Published in final edited form as:
Biol Blood Marrow Transplant. 2010 July ; 16(7): 907–914. doi:10.1016/j.bbmt.2010.02.026.

Frequency of CD4^{+}CD25^{hi}FOXP3^{+} Regulatory T Cells has Diagnostic and Prognostic Value as a Biomarker for Acute Graft-Versus-Host-Disease

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Abstract

The relationship between regulatory T cells (Tregs) and acute graft-versus-host disease (GVHD) in clinical allogeneic bone marrow transplantation (BMT) recipients is not well established. We conducted a prospective analysis of peripheral blood Tregs as determined by the frequency of CD4^{+}CD25^{hi}FOXP3^{+} lymphocytes in 215 BMT patients. Autologous BMT patients (N=90) and allogeneic BMT patients without GVHD (N=65) had similar Treg frequencies, whereas allogeneic patients with GVHD (N=60) had Treg frequencies that were 40% less than those without GVHD. Treg frequencies decreased linearly with increasing grades of GVHD at onset and correlated with eventual maximum grade of GVHD ($p<0.001$). In addition, frequency of Tregs at onset of GVHD predicted the response to GVHD treatment ($p=0.003$). Patients with Treg frequencies less than the median had higher non-relapse mortality than patients with Tregs greater than the median, but experienced equivalent relapse mortality, resulting in an inferior survival at two years (38% vs. 63%, $p=0.03$). Treg frequency may therefore have important prognostic value as a biomarker of acute GVHD.

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AUTHOR CONTRIBUTION J.M.M. and X.Q. performed research, analyzed data, and wrote the paper; C.E.R. designed experiments and performed research; I.T., M.S., D.B., K.L. and P.J. performed research; T.M.B. was the study statistician; D.J. managed human samples database; J.E.L., S.W.C., P.R., C.K. and S.M. contributed to patient accrual and quality control of the clinical endpoints, research discussion, and paper editing. J.E.L. also provided funding. J.L.M.F. and S.P. conceived and planned the study design, analyzed data, wrote the paper and provided funding.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Keywords

Allogeneic BMT; Acute graft-versus-host-disease; Regulatory T cells; Biomarker

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is a curative modality for many hematologic diseases, but its use is limited by mortality due to acute graft-versus-host-disease (GVHD). Naturally occurring regulatory T cells (Tregs) are mediators of immunologic tolerance that attenuate GVHD in experimental models (1,2). Tregs were first defined by a $CD4^+CD25^+$ phenotype (3,4), but subsequent studies identified forkhead box protein 3 (FOXP3) as a highly specific marker in both mouse and human T cells with regulatory function (5-7). High absolute numbers of $CD4^+FOXP3^+$ Tregs in the donor graft inversely correlated with acute GVHD incidence and overall survival of BMT recipients following myeloablative conditioning in one study (8), but not in a second (9). Higher numbers of $CD4^+FOXP3^+$ lymphocytes in stem cell products depleted of T cells have also been associated with a lower risk of developing GVHD (10,11). Experimental models suggest that immunosuppressive therapies may adversely affect Treg reconstitution after BMT (12-14) and studies measuring Tregs following BMT have not consistently correlated with either GVHD occurrence or severity (10,11,15,16); such discrepancies may be due to the sample size analyzed, timing of samples, and to various methodologies to quantify Tregs.

We prospectively analyzed Tregs using the $CD4^+CD25^{hi}FOXP3^+$ phenotype within total lymphocytes in 215 recipients of autologous and allogeneic BMT to determine the utility of this measurement as a cellular biomarker for GVHD. FOXP3 is now accepted as a distinctive molecule for natural and peripherally derived Tregs and is constitutively expressed at high levels in humans (17). Although FOXP3 is expressed in CD4+ cells with low and intermediate levels of CD25, these populations contain effector cells capable of transient FOXP3 expression that may lack equivalent suppressive function compared to $CD25^{hi}$ (17-19). No stem cell sources in this study were manipulated *ex vivo*. The frequency of Tregs was analyzed at the onset of GVHD for its correlation to disease severity, non–relapse mortality (NRM), response to GVHD treatment, and overall survival (OS).

DESIGN AND METHODS

Study Population

Between July 2007 and July 2009, 215 BMT patients (125 allogeneic, 90 autologous) had peripheral blood samples collected under protocols approved by the University of Michigan institutional review board. After providing informed consent, samples were collected within 24 hours of clinical signs of acute GVHD onset and prior to initiation of corticosteroid therapy. In addition, samples were collected on days +20, +30, +60, and +100. Approximately 70% of all possible samples were collected. Reasons for missing samples include patient ineligibility (second BMT / donor lymphocyte infusion, untreated infections, or intercurrent illness requiring intensive care), relapsed leukemia, death, and failure to collect / technical problems. GVHD was histologically confirmed in 88% of patients and was staged per modified Gluksberg criteria (20). All allogeneic BMT patients received calcineurin inhibitors (>95% received tacrolimus) as part of their GVHD prophylactic regimen. Patients received unmanipulated grafts, and no patients analyzed received T cell-depleting antibodies or sera for conditioning.

Flow cytometry of Regulatory T cells

Fresh whole blood sample phenotyping of cell surface markers was performed using CD4-PerCP-Cy5.5 (clone SK3, BD Biosciences, San Jose, CA), CD25-PE (clone M-A251, BD Biosciences), and CD127-FITC (clone eBioRDR5, eBioscience) antibodies. Intracellular staining of FOXP3-APC (clone PCH101, eBioscience, San Diego, CA) was performed after fixation and permeabilization according to the manufacturer's recommendation. We measured Treg frequency by three colors (CD4, CD25, FOXP3) or four colors (CD127, CD4, CD25, FOXP3) in 25% of samples. FACS analysis was performed using a Canto II flow cytometer (BD Biosciences) with FacsDiva software (BD Biosciences) to determine $CD4^{+}CD25^{hi}FOXP3^{+}$ or $CD127^{dim}CD4^{+}CD25^{hi}FOXP3^{+}$ staining within total lymphocytes. Absolute Treg numbers were calculated by multiplying Treg frequency by the absolute lymphocyte count obtained by automated differential. Values are expressed as mean ± SEM.

Statistical Analysis

Differences in characteristics between patient groups were assessed with a Kruskal-Wallis test for continuous values and Fisher's Exact Test for categorical values. Median frequencies of Tregs were compared using a Kruskal-Wallis test. Survival was modeled using Cox regression methods. Relapse mortality and NRM were modeled using cumulative incidence regression methods as described in Fine *et al* (21). The association between Treg frequency and response to therapy was calculated using chi-squared analysis. Wilcoxon signed rank tests were used to compare the Treg frequency at onset of GVHD and 4 weeks after treatment for 25 responders and 15 non-responders.

RESULTS

Phenotypic characterization of Tregs

The population of CD4 lymphocytes expressing very high levels of CD25 has been shown to express high levels of FOXP3 and exert dose dependent inhibition on $CD4^{+}CD25^{-}$ T cells (22). As expected, we observed the highest FOXP3 expression among the $CD25^{+++}$ ($CD25^{hi}$) lymphocyte subset, compared to $CD25^{++}$ and $CD25^{+}$ lymphocyte subsets in autologous and allogeneic patient samples (Figure 1). The mean FOXP3 expression in $CD4^{+}CD25^{hi}$ cells was similar among recipients of autologous BMT (71.9 % ± 2.1), allogeneic BMT with no GVHD (68.9 % ± 2.5), and allogeneic BMT with GVHD (64.1% ± 3.2) (Figure S1). The $CD4^{+}CD25^{hi}FOXP3^{+}$ phenotype had dim CD127 expression in greater than 95% of cells, consistent with prior reports (23,24). But $CD4^{+}CD25^{+}CD127^{dim}$ T cells include cells that lack suppressive function and produce IL-17, IL-2 and IFN-γ (18,25). We therefore used the $CD4^{+}CD25^{hi}FOXP3^{+}$ phenotype to define the Treg population.

Treg frequencies following autologous and allogeneic BMT without GVHD are similar

We first compared Tregs by measuring $CD4^{+}CD25^{hi}FOXP3^{+}$ cells from freshly acquired peripheral blood samples in autologous BMT patients (N = 90) and allogeneic BMT recipients without GVHD (N = 65). Characteristics of autologous and allogeneic populations are shown in Table 1A. High risk malignancy and age were significantly different between groups, but days to sample were similar. Frequencies of Tregs were similar in patients who did not develop GVHD after either autologous (1.09 ± 0.10) or allogeneic BMT (1.06 ± 0.10) (p = 0.84; Figure 2A). These frequencies were also similar to those obtained from six healthy donors (1.17 ± 0.16). However, absolute Treg numbers were reduced in allogeneic BMT patients with no GVHD compared to autologous BMT patients (p = 0.04, Figure 2B), as a result of lower absolute lymphocyte counts (ALC) in allogeneic BMT patients (Figure S2).

Decreased Treg frequencies at time of GVHD onset and 3-14 days prior to GVHD

We compared samples at GVHD onset to samples from patients without GVHD, such that both groups were balanced for time of acquisition. Characteristics of allogeneic patients according to GVHD status are shown in Table 1B. Patients were not significantly different for age, nonmalignant disease, conditioning intensity, and median day of sample acquisition. As expected, recipients of grafts from donors who were not family members or who were not HLA-matched were overrepresented in the GVHD group (Table 2). Patients with all grades of GVHD had a 40 % lower Treg frequency than patients without GVHD ($p < 0.001$, Figure 2A). We calculated the absolute numbers of Tregs by multiplying the frequency of $CD4^+CD25^{hi}FOXP3^+$ cells by the ALC, which was slightly higher in GVHD patients (1.13 ± 1.15) compared to patients without GVHD (Figure S2). The absolute Treg numbers remained lower in patients with GVHD compared to patients without GVHD ($p = 0.01$, Figure 2B). We also analyzed the ratio of Tregs to conventional T cells (Tconv) by dividing the $CD4^+CD25^{hi}FOXP3^+$ frequency by the CD4+CD25-FOXP3- frequency. Tconv frequencies were similar in the two groups (11.8 ± 0.97 vs. 10.5 ± 0.91). The mean Treg/Tconv ratio was significantly lower in patients with GVHD ($p < 0.001$, Figure 2C).

Fourteen patients had paired samples available 3-14 days prior to GVHD onset, and these were compared to fifteen paired samples in patients without GVHD. Treg frequencies prior to acute GVHD significantly decreased in the majority of patients ($p = 0.003$, Figure 3A), while no decrease occurred over a similar time interval in allogeneic patients without GVHD ($p = 0.7$, Figure 3B).

Treg Frequency is correlated with GVHD severity at onset and eventual maximum GVHD grade

We next hypothesized that Treg frequency would correlate with GVHD severity at onset. Frequencies of Tregs decreased in an almost linear fashion with each increasing grade of GVHD, and were significantly reduced in patients with ≥ grade II GVHD compared to patients without GVHD ($p < 0.001$, Figure 4A). A similar pattern was observed with absolute Treg numbers, but the variability within groups was greater, reducing the statistically significant differences between groups (Figure 4B). We also assessed whether Treg frequency at onset correlated with eventual maximum overall GVHD. The median interval between GVHD onset and eventual maximum overall GVHD grade was 14 days, with 11/28 (39%) patients progressing from grade I to higher GVHD grades. Treg frequency at GVHD onset was significantly reduced in patients who eventually developed maximum grade II-IV GVHD compared to patients without GVHD or who remained at grade I GVHD ($p<0.001$, Figure 4C).

Treg frequency has prognostic value at GVHD onset

Given these correlations, we next evaluated whether Treg frequency at onset would predict outcomes in the 60 patients with GVHD by dividing patients according to their median Treg frequency (0.5%). As shown in Figure 5A, patients with GVHD whose Treg frequency was less than the median had a significantly greater NRM at one year (41% [95%CI, 23%-61%]) than patients with Treg frequencies equal to or greater than the median (7% [95%CI, 2%-24%]), $p = 0.01$. Acute GVHD was the major cause of death in patients with low Treg frequencies (27% vs. 3%, $p = 0.03$, Table S1).

Relapse mortality was similar: 21% (95% CI, 9-40) in patients with low Treg frequencies compared to 30% (95% CI, 12-57) in patients with high Treg frequencies ($p=0.86$, Figure 5B). The overall survival at two years was significantly less in patients with low Treg frequencies (38%, [95%CI, 23%-63%]) than in patients with high Treg frequencies (63%, [95%CI, 43%-91%])($p = 0.03$, Figure 5C).

The difference in overall survival according to Treg frequency at GVHD onset remained significant after adjusting for absolute lymphocyte count at time of sample collection and other important prognostic factors such as, age, donor type, degree of HLA-match, and conditioning intensity (hazard ratio of 2.43 [95%CI: 1.01-5.87], p = 0.05). When both Treg frequency (≥ 0.5% vs. < 0.5%) and maximum GVHD grade (Grades I-II vs. III-IV) were analyzed in a simultaneous Cox regression analysis the Treg frequency remained significant (hazard ratio of 2.33 [95%CI: 0.96-5.66], p = 0.05).

Treg frequency and response to treatment

Since Treg frequency had prognostic value in GVHD, we analyzed if Treg frequency at GVHD onset predicted response to treatment. Four weeks following GVHD onset, patients with no GVHD (N = 29) or stage I GVHD of the skin (N =8) were classified as complete responders (N = 37), while patients with more advanced GVHD were classified as non-responders (N = 20). Three patients were not evaluable due to death or progressive disease. In patients with high Tregs (N = 28) at GVHD onset, 24 (86%) had complete responses, whereas in patients with low Tregs (N = 29) only 13 (45%) had complete responses (p=0.003). However, responders had more GVHD grade I at diagnosis (54% vs. 25%, p=0.05). When patients achieving a complete resolution of GVHD (N = 29) were evaluated, Treg frequency remained associated with treatment response (p=0.005). Paired samples at onset of GVHD and four weeks following treatment were available for 25/37 responding patients and 15/20 non-responding patients. As shown in Figure 6, responding patients showed a significant increase of their Treg frequency 4 weeks after treatment (p=0.02).

DISCUSSION

In this study we evaluated Treg as measured by the $CD4^{+}CD25^{hi}FOXP3^{+}$ phenotype for its value as a cellular biomarker at GVHD onset. Because Tregs rely on exogenous IL-2 in order to function (17), situations characterized by impaired IL-2 production might alter Treg frequency. Calcineurin inhibitors, are frequently administered to allogeneic BMT patients as in this study, and have been shown to impair the expansion and suppressive function of Tregs in experimental models (12-14). Treg frequency at day + 30 after autologous and after allogeneic BMT in patients without GVHD were similar, suggesting that allogeneic transplant recipients receiving tacrolimus have similar Treg recovery to autologous transplant recipients without tacrolimus. However, absolute Tregs were less in allogeneic BMTs patients with no GVHD compared to autologous BMT patients, an effect attributed to lower absolute lymphocyte counts. This supports a study in which low dose cyclosporine had no effect on $CD4^{+}FOXP3^{+}$ cell frequencies at day +30 following T cell depleted BMT (10).

Previous studies measuring Tregs following BMT have not analyzed their frequency at time of GVHD onset. We observed that Treg frequencies measured within 24hrs of GVHD diagnosis were significantly less than allogeneic patients without GVHD, and correlated inversely with GVHD severity. This association supports an earlier report from Johns Hopkins University that demonstrated a correlation of FOXP3 mRNA with GVHD severity when using samples at GVHD onset (26). This finding has not been consistently confirmed in other studies (10,11). As a single biomarker, Treg frequency at GVHD onset had only modest diagnostic value (AUC = 0.69), but the inverse relationship between Treg frequency and GVHD severity demonstrates its utility. It should also be noted that we measured Tregs in the peripheral blood, not in the target tissue, where they presumably exert their greatest effect. Interestingly, in a small cohort of patients we observed an overall decrease in Tregs prior to clinical onset of GVHD, suggesting that a decline in Treg frequency may herald onset of the disease. Although these analyses are correlative in nature, this observation indicates that systematic measurements of Treg frequencies may be able to predict the

occurrence of GVHD and extends the findings of a previous study in which the frequency of $CD4^+CD25^+$ T cells decreased during the initial phases of GVHD (27).

Importantly, Treg frequency measured at GVHD onset may have prognostic value, which is a major feature of clinically relevant biomarkers (28). In our study, Treg frequency of less than 0.5% correlated with poor outcomes (maximum overall GVHD grade, NRM, OS). This finding suggests that prospective measurement of Treg frequency at GVHD onset may improve standard clinical GVHD grading and further risk stratify groups of patients. Identification of patients at high risk for severe GVHD early in their transplant course may impact clinical decisions to include more stringent monitoring and/or intense treatment.

Consistent with its prognostic value, Treg frequency at GVHD onset correlated with eventual treatment response at four weeks. It is not known whether altering immunosupression in patients with low Treg frequencies might subsequently improve outcomes through the *in vivo* expansion of Tregs. Recently, treatments such as rapamycin and extracorporeal photophoresis have been shown to increase Tregs and protect from experimental GVHD (29-31). Prospective studies of Treg expansion for the treatment and prevention of GVHD in high risk patients are therefore warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by grants from the National Institutes of Health P01-CA039542-20 and RCI-H2-101102, Cancer center care grant P30 CA46592, the American Cancer Society (CRP 08-224-01), the Leukemia and Lymphoma Society (#6013-09).

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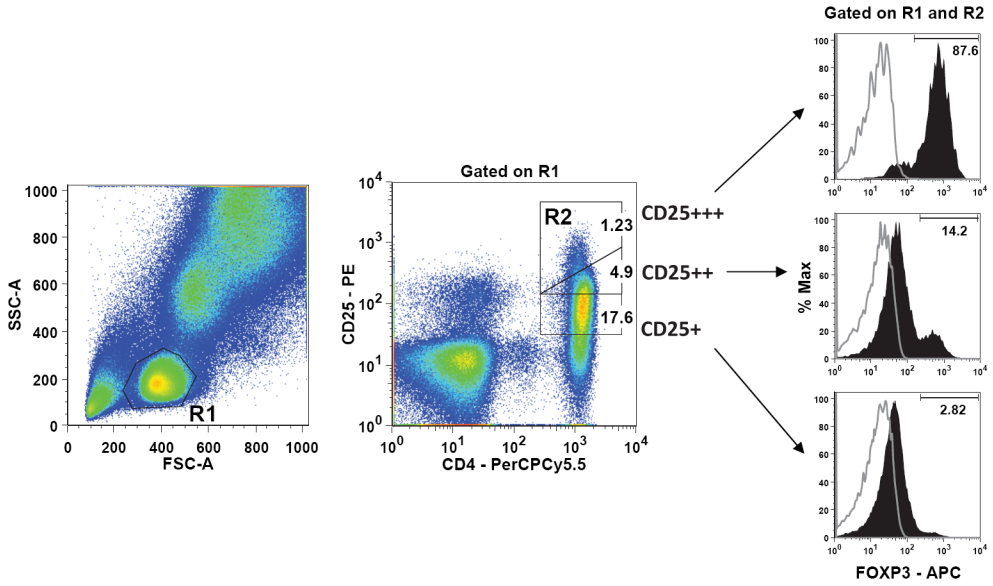

Figure 1. Phenotypic characterization of Tregs

Representative flow data from a patient following allogeneic transplantation. Fresh whole blood was stained with CD4-PerCP-Cy5.5 and CD25-PE antibodies followed by intracellular staining for FOXP3-APC. Treg frequency was determined using a Canto II flow cytometer (BD Biosciences) to identify $CD4^{+}CD25^{hi}FOXP3^{+}$ triple positive cells within the total lymphocyte population. Treg frequency was performed in triplicate for each sample. (A) The total lymphocyte population (R1) was identified by light scatter after backgating on the CD4+ population. On right are histograms of $FOXP3^{+}$ expression (red) gated on $CD4^{+}CD25^{+}$, $CD4^{+}CD25^{++}$, $CD4^{+}CD25^{+++}$ ($CD4^{+}CD25^{hi}$) lymphocyte subsets. Analysis was restricted to the $CD4^{+}CD25^{hi}$ lymphocyte subset (R2) which was identified in R1. The frequency of $CD4^{+}CD25^{hi}FOXP3^{+}$ lymphocytes was calculated by multiplying the percentage of $CD4^{+}CD25^{hi}$ cells by the percentage of $FOXP3^{+}$ cells within $CD4^{+}CD25^{hi}$.

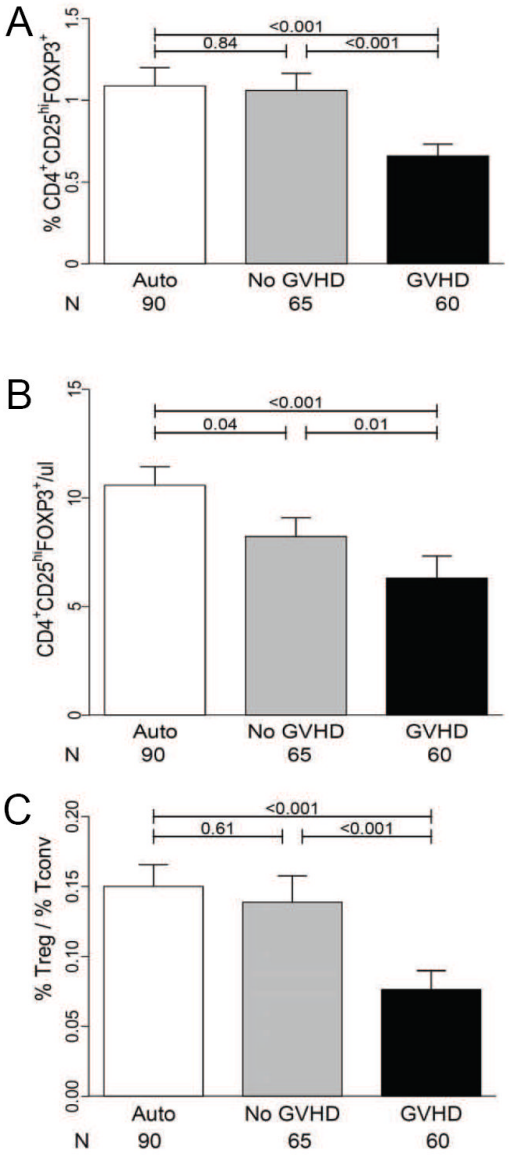

Figure 2. Regulatory T cells (Tregs) at GVHD onset

Fresh blood samples from autologous and allogeneic transplant patients (N=215) were acquired within 24hrs of acute GVHD onset or at equivalent timepoints following transplantation. Mean (A) Treg frequencies (B) Absolute Treg numbers and (C) Treg / Tconv frequencies for autologous BMT patients, allogeneic BMT patients with no GVHD, and allogeneic BMT patients with GVHD. Error bars represent the SEM.

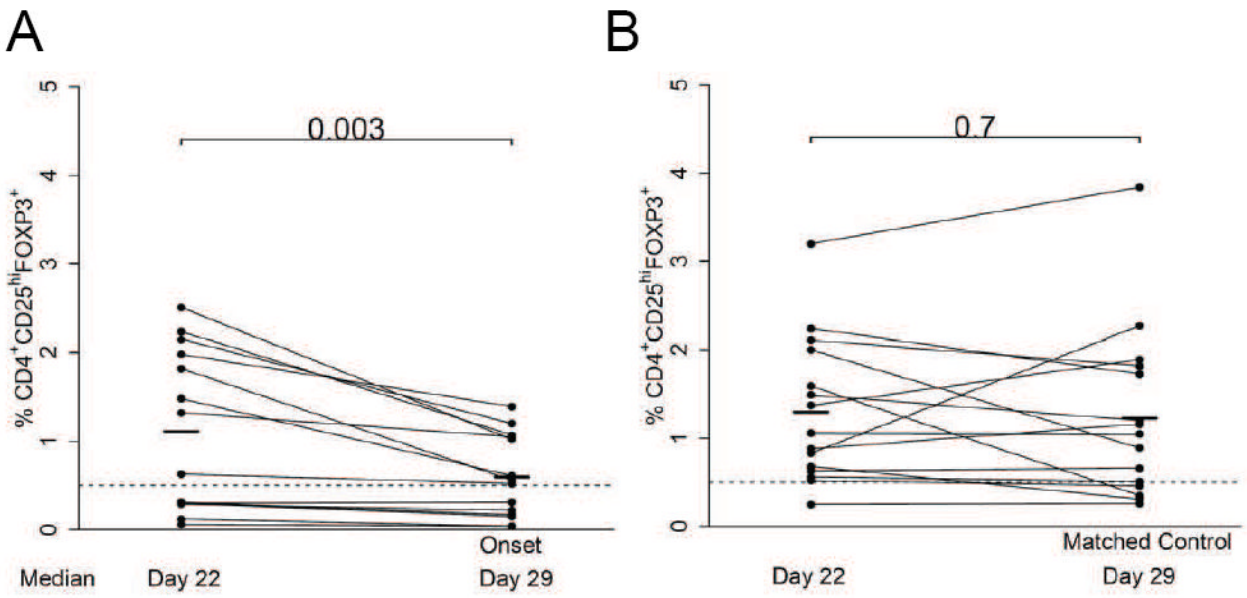

Figure 3. Treg frequencies prior to GVHD onset

(A) Paired analysis in GVHD patients (N = 14) comparing Treg frequencies at GVHD onset to prior timepoints (3 to 14 days). (B) Treg frequencies from matched controls with no GVHD (N = 15) over an equivalent 3-14 day interval. Bars denote mean values.

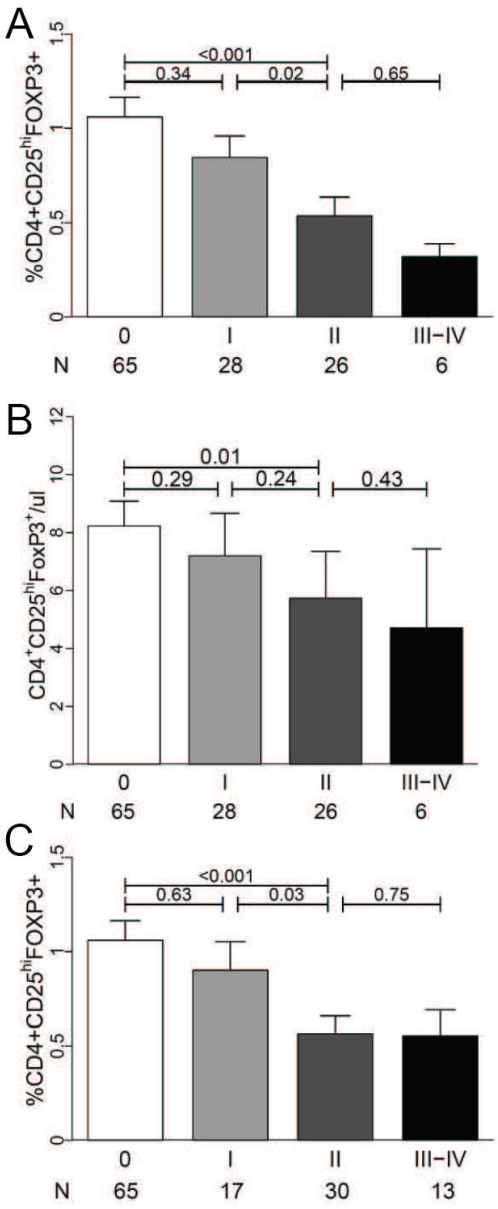

Figure 4. Treg frequencies and GVHD severity

Fresh blood samples from allogeneic transplant patients with GVHD (N = 60) were acquired within 24hrs of acute GVHD onset and analyzed according to GVHD severity. (A) Mean Treg frequencies, (B) absolute Treg numbers by grade of GVHD at onset, and (C) mean Treg frequencies by eventual maximum overall GVHD grade. The median interval between GVHD onset and eventual maximum overall GVHD grade was 14 days. Error bars represent the SEM.

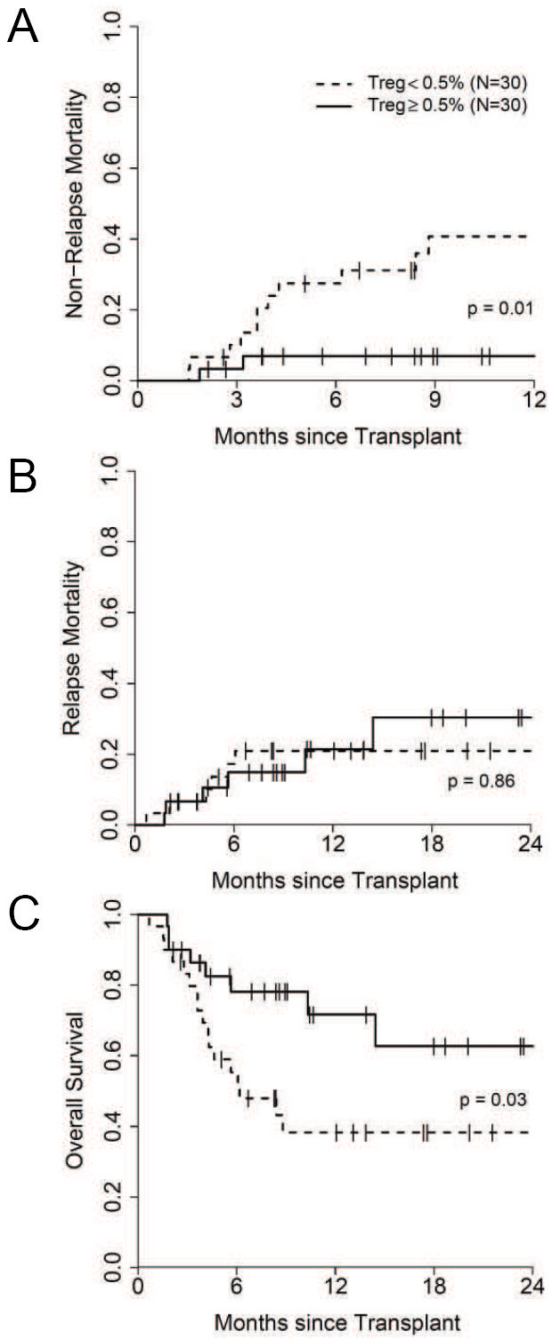

Figure 5. Treg frequencies are correlated with clinical outcomes

The median T reg frequency was 0.5% in allogeneic BMT patients at time of GVHD onset (N = 60). (A) non-relapse mortality, (B) relapse mortality and (C) overall survival in patients with GVHD divided according to the median Treg frequency (high Treg ≥ 0.5% (N=30) or low Treg < 0.5 (N=30).

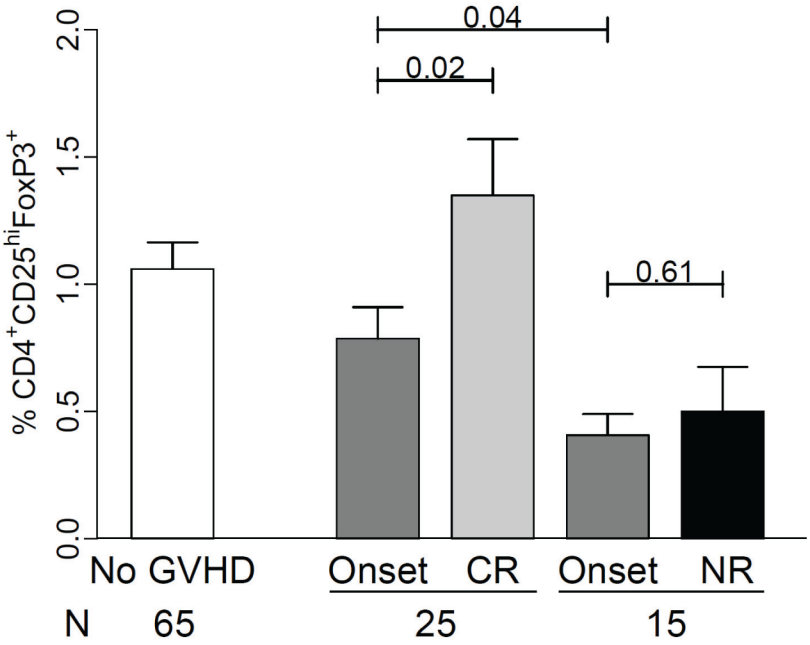

Figure 6. Treg frequencies at 4 weeks according to treatment response

Mean (±SEM) Treg frequencies were measured on paired samples at onset and 4 weeks after treatment in 25 patients with complete response or near complete response (CR) and 15 non-responders (NR).

Table 1

A. Characteristics of autologous and allogeneic patients with no GVHD				
Characteristic		**Auto**	**Allo**	**p-value**
Patients		90	125	
Age, yr	Median	55	50	0.004
	Range	6-70	1-67	
Days to sample	Median	32	29	0.14
	Range	14-119	14-111	
Malignant disease, % (no.)[1]		98% (88)	95 % (119)	0.47
High risk malignancy, % (no.)[2]		14% (13)	39% (49)	<0.001
B. Characteristics of GVHD positive and GVHD negative patients				
Characteristic		**No GVHD**	**GVHD**	**p-value**
Patients		65	60	
Age, yr	Median	50	52	0.24
	Range	1-67	1-66	
Related donor, % (no.)		60% (39)	33% (20)	0.004
Matched donor, % (no.)		86% (56)	67% (40)	0.01
Stem cell source, % (no.)				
Peripheral blood, % (no.)		86% (56)	78% (47)	
Bone marrow, % (no.)		11% (7)	8% (5)	0.12
Cord blood, % (no.)		3% (2)	13% (8)	
Full intensity conditioning, % (no.)		74% (48)	70% (42)	0.69
Malignant disease, % (no.)		92% (60)	98% (59)	0.21
High risk malignancy, % (no.)		32% (21)	47% (28)	0.12
GVHD prophylaxis, % (no.)				
TAC / MMF		37% (24)	42% (25)	
TAC / MTX		38% (25)	25% (15)	0.41
TAC / MMF or MTX / Etanercept		11% (7)	17% (10)	
Other [3]		14% (9)	17% (10)	
Days to sample	Median	29	29	0.29
	Range	16-106	14-111	
Days to GVHD onset	Median		29	
	Range		14-111	
Onset GVHD grade, % (no.)				
Grade I			46% (28)	
Grade II			44% (26)	
Grade III-IV			10% (6)	
Maximum GVHD grade, % (no.)				
Grade I			28% (17)	
Grade II			50% (30)	
Grade III-IV			22% (13)	

A. Characteristics of autologous and allogeneic patients with no GVHD

Characteristic		Auto	Allo	p-value
Organ target at GVHD onset, % (no.)				
Skin only			68% (41)	
Gut only			21% (13)	
Liver only			2% (1)	
Skin + gut / liver			9% (5)	

[1]Malignant diseases include hodgkins disease (HD), chronic lymphocytic leukemia (CLL), acute myelogeneous leukemia (AML), myelodysplastic syndrome (MDS), non-hodgkin lymphoma (NHL), acute lymphoblastic leukemia (ALL), multiple myeloma (MM) and myeloproliferative disease (MPD).

[2]AML and ALL >CR2, induction failure or refractory, AML arising from antecedent MDS, CML in blast or accelerated phases, HD and NHL > CR2 or refractory, progressive multiple myeloma.

[3]Other GVHD prophylaxis include: TAC/SIRO (n = 3), TAC/MTX/SIRO (n=1), TAC/MMF/MTX (n=11), MMF/Cyclosporin (n=3), MTX/ cyclosporin (n=1). TAC = Tacrolimus, MTX = Methotrexate, SIRO = Sirolimus, MMF = mycophenolate mofetil.