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ORIGINAL ARTICLE

Stem cell source-dependent reconstitution of FOXP3 + T cells after pediatric SCT and the association with allo-reactive disease

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In adult patients, regulatory CD4 + FOXP3 + T cells are suggested to have a role in the control of allo-reactive disease after hematopoietic SCT (HSCT). We compared CD4 + FOXP3 + T-cell reconstitution after unrelated cord blood (UCB), matched unrelated donor (MUD) and matched sibling donor (MSD) HSCT in children, starting as early as 1 week after transplantation, and analyzed the association with allo-reactive disease. A total of 30 children were included who underwent a myeloablative-conditioning regimen followed by UCB (12/30), MUD (7/30) or MSD (11/30) HSCT. These three patient groups showed significant differences in FOXP3 + T-cell reconstitution pattern. Early after UCB and MSD, but not after MUD, HSCT a peak in FOXP3 + T cells was observed. There were significant differences in activation status and Ki67 expression of the FOXP3 + T cells after UCB and MSD, respectively. FOXP3 + T-cell proportions early after HSCT and in the graft were inversely correlated with allo-reactivity. This study indicates that FOXP3 reconstitution after HSCT is dependent on the type of graft used. Furthermore, in children evaluation of FOXP3 + T-cell numbers early after HSCT and in the graft may be used to judge the risk of developing allo-reactivity after HSCT.

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Keywords: hematopoietic SCT; GVHD; reconstitution; FOXP3; children

INTRODUCTION

Allogeneic hematopoietic SCT (HSCT) may provide a curative therapy for hematological malignancies, BM failure syndromes and inborn errors (for example, immunodeficiencies, hemoglobinopathies and metabolic conditions). Different stem cell sources are currently used for HSCT, BM-derived stem cells from related (matched sibling donor (MSD)), or unrelated donors (matched unrelated donor (MUD)) and unrelated cord blood cells (UCB). Cord blood sources are on the rise because of the rapid availability and the necessity of fewer matching criteria for successful HSCT.

The downsides of HSCT are the life threatening early allo-reactive complications of acute GVHD (aGVHD) and the related idiopathic pulmonary syndrome (IPS), which limit the use of HSCT as standard therapy for severe disorders. Although the pathogenesis of aGVHD and IPS is unclear, based on recent studies it is plausible that the pathogenesis of both syndromes is similar.¹ Tissue damage, attributable to previous therapy, underlying disease and conditioning regimens is assumed to be the initial trigger for the development of allo-reactive T-cell clones inducing both syndromes.^{2,3} Currently, preventive and therapeutic allo-reactive disease treatment protocols are based on nonspecific immunosuppressive therapy that suppresses the graft-versus-tumor effect and causes severe side effects including infections and viral reactivations.⁴ Insight into the dynamics of early immune reconstitution after HSCT is essential for unraveling the mechanisms that may be involved in allo-reactive disease and may also lead to the development of new and more specific therapeutic approaches.

CD4 + FOXP3 + regulatory T cells (FOXP3 + Treg) are well known to have an important role in establishing and maintaining immunotolerance.^{5–7} FOXP3 + Treg have been shown to be important in prevention of autoimmunity, asthma, allergy and immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome.^{8–11} Interestingly, in preclinical studies, infusion of FOXP3 + T cells at the time of HSCT or immediately following HSCT successfully prevented allo-reactive disease.^{6,7,12,13} A recent study in which Treg were preferentially expanded *in vivo* via daily s.c. low-dose IL-2 in chronic GVHD patients showed promising results.¹⁴ However, further studies are needed to optimize this treatment and evaluate the safety and impact on prevention and/or efficacy in treatment of allo-reactivity.

In addition, as Treg treatment is not without risks it may be desirable to selectively identify and treat only those patients at high risk of developing allo-reactivity with Treg therapy.^{15,16} In this context, the stem cell source and level of mismatch between graft and host are important determinants. Clinical studies in adult patients have shown increased FOXP3 + Treg proportions in the donor graft or FOXP3 + Treg proportions early after HSCT to be negatively correlated with aGVHD.^{5,17–21} In pediatric patients, data on such a correlation are very few.²²

The aim of this single-center study is to obtain a clearer insight into the reconstitution pattern of FOXP3 + T cells in pediatric MSD, MUD and UCB HSCT recipients, and the possible relationship to the development of early allo-reactive disease. This study shows important differences between stem cell sources in early FOXP3 + T-cell reconstitution patterns and suggests that these patterns

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have evolved via different mechanisms. In addition, as is seen in adult patients, the FOXP3 + T-cell proportions in the graft and early after HSCT correlate with the risk of developing allo-reactive disease.

MATERIALS AND METHODS

Study design, study population and data collection

Pediatric HSCT patients (aged 0–20 years), who received a first allogeneic HSCT in the University Medical Center of Utrecht, irrespective of the indication for HSCT, were included between July 2007 and December 2008. The stem cell source for the allogeneic HSCT was either (1) UCB, (2) MSD or (3) MUD (either BM or PBSC derived). Patients were enrolled in the HSCT and research protocol after the patients and/or parents had provided written informed consent. The study was approved by the Ethics Committee of the University Medical Center of Utrecht, The Netherlands. Characteristics of the study patients are shown in Table 1.

Table 1. Patient characteristics

Variable	N = 30
Median age (range) in years	5.8 (0.3–20.1)
Follow-up in months	15 (3–22)
Gender	
Male	21
Female	9
Conditioning ^a	
Chemo-based	21
TBI-based	9
HLA-matched ^b	
No	7
Yes	23
Indication ^c	
Non-malignant	13
Malignant	17
Donor source	
Family	
MSD (BM)	11
Unrelated	
MUD	
BM	5
PBSC	2
UCB	12
Allo-reactive disease ^d	
No	18
Yes	12
Survival	
Alive	28
Deceased	2

Abbreviations: MSD = matched sibling donor; MUD = matched unrelated BM donor; UCB = unrelated cord blood. Numbers represent total number of patients. ^aAll patients received either a chemotherapy-based (Busilvex, Pierre Fabre, Boulogne, France) or TBI-based myeloablative-conditioning regimen. ^bHLA matching was based on high-resolution (HR)-typing for class I and class II (10 antigens: A, B, C, DR and DQ) for MSD and MUD donors. For UCB donors intermediate-resolution criteria were used (loci A and B by intermediate resolution and DRB1 by HR typing).²⁵ ^cNon-malignant = inborn errors of metabolism, immune deficiencies, auto-immune disorders, BM failure syndromes; Malignant = acute lymphoblastic leukemia, acute myeloid leukemia, myelodysplastic syndrome. ^dAllo-reactive disease was defined as the development of grade ≥ 2 aGvHD or having idiopathic pulmonary syndrome (IPS). aGvHD was diagnosed and graded according to Glucksberg *et al.*²³ IPS was diagnosed and graded according to Clark *et al.*²⁴

Conditioning regimens, supportive care and treatment

All patients received myeloablative-conditioning regimens based on chemotherapy (Busilvex: Pierre Fabre, Boulogne, France) or TBI. In case of an unrelated donor, patients first received serotherapy with antithymocyte globulin (ATG)-rabbit (Thymoglobulin, Genzyme, Cambridge, MA, USA). As supportive care, anti-emetic drugs (Ondansetron) and prophylactic anticonvulsive therapy (clonazepam) during the days receiving Busilvex was given. Antimicrobial prophylaxis was administered according to the institutional protocol: ciprofloxacin and fluconazol were administered from the start of ablative medication until the end of the neutropenic period, which was defined as <500 neutrophils/ μL . In addition, cefuroxim (as *Streptococcus viridans* prophylaxis) was administered from day 0 until the end of the neutropenic period. Co-trimoxazol was administered from day +28 after HSCT as pneumocystis pneumonia prophylaxis. Patients who were seropositive for HSV before HSCT received prophylactic treatment of low-dose acyclovir (500 mg/ m^2 /day) until at least day +60 after HSCT. Furthermore, cord blood recipients were treated with filgrastim from day +7 after HSCT until neutrophils exceeded 2000/ μL .

Cyclosporine-A (dose based on plasma levels of 100–250 $\mu\text{g/L}$) was given to all patients as prophylaxis for GvHD. In case of a MSD, the patients received cyclosporine-A as a single agent. In recipients of unrelated marrow donor cells, MTX (short course: 10 mg/ m^2 on day +1, +3 and +6 after HSCT) was added. In cord blood recipients, prednisolone (1 mg/kg until day +28 after HSCT, with a taper in 14 days) was added.

Early allo-reactive disease was defined as the development of grade ≥ 2 aGvHD or having IPS. aGvHD was diagnosed and graded according to Glucksberg *et al.*²³ IPS was diagnosed according to Clark *et al.*²⁴ HLA matching was based on high-resolution typing for class I and class II antigens (10 antigens: A, B, C, DR and DQ) for BM donors. For cord blood (UCB) donors intermediate-resolution criteria were used (loci A and B by low resolution and DRB1 by high-resolution typing).²⁵

Immunophenotyping

Immunophenotyping on fresh whole blood was performed every other week until 4 months after HSCT and approximately monthly until 12 months after HSCT. Total numbers of CD4 + T lymphocytes were determined by using TruCOUNT technology (Becton Dickinson (BD) Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.²⁶ In addition, after erythrocyte lysis, cells were stained with PerCp-conjugated anti-CD4 monoclonal antibodies (BD Biosciences). After staining of surface markers, cells were fixed and permeabilized using Fixation/Permeabilization buffer (eBiosciences, San Diego, CA, USA) and stained with APC-conjugated anti-FOXP3 Mab (clone PCH 1.01, eBiosciences) to determine the proportion of FOXP3 + T cells. In addition, a small aliquot of donor cells was directly stained as well, with these same markers. Stained samples were acquired on the LSR II flow cytometer and data were analyzed using BD FACSDiva software (BD Biosciences). Further phenotypic characterization of FOXP3 + T cells was performed on frozen PBMC samples of a subset of patients. The following monoclonal antibodies were used: anti-CD25 PE-Cy7 anti-CD45RO Pacific Blue (Biolegend, San Diego, CA, USA), anti-Ki67 FITC, anti-CTLA4 APC (BD Biosciences) and anti-GITR FITC (R&D systems, Minneapolis, MN, USA). Staining for flow cytometry was performed as described above.

Statistical analysis

Differences in T-cell numbers and FOXP3 + T-cell proportions between recipients of different stem cell sources (UCB, MSD and MUD), differences in FOXP3 + T-cell proportions in the graft versus peripheral blood after HSCT and differences in the proportion of FOXP3-expressing CD4 + T cells in relation to allo-reactivity were analyzed using Kruskal Wallis and Mann-Whitney tests, respectively. Data were merged in four time periods: 1–6, 6–12, 12–24 and >24 weeks after HSCT. Correlations were tested using Spearman's non-parametric correlation tests with significance defined by $P < 0.05$ using a two-tailed test. In all analyses, median values were analyzed. All analyses were performed using software program SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient characteristics

A total of 30 patients were included with a median age of 5.8 years (range 0.3–20.1, Table 1). Median follow-up was 15 months (range 3–22) and overall survival was 93.4%. In this cohort, 2/30 (6.6%)

patients died owing to relapse. For the T-cell reconstitution analyses, we divided the patients into UCB, MSD and MUD HSCT recipients. All MSD and MUD recipients received HLA-matched grafts, while 7 of the 12 UCB recipients received an HLA-mismatched graft (Table 1). All MSD and five MUD recipients received BM, and two MUD recipients received PBSC.

Inverse relationship between FOXP3 + T-cell frequencies and allo-reactive disease

During follow-up, 12/30 patients (40%) developed allo-reactive disease (aGvHD and/or IPS) at a median time of 9 weeks (range 5–13) after HSCT. The proportion of FOXP3 + T cells (Figure 1a) in the different grafts and within the first 6 weeks after HSCT was analyzed. In all cases, the analysis was performed before the development of allo-reactive disease. Patients without sufficient T-cell numbers (≤ 20 cells/ μ L) or on high-dose corticosteroids in the first week were excluded from further analysis ($n = 12$); two of the patients without sufficient T-cell numbers developed allo-reactive disease (IPS). Consequently, the proportion of FOXP3 + T cells within the first 6 weeks was analyzed in 8 patients with allo-reactive disease (3 patients developed IPS and 5 patients aGvHD) and in 10 patients without allo-reactive disease, irrespective of the stem cell source. The proportion of FOXP3 + T cells in the graft was determined in the original graft material for 18 patients, 7 of whom developed allo-reactive disease (3 patients developed IPS and 4 patients developed aGvHD).

As has previously been shown in adult patients,²⁰ pediatric patients who developed allo-reactive disease showed lower proportions of FOXP3 + T cells before the development of allo-reactive disease than patients who did not develop allo-reactive disease ($P = 0.01$, Figure 1b). Owing to the low number of patients

per subgroup, analysis according to stem cell source was not possible. Also the proportion FOXP3 + T cells within the graft negatively correlated with the development of allo-reactive disease, irrespective of stem cell source ($P = 0.02$, Figure 1c).

Differences in kinetics of FOXP3 + T-cell reconstitution according to stem cell source

Probability of neutrophil engraftment was 95% at day 60 and did not differ among the UCB, MSD and MUD HSCT patients ($P = 0.3$, Figure 2a). In line with literature,²⁷ CD4 + T-cell reconstitution, however, tended to be delayed in patients after UCB HSCT, compared with MSD or MUD HSCT (1–6 weeks, $P = 0.158$; 6–12 weeks, $P = 0.831$; 12–24 weeks, $P = 0.308$ and > 24 weeks, $P = 0.161$, respectively; Figure 2b top graphs).

When a comparison was made between the mean proportion of FOXP3 + T cells in the graft, before infusion, and mean FOXP3 + T-cell proportions measured in peripheral blood in the first 1–6 weeks after transplantation, steep increases were found in UCB and MSD recipients (factor increase UCB 3.5, MSD 3.4, MUD 1.4). For UCB and MSD recipients, but not for MUD recipients, this increase was significant ($P < 0.001$, $P = 0.01$, $P = 0.9$, respectively, Figure 2b lower graphs).

Differences in phenotypic characteristics of reconstituting FOXP3 + T cells according to stem cell source

In order to gain insight into the mechanism of reconstitution of the FOXP3 + T cells within the different graft recipient groups, we performed additional T-cell phenotyping during the first 12 weeks after HSCT.

First, we characterized the reconstituting FOXP3 + T cells by measuring the expression of CD45RO, GITR, CD25 and CTLA4.

Figure 1. The proportion of FOXP3 + T cells according to allo-reactive disease. (a) Flow cytometry analysis of FOXP3 expression in CD4 + cells (black line) and CD4 – cells (gray shaded histogram) in whole-blood samples after HSCT. (b, c) Dots represent the proportion of FOXP3 + T cells of total CD4 + T cells in the first 6 weeks after HSCT with different stem cell sources (▲ MSD, ■ UCB and ● MUD) (b) and in the graft (c) for patients with or without allo-reactive disease. *Calculated with Mann–Whitney U test; **Patients without sufficient CD4 + T-cell numbers excluded ($n = 4$); ***Patients without sufficient CD4 + T-cell numbers excluded ($n = 8$).

Figure 2. Neutrophil, CD4+ and FOXP3+ T-cell reconstitution after HSCT. **(a)** Kaplan-Meier curve showing the probability (log-rank $P=0.315$) of neutrophil engraftment (y axis) according to donor graft. **(b)** Box and whisker diagrams representing the total CD4+ T cells/ μ L (upper panel; 1–6 weeks, $P=0.158$; 6–12 weeks, $P=0.831$; 12–24 weeks, $P=0.308$ and >24 weeks, $P=0.161$, respectively) and the proportion of FOXP3+ T cells within the total CD4+ T cells (lower panel) according to donor source. Also the proportion of FOXP3+ T cells within the different grafts is shown (white boxes; $P=0.1$). Horizontal lines represent median values, boxes represent interquartile ranges and lines represent minimum and maximum value. * $P=0.01$; *** $P<0.001$.

In addition, we measured Ki67 expression, which is expressed exclusively by cells that are in cell cycle²⁸ and is a protein pivotal for cell division. Interestingly, the FOXP3+ T cells that came up in the first 6 weeks after UCB HSCT clearly showed a more activated phenotype than after MSD or MUD HSCT, as indicated by the relatively high expression levels of CD25, CD45RO, CTLA4 and GITR (Figure 3a). Besides higher expression levels of activation markers reconstituting FOXP3+ T cells in UCB recipients expressed higher levels of Ki67. In contrast, the early reconstituting FOXP3+ T cells in MSD recipients expressed relatively low levels of activation markers and Ki67 (Figure 3a). However, the difference in Ki67 expressed on FOXP3+ T cells compared with FOXP3– T cells was clearly more prominent in MSD recipients compared with UCB or MUD recipients (Figure 3b).

DISCUSSION

This is the first single-center cohort study in children in which FOXP3+ T-cell reconstitution is determined starting as early as 1 week after HSCT and in which three different stem cell sources (UCB, MSD and MUD) were compared. The study shows that the

FOXP3+ T-cell reconstitution pattern strongly depends upon the stem cell source. First, early after UCB and MSD, but not after MUD, HSCT a peak in FOXP3+ T cells is seen. Second, the peak after UCB consists of FOXP3+ T cells with a significantly higher activation status and Ki67 expression than the FOXP3+ T-cell peak after MSD. FOXP3+ T-cell frequencies early after pediatric HSCT showed a strong inverse correlation with the development of early allo-reactive disease, this is similar to what has been shown in adult patients.^{17,19,20,29}

In the first weeks following HSCT, the T-cell compartment repopulates rapidly through lymphopenia-induced expansion of mature T cells contained within the stem cell allograft. At this early stage, the thymus has a minor role in T-cell reconstitution also in children.^{30,31} Therefore, we reasoned that the significant enrichment of FOXP3+ T cells early after UCB and MSD HSCT (compared with graft content) evolved either from preferential proliferation of FOXP3+ Treg compared with FOXP3– T cells¹⁹ or from the conversion of FOXP3– T cells into FOXP3+ T cells upon activation.³² We observed that the FOXP3+ T cells that arose early after UCB HSCT had a significantly higher activation status, in terms of GITR, CTLA4, CD25, CD45RO and Ki67

Figure 3. Phenotypic characteristics of FOXP3⁺ T cells after HSCT. FOXP3⁺ T cells emerging in the first 6 weeks after HSCT were characterized by expression of surface (CD25, GITR and CD45RO) and intracellular (Ki67) markers. **(a)** Mean percentage $\pm$ s.e.m. of Ki67⁺, CD25⁺, CD45RO⁺ and GITR⁺ cells, and mean of the mean fluorescence intensity $\pm$ s.e.m. of CTLA4 expression (right) within the FOXP3⁺ T cells after HSCT with indicated donor type are shown ($n=2-3$ patients per group). **(b)** Mean percentage $\pm$ s.e.m. of Ki67⁺ cells is shown within the CD4⁺FOXP3⁺ (black bars) and CD4⁺FOXP3⁻ (white bars) T cells after HSCT with indicated donor type ($n=2-3$ patients per group); *** $P<0.0001$.

expression, than after MSD and MUD HSCT. It therefore seems likely that the high FOXP3 expression early after UCB HSCT is caused by activation-induced conversion of FOXP3⁻ T cells into FOXP3⁺ T cells. Indeed, recent data by our group³³ and others³⁴ have shown that CB-derived naïve FOXP3⁻ T cells upon activation *in vitro* convert more readily into suppressive FOXP3⁺ T cells than adult blood-derived naïve T cells. All together, our data indicate that the expansion of FOXP3⁺ T cells early after the highly mismatched setting of UCB HSCT is specific for CB and is most likely caused by a preferential differentiation of FOXP3⁻ into FOXP3⁺ T cells upon activation.

Following MSD HSCT, the FOXP3⁺ T cells showed a less-activated phenotype compared with UCB HSCT, suggesting a different mechanism for expansion of this subset than in UCB recipients. Although early after MSD HSCT, FOXP3⁺ T cells showed lower Ki67 levels compared with UCB HSCT, the difference in Ki67 levels between FOXP3⁺ and FOXP3⁻ T cells was clearly more pronounced following MSD HSCT. This may suggest that the high number of FOXP3-expressing T cells seen after MSD HSCT is caused by a more pronounced proliferation of FOXP3⁺ compared with FOXP3⁻ T cells. A similar observation has been described in a previous publication by our group on Treg recovery after Auto-SCT (ASCT) for autoimmune disease.³⁵ As lymphopenia-induced proliferation is in part driven by TCR signals^{36,37} and the TCR repertoire of FOXP3⁺ Treg is skewed to a self-reactive repertoire,³⁸ such preferential proliferation is more likely to occur in the HLA-matched settings of MSD HSCT and ASCT.

We also observed differences in the dynamics of FOXP3⁺ T-cell reconstitution between UCB, MSD and MUD HSCT, during later follow-up. While in MSD and MUD the proportion of FOXP3⁺

T cells remained relatively stable, the proportion of FOXP3⁺ T cells dropped in the period of 6–12 weeks after UCB HSCT and increased to normal proportions after 12 weeks. We do not have a clear explanation for this temporary drop in the proportion of FOXP3⁺ T cells, but three non-mutually exclusive mechanisms can be formulated. First, it has been described that activation-induced FOXP3⁺ T cells are more prone to apoptosis.¹⁹ Second, activation-induced FOXP3 is less stable than FOXP3 expressed in thymus-derived Tregs.³⁹ And third, weaning of the steroids may have reduced the therapy-induced FOXP3⁺ T cells in UCB recipients. We hypothesize that, independent of the mechanism, the drop in FOXP3⁺ cells is a retraction in the size, rather than the number, of the individual activation-induced FOXP3⁺ T-cell clones.

In line with several previous studies in adult patients^{19,20} and with one other pediatric study,²² we found in our pediatric patient group a strong inverse correlation between FOXP3⁺ T-cell frequencies early after HSCT and the development of allo-reactive disease. This correlation also held true for the number of FOXP3⁺ T cells within the grafts and alloreactivity. The one pediatric study available reports no significant differences in FOXP3 expression between the recipients of sibling and unrelated grafts 3 month post HSCT.²² However, from our study, it now became clear that at earlier time points post-HSCT substantial differences in frequencies and activation status of FOXP3⁺ T cells can be seen between stem cell sources. These findings suggest that the expansion of FOXP3⁺ T cells early after UCB, MSD and MUD HSCT is driven by different mechanisms.

We conclude that the FOXP3⁺ T-cell reconstitution pattern after pediatric HSCT is stem cell source dependent. Irrespective of

stem cell source, the proportion of FOXP3 + T cells in the graft and early after HSCT is negatively correlated to the risk for allo-reactive disease in pediatric patients and might be used to guide administration of Treg and/or immunosuppressive therapy. A larger prospective follow-up study is needed to analyze the effect of the differences in FOXP3 + T-cell reconstitution on the development of aGvHD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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