Reduced Frequency of FOXP3+ CD4+CD25+ Regulatory T Cells in Patients with Chronic Graft-Versus-Host Disease

Short Title: Regulatory T Cells in cGVHD

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Abbreviations: Treg, Regulatory T Cell; HSCT, Hematopoietic Stem Cell Transplantation; cGVHD, chronic Graft-Versus-Host Disease; TREC, T-Cell Receptor Excision Circle;

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Abstract

Chronic graft-versus-host disease (cGVHD) is a major complication of allogeneic hematopoietic stem cell transplantation but the immune mechanisms leading to the diverse clinical manifestations of cGVHD remain unknown. In this study, we examined regulatory T cells (T_{reg}) in 57 transplant recipients (30 with cGVHD and 27 without active cGVHD) and 26 healthy donors. Phenotypic studies demonstrated decreased frequency of CD4+CD25+ T cells in patients with cGVHD compared to patients without cGVHD (p<0.0001) and healthy individuals (p<0.0001). Gene expression of T_{reg} transcription factor FOXP3 was reduced in cGVHD patients compared to patients without cGVHD (p=0.009) or healthy donors (p=0.01). T-cell-receptor-excision-circle (TREC) assays for the evaluation of thymus activity revealed fewer TREC in both transplant groups compared to healthy donors (p<0.001 and p=0.02, respectively) although no difference was observed between patients with or without cGVHD (p=0.13). When tested in functional assays, T_{reg} from both patient cohorts and normal individuals mediated equivalent levels of suppression. Collectively, these studies indicate that patients with active cGVHD have reduced frequencies of T_{reg} but the function of these cells remains normal. These findings support the development of new strategies to increase the number of T_{reg} following allogeneic HSCT to prevent or correct cGVHD.
Introduction

Chronic graft versus host disease (cGVHD) is a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT)\(^1\)-\(^3\). In most studies, between 30 and 75% of patients who survive after day-100 develop some clinical manifestations of cGVHD\(^1\). Chronic GVHD occurs following engraftment of HLA-identical or HLA-mismatched stem cells and after non-myeloablative and ablative conditioning. Chronic GVHD is presumably mediated primarily by effector T cells and increasing degrees of HLA disparity between recipient and donor contribute to the incidence and severity of disease. However, the pathophysiology of cGVHD remains poorly understood. A paradoxical hallmark of cGVHD is the concurrent manifestation of autoimmunity, alloimmunity and immunodeficiency\(^1\)-\(^3\). These clinical observations suggest that systemic dysregulation of the immune system may play a significant role in this disease.

CD4+CD25+ regulatory T cells (T\(_{\text{reg}}\)) are naturally occurring T cells whose function is to suppress autoreactive lymphocytes and control normal immune responses\(^4\),\(^5\). T\(_{\text{reg}}\) develop in the thymus and in mouse models, depletion of this subset of cells leads to proliferation of normal lymphocytes and autoimmune destruction of various tissues and organs\(^6\). Allogeneic HSCT is known to affect thymic function and the reconstitution of T cell lineages. Poor reconstitution of the T\(_{\text{reg}}\) subset after HSCT could lead to the inability to suppress autoreactive and alloreactive immune cells and contribute to cGVHD. The ability of T\(_{\text{reg}}\) to delay the onset of acute GVHD or reverse established GVHD has previously been reported in several murine models\(^7\)-\(^12\). In humans, recent investigations of T\(_{\text{reg}}\) after HSCT have generated conflicting results. One phenotypic study
suggested that cGVHD is associated with higher absolute numbers of circulating CD4+CD25+ T cells\textsuperscript{13}. In contrast, molecular studies using quantitative PCR demonstrated lower expression levels of the T\textsubscript{reg} specific transcription factor FOXP3 in patients with both acute and chronic GVHD\textsuperscript{14}. Another study found that CD4+CD25+ T\textsubscript{reg} cells were significantly reduced relative to the number of OX40+ activated cells in patients with cGVHD\textsuperscript{15}.

Using phenotypic, molecular and functional methods, we examined CD4+CD25+ T\textsubscript{reg} cells in 30 patients with cGVHD after allogeneic HSCT, 27 patients without active cGVHD and 26 healthy controls. The diagnosis of cGVHD was established using clinically and histologically accepted criteria\textsuperscript{16}. Both phenotypic and molecular assays indicated that patients with active cGVHD have a reduced frequency of circulating T\textsubscript{reg} cells compared to patients without active cGVHD and healthy controls. However, when isolated in vitro, T\textsubscript{reg} from patients and normal individuals appeared to exhibit similar levels of immune suppressive function. We also assessed thymic activity in patients and controls using a quantitative PCR assay for T cell receptor excision circles (TREC). The 2 patient groups had lower TREC values compared to healthy donors indicating that allogeneic HSCT substantially reduces thymic activity and this may contribute to T\textsubscript{reg} imbalance in these patients.
Patients, Materials and Methods

Patients and samples

All patients included in this study were enrolled in clinical protocols approved by the Dana-Farber/Harvard Cancer Center Investigational Review Board. Details of patient clinical history, treatment, HSCT outcome and symptoms of cGVHD are summarized in Table 1. Written informed consent was obtained from each patient prior to sample collection. Patient PBMC were isolated from blood samples by density gradient centrifugation and cryopreserved in aliquots before being analyzed.

Flow cytometry

Thawed PBMC were analyzed by flow cytometry using a series of monoclonal antibodies (Beckman Coulter, Fullerton CA) including anti-CD3-PC5, anti-CD4-PE, anti-CD8-FITC and anti-CD25-FITC (clone 1H4T4H3). The frequency of each cell subset was calculated as a percentage of positive cells in the total lymphocyte gate.

Quantitative PCR for FOXP3 expression

Total RNA was extracted from PBMC using Trizol reagent according to the manufacturer’s guidelines (INVITROGEN, Carlsbad, CA). Complementary DNA was then prepared using 2μg of RNA using the Superscript III cDNA first-strand synthesis kit (INVITROGEN). Real-time PCR was performed in triplicates using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the following primer pairs: FOXP3-forward 5’-TGCCTCCTCTTTCTTTGAAC-3’, FOXP3-reverse 5’-GGGCGTGGGCATCCA-3’;

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GAPDH-forward 5’-CCACCCATGGCAAAATTCC-3’, GAPDH-reverse 5’-GATGGGATTTCATTGATGACA-3’ on a ABI 7700 Sequence Detector (Applied Biosystems). Aliquots of a standard cDNA prepared from PBMC collected from a healthy donor were used as standards in each plate to normalize for inter-plate variability. Results for the expression of FOXP3 were normalized using GAPDH signals, linearly transformed and readjusted to the percentage of PBL in total PBMC. The final value reflects the expression level of FOXP3 within total PBL.

*TREC assay*

A Taqman quantitative PCR method was used to quantify TCR rearrangement excision circles (TREC) in genomic DNA extracted from patient and donor samples. The method has been described in detail elsewhere\(^\text{17}\). Briefly the method consists of amplifying TREC with specific primers (sense 5’-CGTGAGAACGTTGAATGAGACAGACA-3’, antisense 5’-CATCCCTTTCAACCATGCTGACCTCT-3’) in the presence of an internal fluorochrome/quencher-labeled probe (5’-VIC-TTTTTGTAAAGGTGCCCACCTCTGTGCACGGTGTA-TAMRA-3’). Each PCR reaction was performed in a 50µL volume containing 0.075µg genomic DNA, 1X Taqman buffer A (Perkin Elmer Cetus Institute), 3mM MgCl2, 300nM each primer, 100nM probe, 200nM dATP, 200nM dCTP, 200nM dGTP, 400nM dUTP, 17 units UNG, and 2 units AmpliTaq Gold DNA polymerase (Perkin Elmer). A series of standard dilutions of plasmid containing the signal-joint breakpoint was used to quantify TREC in each patient and control DNA sample. Each patient and control DNA sample was run in triplicate on a 96-well plate along with the dilution series of
the TREC plasmid. Data were readjusted based on the percentage of CD3+ T cells in total PBMC and calculated as number of TREC per $10^5$ CD3+ cells.

$T_{reg}$ suppression assays

Suppression of anti-viral T cell reactivity was evaluated using ELISPOT assays for the secretion of IFNγ. Briefly, $10^5$ PBMC obtained from cGVHD patients were incubated in duplicate cultures in multiscreen IP-microplates coated with anti-IFNγ mAB (Mabtech, Mariemont OH) in the presence of $10\mu$g/ml of pooled common viral antigenic peptides presented by frequent HLA class-I molecules. Anti-viral T cell reactivity was assessed before and after complete depletion of CD25+ cells using anti-CD25 microbeads (Miltenyi). After overnight incubation, plates were successively labeled with a biotinylated anti-IFNγ secondary mAB (Mabtech) followed by streptavidin alkaline phosphatase (Mabtech). Spots were revealed using nitroblue tetrazolium and brom-chlor-indolyl phosphate (Promega). Percent inhibition due to the CD25+ cells was calculated as follows:

$$\% \text{ inhibition} = \frac{# \text{ spots using CD25 depleted cells} - # \text{ spots using undepleted cells}}{# \text{ spots using CD25 depleted cells}} \times 100$$

Suppression of T cell proliferation by $T_{reg}$

Standard assays for the suppression of proliferation were performed as previously described with minor modifications. Briefly, CD4+CD25- and CD4+CD25+ cells were purified from patient and donor PBMC using the Miltenyi magnetic separation system according to the manufacturer’s instructions. $1 \times 10^4$ CD4+CD25- responder cells/well were incubated either alone or in the presence of $1 \times 10^4$ CD4+CD25+ $T_{reg}$ cells/well (ratio 1:1) in round bottom microplates coated
with OKT3 (1µg/ml) in the presence of 1x10⁴ irradiated allogeneic PBMC as feeder cells. After 6 days, cells were pulsed overnight with ³H thymidine. Incorporation of radioactivity was measured using a scintillation counter. Percent inhibition of proliferation was calculated as follows:

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\% \text{ Inhibition} = \frac{(\text{cpm responder cells alone} - \text{cpm responder/T}_{\text{reg}})}{\text{cpm responder alone}} \times 100
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**Statistical methods**

For 2x2 table analysis, a two-sided Fisher’s exact test was performed. For two-sample comparison of continuous variables, a two-sided Wilcoxon-Rank-Sum test was performed. Pairwise group comparisons are not adjusted for multiple comparisons. Measure of correlation was calculated and tested using the Spearman rank method. Smoothing spline curve estimation technique was used to characterize the pattern of correlation. Several statistical approaches were explored to establish cut-off points for %CD4+CD25+. These approaches included taking the 95th or 99th percentile value, taking the mean +2*STD value from the log-transformed distribution and a classification and regression trees. The cut-off point of 3.0% was established from these approaches. Linear and logistic regression analyses for T_{reg} were performed to adjust differences in patient baseline characteristics between patients with cGVHD and without cGVHD.
Results

Patients

Fifty-seven patients and 26 healthy donors were analyzed in this retrospective study. Patient characteristics are summarized in Table 1. Thirty patients had active mild (n=23), moderate (n=6) or severe (n=1) cGVHD at the time of sample collection. These clinical grades were assigned by one author (SJL) blinded to T_{reg} results using criteria of the Center for International Blood and Marrow Transplant Research (CIBMTR). Patients with cGVHD were studied at a median of 24 months after transplant and 29 of 30 were receiving immunosuppressive drugs. Patients without active cGVHD were studied at a median of 19 months after transplant and only 12 of 27 were receiving immune suppressive agents at this time. Sixteen of 30 patients with active cGVHD had previously had grade 2-4 acute GVHD but only 5 of 27 patients without active cGVHD had a history of prior acute GVHD (p=0.01). Within the group of patients without active cGVHD, 12 had previously had chronic GVHD that had resolved by the time of analysis for this study. Post-transplant GVHD prophylaxis consisted of cyclosporine/prednisone/mycophenolate mofetil or tacrolimus/methotrexate ± rapamycin. Twenty six of 30 patients with cGVHD and 10 of 27 patients without active cGVHD (p=0.0001) had received bone marrow stem cell grafts. All other patients received G-CSF mobilized peripheral blood stem cells. All 26 normal donors were adults.

Decreased frequency of CD4+CD25+ T cells associated with cGVHD

Percentages of CD4+CD25+ in total PBL were determined by flow cytometry to evaluate the frequency of T_{reg} relative to other blood lymphocytes. As shown in Figure 1, patients with
cGVHD had significantly lower percentages of CD4+CD25+ in total PBL compared with patients without active cGVHD (p=0.007). The median value for the cGVHD group was also lower than that observed for healthy donors however this difference did not reach statistical significance (p=0.29). Patients without active cGVHD also had significantly higher CD4+CD25+ values than the control population (p=0.0005). Interestingly, both patient groups had decreased percentages of CD3+CD4+ in total PBL compared to healthy individuals (p<0.0001, data not shown). These results support the view that CD4+CD25+ cells are independently regulated among total CD3+CD4+ cells. Because of the limited number of patients with moderate or severe cGVHD, we were not able to evaluate the association of CD4+CD25+ cells with severity of cGVHD.

Since CD25 is not a unique marker of T_{reg} and can also be expressed on activated T cells, the wide range of percent CD4+CD25+ values shown in Figure 1 could reveal the presence of effector rather than regulatory T cells in some samples. We therefore considered alternative methods of comparing different groups. Based on the distribution of the normal donors, we established a cut-off point of 3.0 to use in comparing different groups. As seen in Table 2, using this cut-off point, 50% (15/30) of patients with cGVHD had low %CD4+CD25+ (≤3.0%) whereas only 3.7% (1/27) of patients without active cGVHD (p<0.0001) and 8% (2/26) of normal individuals had low %CD4+CD25+ (p<0.0001). As the stem cell source, prior acute GVHD and frequency of related donors appeared to be different between patients with cGVHD and patients without cGVHD, we performed a linear regression model for %CD4+CD25+ on continuous scale and a logistic model for dichotomous %CD4+CD25+ (>3.0% vs. <=3.0%) after adjusting all these factors and age but excluding patient treatment. Immunosuppressive therapy
was not adjusted because nearly all patients with cGVHD received immunosuppressive drugs. Active cGVHD was the only significant factor for lower frequency of CD4+CD25+ in PBL (p=0.01 from a logistic regression model and p=0.049 from a linear regression model). There was no correlation between the time of sample collection post-transplant (p=0.29) or sex matching (p=0.29). Likewise there was no correlation between %CD4+CD25+ and the source of stem cells among patients without cGVHD (p=0.82).

**Decreased expression of FOXP3 in PBL from patients with cGVHD**

To assess T\(_{\text{reg}}\) in patient and donor samples we also measured the expression of the T\(_{\text{reg}}\) specific transcription factor FOXP3 using a quantitative PCR assay. Figure 2A summarizes the expression of FOXP3 transcripts in PBL for both patient groups and healthy donors. Patients with active chronic GVHD have significantly lower expression of FOXP3 when compared to patients without cGVHD (p=0.009) or healthy donors (p=0.01). As with phenotypic results, occurrence of cGVHD was the only risk factor for lower levels of FOXP3 when all other factors such as age, source of stem cell, grade 2-4 acute GVHD, and donor type but excluding immunosuppressive therapy were adjusted in a linear model (p=0.02). There was no correlation between FOXP3 values and time of sample collection (p=0.62) or sex matching (p=0.92). In addition we did not observe any correlation between FOXP3 expression and the source of stem cells in patients without cGVHD (p=0.40). We next examined whether phenotypic assessment of CD4+CD25+ T cells correlated with molecular assessment of FOXP3 in samples from patients with cGVHD. As shown in Figure 2B, there is a significant correlation between percentages of circulating CD4+CD25+ T cells determined by flow cytometry and the level of FOXP3 transcripts measured by quantitative PCR. We confirmed the correlation using a rank-based
Spearman’s Correlation test ($r_s=0.60$, $p=0.0007$). Acute GVHD (aGVHD) is a predominant risk factor for cGVHD. We next analyzed whether FOXP3 expression correlated with cGVHD independently of previous occurrence of aGVHD. As shown in Figure 2C, the lowest levels of expression of FOXP3 were observed in patients who had cGVHD after aGVHD. Remarkably, patients with de novo cGVHD (no previous aGVHD) had reduced FOXP3 expression levels compared to patients without acute or chronic GVHD. These results indicate that reduced T$_{reg}$ cells in these patients correlates with the development of cGVHD independently of aGVHD.

As mentioned previously, within the no-cGVHD group 12 patients previously had cGVHD that had resolved at the time of sample collection. We compared flow cytometric and molecular T$_{reg}$ measurements for these patients to those who had never developed cGVHD. As reported in Table 3, there was no significant difference between the 2 subgroups, strongly suggesting that resolution of cGVHD is accompanied by a readjustment of T$_{reg}$ values towards normal values.

**Inverse correlation between CD4+CD25+ T$_{reg}$ and CD3+CD8+ T cells**

In addition to CD4+CD25+ T$_{reg}$, we also examined other lymphocyte populations in all samples from patients and healthy controls. Effector T cells are predominately CD3+CD8+ T cells and phenotypic studies revealed a highly heterogeneous distribution of CD3+CD8+ values for the cGVHD group (Figure 3 A). In contrast, patients without cGVHD showed little variation of CD3+CD8+ T cells compared to healthy individuals. As shown in Figure 3 B, we found a striking inverse correlation between the percentages of CD4+CD25+ and CD3+CD8+ T cells in samples from patients with cGVHD ($r_s= -0.81$, $p<0.0001$), providing further evidence for a physiological interaction between these 2 subpopulations. This correlation was also highly
significant when results from all patients were combined and analyzed (p<0.0001, data not shown). Remarkably, there was no correlation between percent CD3+CD8+ cells and total CD3+CD4+ cells in these patient samples (data not shown).

**Patient CD4+CD25+ T_{reg} frequency correlates with their suppressive function**

Based on the strong correlation between CD4+CD25+ and CD3+CD8+ T cells, we developed an in vitro assay to measure the suppressive function of patient T_{reg} on autologous antigen specific CTL responses. In this assay, patient PBMC are stimulated with a panel of 17 previously characterized class-I restricted viral epitopes from CMV, EBV and influenza. Specific CTL reactivity is measured using an IFN\(\gamma\) secretion ELISPOT assay before and after depletion of CD25+ T_{reg}. Depletion of CD25+ regulatory T cells increases the number of effector T cells responding to the pool of viral peptides. The difference between the reactivity obtained in the presence and absence of CD25+ cells reflects the level of suppression of anti-viral CTL due to T_{reg}. Figure 4A shows the results obtained using samples from 15 patients with cGVHD tested in this assay. In these experiments, percent inhibition due to the presence of CD25+ cells was calculated to be between 0% (2 samples) and 59.2%. Figure 4B summarizes the inhibitory activity measured for these 15 patients with cGVHD plotted along with the percentages of CD4+CD25+ T cells in these samples. As shown in this Figure, there is a significant correlation between T_{reg} frequencies measured by flow cytometry and their suppressive function measured in this in vitro assay (p=0.02; r_s=0.59).
Patient $T_{reg}$ cells exert normal levels of suppression

The previous series of experiments established a correlation between the number of $T_{reg}$ and their level of suppressive activity. We next investigated whether $T_{reg}$ isolated from patients with or without cGVHD as well as from healthy donors displayed similar levels of suppressive activity. Using antibody coated magnetic particles, CD4+CD25+ $T_{reg}$ were purified from patients and donors and tested for their capacity to inhibit the proliferation of autologous CD4+CD25- T cells stimulated with anti-CD3. Results obtained from 5 patients with cGVHD, 7 patients without cGVHD and 5 controls are shown in Figure 5. Although this analysis was limited by the number of samples available, we could not detect any significant differences between the suppressive activities of $T_{reg}$ cells isolated from patient and donor groups. These findings suggest that $T_{reg}$ isolated from cGVHD patients exert similar levels of suppression as $T_{reg}$ purified from patients without cGVHD or healthy donors.

Decreased thymic activity following allogeneic HSCT

To examine thymic activity, TREC copy numbers in DNA extracted from patient and control samples were measured using quantitative RT-PCR. As shown in Figure 6, patients with or without chronic GVHD showed significant decrease in TREC compared to healthy donors ($p<0.001$). These data support previous reports that thymic function is substantially impaired following allogeneic HSCT. However, in contrast to other published studies we could not find any significant difference between the 2 patient groups$^{20,21}$. In addition, there was no correlation between TREC values and either %CD4+CD25+ cells or FOXP3 levels (data not shown).
Discussion

Several recent studies in murine models have demonstrated the critical role of $T_{\text{reg}}$ in controlling GVHD after allogeneic HSCT\textsuperscript{7-12}. In humans, it is still unclear whether impairment of $T_{\text{reg}}$ populations post-transplant contributes to the development of acute or chronic GVHD. Thus far, only 3 studies have examined $T_{\text{reg}}$ following allogeneic HSCT, using either phenotypic or molecular techniques and these studies have generated conflicting results\textsuperscript{13-15}. To address this issue we performed a retrospective analysis of $T_{\text{reg}}$ following allogeneic HSCT using independent phenotypic markers as well as functional assays. Moreover, we focused entirely on chronic GVHD and results in patients with active clinical manifestations of disease were compared to patients without active disease as well as healthy controls.

$T_{\text{reg}}$ are described through their capacity to suppress other immune cells including CD4+CD25- T cells, CD3+CD8+ T cells as well as NK cells\textsuperscript{22-26}. Based on the wide range of $T_{\text{reg}}$ target immune cells we chose to examine the frequency of CD4+CD25+ cells over total lymphocytes as a quantitative measurement of these cells. Our approach to analyze $T_{\text{reg}}$ populations differed from that of Clark et al. who examined absolute CD4+CD25+ cell counts in the blood of patients with cGVHD. Remarkably, the 2 different methods generated conflicting results. In contrast to absolute numbers, we believe frequency measurements reflect the ratio of $T_{\text{reg}}$ to the total number of cells to be regulated and thus represent a more accurate reflection of the function of these cells in vivo. Most assays used to demonstrate the suppressive activity of $T_{\text{reg}}$ in vitro are based on the same principle of measuring function for defined regulatory/responder cell ratios. In addition, we confirmed our flow cytometry results by assessing FOXP3 gene expression as a complementary method to measure $T_{\text{reg}}$. 
IL-2 plays a critical role in the development and maintenance of T_{reg} and the constitutive expression of IL-2 receptor α chain (CD25) is widely used as a phenotypic marker of these cells. However, CD25 is expressed on other functional T lymphocytes upon activation and is therefore not specific to T_{reg}. Accordingly, we considered whether the CD25+ cells identified by flow cytometry in some transplant patients could represent activated effector T cells as well as T_{reg}. This was consistent with the heterogeneous number of CD4+CD25+ cells found in the cGVHD patient group. In our studies, analysis of dichotomized populations underscored the observation that a large fraction of cGVHD patients (50%) had low values (≤3%), which necessarily revealed reduced T_{reg}. The fact that we observed such low values almost exclusively in patients with active cGVHD supports our main hypothesis. Interestingly, even if CD4+CD25+ cell frequencies were respectively low and high for patients with or without cGVHD compared to healthy donors, percentages of total CD4+ T cells were lower for both patient groups. This indicates that frequency of CD4+CD25+ does not follow values for total CD4+ cells and further suggests that T_{reg} constitute an independently regulated T cell subset among CD4+ cells.

In several independent studies, the expression of FOXP3 gene has been associated with the functional suppressive capacity of T_{reg}. Our results, showed decrease in FOXP3 levels in cGVHD patients compared to control groups confirming a quantitative defect in T_{reg} populations. The correlation between the expression of FOXP3 and the number of CD4+CD25+ cells in cGVHD patient samples was also instrumental in validating both parameters for the detection of T_{reg}. As T_{reg} may be composed of distinct subsets of cells expressing different markers, the use of complementary methods is better suited to quantify these regulatory cells in patient blood
samples. Although apparently moderate, the difference in both molecular and phenotypic $T_{\text{reg}}$ values was statistically significant. On average, $T_{\text{reg}}$ frequency was reduced by half in patients with cGVHD compared with control patients. Likewise median for FOXP3 values in patients with cGVHD was approximately half the median for the no cGVHD group. We believe this difference to be physiologically relevant. Naturally occurring $T_{\text{reg}}$ are present at low frequency in the blood of normal individuals. Yet their critical role in controlling a wide range of immune responses has been demonstrated in many model systems. Even a modest decrease in the pool size of these cells can have considerable implications for the balance of immune responsiveness post-transplant. Similar levels of reduction have also been reported in other clinical studies in different diseases$^{31,32}$.

CD3+CD8+ cytolytic T cells are important effector cells in both cute and chronic GVHD$^{33-39}$. In our studies, the frequency of CD3+CD8+ cells in PBL was inversely correlated with the frequency of CD4+CD25+ cells, suggesting a direct physiological relation between $T_{\text{reg}}$ and CTL. This association might reflect the balance of the post-transplant immune response tipping between 2 extremes: Increased $T_{\text{reg}}$ associated with decreased cytolytic effector T cells, corresponding to a controlled immune response, or decreased $T_{\text{reg}}$ associated with larger numbers of CTL, resulting in escalating immune responses. Further studies will be required to confirm this notion.

In addition to phenotypic analysis, we undertook a functional assessment of $T_{\text{reg}}$ in our patients and donor samples. In most studies, the functional suppression of immune responses has correlated well with the phenotypic assessment of $T_{\text{reg}}$. However, there are also well-documented
examples of phenotypically normal T\textsubscript{reg} that exhibit impaired suppressive function\textsuperscript{19}. Our experiments confirmed that the overall suppression activity is primarily a function of the percentage of T\textsubscript{reg} in the blood in cGVHD patients. Additional functional assays indicated that T\textsubscript{reg} isolated from both patient groups exhibited comparable suppressive capabilities as T\textsubscript{reg} purified from healthy donors. Although we could only perform this assay with a small number of samples, these findings confirm a previous report by Clark et al. demonstrating that T\textsubscript{reg} from cGVHD patients are not impaired in their suppressive function\textsuperscript{13}.

Almost all cGVHD patients as well as a significant number of patients without cGVHD had received various combinations of immunosuppressive drugs. From our data we cannot entirely exclude the possibility that these treatments affected the frequency and function of T\textsubscript{reg} populations in vivo. However there was no significant difference in values for either the phenotypic or the molecular T\textsubscript{reg} marker (p=0.26 and p=0.9 respectively) between patients treated with immunosuppressive drugs and patients not treated within the no-cGVHD group. This demonstrated that immunosuppressive medication alone cannot explain reduction in T\textsubscript{reg} frequencies. Moreover, results from our cell-based assays did not show any significant variations in T cell function that could reflect such modulation. On the contrary, cells isolated from patients with or without cGVHD as well as control healthy donors exhibited similar levels of reactivity in vitro even though all cGVHD patients were receiving immune suppressive therapy at the time of analysis.

Our comprehensive series of experiments provide substantial evidence that active cGVHD correlates with decreased T\textsubscript{reg} populations after allogeneic HSCT but not impaired function of
these cells. The cause of the T_{reg} reduced frequency remains unclear. Previous development of acute GVHD is well recognized as a major risk factor for cGVHD and could have contributed to immune imbalance or thymic injury and subsequent T_{reg} quantitative defects. However, patients with “de novo” cGVHD also had reduced T_{reg} frequency, indicating that the defect is independent of previous aGVHD. Alternatively, it is conceivable that lower T_{reg} frequency results from a poor reconstitution of T cell lineages in transplant recipients. Following HSCT, T cell lineages are reconstituted through combined expansion of mature peripheral cells and the export of new T cells from the thymus. Reduced thymic activity after HSCT would result in impaired reconstitution of T_{reg} as well as naïve T cells. However, in our samples we could not detect any association between TREC values, T_{reg} frequency and cGVHD. Regardless of their GVHD status, all transplant patients had lower TREC copy numbers. Remarkably, the TREC assay we used in these experiments measures the abundance of newly differentiated T cells among all CD3+ cells in the periphery without any distinction between effector and T_{reg}. Therefore, this assay does not account for any defined subset of T cells. Further studies will be required to assess the reconstitution of specific T cell subpopulations, and especially T_{reg}, following allogeneic HSCT. Likewise longitudinal follow-up of transplant patients will be required to establish whether improved T_{reg} reconstitution correlates with the resolution of active cGVHD and a better clinical outcome. Nevertheless our findings give further support for new treatment aimed at reconstituting or enhancing T_{reg} populations in an attempt to correct cGVHD in patients with active disease.
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References

**Figure Legends**

**Figure 1, Decreased frequency of circulating CD4+CD25+ T cells in patients with cGVHD.**
Percent CD4+CD25+ in PBL was measured by flow cytometry in patient and donor samples. Values represent total positive cells including CD4+ CD25+dim and CD25+high cells. Box plots define the values for median, range, 25th and 75th percentiles. One outlier with a value of 37.5% in the group of cGVHD patients is not shown on the figure. P-values were calculated using the Wilcoxon-rank-sum test; cGVHD-vs-no cGVHD, p=0.007; cGVHD-vs-healthy donors, p=0.29; No cGVHD-vs-healthy donors, p=0.0005.

**Figure 2, Decreased expression levels of FOXP3 in patient with cGVHD**
A, Expression levels of the T_{reg} specific transcription factor FOXP3 was assessed by quantitative PCR in patient and donor samples, normalized and reported as a function of total lymphocytes (AU, arbitrary units). Box plots define the values for median, range, 25th and 75th percentiles. P-values were calculated using the Wilcoxon-rank-sum test; cGVHD-vs-no cGVHD, p=0.009; cGVHD-vs-healthy donors, p=0.01; No cGVHD-vs-healthy donors, p=0.34. B, Correlation between CD4+CD25+ phenotypic values and FOXP3 expression levels in patients with cGVHD was calculated using a rank-based Spearman’s test (p=0.0007; r_s=0.60). C, cGVHD patients were subdivided according to whether they previously developed aGVHD. Box plots define the values for median, range, 25th and 75th percentiles. P-values were calculated using the Wilcoxon-rank-sum test; aGVHD & cGVHD-vs-no aGVHD & cGVHD, p=0.29; aGVHD & cGVHD-vs-no aGVHD & no cGVHD, p=0.009; no aGVHD & cGVHD-vs-no aGVHD & no cGVHD, p=0.005.
Figure 3, Correlation between CD3+CD8+ and CD4+CD25+ T cells in active cGVHD

A, percent CD3+CD8+ in PBL was measured by flow cytometry in patient and donor samples. Lines represent median values. B, percent CD4+CD25+ and CD3+CD8+ T cells in total PBLs are shown for cGVHD patients. Correlation between the 2 variables was calculated using a rank-based Spearman’s test ($r_s=-0.81$, p<0.0001).

Figure 4, Percent CD4+CD25+ in PBL correlates with $T_{reg}$ suppressive function in cGVHD

A, CTL reactivity against a pool of class-I restricted common viral peptides was assessed by IFNγ ELISPOT assays before and after CD25+ T cell depletion using 15 samples collected from cGVHD patients. Depletion of CD25+ cells was confirmed by flow cytometry for each sample. B, percent inhibition was measured for 15 cGVHD patients and values are plotted together with percent CD4+CD25+. One sample generated a negative value and was interpreted as null. Correlation between the 2 variables was calculated using a rank-based Spearman’s test ($r_s=0.59$, p=0.02).

Figure 5, Patient $T_{reg}$ express normal levels of suppression

CD4+CD25+ $T_{reg}$ were immuno-purified from PBMC and assessed for their capacity to suppress the proliferation of autologous CD4+CD25- cells at a responder/$T_{reg}$ ratio of 1/1. Percent inhibition of responder cell proliferation due to the presence of $T_{reg}$ was measured using 17 samples collected from 5 cGVHD patients, 7 patients without cGVHD and 5 healthy individuals. The solid lines represent the median values.
Figure 6, Allo-transplant patients have impaired thymus function

Thymic activity was assessed in patient and donor samples using the TREC assay and represented as TREC copy number per $10^5$ CD3+ T cells. Box plots define the values for median, range, 25th and 75th percentiles. One outlier with a value of 1292 in the group of no-cGVHD patients is not shown on the figure. P-values were calculated using the Wilcoxon-rank-sum test; cGVHD-vs-no cGVHD, $p=0.13$; cGVHD-vs-healthy donors, $p<0.001$; No cGVHD-vs-healthy donors, $p=0.02$. 


Table 1. Patient characteristics

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<tr>
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<tr>
<td>Median age (years)</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
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<tr>
<td>M</td>
<td>17</td>
<td>12</td>
<td>0.43</td>
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<tr>
<td>F</td>
<td>13</td>
<td>15</td>
<td></td>
</tr>
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</tr>
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<tr>
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<td>Range (mo.)</td>
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<td>5-127</td>
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Table 2. CD4+CD25+ $T_{reg}$ in peripheral blood

<table>
<thead>
<tr>
<th></th>
<th>cGVHD</th>
<th>No cGVHD</th>
<th>Healthy Donors</th>
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</thead>
<tbody>
<tr>
<td>CD4+CD25+/PBL $\leq$ 3%</td>
<td>50.0% (15/30)</td>
<td>3.7% (1/27)</td>
<td>7.7% (2/26)</td>
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<tr>
<td>CD4+CD25+/PBL $&gt;$ 3%</td>
<td>50.0% (15/30)</td>
<td>96.3% (26/27)</td>
<td>92.3% (24/26)</td>
</tr>
</tbody>
</table>

cGVHD vs. No cGVHD: p<0.0001

cGVHD vs. Healthy donors: p<0.0001

No cGVHD vs. Healthy donors: p=0.61
Table 3. Phenotypic and molecular $T_{\text{reg}}$ markers in patients without cGVHD

<table>
<thead>
<tr>
<th>Previous occurrence of cGVHD</th>
<th>No</th>
<th>Yes (resolved)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD4+CD25+/PBL</td>
<td>8.3 (2.6-12.4)*</td>
<td>8.7 (4.0-16.6)</td>
<td>p=0.44</td>
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<tr>
<td>FOXP3 expression</td>
<td>17548 (268.5-48327)</td>
<td>14872 (4916-51029)</td>
<td>p=0.94</td>
</tr>
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</table>

* Median (min.-max.)
Figure 1
Figure 2 A

FOXP3 Expression in PBL (AU)
cGVHD  no cGVHD  healthy individuals

0
10000
20000
30000
40000
50000
60000
70000

For personal use only.
Figure 2 B

% CD4+CD25+ in PBL

FOXP3 Expression in PBL (AU)
Figure 2 C

FOXP3 Expression in PBL (AU)

- aGVHD & cGVHD
- no aGVHD & cGVHD
- no aGVHD & no cGVHD
Figure 3 A
Figure 3 B
Figure 4 A
Figure 4 B
Figure 5
Figure 6

TREC copy number per $10^5$ CD3+ cells

- cGVHD
- no cGVHD
- healthy individuals

Figure 6
Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease

Emmanuel Zorn, Haesook T Kim, Stephanie J Lee, Blair H Floyd, Despina Litsa, Sankari Arumugarajah, Roberto Bellucci, Edwin P Alyea, Joseph H Antin, Robert J Soiffer and Jerome Ritz