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Persistent changes in the immune system 4–10 years after ABMT

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Summary:

The aim of the present study was to investigate whether the early changes in the immune system observed after ABMT would persist over years. Eighty-five patients with malignant lymphoma were treated with ABMT in Norway from 1987 until 1993. Of the 46 patients in CR by 1997, 36 were enrolled in our study. Median time from ABMT was 5 years (4–10 years). Immunophenotyping showed an increase in the median number of B cells (0.35×10^9/l in patients *vs* 0.28×10^9/l in controls), and a decrease in T cells (1.08 *vs* 1.35×10^9/l). Furthermore, a lower median count of $CD4^+$ T cells (0.54×10^9/l in patients *vs* 0.87×10^9/l in controls) resulted in reduced CD4/CD8 ratios (0.8 in patients *vs* 1.6 in controls). The subgroup of $CD4^+$ T cells expressing the 'naive' phenotype CD45RA was 19.5% in patients *vs* 38% in controls. In contrast, the fraction expressing the 'memory' phenotype CD45RO was higher in the ABMT group (76% *vs* 54%). When stimulated, larger fractions of $CD3^+CD4^+$ cells in patients produced IFN-γ (32% *vs* 16%) or IL-4 (7% *vs* 1%) compared to controls; thus a differentiation into the functionally separate subgroups Th1 and Th2, with a dominant Th2 response. Our data further suggest that the decrease in $CD4^+$ T cell counts and the imbalance between $CD45RA^+$ and $CD45RO^+$ subsets persists 4–10 years after ABMT.

Keywords: ABMT; malignant lymphoma; immune reconstitution

Myeloablative therapy supported by autologous bone marrow or peripheral blood progenitor cells has proven an effective treatment for certain hematological malignancies including relapsed intermediate grade non-Hodgkin's lymphoma,[1] multiple myeloma[2] and acute myelogenous leukemia.[3] The role of this treatment in solid tumors is still not clear. At present peripheral blood progenitor cells are the most frequent source of stem cells, replacing bone marrow. Hematologic recovery of neutrophils and platelets appears almost consistently within 2 weeks after reinfusion of PBSC. Reconstitution of lymphoid and immune effector cells occur more slowly, and may take months to years. It has been a consistent finding that counts of B lymphocytes, NK cells and $CD8^+$ T lymphocytes reach normal levels and function within the first months following ABMT. In contrast, the $CD4^+$ T lymphocytes have been found not to recover completely, even as long as 2 years post transplant in many adult patients.[4] The persisting subnormal $CD4^+$ T cell counts and CD4/CD8 ratios may put patients at risk of developing opportunistic infections and even recurrence of their malignancy. The changes in the immune system over the first few months and years after ABMT and PBSCT have been extensively studied.[5,6] It is, however, still an open question whether complete normalization of $CD4^+$ T cells will occur in these patients.

The role of purging bone marrow or peripheral progenitor cells remains a controversial issue. It is not clear whether purging is of clinical benefit to the patient. Furthermore, purging may influence the recovery of lymphoid cells post transplant.[7]

In the present study we investigated immunological recovery in an unselected population of lymphoma patients, including 36 out of 46 patients in complete remission who received ABMT in Norway between April 1987 and December 1993. Nearly all the patients were treated with a TBI-containing conditioning regimen supported by purged autologous bone marrow. Our data indicate that changes in the immune system previously reported early after ABMT persist even 4–10 years post-transplant.

Materials and methods

Patients and controls

From April 1987 to December 1993 all 85 cases of malignant lymphoma requiring ABMT in Norway were treated at the Norwegian Radium Hospital. In January 1997, 46 patients were alive and in complete remission (CR) and 39 of these agreed to participate in this study. Three patients were withdrawn from the examination due to: an intra-cranial insult; another was hospitalized with a severe bacterial infection and myelodysplasia and the third had psychological problems. Thus, 36 patients were enrolled. The median age was 39.5 years (18–59 years) and median time since ABMT was 5 years (4–10 years). Twenty-eight patients received bone marrow purged by monoclonal antibodies and magnetic beads. This method is described elsewhere.[8,9] Each patient was compared with an age- and sex-matched control recruited from blood donors. Patient characteristics are summarized in Table 1.

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Table 1 Patient characteristics[a]

Male/female	20/16
Age at diagnosis	32 (14–51) years
Age at ABMT	34 (14–54) years
Age at study	39 (18–59) years
Time after ABMT	5 (4–10) years
Diagnosis[b]	
T lymphoblastic lymphoma, 1st remission	7 patients
B lymphoblastic lymphoma, 1st remission	8 patients
Burkitt's lymphoma, 1st remission	3 patients
Low grade follicular non-Hodgkin's lymphoma, 2nd and later remissions	8 patients
High-grade non-Hodgkin lymphoma, 2nd or later remission	5 patients
Hodgkin's lymphoma, 2nd or later remission	5 patients
Preparative regimen	
TBI and cyclophosfamide	33 patients
BEAC (carmustine, etoposid, cytarabin and cyclophosphamide)	3 patients
Bone marrow purging	
Negative selection of T cells	8 patients
Negative selection of B cells	20 patients

[a]Data are given as median and ranges.
[b]Kiel classification of lymphomas.
Six out of 36 patients received radiotherapy some time during treatment. Three of these received mantle field irradiation covering mediastinum/thymus.

Reagents/Antibodies

Phorbol-12-myristate 13-acetate (PMA), ionomycin and brefeldin-A (BFA) were purchased from Sigma (St Louis, MO, USA). MoAbs which were used in flow cytometry were FITC-conjugated anti-CD3, -CD4, -CD16, -CD45, -CD45RO, -kappa, and -lambda; PE-conjugated anti-CD8, -CD14, -CD19, -CD20, -CD45RO, -CD45RA, -CD69, -IL-4, and -IFN-γ; PerCP-conjugated anti-CD3 and -CD4. All antibodies were obtained from Becton Dickinson (San Jose, CA, USA)/PharMingen (San Diego, CA, USA).

Immunophenotyping

A complete blood count including differentials, was performed within 6 h after drawing of EDTA anticoagulated blood. Within 24 h, whole blood was used for immunophenotyping. For most samples, antibodies were added to whole blood. After incubation, the erythrocytes were lysed and the samples run in the flow cytometer without further washing. When testing for immunoglobulin light chains on B lymphocytes, the erythrocytes were lysed and the sample washed before staining. Two-color data were acquired on FACSscan flow cytometer equipped with a 488 nm argon laser (Becton Dickinson). A tight light scatter gate was drawn around the lymphocytes and these cells were analyzed using CellQuest software on a Power Macintosh computer. Counts of B cells ($CD20^+$), T cells ($CD3^+$), $CD4^+$ and $CD8^+$ cells were calculated, using the total lymphocyte count and the percentage of cells with the different phenotypes. The CD4CD8 double-positive group generally constituted a small population ($<3\%$) of the cells and was not counted. The CD4/CD8 ratio was calculated.

Intracellular cytokine analysis

PBMC from patients and controls were separated from whole blood using vacutainer cell preparation tubes (CPT; Becton Dickinson). Cells were washed twice in PBS with 0.1% BSA, and resuspended at 2×10^6 cells/ml in RPMI-1640 supplemented with 5% AB serum, streptomycin and penicillin. The cells were stimulated for 4 h with PMA (25 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin-A (10 μg/ml), an inhibitor of protein secretion inducing cytoplasmic accumulation of cytokines. This technique was originally described by Jung and co-workers,[10] modified by Picker *et al*.[11] Cultured cells were washed twice in PBS with BSA and stained with MoAbs to the following cell-surface markers: CD3, CD4 and CD45RO. The cells were then fixed and permeabilized with FACS permeabilizing solution according to the manufacturer's instruction (FastImmune Kit; Becton Dickinson), followed by staining with MoAbs specific for IL-4 or IFN-γ.

The samples were analyzed on a FACScan flow cytometer using Lysis II or CellQuest software (Becton Dickinson) on HP 340 or Macintosh Quadra workstation. A tight light scatter gate was drawn around the major lymphocyte population. Cells in this gate, staining double positive for CD4 and CD3, or double positive for CD4 and CD45RO were studied. Ten thousand events were acquired and analyzed from each sample.

Statistical methods

For comparison of unpaired data between two groups the Mann–Whitney *U* test was applied. Kruskal–Wallis one-way analysis of variance was used for comparison between several groups. Coefficients of correlation were calculated by the Spearman rank test. Data are given as medians and ranges if not otherwise stated. *P* values are two-sided and considered significant when <0.05.

Results

Median counts of peripheral blood cell

There was no significant difference between patients and controls in numbers of leukocytes, thrombocytes or red blood cells. The numbers of granulocytes were similar, but the numbers of monocytes and lymphocytes were lower in patients (Table 2). The monocyte count was 0.40×10^9/l in patients *vs* 0.48×10^9/l in controls. Lymphocytes were 1.60×10^9/l in patients and 1.95×10^9/l in controls. The difference observed regarding lymphocytes was caused by decreased level of T cells ($CD3^+$) in patients (1.08 *vs* 1.35×10^9/l), while numbers of B cells ($CD20^+$) were increased (0.35 *vs* 0.28×10^9/l). The numbers of natural killer (NK) cells were similar in the two groups.[16]

T lymphocytes

Among $CD3^+$ lymphocytes $CD4^+$ and $CD8^+$ cells constitute the two major subsets. The numbers of $CD8^+$ cells were similar in patients and controls (0.65×10^9/l *vs*

Table 2 Median counts ($\times 10^9$/l) and ranges for cells in peripheral blood of lymphoma patients 4–10 years after ABMT and controls

Phenotype	*Patients n = 36 (*31)*	*Controls n = 36 (*32)*	*P value*
Granulocytes	2.90 (1.40–6.90)	2.86 (1.47–10.35)	0.9
Monocytes	0.40 (0.20–0.70)	0.48 (0.20–1.12)	0.02
NK cells	0.19 (0.13–0.20)	0.22 (0.10–0.61)	0.28
Lymphocytes	1.60 (0.9–5.7)	1.95 (1.2–3.7)	0.02
B cells ($CD20^+$)[a]	0.35 (0.16–2.45)	0.28 (0.15–0.61)	0.013
T cells ($CD3^+$)[a]	1.08 (0.33–3.62)	1.35 (0.85–2.77)	0.001
$CD4^+$ cells[a]	0.54 (0.26–1.43)	0.87 (0.31–1.9)	<0.001
$CD8^+$ cells[a]	0.65 (0.24–1.70)	0.56 (0.31–1.08)	0.66
CD4/CD8 ratio[a]	0.8 (0.4–1.7)	1.6 (0.5–2.8)	<0.001

[a]Immunophenotyping successful in 31 patients and 32 controls.

0.56×10^9/l). In contrast, there was a marked difference in $CD4^+$ counts, 0.54×10^9/l in patients and 0.87×10^9/l in controls (Figure 1a). A tendency towards higher CD4 counts in patients transplanted 7–10 years ago (0.57×10^9/l) compared to patients transplanted 4–6 years ago (0.47×10^9/l), was observed. Due to low $CD4^+$ cell number, the CD4/CD8 ratio was significantly lower among ABMT patients (0.8) than controls (1.6) (Table 2).

Expression of CD45 isoforms on $CD4^+$ T cells

Naive $CD4^+$ T cells generally express the high molecular weight CD45 isoform, CD45RA, whereas memory $CD4^+$ T cells have the low molecular weight isoform CD45RO. The transplanted patients had a significantly lower fraction of $CD4^+CD45RA^+$ cells compared with controls, 19.5% *vs* 38% (Figure 1b). The fraction of $CD4^+CD45RO^+$ cells was higher in patients (76%) than controls (54%) (Figure 1c). In the control group we found a correlation between increasing age and decreasing fraction of $CD45RA^+$ cells, whereas in patients there was no such correlation (Figure 2).

Immune reconstitution and bone marrow purging

Twenty-eight of 36 patients received bone marrow purged by negative selection using MoAbs to B cell antigens (20 patients) or T cell antigens (eight patients) and immunomagnetic beads. There were no significant differences in counts of $CD4^+$ cells, or $CD8^+$ cells observed between patients who received T cell-depleted (eight patients), B cell-depleted (17 patients), or unpurged bone marrow (six patients) (Table 3).

Immunophenotyping was successful in three out of five patients with Hodgkin's lymphoma. Numbers of T cells were not lower among these patients. Due to small numbers of patients with different subtypes of lymphoma, results comparing different groups are not presented.

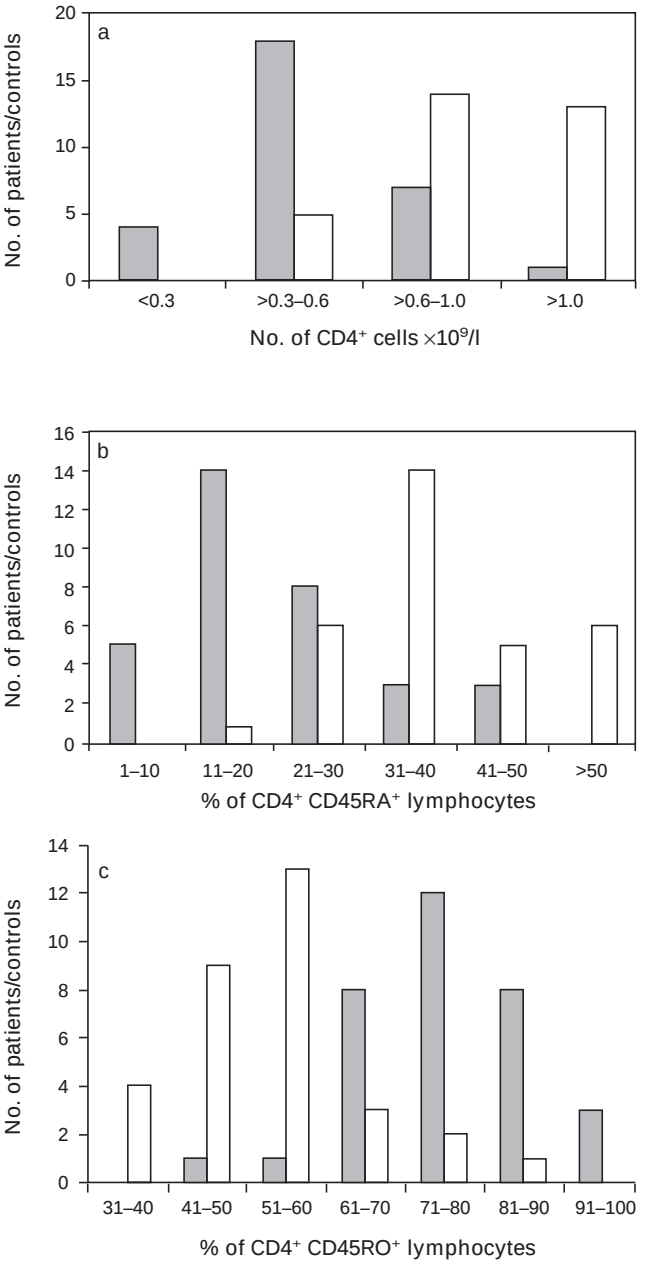

Figure 1 Distribution of $CD4^+$ cells among patients and controls: (**a**) total numbers of $CD4^+$ cells; (**b**) $CD4^+CD45RA^+$ cells; and (**c**) $CD4^+CD45RO^+$ cells. (▩) Patients; (□) controls.

Production of intracellular cytokines

Cells from both patients and controls were successfully stimulated by PMA and ionomycin detected by CD69 expression in more than 90% of the $CD4^+CD3^+$ cells. There was a significant difference between patients and controls in the percentage of $CD4^+CD3^+$ cells producing IFN-γ or IL-4 after stimulation. In the ABMT group 31.9% (10.2–66.2%) of $CD3^+CD4^+$ cells produced IFN-γ, compared to 15.9% (3.7–32.2%) in the control group (Figure 3).

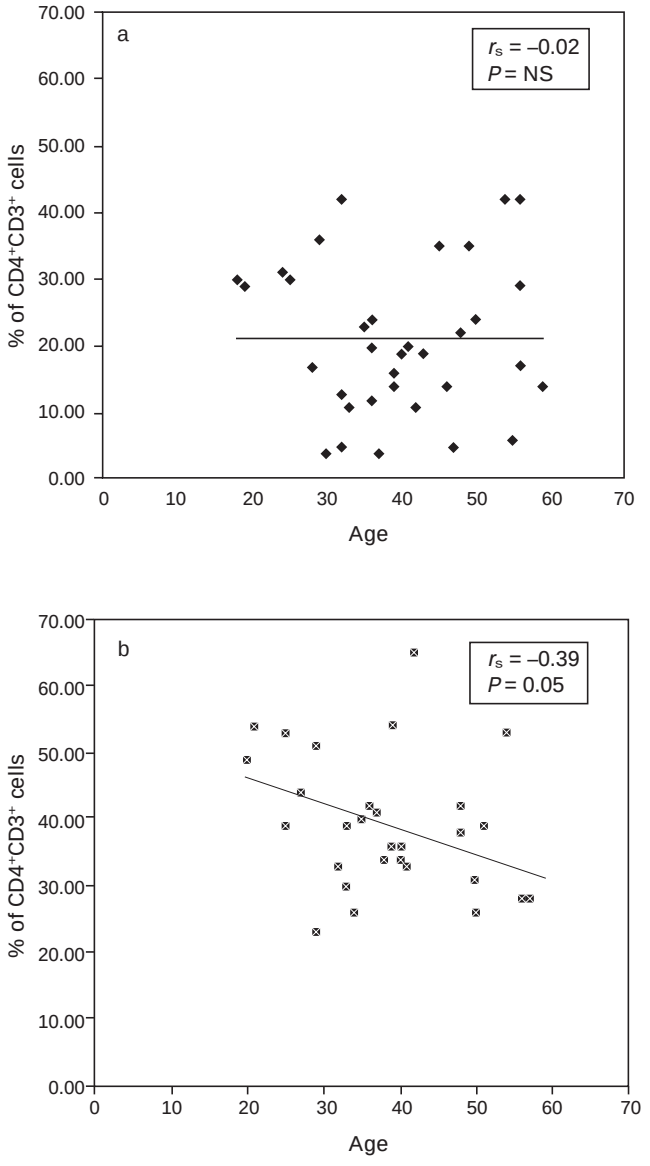

Figure 2 Correlation between age and percentage of $CD4^+CD45RA^+$ cells in (**a**) patients and (**b**) controls. r_s = Spearman correlation coefficient.

Table 3 Median cell counts ($\times 10^9$/l) and ranges in ABMT patients who received T or B cell purged or unpurged marrow ($n = 31$[a])

Cell type	*B cell purged n = 17*	*T cell purged n = 8*	*No purge n = 6*	*P value*
B cells ($CD20^+$)	0.35 (0.16–2.45)	0.32 (0.16–1.47)	0.41 (0.31–0.78)	0.4
T cells ($CD3^+$)	1.03 (0.33–2.60)	1.09 (0.67–2.52)	1.10 (0.58–1.18)	0.95
$CD4^+$ cells	0.54 (0.26–1.20)	0.56 (0.35–1.43)	0.41 (0.28–0.61)	0.4
$CD8^+$ cells	0.65 (0.24–1.70)	0.54 (0.31–1.09)	0.61 (0.34–0.77)	0.7
CD4/CD8 ratio	0.83 (0.40–1.68)	1.10 (0.64–1.3)	0.69 (0.50–0.82)	0.06

[a]n = 31, immunophenotyping successful in 31 patients.

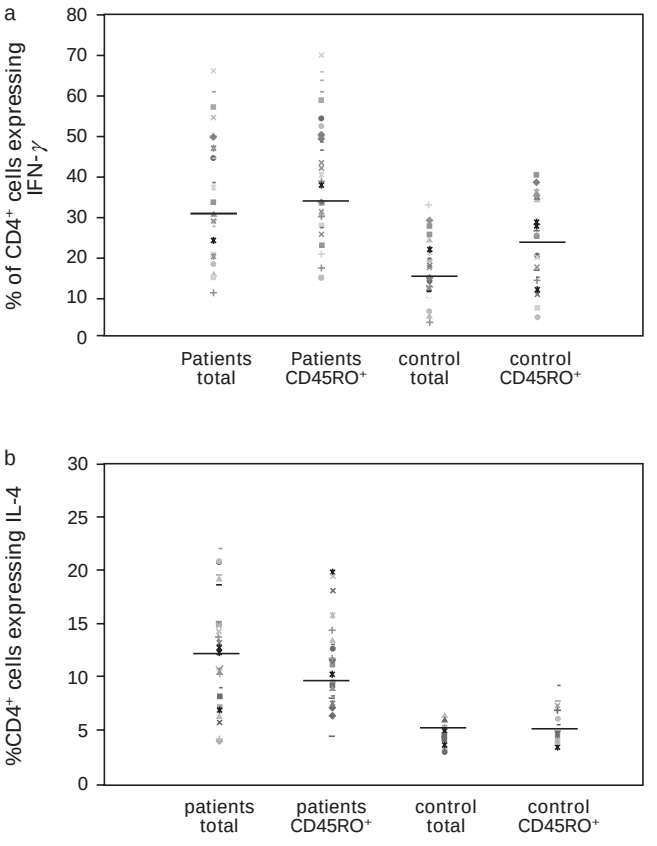

Figure 3 Percentage of $CD3^+CD4^+$ and $CD4^+CD45RO^+$ cells in patients and controls producing (**a**) IFN-γ and (**b**) IL-4 after stimulation with PMA and ionomycin in the presence of brefeldin-A.

A similar and even larger difference was observed regarding production of IL-4 by $CD3^+CD4^+$ cells, 6.9% (0.8–16.6%) *vs* 1.1% (0.1–14.1%). The patients had a higher fraction of cells of the $CD4^+CD45RO^+$ phenotype. To investigate whether over-representation of these cells could explain the difference in cytokine profile, we selectively looked at production of cytokines in the $CD4^+CD45RO^+$ subset. The differences became less pronounced but were still significant. Thirty-five percent (14.4–69.9%) in patients *vs* 25% (4.2–33.9%) in controls produced IFN-γ, and 6.8% (1.3–27.1%) *vs* 1.7% (0.5–11.2%) produced IL-4 (Figure 3).

Discussion

It has been a consistent finding that the numbers of NK cells, B lymphocytes and $CD8^+$ T lymphocytes return to normal levels within the first months following ABMT. In contrast, $CD4^+$ T lymphocytes remain subnormal for a longer time period. This generally results in a decreased CD4/CD8 ratio for at least 2 years post transplant.[12,13] Similar results have been reported in patients undergoing intensive chemotherapy without bone marrow or stem cell support.[14,15] Pedrazzini and colleagues[4] reported that only 14 out of 79 ABMT patients had recovered to 40% of normal $CD4^+$ T cell counts at a median follow-up of 24 months. They estimated the time to recovery of normal levels of

$CD4^+$ cells to be 3–4 years or more. Our data with a median follow-up of 5 years show that the $CD4^+$ counts still remain low, at approximately 2/3 of control levels. We observed a tendency towards higher CD4 counts among those who were transplanted 7–10 years earlier compared to the patients transplanted 4–6 years earlier. This difference was not significant, but might indicate that the process towards normalization of this population of cells continues even after many years.

$CD4^+$ T lymphocytes generally express either a CD45 high molecular weight isoform, CD45RA, or a low molecular weight isoform CD45RO. The $CD4^+CD45RA^+$ cells are regarded as 'naive' T cells recently issued from the thymus, whereas the $CD4^+CD45RO^+$ population responds to recall antigens and represents 'memory' cells.[17] However, a new model for immunological memory has recently been presented, postulating that 'memory' T cells can revert from the $CD4^+CD45RO^+$ to the $CD4^+CD45RA$ 'naive' phenotype in the absence of antigen.[18] Previous reports have shown that the $CD4^+CD45RA^+$ subset is markedly reduced during the first months following ABMT and recovers slowly.[5,19] Hakim and co-workers[15] clearly demonstrated that these cells are more sensitive to chemotherapy; their numbers dropped severely during and after treatment in breast cancer patients receiving the FLAC (5-FU, leucovorin, doxorubicin, cyclophosphamide) regimen. After 1 year the number of $CD4^+CD45RA^+$ cells remained less than one third of pretreatment levels. The $CD4^+CD45RO^+$ subset showed only a modest decrease and recovery was faster. Our results show that even 4–10 years after ABMT, the percentage of $CD4^+CD45RA^+$ cells accounted for 19.5% of $CD4^+$ cells in patients compared to 38% in controls. On the other hand, the $CD4^+CD45RO^+$ subset was increased in the ABMT group. Maturation of T cells has been shown to decrease as early as in young adulthood due to involution of the thymus. It has been postulated that this prohibits the regeneration of naive $CD4^+CD45RA^+$ cells after ABMT.[20] In the non-transplant setting Mackall and colleagues[21] observed that in adult patients, depletion of $CD4^+$ T cells was prolonged, compared to pediatric patients. This suggested an inverse correlation between age and the ability to regenerate 'naive' T cells. Mackall and colleagues[22] have recently published data from mouse models showing that involuted thymuses from aged animals could regenerate a significant number of T cells. They argue that accumulation of 'memory' type $CD4^+$ cells after bone marrow transplantation is not only caused by reduced thymic function in adults, but also increased antigen-driven peripheral expansion of naive $CD4^+CD45RA^+$ thymic emigrants into the $CD4^+CD45RO$ subset. Our data confirm that the imbalance between the subsets of $CD4^+$ cells persists for many years after ABMT, and it is still an open question whether normal numbers of the naive and memory $CD4^+$ T cells can ever be restored.

$CD4^+$ T cells produce different cytokines, and based on the production profiles two functionally different subgroups, Th1 and Th2, can be defined. Th1 cells mainly produce IFN-γ, IL-2 and TNF-α, whereas Th2 cells produce IL-4, IL-5 and IL-10.[23] Guillaume *et al*[24] and others[25] have reported a defect in the production of several cytokines, particularly IL-2, early after ABMT. In our study a higher proportion of $CD4^+$ T cells produced IL-4 or IFN-γ after stimulation with PMA and ionomycin in ABMT patients than in controls. Previous studies have indicated that IFN-γ and IL-4 are mainly produced by the $CD45RO^+$ fraction of T cells.[11,26] When adjusting for the higher fraction of $CD4^+CD45RO^+$ cells in our patients, the difference became less, but was still significant. The reduced number of $CD4^+CD45RA^+$ cells does not necessarily indicate immune competence, the diversity of the T cell repertoire might be equally or more important. Jerne[27] postulated that 10% of the total repertoire is sufficient for immune competence. Thus, our patients might be immune reconstituted despite a low number of $CD4^+$ T cells.

The most physiological stimulation of T cells appears to occur via the T cell receptor (TcR) in the presence of an antigen presenting cell. PMA and ionomycin stimulate all T cells regardless of their TcR specificity. Functional studies of lymphocytes several years post-ABMT are scarce. As far as we know, our study is the first to investigate intracellular cytokine production at this time point. The data indicate that a higher fraction of $CD4^+$ T cells post ABMT are activated and/or have differentiated to become Th1 or Th2 cells. The three to four times increase in IL-4 producing cells compared to a 50% increase in IFN-γ producing cells in patients indicates a dominant Th2 response, at least after stimulation with PMA and ionomycin. Th2 cells are responsible for induction of humoral immunity. We found that serum levels of IgG, IgA and IgM were normal in patients (data not shown). However, the numbers of B cells were increased. The increase in fractions of Th1 and Th2 cells in patients post-ABMT could explain why serious opportunistic infections were rare (patient self reported questionnaire, data not shown), even though the counts of $CD4^+$ cells were significantly less that observed in controls.

The patient material in this study is of particular interest, because the bone marrows were purged by negative selection in most cases, using monoclonal antibodies and immunomagnetic beads. Furthermore, all except three patients received a conditioning regimen containing TBI (13 Gy) and cyclophosphamide (120 mg/kg). This regimen is probably more effective in destroying residual T cells and B cells in the host than are other regimens based on combination chemotherapy. Anderson and co-workers[28] reported that patients who underwent T cell-depleted ABMT had a profound immunodeficiency not reflected in their phenotypic reconstitution. Pedrazzini *et al*[4] reported that purging autologous marrow with monoclonal antibodies to B cell antigens delayed B cell recovery. Keever *et al*[7] found only modest quantitative and temporal differences in immune reconstitution in recipients of T cell-depleted marrow compared to recipients of conventional marrow grafts. We could not find any significant differences between patients who received B or T cell-depleted or unpurged bone marrow in median counts of B cells, $CD4^+$ cells, $CD8^+$ cells. Patient numbers are low, but the data indicate that lymphocytes present in the graft may play a minor role in the long-term immune reconstitution of these patients.

In conclusion, lymphoma patients in complete remission 4–10 years after ABMT have a markedly reduced number of $CD4^+$ T cells, and especially the $CD4^+CD45RA^+$

'naive' T cell compartment. In addition, a larger fraction of the $CD4^+$ T cells produce IFN-γ or IL-4 upon stimulation with PMA and ionomycin, suggesting functional differences compared to normal controls.

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References

1 Philips T, Guglielmi C, Hagenbeek A *et al.* Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy sensitive non Hodgkin's lymphoma. *New Engl J Med* 1995; **333**: 1540–1545.
2 Attal M, Harrousseau JL, Stoppa AM *et al.* A prospective randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. *New Engl J Med* 1996; **335**: 91–97.
3 Zitton RA, Mandelli F, Willemze R *et al*. Autologous or allogeneic bone marrow transplantation compared with intensive chemotherapy in acute myelogenous leukemia. *New Engl J Med* 1995; **332**: 217–223.
4 Pedrazzini A, Freedman AS, Andersen J *et al*. Anti-B-cell monoclonal antibody-purged autologous bone marrow transplantation for B-cell non-Hodgkin's lymphoma; phenotypic reconstitution and B-cell function. *Blood* 1989; **74**: 2203–2211.
5 Storek J, Witherspoon RP, Storb R. T cell reconstitution after bone marrow transplantation into adult patients does not resemble T cell development in early life. *Bone Marrow Transplant* 1995; **16**: 413–425.
6 Talmadge JE, Reed E, Ino K *et al.* Rapid immunologic reconstitution following transplantation with mobilized peripheral blood stem cells as compared to bone marrow. *Bone Marrow Transplant* 1997; **19**: 161–172.
7 Keever CA, Small TN, Flomenberg N *et al*. Immune reconstitution following bone marrow transplantation: comparison of recipients of T-cell depleted marrow with recipients of conventional marrow grafts. *Blood* 1989; **73**: 1340–1350.
8 Wang MY, Kvalheim G, Kvaloy S *et al*. An effective immunomagnetic method for bone marrow purging in T cell malignancies. *Bone Marrow Transplant* 1992; **9**: 319–323.
9 Kvalheim G, Holthe H, Jakobsen E *et al.* Immunomagnetic purging of lymphoma cells from autografts. *J Hematother* 1996; **5**: 561–562.
10 Jung T, Schauer U, Heusser C *et al*. Detection of intracellular cytokines by flow cytometry. *J Immunol Meth* 1993; **159**: 197–207.
11 Picker LJ, Singh MK, Zdraveski Z *et al.* Direct demonstration of cytokine synthesis heterogeneity among human memory/effector T cells by flow cytometry. *Blood* 1995; **86**: 1408–1419.
12 Roberts MM, To LB, Gillis D *et al*. Immune reconstitution following peripheral blood stem cell transplantation, autologous bone marrow transplantation and allogeneic bone marrow tranplantation. *Bone Marrow Tranplant* 1993; **12**: 469–475.
13 Scheid C, Pettengell R, Ghielmini M *et al*. Time-course of the recovery of cellular immune function after high-dose chemotherapy and peripheral blood progenitor cell transplantation for high-grade non-Hodgin's lymphoma. *Bone Marrow Transplant* 1995; **15**: 901–906.
14 Mackall CL, Fleisher TA, Brown MR *et al*. Lymphocyte depletion during treatment with intensive chemotherapy for cancer. *Blood* 1994; **84**: 2221–2228.
15 Hakim FT, Cepeda R, Kaimei S *et al.* Constraints on CD4 recovery postchemotherapy in adults: thymic insufficiency and apoptotic decline of expanded peripheral CD4 cells. *Blood* 1997; **90**: 3789–3798.
16 Hulstaert F, Hannet I, Deneys V *et al*. Age-related changes in human blood lymphocyte subpopulations. *Clin Immunol Immunopathol* 1994; **70**: 152–158.
17 Merchensclager M, Terry L, Edwards R, Beverly PC. Limiting dilution analysis of proliferative responses in human lymphocyte populations defined by the monoclonal antibody UCHL 1: implications for differential CD45 expression in T cell memory formation. *Eur J Immunol* 1988; **18**: 1653–1661.
18 Bell E, Sparshott SM, Bunce C. CD4+ T-cell memory, CD45R subsets and the persistence of antigen – a unifying concept. *Immunol Today* 1998; **19**: 60–64.
19 Sugita K, Soiffer RJ, Murray C *et al*. The phenotype and reconstitution of immunoregulatory T cell subsets after T cell-depleted allogeneic and autologous bone marrow transplantation. *Transplantation* 1994; **27**: 1465–1473.
20 Hirokawa K, Utsuyama M, Kasai M *et al*. Understanding the mechanism of the age-change of thymic function to promote T cell differentiation. *Immunol Lett* 1994; **40**: 269–277.
21 Mackall CL, Fleisher TA, Brown MR *et al.* Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. *New Engl J Med* 1995; **332**: 143–149.
22 Mackall CL, Punt JA, Morgan P *et al.* Thymic function in young/old chimeras: substantial thymic T cell regenerative capacity despite irreversible age-associated thymic involution. *Eur J Immunol* 1998; **28**: 1886–1893.
23 Romagnani S. Human TH1 and TH2 subsets: doubt no more. *Immunol Today* 1991; **12**: 256–257.
24 Guillaume T, Sekhavat M, Rubinstein DB *et al*. Defective cytokine production following autologous stem cell transplantation for solid tumors and hematologic malignancies regardless of bone marrow or peripheral origin and lack of evidence for a role for interleukin-10 in delayed immune reconstitution. *Cancer Res* 1994; **54**: 3800–3807.
25 Cayeux S, Meuer S, Pezzutto A *et al*. T-cell ontogeny after autologous bone marrow transplantation: failure to synthesize interleukin-2 (IL-2) and lack of CD2- and CD3-mediated proliferation by both CD4– and CD8+ cells even in the presence of exogeneous IL-2. *Blood* 1989; **74**: 2270–2277.
26 Akbar AN, Salmon M, Janossy G. The synergy between naive and memory T cells during activation. *Immunol Today* 1991; **12**: 184–188.
27 Jerne N. Idiotype networks and other preconceived ideas. *Immunol Rev* 1984; **79**: 5–24.
28 Anderson KC, Soiffer R, DeLage R *et al*. T-cell depleted autologous bone marrow transplantation therapy: analysis of immune deficiency and late complications. *Blood* 1990; **76**: 235–244.