Expression of the putatively regulatory T-cell marker FOX3 by CD4+CD25+ T cells after pediatric hematopoietic stem cell transplantation

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FOX3 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 FOX3-mRNA expression in highly enriched CD4+CD25+ cells were identical to those in healthy controls irrespective of GvHD status. Moreover, FOX3-mRNA was abundant in recently in vitro stimulated CD4+CD25+ cells that lacked regulatory function. Together these findings suggest that FOX3-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, FOX3 expression, scurfin.

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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 increased1 and this increase was reported to correlate with a lack of Treg cells,2 and overexpression increases the number of Treg cells.3 Hence, FOX3 is considered necessary and sufficient for Treg activity.4 Here, we investigated whether different levels of FOX3 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 FACScan Calibur 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.5 CD4+ T cells were identified as a CD45+CD25+CD127low cell population, 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)6 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

FOX3-mRNA molecules were detected by quantitative real-time reverse transcription PCR (TaqMan7, Applied Biosystems, Foster City, CA, USA), related to a FOX3 standard (pIRES2-eGFP-hFOX3 provided by S.E.Ziegler)8 and to β-2-microglobulin (β2m) and β-glucuronidase genes (done in parallel in
and the following cells >100/µL T cells (Figure 1A), to T-cell compartment by flow cytometry. A. Gating strategy for identifying CD4+CD25hi T cells derived from a healthy donor by their different expression levels of cell surface CD25. B. The proportions of total CD4+ T cells of pediatric patients after HSCT at onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1B) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor to T-cell compartment was increased in CD4+ T cells at the onset of GvHD (Figure 1C) nor

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+CD25hi 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, 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. A separate analysis of CD4+CD25hi 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 ~10-fold more abundant in CD4+CD25+ than in CD4+CD25- T cells (Figure 2A, first column). In subsequent studies the levels of FOXP3-mRNA were only insignificantly higher in CD4+CD25hi T cells than in the CD4+CD25mid T-cell population (Figure 2A second 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), we then calculated the ratio of total FOXP3 to \( \beta2m \) of total WBC in relation to the proportion of CD4+CD25+ cells (FOXP3/\( \beta2m \) of CD4+CD25+ cells). 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 Treg cells.

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+CD25lo and CD4+CD25hi cells derived from a healthy donor by their different expression levels of cell surface CD25. B. The proportions of total CD4+ 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; filled bar, n=28). Data from a total of 21 patients with and without GvHD (Figure 2A, third 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 Treg cells.
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; these cells do not suppress naive 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 approached the sum of the uptake of CD25+ plus CD25− cells (Figure 3C), corroborating that these cells did not act as regulatory cells. Thus, CD4+CD25+ T cells may acquire a stage of differentiation at which FOXP3 expression is high but regulatory function is lacking.

**Figure 2.** 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), CD25+ (shaded diamonds) and a CD25− (black diamonds) cell fractions. The grade of acute GvHD is indicated by circles (GvHD 0-1) and squares (GvHD grade 2-4; grade 2[1 gut); n=1; grades 2-3[1 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 ~5×10^6 cells (Figure 3C), corroborating that these cells do not act as regulatory cells.

**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 5×10^5 CD25− cells/well (open bar), co-cultured with 2.5×10^5 or 5×10^5 CD25+ cells/well (2:1 and 1:1, respectively; shaded bars), or 5×10^5 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 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 lymphocytes²⁴,²⁵ or T cells 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.²⁴,²⁵ Given the close association of FOXp3 expression and CD4+CD25+ T cells in patients after HSCT, CD4+CD25+ FOXp3+ T cells may comprise constitutive Treg cells or peripheral naïve T cells that converted to this phenotype after exposure to activation stimuli (allo-reactivity or infection).²²,²³ Although, when examined as a total population, these peripheral post-HSCT CD4+CD25+ FOXp3+ T cells appeared similar to classical Treg cells, they may also, to at least 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 Treg cells in local target tissues rather than in the circulation may be critical to control inflammation and GvHD.²²,²³ Functionally, the regulatory capacity of post-HSCT CD4+CD25+ cells may be altered by GvHD-prophylactic immunosuppressive medication²⁵ or by a functional defect, in analogy to what has been described in multiple sclerosis.²⁴ Thus, in line with a recent observation,²⁶ 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.

References