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Relationship of post-transplant thymopoiesis with CD4⁺FoxP3⁺ regulatory T cell recovery associated with freedom from chronic graft versus host disease

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Recovery of CD4⁺FoxP3⁺ regulatory T cells (Tregs) has been associated with protection from graft versus host disease (GVHD) in adult hematopoietic stem cell transplantation (HSCT), though significant debate exists as to the extent of this influence [1–7]. The lack of a clearly defined relationship between Tregs and GVHD may be related to heterogeneity of Treg populations, which can differ significantly in their functional responses, proliferative capabilities, tissue homing, and antigenic reactivity [4, 8]. Mechanisms regulating Treg compartment regeneration and function after HSCT are not well-defined, though studies in adult HSCT patients posited that post-transplant Tregs were primarily derived from peripheral expansion of circulating Tregs with minimal contribution from thymopoiesis. The ability of the thymus to reconstitute a functionally self-tolerant T cell compartment is compromised by age-related thymic involution, thymic GVHD, and radiation and chemotherapy. We hypothesized that post-transplant thymopoiesis may be an important factor in restoration of Treg-mediated protection from chronic GVHD (cGVHD), and may be a potential mechanistic explanation for disparate observations of the relationship between Tregs and cGVHD.

As thymus-dependent restoration of T lymphocyte compartments has a larger role in pediatric HSCT owing to lower-thymic function in adults due to age-related thymic involution [9], we examined pediatric HSCT recipients. We examined longitudinal peripheral blood samples and clinical data from 19 pediatric allogeneic HSCT patients (Table S1) collected at 14, 30, 60, 90, 180, 270, and 360 days post transplant. Patients were followed clinically for a mean duration of 16 months post transplant (range 3–24 months), with three patients excluded from analysis due to disease relapse leading to re-transplantation or death within 100 days of HSCT. Patients were divided into two groups, patients that developed cGVHD ($n = 7$) (Table S2) and patients that remained cGVHD-free for the study duration of at least 12 months ($n = 12$). Mean time to first diagnosis of cGVHD was 133 days post-transplant (range 101–189 days post-transplant). T cell reconstitution after HSCT was initially assessed by absolute lymphocyte count, and measurement of naive (CD45RA⁺) and memory (CD45RO⁺) helper CD4⁺ and cytotoxic CD8⁺ T cells by flow cytometry (Fig. S1). These parameters did not differ significantly between patients developing cGVHD and those remaining cGVHD-free.

Based on the hypothesis that the defect in T cell repopulation associated with cGVHD was specific for Tregs, we examined Tregs in peripheral blood by flow cytometry. Tregs were identified among CD4⁺ T cells by expression of the transcription factor FoxP3 (Fig. 1a–c), the lineage determining transcription factor, for Treg development and function. Longitudinal measurement of patients after HSCT identified differences in repopulation of Tregs associated with development of cGVHD. Interestingly, the dynamics of Treg repopulation appeared to occur in two phases; during the first 60 days post-transplant Tregs increased in all patients. However, only patients that remained cGVHD-free demonstrated continued increases in Tregs, while patients that subsequently developed cGVHD did not.

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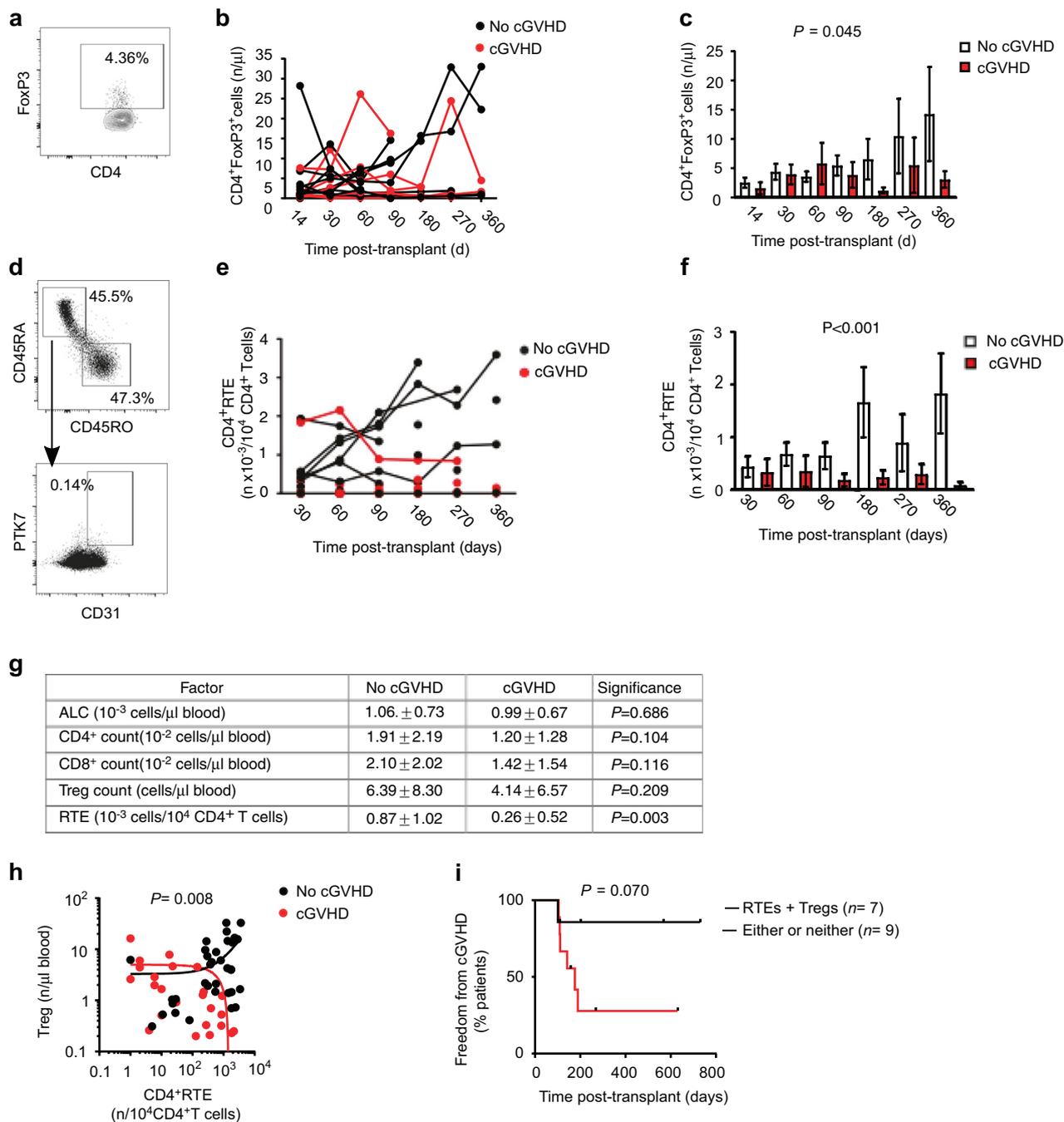


Fig. 1 Associations between Tregs and thymopoiesis and cGVHD in pediatric HSCT patients. **a** Tregs were identified by flow cytometry for CD4⁺FoxP3⁺ T cells. Representative example shown. **b** Enumeration of Tregs in peripheral blood samples, calculated from frequency of CD4⁺FoxP3⁺ Tregs and ALC, for patients developing cGVHD ($n = 7$) and patients remaining cGVHD-free for the study duration ($n = 12$). **c** Comparison of mean \pm sem Tregs at each timepoint for patients developing cGVHD and patients remaining cGVHD-free. Groups compared by 2-way ANOVA. **d** RTEs were identified among CD4⁺ T cells as CD45RA⁺CD31⁺PTK7⁺ cells. **e** Enumeration of RTEs in peripheral blood samples from patients developing cGVHD ($n = 7$) and patients remaining cGVHD-free for the study duration ($n = 9$). **f** Comparison of mean \pm sem RTE frequencies at each timepoint for

patients developing cGVHD and patients remaining cGVHD-free. Groups compared by 2-way ANOVA. **g** Multivariate analysis of measured factors for T lymphocyte reconstitution as predictors of cGVHD. Mean \pm sd values for each variable shown. **h** Treg and RTE frequencies in peripheral blood samples from patients ≥ 60 days post-HSCT were correlated for patients developing cGVHD ($n = 7$) and patients remaining cGVHD-free ($n = 9$). Correlations for each group were analyzed by linear regression. Linear regression model slopes were compared by sum-of-squares analysis. **i** Peripheral blood samples from day 60 post-transplant were grouped based upon the presence ($n = 7$) or absence ($n = 9$) of ≥ 200 RTEs/ 10^4 CD4⁺ T cells and ≥ 1 Treg/ μ l blood and subsequent cGVHD development was monitored. Data presented as Kaplan–Meier survival curve with log-rank analysis

The emergence of differences in Treg frequencies between 60 and 90 days post-transplant suggested that this may be related to thymopoiesis, which begins in a similar time period after HSCT [9, 10]. To examine whether Tregs were of thymic origin, we measured expression of Helios, a transcription factor associated with Tregs generated by thymopoiesis [11], in Tregs present after 60 days post-transplant. The percentage of Tregs expressing Helios in patients with cGVHD and cGVHD-free patients was similar and consistent with reported frequencies in healthy adults [11] (Figure S2). However, it is established that Helios expression is not limited to thymically-derived Tregs, and thus may not adequately define the relationship between thymopoiesis and Tregs [11].

To examine thymopoiesis, we measured frequencies of CD4⁺CD45RA⁺CD31⁺PTK7⁺ recent thymic emigrant (RTE) T cells in peripheral blood. Several markers, including CD45RA, CD31, and PTK7 have been implicated as identifying human RTEs [12–14]. To ensure accurate identification of RTEs, we first validated the flow cytometry strategy by quantifying T cell receptor excision circles (TRECs), small extrachromosomal circular DNA fragments generated by T cell receptor gene rearrangement during thymic development [15] in putative RTEs defined by expression of CD45RA, CD31, and PTK7. Using a real-time PCR assay, we observed the absence of TRECs in CD4⁺CD45RO⁺ cells and confirmed the highest-TREC concentrations among CD4⁺CD45RA⁺ cells in CD31⁺PTK7⁺ cells (Figure S3).

Using this flow cytometry-based strategy, we enumerated RTEs in longitudinal samples beginning 30 days after transplantation (Fig. 1d-f). Patients that subsequently developed cGVHD demonstrated persistently low frequencies of RTEs as compared to patients that remained cGVHD-free. RTE production in cGVHD-free patients began to increase at 60 days post-transplant, with continuing increases throughout the study duration. Multivariate analysis of all measured parameters of T cell reconstitution (ALC, peripheral numbers of CD4⁺ cells, CD8⁺ cells, and Tregs, and RTE frequency) indicated that RTE frequency was independently predictive of freedom from cGVHD (Fig. 1g). These data support the hypothesis that restoration of a functionally self-tolerant T lymphocyte compartment after HSCT requires effective thymopoiesis.

Given data indicating that both Tregs and thymic activity correlated with freedom from cGVHD, we examined their correlation. Among all patients, peripheral-blood Treg numbers did not correlate strongly with thymic function as evidenced by RTE frequency (Fig. 1h-i). However, patients that remained cGVHD-free for the study duration demonstrated a significantly different relationship between Treg numbers and RTE frequency compared to patients that developed cGVHD (comparison of slopes of linear-

regression models, $P=0.008$). Patients who did not develop cGVHD demonstrated positive correlation between thymic function and increased numbers of Tregs, unlike patients developing cGVHD. This further indicated that the presence of relatively high numbers of Tregs in the absence of thymic function, or conversely high frequencies of RTEs without restoration of the Treg compartment was insufficient to restore a self-tolerant immune system and protect from cGVHD development. Demonstration of a relationship between Treg restoration and RTE frequency among patients remaining free from cGVHD, prompted us to evaluate whether either RTE frequency (≥ 200 RTEs/ 10^4 CD4⁺ T cells), Treg abundance (≥ 1 Treg/ μ l blood), or both were predictive of cGVHD development. While not reaching statistical significance ($P=0.070$), patients with restoration of the Treg compartment and increased thymic function by 60 days post-transplant ($n=7$) were more likely to remain cGVHD-free compared to patients who did not ($n=10$).

Our data indicate that both Treg numbers and effective thymopoiesis are important factors related to protection from cGVHD. The distinct correlations between Tregs and RTEs in patients with and without cGVHD, as well as multivariate analysis indicating that Treg frequency in the absence of effective thymopoiesis is not sufficient to protect from cGVHD, provides an important clue of a potential mechanistic explanation for the conflicting descriptions of the associations between Treg frequency and cGVHD [1, 3, 5, 7]. Naive phenotype CD45RA⁺ Tregs have been specifically identified as associated with freedom from GVHD [4], supporting the concept that donor-derived Tregs and Tregs generated de novo after HSCT may provide differential contribution of self-tolerance.

While our data indicate that Tregs and RTEs are potentially useful biomarkers for prediction of cGVHD, limitations in the study bear consideration. Foremost, our study is of a relatively small number ($n=19$) of patients. Study inclusion was limited by the number of pediatric transplants performed at our center, as well as the need for extended clinical follow-up. Additionally, our small sample size precluded precise definition of optimal diagnostic cutoff values for potential clinical assays. Validation of the utility of Treg and RTE enumeration in cGVHD prognosis will require a larger-validation cohort. Additionally, while our data demonstrate correlation between Treg reconstitution, post-transplant thymopoiesis, and protection from cGVHD, they do not necessarily prove causality. The low frequencies of Tregs and RTEs, as well as the limited utility of markers such as CD31 and Helios in identifying thymic-derived Tregs, prevented us from directly assessing this question. Additionally, it is not possible to fully disentangle the efficacy of post-HSCT thymopoiesis from the effects of GVHD, particularly thymic GVHD, which likely has

significant consequences for subsequent thymopoiesis. Future studies utilizing methods such as single-cell RNA sequencing and animal models of HSCT will be necessary to understand the mechanistic relationship between post-transplant thymopoiesis, Treg reconstitution, and immunologic self-tolerance protecting against cGVHD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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