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Tissue-specific regulatory T cells: biomarker for acute graft-vs-host disease and survival

Brian G. Engelhardt[a], **Salyka M. Sengsayadeth**[a], **Madan Jagasia**[a], **Bipin N. Savani**[a], **Adetola A. Kassim**[a], **Pengcheng Lu**[b], **Yu Shyr**[b], **Sandra M. Yoder**[c], **Michael T. Rock**[c], and **James E. Crowe Jr**[c,d]

[a]Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

[b]Department of Biostatistics, Vanderbilt University Medical Center, Nashville, TN, USA

[c]Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN, USA

[d]Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN, USA

Abstract

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that are characterized by the expression of CD25 and Foxp3 and are capable of suppressing alloimmune responses. We assessed whether high frequencies of circulating skin or gut tissue–specific Tregs at engraftment could predict acute graft-vs-host disease (aGVHD) incidence and survival in a cohort of hematopoietic cell transplant (HCT) recipients. Tregs were analyzed at engraftment in 74 patients receiving HCT. Treg skin-homing (CLA^+) or gut-homing ($\alpha_4\beta_7^+$) subsets were identified by flow cytometry, and patients were divided into high CLA^+ Tregs or high $\alpha_4\beta_7^+$ Tregs groups, using the 75th percentile of tissue-specific Treg percentages as a threshold. At day +100 post-HCT, the cumulative incidence of any stage skin or gut aGVHD was significantly lower in those patients with high CLA^+ Tregs or high $\alpha_4\beta_7^+$ Tregs at engraftment, respectively (high CLA^+ Tregs, 24.0% vs low CLA^+ Tregs, 55.1%; $p = 0.011$ for skin aGVHD or high $\alpha_4\beta_7^+$ Tregs, 47.3% vs low $\alpha_4\beta_7^+$ Tregs, 74.5%; $p = 0.029$ for gut aGVHD). The 2-year probabilities of overall survival and nonrelapse mortality were 73.4% and 7.5% among patients with high frequencies of tissue-specific Tregs vs 49.4% and 36.1% for those with both low CLA^+ Tregs and low $\alpha_4\beta_7^+$ Tregs ($p = 0.039$, $p = 0.010$). These results suggest that a threshold value for CLA^+ or $\alpha_4\beta_7^+$ Tregs could be used to predict important HCT outcomes, and to direct the rationale use of tissue-specific pre-emptive therapies to decrease clinical aGVHD and improve HCT survival.

Acute graft-vs-host disease (aGVHD) is a significant and unpredictable complication after allogeneic hematopoietic cell transplantation (HCT). aGVHD occurs when donor T cells recognize incompatibilities in recipient major or minor histocompatibility antigens. After cell activation and up-regulation of tissue-specific homing receptors, donor T cells migrate from secondary lymphoid organs to recipient target tissues, leading to the clinical manifestations of aGVHD, e.g., dermatitis, gastroenteritis, and cholestatic hepatitis [1–5]. Despite prophylaxis strategies with calcineurin inhibitors and methotrexate or

Offprint requests to: James E. Crowe Jr., M.D., Department of Pediatrics, Vanderbilt University Medical Center, 11475 MRB IV, 2213 Garland Avenue, Nashville, TN 37232-2905; james.crowe@vanderbilt.edu.

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mycophenolate mofetil, about 50% of patients undergoing HCT will develop moderate to severe (Glucksberg grade II–IV) aGVHD [1,2]. Standard treatment of aGVHD with high-dose corticosteroids produces a complete response in only 25% to 50% of patients, with about one third of individuals having persistent symptoms [1,2,6,7]. For patients with steroid refractory aGVHD, there is no standard second-line treatment, and overall prognosis of such individuals is guarded. Due to the unpredictable nature of aGVHD, inferior survival of Glucksberg grade III–IV aGVHD, and the heterogeneous response to initial therapy, the development of a laboratory test that can predict incidence and target organ involvement and severity of aGVHD would be clinically important, and could be used to improve HCT outcomes.

$CD25^+Foxp3^+$ regulatory T cells (Tregs) are an important subset of $CD4^+$ T cells that maintain immunological tolerance [8–11]. Tregs are associated with the prevention of aGVHD in murine models of transplantation and in human HCT [12–16]. Similar to conventional T cells, Tregs localize to host secondary lymphoid organs early after HCT and then migrate to the skin, gut, or liver via the expression of selectins, integrins, and chemokine receptors [17, 18]. The expression of these cell-surface molecules can be used to identify unique subsets of Tregs with either a skin-homing ($CLA^+/CCR4^+$) or a gut-homing ($\alpha_4\beta_7^+/CCR9^+$) phenotype [19, 20]. In a limited cohort of 43 patients, our group has shown a relationship between tissue-homing Treg subsets with skin or gut aGVHD [21]. To facilitate future clinical trials, determination of a threshold or cutoff value corresponding to high or protective Treg percentages would be clinically useful. Ideally this biomarker would predict both aGVHD incidence and survival.

We hypothesized that high frequencies of circulating CLA^+ or $\alpha_4\beta_7^+$ Tregs would be associated with decreased skin or gut aGVHD, respectively, and improved post-HCT survival. The data showed that high or low percentages of CLA^+ or $\alpha_4\beta_7^+$ Tregs at the time of neutrophil engraftment can be used to predict both aGVHD target organ involvement and post-transplantation survival. These laboratory parameters represent a novel, easily accessible, and pertinent clinical test that can be used to predict aGVHD onset and mortality before their clinical occurrence. Furthermore, this Treg subset threshold represents a target that is potentially modifiable through pharmacological intervention or cellular therapy.

Material and methods

Study design

Patients undergoing HCT were enrolled in a Vanderbilt Institutional Review Board–approved protocol after written informed consent was obtained. All analyzed patients were diagnosed with a hematological malignancy and received either a myeloablative or reduced-intensity conditioning regimen, followed by a T-cell replete related donor or unrelated donor transplant. Patients receiving in vivo T-cell depletion with anti-thymocyte globulin were excluded due to low lymphocyte numbers, which interfered with flow cytometric analysis. All patients received aGVHD prophylaxis with a calcineurin inhibitor and either methotrexate or mycophenolate mofetil. Sirolimus was not administered routinely. No patients received donor lymphocyte infusions during the study period, defined as the first 100 days of transplant. Clinical features of aGVHD were assessed weekly for the first 100 days after HCT by a single individual blinded to the Treg data (M.J.). The recorded features included aGVHD timing, incidence, organ involvement, severity, and recurrence rates. Histologic confirmation of clinical aGVHD was performed for clinical purposes when feasible. Recurrent aGVHD was defined as any increase in aGVHD symptoms or therapy during the first 100 days of transplant after an initial response to treatment or during steroid taper. Recurrent aGVHD was not analyzed beyond day +100. The clinical severity of

aGVHD was determined by the overall grade (0–IV) and the individual organ stage (0–4), as defined by the 1994 consensus conference criteria [22].

Blood cell isolation and flow cytometric analysis

The procedures for collection and storage of peripheral blood mononuclear cells for T-cell immunophenotyping were reported previously [21]. Samples were collected at two time points: neutrophil engraftment (defined as absolute neutrophil count $\geq 0.5 \times 10^9$/L for 3 days) and at day$^+$30 after HCT. After thawing, cells were analyzed using a 10-color multiparametric flow cytometric panel including the following antibodies or dye: CD3-peridinin-chlorophyllprotein complex-Cy5.5, CD4-Alexa700, CD25-allophycocyanin-Cy7, CD45RO-phycoerythrin (PE)-Cy7, CLA-fluorescein isothiocyanate (BD Biosciences, San Jose, CA, USA), CD8-PE-Cy5, CD14-PE-TR, amine viability dye (Invitrogen, Carlsbad, CA, USA), CD127-Pacific Blue (eBioscience, San Diego, CA, USA), and α4β7-PE (kind gift from Millennium Pharmaceuticals, Inc., Cambridge, MA, USA and commercially conjugated by Chromaprobe, Inc., Maryland Heights, MO, USA). After surface staining, cells were fixed and permeabilized with the Human Foxp3 Buffer Set (BD Biosciences, San Jose, CA, USA) as per manufacturer's instructions followed by intracellular staining with Foxp3-Alexa 647 (clone 259D/C7; BD Biosciences, San Jose, CA, USA). Stained cells were analyzed with a LSRII flow cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis was performed using FlowJo software version 8.0 (Tree Star, Ashland, OR, USA) by a single expert cytometrist who was blinded to the transplant data. A minimum of 100,000 CD4$^+$ events was acquired. To increase specificity, multiple markers were used to indentify Tregs, including CD4$^+$CD45RO$^+$CD25$^+$Foxp3$^+$CD127^{lo}, and their frequency was expressed as the percentage of positive cells in the total CD4$^+$ gate. CLA$^+$ (considered skin homing) or $\alpha_4\beta_7^+$ (considered gut homing) Tregs were identified and quantified as their respective subpopulations within the total population of Tregs (Supplementary Figure E1; online only, available at www.exphem. org). Treg percentages rather that absolute numbers were analyzed due to the infrequency of the T-cell subsets studied and the severity of the lymphopenia at the time of sample collection.

Statistical analysis

Continuous variables were summarized using the mean, median, quartiles, or range. For continuous variables, the mean difference between two independent groups was compared using the Mann-Whitney *U* test. Categorical variables were described by the percentage or frequency and were compared by the χ^2 or Fisher's exact test. Correlation between continuous variables was determined by Spearman's rank correlation. The primary aims of this study were to determine if there was an association between high or low percentages of circulating blood CLA$^+$ and $\alpha_4\beta_7^+$ Tregs with the occurrence of skin or gut aGVHD, respectively, and to identify if these subsets predict post-HCT survival. Various statistical methods were used to establish a cutoff for high or low Treg percentages, including taking the median and grouping by quartiles. In univariate analysis, χ^2 was used to test the association between high or low Treg subset percentages with organ-specific aGVHD incidence or recurrence during the first 100 days of HCT. Multivariate logistic regression was used to evaluate whether high or low Treg subset percentages could independently predict aGVHD outcomes during the first 100 days of HCT, after adjusting for potential confounding variables, including intensity of conditioning regimen (i.e., myeloablative vs reduced-intensity conditioning), donor type (i.e., related vs unrelated), and stem cell source. These clinically important transplant characteristics were selected as covariates for the logistic regression models a priori. Overall survival and disease-free survival were estimated using the Kaplan-Meier method, and cumulative incidence was used to estimate the probability of nonrelapse mortality (NRM). NRM was defined as death in the absence of disease relapse or progression. NRM and relapse were considered competing risks for

disease-free survival and NRM, respectively. Survival outcomes between groups were compared with a log-rank test for univariate analysis and a Cox proportional hazards regression for multivariate analysis. The p values were two-tailed and considered significant at $p < 0.05$. Analyses were performed using SPSS version 18 (SPSS Inc, Chicago, IL, USA) and R version 2.7.0 (Free Software Foundation, Boston, MA, USA).

Results

Patients

From February 2, 2007 to March 3, 2009, 130 patients (aged 18 years or older) underwent HCT at a single institution (Vanderbilt University Medical Center, Nashville, TN, USA) and 74 of these patients had peripheral blood mononuclear cells collected for Treg analysis (Fig. 1). The clinical characteristics of this cohort are summarized in Table 1. aGVHD events were recorded until day +100. Grade II to IV aGVHD occurred in 57 (77%) patients at a median of 28 days post-HCT (range, 7–93 days) and was biopsy proven in 51 (91%) of those individuals. aGVHD affected the skin, gut, and liver in 33 (44.6%), 50 (67.6%), and 2 (2.7%) subjects, respectively and was grade III–IV in 13 (17.6%) of the HCT recipients. Forty-five (60.8%) patients received systemic corticosteroids as part of their aGVHD treatment. Despite treatment, grade II–IV aGVHD recurred in 32 (68%) of these individuals during the first 100 days. Actuarial survival at day +100 was 95.9%; causes of death included relapse of malignancy (n = 2) and infection (n = 1). No patients died from complications related to aGVHD during the first 100 days.

Treg analysis

Median time from stem cell infusion to initial Treg analysis was 19 days (range, 10–34 days). Treg subsets could be quantified 7–10 days before development of grade II–IVaGVHDor the start of systemic steroids. Using multiparametric flow cytometry, we were able to identify populations of CLA^+ (skin homing) or $\alpha_4\beta_7^+$ (gut homing) Tregs in all patients at the time of neutrophil engraftment and as early as 10 days post-HCT (Supplementary Figure E1; online only, available at www.exphem.org). As expected, these $Foxp3^+$ tissue-homing subsets were primarily $CD45RO^+$ and $CD127^{lo}$. These subpopulations of Tregs appeared mutually exclusive with few cells positive for both CLA^+ and $\alpha_4\beta_7^+$ (median percentage of $CLA^+\alpha_4\beta_7^+$ Tregs at engraftment and at day +30 was 0.13% [range, 0–1.12%] and 0.12% [range, 0–1.10%], respectively). Treg expression of CLA was inversely related to expression of $\alpha_4\beta_7$, with a negative correlation noted at engraftment ($r_s = -0.40$; $p < 0001$) (Fig. 2C). Thus, it appeared that some patients exhibited preferential expansion of a single organ-specific subset of Tregs (Fig. 2A, B).

Donor chimerisms were not studied at the time of neutrophil engraftment. Day +30 restriction fragment length polymorphism data were obtained from bone marrow in all evaluable patients (n = 73), and circulating $CD3^+$ sorted T cells in patients undergoing cord blood or reduced-intensity conditioning HCT (n = 33). The median percentage of donor cells in the marrow or within the peripheral blood T-cell compartment was 99% (range, 50–100%) and 83.5% (range, 39–100%), respectively. These data indicate that the majority of circulating hematopoietic cells early after transplantation were derived from the donor.

Treg subsets and organ-specific aGVHD outcomes

Because the majority of initial aGVHD events occurred before day +30, we focused our analysis on the frequency of Tregs at engraftment as predictive markers for development of aGVHD. After examining the distribution of Treg subset percentages among patients with or without aGVHD in our original cohort, the 75^{th} percentile or higher was established as the cutoff point to define high or protective percentages of Treg cell subsets (Tregs/$CD4^+$

≥14.0%), CLA^+ Tregs (CLA^+ Tregs/Tregs ≥3.25%), or $\alpha_4\beta_7^+$ Tregs ($\alpha_4\beta_7^+$ Tregs/Tregs ≥21.8%) [21]. This cutoff was then applied to the total cohort (n = 74) for analysis, and 13 (17.6%), 25 (33.8%) and 19 (25.7%) patients had high frequencies of Tregs, CLA^+ Tregs, or $\alpha_4\beta_7^+$ Tregs, respectively.

We noted a reciprocal regulation of the skin and gut-coming subsets in peripheral blood. Consistent with this trend, only one patient in the cohort was classified as having both high CLA^+ and high $\alpha_4\beta_7^+$ circulating Tregs. Next, we examined whether the clinical characteristics as outlined in Table 1 influenced the occurrence of high CLA^+ or high $\alpha_4\beta_7^+$ Treg cell subsets. Patients classified as high $\alpha_4\beta_7^+$ Treg producers were younger (median 5 42 vs 47 years; $p = 0.047$) and were more likely to have received human leukocyte antigen–mismatched grafts (31.6% vs 3.64%; $p = 0.001$). Patients with high $\alpha_4\beta_7^+$ Tregs also tended to have received myeloablative conditioning (78.9% vs 54.5%; $p = 0.060$) and transplants using bone marrow or cord blood (42.1% vs 20%; $p = 0.057$). Definite associations were not found between high CLA^+ Tregs and the clinical characteristics listed in Table 1.

The proportion of patients developing any skin aGVHD during the first 100 days of HCT was significantly lower in those with high CLA^+ Tregs vs those with low CLA^+ Tregs (24.0% [6/25] vs 55.1% [27/49]; $p = 0.011$). Similar results were found with respect to high $\alpha_4\beta_7^+$ Tregs with any stage gut aGVHD occurring in 47.3% (9 of 19) as compared to 74.5% (41 of 55) in patients with low $\alpha_4\beta_7^+$ Tregs ($p = 0.029$). The analysis was repeated after excluding the three patients who died before day +100 and similar results were found with high CLA^+ Tregs and high $\alpha_4\beta_7^+$ Tregs associated with the prevention of skin aGVHD ($p = 0.009$) or gut aGVHD ($p = 0.043$), respectively (data not shown). During the first 100 days after transplantation, high frequencies of circulating tissue-specific Tregs at engraftment were also associated with prevention of repeat episodes of aGVHD involving the same organ. Among patients with a history of aGVHD involving the skin (n = 33) or gut (n = 50), 20 (60.6%) and 19 (38%) of those individuals had recurrent symptoms of either skin or gut aGVHD, respectively. Patients with high $\alpha_4\beta_7^+$ Tregs had decreased recurrent gut aGVHD episodes (high $\alpha_4\beta_7^+$ Tregs, 0% [0 of 19] vs low $\alpha_4\beta_7^+$ Tregs, 34.5% [19 of 55]; $p = 0.003$), while HCT recipients with high CLA^+Tregs had a nonsignificant decrease in recurrent skin aGVHD (high CLA^+Tregs, 16% [4 of 25] vs low CLA^+ Tregs, 32.6% [16 of 49]; $p = 0.127$). Thus, the frequency of Treg subsets at engraftment predicted not only the incidence of organ-specific aGVHD, but also identified individuals who may require prolonged exposure to systemic steroids, which in turn could affect survival. In contrast to the organ-specific Treg percentages, the total number of circulating Treg cells (not accounting for homing properties) was not a predictive factor. Total Treg percentages were not associated with grade II–IV aGVHD ($p = 0.474$), any stage skin aGVHD ($p = 0.176$), or any stage gut aGVHD ($p = 0.888$) (data not shown).

In multivariate logistic regression, patients with high percentages of CLA^+ Tregs or $\alpha_4\beta_7^+$ Tregs at the time of neutrophil engraftment continued to have a significantly decreased odds of skin (odds ratio [OR] = 0.27; 95% confidence interval [CI], 0.09–0.81; $p = 0.020$) or gut (OR = 0.20; 95% CI, 0.06–0.69; $p = 0.011$) aGVHD, respectively, during the first 100 days of transplant (Table 2).

Treg subsets and survival analysis

Median follow-up was 2.5 years (range, 0.5–4 years) from time of HCT for surviving patients (n = 45). The major cause of death was relapse or progression of malignancy (n = 19). NRM occurred in 10 (13.5%) patients. Causes of death included infection (n = 5), acute blood loss from gastrointestinal GVHD (n = 1), diffuse alveolar hemorrhage (n = 1), thrombotic thrombocytopenic purpura (n = 1), secondary malignancy (n = 1), and myocardial infarction (n = 1). The majority of patients with NRM were on systemic

immunosuppression (n = 9), had active GVHD (overlap chronic GVHD, n = 5; recurrent aGVHD, n = 2; and classic chronic GVHD, n = 1), and had low frequencies of CLA^+ Tregs and $\alpha_4\beta_7^+$ Tregs (n = 8).

To determine whether tissue-specific Tregs were associated with survival after HCT, patients were stratified into two groups. Patients with the favorable phenotype of either high CLA^+ Tregs or high $\alpha_4\beta_7^+$ Tregs were collapsed into a single group (n = 43) and were compared to patients with both low CLA^+ Tregs and low $\alpha_4\beta_7^+$ Tregs (n = 31). The estimated 2-year overall survival was 73.4% (95% CI, 59.7–87.1%) for patients with either high CLA^+ Tregs or high $\alpha_4\beta_7^+$ Tregs and was 49.4% (95% CI, 31.2–67.6%) for individuals with decreased frequencies of tissue-specific Tregs (p =0.039). This survival benefit was primarily due to a decreased 2-year NRM among patients with the favorable Treg phenotype when compared to patients with both low CLA^+ Tregs and low $\alpha_4\beta_7^+$ Tregs (7.5%; 95% CI, 2.7–17.7% vs 36.1%; 95%CI, 14.1–58.1%; p = 0.010, respectively). No difference in disease-free survival was seen between the groups (Fig. 3).

Cox proportional hazards regression models were constructed utilizing the same covariates used in the previous logistic regression models, along with adjustment for grade II–IV aGVHD. In multivariate analysis, patients with either high CLA^+ Tregs or high $\alpha_4\beta_7^+$ Tregs had a 56% reduction in the risk for all-cause mortality (hazard ratio = 0.44; 95% CI, 0.20–0.99; p = 0.046) and specifically an 88% decrease in the risk for NRM (hazard ratio = 0.12; 95% CI, 0.02–0.61; p = 0.011) (Table 3).

Discussion

Tregs are recognized as an important lymphocyte population for the prevention of aGVHD in human HCT [16, 19]. Our data support this concept while further exploring the role of Treg tissue-homing subsets with organ-specific aGVHD incidence and transplant survival. The data show that increased frequencies of circulating, skin-homing (CLA^+) or gut-homing ($\alpha_4\beta_7^+$) Tregs at engraftment are associated with a reduced incidence of skin or gut aGVHD, respectively. Furthermore, the level of expansion of either CLA^+ or $\alpha_4\beta_7^+$ Tregs appears to be inversely related to each other, and the relative proportions of these subsets may represent an individualized set point for Treg frequencies post-transplantation. This Treg set point represents a target that is potentially modifiable via pharmacologic intervention or by cellular therapy. Indeed, our data suggested that $\alpha_4\beta_7^+$ Tregs subsets could be influenced by human leukocyte antigen disparity and stem cell graft source. Furthermore, these novel results also demonstrated that early expansion of CLA^+ or $\alpha_4\beta_7^+$ Tregs was associated with improved survival and decreased NRM in patients receiving T cell replete HCT. Remarkably, the biomarkers predicted both aGVHD incidence and survival in a heterogenous patient population and the results remained significant, even after adjustment for important transplant characteristics.

These data suggest that Tregs are not a single, uniform subset of T cells, but rather a highly diversified cell population, each with distinct characteristics and functional properties [19, 20,23]. Similar to other T cell subsets, the current studies indicate that Tregs can be divided into several tissue-specific groups, including skin- or gut-homing populations. This compartmentalization appears to occur during the early phases of immune reconstitution, which may have important implications for determining aGVHD organ involvement and long-term morbidity and mortality after HCT.

It is increasingly clear that Tregs must migrate from the circulation to either secondary lymphoid organs or to other peripheral tissues to maintain their regulatory control over the immune system. Currently, there is ongoing debate about where Tregs exert their

suppressive effect during HCT. In animal models of transplantation, Tregs initially accumulate in secondary lymphoid organs and prevent proliferation of alloreactive T cells there, and then they migrate to aGVHD target tissues [17]. In addition, it was previously shown that only the $CD62L^+$ (lymph node– homing) population of Tregs prevented aGVHD in the mouse, thus further supporting the lymph node as the principal biological site for aGVHD suppression [13, 14].

Human studies suggest that Treg localization in cutaneous or gut tissues is important for the suppression of aGVHD at those sites [24, 25]. Presumably, these cells migrated from secondary lymphoid organs to skin or gut as directed by expression of adhesion molecules, however, tissue-specific homing markers were not specifically analyzed in those studies. In our current analysis, high frequencies of circulating CLA^+ or $\alpha_4\beta_7{}^+$ Tregs were associated with the prevention of skin or gut aGVHD, which supports the hypothesis that aGVHD suppression occurs, at least in part, at a local tissue level in humans. Alternatively, because Tregs activated in peripheral lymph nodes up-regulate CLA, while those activated in mesenteric lymph nodes more often express $\alpha_4\beta_7$our analysis may simply reflect the secondary lymph node location in which the Treg was initially induced to acquire suppressive function, as opposed to the final destination of the Treg. To help answer this question, future experiments should determine whether circulating CLA^+ or $\alpha_4\beta_7{}^+$ Tregs in the blood of HCT recipients correlate with the proportion of $Foxp3^+$ cells infiltrating skin or gut biopsies of patients with aGVHD, respectively. Adding another layer of complexity, it was shown recently in an islet allograft model that Tregs needed to migrate from the transplanted tissue to the draining lymph node to suppress graft rejection [26]. Tregs failing to first localize in the transplanted tissue due to lack of homing receptors did not prolong graft survival. These data imply that Treg trafficking to both secondary lymphoid organs and to peripheral tissues could be important for determining aGVHD outcomes. The molecular basis for control of this migration requires further elucidation.

Although we identified a relationship between Tregs and organ-specific aGVHD outcomes, not all studies have arrived at the same conclusions. In a recent report, neither the circulating percentage of Tregs nor the infiltration pattern of $Foxp3^+$ cells in gut biopsies correlated with the prevention of gastrointestinal aGVHD [27]. There were significant differences between the studies with respect to Treg subset identification. We focused our analysis on organ-specific subsets of Tregs to identify the association. However, when investigating the total number of circulating Treg cells in a way that did not account for homing properties, the results were similar with the other study. No relationship was found for total number of circulating Tregs with either skin or gut aGVHD. As we move forward with therapeutic clinical trials aimed at increasing Treg numbers post-transplantation as a means to prevent or treat aGVHD, it will be important that we understand how specific Treg populations influence aGVHD outcomes and long-term survival.

The majority of adult patients undergoing HCT at this institution were entered into the trial. Within this population, we report a high incidence of grade II–IV aGVHD with a predominance of gastrointestinal involvement. Other groups also have reported increasing diagnosis of gut aGVHD, likely due to early esophagogastroduodenoscopy and less reliance on total parenteral nutrition [28, 29]. However, the possibility that gut aGVHD was overdiagnosed while cutaneous aGVHD was underdiagnosed in this study certainly exists, thus underscoring the need for prospective multicenter trials in aGVHD.

Conclusions

These data supports our hypothesis that Treg tissue compartmentalization is important for determining both organ-specific aGVHD incidence and survival after T-cell replete HCT. In

this analysis, high frequencies of CLA^+ or $\alpha_4\beta_7^+$ Tregs at engraftment were associated with prevention of skin or gut aGVHD during the first 100 days of transplant, respectively. Despite no definite correlation between Tregs and aGVHD severity, high frequencies of CLA^+ or $\alpha_4\beta_7^+$ Tregs continued to be associated with improved survival and decreased NRM. If validated, a level of < or $\geq 75^{th}$ percentile of CLA^+ or $\alpha_4\beta_7^+$ Tregs could be used to stratify a patient's risk for either skin or gut aGVHD, or even NRM. The mechanism by which Tregs may decrease mortality is not clear because not all studies have shown a correlation between reduced aGVHD incidence and superior survival [30, 31].

The purpose of the current study was to identify a cutoff value corresponding to high or protective Treg frequencies to facilitate future clinical trials examining Tregs in the post-transplantation setting. Ideally, if subclinical aGVHD could be identified, then prophylaxis strategies could be intensified or directed to a particular tissue at an earlier stage, thereby preventing clinical manifestations of aGVHD, limiting patient exposure to high-dose corticosteroids, and improving transplant outcomes. In addition, a level of $\geq 75^{th}$ percentile of CLA^+ or $\alpha_4\beta_7^+$ Tregs at engraftment could be used as an end point or benchmark in future clinical trials examining novel therapeutic strategies aimed at increasing Tregs, preventing aGVHD, and improving HCT survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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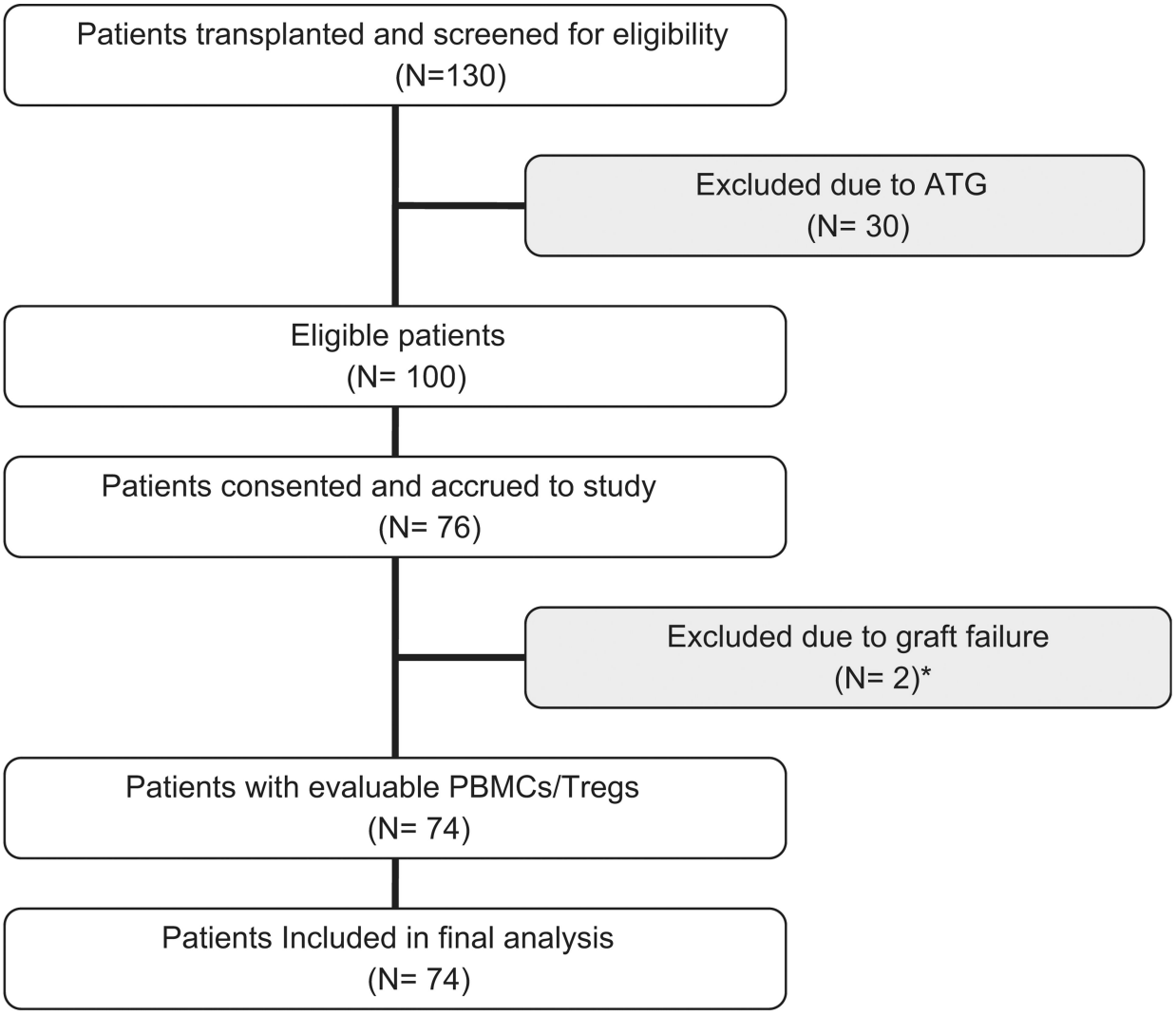

Figure 1.
Number of patients who underwent allogeneic hematopoietic cell transplantation, accrued to study, and were included in the final Treg analysis. ATG, antithymocyte globulin; PBMCs, peripheral blood mononuclear cells.

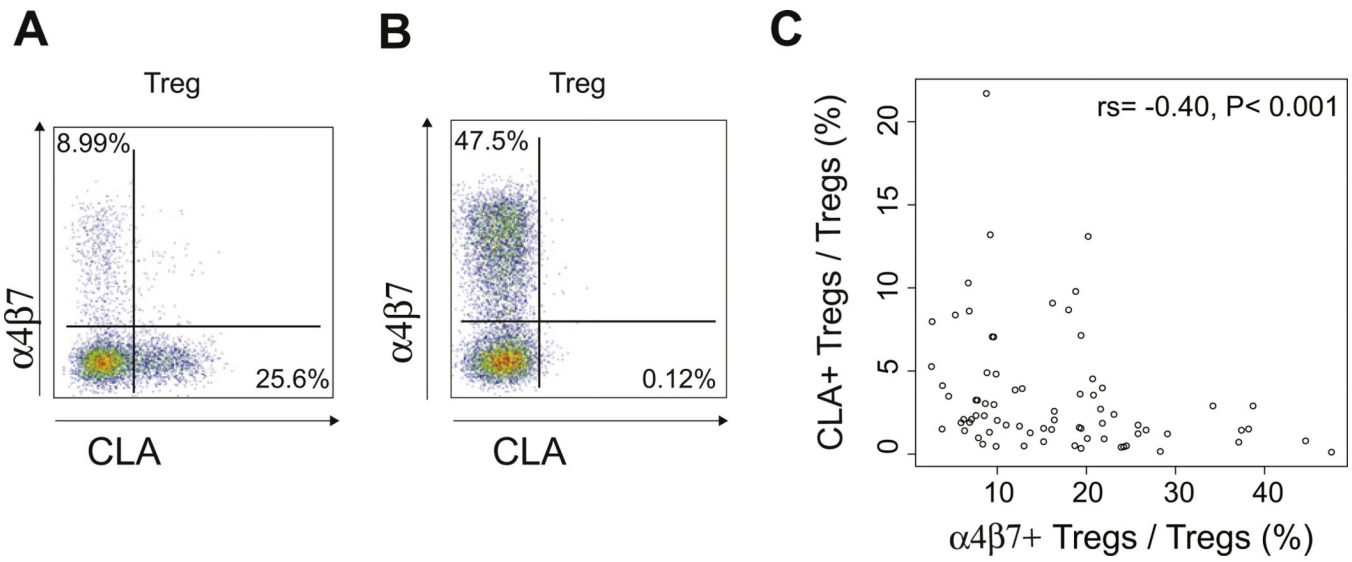

Figure 2.

CLA^+ and $\alpha_4\beta_7^+$ Tregs can be identified at the time of neutrophil recovery after allogeneic hematopoietic cell transplantation. (**A**) Patient with preferential expansion of skin-homing (CLA^+) Tregs ($CD4^+CD45RO^+CD25^+Foxp3^+CD127^{lo}$ cells). (**B**) Patient with preferential expansion of gut-homing ($\alpha_4\beta_7^+$) Tregs ($CD4^+CD45RO^+CD25^+Foxp3^+CD127^{lo}$ cells). (**C**) Treg expression of CLA and $\alpha_4\beta_7$ are inversely related.

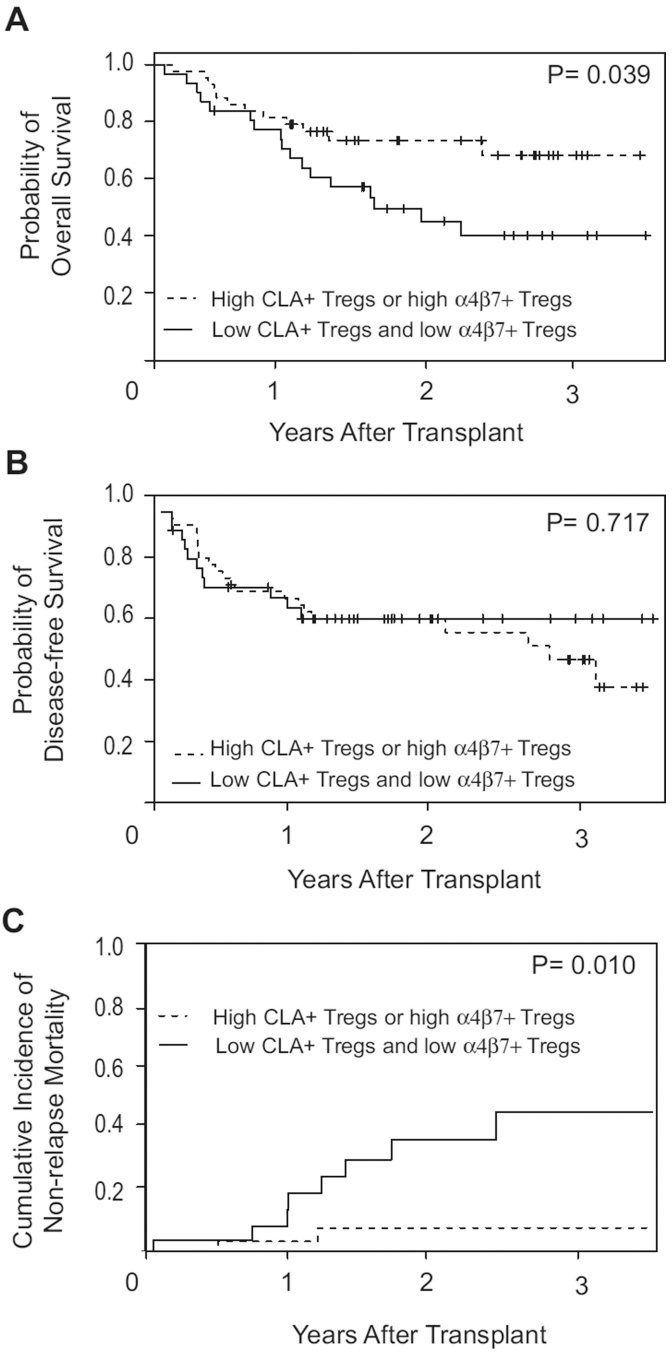

Figure 3.

Clinical outcomes stratified by Treg tissue-homing subsets. Probabilities of (**A**) overall survival, (**B**) disease-free survival, and (**C**) non-relapse mortality based on high CLA^+ or high $\alpha_4\beta_7^+$ Tregs (n = 43) vs low CLA^+ and low $\alpha_4\beta_7^+$ Tregs (n = 31). High CLA^+ Tregs: CLA^+ Tregs/Tregs ≥3.25%; High $\alpha_4\beta_7^+$ Tregs: $\alpha_4\beta_7^+$ Tregs/Tregs ≥21.8%.

Table 1

Characteristics of total cohort, 74 patients undergoing HCT

Characteristic	Patients with characteristic (n), stratified by aGVHD organ involvement (%)			
	Skin only aGVHD	Gut only aGVHD	Multi-organ* aGVHD	No aGVHD
Total no. of patients	11	29	22	12
Age (y)				
Median	43	47	44	49
Range	33–65	24–61	21–70	34–65
Sex				
Male	6 (55)	13 (45)	9 (41)	8 (67)
Female	5 (45)	16 (55)	13 (59%)	4 (33)
Diagnosis				
Acute leukemia + MDS	6 (55)	18 (62)	14 (64)	3 (25)
CML + MPD	—	3 (10)	—	2 (17)
NHL + HL + CLL + MM	5 (45)	7 (24)	7 (32)	7 (58)
Other	—	1 (4)	1 (4)	—
Conditioning regimen				
Myeloablative	6 (55)	20 (69)	14 (64)	5 (42)
Reduced intensity	5 (45)	9 (31)	8 (36)	7 (58)
Donor				
Related	8 (73)	21 (72)	11 (50)	10 (83)
Unrelated	3 (27)	8 (28)	11 (50)	2 (17)
Stem cell source				
Peripheral blood	8 (73)	24 (83)	13 (59)	10 (83)
Other	3 (27)	5 (17)	9 (41)	2 (17)
HLA				
Matched	8 (73)	27 (93)	20 (91)	11 (92)
Mismatched	3 (27)	2 (7)	2 (9)	1 (8)
Donor/recipient sex				
Matched	8 (73)	13 (45)	15 (68)	9 (75)
Mismatched	3 (27)	16 (55)	7 (32)	3 (25)
Female to male	1 (9)	4 (14)	1 (5)	1 (8)
CMV serostatus				
Recipient/donor				
Negative/negative	4 (36)	8 (28)	2 (9)	2 (17)
Positive/negative	3 (27)	10 (34)	8 (36)	2 (17)
Negative/positive	1 (9)	2 (7)	3 (14)	4 (33)
Positive/positive	3 (27)	9 (31)	9 (41)	4 (33)
$CD34^+$ ($\times 10^6$/kg)				
Median	5.56	5.94	6.34	6.23
Range	0.04–9.68	0.16–10.1	0.09–9.99	0.56–10.4

Characteristic	Patients with characteristic (n), stratified by aGVHD organ involvement (%)			
	Skin only aGVHD	Gut only aGVHD	Multi-organ* aGVHD	No aGVHD
aGVHD prophylaxis				
CSA + methotrexate	5 (46)	18 (62)	13 (59)	5 (42)
CSA/FK506 + MMF	6 (54)	11 (38)	9 (41)	7 (58)
Day + 100 disease status				
CR or PR	8 (73)	26 (90)	18 (82)	9 (75)
Relapse or progression	3 (27)	3 (10)	4 (18)	3 (25)
Day + 100 survival				
Alive	10 (91)	28 (97)	22 (100)	11 (92)
Dead	1 (9)	1 (3)	—	1 (8)

CLL = chronic lymphocytic leukemia; CML = chronic myeloid leukemia; CMV = cytomegalovirus; CR = complete response; CSA = cyclosporine; FK506 = tacrolimus; HL = Hodgkin's lymphoma; MDS = myelodysplastic syndrome; MM = multiple myeloma; MMF = mycophenolate mofetil; MPD = myeloproliferative disorder; NHL = non-Hodgkin lymphoma; PR = partial response.

* Liver aGVHD occurred in two patients with multi-organ aGVHD involvement.

Table 2

Logistic regression models for development of any stage skin or gut aGVHD

Target organ	Factor	aGVHD/patients at risk	Odds ratio (95% CI)	*p* Value
Skin aGVHD				
	Low CLA^+ Tregs	27/49	1	0.020
	High CLA^+ Tregs *	6/25	0.27 (0.09–0.81)	
	Myeloablative	20/45	1	0.430
	Reduced intensity	13/29	0.63 (0.20–1.97)	
	Related donor	19/50	1	0.750
	Unrelated donor	14/24	1.32 (0.24–7.36)	
	Peripheral blood stem cells	21/55	1	0.300
	Bone marrow or cord blood	12/19	2.53 (0.44–14.6)	
Gut aGVHD				
	Low $\alpha_4\beta_7^+$Tregs	41/55	1	0.011
	High $\alpha_4\beta_7^+$ Tregs †	9/19	0.20 (0.06–0.69)	
	Myeloablative	33/45	1	0.145
	Reduced intensity	17/29	0.41 (0.12–1.36)	
	Related donor	32/50	1	0.716
	Unrelated donor	18/24	1.43 (0.21–9.69)	
	Peripheral blood stem cells	36/55	1	0.833
	Bone marrow or cord blood	14/19	1.24 (0.17–9.16)	

* High CLA^+ Tregs: CLA^+ Tregs/Tregs ≥3.25%.

† High $\alpha4\beta7^+$ Tregs: $\alpha4\beta7^+$ Tregs/Tregs ≥21.8%.

Table 3

Cox proportional hazard regression models for overall survival and nonrelapse mortality

Factor	Overall survival			Nonrelapse mortality		
	Deaths/patients at risk	HR (95% CI)	*p* Value	Deaths/patients at risk	HR (95% CI)	*p* Value
Low CLA^+ and low $\alpha_4\beta_7^+$ Tregs	17/31	1		8/31	1	
High CLA^+ [*] or high $\alpha_4\beta_7^+$ Tregs [†]	12/43	0.44 (0.20–0.99)	0.046	2/43	0.12 (0.02–0.61)	0.011
Myeloablative	14/45	1		6/45	1	
Reduced intensity	15/29	1.23 (0.53–2.90)	0.630	4/29	1.09 (0.22–5.53)	0.918
Related donor	22/50	1		6/50	1	
Unrelated donor	7/24	0.24 (0.05–1.22)	0.085	4/24	0.22 (0.02–3.02)	0.255
Peripheral blood stem cells	22/55	1		6/55	1	
Bone marrow or cord blood	7/19	3.41 (0.67–17.4)	0.140	4/19	9.78 (0.68–140)	0.093
Grade 0–I aGVHD	8/17	1		2/17	1	
Grade II–IV aGVHD	21/57	0.65 (0.28–1.52)	0.324	8/57	0.70 (0.14–3.60)	0.667

HR 5 hazard ratio.

[*] High CLA^+ Tregs: CLA^+ Tregs/Tregs ≥3.25%.

[†] High $\alpha4\beta7^+$ Tregs: $\alpha4\beta7^+$ Tregs/Tregs ≥21.8%.