The Functional Capacities of Peripheral Blood Regulatory T Cells after an Allogeneic Stem Cell Transplant Correlate with the Risk of Graft-Versus-Host Disease

Nadia Peragine, Anna P. Iori, Walter Barberi, Maria S. De Propris, Anna Guarini, Robin Foà and Giovanni F. Torelli*

Division of Hematology, Department of Cellular Biotechnologies and Hematology, "Sapienza" University, Via Benevento 6, 00161 Rome, Italy

Abstract: The immunologic reconstitution is ultimately responsible of the clinical outcome of patients who have undergone an allogeneic stem cell transplantation (SCT). The occurrence of graft-versus-host disease (GVHD), which represents the major cause of morbidity and mortality after the transplant correlates with the concentration in the peripheral blood (PB) of regulatory T cells (Tregs). In this study we aim at demonstrating that not only the concentration but also the functional capacities and the degree of activity of Tregs act as an important regulator of alloreactivity and may help to predict the risk of acute and chronic GVHD in the post-transplant period. Sixteen patients who underwent an allogeneic SCT were evaluated at 1 year from transplant. Tregs were expanded from the PB of these patients and from 8 normal donors; their expansion capacity, phenotype, suppressor activity and IL-10 production were measured. Tregs expanded from patients without GVHD exerted a higher suppressive function on the proliferative reaction of T cells and showed a higher IL-10 production capacity compared to patients with acute or chronic GVHD. These results document that the functional activity and the suppressor capacity of Tregs after an allogeneic SCT may protect from GVHD, and support the design of clinical protocols based on the infusion of expanded and activated Tregs.

Keywords: Allogeneic stem cell transplant, graft-versus-host disease, regulatory T cells, mixed lymphocyte reaction, Interleukin-10.

INTRODUCTION

Allogeneic hematopoietic stem cell transplant (SCT) represents the treatment of choice for many hematologic malignancies and for different non-neoplastic hematologic disorders [1]. This therapeutic procedure implies the development of a new immunologic configuration in which donor lymphocytes recognize recipient histocompatibility antigens. After the transplant donor T cells play an important role in lowering the risk of tumor recurrence by inducing the so-called graftversus-leukemia (GVL) effect [2] and in reducing the incidence of infections; at the same time this cell population is responsible for the occurrence of the graft-versus-host disease (GVHD) phenomenon, which represents the major cause of morbidity and mortality [3,4]. GVHD, both in its acute and chronic presentation, is in fact thought to be primarily mediated by effector T cells of donor origin along with antigen presenting cells (APCs) and B cells which have been addressed as key mediators of T-cell activation in this setting [5-9].

Many reports have focused on the possibility that natural occurring regulatory T cells (Tregs), a subpopulations of thymus-derived CD4+ T cells that constitutively express the interleukin (IL)-2 receptor α chain (CD25), the cytotoxic T lymphocyte (CTL)-associated protein 4 (CTLA-4) and the forkhead box P3 (FoxP3) gene product, may play a central role in the suppression of T-cell functions in the allogeneic SCT context [10-13] due to their capacity of producing regulatory cytokines such as IL-4, IL-10 and transforming growth factor (TGF-α) [14-16] and their interaction with different cell types, including dendritic cells (DCs) and B cells [17, 18]. In addition, it has been reported that Tregs can inhibit the GVHD phenomenon [19-21] while apparently retaining the GVL effect [13, 22]. Recently, two clinical studies performed in the double umbilical cord blood and haploidentical transplantation setting confirmed the possible role of Tregs infusion on preventing the onset of GVHD [23, 24].

We hereby hypothesized that this cell population may act as an important regulator of alloreactivity and that not only its content but also its degree of activity may help to predict the risk of GVHD. Aim of the present study was to verify whether the functional activity and suppressive capacity of peripheral blood (PB) Tregs correlates with the presence of acute and chronic GVHD.

PATIENTS AND METHODS

Patients

Sixteen adult patients suffering from hematologic malignancies who consecutively underwent an

*Address correspondence to this author at the Division of Hematology, Department of Cellular Biotechnologies and Hematology, "Sapienza" University, Via Benevento 6, 00161 Rome, Italy; Tel: +390649974321; Fax: +390644241984; E-mail: torelli@bce.uniroma1.it

E-ISSN: 2312-5411/15

allogeneic SCT at our Institute were investigated after having given written informed consent for participating to the study. Eight adult normal donors of the Transfusional Center of our University Hospital served as controls.

Cell Purification and Expansion

Tregs were purified from mononuclear cells obtained from 50 cc PB of patients who had undergone an allogeneic SCT or of normal donors using the CD4+CD25+ regulatory T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer instructions. Briefly, CD4+ T cells were negatively isolated by magnetic labeling of non-CD4+ T cells with a biotin-Ab cocktail and antibiotin MicroBeads using a LD column. Thereafter, the effluent preenriched CD4+ cells were labeled directly with CD25 Micro-Beads and separated using a MS column. For *in vitro* Treg expansion, 96-well U-Bottom plates were used. Each plate was coated overnight with 5g/ml of anti-CD3 and 5g/ml of anti-CD28 at 4°C. Purified Tregs were then cultured at a median of 2.5 x 10^4 cells/well (range: 2.1 x 10^4 - 3.0 x 10^4 cells/well) in complete RPMI supplemented with 10% heatinactivated fetal bovine serum (FBS, HyClone, South Logan, UT), 0.3mg/ml L-glutamine and 1% Pen-strepto (Euro-Clone, Milano, Italy), for 6 days. IL-2 was added on days 1 and 3 of the Treg cultures at a final concentration of 100U/ml.

Cytofluorimetric Analysis

Expanded Tregs were stained with four color (FITC/PE/PerCP/TC) combinations of conjugated monoclonal Ab (mAb) against the surface antigens CD3, CD4, CD25 and CD62L and for the cytoplasmic antigens CTLA-4 and FoxP3 (all from Becton Dickinson, Mountain View, CA, except for CD62L, CTLA-4 and FOXP3 which were from eBioscience, San Diego, CA). All marked cells, after PBS washing, were acquired using the FACSCalibur flow cytometer and analyzed with the CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Mixed Lymphocyte Reaction (MLR)

To assess the suppressive capacity of Tregs over the proliferative reaction of autologous peripheral T lymphocytes induced by allogeneic DCs, expanded CD4+CD25+ Tregs were seeded in 96-well U-Bottom plates at a 1:1 ratio together with naïve autologous effector cells at a final volume of 0.2 ml/well. Effector cells were previously stimulated at a 1:5 ratio with allogeneic DCs pulsed with apoptotic leukemic blasts (Apo-DCs). Expanded Tregs and autologous effector cells were seeded at a median of 7.2 x 10_4 cells/well (range: 2.5 x 10_4 – 1.0 x 10_5 cells/well), while Apo-DC were seeded at a median of 14.3 x 10_3 cells/well (range: 5.0 x 10_3 – 2.0 x 10_4 cells/well). After 6 days of culture, cells were incubated with 1 Ci of [^{3}H]-thymidine/well for 18 hours, harvested and counted using a beta-counter (Packard Bioscience, Groningen, The Netherlands). Suppressor activity was measured as [^{3}H]-thymidine incorporation in the presence or absence of Tregs. All experiments were set up in triplicate.

ELISA Assays

After the 6-day culture period required for Treg expansion, supernatants were harvested and tested for IL-10 production using the commercial quantitative sandwich immunoassay Quantikine kit (R&D System, Minneapolis, MN).

Statistical Analysis

The two-sided Student *t* test was used to evaluate the significance of differences between groups for expansion, suppressive and IL-10 production experiments. These results are expressed as the means plus or minus standard deviation (SD).

RESULTS

Expansion Capacity

Tregs purified from the PB of patients at 1 year from transplant without signs of GVHD (n = 9), with GVHD (n = 7: acute GVHD n = 2, chronic GVHD n = 3, acute and chronic GVHD n = 2), or from normal donors (n = 8) showed an equivalent expansion capacity: mean fold expansion (range), PB of patients without GVHD 7.0 (1.4-13), PB of patients with GVHD 7.8 (1.6-16.5), normal donors 4.6 (1.5-10) (Table **1**).

Table 1: Expansion Capacity of Tregs Origin of Tregs Fold Increase (Range)

Origin of Tregs	Fold increase (range)
PB patients with GVHD (n=7)	7.8 (1.6-16.5)
PB patients without GVHD (n=9)	7 (1.4-13)
Normal donors (n=8)	4.59 (1.5-10)

Cytofluorimetric Analysis

No differences were observed when the immunophenotypic analysis of expanded Tregs was

carried out, in terms of surface expression of CD4, CD25, CD62L, cytoplasmic CTLA-4 and Foxp3, from the two groups of patients and normal donors (data not shown).

Suppressive Function

Tregs expanded from the PB of patients without signs of GVHD (n = 5) exerted a significantly higher suppressive function on the proliferative reaction of T cells stimulated by allogeneic DCs compared to Tregs expanded from the PB of patients with GVHD (n = 5: acute GVHD n = 2, chronic GVHD n = 2, acute and chronic GVHD n = 1): mean fold reduction (range), PB of patients without GVHD 10.9 (5.28-21.82), PB of patients with GVHD 3.9 (1.49-6.34), p 0.05 (Figure **1**).

Cytokine Production

Tregs expanded from patients without GVHD (n = 3) showed a significantly higher *in vitro* IL-10 production capacity compared to patients with GVHD (n = 3: acute GVHD n = 1, chronic GVHD n = 1, acute and chronic GVHD n = 1): mean pg/ml (range), patients without GVHD 626 (461-731), patients with GVHD 245 (125-355), p 0.02 (Figure **2**).

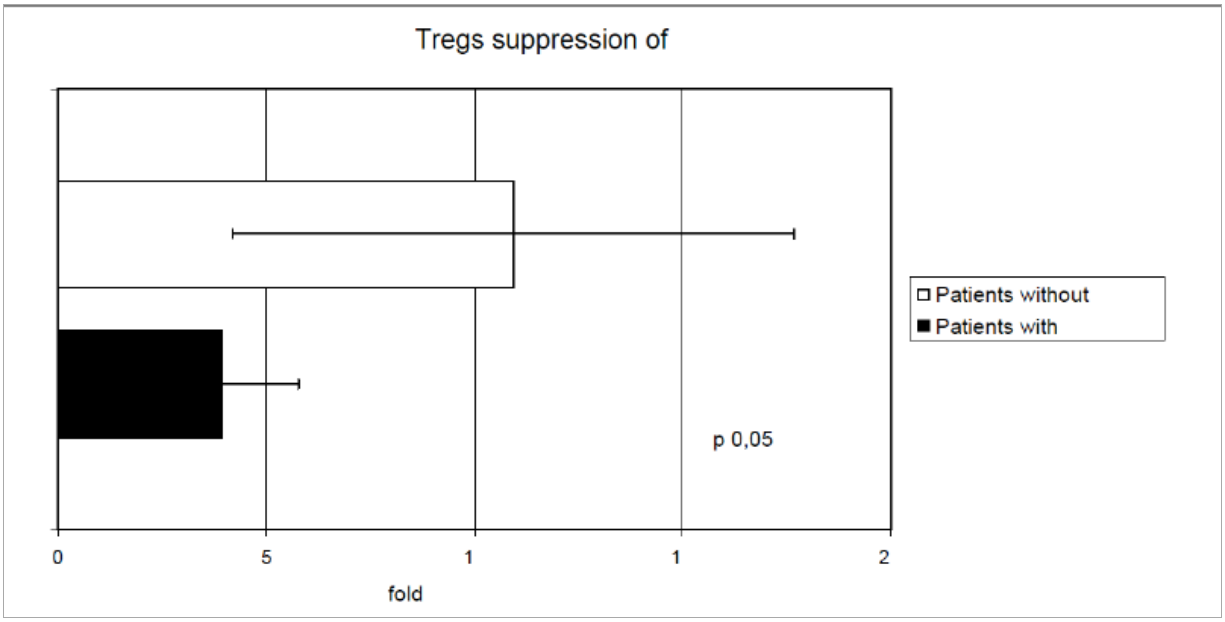

Figure 1: Suppressive capacity of MLR exerted by Tregs expanded from the PB of patients with or without GVHD.

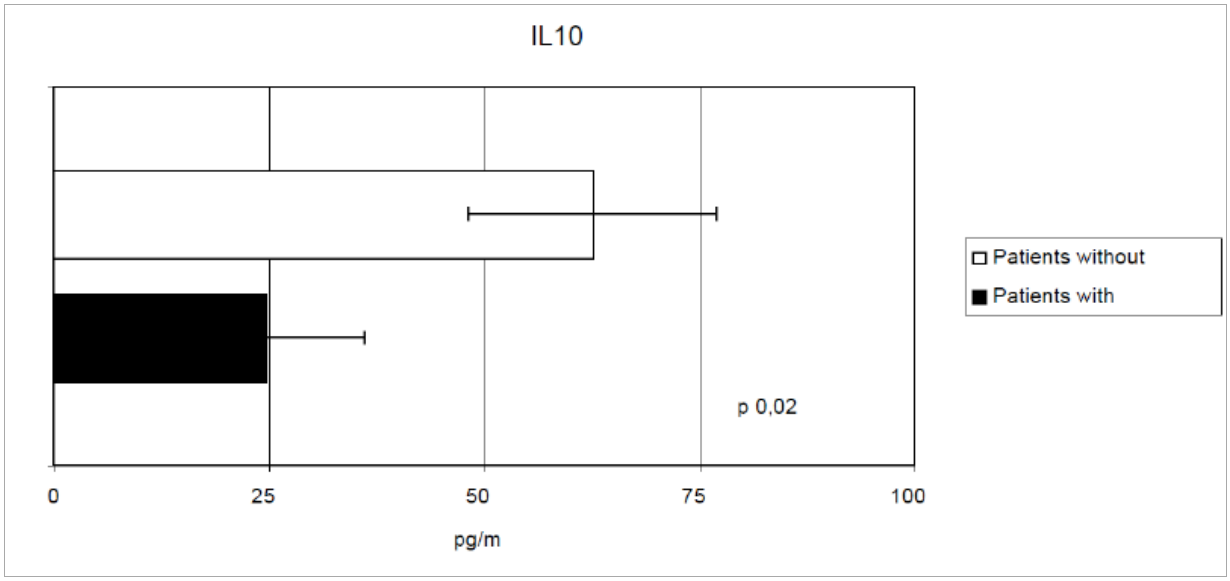

Figure 2: IL-10 production capacity of Tregs expanded from the PB of patients with or without GVHD.

DISCUSSION

The results of this study performed on patients suffering from hematologic malignancies who underwent an allogeneic SCT show that Tregs expanded from the PB of allotransplanted patients without any signs of GVHD display a higher *in vitro* suppressive capacity which correlates with a higher *in vitro* IL-10 production, when compared to Tregs expanded from patients who presented in their clinical courses an acute or chronic GVHD; in addition, no differences have been observed in the expansion capacity and immunophenotypic analysis between Tregs expanded from the PB of allotransplanted patients with or without signs of GVHD.

GVHD is one of the most important causes of morbidity and mortality following an allogeneic SCT, and it accounts for the reduced quality of life of these patients. The reports present in the literature that associate the content of Tregs with the onset of GVHD [11,12,25] have suggested the possibility of utilizing *in vivo* Tregs in the allogeneic SCT context for the prevention and treatment of GVHD [23,24]. Most interestingly, the results hereby presented indicate that not only the content but also the degree of Treg activity plays a decisive role towards the onset of the GVHD phenomenon. In fact, we could show that the *in vitro* suppressive capacity of expanded Tregs and their *in vitro* IL-10 production ability correlate with the onset of acute and/or chronic GVHD.

In order to more finely define the functional role played by Tregs in the protection from GVHD further studies, though more invasive, will need to investigate in individual patients the Treg tissue content at the site of the GVHD phenomenon; it would also be of interest to analyze the degree of activity of Tregs at the onset of GVHD, although the large amount of PB required to perform the analysis in patients presenting an acute GVHD may be a limiting factor.

In conclusion, the findings here reported confirm the importance of Treg activity for the modulation of the immune system in the transplant setting and support the design of therapeutic and/or preventive GMP protocols based on the *in vivo* infusion of *ex vivo* expanded and activated Tregs, aiming at modulating the activity of the immune system after an allogeneic SCT.

ACKNOWLEDGEMENTS AND DECLARATION OF INTERESTS

Research grant support: Istituto Superiore di Sanità (ISS), Rome, n. 7OAF3, Progetto “Oncologia”, Ministero della Salute, Fondazione Italiana di Ricerca in Medicina Sperimentale (FIRMS), Turin. Compagnia di San Paolo, Turin and Associazione Italiana per la Ricerca sul Cancro (AIRC), Milan, Italy.

The authors report no declarations of interest.

REFERENCES

[1] Thomas ED, Storb R, Clift RA, Fefer A, Johnson L, Neiman PE, *et al.* Bone Marrow Transplant. N Engl J Med 1975; 292: 895-902. http://dx.doi.org/10.1056/NEJM197504242921706

[2] Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, *et al.* Graft-versus-leukemia reactions after bone marrow transplantation. Blood 1990; 75: 555-62.

[3] Blazar BR, Korngold R and Vallera DA. Recent advances in graft-versus-host disease (GVHD) prevention. Immunol Rev 1997; 157: 79-109. http://dx.doi.org/10.1111/j.1600-065X.1997.tb00976.x

[4] Cutler C, Giri S, Jeyapalan S, Paniagua D, Viswanathan A and Antin JH. Acute and chronic graft-versus-host disease after allogeneic peripheral-blood stem-cell and bone marrow transplantation: a meta-analysis. J Clin Oncol 2001; 19: 3685-3691.

[5] Manjili MH and Toor AA. Etiology of GVHD: alloreactivity or impaired cellular adaptation? Immunol Invest 2014; 43: 851-857. http://dx.doi.org/10.3109/08820139.2014.953636

[6] Choi SW and Reddy P. Current and emerging strategies for the prevention of graft-versus-host disease. Nat Rev Clin Oncol 2014; 11: 536-547. http://dx.doi.org/10.1038/nrclinonc.2014.102

[7] Socié G and Ritz J. Current issues in chronic graft-versus-host disease. Blood 2014; 124: 374-384. http://dx.doi.org/10.1182/blood-2014-01-514752

[8] Markey KA, MacDonald KP and Hill GR. The biology of graftversus-host disease: experimental systems instructing clinical practice. Blood 2014; 124: 354-362. http://dx.doi.org/10.1182/blood-2014-02-514745

[9] Iori AP, Torelli GF, De Propris MS, Milano F, Pupella S, Gozzer M, *et al.* B-cell concentration in the apheretic product predicts acute graft-versus-host disease and treatmentrelated mortality of allogeneic peripheral blood stem cell transplantation. Transplantation 2008; 85: 386-390.

[10] Issa F, Robb RJ and Wood KJ. The where and when of T cell regulation in transplantation. Trends Immunol 2013; 34: 107-113. http://dx.doi.org/10.1016/j.it.2012.11.003

[11] Rezvani K, Mielke S, Ahmadzadeh M, Kilical Y, Savani BN, Zeilah J, *et al.* High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. Blood 2006; 108: 1291-1297. http://dx.doi.org/10.1182/blood-2006-02-003996

[12] Zorn E, Kim HT, Lee SJ, Floyd BH, Litsa D, Arumugarajah S, *et al.* Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. Blood 2005; 106: 2903-2911. http://dx.doi.org/10.1182/blood-2005-03-1257

[13] Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, *et al.* CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med 2003; 9: 1144-1150. http://dx.doi.org/10.1038/nm915

[14] Nagler-Anderson C, Bhan AK, Podolsky DK and Terhorst C. Control freaks: immune regulatory cells. Nat Immunol 2004;

5: 119-22.
http://dx.doi.org/10.1038/ni0204-119

[15] Fehervari Z and Sakaguchi S. CD4+ Tregs and immune control. J Clin Invest 2004; 114: 1209-1217.
http://dx.doi.org/10.1172/JCI200423395

[16] Fehervari Z and Sakaguchi S. Development and function of CD25+CD4+ regulatory T cells. Curr Opin Immunol 2004; 16: 203-208.
http://dx.doi.org/10.1016/j.coi.2004.01.004

[17] Lamioni A, Parisi F, Isacchi G, Giorda E, Di Cesare S, Landolfo A, *et al.* The immunological effects of extracorporeal photopheresis unraveled: induction of tolerogenic dendritic cells in vitro and regulatory T cells in vivo. Transplantation 2005; 79: 846-850.
http://dx.doi.org/10.1097/01.TP.0000157278.02848.C7

[18] Lim HW, Hillsamer P, Banham AH and Kim CH. Cutting Edge: direct suppression of B cells by CD4+CD25+ regulatory T cells. J Immunol 2005; 175: 4180-4183.
http://dx.doi.org/10.4049/jimmunol.175.7.4180

[19] Taylor PA, Lees CJ and Blazar BR. The infusion of ex vivo activated and expanded CD4+CD25+ immune regulatory cells inhibits graft-versus-host disease lethality. Blood 2002; 99: 3493-3499.
http://dx.doi.org/10.1182/blood.V99.10.3493

[20] Hoffmann P, Ermann J, Edinger M, Fathman CG and Strober S. Donor-type CD4(+) D25(+) regolatory T cells suppress lethal acute graft-versus-host-disease after allogeneic bone marrow transplantation. J Exp Med 2002; 196: 389-399.
http://dx.doi.org/10.1084/jem.20020399

[21] Cohen JL, Trenado A, Vasey D, Klatzmann D and Salomon BL. CD4+CD25+ immunoregulatory T cells: new therapeutics for graft-versus-host disease. J Exp Med 2002; 196: 401-406.
http://dx.doi.org/10.1084/jem.20020090

[22] Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, *et al.* Recipient-type specific CD4+CD25+regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versusleukemia. J Clin Invest 2003; 112: 1688-1696.
http://dx.doi.org/10.1172/JCI17702

[23] Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, Curtsinger J, *et al.* Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood 2011; 117: 1061-1070.
http://dx.doi.org/10.1182/blood-2010-07-293795

[24] Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, *et al.* Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood 2011; 117: 3921-3928.
http://dx.doi.org/10.1182/blood-2010-10-311894

[25] Schneider M, Munder M, Karakhanova S, Ho AD and Goerner M. The initial phase of graft-versus-host-disease is associated with decrease of CD4+CD25+ regolatory T cells in the peripheral blood of patients after allogeneic stem cell transplantation. Clin Lab Haematol 2006; 28: 382-390.
http://dx.doi.org/10.1111/j.1365-2257.2006.00825.x

Received on 12-12-14 Accepted on 18-01-15 Published on 31-12-2015

DOI: http://dx.doi.org/10.12974/2312-5411.2015.02.01.4