In vitro and *in vivo* T-cell depletion with myeloablative or reduced-intensity conditioning in pediatric hematopoietic stem cell transplantation

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Background and Objectives. Anti-thymocyte globulin (ATG) is given in various conditioning regimens for children and young adults undergoing hematopoietic stem cell transplantation (HSCT) from HLA non-identical donors in order to reduce the risks of graft-versus-host disease (GvHD) and rejection after the transplant. The aim of this study was to define the effect of *in vitro* T-cell depletion in addition to ATG on the reconstitution of T-cell-mediated immunity.

Design and Methods. We retrospectively analyzed the engraftment kinetics and clinical performance of 134 patients (median age 5.80 years) who received ATG during myeloablative or reduced intensity conditioning, and either *in vitro* T-cell-depleted or unmanipulated grafts.

Results. T-cell reconstitution was significantly delayed after T-cell-depleted grafts, irrespectively of the conditioning intensity ($p<0.001$). The incidence of fatal viral and fungal infections was higher in recipients of T-cell-depleted grafts than in those receiving unmanipulated grafts (26.6-29% versus ≤13%). The rejection rate was likewise higher in recipients of T-cell-depleted grafts whether following myeloablative or reduced intensity conditioning (37% and 18%, associated with recipient chimerism or non-engraftment, respectively) versus ≤4% without graft T-cell depletion. Grades 3 and 4 acute GvHD (CI±SE ≤0.11±0.03) and severe Epstein-Barr virus lymphoproliferative disease (1.49% fatal) were rare.

Interpretations and Conclusions. A combination of *ex vivo* and *in vivo* T-cell depletion, although inhibiting GvHD, prolongs the interval of T cell deficiency after HSCT and favors rejection and infections, implying that the requirement for this type of extensive T-cell depletion should be carefully re-evaluated.

Key words: anti-lymphocyte globulin (ATG), graft-versus-host disease, transplantation immunology, engraftment kinetics, T-cell reconstitution.

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Both *in vitro* T-cell depletion of grafts, by immunologic CD34-positive or T-negative selection, and *in vivo* T-cell depletion, by administering anti-thymocyte globulin (ATG) before hematopoietic stem cell transplantation (HSCT), are standard procedures to prevent or reduce undesired histo-incompatibility immune reactions. However, since T lymphocytes are the main effectors of cellular immunity and, in the setting of transplantation, are primarily responsible for mediating antiviral, antifungal, and tissue incompatibility immune responses, T-cell function after HSCT is crucial for survival.

Anti-thymocyte globulin, a polyclonal rabbit- or horse-derived antibody preparation directed against more than 23 lymphocyte epitopes, is intended to reduce effector T-cell activity.[1-4] Anti-lymphocyte globulin had been administered successfully in the treatment of severe aplastic anemia prior to its use in HSCT.[5,6] ATG induces Fas/FasL-mediated apoptosis,[7] complement- and antibody-dependent cytotoxicity, and it impairs the expression of functional molecules (i.e. CD2, CD3, CD4, CD8) and co-activation signals.[8-11] Matched cohort studies have shown that the use of *in vivo* T-cell depletion by various ATG preparations may reduce the incidence and severity of graft-versus-host disease (GvHD) and non-relapse mortality.[12-15] T-cell depletion is often necessary in transplantations from donors with multiple HLA mismatches e.g. haplo-identical parents, and is frequently performed in peripheral blood HSCT. The effects of *ex vivo* and *in vivo* T-cell depletion might be additive in these high-risk patients, and prolonged T-cell insufficiency could increase the susceptibility to severe viral or fungal infections or Epstein Barr virus-linked lymphoproliferative disorder (EBV-LPD).[16-21]

There is little published clinical experience of the combination of *in vivo* T-cell depletion and graft T-cell depletion in pedi-

atric HSCT; most studies compare one procedure to another or to none at all.[12-14,22] We aimed to define the effects of *in vitro* T-cell depletion by CD34+ selection and concurrent *in vivo* ATG pre-treatment on immune reconstitution and clinical outcome in children. Thus, we performed a single center retrospective analysis of 134 transplantations in ATG-pretreated patients who received either unmanipulated grafts (n=68) or *in vitro* T-cell-depleted stem cells (n=66). To include the effect of conditioning intensity on hematopoietic and immunologic reconstitution, we subsequently divided the groups according to whether myeloablative (n=81) or non-ablative conditioning regimens (n=53) had been used. We analyzed the engraftment time of all blood cell types as well as the transplant outcome and incidence of adverse events such as drug toxicities, rejection, acute and chronic GvHD, infections, and EBV-LPD in relation to the intensity and mode of T-cell depletion and conditioning.

Design and Methods

Patients

Informed consent was obtained from patients or their representatives for prospective data and serum sample acquisition. The study population comprised 134 patients who underwent allogeneic HSCT from HLA-non-identical related or unrelated donors at St. Anna Children's Hospital, Vienna, between 1994 and 2003. The characteristics of these patients are shown in Table 1. Their median age was 5.80 years (range 0.14-28 years) and 61% were males. The mean follow-up was 1.94 years. The indication for transplantation was malignant and non-malignant disease in 51% and 49%, respectively. To allow a comparison of the effect of *ex vivo* graft T-cell depletion in patients who had received myeloablative or non-myeloablative conditioning, we divided the population into four groups: group 1 (n=53, median age 7.9 years) received myeloablative conditioning and an unmanipulated graft; group 2 (n=15, median age 5.5 years) received reduced intensity conditioning and an unmanipulated graft; group 3 (n=28, median age 5.01 years) received myeloablative conditioning and a T-cell-depleted graft; group 4 (n=38, median age 2.13 years) received reduced intensity conditioning and a T-cell-depleted graft (Table 1). All the patients in the cohort received ATG (Sangstat, Lyon, France) during conditioning due to the transplant setting and/or underlying disease according to the relative EBMT protocols. ATG was either horse-derived (during the first six years of the study) or rabbit-derived (predominantly from 1998-2003). Due to differing pharmacology, the ATG dose depended on the source (Table 1), i.e. lower doses of the rabbit-derived compound were given to achieve similar effects in accordance with the manufacturer's instructions. ATG was administered as one-hour intravenous infusions for 3-5 days before transplant (day –5 or –3 until –1). Because of possible biases from improvements in supportive care and HLA typing over the long time frame of the study, we avoided a comparison between horse- and rabbit-ATG-treated patients. Similarly, quantitative evaluation of viral infections on a molecular level (viral load, *see below*) was only available after August 1998. Thus, we included 74 (of 134) patients in the sub-analysis of the severity of viral infections (Figure 5).

Table 1. Patients' data. All patients received ATG for 3-5 days prior to HSCT.

Recipients	*MA/noTCD (n=53)*	*RIC/noTCD (n=15)*	*MA/TCD (n=28)*	*RIC/TCD (n=38)*	*Total (n=134)*
Myeloablative conditioning	Yes	No	Yes	No	81 yes/53 no
Graft T-cell depletion	No	No	Yes	Yes	66 yes/68 no
Median age (years)	7.9 (0.59-23.57)	5.50 (0.95-23.33)	5.01 (0.52-20.77)	2.13 (0.14-28.07)	5.80 (0.14-28.07)
Median CD34/CD3 cell doses infused ($\times 10^6$/kg)	3.9/93.5	3.9/90	10.6/0.055	19/0.1	–
TRM at 1 year, CI±SE	0.28±0.06	0.13±0.09	0.57±0.09	0.37±0.08	0.36±0.04
Relapse at 1 year, CI±SE (n=malignant diseases)	0.10±0.04 (n=50)	0.17±0.15 (n=4)	0.16±0.07 (n=25)	0 (n=10)	0.11±0.03 (n=89)
Rejection	4%	0	18%	37%	17%
Male/female	61/38%	73/27%	64/36%	53/47%	61/39%
Malignant/ non-malignant	94/6%	40/60%	89/11%	29/71%	51/49%
MFD	2	1	0	1	4
MMFD	1	1	18*	19#	40
MUD	46	12	9	15	81
MMUD	4	1	1	3	9
BMT/PBSCT	47/6	14/5	3/25	0/38	60/74
Rabbit vs horse ATG.	30/70%	73/27%	64/26%	71/29%	54/46%

MA: myeloablative conditioning; RIC: reduced intensity conditioning; noTCD: no ex vivo T-cell depletion; TCD: ex vivo T- cell depletion; TRM: transplant-related mortality; MFD: matched family donor; MMFD, class I or class II mismatched family donor, including (*) in group MA/TCD: 17 parental (haplo-identical) and one brother as MMFD, and in RIC/TCD (#): 2 cousins, 1 brother, 16 parents as MMFD; MUD, matched unrelated donor; MMUD, unrelated donor with ≥3 HLA mismatches; BMT, bone marrow transplantation; PBSCT: peripheral blood stem cell transplantation.

Conditioning

Depending on their underlying disease, patients were conditioned with either high-dose myeloablative cytostatic drugs with or without total body irradiation, or with non-ablative conditioning regimens. The condi-

tioning in addition to ATG consisted of total body irradiation/cyclophosphamide in patients with chronic myeloid leukemia, and of total body irradiation/etoposide with or without cyclophosphamide in patients with acute lymphocytic leukemia or non-Hodgkin's lymphoma, and of busulfan/cyclophosphamide with or without melphalan in patients with myelodysplastic syndrome, acute myeloid leukemia or secondary leukemia. Conditioning regimens for patients with haplo-identical donors and for patients with juvenile myelomonocytic leukemia are described elsewhere.[23,24] Patients with phagocytic disorders, storage diseases and hemoglobinopathies who underwent myeloablative therapy received a combination of busulfan/cyclophosphamide. Total body irradiation was not administered to children below two years of age or to patients who had received high irradiation pre-treatment. Patients with severe combined immunodeficiency did not receive any cytostatic chemotherapy before HSCT. Patients with severe aplastic anemia or Fanconi's anemia underwent conditioning with cyclophosphamide/ATG according to the then current EBMT protocols. From 1999 all patients with non-malignant disorders received reduced intensity conditioning to avoid severe late effects. This conditioning consisted predominantly of a combination of fludarabine/melphalan with or without busulfan or total lymphoid irradiation.[25,26]

HLA-typing

Donors were assessed for the degree of mismatch by HLA-typing and mixed lymphocyte cultures. Until 1997 HLA class I was typed by serology; subsequently class I alleles were determined by nucleotide sequencing of HLA-A, B and C alleles. HLA class II typing was performed since 1994 at low resolution by a PCR-SSOP procedure and at high resolution by nucleotide sequencing of DRB1, DRB3/4/5 and DQB1 alleles.

GvHD prophylaxis

Graft T-cell depletion, when indicated, was performed using a two-step $CD34^+$ selection on two different magnetic bead devices (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) as described elsewhere.[24] Leukemia patients receiving T-cell-depleted grafts did not receive pharmacological GvHD prophylaxis. All other patients received 1.5 mg/kg cyclosporine A twice daily intravenously commencing on day -1. Oral administration was preferred (3 mg/kg/day) and was reduced if no GvHD occurred after T-cell engraftment. Methotrexate (10 mg/m^2) was also administered on days +1, +3, +6 and +11 in patients transplanted from unrelated donors or from phenoidentical family members.

Viral and fungal infection monitoring

Molecular screening for viral infections was carried out as described previously.[27] In brief, DNA was extracted from largely cell-free liquids except urine, and from peripheral blood leukocytes for detection of intracellular virus particles using QIAamp DNA and RNA Mini Kits (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. Amplification was carried out using the ABI Prism 7700 or 7900 Sequence Detector (Applied Biosystems, Foster City, Ca, USA) as described elsewhere.[28] External standard curves for each species were established by making serial dilutions of quantified viral preparations derived from reference strains. For assessment of virus copies per cell, a single copy gene (β2-microglobulin) was quantified in parallel by real-time polymerase chain reaction (PCR).[29] Fungal infections were diagnosed by histological and microbiological tests and/or pan-fungal qualitative PCR.

Detection of chimerism

The samples from patients with sex-matched donors underwent PCR analysis[30] prior to transplantation using a panel of seven highly polymorphic short tandem repeat (STR) markers in order to select an informative primer set suitable for chimerism monitoring during the post-transplant course. Alleles were quantified by capillary electrophoresis and fluorescence-based quantification using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Specimens from patients transplanted from sex-mismatched donors were analyzed by fluorescent *in situ* hybridization according to standard procedures with commercially available probes specific for the centromeric and heterochromatic regions of the X and Y-chromosomes, respectively.

FACS analysis of differential blood counts

Flow cytometric monitoring of hematopoietic reconstitution was performed as described elsewhere.[31,32] In brief, circulating leukocytes were analyzed before and during regeneration after HSCT with a four-color FACS Calibur (Beckton Dickinson [BD], Sunnyvale, CA, USA). Data acquisition and analysis was performed with CellQuest (BD) software, and Paint-a-gate Pro (BD) software. The monoclonal antibodies used were CD3(UCHT1), CD4 (MT310), CD8 (DK25), CD14 (TÜK4), CD15 (C3D1), CD19 (HD37), CD45 (T29/33; all from Dako, Glostrup, Denmark), CD3 (SK7), CD14 (MoP9), CD33 (P67.6), CD38 (HB-7), CD45 (2D1), CD56(NCAM16.2; all from BD), and CD19 (J4.119) and CD45RA (2H4, both from Coulter, Krefeld, Germany). CD34 class III antibodies were from Dako (Birma K3), BD (HPCA-2), and Coulter (581).

Statistics

Cumulative incidence estimates are used to describe the time to reach the indicated number of leukocyte

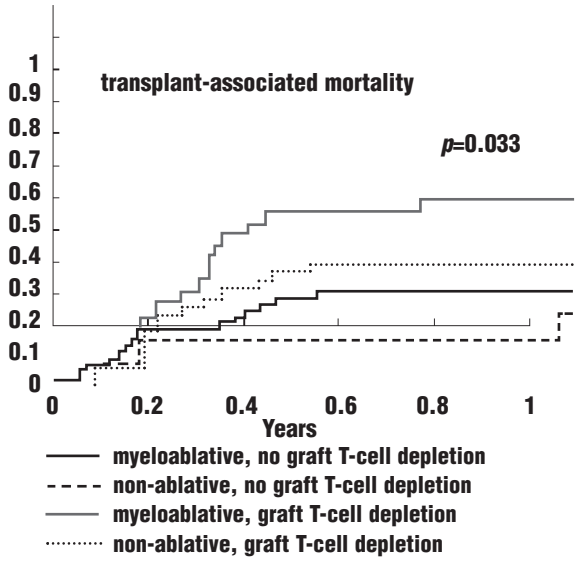

Figure 1. Transplant-associated mortality until one year after HSCT (non-relapse) is plotted as cumulative incidence curves for the four groups of patients described in Table 1. The group with graft T-cell depletion and myeloablative conditioning performed significantly worse than did the other groups (1 year transplant-associated mortality CI±SE: 0.57±0.09 versus 0.13-0.37; p=0.033). The causes of death were analyzed and are shown in Figure 4.

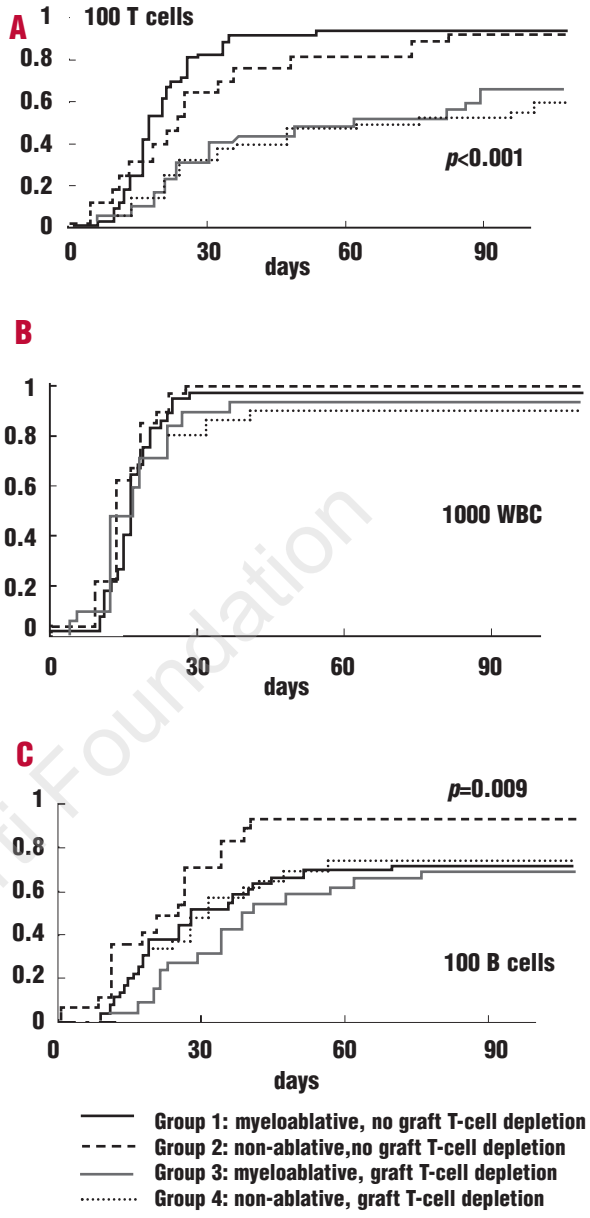

Figure 2 [right column]. Engraftment times of blood cell types are shown as the time interval from HSCT to first appearance of donor blood cells in the peripheral blood (per µL, cell type as indicated) until day +100 according to the four groups as described in Table 1. A. Reconstitution of $CD3^+$ T cells was significantly delayed in groups with *ex vivo* T-cell depletion, irrespective of the conditioning regimen. B. 1000 leukocytes were detectable at the same time in all 4 groups. C. $CD19^+$ B-cell recovery was significantly better in the group that received reduced intensity conditioning and an unselected graft, but showed equal engraftment kinetics in the other three groups. NK cell ($CD56^+$) and monocyte ($CD33^+$) appearance was independent of graft T-cell depletion and/or conditioning regimen (*not shown*).

subtypes, taking into account the competing risk of death. The same approach was used to estimate the cause-specific failure probability for each cause of death separately. Log-rank tests were used for comparisons between different subgroups. A p value of <0.05 was considered statistically significant.

Results

Survival

The one-year survival and non-relapse transplant-related mortality differed according to the underlying disease, donor status and transplantation conditions among the four groups (Table 1 and Figure 1). The transplant-related mortality rate in patients who received non-myeloablative conditioning without graft T-cell depletion was 13%, in patients who underwent myeloablative conditioning and received unselected grafts it was 28%, and in patients who received a T-cell-depleted graft after non-ablative conditioning it was 37%. The significantly highest one-year transplant-related mortality was observed in the group of patients who received myeloablative conditioning and T-cell-depleted grafts, which included many patients at high-risk according to their pre-HSCT disease, remission status and donor type (cumulative incidence±SE: 0.57±0.09; p=0.033; *see Tables and below for patient statistics and remission status*).

Engraftment kinetics of white blood cell subtypes

The first occurrence of 100/µL $CD3^+$ T cells in the peripheral blood after HSCT was significantly delayed in patients receiving T-cell-depleted grafts (p<0.001, corrected for earlier deaths; Figure 2A). This delay was

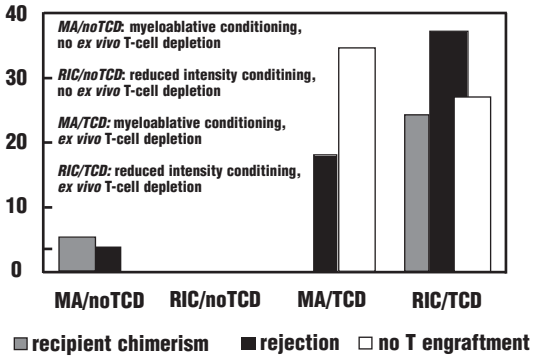

Figure 3. T-cell recipient chimerism or non-engraftment and the incidence of rejection differed between the groups without or with graft T-cell depletion and myeloablative or reduced-intensity conditioning. Rejection occurred in 18% of the patients in the MA/TCD group, in 37% of the RIC/TCD group, and in 4% of the MA/noTCD group. T-cell non-engraftment was restricted to recipients of T-cell depleted grafts and observed in 35% of MA/TCD and 27% of RIC/TCD patients; whereas recipient T-cell chimerism was detected in 24% of RIC/TCD patients, in 5% of the MA/noTCD group, and in none of the patients in the RIC/noTCD and MA/TCD groups.

independent of the conditioning intensity (myeloablative vs. non-ablative). Identical repopulation kinetics were observed in both the $CD4^+$ and $CD8^+$ T-cell fraction (*data not shown*). Additionally, the proportion of patients showing no T-cell engraftment at all was higher in both groups of T-cell-depleted graft recipients than in patients receiving unselected grafts (27-35% vs. 0%; Figure 2A, Figure 3). Among the patients who underwent reduced intensity conditioning and received a T-cell depleted graft, 24% were reconstituted with autologous T cells (full recipient chimerism; Figure 3); however, all patients who underwent reduced intensity conditioning but received unselected grafts engrafted with T cells of donor origin. In contrast to T-cell reconstitution, engraftment times of whole white blood cells (WBC; time after HSCT until 1000/μL; Figure 2B), neutrophils (first appearance of 500/μL, *data not shown*), B cells (Figure 2C), natural killer cells, and monocytes (*not shown*) were not negatively affected by T-cell depletion.

Transplant-associated mortality and relapse

We analyzed whether the delay in T-cell recovery after HSCT in patients receiving *in vivo* and *in vitro* (graft) T-cell depletion had an influence on the causes of transplantation-associated deaths among the groups of patients. We plotted each death (over time, in cumulative incidence curves), according to its cause, for all four groups of transplant recipient (shown until 360 days in Figure 4). Of note, 25% of all patients treated with myeloablative conditioning and a T-cell-depleted graft had died of a viral infection by day +180, in contrast to 0-13% of the patients in the other groups (Figure 4C; cytomegalovirus, adenovirus, and EBV). In the second

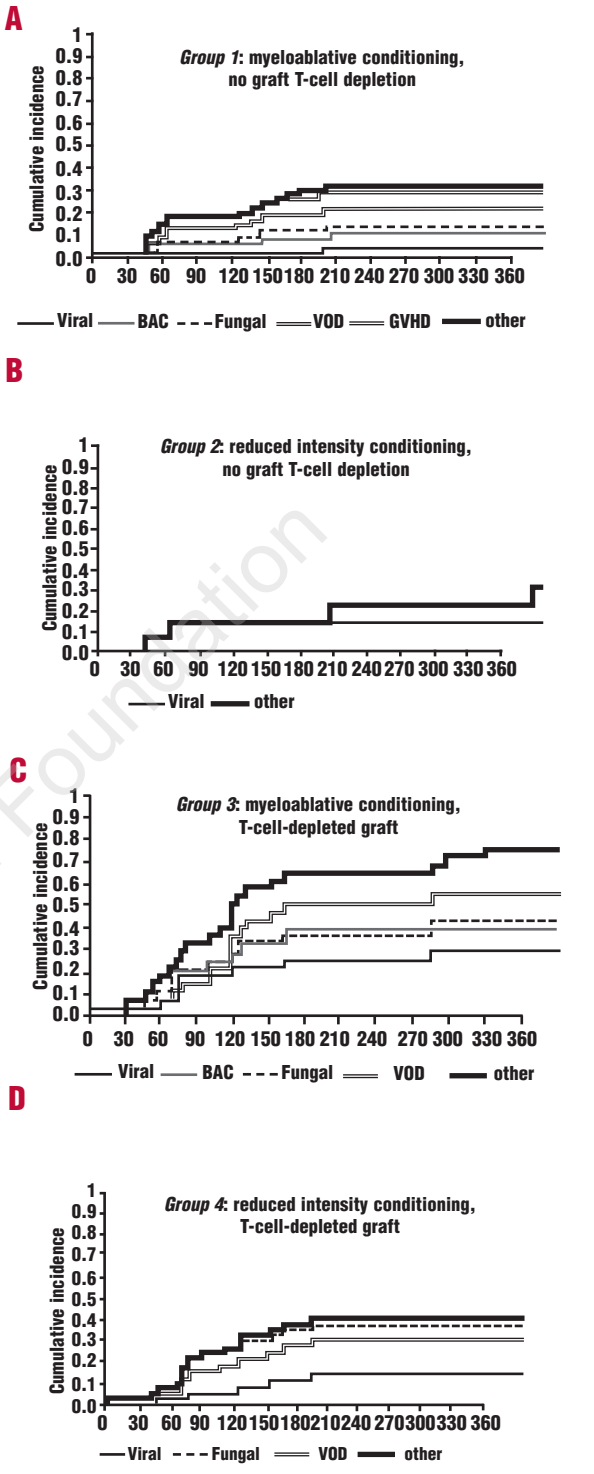

Figure 4. Causes of deaths are plotted by their relative incidence over time. Overlaid curves show infections (viral; bac; bacterial; fungal), veno-occlusive disease (VOD), graft-versus-host disease (*GvHD*), and *other* causes of deaths (including relapses, toxicities, etc.) as a sum (total mortality) until day +360 after HSCT in each group of patients (see Table 1 for a description of the groups). The disproportionally higher incidence of *other* causes of death in group 3 is due to a higher relapse rate (6 out of 28 patients, most of whom were transplanted in second or third complete remission, see *Results* section) in this group than in the other groups. If there was no death due to one of the above causes in a particular group of patients, the corresponding curves and legends are not shown.

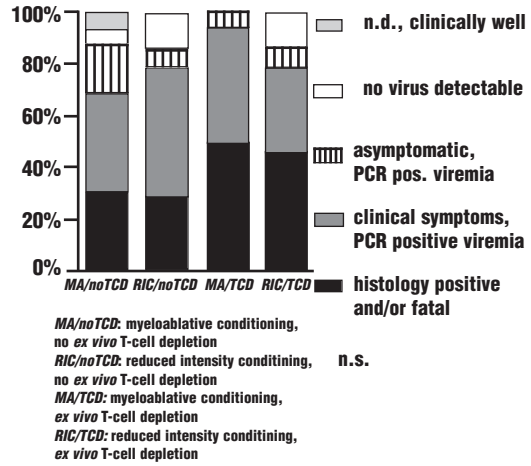

Figure 5. The occurrence and grade of viral infections in subgroups of patients according to their T-cell depletion and conditioning status (transplanted after September 1998; n_1=16, n_2=14, n_3=16, n_4=28; in cases in which diagnosis by quantitative polymerase chain reaction (PCR) was available) with or without clinical symptoms or histological confirmation of reactivated or de novo infection. no virus, no clinical symptoms and PCR negative; n.d., not determined (one patient in group MA/noTCD was clinically asymptomatic and not tested by PCR). Results show a trend but were not significantly different (p=n.s.).

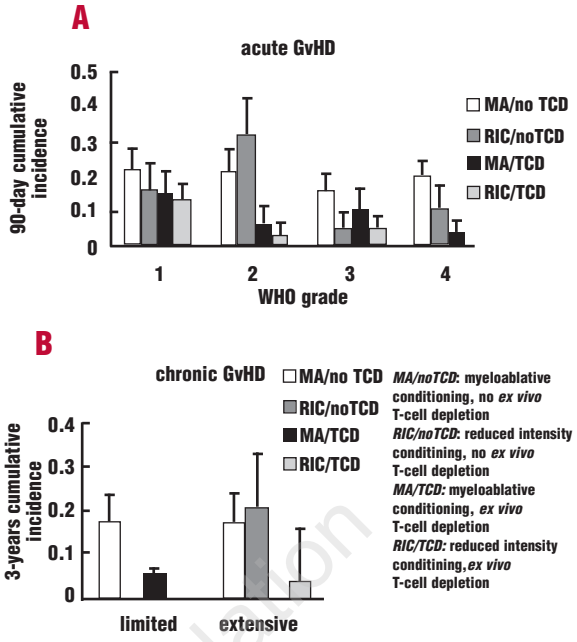

Figure 6. The cumulative incidence of A) acute GVHD (90 days CI±SE) WHO grades 1-4 and B) chronic GVHD (3 years CI±SE) limited vs. extensive is shown for the groups of patients with myeloablative or non-ablative conditioning and with or without graft T-cell depletion.

group with delayed T-cell reconstitution, the patients who had undergone reduced intensity conditioning and had received a T-cell-depleted graft, who had a different distribution of underlying diseases than the T-cell-depleted graft recipients who had had myeloablative conditioning (Table 2), the proportion of fatal fungal infections (16% until day +180, as compared to 0-5% in the other three groups) was higher than that of viral infections (11%; Figure 4D). Together, the proportion of deaths due to viral and fungal infections was more than double (29 and 27% of all patients) in groups with delayed T-cell recovery than in groups receiving unselected grafts (Figures 4A-B; myeloablative conditioning plus unselected graft and non-myeloablative conditioning plus unselected graft, 5.5 and 13%, respectively). To assess whether there was a difference in susceptibility to viral infections between the four groups, we divided the severity of viral infections in patients transplanted after September 1998 (n=74; for reasons of technical comparability) into four grades (no virus detectable, positive for viral nucleic acid (by quantitative PCR [qPCR]; see *Design and Methods*) but clinically asymptomatic, qPCR-positive with clinical symptoms, and histology-positive or fatal). Nucleic acid detection was performed for EBV, CMV, various strains of adenovirus, HHV6-7, HSV1-2, rotavirus, enterovirus, BKV, and PVB19. According to this grading, there was a similar frequency of viral disease in the two groups receiving unselected grafts and that treated with reduced intensity conditioning and a T-cell-depleted graft but a higher frequency in the group that underwent myeloablative conditioning and received a T-cell-depleted graft (100% vs. <83%), and an increased severity in both groups of patients whose graft had been T-cell-depleted (histology positive or fatal viral disease: 46.4-50% vs. 28.6-31% in groups receiving unselected grafts; Figure 5). These differences between groups represent a trend but were not statistically significant. EBV-reactivation as assessed by qualitative EBV-PCR positivity was detected in 2 of 53 patients treated with myeloablative conditioning and unselected grafts, 3 of 15 with non-myeloablative conditioning and unselected grafts, 4 of 28 with myeloablation and T-cell depletion and 10 of 38 with non-myeloablative conditioning and T-cell depletion.

The incidence of relapse was only insignificantly different between myeloablatively conditioned patients with malignancies who received an unselected graft (n=50), and those whose graft was T-cell-depleted (n=25; relapse incidence corrected for earlier deaths: 1-year CI±SE 0.10±0.04 and 0.16±0.07, respectively; p=0.123; Table 1) although the ratio of patients in second or more complete remission (CR2-3, including one non-responder in the T-cell depleted group) versus first complete remission was higher in group receiving T-cell-depleted grafts than in the group receiving unselected grafts (75% as compared to 56%, *not shown*). Among the patients treated with non-myeloablative conditioning there were no relapses in patients receiving T-cell-depleted grafts (n=11), but one relapse among

Table 2. Diagnoses.

Diagnoses (including relapsed disease)	*MA/noTCD (n=53)*	*RIC/noTCD (n=15)*	*MA/TCD (n=28)*	*RIC/TCD (n=38)*	*Total (n=134)*
Acute lymphocytic leukemia	24 (47.2%)	1 (6.7%)	10 (35.7%)	1 (2.6%)	36 (26.9%)
AML, MDS, biphenotypic hybrid, undifferentiated AL	13 (24.5%)	2 (13.3%)	6 (21.4%)	4 (10.5%)	25 (18.7%)
Chronic myeloid leukemia	4 (7.5%)	0	4 (14.3%)	1 (2.6%)	9 (6.7%)
CMML/JMML	6 (11.3%)	0	0	0	6 (4.5%)
Malignant lymphoma	0 (6.7%)	1	0	0 (0.7%)	1
Hemophagocytic lymphohistiocytosis FHL	3 (5.7%)	0	5 (17.9%)	1 (2.6%)	9 (6.7%)
Langerhans' cell histiocytosis	0	0	0	3 (7.9%)	3 (2.2%)
Fanconi's anemia	0 (13.3%)	2	0 (7.9%)	3 (3.7%)	5
β-thalassemia	0	0	0 (2.6%)	1 (0.7%)	1
Severe aplastic anemia	0 (13.3%)	2	0 (15.8%)	6 (6.0%)	8
Severe combined immune deficiency	0	1 (6.7%)	0	9 (23.7%)	10 (7.5%)
X-linked lymphoproliferative disease, septic granulomatosis, Wiskott-Aldrich or Kostmann syndrome	0	4 (26.7%)	1 (3.6%)	2 (5.3%)	7 (5.2%)
Others mucopolysaccharidoses, Wolman's disease, Krabbe's disease, adrenoleukodystrophy, lysinuric protein intolerance, Omen syndrome, hyper-IgM-syndrome + Ewing's sarcoma	3 (5.7%)	2 (13.3%)	2 (7.1%)	7 (18.4%)	14 (10.4%)

MA: myeloablative conditioning; RIC: reduced intensity conditioning; noTCD: no ex vivo T-cell depletion; TCD: ex vivo T- cell depletion; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; AL: acute leukemia; CMML: chronic myelomonocytic leukemia; JMML: juvenile myelomonocytic leukemia.

those receiving an unselected graft (n=6; CI±SE: 0.17±0.15; n.s.).

Graft-versus-host disease and rejection

Acute or chronic GvHD was a cause of death only among patients who received unselected grafts, being responsible for 7% of the deaths in the myeloablated group, while only the acute form of GvHD caused deaths in 5% of patients who had undergone non-myeloablative conditioning (Figures 4A-B and 6A-B). No deaths due to either acute or chronic GvHD were observed in patients receiving a T-cell-depleted graft. We detected no grade 4 acute GvHD in patients with non-ablative conditioning and T-cell-depleted grafts (Figure 6A), and a low incidence of grade 4 acute GvHD in the other group with T-cell-depleted grafts (with myeloablative conditioning). Similar results were obtained for chronic GvHD, which was more frequent and severe in patients transplanted with unselected grafts (Figure 6B; limited versus extensive chronic GvHD).

In contrast to GvHD, the incidence of graft rejection was highest in non-myeloablatively treated patients who received a T-cell depleted graft (37%; Figure 3), followed by patients with myeloablative conditioning and graft T-cell depletion (18%) and substantially lower in patients without graft T-cell depletion (no rejection in the group with non-myeloablative conditioning, and 4% in the group with myeloablative conditioning; Figure 3 and Table 1). While there was a strong association between rejection (37%) and completely autologous T cell reconstitution or T-cell non-engraftment (24% or 27%, respectively) in reduced intensity conditioning, T-cell-depleted recipients we detected an increased proportion of T- cell non-engraftment (35%) but no case of autologous T-cell reconstitution in the myeloablative conditioned, T-cell-depleted graft recipients.

EBV-lymphoproliferative disease

We observed a low incidence of EBV-LPD, defined as monoclonally derived systemic disease and lymphoma, with two fatal cases within the population of 134 pediatric patients who all received ATG during pre-transplant conditioning (1.49%). Although beyond the limits of statistical value, it is of note that both patients who succumbed to monoclonal EBV-LPD, belonged to a subgroup of patients who had received T-cell-depleted grafts and *high-dose* (i.e. 30 mg/kg) rabbit-derived ATG, a treatment associated with a higher frequency of EBV-PCR positivity and EBV-linked clinical symptoms (virus-nucleic acid-positive polyclonal disease with tonsillitis, fever, adenopathies, mononucleosis) than *low-dose* ATG (≤10mg/kg cumulative dose; *data not shown*).

Discussion

Here we show in a large clinical analysis of pediatric HSCT that extensive T-cell depletion, by combining *ex vivo* graft T-cell depletion and *in vivo* ATG treatment significantly inhibits $CD4^+$ and $CD8^+$ T-cell reconstitution irrespectively of the conditioning intensity. We suspect that the delay of T-cell engraftment associated with graft T-cell depletion results in a substantial cellular immune defect, leading to the increased incidence of

fatal viral and fungal infections seen in these patients.

Grouping patients according to conditioning intensity and graft T-cell depletion resulted in clustering of patients with regard to certain prognostic factors (e.g. haplo-identical donor transplantations fell almost exclusively into the two T-cell-depleted groups, patients with malignant diseases clustered in the groups receiving myeloablative conditioning; for diagnoses see Table 2). The variety of underlying diseases in each of the four subgroups of patients predisposed the individuals to certain infectious and immunologic complications as well as toxicities independently of the HSCT setting. However, this grouping appeared to be adequate to analyze the effect of combined T-cell depletion approaches on T-cell reconstitution and its consequences on histo-incompatibility and on third-party cellular immune reactions. Although, due to diagnoses and remission status, patients who had myeloablative conditioning and graft T-cell depletion had the worst prognosis when entering HSCT and the worst outcome thereafter, the similarity of delayed T-cell reconstitution and increased incidence of viral and fungal infections between these patients and those who received reduced intensity conditioning and graft T-cell depletion supports the conclusion that adding *in vitro* T-cell depletion to *in vivo* T-cell depletion has adverse effects. Despite this not being a randomized prospective study, it should be stated that the retrospectively analyzed cohort of patients consisted of unselected, consecutive ATG-pretreated HLA-non-identical allogeneic HSCT recipients managed in single center over a period of ten years. Given the long time frame of the study, various ATG preparations and doses were used, according to the then current treatment protocols, but this does not appear to have had an influence on the reduction of the incidence of GvHD. Similarly, the GvHD prophylactic regimens differed somewhat during the study period, but again this does not appear to have affected the benefit of T-cell depletion on the risk of GvHD. The distribution of patients with earlier as compared to more recent transplants was similar in all four groups. Hence, no bias from modified GvHD prophylactic regimens or changes in supportive care was to be expected. There were no significant differences in the clinical course among the small number of sex-mismatched transplantations in any subgroup of the study cohort (*data not shown*), so no judgment could be made on the clinical relevance of DBY or other sex-dependent minor histo-compatibility antigens for immune reconstitution and the sex-mismatch-associated risk of infections, rejection, and GvHD in the context of this study.[33,34]

The high rate of rejection in both groups with T-cell-depleted grafts (reduced intensity conditioning: 37%; myeloablative conditioning: 18%) is associated with a notable number of patients with autologous T-cell regeneration after reduced-intensity conditioning (i.e. one third of the T-cell-reconstituted patients in the reduced intensity conditioning, T-cell-depleted group) and of T-cell non-engraftment in the myeloablatively conditioned, T-cell-depleted group. Thus, while preventing GvHD, the combination of graft T-cell depletion and ATG might favor: i) T-cell recipient chimerism-associated rejection in non-myeloablative regimens, and ii) T-cell non-engraftment and rejection after myeloablative conditioning. These phenomena might be because the removal of potentially alloreactive T cells from the graft also removes part of the conditioning of the patient, thus requiring intensification of the non-myeloablative regimen as compared to that necessary in patients receiving unselected grafts.[35] All patients included in our study underwent *in vivo* T-cell depletion by ATG, which supposedly inhibited endogenous and allogeneic T cells and thus alloreactivity in both directions equally. The increased rate of rejection in patients receiving a T-cell-depleted graft appears to suggests that the effect of graft T-cell depletion on T-cell suppression might dominate over ATG with respect to rejection. However, it should be stated that haplo-identical HSCT recipients, who almost exclusively fell in groups who received T-cell-depleted grafts, are *per se* at much higher risk of rejection than are the others, which might also, at least in part, be responsible for the increased incidence of rejection in both groups who received T-cell-depleted grafts.

The incidence of severe acute and chronic GvHD was low in our whole patient population, which we ascribe to the fact that a large proportion of the patients received HLA-matched grafts that had been selected after high-resolution typing of class I and II alleles. Clinical evaluation of the effect of modern techniques of HLA typing on a genomic level will most likely alter the requirements and indications for GvHD prophylaxis,[36] allowing for a reduction of T-cell depletion in many patients. Additionally, the quality of high-resolution HLA-typing might have contributed to the low incidence of post-HSCT EBV-LPD (1.49%). Indeed, this incidence was lower than would have been expected from the results of other studies[19-21] that indicated HLA disparities, graft T-cell depletion and ATG or anti-CD3 treatment as primary risk factors imposing a relative risk of 3.7 (≥2 antigen HLA mismatch), 9.1 (T-cell depletion), 5.5 (ATG/antilymphocyte globulin) and 35.9 (anti-CD3 monoclonal antibody) on the overall less than 1% risk in HSCT.[19] We hypothesize that primarily the early diagnosis and pre-emptive treatment of EBV-viremia with ganciclovir (even without rituximab) were responsible for the low incidence of EBV-LPD in our cohort of patients.

The effect of *ex vivo* graft T-cell depletion by $CD34^+$-positive or $CD3^+$-negative selection on T-cell reconstitution and clinical outcome has been evaluated in sep-

arate studies: although the striking benefit of graft T-cell depletion on GvHD prevention has been confirmed in adults and children,[16,37,38] disadvantages such as delayed immune recovery, reduced graft-versus-leukemia effect, and higher incidence of rejection are becoming increasingly acknowledged.[39-45] Attempts to select T-cell subsets negatively or to specifically eliminate alloreactive T cells from the graft have also demonstrated this trend.[35,46-51] We show here that T-cell reconstitution is delayed after *ex vivo* graft T-cell depletion independently of the conditioning regimen intensity. The consequences of compromised T-cell immunocompetence in recipients of T-cell-depleted grafts (such as higher frequencies of severe viral and fungal infections and rejection) than in patients receiving unselected grafts, predominantly contribute to the increased transplant-related mortality in these groups of patients. This observation is in contrast to the hypothesis that non-myeloablative chemotherapy leads to earlier and functionally better immune recovery than does myeloablative treatment, and it underlines the concern of exchanging the risk of GvHD for the risk of infections and rejection. In the light of the striking increase of transplant-associated mortality due to significantly delayed T-cell reconstitution in patients with combined *in vivo* and *in vitro* T-cell depletion, the results of our study strongly imply that such extensive depletion is not recommendable in highly matched unrelated donor transplants.

The increasing evidence that alloreactive T-cell subsets are responsible for GvHD[48,51,52] and that regulatory T cells are involved in GvHD-suppression;[53,54] (*for reviews see also Sakaguchi[55] and Shevach[56]*) suggests that it will be critical to find means to distinguish the dangerous from the beneficial T cells by phenotypic and functional parameters (e.g. CD25, CD62L, FoxP3, ROG/RGS-1, MLR, CFSE), in order to provide optimally prepared grafts. Eliminating subsets of alloreactive T cells rather than *all* T cells from the graft might be a way to avoid the disadvantages of T-cell depletion, although definitive identification and permanent deletion of these activated effector T cells are still unresolved issues in transplantation immunology. An alternative may be the expansion or specific generation of regulatory and third party-(e.g. virus)-effective T cells to induce tolerance but bolster immune competence during the critical period of post-transplantation deficient T-cell function.

GF and TL contributed substantially to data acquisition and analysis (FACS, chimerism, viral and fungal infection monitoring); UP conducted all the statistical analyses; CP, SMM and AL were responsible for clinical data acquisition, treatment of the patients, and follow-up documentation; AR and GF contributed to HLA-typing and, in part in co-operation with GF, to the preparation of stem cell products; HG, CP and MGS are responsible for the general concept and design of the study; in addition, CP supervised both the clinical performance, documentation of patients as well as the methodological design, and MGS supervised data analyses and prepared the article. All authors contributed to the interpretation of the data, revised the manuscript critically for intellectual content, and approved its final version. The authors declare that they have no potential conflict of interest.

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