Expression of the putatively regulatory T-cell marker *FOXP3 by* CD4⁺CD25⁺ T cells after pediatric hematopoietic stem cell transplantation

Markus G. Seidel
Ursula Ernst
Dieter Printz
Birgit Juergens
Josefa Pichler
Andishe Attarbaschi
Gerhard Fritsch
Helmut Gadner
Andreas Heitger

FOXP3 has been proposed to be critical for the regulatory function of CD4⁺CD25⁺ T cells and it has been reported that its expression correlates with protection from graft-versus-host-disease (GvHD) after allogeneic hematopoietic stem cell transplantation (HSCT). Here, by monitoring 28 pediatric HSCT recipients, we found that the levels of *FOXP3*-mRNA expression in highly enriched CD4⁺CD25⁺ cells were identical to those in healthy controls irrespective of GvHD status. Moreover, *FOXP3*-mRNA was abundant in recently *in vitro* stimulated CD4⁺CD25⁺ cells that lacked regulatory function. Together these findings suggest that *FOXP3*-mRNA expression primarily reflects CD4⁺CD25⁺ cell frequency rather than defining the regulatory potential of CD4⁺CD25⁺ T cells and GvHD risk after HSCT.

Key words: hematopoietic stem cell transplantation, tolerance, graft-versus-host disease, CD4⁺CD25⁺ T cells, *FOXP3* expression, scurfin.

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All authors from the St. Anna Children's Hospital and Children Cancer Research Institute, Children's Cancer Research Institute (CCRI), Kinderspitalgasse 6, A-1090 Wien, Austria.

Correspondence:
Andreas Heitger, M.D., Associate Professor of Pediatrics, St. Anna Kinderspital, Children's Cancer Research Institute (CCRI), Kinderspitalgasse 6, A-1090 Wien, Austria.
E-mail: Andreas.Heitger@ccri.at

CD4⁺CD25⁺ T cells have been implicated in two apparently opposing functions, namely an *activated effector* function but also a *regulatory* function. In human hematopoietic stem cell transplantation (HSCT), the frequency of CD4⁺CD25⁺ T cells is increased[1] and this increase was reported to correlate with graft-versus-host disease (GvHD),[2] suggesting that the *effector* function predominates. In mice, CD4⁺CD25⁺ cells have been proposed to maintain self-tolerance and protect from GvHD, thus representing regulatory (T_{reg}) cells.[3] *FOXP3*, a transcriptional repressor in T_{reg}-cells, was suggested to specifically identify CD4⁺CD25⁺ cells as *regulatory* cells, because its deficiency leads to a lack of T_{reg} cells,[4,5] and overexpression increases the number of T_{reg} cells.[6] Hence, *FOXP3* is considered necessary and sufficient for T_{reg} activity.[7-9] Here, we investigated whether different levels of *FOXP3* expression in highly purified CD4⁺CD25⁺ cells after allogeneic HSCT would indicate the regulatory capacity of a given CD4⁺CD25⁺ cell population and thus be related to the risk of GvHD.

Design and Methods

Patients

Pediatric patients (n=28, median age 11.0 years, 17 males, 11 females) underwent HSCT from HLA-identical family (n=13) or matched unrelated donors (n=15) for relapsed or high-risk leukemia or lymphoma and received myeloablative conditioning with chemotherapy only (n=23) or additional total body irradiation (1.2 Gy, n=5). Anti-thymocyte globulin was given in six cases. Cyclosporin-A was tapered after T-cell engraftment. The transplant recipients or their representatives and the healthy donors gave informed consent to investigations of their T cells.

T-cell reconstitution

Lymphocyte reconstitution was monitored by flow cytometry (FACS) using a FACSCalibur flow cytometer, (Beckton-Dickinson [BD], Franklin Lakes, NJ, USA). Monoclonal antibodies were obtained from several companies (Dako, Glostrup, Denmark or BD; and Coulter, Krefeld, Germany) as described elsewhere.[10] CD4⁺ T cells were identified as a CD45⁺CD3⁺CD4⁺ cell population, and gated and assessed for CD25 expression. CellQuest-software (BD) was used for data acquisition and Paint-a-Gate-Pro (BD) for evaluation.

Chimerism analysis

The samples from patients with sex-matched donors were analyzed by polymerase chain reaction (PCR)[11] using highly polymorphic short tandem repeat (STR) markers. Specimens from patients with sex-mismatched donors were analyzed by fluorescence *in situ* hybridization. All patients had ≥99% donor T-cell chimerism, except for one with ≥83% donor T cells at the time of investigation.

Functional assays

CD4⁺CD25⁺ or CD4⁺CD25⁻ cells were purified by FACS (purity ≥99%, median) and were either cultured in RPMI-1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with protein and antibiotics for functional assays, or lysed for RNA extraction using the Blood-Mini-Kit (QIAGEN, Hilden, Germany). Polyclonal T-cell stimulation was performed with immobilized anti-CD3 (250 ng/mL, Sigma-Aldrich, St. Louis, MO, USA), soluble anti-CD28 (2 μg/mL, Sigma) and proliferation was measured by [³H]-thymidine incorporation (Amersham, UK).

Gene expression

FOXP3-mRNA molecules were detected by quantitative real-time reverse transcription PCR (TaqMan™, Applied Biosystems, Foster City, CA, USA), related to a *FOXP3* standard (pIRES2-eGFP-hFOXP3 provided by S.F.Ziegler)[12] and to β-2-microglobulin (β2m) and β-glucuronidase genes (done in parallel in

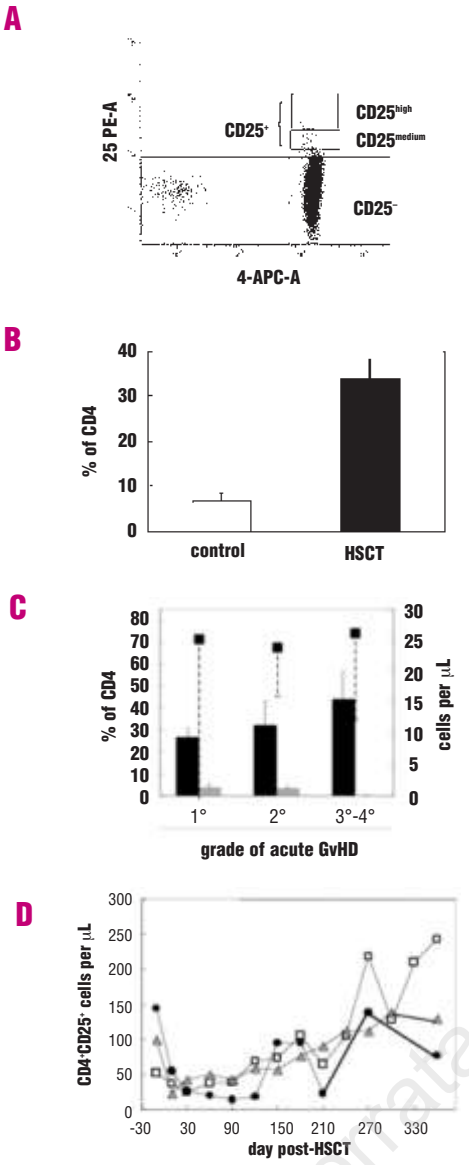

Figure 1. CD4+CD25+ cell frequency in healthy donors and pediatric patients after HSCT. Total white blood cells were analyzed for the content of CD25+ cells within the CD4+ T cell compartment by flow cytometry. **A.** Gating strategy for identifying CD4+CD25medium and CD4+CD25high cells derived from a healthy donor by their different expression levels of cell surface CD25. **B.** The proportions of total CD25+ T cells of CD4+ T cells are shown in control subjects (blank bars, n=8) and pediatric patients after HSCT at the time of T-cell engraftment (first day of CD3+ cells >100/µL; filled bar, n=28). Data represent the median ± standard errors of the mean (**B** and **C**). **C.** The fraction of CD4+CD25+ T cells of total CD4+ T cells of pediatric patients after HSCT at onset of acute GvHD are shown according to the grade of severity of the GVHD (grade 1, n=12; grade 2, n=7; grades 3 or 4, n=5; occurring at a median of day +20, +10, +17, respectively). Blood samples were obtained before initiation of GvHD-specific treatment. The shaded bars indicate the proportions of putatively naïve T cells (CD45RA+CD62L+) within the CD4+ compartment. Squares indicate the absolute count of CD4+CD25+ cells per microliter of whole blood. **D.** Absolute numbers of CD4+CD25+ cells/µL blood from 28 HSCT recipients were monitored from day –30 until +360 after HSCT and related to the development of GvHD. Medians are shown for three patient groups, defined by the maximum grade of acute GvHD diagnosed during the first year post-HSCT: grade 0-1 acute GvHD, n=16, open squares; grade 2, n=7, shaded triangles; grades 3-4, n=5, filled circles.

22 specimens, *not shown*) using an ABI-Prism-7700 (Applied Biosystems) as described previously[13] and the following probes FOXP3-7fw: 5'GAGAAGCTGAGTGCCATGCA, FOXP3-8rev: 5'GGAGCCCTTGTCGGATGAT: FOXP3-7revProbe: 5'FAM-CACAGATGAAGCCTTGGTCAGT-GCCAT-TAMRA: β-2-m-2fw: 5'TGAGTATGCCTGC-CGTGTGA, β2m-3rev: 5'TGATGCTGCTTACATGTCTC-GAT, β2m-probe: 5'FAM-CCATGTGACTTTGTCACA-GCCCAAGATAGTT-TAMRA: β-glucuronidase: GUS-fw: 5'GAAAATATGTGGTTGGAGAGCTCATT, GUS-rev: 5'CCGAGTGAAGATCCCCTTTTTA, GUS-probe: 5'FAM-CCA-GCACTCTCGTCGGTGACTGTTCA-TAMRA.

Statistics

Wilcoxon's exact test was used to compare groups. A *p*-value of <0.05 was considered statistically significant.

Results and Discussion

Total CD4+CD25+ and CD4+CD25high T cells (Figure 1A), were analyzed in 28 pediatric patients post-HSCT first at T-cell engraftment (>100/µL total CD3+ cells) and subsequently at 2-weekly intervals during the first year post-HSCT. As in previous studies,[1] in this series the fraction of CD4+CD25+ T cells within the CD4+ T-cell compartment was increased as compared to that in healthy subjects (32% vs. 8% median, $p<0.001$; Figure 1B). However, neither the number of CD4+CD25+ T cells at the onset of GvHD (Figure 1C) nor monitoring CD4+CD25+ T cells for up to one year after HSCT (Figure 1D) was related to subsequent GvHD severity.[14] A separate analysis of CD4+CD25high T-cells yielded parallel-shifted but otherwise identical results (*not shown*). In subsequent studies the levels of *FOXP3* expression were first determined in fresh highly purified CD4+CD25+ and CD4+CD25- cells obtained from healthy subjects (n=22) to define a range of normal. *FOXP3*-mRNA was consistently ~100-fold more abundant in CD4+CD25+ than in CD4+CD25- T cells (Figure 2A, first column.[7-9,12] *FOXP3*-mRNA levels were only insignificantly higher in CD4+CD25high T cells than in the CD4+CD25med T-cell population (Figure 2A second column). Remarkably, samples obtained from patients after HSCT (n=7) showed *FOXP3* levels in the same range as in controls, irrespective of the presence of grade 2-4 or no acute GvHD (Figure 2A, third column). Repetitive monitoring of CD4+CD25+ T cells in individual patients in stable clinical conditions within a one-month period after HSCT revealed a ±9% oscillation of the CD4+CD25+ T-cell fraction accompanied by parallel changes of *FOXP3* (Figure 2B). The same range was found in repetitive analyses of controls (*not shown*). Since we found abundant *FOXP3*-mRNA being restricted to CD4+CD25+ T cells (Figure 2A),[7-9] we then calculated the ratio of total *FOXP3* to β2m of total WBC in relation to the proportion of CD4+CD25+ cells ($(FOXP3/\beta2m)_{CD25+}=FOXP3_{total}/(\beta2m_{total}*[\%CD4^+CD25^+])$). These analyses, requiring only small amounts of blood, enabled us to study a further 36 specimens from a total of 21 patients with and without GvHD (Figure 2A, fourth column) yielding an identical range of *FOXP3*-mRNA content as in FACS-sorted pure CD4+CD25+ cell specimens. These findings indicated that *FOXP3*-mRNA expression by CD4+ T cells in pediatric HSCT was closely associated with the CD4+CD25+ cell compartment.

In contrast to patients' CD4+CD25- T cells, post-HSCT CD4+CD25+ cells were unresponsive to polyclonal stimulation (Figure 3A), and thus functionally resembled T_{reg} cells.

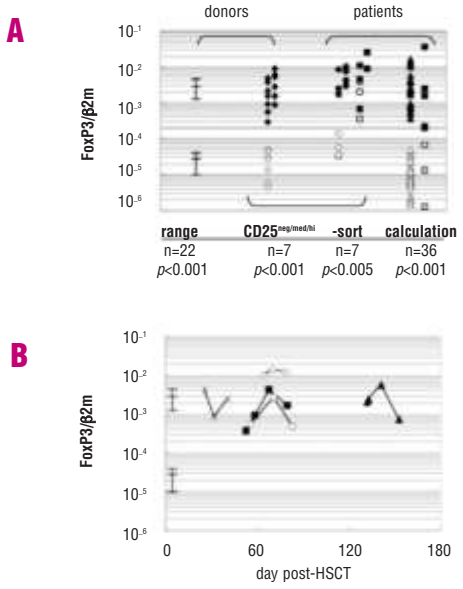

Figure 2. A. *FOXP3*-mRNA expression levels correlate with the frequency of CD4⁺CD25⁺ white blood cells in HSCT patients with and without GvHD. A, First column from left; the range of FOXP3 mRNA expression in CD4⁺CD25⁺ and CD4⁺CD25⁻ cells of healthy control subjects (n=22) was determined by rtPCR (ratio of *FOXP3* to β2m) of freshly isolated CD4⁺ cells separated into CD4⁺CD25⁺ and CD4⁺CD25⁻ fractions by flow cytometry (purity 99%). The vertical line indicates the median and the 25th and 75th percentiles. Second and third columns from left; in selected experiments, CD4⁺ cells from controls (second column, n=7) and from patients after HSCT (third column, n=7) were sorted into CD25⁻ (open diamonds), CD25med (shaded diamonds) and a CD25hi (*black* diamonds) cell fractions. The grade of acute GvHD is indicated by circles (GvHD 0-1) and squares (GvHD grade 2-4; grade 2[gut]:n=1; grades 2-3[gut, liver]: n=1; grade 4 [skin]: n=1). Although our data do not show *FOXP3* expression on a per-cell basis, the fact that FACS-sorted pure cell populations of ~5×10² cells up to >1×10⁵ cells showed an identical range of absolute copy numbers of *FOXP3*-mRNA relative to the cell numbers used suggests that the CD4⁺CD25⁺ cell compartment was homogenous regarding the level of expression of *FOXP3*. *The right column* indicates the range of *FOXP3* expression corresponding to the CD4⁺CD25⁺ cell fraction of WBC specimens (n=36; see *Results section for calculation formula*) obtained from 21 individual patients at one or more time points after HSCT without GvHD (*triangles*) and from four patients with grade 2-4 acute GvHD (squares; skin and gut GvHD). *p* values indicate statistical comparisons of *FOXP3* between CD25⁺ and CD25⁻ cells by Wilcoxon´s exact test. B. Repeated analyses of calculated *FOXP3*/β²mCD25⁺ ratios (as in the right column of A) of CD4⁺CD25⁺ T cells from five HSCT recipients in a stable clinical condition within 3-4 weeks after transplantation.

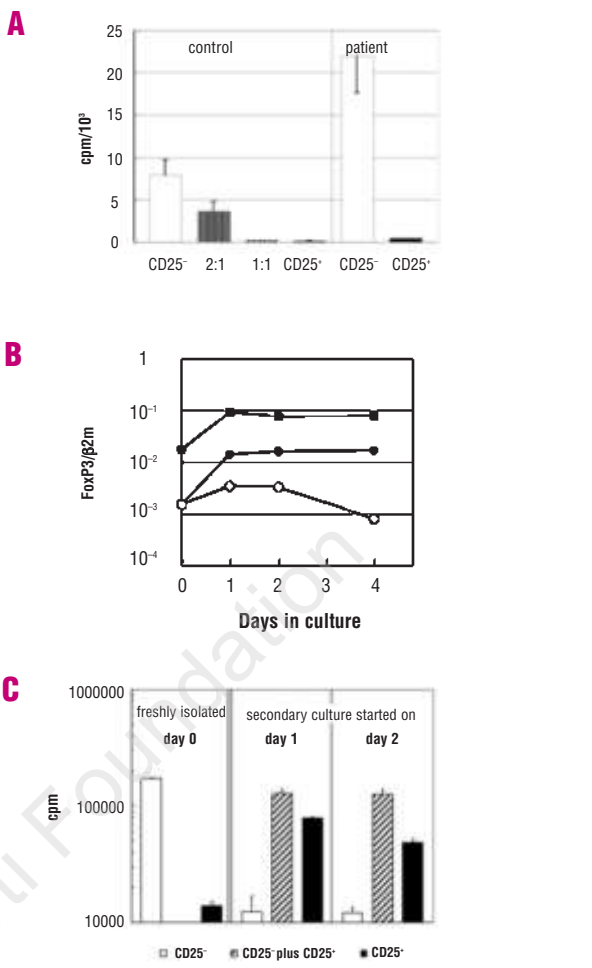

Figure 3. FOXP3 expression and functional characteristics of freshly isolated or activation-induced CD4⁺CD25⁺ cells. A. Control and post-HSCT CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were stimulated with immobilized anti-CD3 and anti-CD28 monoclonal antibodies and cultured for 72 hours. DNA synthesis was determined by [³H]-thymidine incorporation. Relative cpm are shown for 5×10⁴ CD25⁻ cells/well (open bar), co-cultured with 2.5×10⁴ or 5×10⁴ CD25⁺ cells/well (2:1 and 1:1, respectively; *shaded bars*), or 5×10⁴ CD25⁺ cells/well alone (*filled bar*). Error bars show the standard error of means of triplicates. The results shown are of one experiment, representative of four performed. In two experiments the cells were obtained from a patient with grade 2 GvHD. B and C, Freshly isolated CD4⁺CD25⁺ and CD4⁺CD25⁻ cells from healthy donors were highly purified by flow cytometry and were separately stimulated with immobilized anti-CD3 and anti-CD28 for 72 hours as in A. The cultures that originally contained only CD4⁺CD25⁻ cells were re-sorted into a (declining) population that remained CD25⁻ and another population that had acquired CD25 expression (*induced* CD4⁺CD25⁺ cells). At the beginning (indicated as day 0) and after one to four days of stimulation, CD4⁺ T cells were analyzed daily for changes of CD25 and *FOXP3*-mRNA expression as well as for proliferation and suppressive function. *FOXP3*-mRNA expression of constitutive CD4⁺CD25⁺ cells (*filled squares*) and of re-sorted CD4⁺CD25⁻ cells (*open circles*) before and after *in vitro* stimulation as well as of *induced* CD4⁺CD25⁺ (*filled circles*) cells are shown in B. C. Proliferation and co-culture suppression assays were performed as in A with freshly isolated cells (=day 0; left panel, *co-culture not shown*; CD4⁺CD25⁻ cells: *open bars*, CD4⁺CD25⁺ cells: *filled bars*) and daily re-sorted *induced* CD25⁺ and CD25⁻ cells (days 1 and 2; *right panel*). Because only a minute fraction of cells remained CD25⁻ after 24-72 hours of stimulation in these culture conditions, responder CD4⁺CD25⁻ T cells for subsequent proliferation/suppression assays were obtained from CD4⁺CD25⁻ cells frozen on day 0 and thawed for co-cultures with *induced* CD4⁺CD25⁺ T cells (*hatched bars*). The data shown are from one representative experiment of three performed.

The low number of circulating T cells after HSCT and the limited access to quantities of blood in pediatric patients precluded a direct investigation of the suppressor activity of post-HSCT CD4⁺CD25⁺ T cells.

Insofar as high proportions of CD4⁺CD25⁺ cells were present in patients with GvHD and abundant *FOXP3* expression was a constant finding in CD4⁺CD25⁺ cells, we questioned whether *FOXP3* might be present in CD4⁺CD25⁺ cells that do not act as regulatory cells. One CD4⁺CD25⁺ cell population without regulatory activity is represented by recently activated CD4⁺CD25⁻ cells that convert into a CD4⁺CD25⁺ cell population;[15] these cells do not suppress naïve T cells and proliferate rapidly. To test this possibility, highly purified CD4⁺CD25⁺ and CD4⁺CD25⁻ cell populations from controls were stimulated *in vitro* and monitored in parallel for CD25 and *FOXP3*-mRNA (Figure 3B). The acquisition of CD25 cell surface expression was accompanied by a parallel increase of *FOXP3*-mRNA expression. Furthermore, when highly enriched by FACS-sorting, these *FOXP3*⁺ *induced*-CD25⁺ T cells proliferated rapidly and, when co-cultured with CD25⁻ cells, [³H]-thymidine uptake of the total culture approximated the sum of the uptake of CD25⁻ plus CD25⁺ cells (Figure 3C), corroborating that these cells did not act as regulatory cells.[15] Thus, CD4⁺CD25⁺ T cells may acquire a stage of differentiation at which *FOXP3* expression is high but regulatory function is lacking.

The most important observation we made in this cohort of pediatric patients was that after HSCT *FOXP3* expression was closely linked to the CD4+CD25+ T-cell population irrespective of the presence of GvHD. These data are in conflict with previous reports which claimed that *FOXP3*-mRNA expression when measured in total peripheral blood mononuclear cells or lymphocytes[16,17] or T cells[2] represented a biomarker for determination of GvHD risk after allogeneic HSCT. Moreover, our finding that abundant *FOXP3*-mRNA was detectable in recently activated CD4+CD25+ T cells lacking regulatory function indicate that in humans the determination of *FOXP3* alone may not be sufficient to indicate regulatory activity of CD4+CD25+ T cells. Thus, despite the limitations of this study (small sample size, only pediatric patients investigated, low incidence of GvHD), these findings instigate caution concerning measurements of *FOXP3* to estimate the risk of GvHD.[2,14,16-19] Given the close association of *FOXP3* expression and CD4+CD25+ T cells in patients after HSCT, CD4+CD25+*FOXP3*^{high} T cells may comprise constitutive T_{reg} cells[16-18] or peripheral naïve T cells that converted to this phenotype after exposure to activation stimuli (allo-reactivity or infection).[12,20] Although, when examined as a total population, these peripheral post-HSCT CD4+CD25+*FOXP3*^{high} T cells appeared similar to classical T_{reg} cells, they may also, at least to some extent, contain recently activated non-regulatory cells, which may affect their total regulatory capacity. The value of measuring circulating CD4+CD25+*FOXP3*+ cells to predict GvHD risk may further be limited, as the frequency and function of T_{reg}-cells in local target tissues rather than in the circulation may be critical to control inflammation and GvHD.[21,22] Functionally, the regulatory capacity of post-HSCT CD4+CD25+ cells may be altered by GvHD-prophylactic immunosuppressive medication[23] or by a functional defect, in analogy to what has been described in multiple sclerosis.[24] Thus, in line with a recent observation,[25] the determination of *FOXP3*, which we found to be expressed by post-HSCT CD4+CD25+ T cells irrespective of GvHD status, appears of limited value for estimating the overall CD4+CD25+ T-cell regulatory capacity and GvHD risk.

DP, JP and GF contributed substantially to patient data acquisition and analysis (FACS differential blood counts, chimerism analyses): MGS, UE and BJ performed in vitro laboratory experiments: AA, AH, and MGS were responsible for clinical data acquisition, treatment of HSCT patients, and follow-up documentation: HG, AH and MGS are responsible for the general concept and design of the study: in addition, AH supervised both the methodological and theoretical composition and the preparation of the article by MGS. All authors contributed to the interpretation of the data, revised the manuscript critically for intellectual content, and approved its final version.

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