Supplementary Material

Methods

Transplant details

Eighty (85%) transplants were performed using a reduced-intensity conditioning (RIC) regimen containing Fludarabine 125 mg/m^2 and Melphalan 140 mg/m^2; or Fludarabine 120 mg/m^2 and Cyclophosphamide 1200 mg/m^2. Fourteen (15%) used myeloablative conditioning (MAC) with Cyclophosphamide 120 mg/kg and 14.4 Gy TBI; or Cyclophosphamide 120 mg/kg and Busulphan 16 mg/kg. Patients with MAC received Methotrexate 15 mg/m^2 on day +1 and 10 mg/m^2 on day +3, +6, and +11, whilst Fludarabine/Melphalan sibling allografts received Methotrexate 5 mg/m^2 on day +1, +3, +6, and +11.

Flow cytometry

PBSC grafts were analysed by flow cytometry using CD45/CD3/CD4/CD8 Multitest with Trucount tubes (342447) and anti-CD3 PE-Cy7 (557851), CD4 FITC (555346), CD19 PE (555413), CD56 APC (555518) [BD Biosciences, Oxford, UK], and CD8 APC Alexa-Fluor 750 (27-0088, eBiosciences, Hatfield, UK). To quantify Tregs, defined phenotypically as $CD3^+CD4^+CD8^-CD25^+FOXP3^+CD127^{dim/-}$ cells, peripheral blood mononuclear cells (PBMC) were isolated from PBSC grafts using Ficoll-Paque and stained using anti-CD3 PE-Cy7 (557851), CD4 FITC (555346), CD25 PE (557741) [BD Biosciences]; CD8 APC-Alexa-Fluor 750 (27-0088), and CD127 PerCP-Cy5.5 (45-1278) [eBiosciences]. Stained PBMC were washed, fixed and permeabilized, and stained with anti-FOXP3 APC (130-093-013, Miltenyi Biotech, Bisley, UK). Flow cytometry was performed on an LSR II Flow Cytometer

using FACS Diva software (BD Biosciences). Staining controls were fluorescence-minus-one, isotype controls, and antigen negative cells.

Statistics

Sample size was calculated with overall survival as the primary outcome. The historical 3-year overall survival for allogeneic HSCT for Oxford University Hospitals NHS Trust is 60-65%. Based upon these data, this study was designed to detect a difference of 25%, with an estimated 3-year overall survival of 50% and 75% in the low and high Treg/$CD4^+$ T-cell groups, respectively. With a one-sided model using an α value of 0.05 and β value of 0.2, the estimated sample size was 45 in each arm.

Variables initially considered in univariate analysis were recipient and donor age; sex mismatch (female donor to male recipient vs other); ABO mismatch (matched/minor vs major/bidirectional); recipient and donor CMV serology; disease (acute leukaemia vs other); disease stage (Early (CR1/chronic phase/untreated) vs Other (≥ CR2/partial response/active disease); conditioning (RIC vs MAC); donor (sibling vs unrelated). Graft variables considered were $CD34^+$, $CD3^+$, $CD19^+$, $CD3^-CD56^+$, Treg ($CD3^+CD4^+CD25^+FOXP3^+CD127^{dim/-}$) dose, and the Treg/$CD4^+$ T-cell ratio.

Table S1. Analysis of the PBSC graft contents.

A. Cell type	**Treg dose**		**Treg/CD4$^+$ T-cell ratio**	
	r	***P***	***r***	***P***
TNC	0.45	**< 0.0001**	0.03	0.78
CD34$^+$	0.13	0.22	0.02	0.86
CD3$^+$	0.65	**< 0.0001**	−0.10	0.35
CD19$^+$	0.53	**< 0.0001**	0.12	0.25
CD3$^-$CD56$^+$	0.50	**< 0.0001**	0.12	0.25

B. Univariate	**Variable**		**Median**	**Range**	***P***
Treg Counts ($\times 10^8$)	Donor	Sibling	4.35	1.73-13.78	**0.01**
		Unrelated	3.48	0.48-10.54	
	Harvest	One day	3.51	0.48-9.62	**< 0.001**
		Two days	6.76	2.36-13.87	
Treg/CD4$^+$ T cells	Donor	Sibling	0.033	0.011-0.086	**0.04**
		Unrelated	0.026	0.008-0.061	
	Gender	Male	0.030	0.008-0.086	0.07
		Female	0.028	0.014-0.045	

C. Multivariate	**Variable**	**B**	**SE B**	**β**	***P***	R^2
Treg Counts ($\times 10^8$)*	Constant	0.84	0.72		0.24	0.24
	Harvest	3.02	0.56	0.49	**< 0.001**	
Treg/CD4$^+$ T cells$^{\#}$	Constant	−1.44	0.04			0.09
	Donor	−0.10	0.04	−0.28	**0.01**	
	Gender	−0.10	0.04	−0.24	**0.03**	

(A) Spearman correlation (*r*) between Treg dose ($\times$ 10^6/kg) or proportion of Tregs (Tregs/CD4$^+$ T cells), and the dose ($\times$ 10^6/kg) of the main cell populations in the graft. (B) Univariate analysis of the Treg counts ($\times$ 10^8) and proportion of Tregs (Tregs/CD4$^+$ T cells) in the graft. Analysed by Mann-Whitney test. (B) Multivariate linear regression of Treg counts ($\times$ 10^8) and proportion of Tregs (Tregs/CD4$^+$ T cells)) in the graft. *Data were transformed using a $\sqrt{}$ transformation; $^{\#}$Data were transformed using a $\log_{10}$ [x/(1−x)] transformation; Tregs, regulatory T cells.

Table S2. Timing and cause of mortality.

Low Treg/CD4$^+$ T-cell ratio		**High Treg/CD4$^+$ T-cell ratio**	
Day	*Cause*	*Day*	*Cause*
10	Infection	18	Venoocclusive disease
16	Infection	20	Infection
35	GvHD	25	Infection
43	GvHD	59	Pneumonitis
44	Relapse	146	Relapse
74	GvHD	157	Relapse
116	Relapse	171	Relapse
143	Relapse	199	Relapse
145	Thrombosis	241	GvHD
161	Infection	304	Relapse
163	GvHD	339	PTLD
198	Relapse	427	Relapse
221	Relapse	1484	Relapse
226	GvHD		
240	Neuropathy		
245	GvHD		
252	Infection		
256	GvHD		
258	Relapse		
315	GvHD		
317	GvHD		
566	Relapse		
747	Intracranial haemorrhage		
845	Relapse		

Tregs, regulatory T cells; PTLD, post-transplant lymphoproliferative disorder.

Table S3. Overall survival according to the proportion of Tregs (Tregs/$CD4^+$ T cells) in the PBSC grafts (quartiles).

A. Univariate analysis

Group	Tregs/$CD4^+$ T cells	3-year OS (%)	95% CI
1st quartile	0.0081-0.0222	42	26-67
2nd quartile	0.0223-0.0295	57	40-81
3rd quartile	0.0296-0.0393	67	50-89
4th quartile	0.0394-0.0856	83	69-100

B. Multivariate analysis

Variable		HR	95% CI	*P*
Tregs/$CD4^+$ T cells	1st quartile	1.00		
	2nd quartile	0.74	0.30-1.82	0.51
	3rd quartile	0.63	0.25-1.56	0.32
	4th quartile	0.22	0.06-0.73	**0.01**
Recipient CMV	Seronegative	1.00		
	Seropositive	2.23	1.07-4.66	**0.03**

(A) Kaplan-Meier estimation of 3-year overall survival according to the proportion of Tregs (Tregs/$CD4^+$ T cells) in the grafts, divided by quartiles. (B) Multivariate analysis of overall survival. Variables included in the initial model were the Treg/$CD4^+$ T-cell ratios in the peripheral blood stem cell grafts (quartiles) adjusting for significant differences between the groups (donor age, donor gender, and $CD3^-CD56^+$ cell dose) and variables with $P < 0.10$ in univariate analysis (recipient age, recipient CMV serology, and HLA-mismatch in HvG direction). Tregs, regulatory T cells; OS, overall survival.

A. No Alemtuzumab (*n*=36) **B. Alemtuzumab (*n*=58)**

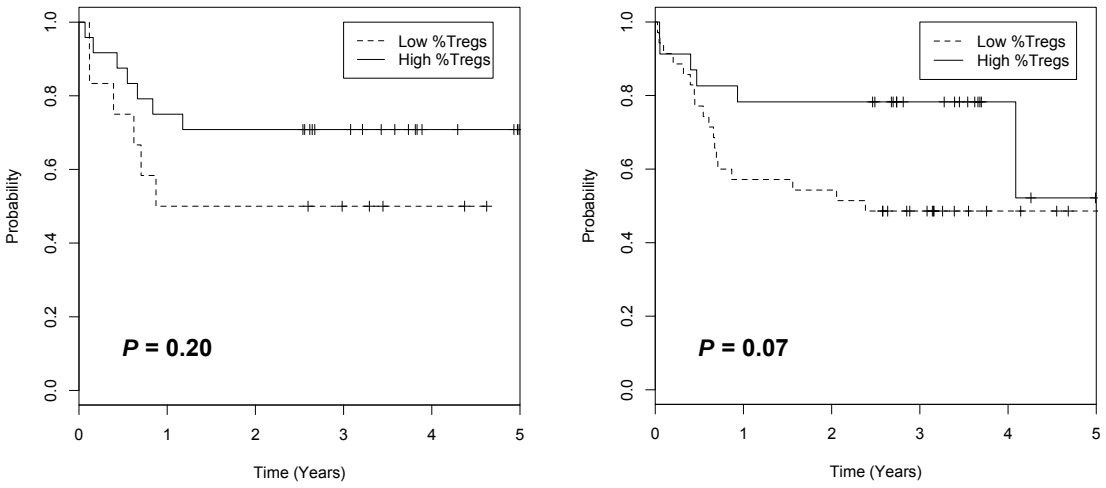

Figure S1. Overall survival in T-replete and T-deplete transplants according to the proportion of Tregs (Tregs/CD4$^+$ T cells) in the graft. (A) No Alemtuzumab conditioning; (B) Alemtuzumab conditioning. Low %Tregs, Tregs/CD4$^+$ T cells below the median (dotted line); High %Tregs, Tregs/CD4$^+$ T cells above the median (solid line); Tregs, regulatory T cells.