Experimental
ONCOLOGY

HELPER T CELLS (CD3+/CD4+) WITHIN THE AUTOLOGOUS PERIPHERAL BLOOD STEM CELL GRAFT POSITIVELY CORRELATE WITH EVENT FREE SURVIVAL OF MULTIPLE MYELOMA PATIENTS

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Background: The microenvironment in the bone marrow — including lymphocytes — is part of the pathophysiology of multiple myeloma (MM). High dose chemotherapy followed by autologous stem cell transplantation is standard of care for younger patients. *Aim:* To determine the influence of reinfused lymphocyte subsets on event free survival (EFS) and overall survival (OS). *Methods:* In peripheral blood (PB) and aphaeresis products (AP) of 41 MM patients lymphocyte subsets were determined by flow cytometry and were correlated with clinical outcome. *Results:* PB lymphocyte subsets did not influence EFS or OS. Residual plasma cells in the AP were not correlated with poor outcome, whereas a high percentage of B cells (CD19+) showed a trend towards reduced EFS ($P = 0.051$). A high amount of CD4 cells and an increased CD4/CD8 ratio were significantly associated with prolonged EFS. In contrast, high percentage of HLA-DR positive lymphocytes showed negative impact on EFS and OS ($P = 0.03$ and 0.02, respectively). *Conclusion:* Obtained data suggest the non-activated (HLA-DR negative) helper CD4+ T cells in the AP to be tumour protective.
Key words: autologous graft, blood stem cell transplantation, correlation, event-free survival, lymphocytes, multiple myeloma.

High dose chemotherapy followed by autologous blood stem cell transplantation (ASCT) is standard treatment for younger patients with symptomatic multiple myeloma (MM). The lymphocytes in the microenvironment of MM cells are an essential part of the pathophysiology of this incurable disease [1]. It has been previously shown that lymphocytes within the graft are linked to the survival of MM patients [2]. However, only little is known which lymphocyte subsets are responsible for this tumor protective effect. In the presented study we have analyzed the lymphocytes by immunophenotyping in the peripheral blood (PB) and in the apheresis product (AP) of 41 consecutive patients.

METHODS

Patients. 41 consecutive patients with multiple myeloma received ASCT between 11/1995 and 07/2000. The study was approved by the Ethical committee of the University Munich and informed consent was obtained from each patient. Mobilization chemotherapy and G-SCF was administered according to the IEV regimen [3]. Peripheral blood stem cells were harvested when the post-nadir absolute leukocyte count rose above 5000/μl using a Cobe Spectra (Cobe, Heimstetten, Germany) or an AS104 (Fresenius, St. Wendel, Germany) cell separator and standard programs. The harvested blood stem cells were mixed with an equal volume of a freezing solution which was prepared with 5% HSA and 100% DMSO (Cryoserv, Tera Pharmaceuticals, Midvale, Utah, USA) (4 : 1). Relapse was defined according to the EBMT criteria [4].

Flow cytometry. Cells in the AP and cells in the PB at the day of apheresis were analyzed. Immunophenotyping was performed with a three-color-fluorescence using an EPICS XL-MCL flow cytometer and the System II software (Beckman Coulter, Krefeld, Germany). 100 μL of sample were incubated 15 min with saturating concentrations of the fluorochrome-conjugated antibodies [5]. IgG1-FITC, IgG1-PE, CD45-FITC, CD3-FITC, CD38-FITC, CD14-PE, CD19-PE, CD4-PE, CD8-PE, CD56/CD16-PE, HLA-DR-PE, and CD138-PR were purchased from Immunotech (Marseille, France).

Statistics. SPSS 11.5 for Microsoft Windows (SPSS Inc., Chicago, USA) was used for statistical analysis. The median number of positive cells was calculated for each marker and patients were assigned to a group either above or below median. To compare survival curves a log-rank test was performed and $P < 0.05$ was considered statistically significant.

RESULTS

Patients and stem cell harvest. The mean age was 56 years (range: 38–68) and 25 of the 41 patients were male (61%). Within the median follow up of 3.9 years 30 pts experienced relapse (73%) and 13 pts died (32%). The median event free survival (EFS) was 858 days (range: 104–2141 days), the median overall survival (OS) was 1406 days (range: 104–2557 days).

Immunophenotyping of AP lymphocytes. A median of 78,000 leukocytes per μl (range: 12,000–370,000/μl) was counted in the APs. 29% (7–63%) were lymphocytes. The table shows the lymphocyte pattern within the AP among the 41 tested patients. The composition of lymphocyte subsets expectantly refers rather well to values of the peripheral blood with 81% T-cells (CD3), 1% B-cells, and 4% NK-cells (CD56/16). A small but still significant MM cell contamination was seen. The median CD4/CD8-ratio was 1.6 (range 0.2–7.0) and 5% of lymphocytes expressed the activation

marker HLA-DR. With respect to the clinical outcome analysis below the displayed median values were used for discrimination between high and low.

Table. Lymphocyte subsets within the peripheral blood stem cell grafts. After IEV mobilization treatment and GCSF stimulation leukapheresis was performed and lymphocyte subsets in the apheresis products of 41 consecutive multiple myeloma patients were determined by flow cytometry as indicated. Median and range are shown [% lymphocytes]. The median was used as cut off to discriminate between high and low for further data analysis

Lymphocyte Subsets	Median	Range
CD3	81	7–63
CD19	1	0–15
CD4	45	14–76
CD8	29	11–63
CD56/16	4	1–19
HLA-DR	5	1–37
CD138/CD38	0.03	0.00–0.84

Survival according to lymphocyte subsets within the graft. First, it was tested whether plasma cells within the AP predict poor outcome. Figure *a* shows that the median EFS was not significantly different in patients with many plasma cells (CD138$^+$CD38$^+$ above median) in the AP (1268 ± 258 *vs* 1066 ± 140 days). This is in accordance to the results from PB. However, the percentage of B cells (CD19$^+$) within the AP had a strong trend towards prediction of poor outcome (*P* = 0.051, see Figure *a*).
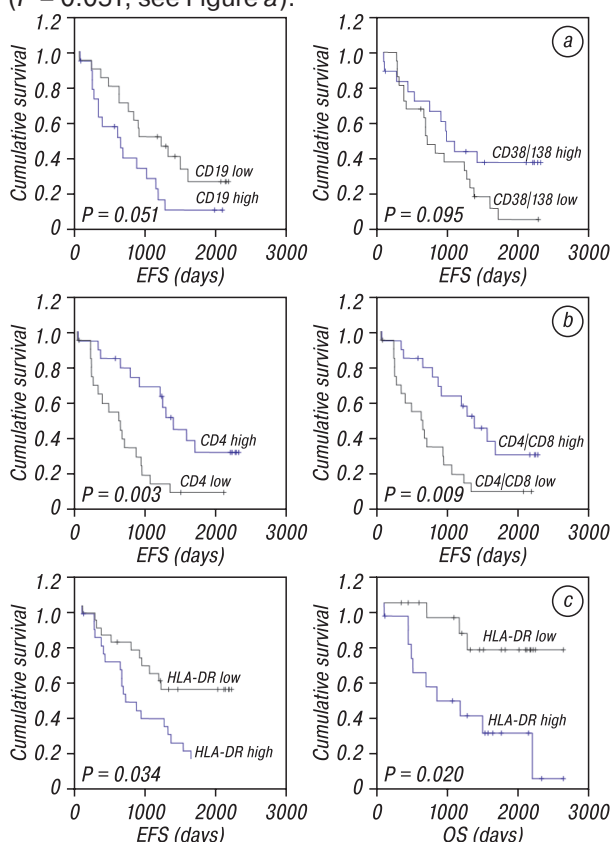

Figure. Correlation of lymphocyte subsets within the peripheral blood stem cell graft with clinical outcome (EFS event free survival, OS overall survival) after high dose melphalan treatment. Patients were grouped according to the amount of specific lymphocyte subsets [% lymphocytes] within the stem cell graft (high versus low defined as above or below median respectively). EFS and OS were determined for the different groups and are shown as Kaplan — Meier blots. *a*, EFS depending on B cells (CD19) and plasma cells (CD138/CD38). *b*, EFS depending on CD4 cells and CD4/CD8 ratio. *c*, EFS and OS depending on HLA-DR positive cells

High leukocyte counts in the AP resulted in apparently longer EFS (1363 ± 164 *vs* 862 ± 111 days) and slightly increased OS (1902 ± 138 *vs* 1663 ± 196 days), although these results did not reach statistical significance (*P* = 0.074 and 0.288, respectively).

A high percentage of CD4 cells was associated with a statistically significant prolongation of EFS (2179 ± 170 *vs* 1670 ± 212 days; *P* = 0.003), which was additionally reflected by the results obtained regarding OS (Figure *b*). A low proportion of CD8 cells and consequently an increased T4/T8 ratio were significantly associated with good EFS (see Figure *b*). In addition, a small amount of activated lymphocytes (HLA-DR$^+$) almost doubled EFS: 1370 ± 173 *vs* 751 ± 104 days (Figure *c*). This was also associated with a significantly prolonged OS (*P* = 0.020).

Finally, none of the lymphocyte subsets in the peripheral blood at the day of aphaeresis correlated with any outcome parameter (OS, EFS).

DISCUSSION

The study was conducted between 1995 and 2000. In the meantime several new drugs and treatment regimens for relapsed patients have been developed. Since probably all patients received these kind of effective salvage treatment OS is most likely a poor parameter to assess the influence of AP lymphocytes on outcome. It seems reasonable to focus on EFS in the discussion of the results.

There is a trend towards a better prognosis with high leukocyte counts in the AP. This may be due to a more pronounced G-SCF responsiveness as an indicator for the quality of the bone marrow microenvironment [6]. However, peripheral blood leukocyte counts on the day of apheresis did not predict for EFS (data not shown). It has been shown previously that the absolute number of infused lymphocytes during ASCT predicts OS and time to progression in myeloma patients [2]. In our study the relative numbers of lymphocytes (CD3$^+$ and CD19$^+$) were associated with a trend towards a worse outcome, but this was due to the high numbers of granulocytes that reflect good bone marrow response to G-CSF. The relative amount of CD4 cells was associated with prolonged EFS in our study. The EFS was the same as the relapse free survival. The positive effect was therefore not due to prevention of infectious complications but improved tumor control. To the current knowledge more likely CD4 helper cells than cytotoxic T cells are capable of tumor surveillance and control [7–9]. Interestingly the most prominent association was found in our study with respect to activated lymphocytes (HLA-DR$^+$). Two similar studies did not reveal significant relations [10–11]. However in the first peripheral blood was examined and in the latter allogeneic transplantations were performed, making both studies only poorly comparable. In the context with the above mentioned results regarding CD4 cells the negative impact of the activation marker HLA-DR may be explained by the fact that activated T cells are

more differentiated and less potent to transfer regulatory immune functions.

The number of plasma cells and as well the number of B cells in the AP do not determine the clinical outcome of MM patients. This is in accordance with the failure of purging strategies [12] and supports the hypothesis that the number of high dose chemotherapy surviving cells within in the body are quite higher than the number of plasma cells reinfused while ASCT. In contrast, the relative B cell amount in the graft was associated with an almost significant worsening of EFS. This corresponds well to the results of B cell purging in MM [13] and supports the hypothesis of precursor B cells as origin of relapse [14–15]. However, clonality was not assessed in the presented study and the sample size were probably too small to reach statistical significance. Further studies are needed to address this topic.

Of note, a similar analysis was performed on lymphocytes in the PB at the day of leukapheresis, but none of the immunophenotyping parameters correlated with the outcome of the patients (data not shown). Most probably the high-dose chemotherapy diminishes the pre-existing immune function. More importantly, the lymphocytes within the graft significantly influence the immune function after ASCT and herewith contribute to disease control [10, 16]. Indeed it has been supposed that the lymphocytes within the bone marrow influence the course of disease [17].

In summary, we suggest that a relative high proportion of non-activated T helper cells within the graft predicts proper tumor control in MM patients after high-dose chemotherapy. Targeted lymphocyte preparation in the AP may further improve the results of ASCT in MM.

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Т-ХЕЛПЕРЫ (CD3$^+$/CD4$^+$) АУТОЛОГИЧЕСКОГО ТРАНСПЛАНТАТА СТВОЛОВЫХ КЛЕТОК КРОВИ КОРРЕЛИРУЮТ С БЕЗРЕЦИДИВНОЙ ВЫЖИВАЕМОСТЬЮ БОЛЬНЫХ С МНОЖЕСТВЕННОЙ МИЕЛОМОЙ

Микроокружение в костном мозге, включая лимфоциты, оказывает влияние на патофизиологию множественной миеломы (ММ). Высокодозовая химиотерапия, за которой следует аутологическая трансплантация стволовых клеток, является стандартным подходом при лечении более молодых пациентов. *Цель:* изучить влияние введенных субпопуляций лимфоцитов на безрецидивную выживаемость (EFS) и общую выживаемость больных с ММ. *Методы:* методом проточной цитофлуориметрии в периферической крови (PB) и продуктах афереза (AP) пациентов с ММ (n = 41) изучали субпопуляции лимфоцитов и возможную корреляцию с исходом болезни. *Результаты:* субпопуляции лимфоцитов PB не влияли на EFS или OS. Остаточные клетки плазмы в AP не коррелировали с плохим прогнозом, в тоже время при высоком содержании В-клеток (CD19$^+$) отмечали тенденцию к снижению EFS (*P* = 0,051). Высокое содержание CD4-клеток и увеличение соотношения CD4/CD8 были достоверно ассоциированы с повышением EFS. В отличие от этого, высокий процент HLA-DR-положительных лимфоцитов имел отрицательное влияние на EFS и OS (*P* = 0,03 и 0,02 соответственно). *Выводы:* полученные данные позволяют предположить, что неактивированные (HLA-DR-отрицательные) хелперные CD4$^+$ Т-клетки в AP могут обладать антиопухолевыми свойствами.

Ключевые слова: аутологический трансплантат, трансплантация стволовых клеток крови, корреляция, безрецидивная выживаемость, лимфоциты, множественная миелома.**