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SHORT COMMUNICATION

Changes in the Number of $CD8^+$ T Lymphocytes in the Peripheral Blood of Patients with Various Autoimmune Diseases after Autologous Hematopoietic Stem Cell Transplantations and their Relations to the Survival Times

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Abstract The changes in the number of $CD8^+$ T lymphocytes were studied before (0 day) and then 30 days after the autologous hematopoietic stem cell transplantations (AHSCT) in 14 therapy refractory patients with autoimmune diseases. The years of survival and the clinical states were also evaluated. The number of $CD8^+$ T cells was determined by an hematologic automat and by flow cytometry. Longer than 5-year survival times were found in 6 cases, whereas there was no progression (improvement) in 2 cases, and 4 patients were lost. The increase in the number of $CD8^+$ cytotoxic T cells was gradual in the first 2 months and reached the significantly highest values among all subtypes of lymphocytes. It was of a special interest that in all the 4 patients who died, the numbers of $CD8^+$ T cells were less than 150/μl on the 30th day after AHSCT, whereas all the 10 patients with a higher cell number survived. These results suggest that the early monitoring of the number (not only the ratio) of regenerating $CD8^+$ T cells in the peripheral blood can be a useful and quantitative laboratory measurement after AHSCT, and it has a significant relation also to the survival times of transplanted patients.

Keywords Autoimmune diseases · Autologous stem cell transplantation · $CD8^+$ T cells

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Introduction

High-dose chemotherapy followed by autologous hematopoietic stem cell transplantation (AHSCT) has become a promising approach to treat patients with severe, refractory autoimmune disorders. More than 1,000 patients have undergone the procedure so far, most of them suffered from systemic lupus erythematosus (SLE), systemic sclerosis (SSc), multiple sclerosis (MS) and rheumatoid arthritis (RA). It has become clear up to now that AHSCT can be beneficial for the patients with severe disease course, with life-threatening complications, but still without irreversible, end-stage organ damages (Nikolov and Pavletic 2008; Passweg and Tyndall 2007; Szodoray et al. 2010; Tyndall and Saccardi 2005). During the conditioning regimen, the patients' immune system is almost entirely eliminated. Earlier observations described that after stem cell transplantations, the first engrafting cells were monocytes, rapidly followed by granulocytes, platelets and natural killer cells, while the adaptive immune system recovered much slower. B- and T-lymphocyte counts normalized during the first months, yet T-cell immunity became impaired for years (Rutella et al. 2000; Storek et al. 2008). Recipients of purified $CD34^+$ progenitors promptly recovered their lymphocyte count, although numbers of circulating T cells and, in particular, of $CD4^+$ T lymphocytes were significantly depressed during the first 2 months after AHSCT. Thereafter, the number of most leukocyte subsets normalizes rapidly. However, T- and B-cell responses will be incomplete for a long period after transplantation (Williams and Gress 2008). This can be explained by the fact that most of the T cells lose the required homing receptors to enter the secondary lymphoid organs to interact with antigen-presenting cells and stimulate B cells (Rutella et al. 2000; Storek et al. 2008). The

recovery time for the $CD56^+$ and $CD8^+$ lymphocyte subsets was about 90 days, whereas it was about 150 days for the $CD4^+$ and B cells (Szodoray et al. 2012).

Alexander et al. (2009) investigated the immunological factors needed for the long-term remission in patients with refractory SLE treated with AHSCT. They found that the depletion of autoreactive immunologic memory subset of $CD4^+$ T lymphocytes and the profound resetting of immune adaptive immune system were required to establish self-tolerance (Alexander et al. 2009). Recently, in a European retrospective multicentric survey, the 5-year overall survival of SLE patients treated with AHSCT was 81 % (Alchi et al. 2013). However, lethal complications also occur rarely (Váróczy et al. 2012).

In our department, the first AHCST was carried out on a 28-year-old woman with therapy refractory SLE in 2006, 7 years ago. Up to now, we have been applying this method altogether in 14 patients with various autoimmune diseases including SLE, SSc, RA, MS and Sjögren's syndrome (SS). In this study, we present the changes in the number of peripheral lymphocyte subsets tested just before AHCST (0 day) and 30 days after the transplantation; in addition, we analyzed their relation to the survival times of patients.

Materials and Methods

Patients

Fourteen patients (9 females, 5 males, range of age 28–63 years, mean 42.2 years) with severe, refractory systemic autoimmune diseases were enrolled in the study carried out at the Division of Clinical Immunology, Institute of Internal Medicine, Medical and Health Science Centre, University of Debrecen in Hungary. All ethical permissions were collected for the AHSCT interventions on the 3 patients with SLE, 3 patients with SSc, 2 patients with RA, 2 patients with MS and 4 patients with "overlap" syndrome (2 autoimmune diseases together). The inclusion criteria were based on the internationally accepted protocols of onco-hematology adapted by the European Group for Blood and Marrow Transplantation and the European League Against Rheumatism. The demographic, clinical and therapeutic data are summarized in Table 1.

For stem cell mobilization, the following protocol was used: cyclophosphamide 2–4 g/m^2 and granulocyte colony-stimulating factor 10 μg/kg. Mononuclear cell apheresis was carried out if the $CD34^+$ cell count was >20/ml (Baxter Amicus cell separator, Baxter International Inc., USA). The grafts were frozen without any manipulation in patients No. 1, 2, 3, 4, 5, 7, 10, 11, 13, 14; however, $CD34^+$ cell selection was applied in the grafts of scleroderma patients (No. 6, 8, 9, 12). Serum blood sampling was also carried out from each patient before transplantation and then subsequently on the 30th days after AHSCT.

Determination of the Number of Peripheral Blood Lymphocyte Subpopulations

The absolute number of lymphocytes with various CD markers were calculated based on the total lymphocyte count (measured by an hematologic automat, Sysmex, Japan), and the percent of various CD^+ subsets was determined by flow cytometry. The results were expressed as "cells/μl."

To determine the ratio (percent) of the various lymphocyte subpopulations in freshly isolated heparinized blood samples, we used monoclonal antibodies against the cell surface markers of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD56^+$ (BD Biosciences, USA; Immunotech, France; Beckman Coulter, USA; Serotec, UK). The samples were processed according to the Coulter Q-PREP protocol (Beckman Coulter, USA). In brief, 100 μl of whole blood was incubated with monoclonal antibodies for 30 min, followed by erythrocyte lysis using an "ammonium chloride-EDTA" solution (Sigma-Aldrich, Hungary). Then the samples were washed by phosphate-buffered saline prepared in the laboratory, containing additionally 10 mg/l of bovine serum albumin and 2 mg/l of sodium azide (both purchased from Sigma-Aldrich, Hungary). Finally, the cells were suspended and fixed by paraformaldehyde (1 %; Sigma-Aldrich, Hungary). The measurements were performed and data collected on a Coulter EPICS XL flow cytometer (Beckman Coulter Inc.). Lymphocytes, monocytes and granulocytes were differentiated based upon their size and granulation patterns. The lymphocyte subsets were further analyzed from the "lymphocyte gate". The ratios of subsets were expressed and documented as "percentage of all lymphocytes".

Statistical Analysis

The results represent the mean ± SD values. The differences were statistically characterized by the non-parametric Mann–Whitney test. For the investigation of survival rates after transplantation, the Kaplan–Meier estimator (plot analysis) was applied (Kaplan and Meier 1958). The calculation was carried out by the statistical software SPSS 20.0. The changes showing a $p < 0.05$ value were regarded as statistically significant.

Results

Demographic, Clinical, Therapeutic and Survival Data of 14 Patients treated with AHSCT

According to the data of Table 1 from the 14 patients (9 women, 5 men) treated with AHSCT, 4 patients (3 women,

Table 1 Demographic, clinical, therapeutic and survival data of 14 patients treated with AHSCT

Patient no.	Gender	Age (years)	Diagnoses	Former treatment	Date of TX	No. of CD34 cells (10^6 cell/bw kg)	Overall survival (months)	Progression free survival (months)	Death	Current therapy
1	F	28	SLE	Steroid, azathiorin, cyclosporin	07 Aug 2006	5.7	1	0	TX + 47 days	–
2	M	34	SLE	Steroid, cyclophosphamid, cyclosporin	04 Sep 2006	2.9	75	21	No	Azathioprin
3	F	59	RA	Methotrexate, leflunomide, cyclosporin, etanercept	22 Nov 2006	6.1	72	48	No	Rituximab
4	F	52	RA + MS	Methotrexate, eflunomide, cyclosporin	15 Jan 2007	3.4	70	35	No	Rituximab
5	F	53	RA + SS	Methotrexate, leflunomide, cyclosporin, etanercept	19 Feb 2007	7.0	69	6	No	Rituximab
6	F	63	SSc	D-penicillamine, plasmapheresis	02 Oct 2007	17.0	62	62	No	–
7	F	51	RA	Methotrexate, leflunomide, sulphasalazin, adalimumab	20 Nov 2007	10.1	61	32	No	Methotrexate
8	F	39	SSc, RA	Methotrexate	22 Apr 2008	12.4	5	0	TX + 162 days	–
9	M	52	SSc	None	03 Jun 2008	7.45	54	54	No	–
10	F	41	SLE	Cyclophosphamid, azathioprin	21 Oct 2008	9.7	12	11	TX + 12 months	–
11	F	52	OL	Methotrexate, leflunomide, cyclophosphamid	20 Jan 2009	10.2	28	20	No	Azathioprin
12	M	28	SSc	Cyclophospamid	27 Apr 2009	3.1	14	13	TX + 14 months	–
13	M	28	MS	Mitoxantrone	10 Jan 2010	6.5	35	27	No	–
14	M	39	MS	Mitoxantrone	10 Nov 2010	5.0	25	6	No	–

M male, *F* female, *SLE* systemic lupus erythematosus, *RA* rheumatoid arthritis, *MS* multiple sclerosis, *SS* Sjögren's syndrome, *SSc* systemic sclerosis, *OL* overlap, *TX* transplantation

1 man) were lost (2 from infections, 2 from relapses) in the last 7 years (28.6 %). The surviving time longer than 5 years is found in 6 (60 %) cases, whereas the surviving time shorter than 5 years found in 4 (40 %). The average time of overall survival is 42.6 months, the average time of progression free survival is 23.9 months. The ratio of relapse incidence is 60 % (6/10), the ratio of non-relapse mortality is 14 % (2/14). The number of responders (with surviving time longer than 1 year) is 12 (86 %), the number of non-responders (with surviving time shorter than 1 year) is 2 (14 %). The establishment and evaluation of relapses took place using the internationally accepted activity indexes of the given diseases, e.g., SLEDAI.

Changes in the Number of the Various Subsets of Lymphocytes in the Peripheral Blood of 14 Patients with various Autoimmune Diseases just before and 30 Days after AHSCT

Table 2 demonstrates and summarizes the data of the changes in the number of all lymphocytes, including the $CD3^+$, $CD4^+$, $CD8^+$, $D19^+$, $CD56^+$, $CD3^+CD56^+$ subsets measured in the peripheral blood of 14 patients just before (0 day) and 30 days after AHSCT, comparing the results of 10 surviving and 4 patients lost. In addition, we compared the median times of polymorphonuclear neutrophil (PMN), lymphocyte and platelet cell recoveries. We have found statistically significant changes in the cell counts of $CD3^+$ and $CD8^+$ T cells measured on the 30th day, and during the recovery time of PMNs. Namely, the number of $CD3^+$ cells at a less level of significance ($p = 0.014$) and the number of $CD8^+$ T cells at a higher level of significance ($p = 0.004$) were lower in the group of lost patients than that in the surviving subjects. It is of a special interest that the number of $CD8^+$ cells in the lost patients never exceeded the value of 150 cells/μl.

Differences in the Median Recovery Times for PMNs, Lymphocytes, Platelets, and in the Ages and Genders of Survived and Lost Patients Treated with AHSCT

In addition, we compared the median times of PMN, lymphocyte and platelet recoveries and the distribution of ages and the genders. The median time of neutrophil (PMN) recovery was significantly longer in this group of patients ($p = 0.02$). Similar tendency was observed also concerning the recovery time of lymphocytes. However, in consequence of the low number of available data, it could not be evaluated statistically. In addition, such tendency appeared in the group of women with younger age that they would probably have shorter survival times than the other patients treated with AHSCT. The recovery times of

Table 2 Changes in the number of the various subsets of lymphocytes in the peripheral blood of 14 patients with various autoimmune diseases just before and 30 days after AHSCT

Patient no.	Lymphocytes		$CD3^+$		$CD4^+$		$CD8^+$		$CD19^+$		$CD56^+$		$CD3^+CD56^+$	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
Surviving patients														
2	350	600	193	180	84	18	116	162	7	3	113	330	3	3
3	1,480	900	1,215	180	710	69	51	122	266	8	190	437	17	5
4	844	653	479	208	311	40	143	174	109	20	177	389	17	27
5	740	930	572	434	349	106	229	208	348	2	86	266	14	36
6	1,160	2,230	668	638	475	154	185	528	162	4	267	1,210	9	9
7	1,580	1,470	1,290	440	705	135	570	308	60	6	75	821	30	16
9	810	640	554	454	361	143	181	192	81	3	80	128	24	2
11	640	890	443	383	167	45	253	318	15	3	142	406	86	53
13	890	725	284	375	135	38	123	306	167	2	110	259	NA	NA
14	648	1,870	355	1,478	179	103	132	1,274	62	7	161	299	NA	NA
Lost patients														
1	200	200	172	79	55	<5	139	77	<5	3	9	17	3	6
8	610	630	350	91	302	72	65	15	68	3	131	408	23	2
10	920	240	582	44	141	18	447	13	8	1	305	848	11	1
12	790	940	624	369	278	276	331	133	92	5	48	308	38	32
p values	0.240	0.106	0.635	0.014*	0.188	0.539	0.839	0.004***	0.188	0.454	0.635	1.000	0.808	0.283

NA not available data

*,*** Significant changes

platelets did not differ significantly. These data are shown in Table 3.

In the healthy volunteer controls, the average numbers of various lymphocyte subsets were as follows (in cells/μl): lymphocytes—3,258, $CD3^+$—2,582, $CD4^+$—1,880, $CD8^+$—702, $CD19^+$—240, $CD3^+/56^+$—90.

Table 3 Differences in the median recovery times for PMNs, lymphocytes, platelets, and in the ages and genders of survived and lost patients treated with AHSCT

Patients no.	PMN (day)	LY (day)	Platelet (day)	Age (years)	Gender (F/M)
Surviving patients					
2	10	NA	25	34	F
3	9	30	14	59	M
4	10	17	15	52	F
5	9	18	14	53	F
6	9	14	14	63	F
7	10	16	15	51	F
9	9	NA	14	52	M
11	10	NA	15	52	F
13	9	35	13	28	M
14	9	14	14	39	M
Lost patients					
1	11	NA	20	28	F
8	11	NA	15	39	F
10	11	45	21	41	F
12	10	30	14	28	M
p values	0.02*		0.240	0.054	

NA not available data, *LY* lymphocyte

* Significant change

Kaplan–Meier Plot Analysis of the Survival Times in the Two Groups of Patients Showing Higher and Lower than 150 cells/μl of $CD8^+$ T Cells 30 Days after AHSCT

Figure 1 shows that the 5-year survival rate is 1.0 (100 %) for the patients whose $CD8^+$ T-cell numbers were higher than 150 cells/μl 30 days after AHSCT, whereas this value is 0.34 (34 %), significantly less ($p = 0.007$) for the patient who had $CD8^+$ T-cell numbers lower than this value. According to this calculation, testing this relatively small number of cases, the chance (probability) for a 5-year survival with a $CD8^+$ T-cell number higher than 150 cells/μl found 30 days after transplantation was almost 100 %; on the other hand, this probability with a lower $CD8^+$ T-cell count was only 34 % after the first 14 months.

Discussion

In the present study, we stress on the following new findings: (a) the determination of the "absolute" $CD8^+$ T-cell count is a practical quantitative laboratory parameter for monitoring of T-cell regeneration after AHSCT in the daily routine; (b) the $CD8^+$ T-cell number less than 150 cells/μl measured on the 30th days after AHSCT shows a rather

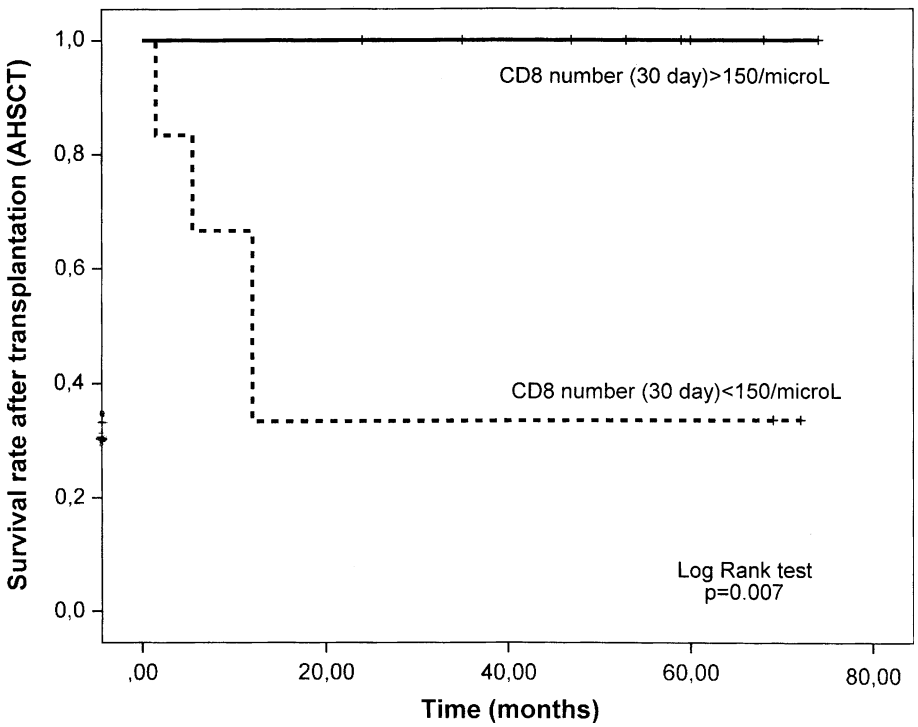

Fig. 1 Kaplan–Meier plot analysis of the survival times in the two groups of patients showing higher and lower than 150 cells/μl of $CD8^+$ T cells 30 days after AHSCT

unfavorable probability for the 5-year survival of the patients, whereas a $CD8^+$ T-cell number higher than 150 cells/μl means a good chance; (c) this observation, however, needs further confirmation on a greater number of patients.

The special importance of $CD8^+$ T cells determinations for the characterization of actual state of immune system in various types of transplanted patients or subjects with various immunological defects was also already observed in several other works as follows: the $CD8^+$ lymphocyte dose in the autograft was critical for early lymphocyte recovery after AHSCT (Atta et al. 2009); higher number of survivors occurred among patients whose $CD8^+CD3^+$ absolute counts rose above the age-matched normal levels in children undergoing allogenic stem cell transplantation (Koehl et al. 2007); the patients with B-cell non-Hodgkin's lymphomas showing $CD8^+$ cell number less than 200 cells/μl were corresponding with a significantly inferior overall survival on the targeted immuno-chemotherapy (Gergely et al. 2011); $CD8^+$ T-cell transcription signature predicted prognosis in autoimmune diseases (McKinney et al. 2010); regulatory T cells increased the avidity of primary $CD8^+$ T-cell responses and promoted memory (Pace et al. 2012).

For a 2-year period, in 4 patients, we had the opportunity to follow up the changes in the number of $CD8^+$ T cells and the serum level of IL-2, IL-3, IL-4, IL-6, IL-7, IL-15 and IL-16 in parallel before and 30 days after AHSCT. Only the level of IL-6 showed a positive correlation with the number of $CD8^+$ T cells (in consequence of the low number of cases, these data are not presented).

In conclusion, these results suggest that the determination of "absolute" number (not only the ratio, percent) of the $CD8^+$ T cells 30 days after AHSCT for monitoring the regeneration of peripheral blood cells can be a practical and quantitative laboratory method and marker, in addition, has a significant relation to the survival times of transplanted patients.

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