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High frequencies of CD62L$^+$ naive regulatory T cells in allografts are associated with a low risk of acute graft-*versus*-host disease following unmanipulated allogeneic haematopoietic stem cell transplantation

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Summary

Regulatory T cells (T_{regs}) play a key role in the prevention of acute graft-*versus*-host disease (aGVHD). To investigate the association between T_{reg} subsets and aGVHD, we prospectively analysed T cell subsets in the allografts of 35 patients undergoing myeloablative unmanipulated haematopoietic stem cell transplantation. Multivariate analysis found that patients infused with less than $0{\cdot}29 \times 10^6$/kg of CD4$^+$CD25^{high}CD45RA$^+$CD62L$^+$ T cells during transplantation exhibited an increased incidence of II–IV aGVHD [hazard ratio (HR) = 0·000, 95% CI = 0·000–0·106, $P = 0{\cdot}013$]. Next, we compared the reconstitution characteristics of T cell subsets between haploidentical haematopoietic stem cell transplantation (HSCT) and sibling HSCT by collecting peripheral blood samples at regular intervals (days 30, 60 and 90) after transplantation. No significant differences were observed in the reconstitution of conventional T cells between haploidentical HSCT and sibling identical HSCT. However, total counts of recovered naiveT_{regs} and CD62L$^+$ naive T_{regs} from haploidentical HSCT were significantly lower compared to sibling identical HSCT; *P*-values were 0·045 and 0·021, respectively. Although total counts of conventional T cells in aGVHD patients reached similar levels compared to non-aGVHD patients before day 60 post-HSCT, total counts of naive T_{regs} and CD62L$^+$ naive T_{regs} in aGVHD patients did not reach similar levels to non-aGVHD patients until 90 days post-HSCT. Taken together, our findings demonstrate that a large population of CD62L$^+$ naive T_{regs} in allografts reduces the incidence of aGVHD. Further, development of aGVHD is related closely to the delayed reconstitution of the naive T_{reg} population.

Keywords: acute graft-*versus*-host disease, allogeneic haematopoietic stem cell transplantation, immune reconstitution, immune tolerance, regulatory T cell

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Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) provides potential curative therapy for patients with both malignant and non-malignant haematological diseases. However, acute graft-*versus*-host disease (aGVHD) remains a major obstacle to a more favourable therapeutic outcome of HSCT, as after HSCT patients tend to lose immune tolerance [1–4]. Immune tolerance is maintained by both central negative selection and a peripheral regulatory system. In HSCT, damage to the thymic epithelium by a pretransplantation-conditioning regimen and host-reactive T cells compromise negative selection in the thymus, which leads to the subsequent release of alloreactive T cells into the periphery. These cells recognize both major and minor histocompatibility antigens on recipient target cells, and an attack on these target cells results in aGVHD [1,2,5,6]. Therefore, the peripheral regulatory system plays a critical role in the establishment of tolerance between host tissue and donor-derived immune cells early after HSCT by suppressing the activity of pathogenic effector T lymphocytes.

Regulatory T cells (T_{regs}) play a key role in the maintenance of peripheral tolerance. Although a variety of T_{reg} populations that ameliorate the development of aGVHD have been described, the majority of studies have focused on CD4$^+$CD25$^+$forkhead box P3 (FoxP3$^+$) T_{regs}, which have been shown to suppress alloreactivity in a cell contact-dependent manner both *in vitro* and *in vivo* [7–11]. Patients who experienced aGVHD had a lower frequency of FoxP3$^+$ T_{regs}

compared to patients without aGVHD [12–15]; further, adoptive transfer of this subset protected against or reduced GVHD in both animal models and humans [16–19]. However, conflicting reports exist regarding the impact of T_{regs} on GVHD in humans. Indeed, several researchers have reported that T_{regs} do not influence the generation of aGVHD after HSCT [20,21]. These findings are supported by our previous work, which found that neither CD4$^+$CD25$^+$FoxP3$^+$ T_{regs} in allografts nor their reconstitution after HSCT correlated with aGVHD [22].

In human peripheral blood, CD4$^+$CD25$^+$FoxP3$^+$ T_{regs} reside mainly within the subset of CD4$^+$ cells that highly express CD25 (CD25^{high}). Hoffmann *et al.* [23] obtained CD4$^+$CD25$^+$FoxP3$^+$ T_{regs} expanded from naive T_{regs} (CD45RA$^+$ CD4$^+$CD25^{high}) in humans; furthermore, the transfer of naive T_{regs} was identified as the best strategy for adoptive therapy. As CD62L (L-selectin) is an important T cell homing receptor, CD62L$^+$ T_{regs} enter preferentially into second lymphoid organs; in particular, entry into lymph nodes via high endothelial venules prevents aGVHD in target organs [24]. Ermann *et al.* [25] demonstrated that the adoptive transfer of CD62L$^+$ but not CD62L$^-$ T_{regs} protected against GVHD in a murine model. Therefore, we hypothesize that a specific subset or subsets of CD4$^+$CD25$^+$FoxP3$^+$ T_{regs} rather than the whole population are correlated with the development of aGVHD.

Previous clinical studies have identified that allograft characteristics such as infusion of increased numbers of total nucleated cells (TNC), CD34$^+$ cells and CD3$^+$ T cells are associated with the development of aGVHD. In contrast, a high percentage of FoxP3$^+$ T_{regs} in allografts is associated with a reduced risk of aGVHD following HSCT with T cell depletion *in vitro* [26–29]. However, we found that the transfer of cells with a higher CD4/CD8 ratio and an increased population of CD4$^+$CD45$^+$CD62L$^+$ in allografts increased the risk of aGVHD. No correlation between FoxP3$^+$ T_{regs} and aGVHD risk was observed in our centre-performed unmanipulated allogeneic HSCT (without T cell depletion *in vitro*) [30,31]. Therefore, we sought to clarify whether the different reports of FoxP3$^+$ T_{regs} in aGVHD were derived from different HSCT methodologies or from differences in T_{reg} subpopulations.

This study analysed prospectively 35 patients undergoing unmanipulated allogeneic HSCT; we detected the absolute numbers and relative proportions of lymphocyte subsets in their allografts and found a strong correlation between the number of CD62L$^+$ naive T_{regs} and the incidence of aGVHD. Our data also demonstrated that no differences were found in conventional T cell reconstitution between sibling identical HSCT and haploidentical HSCT. However, the total counts of naive T_{regs} and CD62L$^+$ naive T_{regs} were decreased substantially following haploidentical HSCT. Analysis of the reconstitution of different T cell subsets between aGVHD patients and non-aGVHD patients showed that aGVHD patients exhibited a delayed reconstitution of naive and CD62L$^+$ naive T_{regs}.

Materials and methods

Patients, samples and grouping

Peripheral blood samples were collected from 23 patients with malignant haematological diseases undergoing HSCT from July to September 2009 to detect the association between FoxP3$^+$ T_{regs} and aGVHD and between FoxP3$^+$ T_{regs} and CD4$^+$CD25^{high} T_{regs}. Blood samples were taken the day before transplantation and 30 days after transplantation. Additionally, we collected allograft samples from 35 patients with malignant haematological diseases undergoing HSCT (haploidentical HSCT $n = 15$, sibling HSCT $n = 20$) from December 2009 to February 2010. Donors received the granulocyte colony-stimulating factor (G-CSF) analogue filgrastim (Kirin Brewery Co, Tokyo, Japan) at 5 μg/kg daily for 5 days. On the fourth day bone marrow cells were harvested, and on the fifth day peripheral blood progenitor cells (PBPCs) were collected for the detection of allograft components. In addition, peripheral blood samples were collected at regular intervals (days 30, 60 and 90) after transplantation to study T cell reconstitution; however, only 20 patients who had not received donor lymphocyte infusion (DLI) until 90 days were included. Patient clinical history, pretransplantation-conditioning regimen, treatment, HSCT outcome, aGVHD symptoms and their donors' information are summarized in Table 1. All patients included in this study were enrolled in clinical protocols approved by the Institutional Review Board of Peking University Institute of Haematology. Written informed consents were obtained from both patients and their donors prior to sample collection.

Transplantation approach

Patients with human leucocyte antigen (HLA)-identical donors received either the myeloablative busulphan/cyclophosphamide (Bu/Cy) regimen or the busulphan/fludalabin (Bu/Flu) regimen. Patients with HLA mismatched or haploidentical donors received the Bu/Cy regimen and porcine anti-human thymocyte immunoglobulin (ATG). Prophylaxis for GVHD included treatment with cyclosporine A (CSA), short-term methotrexate (MTX) and mycophenolate mofetil (MMF), as described previously [3,4].

Engraftment, diagnosis and scoring of aGVHD

Neutrophil engraftment after transplantation was defined as an absolute neutrophil count (ANC) exceeding $0{\cdot}5 \times 10^9$/l for 3 consecutive days; platelet engraftment was defined as a platelet count exceeding 20×10^9/l without transfusion for 7

Table 1. Patient characteristics.

Characteristic	No. of patients (%)
Number	35
Median age, years	31 (range 9–57)
Diagnosis	
Acute myeloid leukaemia	15 (43)
Acute lymphoblastic leukaemia	9 (26)
Myelodysplastic syndrome	4 (11)
Chronic myeloid leukaemia	4 (11)
Lymphoma	3 (9)
Disease status, *n*	
Standard risk	23 (66)
High risk	12 (34)
Conditioning regimen	
Bu/Cy + ATG	15 (43)
Bu/Cy	11 (31)
Bu/Flu	9 (26)
Stem cell source	
G-PB + G-BM	33 (94)
G-PB only	2 (6)
Donor median age, years	40 (range 11–63)
Patient/donor HLA compatibility	
Matched	20 (57)
Two locus mismatched	7 (20)
Three locus mismatched	8 (23)
Sex match, patient/donor	
Male/female	15 (43)
Male/female	7 (20)
Female/male	8 (23)
Female/female	5 (14)
Acute GVHD grade	
0 to I	25 (71)
II to IV	10 (29)
Median follow-up days	334 (range 275–360)
Engraftment	
Neutrophil engraftment, days (range)	13 (range 9–23)
Platelet engraftment, days (range)	14 (range 8–51)

Bu: busulphan; Cy: cyclophosphamide; ATG: anti-human thymocyte immunoglobulin; G-PB: granulocyte colony-stimulating factor (G-CSF)-priming-peripheral blood progenitor cells; Flu: fludarabine.

consecutive days. Clinical aGVHD was graded according to standard criteria [32].

Treatment of aGVHD

aGVHD no less than grade II is usually treated with high-dose methylprednisolone, typically starting at a dose of 2 mg/kg per day. MTX and anti-CD25 monoclonal antibodies (mAb) were used to treat patients not fit for corticosteroid-therapy. aGVHD less than grade II is usually treated by the adjustment of the ongoing immunosuppressive drug therapy [33,34].

Flow cytometry

Samples were stained shortly after collection without further separation to minimize selective loss. Anti-CD4 peridinin chlorophyll protein (PerCP), anti-CD25 phycoerythrin (PE), anti-CD62L allophycocyanin (APC), anti-CD45RA fluorescein isothiocyanate (FITC), anti-FoxP3 PE, anti-CD25 APC and matched mouse isotype control antibodies (Becton-Dickinson, San Jose, CA, USA) were used. Intracellular analysis of FoxP3 expression was assessed using anti-FoxP3-PE (eBioscience, San Diego, CA, USA) and its mouse isotype control antibody; staining was performed after fixation and permeabilization according to the manufacturer's recommendations. Flow cytometry was performed using a BD FACSort.

Statistical analysis

Statistical analysis was performed using SPSS version 16·0. For non-normally distributed values, data were summarized by the median and ranges. For a two-related sample comparison of continuous variables, a two-sided Wilcoxon rank sum test was performed. For a two-unrelated sample comparison of continuous variables, a two-sided Mann–Whitney *U*-test was performed. For correlation analysis between non-normally distributed values, a Spearman correlation analysis was performed; for normally distributed values, a Pearson correlation analysis was performed. One-way univariate analysis using the Welch and Brown-Forsythe test was performed to select allograft components associated with aGVHD. Variates were grouped by median to analyse the association between subsets of allografts and aGVHD by Kaplan–Meier. Multivariate Cox proportional hazards models were assessed to avoid potential confounding factors. All factors with $P < 0{\cdot}05$ in univariate analysis were included as covariates for testing interaction, other factors with $P > 0{\cdot}05$ but that were associated closely with aGVHD were also included. Factors included were donor ages, conditioning regimen, HLA mismatch, dose of total nucleated cells, dose of $CD34^+$ cells, dose of $CD3^+$ T cells, dose of $CD14^+$ cells, dose of $CD4^+$ T cells, dose of $CD8^+$ T cells and dose of $FoxP3^+$ T_{reg} subsets. The final multivariate models were built using a forward stepwise model selection approach. Repeated-measures analysis was performed to compare T cell reconstitution between haploidentical HSCT and sibling HSCT or between patients with aGVHD and those without aGVHD. $P < 0{\cdot}05$ was considered statistically significant.

Results

$CD4^+CD25^+FoxP3^+$ T cell frequencies decrease after HSCT, but do not correlate with aGVHD

A total of 23 patients with malignant haematological disease undergoing HSCT (haploidentical HSCT $n = 16$, sibling HSCT $n = 7$) were monitored for the detection of an association between $FoxP3^+$ T_{regs} and aGVHD and an

association between FoxP3$^+$ T_{regs} and CD4$^+$CD25^{high} T_{regs}. The median age of patients was 39 years (range 22–49 years), and the median age of donors was 44 years (range 20–51 years). Sixteen (69·57%) of the 23 patients were considered standard risk and seven patients (30·43%) were considered high risk. While all 16 patients with HLA mismatched donors received the myeloablative Bu/Cy+ATG regimen, six of seven patients with HLA sibling donors received the myeloablative Bu/Cy regimen; the remaining patient with a HLA sibling donor received the Bu/Flu regimen. All patients exhibited stable neutrophil engraftment prior to day 30 post-HSCT. Nine patients (defined as the aGVHD group) suffered from aGVHD with a grade from II to IV. The median onset time was day 21 post-HSCT and ranged from days 17–89 following HSCT; all patients demonstrated a complete response (CR) to aGVHD following treatment. The other patients were defined as the non-aGVHD group ($n = 14$).

We compared the frequency of T_{regs} in patients prior to HSCT and 30 days after HSCT. Initial samples were collected at the time of hospital admission prior to the administration of any medicine associated with HSCT; post-HSCT samples were collected at day 30 to exclude any influence caused by DLI. Populations of CD4$^+$CD25$^+$FoxP3$^+$ T_{regs} were not significantly different in aGVHD patients *versus* non-aGVHD patients either before HSCT ($P = 0{\cdot}477$) or after HSCT ($P = 0{\cdot}250$) (Fig. 1a); the same results were also observed for CD4$^+$CD25^{high} T cells (Fig. 1b). Both CD4$^+$CD25$^+$FoxP3$^+$ T_{reg} and CD4$^+$CD25^{high} T cell populations decreased after HSCT (Fig. 1c). FoxP3$^+$ T_{regs} decreased from a median of 1·83% (range 0·15–6·08%) to a median of 0·91% (range 0·00–4·47%); similarly, CD4$^+$CD25^{high} T cells decreased from a median of 3·24% (range 0·25–10·28%) to a median of 1·73% (range 0·00–6·25%). To detect an association between FoxP3$^+$ T_{regs} and CD4$^+$CD25^{high} T cells, correlation analysis was performed both before HSCT and after HSCT; a strong association was observed between FoxP3$^+$ T_{regs} and CD4$^+$CD25^{high} T cells, and these results are presented in Fig. 1d,e.

Absolute numbers of CD4$^+$CD25^{high}CD45RA$^+$CD62L$^+$ T cells in allografts are a strong predictor for the development of aGVHD

Naive T_{regs} were defined as described previously [23,35]; details are shown in Fig. 2. Table 2 depicts the subsets of cells present in the allografts of 35 HSCT patients. Patient and donor characteristics are listed in Table 1. Although the median onset time of aGVHD with a grade of II to IV was day 25 post-HSCT, onset ranged from days 15 to 45 post-HSCT. All patients exhibited a complete response (CR) to aGVHD following treatment. Univariate analysis showed that absolute CD4$^+$CD45RA$^+$CD62L$^+$ T cell numbers, absolute CD4$^+$CD25^{high}CD45RA T cell numbers and absolute CD4$^+$CD25^{high}CD45RA$^+$CD62L$^+$ T cell numbers were statistically different between patients with aGVHD of grade 0–I and patients with aGVHD of grades II–IV; *P*-values were 0·038, 0·040 and 0·003, respectively. Variates were grouped by median of absolute numbers of those three subsets, respectively. The comparison of cumulative incidence of aGVHD between groups more than median and groups less than median were performed by Kaplan–Meier and a log-rank test as shown in Fig. 3. However, analysis of the correlation coefficients of various cell components in allografts revealed that many cell subsets showed statistically significant positive linear relationships with one another, indicating that these subsets could not be regarded as completely independent factors (Table 3). Therefore, their relative proportions were selected as factors for multivariate analysis. Finally, we found that patients who received less than $0{\cdot}29 \times 10^6$/kg of CD4$^+$CD25^{high}CD45RA$^+$CD62L$^+$ T cells in their allograft exhibited an increased incidence of II–IV aGVHD [hazard

Table 2. Cellular composition of the mixed allografts of the granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood stem cell grafts and G-CSF-primed bone marrow grafts.

	BM	PB	Total
TNC $\times 10^8$/kg	2·61 (0·94–4·79)	7·71 (2·16–14·59)	9·90 (3·48–16·75)
CD3$^+$ cells $\times 10^8$/kg	0·16 (0·05–0·41)	2·19 (0·87–4·41)	2·47 (1·06–4·69)
CD4$^+$ cells $\times 10^8$/kg	0·08 (0·02–0·21)	1·12 (0·49–2·27)	1·22 (0·60–2·31)
CD8$^+$ cells $\times 10^8$/kg	0·06 (0·03–0·79)	0·94 (0·29–2·17)	0·99 (0·36–2·32)
CD14$^+$ cells $\times 10^8$/kg	0·08 (0·02–0·16)	2·36 (0·71–5·27)	2·44 (0·75–5·27)
CD34$^+$ cells $\times 10^6$/kg	0·70 (0·21–2·26)	2·79 (0·66–9·25)	3·46 (1·45–9·90)
Naive T cells $\times 10^6$/kg	2·62 (0·09–10·61)	40·16 (16·30–87·12)	41·52 (17·95–92·19)
CD62L$^+$ naive T cells $\times 10^6$/kg	0·37 (0·00–1·01)	4·90 (2·14–13·65)	5·62 (2·22–13·65)
CD4$^+$CD25^{high} T cells $\times 10^6$/kg	0·25 (0·00–2·68)	8·86 (0·34–58·34)	9·20 (0·39–58·84)
Naive $T_{regs} \times 10^5$/kg	0.70 (0·00–4·09)	10·99 (4·67–55·35)	11·26 (4·84–58·02)
CD62L$^+$ naive T_{regs} $\times 10^5$/kg	0·09 (0·00–1·08)	2·62 (0·20–35·65)	2·88 (0·41–35·69)

TNC: total nucleated cells; naive T cells: CD4$^+$CD45RA$^+$ T cells; CD62L$^+$ naive T cells: CD4$^+$CD45RA$^+$CD62L$^+$ T cells; naive regulatory T cells (T_{regs}): CD4$^+$CD25^{high}CD45RA T cells; CD62L$^+$ naive T_{regs}: CD4$^+$CD25^{high} CD45RA$^+$CD62L$^+$ T cells; PB: peripheral blood; BM: bone marrow.

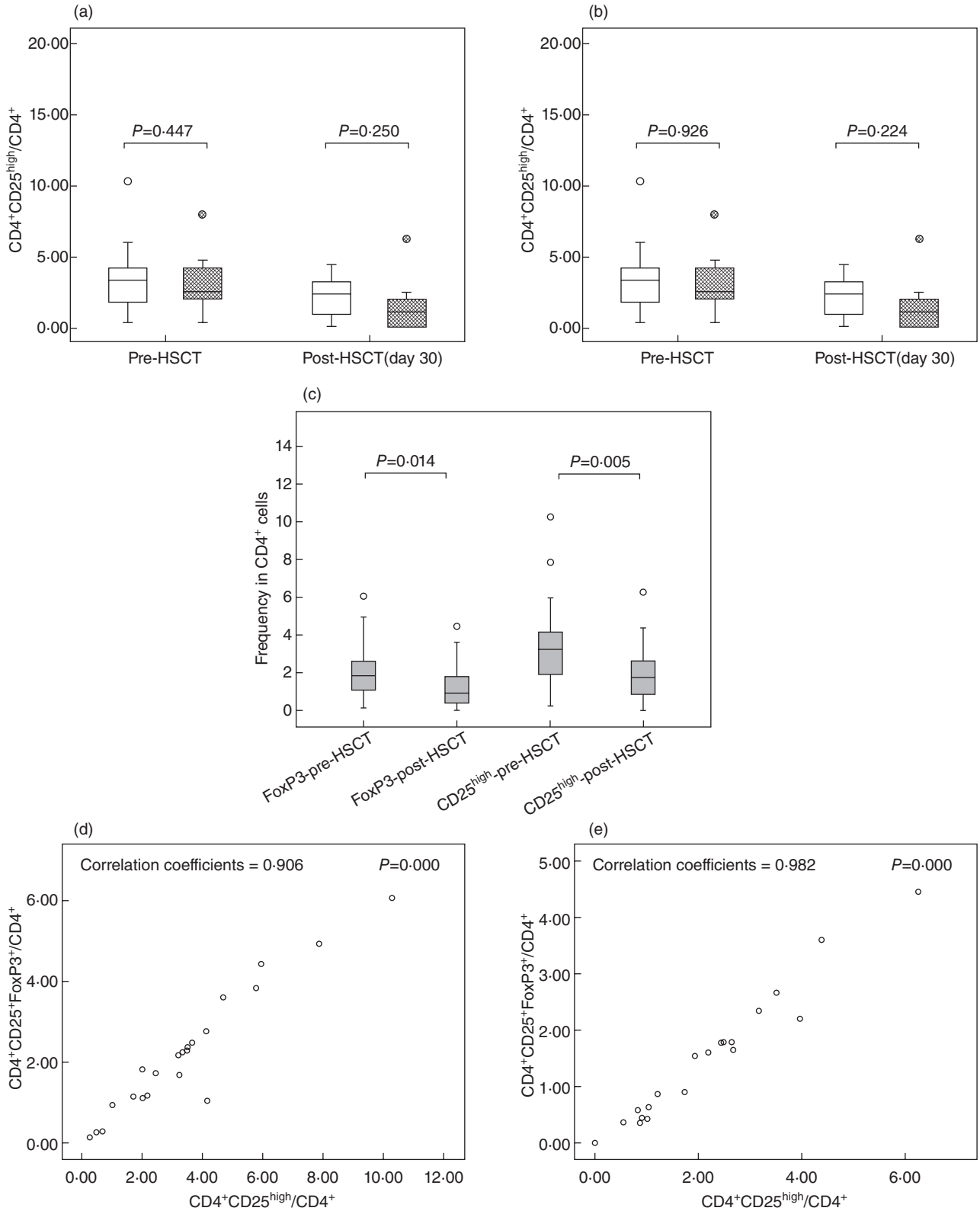

Fig. 1. Frequencies of regulatory T cells (T_{regs}) pre-haematopoietic stem cell transplantation (HSCT) and post-HSCT. (a) Comparison of $CD4^+CD25^+$forkhead box P3 ($FoxP3^+$) T_{reg} frequencies between acute graft-*versus*-host disease (aGVHD) and non-aGVHD (non-aGVHD = white box, aGVHD = grey box). (b) Comparison of $CD4^+CD25^{high}$ T_{reg} frequencies between aGVHD and non-aGVHD (non-aGVHD = white box, aGVHD = grey box). (c) Frequencies of $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^{high}$ T_{regs} decrease post-HSCT. (d) Correlation analysis between $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^{high}$ T_{regs} pre-HSCT. (e) Correlation analysis between $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^{high}$ T_{regs} post-HSCT.

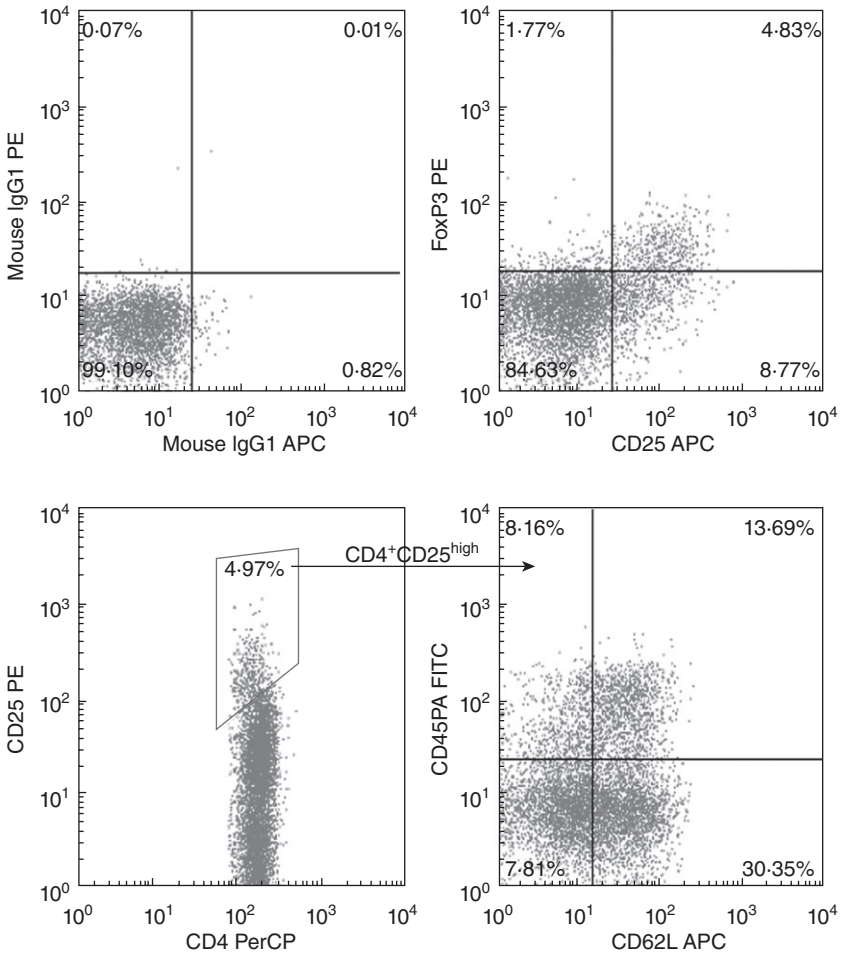

Fig. 2. Flow cytometric analysis of regulatory T cell (T_{reg}) frequencies. The frequency of each cell subset was determined as described in the Materials and methods. Cells were stained from allograft samples. (a) Isotype control. (b) $CD4^+CD25^+$ forkhead box P3 ($FoxP3^+$) T_{regs}. (c) $CD4^+CD25^{high}$ T_{regs}. (d) Subsets of $CD4^+CD25^{high}$ T_{regs}.

Table 3. Correlation coefficients for cell subsets within total allografts.

Cell subsets coefficients *P*-value	$CD3^+$	$CD4^+$	$CD8^+$	$CD14^+$	$CD34^+$	Naive	$CD62L^+$ naive	T_{regs}	Naive T_{regs}	$CD62L^+$ naive T_{regs}
TNC	0·526	0·557	0·436	0·767	0·521	0·320	0·402	0·163	0·334	0·154
	0·001	0·001	0·000	0·000	0·001	0·061	0·017	0·349	0·050	0·379
$CD3^+$		0·872	0·802	0·423	0·336	0·624	0·562	0·241	0·344	0·247
		0·000	0·000	0·011	0·048	0·000	0·000	0·163	0·043	0·153
$CD4^+$			0·539	0·430	0·352	0·592	0·661	0·320	0·328	0·319
			0·001	0·010	0·038	0·000	0·000	0·061	0·055	0·062
$CD8^+$				0·312	0·197	0·496	0·221	0·038	0·150	0·047
				0·068	0·258	0·002	0·202	0·828	0·391	0·788
$CD14^+$					0·439	0·204	0·325	0·046	0·231	0·031
					0·008	0·240	0·057	0·795	0·220	0·859
$CD34^+$						0·229	0·344	0·259	0·374	0·181
						0·187	0·043	0·133	0·027	0·299
Naive							0·468	0·199	0·400	0·152
							0·005	0·251	0·017	0·383
$CD62L^+$ naive								0·583	0·712	0·639
								0·000	0·000	0·000
T_{regs}									0·501	0·773
									0·000	0·000
Naive T_{regs}										0·564
										0·000

TNC: total nucleated cells; T_{regs}: regulatory T cells.

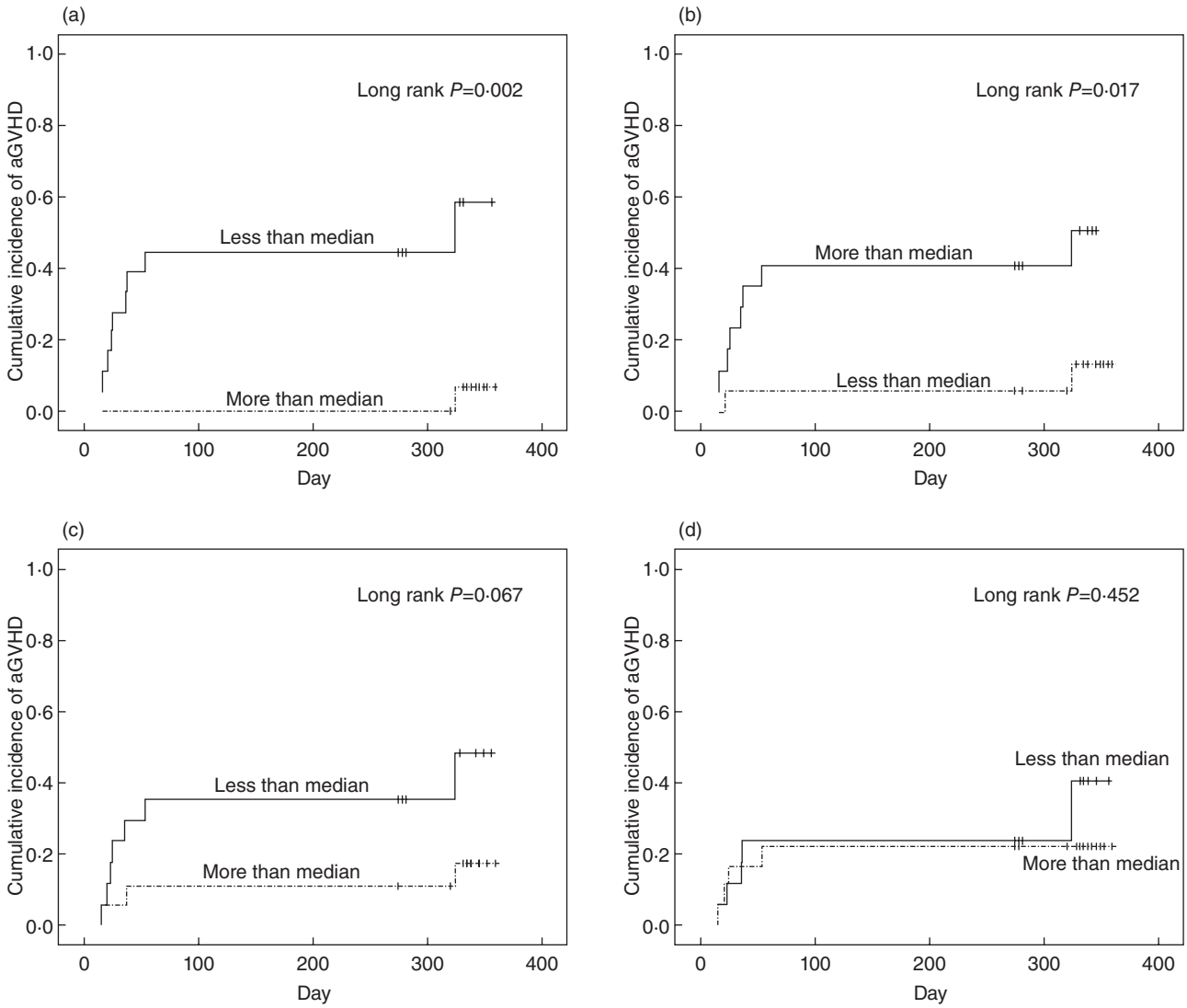

Fig. 3. Association between the absolute number of cells in T cell subsets in allografts and acute graft-*versus*-host disease (aGVHD). (a) The incidence of aGVHD increased in patients infused with an absolute number of $CD4^+CD25^{high}$ $CD45RA^+CD62L^+$ regulatory T cells (T_{regs}) less than $2{\cdot}88 \times 10^5$/kg. (b) The incidence of aGVHD increased in patients infused with an absolute number of $CD4^+CD25^{high}CD45RA$ T_{regs} less than $11{\cdot}26 \times 10^5$/kg. (c) Infusion with absolute number of naive T cells more than $41{\cdot}52 \times 10^6$/kg did not increase the incidence of aGVHD. (d) Infusion with absolute number of $CD4^+CD25^{high}$ T_{regs} less than $9{\cdot}20 \times 10^6$/kg did not increase the incidence of aGVHD.

ratio (HR) = 0·000, 95% confidence interval (CI) = 0·000–0·106, $P = 0{\cdot}013$]).

Kinetics of T cell reconstitution following HSCT

To investigate whether T cell subset reconstitution characteristics were influenced by transplantation method, we compared the kinetics of T cell reconstitution between haploidentical HSCT ($n = 9$) and sibling HSCT ($n = 11$). In total, seven patients (35%) suffered from aGVHD; of these, four received haploidentical HSCT (44·44%) and three received sibling identical HSCT (27·27%). Total counts of all T cell subsets declined after conditioning and were reduced significantly at day 30 post-HSCT both in haploidentical HSCT patients and sibling identical HSCT patients. Thereafter, these subsets expanded dramatically from days 30 to 60 in haploidentical HSCT patients. However, no differences were observed in any of the subsets between days 60 and 90 following haploidentical HSCT. There were no differences in the numbers of recovered $CD3^+$, $CD4^+$, $CD8^+$ or T_{regs} between patients receiving haploidentical HSCT and those receiving sibling identical HSCT (Fig. 4a–d). In contrast, total counts of naive T_{regs} and $CD62L^+$ naive T_{regs} in haploidentical HSCT patients were significantly lower compared to sibling identical HSCT patients (Fig. 4e–f). There were no differences in the frequency of total recovered T cells between haploidentical HSCT patients and sibling identical HSCT patients. The

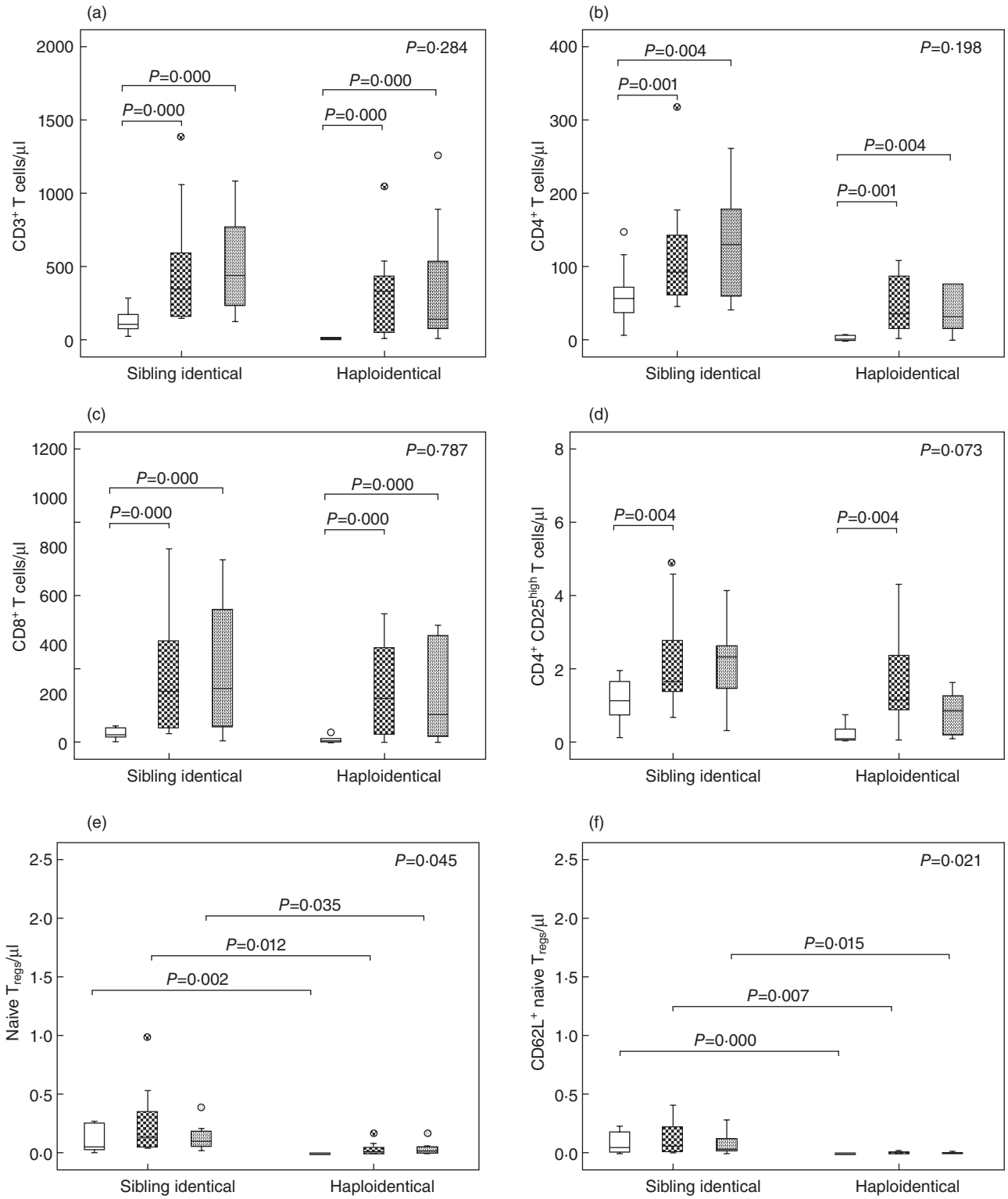

Fig. 4. Comparison of T cell subset reconstitution between sibling identical haematopoietic stem cell transplantation (HSCT) and haploidentical HSCT. (a) Total counts of CD3$^+$ cells. (b) Total counts of CD4$^+$ cells. (c) Total counts of CD8$^+$ cells. (d) Total counts of CD4$^+$CD25^{high} T cells. (e) Total counts of CD4$^+$CD25^{high}CD45RA T cells. (f) Total counts of CD4$^+$CD25^{high} CD45RA$^+$CD62L$^+$ T cells. (g) Frequencies of CD3$^+$ cells in total lymphocytes. (h) Frequencies of CD4$^+$ cells in total lymphocytes. (i) Frequencies of CD8$^+$ cells in total lymphocytes. (j) Frequencies of CD4$^+$CD25^{high} T cells in total lymphocytes. (k) Frequencies of CD4$^+$CD25^{high}CD45RA T cells in CD4$^+$CD25^{high} T cells. (l) Frequencies of CD4$^+$CD25^{high} CD45RA$^+$CD62L$^+$ T cells in CD4$^+$CD25^{high} T cells. +30d = white box, +60d = grid box, +90d = spotted box.

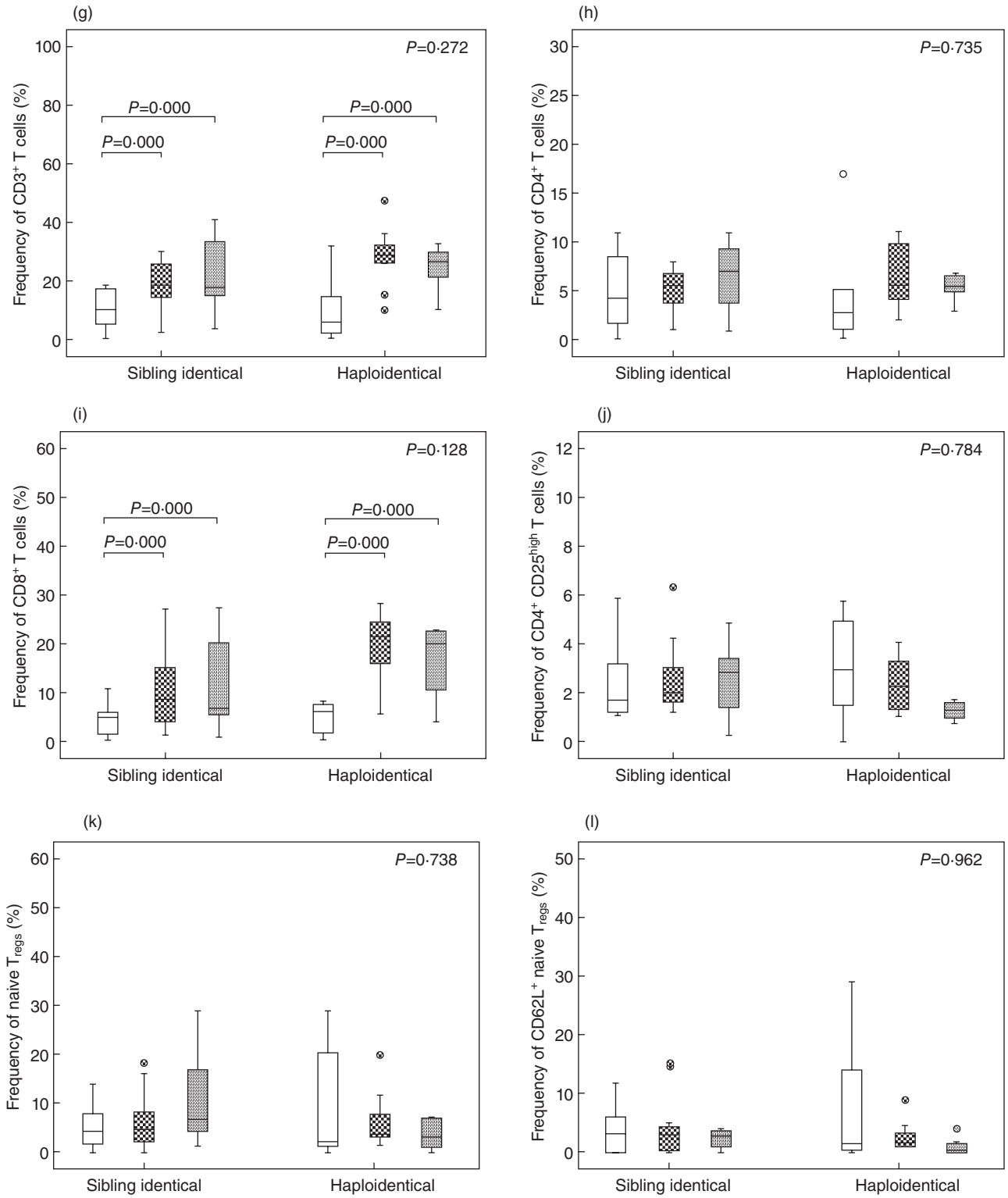

Fig. 4. *Continued*

frequency of T_{regs} and T_{reg} subsets increased from days 30 to 90 after HSCT in sibling identical HSCT patients; however, their frequency declined slightly in haploidentical HSCT patients from days 60 to 90 after HSCT (Fig. 4g–l).

Next, the same samples from the same 20 patients were used to compare T cell reconstitution between patients with aGVHD ($n = 7$) and those without aGVHD ($n = 13$). There were no differences in frequencies of all T cell subsets at

any intervals between patients with aGVHD and those without aGVHD, while total counts of all T cell subsets were significantly lower in aGVHD patients compared to non-aGVHD patients on day 30-post HSCT. However, total counts of CD3$^+$, CD4$^+$, CD8$^+$ and T_{regs} in aGVHD patients reached levels similar to non-aGVHD patients by day 60 post-HSCT. Despite this overall reconstitution, naive T_{regs} and CD62L$^+$ naive T_{regs} in aGVHD patients were significantly lower compared to non-aGVHD patients by day 60 post-HSCT. By 90 days after HSCT, total counts of all T cell subsets in aGVHD patients reached similar levels to non-aGVHD patients (Fig. 5a–f). These data indicate that aGVHD is associated with delayed reconstitution of naive T_{regs}.

Discussion

Here, we provide the first indication that CD62L$^+$ naive T_{regs} in allografts are a strong predictor of aGVHD. As the initial phase of T cell reconstitution depends primarily on the peripheral expansion of donor-derived T cells following myeloablative conditioning and allograft infusion, donor T_{regs} and T_{reg} subsets can influence aGVHD development [36]. Therefore, the transfer of a high percentage of CD62L$^+$ naive T_{regs} can promote reconstitution of naive T_{regs} and reduce the risk of aGVHD.

The T_{reg} compartment in adult peripheral blood is comprised of naive as well as memory cells [35,37]; in fact, CD45RA$^+$ naive T_{regs} account for approximately 42% of the total T_{reg} population. The selective expansion of naive T_{regs} *in vitro* and *in vivo* has demonstrated sustained T_{reg} expression of lymph node (LN) homing receptors [23,38]. As T_{regs} suppress alloreactivity in a cell contact-dependent manner, they require efficient entry to the priming sites of aGVHD. Therefore, it is not surprising that LN homing capacity plays an essential role in suppression of alloresponses in animal models of HSCT [8,24,25]. CD62L is an important T cell homing receptor that is crucial for T cell entry into LN. Ermann *et al.* demonstrated that CD62L$^+$ T_{regs} home more efficiently to secondary lymphoid organs compared to their CD62L$^-$ counterparts; moreover, CD62L$^-$ T cells are less responsive to alloantigen stimulation compared to CD62L$^+$ T cells [39]. Thus, CD62L$^+$ naive T_{regs} with a strong homing capacity respond rapidly after encountering alloantigen in a target organ. It has been demonstrated that T_{reg} entry into lymph nodes is crucial in inhibiting the initial expansion of donor-derived anti-host alloreactive T cells, which prevents aGVHD [40].

Previously, Chang *et al.* showed that the transfer of a large number of CD62L$^+$ naive T cells during HSCT increased the risk of II–IV aGVHD [31]. Although the number of patients was limited, our results are consistent with this finding in univariate analysis but not in multivariate analysis, which indicates that CD62L$^+$ naive T_{regs} may be a more sensitive object. We have also found naive T_{regs} to be a predictor using univariate analysis and the Kaplan–Meier method, but not in multivariate analysis. One plausible interpretation is that CD62L$^+$, which distinguishes central memory and recently activated T_{regs}, was not used and confounded the findings regarding the total CD45RA$^+$ naive T_{reg} population; thus, to ensure population purity, CD62L$^+$ should be used to identify T_{regs} that are capable of LN homing and are sensitive to alloresponses [25,41].

For conventional T cells, haploidentical immune reconstitution is comparable to sibling identical HSCT; however, differences have been observed in the reconstitution of T_{reg} subsets. The reconstitution of T_{reg} subsets following HSCT in our study is consistent with other reports reflecting an important role for lymphopenia-driven peripheral expansion of T_{regs} [42]. These data imply that peripheral tolerance is more important in the prevention of aGVHD than negative selection in the thymus early after HSCT [43,44]. CD4$^+$ lymphopenia selectively induces T_{reg} proliferation early after haploidentical HSCT, and the initial phase of T_{reg} reconstitution is promoted by lymphopenia-related signals and stimulation by alloantigen [36]. Early after HSCT, T_{regs} have a higher chance to encounter abundant recipient alloantigens; however, with the reconstitution of CD4$^+$ lymphocytes and other lymphocyte populations, the frequency of T_{reg} subsets decreases, thus explaining why T_{reg} frequency decreases after 30 days in patients with haploidentical HSCT. Other possibilities to explain the T_{reg} reconstitution difference between haploidentical HSCT and sibling identical HSCT include that ATG, which is not used in sibling identical HSCT, decreases the expression of the main functional molecule cytotoxic T lymphocyte antigen (CTLA)-4 and down-regulates the expansion of FoxP3$^+$ T_{regs} [45]. Additionally, high doses of CSA can cause a reduction in CD4$^+$CD25^{high} T_{regs} by decreasing interleukin (IL)-2 production [46–49]; further, MMF also inhibits T_{reg} proliferation [50]. In our study, the haploidentical HSCT patients were treated with higher doses of CSA and MMF for a longer period of time compared to sibling identical HSCT patients. Our data were similar to findings that the frequencies of FoxP3$^+$ T_{regs} and CD4$^+$CD25^{high} T_{regs} in patients undergoing HSCT were much lower compared to frequencies prior to HSCT (Fig. 1c). Further, the differences in immune reconstitution between aGVHD patients and non-aGVHD patients indicate strongly that the development of aGVHD is correlated highly with the delayed reconstitution of naive T_{regs}.

In summary, our data suggest that naive CD62L$^+$ T_{reg} content in allografts can predict the risk of aGVHD, and that impaired naive T_{reg} reconstitution probably induces aGVHD. This study demonstrates a clinical association between aGVHD and CD62L$^+$ naive T_{regs}. However, further studies will be required to investigate whether functional alteration of T_{reg} subsets exists after allo-HSCT. In particular, longitudinal follow-up studies of post-transplantation

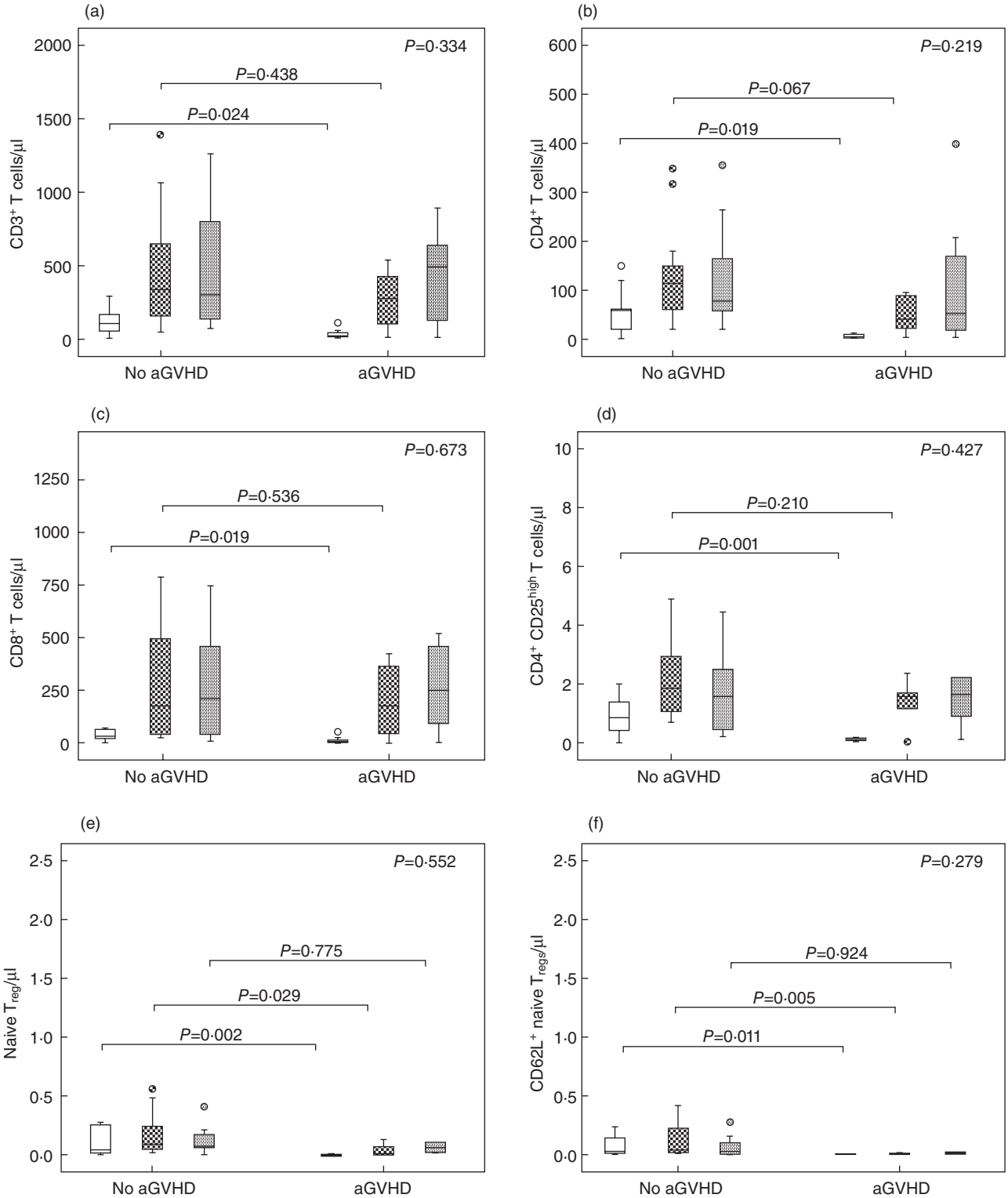

Fig. 5. Comparison of T cell subset reconstitution between patients with and without acute graft-*versus*-host disease (aGVHD). (a) Total counts of $CD3^+$ cells. (b) Total counts of $CD4^+$ cells. (c) Total counts of $CD8^+$ cells. (d) Total counts of $CD4^+CD25^{high}$ T cells. (e) Total counts of $CD4^+CD25^{high}CD45RA$ T cells. (f) Total counts of $CD4^+CD25^{high}$ $CD45RA^+CD62L^+$ T cells. (g) Frequencies of $CD3^+$ cells in total lymphocytes. (h) Frequencies of $CD4^+$ cells in total lymphocytes. (i) Frequencies of $CD8^+$ cells in total lymphocytes. (j) Frequencies of $CD4^+CD25^{high}$ T cells in total lymphocytes. (k) Frequencies of $CD4^+CD25^{high}CD45RA$ T cells in $CD4^+CD25^{high}$ T cells. (l) Frequencies of $CD4^+CD25^{high}$ $CD45RA^+CD62L^+$ T cells in $CD4^+CD25^{high}$ T cells. +30d = white box, +60d = grid box,+90d = spotted box.

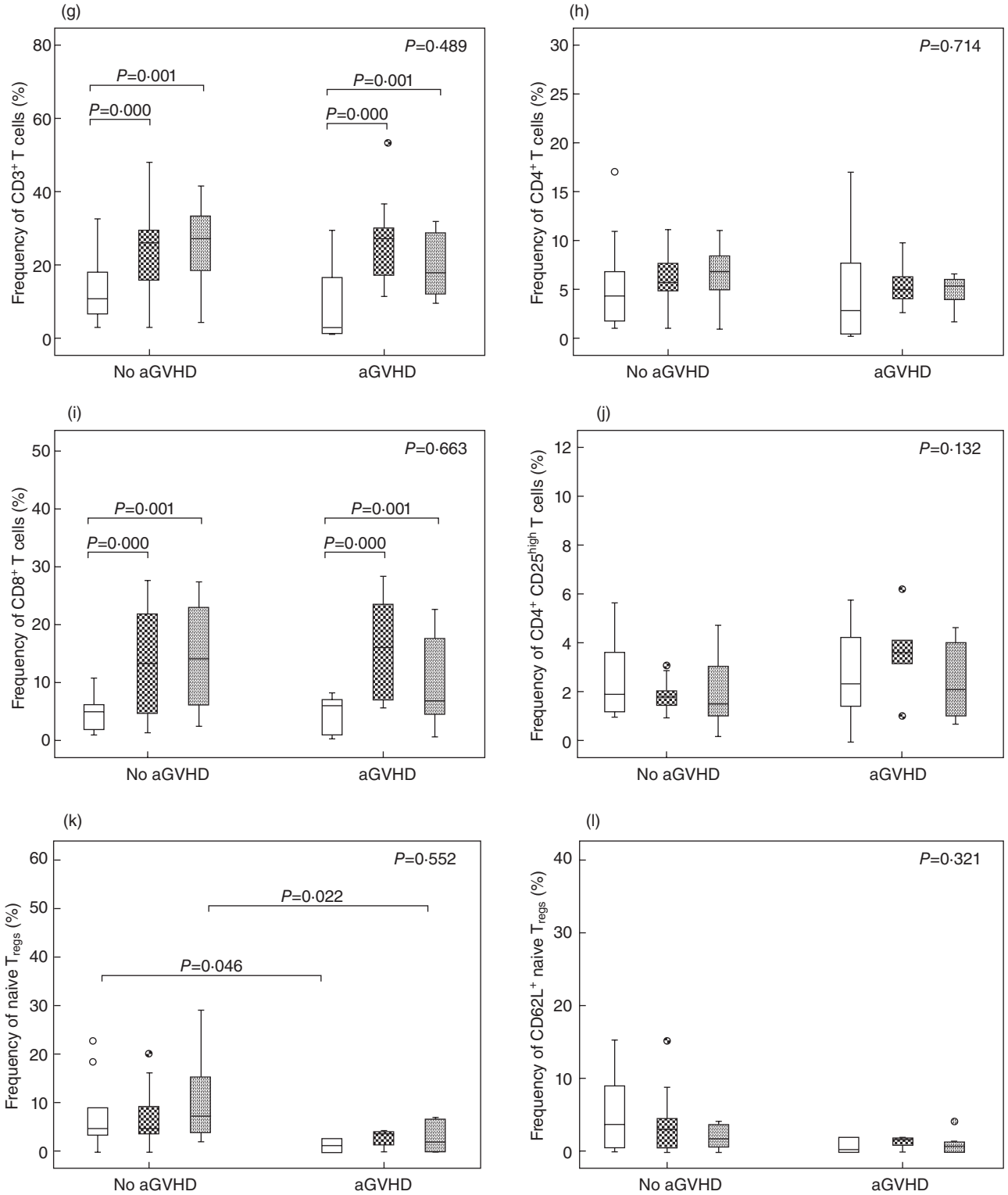

Fig. 5. *Continued*

patients will be required to establish whether the infusion of large numbers of CD62L+ naive T_{reg} correlates with the prevention of cGVHD or with an increased incidence of relapse.

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Disclosure

All the authors declare no competing financial interests.

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