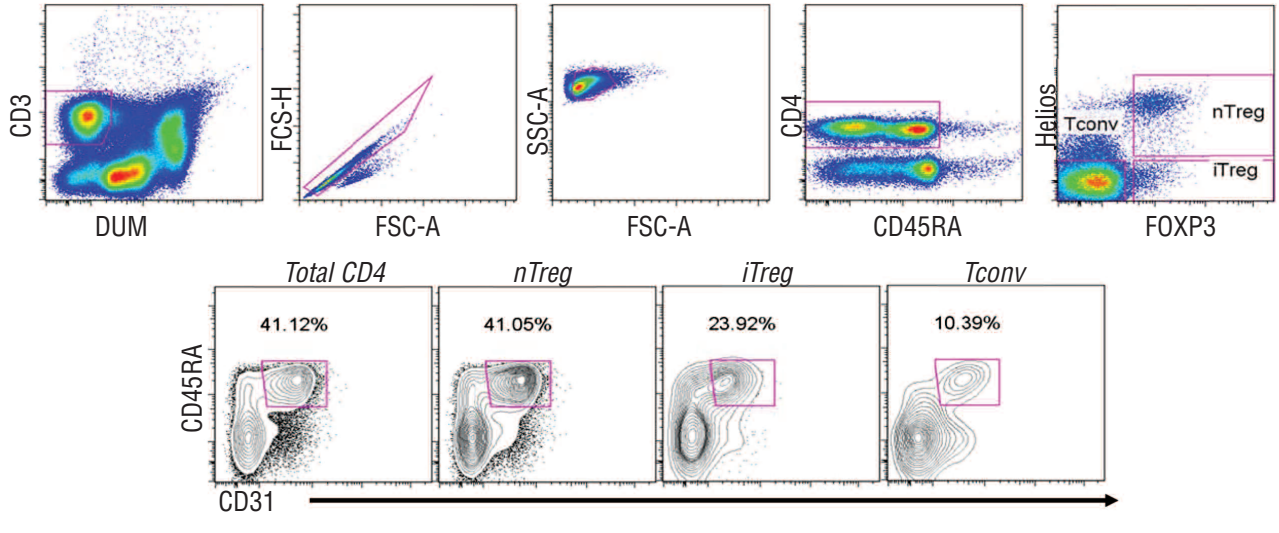

Figure 1. Flow cytometric analysis of CD4$^+$ recent thymic emigrants (RTE) and regulatory T cells (Treg). Donor peripheral blood mononuclear cells were stained, acquired, and analyzed as outlined in the *Design and Methods* section. To identify CD4 subsets, live T cells were discriminated from dead cells, monocytes, and B cells in a dump (CD14$^+$ and CD19$^+$ cells, and dead cells staining brightly with ViViD) *versus* a CD3 bivariate plot. Next, singlets were identified in a forward scatter-area (FSC-A) *versus* –height (FSC-H) plot, and intact cells in a forward scatter-area *versus* side scatter-area (SSC-A) plot. CD4 cells were next gated on a CD45RA *versus* a CD4 plot, which also served to eliminate fluorochrome, in this instance, TxPE, aggregates. Within the CD4 population we identified (i) rRTE as cells co-expressing both CD31 and CD45RA, and (ii) natural and induced Treg (nTreg and iTreg, respectively) and conventional T cells (Tconv) in a FOXP3 *versus* Helios plot. Within the Treg and Tconv subsets, RTE were identified again using a CD31 *versus* CD45RA bivariate plot.

Graft-versus-host disease, relapse, non-relapse mortality, and overall survival

Sixty-five patients (31.1%) developed grade II-IV acute GvHD, and 25 patients (12.0%) developed severe acute GvHD (grade III or IV). Sixty-two patients (28.2%) developed extensive chronic GvHD, with the 3-year probability of this complication being 35.6% (95% CI, 26% to 45.2%). Seventy-three patients experienced relapse of their hematologic disease after HSCT (3-year probability 32.5%; 95% CI, 25.4% to 38.9%). Probabilities of overall survival and non-relapse mortality at 3 years were 44.7% (95% CI, 37.6% to 51%) and 19.5% (95% CI, 13.5% to 25.1%), respectively.

Natural killer and T-cell reconstitution

We compared lymphocyte counts in 220 donors with lymphocyte counts in 220, 201, and 141 survivors on day 30, day 100, and 1 year after HSCT, respectively. Lymphocyte phenotyping by FACS analysis was also performed on all available samples which included 139 donor leukopheresed products, and peripheral blood samples collected from 47, 75, and 87 recipients on day 30, day 100, and 1 year after HSCT, respectively. In donors, the ALC correlated closely with the $CD3^+$ T-cell count (R^2 = 0.87, P<0.001), and with the ALC in the recipient after HSCT (Figure 2A). A donor ALC above the median (>2,110 cells/μL) correlated with day 30 post-HSCT higher natural killer cell count (mean 452 *versus* 305 cells/μL, P=0.04) and $CD3^+$ T-cell count (mean 1171 *versus* 713 cells/μL, P=0.03). A higher than median donor ALC also predicted a higher T-cell count at 1 year after HSCT (mean 1388 *versus* 898 cells/μL, P<0.001). Within the T-cell compartment, patients whose donors exhibited higher than the median $CD3^+$ T-cell counts (>1,645 cells/μL) had higher $CD8^+$ T-cell counts 30 days after HSCT (P=0.01), and higher $CD4^+$ and $CD8^+$ T-cell counts 1 year after HSCT (P<0.01). In comparison, there was no impact of donor ALC on long-term B-cell or natural killer cell recovery.

Donor absolute lymphocyte count and transplant outcome

In univariate analysis, a donor ALC above the median (2,110 /μL) was associated with a decreased incidence of extensive chronic GvHD occurring after HSCT (n=220, HR=2.44, log-rank P<0.001, Figure 2B). In a multivariate Cox regression analysis comparing donor ALC, patient's age, T-cell dose at the time of HSCT, administration of a donor lymphocyte infusion after HSCT, and donor-recipient sex mismatch (female donor to male recipient *versus* other combinations), only donor ALC (HR=0.422, 95% CI 0.244-0.731; P=0.002) and donor-recipient sex mismatch (HR=1.857, 95% CI 1.043-3.304; P=0.035) remained signif-

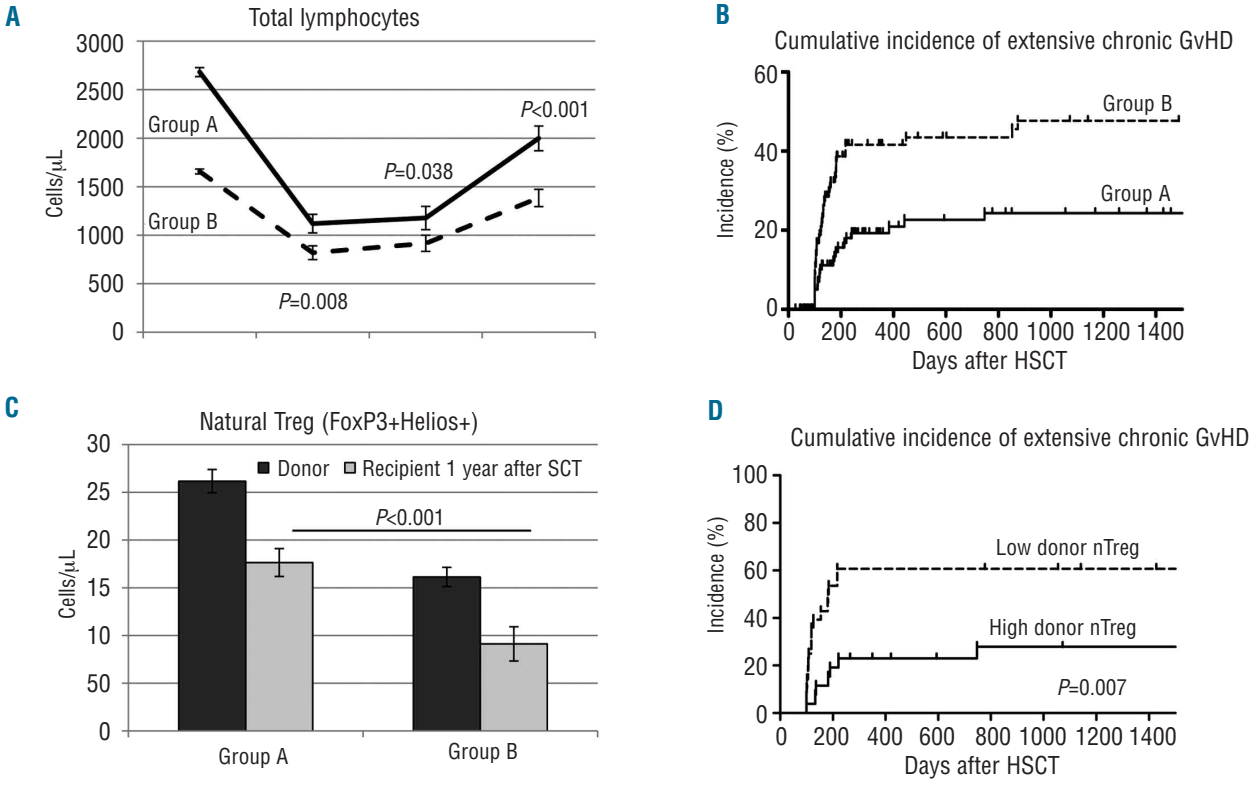

Figure 2. Lymphocyte reconstitution and cumulative incidence of extensive chronic GvHD. The reconstitution of peripheral blood lymphocytes was assessed at 30 days, 100 days, and 1 year after HSCT. Patients were categorized into two groups on the basis of donor ALC. Group A (solid line) consists of patients whose donors had more than the median ALC (>2,110 cells/μL), and group B (dashed line) consists of patients whose donors had less than the median ALC. (A) Mean (+ SEM) ALC is shown for the donors, and for the patients at 30 days, 100 days, and 1 year after HSCT. (B) Patients whose donors had more than the median ALC had a low cumulative incidence of extensive chronic GvHD. (C) The reconstitution of natural Treg (nTreg, $Foxp3^+Helios^+$) was assessed at 1 year after HSCT and compared to the mean donor nTreg count. (D) Cumulative incidence of extensive chronic GvHD by donor nTreg count in a matched analysis performed on donor and 1-year patient samples from 55 donor/patient pairs. *P* values were calculated using a two-sided t-test or a log-rank test.

icant independent risk factors for extensive chronic GvHD. In univariate analysis, donor ALC greater than 2,110/μL also predicted for a lower non-relapse mortality rate (HR 0.51, 95% CI 0.27-0.96; *P*=0.037). In multivariate Cox regression analysis including patient's age, sex, malignancy, risk of relapse (standard *versus* high), administration of a donor leukocyte infusion, and incidence of acute GvHD (grades II – IV), both donor ALC (HR 0.47, 95% CI 0.26-0.90; *P*=0.02) and patient's age (RR 1.03, 95% CI 1.01-1.06; *P*=0.01) remained significant for predicting non-relapse mortality, consistent with the association of ALC and age.

Donor natural regulatory T cells and chronic graft-versus-host disease

We next evaluated the role of specific donor lymphocyte subsets in predicting transplant outcomes. Patients whose donors had more than the median ALC had higher nTreg counts at 1 year after HSCT (Figure 2C). Although a higher incidence of chronic GvHD was noted in patients who returned to the clinic 1 year after HSCT, in a matched analysis performed on samples from 55 donors and their recipients 1-year post-HSCT, we found that the patients whose donors had a higher than median count of nTreg ($CD4^+FOXP3^+Helios^+$) experienced less extensive chronic GvHD (HR 0.333, 95% CI 0.143-0.732; log-rank *P*=0.007, Figure 2D). However, among all donors nTreg counts did not correlate with total $CD4^+$ T-cell thymic output, indicating that thymic production of nTreg varied independently of other $CD4^+$ T cells. To compare thymic production of nTreg, we calculated the ratio of RTE nTreg counts to overall $CD4^+$ T-cell thymic output (RTE nTreg count/percent CD4 RTE). Patients whose donors had more than the median thymic production of nTreg had a significantly lower probability of developing extensive chronic GvHD (HR=0.389, 95% CI 0.198-0.766; log-rank *P*=0.005, Figure 3). In multivariate analysis that included patient's age, T-cell dose received at the time of HSCT, donor-recipient sex mismatch (female donor to male recipient *versus* other combinations), type of malignancy, and administration of a donor lymphocyte infusion after HSCT, thymic production of nTreg (HR=0.597, 95% CI 0.384-0.929; *P*=0.022) and donor-recipient sex mismatch (HR=2.222, 95% CI 1.162 – 4.247; *P*=0.016) remained significant for predicting an increased risk of extensive chronic GvHD.

Donor lymphocyte subsets and overall survival

In univariate Cox regression models, only $CD4^+$ RTE counts were significantly associated with HSCT outcomes. Higher $CD4^+$ RTE counts were associated with better overall survival of patients after HSCT (HR=0.822, 95% CI 0.701-0.964; log-rank *P*=0.016, Figure 4) but not with non-relapse mortality (HR=0.761, 95% CI 0.571-1.010, log-rank *P*=0.057) or relapse (HR=0.869, 95% CI 0.718-1.030; log-rank *P*=0.101). In multivariate Cox regression analysis including patient's age, sex, malignancy, risk of relapse (standard *versus* high), and incidence of acute GvHD (grades II – IV), higher donor $CD4^+$ RTE counts remained significantly associated with better overall survival (HR=0.808, 95% CI 0.665-0.982; *P*=0.032).

Effect of the transplant procedure and acute graft-versus-host disease on lymphocyte reconstitution and transplant outcomes

We evaluated the differences in the transplant procedure specific to each protocol in our study. No associations were noted between dose of total body irradiation (1200 or 1360 cGy), or the inclusion of fludarabine, with post-transplant lymphocyte recovery or transplant outcomes. Although the efficiency of T-cell depletion achieved by the the $CD34^+$ selection systems differed significantly, we found no associations between the $CD34^+$ selection system used on each clinical protocol and post-transplant lymphocyte recovery or outcomes. To evaluate the effects of G-CSF on RTE populations we performed a subset analysis on 38 available donor stem cell products. A significant decrease in the frequency of CD45RA and CD31 co-expression in the total $CD4^+$ T-cell compartment was noted in the G-CSF-mobilized stem cell products compared to unmobilized donor apheresis (mean, 14% *versus* 25%; *P*<0.001). However, this difference was not evident within the nTreg RTE population, and the change in frequency of the CD4 RTE T cells induced by G-CSF did not correlate with HSCT outcomes.

Although donor lymphocyte infusions were not administered to patients who had significant acute GvHD (grade ≥2), there was no association between administration of donor lymphocyte infusions and extensive chronic GvHD, relapse, or survival in our study. In addition, no associations were noted between the presence of any grade of acute GvHD and donor lymphocyte counts. However, lymphocyte counts were lower in patients with acute GvHD 100 days after HSCT, but not at 30 days or 1 year after transplantation. Grade I acute GvHD was associated with the occurrence of extensive chronic GvHD (HR=1.741, 95% CI 1.044-2.901; *P*=0.035), consistent with skin being the common organ involved. Grades III-IV acute GvHD was associated with increased non-relapse mortality (HR=2.611, 95% CI 1.262-5.611; *P*=0.010).

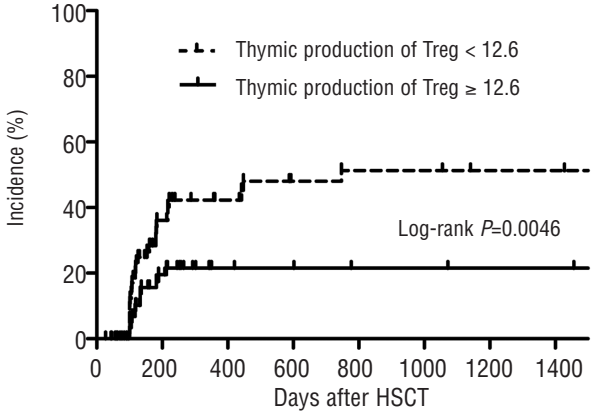

Figure 3. Thymic production of nTreg and risk of extensive chronic GvHD. Patients whose donors had a higher thymic production of nTreg (RTE nTreg count/percent CD4 RTE) had a lower cumulative incidence of extensive chronic GvHD. The *P* value between the two groups was calculated using a log-rank test.

Discussion

Post-transplant patterns of immune recovery and the relationship of this recovery to transplant outcomes are well characterized. In particular, delayed recovery of a broad repertoire of naïve T cells after HSCT leads to an increased

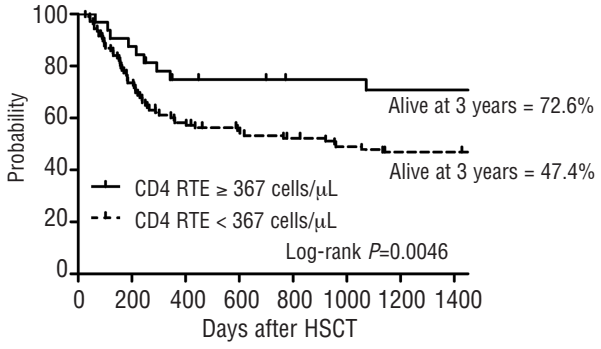

Figure 4. Donor CD4⁺ recent thymic emigrant (RTE) counts and overall survival. Patients whose donors had higher CD4⁺ RTE counts had a better overall survival. The *P* value between the two groups was calculated using a log-rank test.

risk of infection and disease relapse.[1] However, the contribution of the donor's unique immune characteristics to the quality of immune recovery and transplant outcome has not been fully established. Specifically, it is not known how much the naïve T-cell repertoire of the donor can influence immune recovery and transplant outcomes. We found that donors with more than the median total lymphocyte count conferred higher lymphocyte counts to the recipients in the first year after HSCT. A higher lymphocyte count in the donor was associated with a reduced risk of extensive chronic GVHD, and higher RTE CD4⁺ T-cell counts in the donor resulted in better overall survival after HSCT.

Since the CD4⁺ compartment contains the important Treg subset which plays a major role in controlling GvHD, we further studied the association of donor CD4⁺FOXP3⁺ Treg on HSCT outcomes. Tregs can be further divided into naturally occurring thymus-derived Treg (nTreg) and peripherally induced Treg.[27] Both subsets express FOXP3, with high CD25 expression, low CD127 expression, and are CTLA-4-positive.[28-30] However, nTreg possess greater stability of FOXP3 expression and suppressive activity.[31,32] Although FOXP3 DNA methylation patterns and cytokine profiles are different, discrimination between the subsets based on phenotyping alone is difficult. Helios, a member of the Ikaros transcription factor family, has recently been identified as a specific marker of nTreg when expressed in combination with FOXP3.[24,25] In mice, Helios is expressed in 100% of CD4⁺CD8⁻FOXP3⁺ thymocytes, while only 70% of Treg in the periphery were Helios⁺ in both mice and humans.[24] Using this newly identified marker of nTreg, we found that a median of 63% of peripheral Treg expressed Helios in our population of donors. Using CD31 and CD45RA as markers of recent thymic export, we found that the proportion of RTE-marked nTreg did not correlate with overall CD4⁺ T-cell thymic output, indicating that nTreg thymic output was independent of total CD4⁺ T-cell thymic neogenesis.

Previous work has demonstrated that thymic function in the recipient prior to a myeloablative matched-sibling HSCT may also predict clinical outcomes.[33] However, RTE counts on days 30 and 100 after HSCT were barely detectable in our study, consistent with the prolonged thymic failure in adult myeloablative transplant recipients. Assuming a steady-state immune homeostasis in donors prior to stem cell mobilization, our results imply that innate differences in donor thymic function can transfer a favorable pattern of immune reconstitution to the recipient which may be protective against chronic GvHD, and that the predictive value of pretransplant recipient thymic measurements may, in part, result from similarities between the donor and recipient thymic output in the matched-sibling setting.

Thymic output is dependent on a complex process of T-cell production, which includes regular migration of T lymphoid progenitors from the bone marrow to the thymus, the efficient commitment and maturation of these progenitors in the thymus, and a proficient export of mature T cells to the periphery.[25,34] As a result, ALC represents a unique measure of thymic activity by capturing both bone marrow production, as reflected in the absolute concentration of all lymphocyte subsets, and thymic export of all T cells. In explanation of the donor ALC predicting immune tolerance aftert HSCT, we found that absolute concentrations of nTreg correlated with both the size of the T-cell compartment and ALC in donor samples at the time of presentation, both of which predicted the occurrence of extensive chronic GvHD after HSCT.

Lymphocyte counts and thymic activity in healthy individuals are age-related, and recipient age is known to be associated with an increased risk of GvHD and decreased overall survival.[35-37] Since the ages of the patients and donors are highly correlated in HLA-matched sibling transplants, we explored the relationship between patient's age and donor's lymphocyte counts on HSCT outcomes. Although the donor lymphocyte count correlated with the patient's age (R^2 = - 0.15) and both were found to be associated with non-relapse mortality, age was not an independent risk factor for overall survival in our study. In fact higher donor CD4⁺ RTE was associated with better overall survival independently of either the recipient's or donor's age.

These finding suggest that obtaining a graft from a donor with higher lymphocyte counts and markers of superior thymic output, regardless of age, might result in better outcomes in recipients. The impact of the donor's immune profile was even more remarkable in our series of patients because all patients received a TCD transplant, making the effect of donor lymphocytes more related to the quality of the cells infused rather than to their quantity. However, while this may be true for myeloablative TCD HSCT from HLA-matched siblings, our findings cannot be generalized to other transplant settings, such as mismatched or T-cell-replete transplants, in which more powerful alloreactivity may transcend donor immune qualities.

We can only speculate whether the variations in functional lymphocyte subsets found in donors (and in all healthy individuals) leading to different transplant outcomes simply reflect day to day fluctuations, or whether genetically determined immune dynamics of lymphocyte production and loss is responsible. Likewise, the factors underlying an individual's thymic output are likely to be the consequence of multiple gene interactions as well as environmental influences. Long-term surveys of these minor lymphocyte populations in healthy individuals would help to resolve this issue.

In conclusion, we have shown that profiling the spectrum of absolute numbers of donor T cells and the numbers of their subsets using markers for Treg and RTE can predict important HSCT outcomes. Documenting donor lympho-

cyte counts and subsets is easily performed by many transplant centers. It could be of potential prognostic importance as well as illuminating to the biology of immune recovery to perform similar studies in other HSCT series.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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