Combined CD4+ Donor Lymphocyte Infusion and Low-Dose Recombinant IL-2 Expand FOXP3+ Regulatory T Cells following Allogeneic Hematopoietic Stem Cell Transplantation

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Abstract

CD4+CD25+FOXP3+ regulatory T cells (Treg) successfully control graft-versus-host-disease (GVHD) in animal models. In humans, incomplete reconstitution of Treg after allogeneic hematopoietic stem cell transplantation (HSCT) has been associated with chronic GVHD. Recent studies have demonstrated that IL-2 infusions expand Treg in vivo. However, the effectiveness of this therapy depends on the number of cells capable of responding to IL-2. We examined the effect of low-dose IL-2 infusions on Treg populations after HSCT in patients who also received infusions of donor CD4+ lymphocytes. Utilizing *FOXP3* as a Treg marker, we found that patients who received CD4+DLI concomitantly with IL-2 had greater expansion of Treg compared to patients who received IL-2 (p=0.03) or CD4+DLI alone (p=0.001). *FOXP3* expression correlated with absolute CD4+CD25 + cell counts. Moreover, expanded CD4+CD25+ T cells displayed normal suppressive function and treatment with CD4+DLI and IL-2 was not associated with GVHD. This study suggests that administration of low-dose IL-2 combined with adoptive CD4+ cellular therapy may provide a mechanism to expand Treg in vivo.

Introduction

CD4+CD25+FOXP3+ regulatory T cells (Treg) play a critical role in controlling immune reactions following allogeneic hematopoietic stem cell transplantation (HSCT) (1). In mice, infusion of purified Treg at the time of transplant can prevent the development of lethal graft versus host disease (GVHD) while depletion of these cells worsens GVHD (2–5). In most experimental models, prevention of GVHD is achieved while preserving a beneficial graft versus leukemia effect (GVL) (3–5). In humans, poor reconstitution of Treg after HSCT correlates with the subsequent development of chronic GVHD (6,7). Taken together, these findings suggest that methods to increase the number of Treg in vivo may provide a new approach for either prevention or treatment of GVHD following allogeneic HSCT (1,8,9). Based on murine models, several investigators have proposed that adoptive therapy with purified Treg that have previously been expanded in vitro may be able to prevent or treat GVHD

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(10,11). Recent clinical studies have also shown that IL-2 therapy induces the selective expansion of Treg following HSCT as well as in patients with solid tumors (12–14). The effect of IL-2 however, depends on the presence of sufficient numbers of Treg capable of responding to cytokine stimulation. Combining adoptive cellular therapy together with IL-2 administration could therefore represent a strategy to effectively expand Treg in patients where these cells are severely reduced. In the present study, we examined the modulation of Treg in patients who received concomitant CD8-depleted donor lymphocyte infusion (CD4+DLI) together with prolonged low-dose IL-2 following HSCT. We provide evidence that this combined therapy substantially expands Treg in vivo to a level greater than CD4+DLI or low-dose IL-2 alone.

Materials and Methods

Patients and samples

Patients included in this study were enrolled in clinical protocols approved by the Dana-Farber Cancer Institute Investigational review board (IRB). All patients received myeloablative conditioning and peripheral blood mononuclear cells (PBMC) were isolated from blood before and after treatment and cryopreserved. Patient and treatment characteristics are summarized in Table 1.

Quantitative PCR

Quantitative PCR for *FOXP3* has been described previously (7,14). Briefly, experiments were performed using universal PCR Master Mix (Applied Biosystems) and *FOXP3* specific primers and probe. Results were normalized based on the amplification of TFRC (CD71, transferrin receptor).

Immunofluorescence analysis

Intracellular staining of FOXP3, was performed using a commercially available kit (eBioscience, San Diego, CA). Absolute numbers of CD4+ and CD4+CD25+ lymphocytes were calculated based on total lymphocyte counts and phenotypic data.

Suppression of T cell proliferation by Treg

Standard suppression assays were performed as described previously (7). Briefly, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were purified from patient samples by cell sorting and incubated either alone or together (ratio 1:1) in the presence of soluble anti CD3 (OKT3; 1μg/ml) and irradiated allogeneic PBMC (1×10^4 cells/well). Responder cell proliferation was measured after 6 days.

Statistical analysis

FOXP3 expression level was log-transformed (base 10). Student t-test was used for two-sample comparisons. Linear regression models were fit to examine the group effect after adjusting for age, sample collection days, time from HSCT/DLI to therapy, IL-2 dose, duration of IL-2, and DLI dose.

Results

Patient groups

In the present study we investigated whether a combination of IL-2 and adoptive cellular therapy could enhance the expansion of Treg in vivo. We examined changes in circulating Treg in 3 groups of patients who had previously undergone allogeneic HSCT following a myeloablative conditioning regimen consisting of cyclophosphamide and total body irradiation. Patient characteristics are summarized in Table 1. The first group includes 5

patients with relapsed hematologic malignancy who received CD8-T cell depleted DLI (CD4 + DLI) and low-dose IL-2. These patients received defined doses of CD4+ DLI (3×10^7 to 1 $\times 10^8$ CD4+ cells/kg) followed by daily administration of 6×10^5 IU/m^2 IL-2 for 12 continuous weeks. One patient had to discontinue treatment after 5 weeks for accelerated disease (pt. 1). This Phase I trial was designed to evaluate safety of the combined therapy as a primary endpoint and anti-tumor effect of the combined therapy as a secondary endpoint. The second group of 5 patients received lower doses of IL-2 (2×10^5 IU/m^2/day) for 12–13 continuous weeks. For these patients, IL-2 was administered relatively early after CD6 depleted allogeneic transplant when leukemia was in remission with the intent to increase the GVL effect and prevent relapse (15,16). Patients were monitored for safety and the effects of IL-2 therapy on reconstitution of NK cells (CD56+) and T cells (CD3+CD4+ and CD3+CD8+) in peripheral blood. Group 3 was comprised of 4 patients who received CD4+ DLI alone without IL-2 for relapsed leukemia. This Phase I trial was undertaken to evaluate the safety of escalating doses of CD4+ DLI as well as efficacy of DLI. None of the patients included in the 3 groups had prior episodes of acute or chronic GVHD. As shown in Table 1, times from HSCT to therapy varied for the different groups. Patients who received IL-2 alone were treated prophylactically beginning at 6–12 weeks post-transplant. In contrast, patients who received DLI with or without IL-2 were treated for relapsed leukemia at later times post-transplant. Toxicity specifically associated with IL-2 administration has been reported previously (17,18). IL-2 toxicity in these patients was minimal including transient fatigue, fever, nausea, myalgia and rash and was consistent with previous findings (17,18). CD4+ DLI was also well tolerated and did not lead to any severe side effects as previously reported in similar trials (19,20). None of the patients examined in this study developed GVHD following IL-2 infusion or CD4+ DLI. One patient who received CD4+ DLI alone developed GVHD after treatment.

Increase in FOXP3 gene expression following CD4+ DLI and IL-2 therapy

To measure changes in Treg during therapy we assessed *FOXP3* gene expression by quantitative PCR in PBMC collected before and after treatment. Results are reported in Figure 1 for the 3 patient groups. As depicted in this figure, concomitant administration of low-dose IL-2 and CD4+DLI (red lines) induced a significant increase in *FOXP3* gene expression levels when compared to CD4+DLI only (black lines; Student t-test for largest value, p=0.001) or low-dose IL-2 only (blue lines; Student t-test for largest value, p=0.03). Increase in *FOXP3* gene expression following CD4+DLI + IL-2 was also confirmed in a linear regression model for which age, sampling time, time from HSCT to therapy, DLI and IL-2 doses as well as duration of IL-2 therapy were adjusted (p<0.001). Increased *FOXP3* gene expression was not seen in any of the patients who only received CD4+DLI. In addition, compared to previously published reports, IL-2 had a relatively weak effect on *FOXP3* gene expression in the 5 patients who only received this treatment (12–14). This was likely due to low numbers of CD4+CD25 + T cells in these patients prior to therapy early after stem cell transplantation. The median absolute CD4+CD25+ T cell counts in these patients before treatment was only 7.5 +/−3.8 cells/μl compared to approximately 200 CD4+CD25+ cells/μl in the peripheral blood of healthy donors. Only one patient (Pt. 4) did not experience an increase in *FOXP3* gene expression following CD4+DLI + IL-2. This patient had relapsed AML at the time therapy was started.

CD4+ DLI + IL-2 induces peripheral Treg expansion and increased FOXP3 protein expression in peripheral Treg

FOXP3 protein expression in peripheral blood lymphocytes was also examined by flow cytometry before and after CD4+ DLI and IL-2. As shown in Figure 2 for the 4 patients in Group 1 who had increased *FOXP3* gene expression by quantitative RT-PCR, this was confirmed by an expansion of CD4+ T cells expressing intracellular FOXP3 protein. Staining intensity for FOXP3 was increased for all CD4+CD25+ T cells indicating that IL-2 also induced up-regulation of *FOXP3* gene expression at the single cell level.

FOXP3 gene expression levels correlate with absolute CD4+CD25+ T cell counts

Consistent with the IL-2 effect on Treg expansion, we observed a highly significant correlation between *FOXP3* gene expression levels and absolute CD4+CD25+ counts (Spearman correlation coefficient r=0.79, p=0.0003; Figure 3A). These results provided additional support for assessing *FOXP3* gene expression as a surrogate marker for Treg populations. Correlation between *FOXP3* gene expression levels and CD4+ counts was also significant in these patients (r=0.76, p=0.0006: Figure 3B).

CD4+ DLI + IL-2 expanded Treg exhibit normal immune suppressive function in vivo

To determine whether Treg that expanded in vivo after CD4+DLI and IL-2 maintained their suppressive function, we tested purified CD4+CD25+ cells from these patients for their ability to inhibit proliferation of autologous CD4+CD25− T cells in vitro. As shown in Figure 4, Treg purified from patient samples collected during combined therapy at a time when *FOXP3* gene expression was increased, displayed similar levels of suppressive function as Treg isolated from healthy donors.

Discussion

The present studies demonstrate that the addition of low dose IL-2 to CD4+ lymphocyte infusions results in a marked expansion of CD4+CD25+FOXP3+ regulatory T cells. The increase was significantly greater than that observed in patients receiving rIL-2 alone. As this latter group of patients received a lower dose of IL-2 that patients who received both DLI and IL-2, it is possible that the dose of IL-2 contributed to the different effects in these 2 groups of patients. In contrast, patients who received CD8 depleted DLI alone had no increase in circulating CD4+CD25+FOXP3+ regulatory T cells whatsoever. These results provide a framework in which allogeneic Tregs can be expanded in vivo without the need for long term *ex vivo* culture.

It is noteworthy that previous studies from our institution have documented that low dose IL-2 can be safely administered to patients early after T cell depleted allogeneic transplantation without inducing acute or chronic GVHD. We previously demonstrated that such treatment dramatically expands CD56+CD3− NK cells, but also CD4+CD25+FOXP3+ regulatory T cells (14,17,18). The patients receiving CD8+ depleted DLI plus IL-2 also did not develop any GVHD. Nor, however, was any GVL activity noted. None of the patients receiving CD8 depleted DLI plus IL-2 achieved significant tumor responses. It is possible that the anti-tumor effect of DLI was diminished by the expansion of Treg though all of these patients had advanced hematologic malignancies prior to treatment. While the original intent of this clinical trial was to augment cytotoxic effector activity, the combination of CD8 depleted DLI and low dose IL-2 appears to have served to instead expand cells with potent immune suppressive properties. We have reported on the expansion of effector T cells with potent anti-tumoral activity following CD4+ DLI (21). It is possible that expanded Treg in patients who received the combined therapy may have diminished the efficacy of such GVL effector cells.

Other studies published by Ahmadzadeh et al. and Zhang et al. have also demonstrated the capacity of IL-2 therapy to expand human Treg *in vivo* using higher doses and intermittent therapy with IL-2 (12–14). We have also reported similar expansion of Treg in vivo following administration of low doses of IL-2 comparable to the doses given to DLI recipients in this study (14). IL-2 directly induces the up-regulation of *FOXP3* gene expression through the phosphorylation of STAT proteins downstream of the IL-2 receptor that subsequently translocate to the nucleus and bind to regulatory motifs located in the first intron of the *FOXP3* gene (14). This signaling pathway appears active in CD4+CD25+ Treg that already express *FOXP3* but not in CD4+CD25− cells. Consistent with this mechanism, the intensity

of FOXP3 intra-cellular staining in Treg increased following IL-2 treatment, reflecting an up-regulation of gene expression at the single cell level. The effect of IL-2 therapy is therefore dependent on preexisting populations of Treg in treated patients. Following HSCT, T cell populations are often reduced due to the preparative regimen and concomitant administration of calcineurin inhibitors. Our studies suggest that a combination of both CD8-T cell depleted DLI to replenish CD4+ T cell pools together with administration of low dose IL-2 can more effectively expand Treg in vivo.

Recent investigations have revealed a crucial role for Treg in controlling immune responses post-HSCT and especially the development of GVHD. In animal models, infusion of Treg at the time of stem cell infusion or immediately following transplantation successfully prevented GVHD (2,3,5). In most studies, this was achieved while maintaining the beneficial GVL effect. In the autologous setting however, Treg have been shown on numerous occasions to impair anti-tumor immunity (22). In humans, we and others have shown that poor Treg reconstitution correlates with chronic GVHD (6,7,23,24). Treg cellular therapy could thus be utilized to replenish Treg pools in vivo and prevent or even correct GVHD. A number of studies have addressed the feasibility of purifying and expanding human Treg ex vivo in order to use these cells for adoptive therapy (10,25,26). Overall, our retrospective study suggests that combined therapy with CD4+ donor lymphocytes and low-dose IL-2 may be an alternative strategy for expanding Treg in vivo. This strategy does not rely on the purification and selective expansion of Treg in vitro. Furthermore, previous findings that combined CD4+DLI and low-dose IL-2 have not been associated with GVHD suggest that this approach could be safely tested in clinical trials with careful monitoring of immunological effects and toxicity.

Acknowledgements

Abbreviations

Treg

    T Regulatory Cell

HSCT

    Hematopoietic Stem Cell Transplantation

GVHD

    Graft-Versus-Host Disease

DLI

    Donor Lymphocyte Infusion

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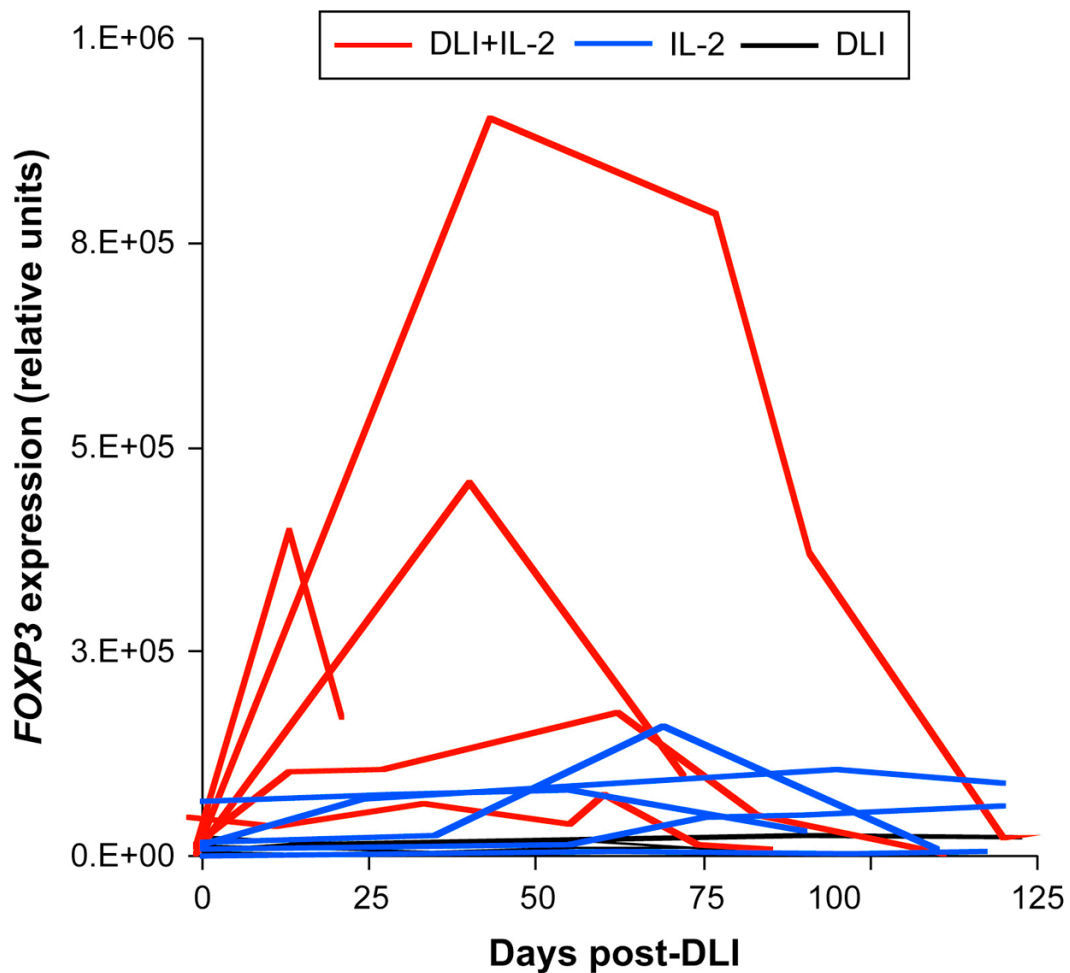
Figure 1. Increased FOXP3 gene expression following CD4+ DLI plus low dose IL-2
FOXP3 expression was assessed by real time PCR in PBMC from patients who received CD4
+ DLI (black lines), low-dose IL-2 (blue lines) or combined CD4+ DLI and low-dose IL-2 (red
lines). FOXP3 expression levels were normalized based on the expression of *TFRC* (transferrin
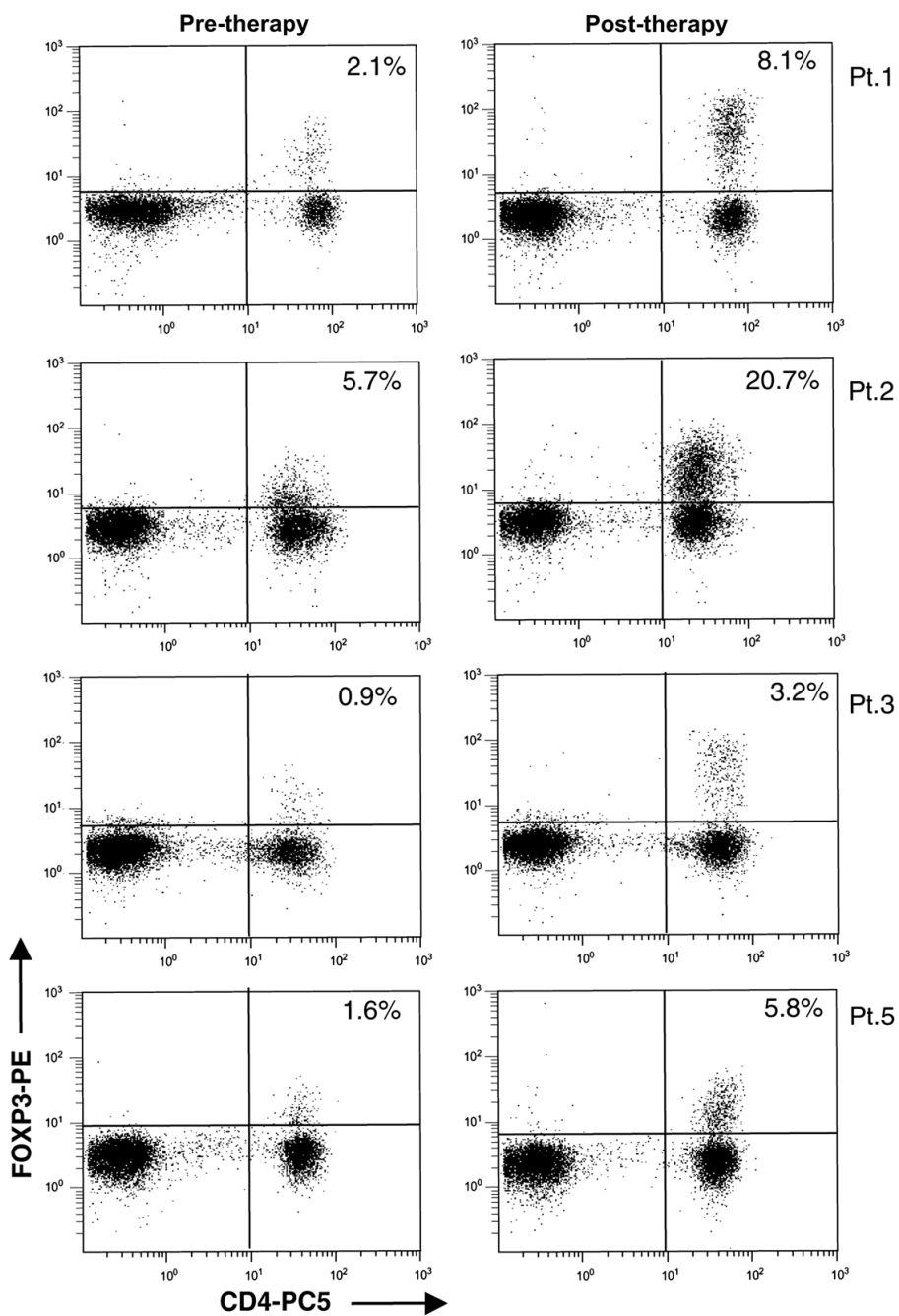receptor).

Figure 2. Intracellular staining for FOXP3 protein in patients who received CD4+ DLI and IL-2
Patient PBMC were tested for expression of cell membrane CD4 and intracellular FOXP3. Samples were collected before patients received CD4+DLI and IL-2 and between 13 to 77 days after starting therapy. Dot-plots are shown after gating on lymphocyte populations.
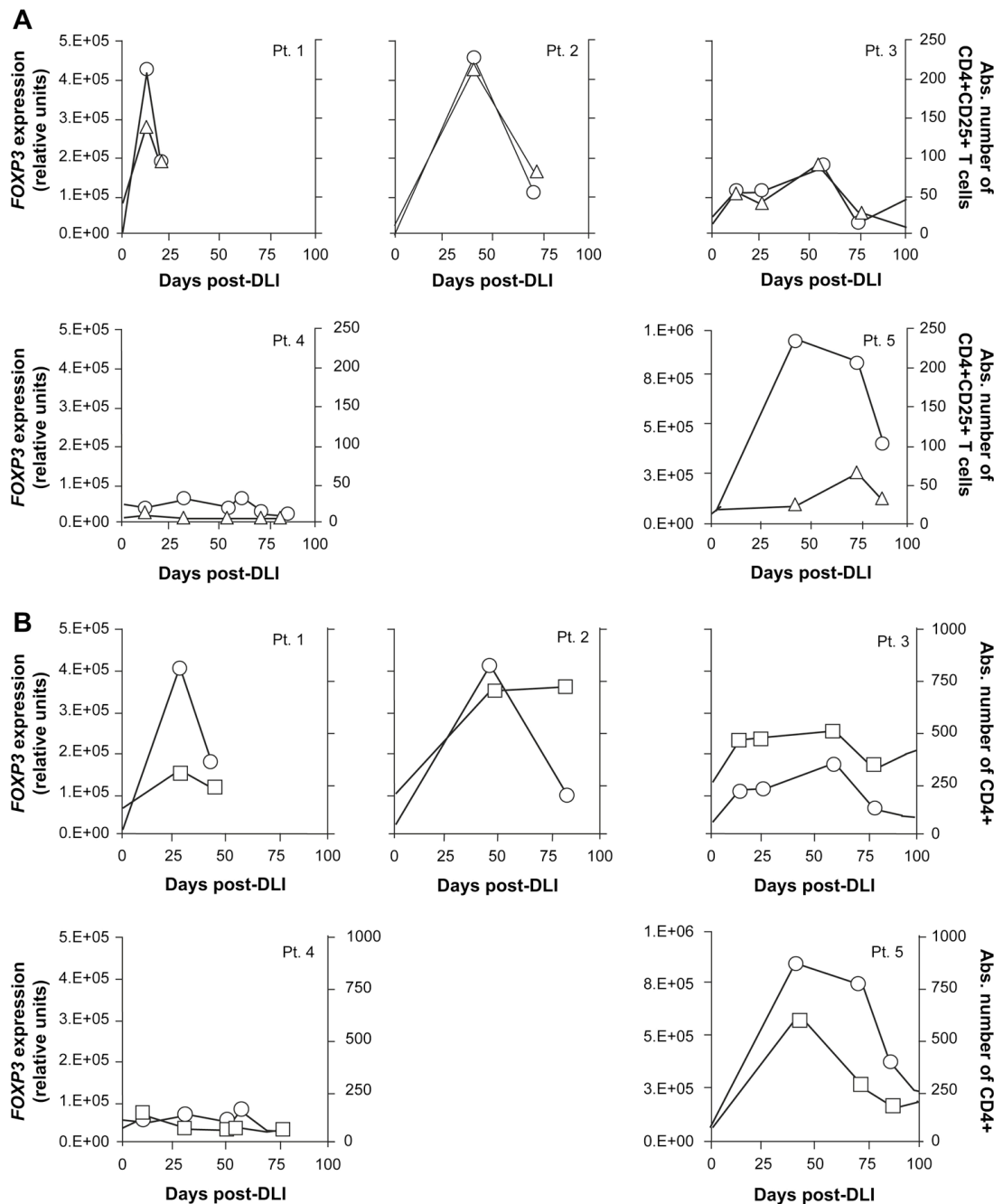
Figure 3. Correlation of FOXP3 gene expression and CD4+CD25+ Treg and total CD4 T cells in peripheral blood after CD4+ DLI and IL-2
A. FOXP3 gene expression (circles) was assessed by quantitative PCR in PBMC collected from patients who received CD4+DLI + IL-2 and is shown together with absolute blood CD4 +CD25+ T cell counts (triangles; cells/μl). Spearman correlation coefficient r=0.79, p=0.0003.
B. FOXP3 gene expression is depicted (circles) together with absolute blood CD4+ T cell counts (squares; cells/μl) for all patients treated with CD4+DLI + IL-2. Spearman correlation coefficient r=0.76, p=0.0006.
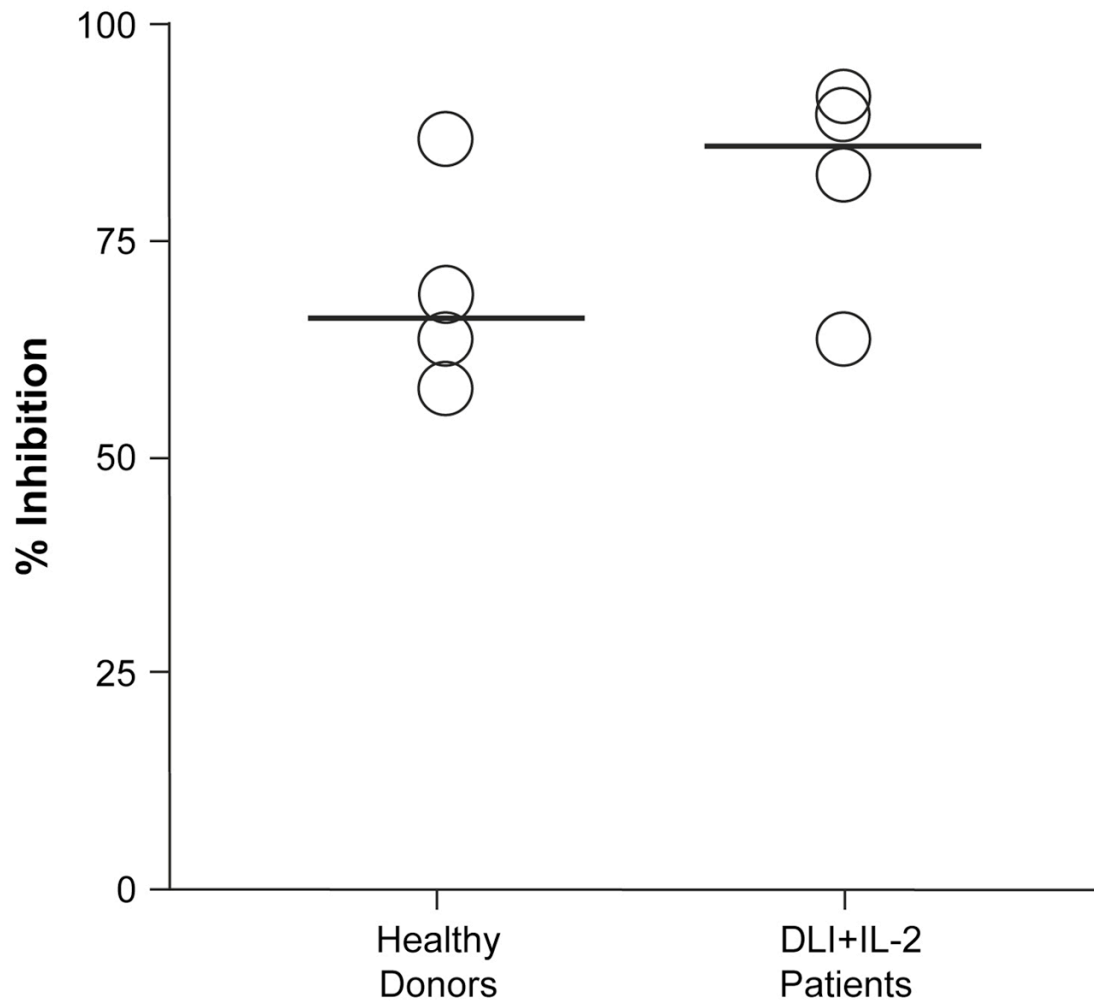
Figure 4. Suppressive activity of CD4+CD25+ Treg following IL-2 and CD4+ DLI combined therapy
Suppressive activity of purified CD4+CD25+ T cells was assessed for patients 1, 2, 3 and 5 using samples collected after DLI and during IL-2 treatment. Suppression by Treg purified from 4 healthy donors was also measured. Solid lines represent median values for the 2 groups.