Imbalance of effector and regulatory CD4 T cells is associated with graft-versus-host disease after hematopoietic stem cell transplantation using a reduced intensity conditioning regimen and alemtuzumab

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ABSTRACT

Background

A variety of immune pathways can lead to graft-versus-host disease. A better understanding of the type of immune response causing graft-versus-host disease in defined clinical hematopoietic stem cell transplant settings is required to inform development of methods for monitoring patients and providing them tailored care.

Design and Methods

Twenty-five patients were recruited presenting with myeloid malignancies and treated with a reduced intensity conditioning transplant regimen with graft-versus-host disease prophylaxis comprising *in vivo* lymphocyte depletion with alemtuzumab and cyclosporin. A prospective study was performed of lymphocyte subset reconstitution in peripheral blood in relation to the incidence of graft-versus-host disease.

Results

Acute graft-versus-host disease was associated with significantly higher numbers of natural killer cells and donor-derived effector CD4 T cells ($CD45RO^{+}$ $CD27^{-}$) early (day 30) after transplantation (p=0.04 and p=0.02, respectively). This association was evident before the emergence of clinical pathology in six out of seven patients. Although numbers of regulatory CD4 T cells ($CD25^{high}$ $Foxp3^{+}$) were similar at day 30 in all patients, a significant deficit in those who developed acute graft-versus-host disease was apparent relative to effector CD4 T cells (median of 41 effectors per regulatory cell compared to 12 to 1 for patients without graft-versus-host disease) (p=0.03). By day 180, a functional regulatory CD4 T-cell population had expanded significantly in patients who developed chronic graft-versus-host disease, reversing the imbalance (median of 3 effectors per regulatory cell compared to 9.6 to 1 for patients without graft-versus-host disease) (p=0.018) suggesting no overt absence of immune regulation in the late onset form of the disease.

Conclusions

Imbalance of effector and regulatory CD4 T cells is a signature of graft-versus-host disease in this transplantation protocol.

Key words: graft-versus-host disease, CD4 T cell, alemtuzumab.

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Introduction

Graft-versus-host disease (GvHD) can be a life-threatening complication of allogeneic hematopoietic stem cell transplantation (HSCT). Both the incidence and severity of GvHD are reduced by lymphocyte-depletion strategies, such as use of the anti-CD52 cytolytic monoclonal antibody called alemtuzumab (Campath 1H), and pharmacological immunosuppression. These non-specific approaches, however, leave patients vulnerable to infection and at increased risk of leukemia relapse due to loss of a beneficial graft-versus-leukemia (GvL) immune response. The problem of disease relapse is especially pertinent for patients treated with reduced intensity conditioning regimens that utilize lower doses of chemotherapy or irradiation and rely, to varying extents, on the GvL response to eliminate disease.[1] Treatment regimens ideally should be based on prudent use of immunosuppression sufficient to prevent GvHD without excessive compromise of the GvL response. At present, the diagnosis of GvHD is mainly based on clinical features. The disease is, therefore, recognized only after emergence of pathology and treatment is reactionary. A better understanding of the immunological mechanisms involved in GvHD is required for the development of predictive diagnostic methods. This would allow segregation of patients according to risk and judicious tailoring of immunosuppression.

GvHD is caused by alloreactive donor lymphocytes that attack patient's cells due to recognition of foreign minor histocompatibility antigens or major histocompatibility antigens in the case of HLA-mismatched transplants.[2] The disease has distinct acute and chronic forms. The physiology of late-onset, chronic GvHD is distinct from that of the early, acute form of disease, the cause of the pathology is poorly understood and the disease does not respond well to conventional therapies.[3]

A variety of immune effector pathways can lead to GvHD pathology. The αβ T cells are principle players, and depletion of either CD4 or CD8 T cells can reduce clinical GvHD[4,5] but few studies have examined the role of T-cell subsets. Chronic GvHD has been associated with an increase in CD4 effector memory,[6] increase in CD8 central memory and decrease in CD4 central memory cells.[7] In acute GvHD, a correlation was found with prevalence of naïve and central memory CD4 T cells in the donor allograft.[8] Naïve human T cells appear more alloreactive than memory T cells *in vitro*[9] but pathogen-specific memory T cells that cross-react with alloantigens have been implicated in the development of GvHD.[10-12] Natural killer (NK) cells also exhibit alloreactivity, but clinical studies indicate an ambiguous role in GvHD. Some reports describe an association of NK cell allo-disparities with an increased incidence of GvHD[13,14] but others found no impact[15,16] and some studies have shown rapid NK recovery can have a beneficial effect on GvHD.[17,18] Involvement of B cells is indicated by a correlation between chronic GvHD and increased levels of antibodies specific for minor histocompatibility antigens[19] together with the demonstration that some patients respond to B-cell depletion therapy.[20]

Failure of regulatory mechanisms may also play a role in GvHD. Studies using mouse models have shown regulatory CD4 T cells can exert a beneficial immune suppressive effect in GvHD.[21-24] Results of clinical studies to assess the role of regulatory T cells in GvHD are less clear. In some studies a deficit of these cells was found in GvHD,[25-27] in other studies no difference was recorded between patients with or without disease[28,29] and yet other studies indicated that regulatory T cells may be increased in patients with chronic GvHD.[30,31]

The redundancy of mechanisms potentially involved in GvHD hinders development of new diagnostic methods and therapies. The type of immune response likely reflects the immune status of the patient and donor, the transplant preparative regimen and underlying diseases. Consequently there is a need to understand the mechanistic basis of GvHD that develops for individual treatment protocols. We performed a longitudinal study of lymphocyte subset reconstitution after allogeneic HSCT in 25 patients, all presenting with myeloid disease, who received peripheral blood stem cell transplants after a uniform reduced-intensity conditioning regimen comprising the chemotoxic agents fludarabine and busulphan.[32] GvHD prophylaxis was based on *in vivo* immune depletion with alemtuzumab together with cyclosporin. After this preparative treatment the incidence of acute GvHD was 36% and that of chronic GvHD was 22%.[33] The dynamics of naïve, memory and effector T cells, regulatory CD4 T cells, NK and B-cell recovery were correlated with GvHD incidence to indicate the key players driving the disease. The information gained provides the essential basis for identifying patients at risk of GvHD and improving disease control by selecting treatments appropriate to the type of immune response involved.

Design and Methods

Patients and transplant regimen

A prospective study was performed of 25 patients who underwent allogeneic HSCT for myeloid malignancies between September 2005 and September 2006 at King's College Hospital. The transplant preparative regimen consisted of fludarabine (30 mg/m^2 daily, administered intravenously from day -9 to day -5), busulphan (3.2 mg/kg body weight, administered intravenously in four divided doses from day -3 to day -2), and alemtuzumab (20 mg/day intravenously on days -8 to day -4). Unselected allogeneic peripheral blood stem cells were infused on day 0. Intravenous cyclosporin was started from day -1 as GvHD prophylaxis at a dose adjusted to achieve plasma trough levels of 150-200 ng/L for all patients. Oral cyclosporin was substituted when a good oral intake was achieved and rapidly tapered to discontinuation from day 60 in the absence of GvHD. Acute and chronic GvHD were graded using standard criteria.[34,35] Recombinant granulocyte colony-stimulating factor (G-CSF) was administered subcutaneously or intravenously from day +7 until neutrophil engraftment. The patients' characteristics are shown in Table 1. Clinical data were censored at May 2007. Peripheral

blood samples were collected immediately prior to conditioning for the transplant and at days 30, 60, 90, 180, 270 and 360 after transplantation. Samples of peripheral blood were also collected from 11 healthy age-matched individuals (median age 51 years; range, 41-56 years). King's College Hospital Research Ethics Committee approved the use of the patients' samples and The Royal Free Hospital Research Ethics Committee approved the use of the samples from healthy volunteers. Written informed consent was obtained from all participants.

Immunophenotypic analysis

Lymphocyte subsets were enumerated in whole peripheral blood using fluorochrome-labeled monoclonal antibodies to CD4 (clone SK3), CD8 (SK1), CD25 (2A3), CD27 (M-T271), CD45RO (UCHL1), CD56 (B159), (BD Biosciences) and CD3 (OKT3), CD19 (HIB19), CD31 (WM59), CD45RA (HI100), CD62L (Dreg 56), FoxP3 (PCH101), and rat IgG2a isotype control (eBR2a) (eBioscience). Cells in 200 μL peripheral blood were stained for surface markers and erythrocytes were removed using FACS lysing solution (BD Biosciences). Intracellular Foxp3 staining was performed after permeabilization (BD Biosciences Cytofix/Cytoperm solution) according to the manufacturer's instructions. Eight-color analysis was performed by flow cytometry using a BD FACSCanto II (BD Biosciences) and results analyzed with FlowJo software (TreeStar). NK cells were defined as $CD3^-$ $CD56^+$. B cells were defined as $CD19^+$. $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T-cell subsets were defined as $CD45RO^-$ $CD27^+$ naïve, $CD45RO^+$ $CD27^+$ $CD62L^+$ central memory, $CD45RO^+$ $CD27^+$ $CD62L^-$ effector memory, $CD45RO^+$ $CD27^-$ effectors and $CD45RO^-$ $CD27^-$ terminal effectors. CD4 regulatory T cells were defined as $CD4^+$ $CD25^{high}$, $Foxp3^+$. CD4 T-cell recent thymic emigrants were defined as $CD4^+$ $CD45RA^+$ $CD31^+$ $CD62L^+$. Cell subset numbers were calculated from percentage values based on an absolute lymphocyte count of the blood sample obtained using an automated leukocyte counter.

Chimerism

Peripheral blood mononuclear cells were purified by density gradient centrifugation on Lympholyte-H (Cedarlane Laboratories) and CD4 T-cell subsets isolated using a FACSAria sorter after surface staining with CD3, CD4, CD45RO and CD27 antibodies. Purity of the populations was >95%. Cells were lysed with proteinase K (0.2 mg/mL in 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 1% Tween-20). Donor and recipient composition was determined by polymerase chain reaction amplification of informative alleles from 15 polymorphic short tandem repeat loci and the sex-determining amelogenin loci (Powerplex®; Promega Corp, Madison, WI, USA). Products were separated by capillary electrophoresis using an ABI 3130XL DNA sequencer and results analyzed using Genemapper 4.0 software (Applied Biosystems). Quantification was based on area under the peaks. The sensitivity of this methodology was previously shown to be 5% by cell dilution studies.[36]

T-cell function

Suppressive activity of CD4 $CD25^+$ regulatory T cells from patients was measured by their ability to inhibit proliferation of CD4 $CD25^-$ T cells from a healthy volunteer. Cell subsets were purified by immunomagnetic cell sorting using a regulatory T-cell isolation kit and an AutoMACS separator (Miltenyi Biotec). CD4 $CD25^-$ cells (5,000) were cultured in RPMI 1640 medium containing 10% human AB serum and stimulated with anti-CD3/CD28 antibody coated beads at a ratio of one bead to five cells (Dynabeads human Treg expander, Invitrogen) in the presence or absence of CD4 $CD25^+$ cells (5,000). After 5 days, 1 μCi of [^{3}H]-labeled thymidine was added and cells harvested after 16 h. Thymidine incorporation was measured using a liquid scintillation counter. Percentage suppression was calculated using the equation 100-[(counts per minute $CD25^-$ plus $CD25^{high}$ co-culture/counts per minute $CD25^-$ alone) x 100].

Statistical methods

Statistical analyses were performed using SPSS 14.0 for Windows software (SPSS Inc.). Non-parametric statistical comparisons were performed using the Mann-Whitney U test. Differences were considered statistically significant when p values were less than 0.05.

Table 1. Patients' characteristics.

	No GvHD (n=16)	GvHD (n=9)
Patient		
Age, median years (range)	52 (34-70)	58 (46-67)
Sex (male/female)	8/8	7/2
Donor		
Unrelated HLA matched[1]	6	6
Unrelated HLA mismatched[2]	5	1
Sibling HLA matched	5	2
Sex mismatched	8	4
Female donor/male patient	2	2
Diagnosis		
Myelodysplastic syndrome	6	5
Acute myeloid leukemia	7	3
Myeloproliferative disorders (MDS/MPD)	2	1
Chronic myeloid leukemia	1	0
Disease stage		
Early	9	2
Advanced	7	7
Disease status at transplantation		
Complete remission	16	9
Stem cell source		
Peripheral blood stem cells	16	9
Acute GvHD (grade II-IV)	0	5
Chronic GvHD	0	1
Acute and chronic GvHD	0	3
Full donor $CD3^+$ chimerism by day 90	3	8
Mixed $CD3^+$ chimerism >day 90	13	1
CMV reactivation after transplantation	10	5
Disease relapse	5	3

[1]HLA match indicates identity at ten alleles (HLA-DP type not determined)
[2]HLA mismatched transplants were mismatched at HLA-A (n=1) at HLA-C (n=1), at HLA-A&C (n=1), at DRB1 (n=1) and at DQB1 (n=2). CMV: cytomegalovirus.

Results

The residual T-cell population after lymphocyte depletion with alemtuzumab is dominated by effector and memory subsets

Alemtuzumab induced profound depletion of all lymphocyte subsets. Serial analysis of peripheral blood to monitor immune reconstitution after treatment showed that NK cells were the main lymphocyte population at day 30, accounting for a median of 24% compared to 12% in healthy age-matched volunteers (*data not shown*). The numbers of NK cells reached the normal range at 90 days whereas B-cell recovery was slowest commencing day 90 (*data not shown*). Figure 1A shows that the number of CD8 T cells recovered rapidly, reaching the normal range by 9 months, whereas the numbers of CD4 T cells were still below the normal range at 12 months. T-cell subset composition was investigated by analyzing expression of CD45RO and the co-stimulatory molecule CD27, which is lost during antigen-induced differentiation.[37,38] The composition of both CD4 and CD8 T-cell populations was skewed toward effectors with a deficit of naïve cells relative to the composition in age-matched healthy volunteers (Figure 1B). Recovery of naïve T cells in patients was not detectable until 6 months and the majority of naïve CD4 T cells expressed CD31 (range, 75-98%) (Figure 1C), which is a marker of recent thymic origin[39] indicating that they arose from renewed thymopoiesis.

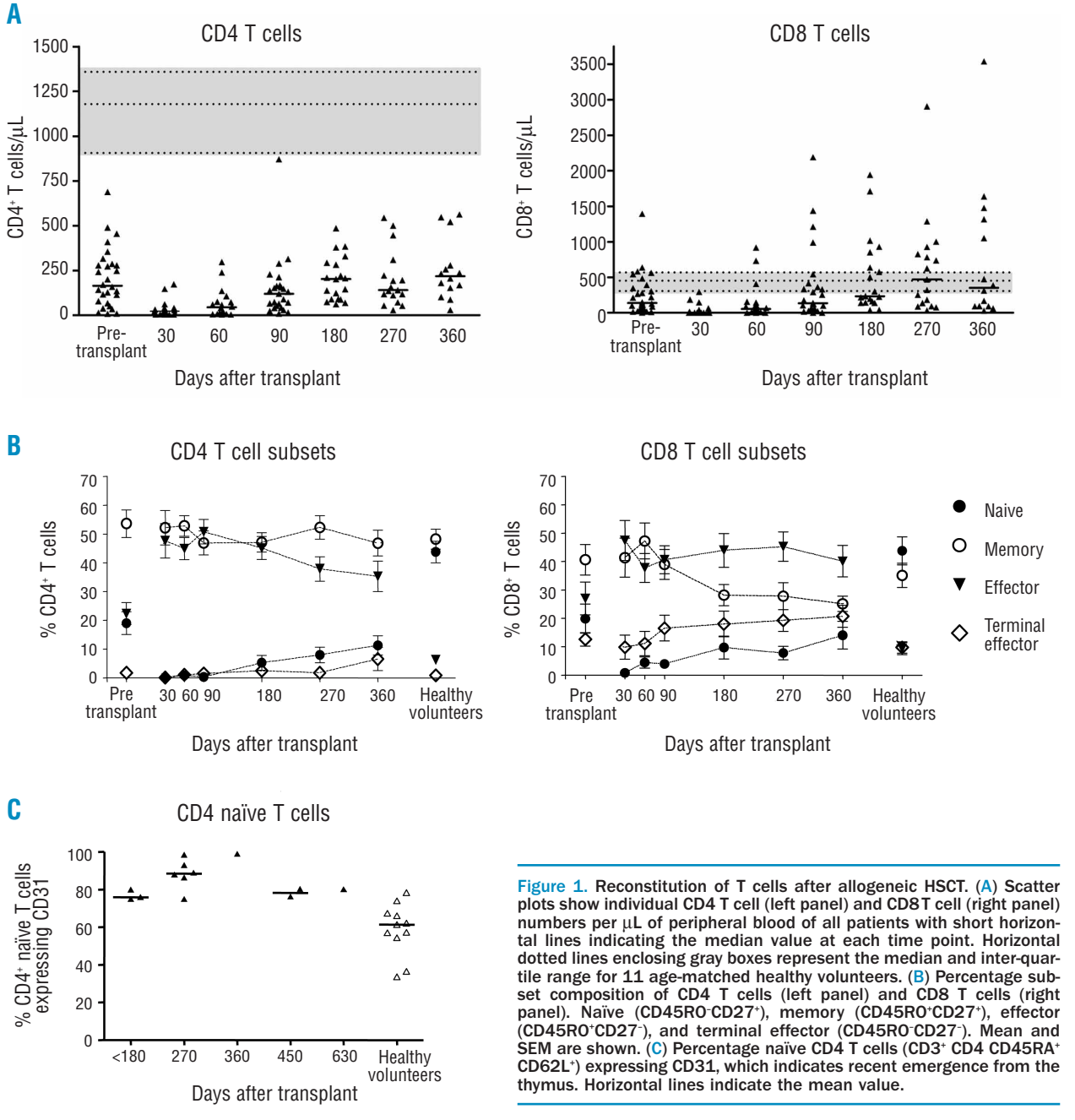

Figure 1. Reconstitution of T cells after allogeneic HSCT. (A) Scatter plots show individual CD4 T cell (left panel) and CD8T cell (right panel) numbers per µL of peripheral blood of all patients with short horizontal lines indicating the median value at each time point. Horizontal dotted lines enclosing gray boxes represent the median and inter-quartile range for 11 age-matched healthy volunteers. (B) Percentage subset composition of CD4 T cells (left panel) and CD8 T cells (right panel). Naïve ($CD45RO^-CD27^+$), memory ($CD45RO^+CD27^+$), effector ($CD45RO^+CD27^-$), and terminal effector ($CD45RO^-CD27^-$). Mean and SEM are shown. (C) Percentage naïve CD4 T cells ($CD3^+$ CD4 $CD45RA^+$ $CD62L^+$) expressing CD31, which indicates recent emergence from the thymus. Horizontal lines indicate the mean value.

Higher numbers of natural killer cells and donor-derived effector CD4 T cells at day 30 after transplantation correlate with acute graft-versus-host disease

The incidence of acute GvHD in the cohort was 28% (n=7) and that of chronic GvHD was 16% (n=4). All cases of GvHD developed prior to administration of donor lymphocytes to treat falling donor chimerism or disease relapse. The characteristics of the individuals who developed and those who did not develop GvHD were equivalent (Table 1). Comparison of lymphocyte subset recovery after transplantation with development of GvHD showed a significant correlation between acute GvHD and higher numbers of NK cells (p=0.04) and CD4 T cells (p=0.02) at day 30 (Table 2). The larger CD4 T population in patients who developed acute GvHD was mainly due to higher numbers of cells with effector (CD45RO$^+$ CD27$^-$ CD62L$^-$) (p=0.02) and effector memory (CD45RO$^+$ CD27$^+$ CD62L$^-$) (p=0.03) phenotypes (Table 2).

This finding indicates that effector CD4 T cells may be key players in GvHD, in which case they are likely to be of donor origin. The transplant preparative regimen used frequently produces a period of prolonged mixed T-cell chimerism after transplantation.[36] The origin of effector CD4 T cells present at day 30 in patients who developed acute GvHD was, therefore, determined by isolating the population and evaluating the percentage composition of donor and patient cells by genetic profiling of pertinent polymorphic loci. Effector CD4 T cells (CD45RO$^+$ CD27$^-$) from four patients who developed acute GvHD were predominantly of donor origin (86% for patient 2, 100% for patient 14, 50% for patient 25, 60% for patient 28) whereas the donor component of the memory CD4 T-cell population (CD45RO$^+$ CD27$^+$) was lower (84% for patient 2, 63% for patient 14, 28% for patient 25, 10% for patient 28). Attempts to determine the donor composition of the CD4 T-cell subset at day 30 for patients who did not develop GvHD were not successful because of insufficient numbers of cells. Analysis, at later time points, of four patients who did not develop GvHD showed that they had lower percentages of donor-derived cells and this was more pronounced in the effector than the memory CD4 T-cell population, contrasting with the findings in patients who did develop GvHD (donor-derived cells accounted for 50% of the memory and 38% of the effector populations at day 60 in patient 4, for 10% of the memory and 0% of the effector populations at day 60 in patient 7, for 16% and 5% at day 60 in patient 11 and for 55% and 60% at day 90 in patient 15). These results provide support for donor-derived effector CD4 T cells being key players driving GvHD in this cohort of patients.

The median onset of acute GvHD symptoms was 63 days (range, 12-120). Given this variation in time of disease onset, representative examples of NK and T cell population dynamics during GvHD progression are shown individually for three patients (Figure 2). The association of high NK cell numbers with GvHD was only seen at the early, day 30 time-point. Thereafter, the NK cell population size remained relatively static in patients who developed GvHD while continued expansion was seen in patients without GvHD (Figure 2A). The initial expansion of the effector CD4 T cell population at day 30 continued in all patients who developed GvHD suggesting that these cells are the key players mediating pathology in this cohort of patients (Figure 2B upper panel). In patients 12 and 14, the numbers of effector CD4 T cells overshot the normal range in the healthy age-matched volunteers, but patient 25 illustrates that the expansion can be more modest. There was no consistent increase in effector CD8 T cell numbers in patients with GvHD (Figure 2B lower panel). A large increase in the number of effector CD8 T cells was observed in patient 12, perhaps associated with a concurrent episode of cytomegalovirus reactivation, but numbers for patients 14 and 25 were below the average for patients who did not develop GvHD. No correlation between B-cell reconstitution and GvHD was seen at any time point (*data not shown*).

The frequency of regulatory CD4 T cells relative to effector CD4 T cells is reduced at day 30 in patients who develop acute graft-versus-host disease

Regulatory CD4 T cells suppress the activity of effector T cells and, consequently, could have a beneficial role in the control of GvHD. Enumeration of CD4 CD25^{high} Foxp3$^+$ T cells showed that the numbers of these putative regulatory cells at the early, day 30 time-point were similar in patients who did and did not develop acute GvHD (Figure 3A left panel). However, a significant deficit in the group that developed acute GvHD was apparent when numbers were considered relative to effector CD4 T cells at day 30. The median ratio of effector CD4 T cells per regulatory CD4 T cell in the acute GvHD group was 41 to 1 compared to 12 to 1 for patients without GvHD (p=0.03) (Figure 3A right panel).

The regulatory CD4 T-cell population expands in patients with chronic graft-versus-host disease

At later time points, the CD4 CD25^{high} Foxp3$^+$ T-cell population gradually increased in all patients with no difference in numbers (Figure 3B) or percentage composition (*data not shown*) between patients with and without GvHD up to and including day 90. At day 90, the difference in the

Table 2. Univariate analysis of lymphocyte subset numbers at day 30 after transplantation and GvHD.

Cell type	Acute GvHD (n=7)	No GvHD (n=12)	p value
Lymphocytes	290 (180-810)	135 (60-620)	0.04
CD4 T cells	36 (13-170)	8 (1-150)	0.02
CD4 memory T cells	11 (7-89)	4 (1-110)	0.05
CD4 central memory T cells	4 (1-14)	1 (1-47)	0.15
CD4 effector memory T cells	5 (4-75)	2 (0.19-60)	0.03
CD4 effector T cells	25 (6-84)	5 (0-40)	0.02
CD8 T cells	3 (0.67-293)	2 (0.26-147)	0.34
NK cells	115 (55-186)	44 (5-219)	0.04
B cells	0.6 (0-30)	0.9 (0-118)	0.96

Median cell number/μL blood (range). Bold values indicate p<0.05. No significant differences detected for CD8 T-cell subsets (data not shown).

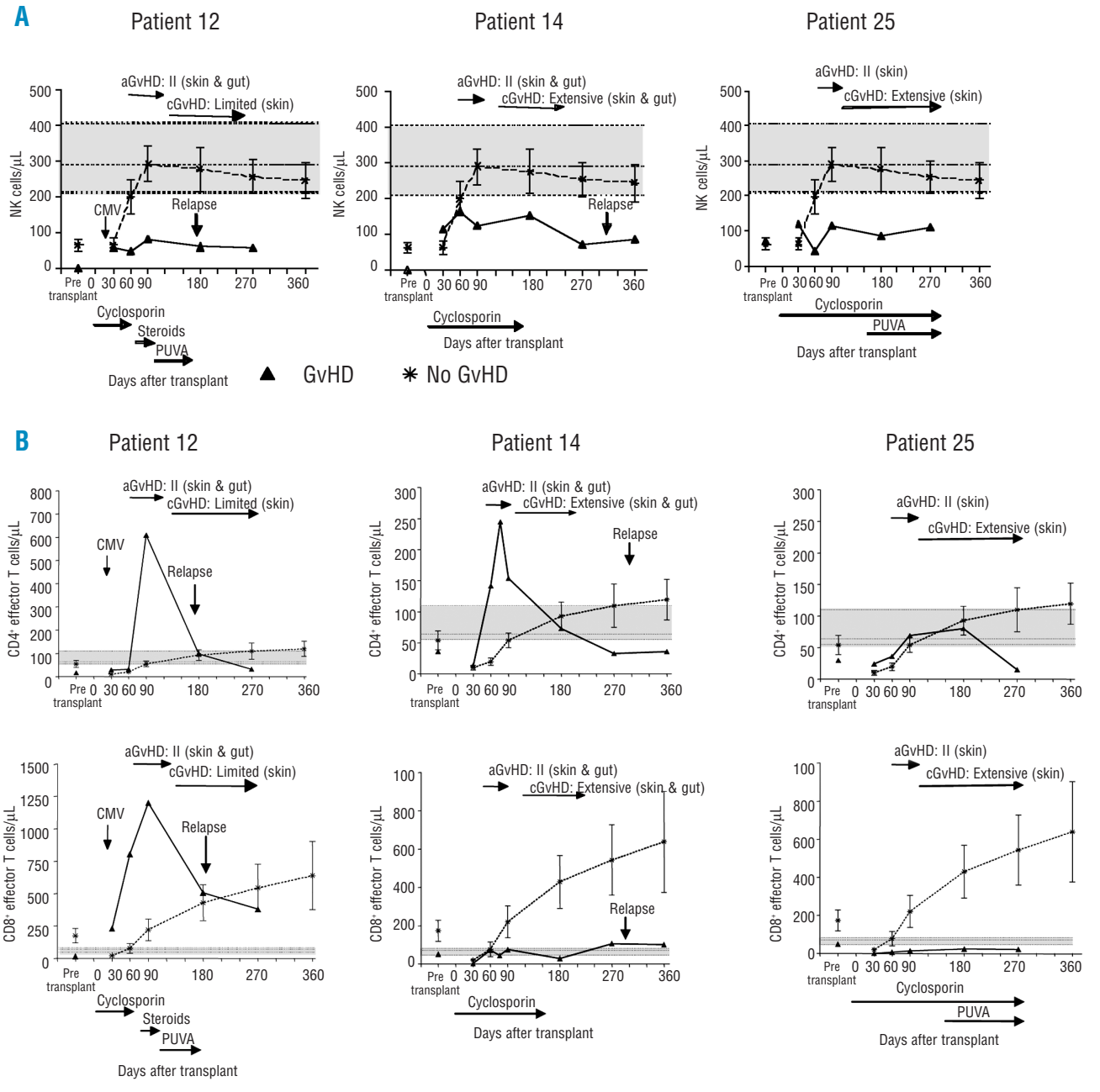

Figure 2. A-B Reconstitution of NK and effector T cells after allogeneic HSCT in three patients who developed GvHD. (A) NK cell dynamics. (B) Effector T-cell (CD45RO$^+$ CD27$^-$ and CD45RO$^-$ CD27$^-$) dynamics with CD4 and CD8 populations in the upper and lower panels, respectively. Mean and SEM are shown for patients who did not develop GvHD. Horizontal dotted lines enclosing gray boxes represent the median and inter-quartile range for 11 age-matched healthy volunteers. Patient 12: female with an HLA-matched unrelated male donor, who received donor lymphocyte infusions beginning day 228. Patient 14: female with an HLA-matched sibling male donor, who received donor lymphocyte infusions beginning day 321. Patient 25: male with an HLA-matched unrelated male donor. GvHD therapy regimens are indicated for each patient (aGvHD is acute GvHD, cGvHD is chronic GvHD, PUVA is psoralen ultra violet A).

median ratio of effector CD4 T cells to CD4 CD25^{high} Foxp3$^+$ T cells in the patients with or without GvHD had narrowed to 15 to 1, and 13.5 to 1 respectively (*data not shown*). Beyond day 100, there was a striking increase in the CD4 CD25^{high} Foxp3$^+$ T-cell numbers in four patients who developed chronic GvHD (indicated by large white triangles in Figure 3B). These cells constituted a higher percentage of the CD4 T-cell population (range, 13-31%) at day 180 compared to the median of 5% in healthy age-matched volunteers and 4.03% among patients who did not develop GvHD. The number and percentage composition of CD4 CD25^{high} Foxp3$^+$ cells at day 180 and beyond was not significantly different in patients without GvHD and in the five patients who had acute but not chronic GvHD (*data not shown*).

There are no unique markers for regulatory CD4 T cells

and expression of intracellular Foxp3 by activated human CD4 T cells[40-42] potentially confounds enumeration of regulatory T cells in inflammatory diseases such as GvHD. To substantiate our interpretation of regulatory CD4 T-cell recovery, CD27 was analyzed as an additional marker in some samples. CD27 is constitutively expressed on functional regulatory T cells[43,44] but is lost on effector T cells.[37,38] At day 30, the median ratio of effector CD4 T cells per CD4 CD25^{high} Foxp3^{+} CD27^{+} T cell in five patients who subsequently developed acute GvHD was 58 to 1 whereas it was 6.8 to 1 (p=0.03) for three patients who did not develop GvHD, providing additional support that regulatory CD4 T cells are deficient relative to effector CD4 T cells early after transplantation in patients who develop acute GvHD. Analysis of CD4 CD25^{high} Foxp3^{+} CD27^{+} T-cell dynamics in patients 12, 14 and 25 (Figure 3C) revealed that expansion of the regulatory CD4 T-cell population during chronic GvHD comprises a delayed wave following the spike in effector CD4 T-cell numbers. We verified that regulatory CD4 T-cell numbers were increased in patients with chronic GvHD by analysis of samples from an additional six patients with disease at day 180 (Figure 3D). Effector CD4 T-cell numbers were similar in all patients but significantly more regulatory CD4 T cells were present in the ten patients with chronic GvHD (p=0.008). Consequently the median ratio of effector per regulatory CD4 T cell in the chronic GvHD group was 3 to 1 compared to 9.6 to 1 for patients without GvHD (p=0.018).

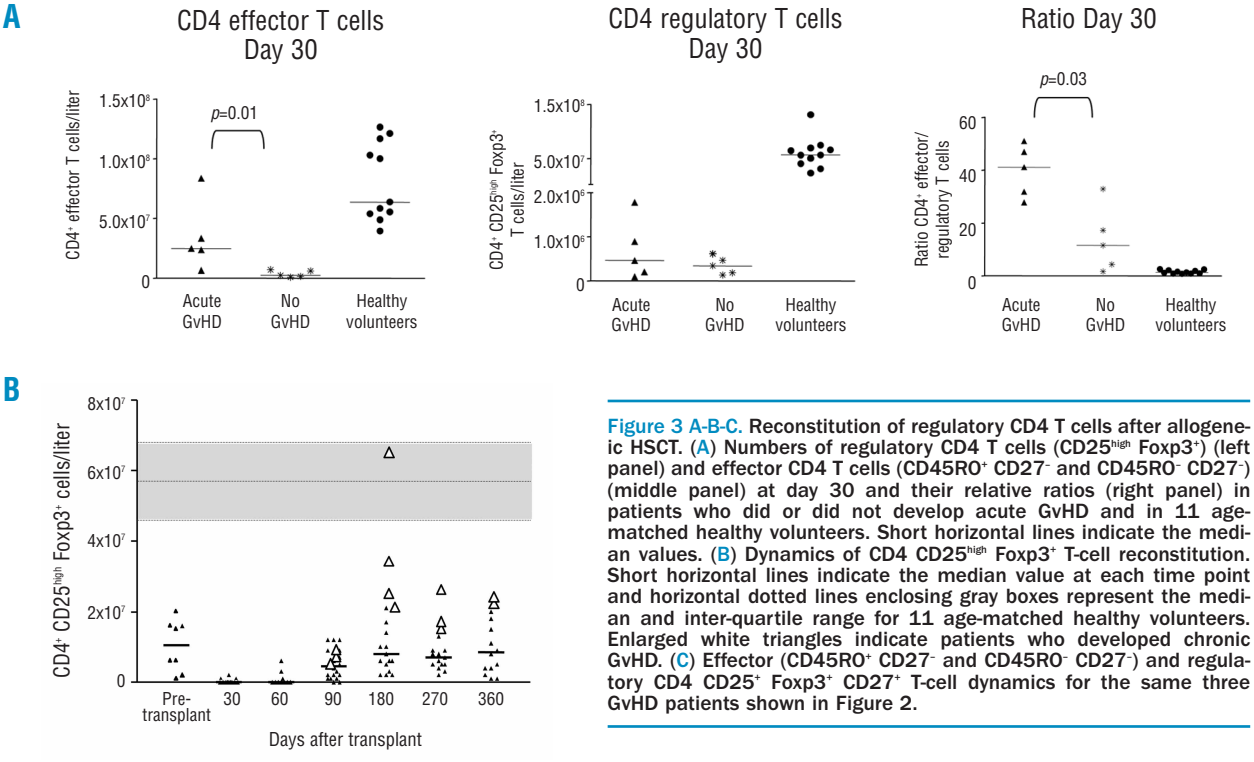

Figure 3 A-B-C. Reconstitution of regulatory CD4 T cells after allogeneic HSCT. (A) Numbers of regulatory CD4 T cells (CD25^{high} Foxp3^{+}) (left panel) and effector CD4 T cells (CD45RO^{+} CD27^{-} and CD45RO^{-} CD27^{-}) (middle panel) at day 30 and their relative ratios (right panel) in patients who did or did not develop acute GvHD and in 11 age-matched healthy volunteers. Short horizontal lines indicate the median values. (B) Dynamics of CD4 CD25^{high} Foxp3^{+} T-cell reconstitution. Short horizontal lines indicate the median value at each time point and horizontal dotted lines enclosing gray boxes represent the median and inter-quartile range for 11 age-matched healthy volunteers. Enlarged white triangles indicate patients who developed chronic GvHD. (C) Effector (CD45RO^{+} CD27^{-} and CD45RO^{-} CD27^{-}) and regulatory CD4 CD25^{+} Foxp3^{+} CD27^{+} T-cell dynamics for the same three GvHD patients shown in Figure 2.

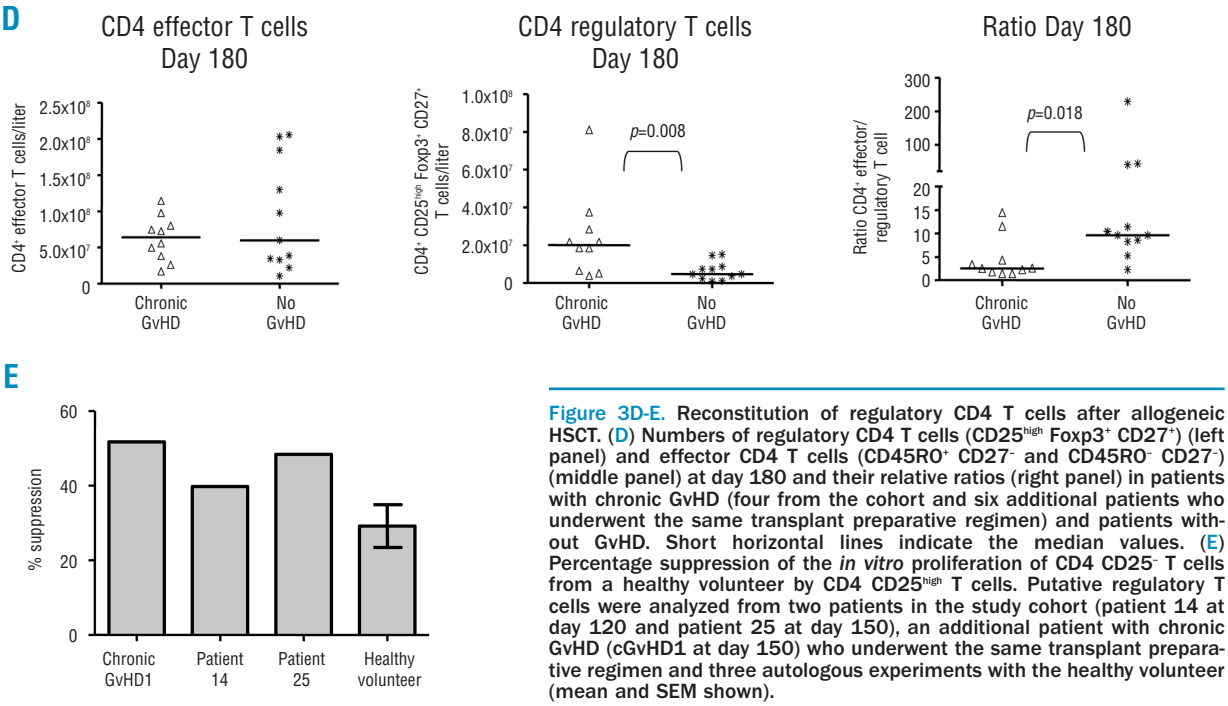

Figure 3D-E. Reconstitution of regulatory CD4 T cells after allogeneic HSCT. (D) Numbers of regulatory CD4 T cells ($CD25^{high}$ $Foxp3^{+}$ $CD27^{+}$) (left panel) and effector CD4 T cells ($CD45RO^{+}$ $CD27^{-}$ and $CD45RO^{-}$ $CD27^{-}$) (middle panel) at day 180 and their relative ratios (right panel) in patients with chronic GvHD (four from the cohort and six additional patients who underwent the same transplant preparative regimen) and patients without GvHD. Short horizontal lines indicate the median values. (E) Percentage suppression of the *in vitro* proliferation of CD4 $CD25^{-}$ T cells from a healthy volunteer by CD4 $CD25^{high}$ T cells. Putative regulatory T cells were analyzed from two patients in the study cohort (patient 14 at day 120 and patient 25 at day 150), an additional patient with chronic GvHD (cGvHD1 at day 150) who underwent the same transplant preparative regimen and three autologous experiments with the healthy volunteer (mean and SEM shown).

Definition of regulatory status requires demonstration of functional suppressive activity. Expansion of the putative regulatory CD4 T-cell population in chronic GvHD patients provided sufficient numbers of cells for assessment of their ability to inhibit *in vitro* proliferation of CD4 $CD25^{-}$ cells. Effector T-cell function was variable among patients, probably reflecting the extent and type of immunosuppression administered to control GvHD. Therefore criss-cross assays were performed in which CD4 $CD25^{+}$ cells from patients were co-cultured with CD4 $CD25^{-}$ cells from a healthy volunteer representing a uniform response with which to assess suppressive activity. Results in Figure 3E show that CD4 $CD25^{high}$ cells from three patients with chronic GvHD all inhibited proliferation and, therefore, possessed regulatory function. We attribute the relatively low percentage suppression to some loss of regulatory activity after freezing and thawing cells as this has been observed in comparative studies performed with samples from healthy volunteers (*data not shown*).

Discussion

The incidence of GvHD in HSCT patients treated using a reduced intensity conditioning regimen with alemtuzumab and cyclosporin for GvHD prophylaxis was shown to correlate with higher numbers of effector CD4 T cells. A significant association was detected early (day 30) after transplantation, preceding the emergence of clinical pathology, for six of seven patients who developed acute GvHD. No correlation with CD8 T cells and GvHD was observed. There was no gross deficiency of regulatory CD4 T cells with numbers up to day 100 being similar in patients who developed GvHD and those who did not. However, the ratio of effector to regulatory CD4 T cells was significantly lower at day 30 in patients who developed acute GvHD suggesting that a diminished quantitative potential to control activity of the larger population of effectors early after transplantation may contribute to disease development. This result emphasizes the need for knowledge of the global immune response and the relationship between populations in order to understand the basis of the pathology.

At later time points, the gap between effector and regulatory CD4 T cell numbers closed in patients who developed GvHD. The kinetics of reconstitution of these cell populations in patients who recovered from acute GvHD and did not progress to develop chronic GvHD aligned with the pattern seen in patients who never developed GvHD. In contrast, the regulatory CD4 T-cell population expanded in patients who developed chronic GvHD, producing an imbalance relative to effectors that appeared to favor regulation. This observation seems counter-intuitive. Inflammatory conditions are typically associated with reduced regulatory T cells and this has indeed been reported in the context of clinical chronic GvHD.[25,26] However, some reports have described relatively high numbers of putative regulatory $CD25^{high}$ CD4 T cells in patients with chronic GvHD,[30,31] similar to our observations made using a reinforced definition of regulatory cells by considering $Foxp3^{+}$ and $CD27^{+}$ status and demonstration of suppressive activity. The longitudinal analysis of effector and regulatory CD4 T-cell dynamics performed in our study revealed that the expansion of regulatory T cells in chronic GvHD patients in this cohort

occurred as a delayed wave after the earlier expansion of effector CD4 T cells (Figure 3C). Seen in this context, the CD4 T-cell-mediated immune response in these GvHD patients is consistent with evidence of the sequential co-evolution of memory and regulatory CD4 T cells in the periphery in response to antigen stimulus. An initial phase of CD4 T-cell expansion that mediates pathology is typically followed by recovery dependent upon generation in the periphery of regulatory $CD25^{high}$ $Foxp3^{+}$ CD4 T cells from the memory population.[45] Regulatory CD4 T cells from patients in our study did not express CD31 (*data not shown*) indicating they were not thymus-derived but may indeed have developed *de novo* from the effector CD4 T-cell population. It is also noteworthy that three of the four patients with chronic GvHD with expanded regulatory CD4 T-cell populations subsequently experienced leukemia relapse. This trend, with its implication that anti-leukemia immunity may be suppressed, has been reported previously after HSCT for chronic myeloid leukemia.[46]

Correlation of higher effector CD4 T-cell numbers prior to emergence of GvHD pathology, expansion of the population during disease development and imbalance relative to regulatory CD4 T cells shows that acute GvHD pathology in these patients is primarily mediated by CD4 T cells. Alemtuzumab, used in the treatment regimen, is a humanized form of the monoclonal antibody Campath specific for CD52 which is expressed by lymphocytes, monocytes and some subtypes of dendritic cells and results in extensive depletion of all lymphocyte subsets. The T-cell population that recovered seems to have originated from residual mature cells because very few naïve T cells were present and there was little evidence of thymopoiesis prior to day 180. Studies of lymphocyte repopulation after lymphocyte depletion with alemtuzumab in the clinical setting of autoimmune disease,[47,48] and solid organ transplantation[49,50] all report a predominance of memory CD4 T cells in the residual population. *In vitro* studies suggest that alemtuzumab is less efficient at depleting memory CD4 T cells.[49,50] Differential susceptibility of T-cell subsets to alemtuzumab-mediated elimination may, therefore, underlie the skewed CD4 T-cell population that emerges in patients who develop GvHD.

The other lymphocyte population whose numbers, at day 30, correlated with acute GvHD in the patients was NK cells, perhaps suggesting that these cells play a role in initiation of disease. It has been reported that rapid NK cell recovery can be associated with a lower incidence of GvHD,[18] attributed to elimination of the patients' antigen-presenting cells by alloreactive donor-derived NK cells. This would remove both the allostimulus for donor T cells and the potential reservoir of malignant cells, explaining the also reported lower risk of leukemia relapse.[18] We did not observe this beneficial effect of NK cells in our cohort of patients. This may be because complete depletion of T cells seems to be required for the positive effect of NK cells to be apparent.[13] It is also possible that disease control early after transplantation in our cohort of patients was primarily determined by the chemotoxicity of the regimen used with a relatively minor role for the GvL response. Consistent with this view, the increased numbers of effector CD4 T cells and NK cells in patients who developed acute GvHD were not associated with a beneficial impact on incidence of disease relapse (*data not shown*) despite the related nature of the GvHD and GvL alloresponses.

The increased numbers of NK cells, effector CD4 T cells and relative deficit of regulatory CD4 T cells to effectors in patients who developed acute GvHD was evident at day 30 after transplantation. The risk of acute GvHD is thus pre-determined early after a transplant. These phenotypic characteristics preceded emergence of pathology in the majority of patients suggesting further studies are warranted to assess the prognostic utility. Furthermore the signature appears GvHD-specific because it did not correlate with other complications after HSCT such as cytomegalovirus reactivation (*data not shown*). Reliable clinical indicators would enable safe early withdrawal of immunosuppression for patients not at risk of GvHD, identification of patients requiring a more intensive regimen for protection against GvHD, and could be used to guide response to therapy in patients who develop disease.

Knowledge of the type of immune response that produces GvHD is also a prerequisite for the improvement and development of new strategies to treat GvHD. The relative deficit of regulatory to effector CD4 T cells at day 30 in patients who developed acute GvHD suggests a need to modify treatment protocols to favor balanced early reconstitution of these subsets. This could, perhaps, be achieved by changes to the immunosuppressive regimen that aim to preserve regulatory T cells.[44] Adoptive transfer of regulatory T cells is also being evaluated as a therapy for GvHD.[51] Numbers of regulatory CD4 T cells were, however, not deficient in our GvHD patients and were actually increased in those with the chronic form of disease; furthermore, these cells exhibited suppressive activity *in vitro*. Additional studies are, therefore, required to understand why chronic GvHD pathology persists. Experimental mouse models of GvHD indicate regulatory T-cell suppressive activity has greatest efficacy when the alloresponse has not fully developed.[52,53] The type of regulatory T cell is also likely to be important. $CD103^{+}$ regulatory T cells have been shown to suppress active chronic GvHD in mice,[54] perhaps because they can traffic to sites of pathology.

Studies correlating immune reconstitution following HSCT with GvHD development have typically focused on tracking one lymphocyte subset, often in a cohort of patients treated with a variety of conditioning regimens. In contrast, we have undertaken a longitudinal study characterizing recovery of the mixture of lymphocyte populations in patients all treated with the same regimen that incorporates the widely used combination of alemtuzumab and cyclosporin for GvHD prophylaxis. In addition to identifying the key players in the immune response that causes GvHD in this treatment setting, our study revealed the importance of the balance between effector and regulatory CD4 T-cell populations in determining immune reactivity. Global immune profiling studies of this type provide information essential for improvements in diagnosis and treatment of GvHD.

Authorship and Disclosures

KM designed the study, planned and performed experiments, analyzed data and edited the manuscript. ZL designed the study, collected patients' samples and clinical data, and edited the manuscript. BA planned and performed experiments, analyzed data and edited the manuscript. LP, AA and SK planned and performed experiments and analyzed data. AP contributed to research discussion and reviewed the manuscript. GL planned experiments, analyzed data and edited the manuscript. JAM and GJM designed the study and contributed to research discussion. LDB designed the study, planned and performed experiments, analyzed data and wrote the manuscript.

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