Ageing of long-term allogeneic hematopoietic cells recipients compared to their donors

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Abstract

Background: Ageing is a complex phenomenon that leads to decreased proliferative activity, loss of function of the cells, and cellular senescence. Senescence of the immune system exacerbates individual's immune response, both humoral and cellular but increases the frequency of infections. We hypothesized that physiological ageing of adaptive immune system occurs in recipients of allogeneic hematopoietic cells transplant (allo-HCT) at faster rate when compared to their respective donors since the small number of donor cells undergo immense proliferative stress restoring recipients hematopoiesis. We compared molecular characterizations of ageing between recipients and donors of allo-HCT: telomeric length, proinflammatory cytokines concentration, and immunophenotypic changes in main lymphocyte subsets – $CD4^+$, $CD8^+$, $CD19^+$, $CD56^+$.

Results: Median telomeric length (TL) of $CD8^+$ lymphocytes was significantly longer in donors compared to recipients (on average 2,1kb and 1,7kb respectively, p = 0,02). Similar trends were observed for $CD4^+$ and $CD19^+$ although the results did not reach statistical significance. We have also found trends in the immunophenotype between recipients and donors in the subpopulations of $CD4^+$ (naïve and effector memory), $CD8^+$ $Eomes^+$ and B-lymphocytes (B1 and B2). When grouped according to the history of infections, there were differences between recipients in the TNF-a and Il-4 concentrations. They were higher in the recipients with lower infection number p=0,05 and p=0,003 respectively. Lower infection risk recipients had also significantly greater percentage of NK cells (22,3%) than high-risk patients (9,3%) p=0,04.

Conclusions: Our results support the hypothesis of accelerated ageing of the recipients lymphocytes observed mainly in CD8 subset compared to their respective donors. Quantitative changes in recipients immunophenotype in some lymphocyte subsets resemble physiological ageing-associated changes. No difference in cytokine concentrations suggests that inflammageing does not increase in allo-HCT recipients, however a lower infection numbers in HCT recipients seems to be associated with increased concentration of proinflammatory cytokines and increased percentage of NK cells. The GVHD does not affect the rate of ageing. Therefore, the differences between recipients and donors cells may result from the proliferative stress in the early period after allo-HCT and the difference between hosts and recipients microenvironments, the only other variable that may influence the identical cells originating from donor hematopoiesis.

Background

Ageing involves a series of biological processes that lead to gradual loss or change in the function of body cells. Although many questions remain unanswered, some molecular mechanisms of ageing have already been identified. They include telomeric shortening, the phenomenon of "inflammageing" and age-associated changes in immunophenotype.

Telomeric shortening occurs with every cellular division. After reaching the critical length of telomeres a cell enters the senescent phase or undergoes apoptosis (1). The most pronounced telomeric shortening throughout a life happens in lymphocytes. At birth mean telomeric length for lymphocytes is ~ 11kb and decreases to ~ 4kb at the age of 100 years. With ageing, telomeric shortening gradually decelerates. The average annual rate of telomeric shortening for human lymphocytes is 1190bp in the first year of life, than 126bp/year in childhood and 43bp/year through the rest of adult life (2).

Physiologically, ageing is associated with a peculiar phenomenon of low-grade chronic sterile inflammatory process termed as "inflammageing". It results from the chronic physiological stimulation of the innate immune system, and is characterized by a moderately increased concentration of proinflammatory cytokines (2). Immunophenotypic changes during inflammageing include, among others, an increase in the proportion of anergic $CD8^+$ lymphocytes leading to a decreased ratio of $CD4^+/CD8^+$ lymphocytes, an increased proportion of Treg and Th2 lymphocytes, and loss of CD28. (3)(4). $CD28^-$ T-cells are characterized by reduced replicative lifespan and decreased proliferative capacity, as well as by reduced response for antigen stimulation while exhibiting increased cytotoxic activity (5). Inflammageing is the major cause of morbidity and mortality in the elderly. (6)(7)

The key aspect of allogeneic hematopoietic cells transplantation (allo-HCT) is the restoration of the whole hematopoiesis in the recipient from the relatively small 2–5 x 106/kg number of donor stem cells. Thus, the transplanted cells are exposed to immense proliferative stress compared to identical cells that remain in the donor system. (8). The immune part of the hematopoietic system is particularly exposed to the proliferative stress since it is also stimulated by the differences between recipient donor minor histocompatibility antigens (MiHAs) leading to the graft versus host reaction which clinically is manifested as graft versus host disease (GvHD) (9). Moreover, recipients of allo-HCT are susceptible to infectious complications that cause additional proliferative stress to immune cells (10). Consequently, we have hypothesized that progeny of the donor HSC in the recipients of allo-HCT undergoes accelerated aging, which may be responsible for these clinical consequences.

Thus, in our study we compared 1) the magnitude of telomeric shortening of the transplanted donor cells subpopulation to the same cells subpopulations that remained intact in the donor 2) immunophenotypic changes of respective lymphocyte subpopulations and 3) the proinflammatory cytokine concentrations reflecting the postulated phenomenon of inflammageing between donors and their respective recipient.

Methods

Patients

We enrolled 20 pairs of donors (D) and their related recipients (R) undergoing the allo-HCT at least more than 12 years ago (long-term survivors) at the University Clinical Center, Medical University of Gdańsk, Gdańsk, Poland (EBMT accredited center 799). The number of pairs (20) was limited by overall mortality related to the procedure and availability of long-term survivors. For every recipient-donor pair sample of 50ml of full venous blood were collected with anticoagulant (EDTA), at single timepoint.

GvHD and infectious status assessment

Patients were stratified according to chronic GvHD status (Yes versus No) and infectious complications according to an infection risk status score (Table 1.) that was based on the number of infections in the last year and the need for antibiotic usage or hospitalization.

Table 1
Infection risk status

Infection risk status			
No of episodes of infections (during last year)	**SCORE**		
	Without antibiotic	With antibiotic	Hospitalization
0	0	0	0
1	1	2	3
2	2	4	6
≥3	3	6	9
			TOTAL
Low risk			< 3
High risk			>=3

Peripheral blood mononuclear cells (PBMC) and lymphocyte isolation

PBMC were obtained from venous blood and centrifugation over a Ficoll-Hypaque (Ficoll-Paque PLUS assay (GE Healthcare, USA) gradient. Lymphocytes were isolated from PBMC by immunomagnetic positive separation technique with magnetic particles (EasySep Kit III from STEMCELL™ Technologies) recognizing respective $CD4^+$, CD8+, CD19 + or CD56 + antigens. The purity of each cell population was > 90% (assessed by flow cytometry), sufficient for further parts of the experiment. (4) (11) Isolated lymphocyte subpopulations were pelleted by centrifugation and stored at -80^{o}C for further processing.

Telomeric length measurement

Determination of the average telomeric length was performed using quantitative polymerase chain reaction (qPCR) applying the commercially available Absolute Human Telomere Length Quantification qPCR Assay Kit (from ScienCell Research Laboratories). The single-copy reference primers (included in the kit), recognizing and amplifying a 100bp sequence of chromosome 17 were used as a reference for data normalization. The reference DNA sample with established telomere length (also included in the kit) served as a reference for the assessment of the telomeric length. Acquired results for every sample were then computed according to the manufacturer's instructions. The total length of all telomere ends in a single cell was divided by the number of telomeric ends (92) which is the final result shown in the Fig. 1. The final result is a median of two independent measurements per individual sample.

Immunophenotyping

Stored lymphocytes obtained as above were thawed and their viability was checked with trypan blue assay using TC20 Automated Cell Counter (Bio-Rad Laboratories, USA. The viability cut-off was set to 80%. Next, samples of $2x10^5$ cells were stained with anti-CD45 (clone HI30), anti-CD3 (clone OKT3), anti-CD4 (clone MEM-241), anti-CD19 (clone HIB19), anti-CD5 (clone UCHT2), (all from Thermo Fisher Scientific, USA) and anti-CD8 (clone RPA-T8), anti-CD56 (clone NCAM16.2) (all from BD Bioscience, USA). For intracellular staining anti-Foxp3 (clone PCH101), and anti-Helios (clone 22F6) were used with Foxp3 / Transcription Factor Staining Buffer Set (all from Thermo Fisher Scientific, USA). Samples were read out with LSRFortessa flow cytometer (BD Bioscience, USA) and for every sample, a minimum of 75.000 events was recorded.

Flow cytometry data were analyzed with Kaluza 1.2 software (Beckman Coulter, USA). First, doublets were excluded by FSC area (FSC-A) and FSC height (FSC-H) discrimination, and then lymphocytes were identified upon SSC/$CD45^+$ gating. Major lymphocytes subsets were identified as: lymphocytes T, both $CD3^+/CD4^+$, and $CD3^+/CD8^+$, lymphocytes B, $CD19^+$, NK cells $CD3^-/CD56^+$, B1 B cells, $CD5^+/CD19^+$, B2 B cells, $CD5^-/CD19^+$, and regulatory T cells, $Foxp3^+/CD4^+/CD3^+$. Gating was done upon FMO (fluorescence minus one) approach. The absolute count of $CD4^+$ and $CD8^+$ was calculated using a percentage of $CD4^+$ and $CD8^+$ from immunophenotyping and absolute lymphocyte count (ALC) obtained from Sysmex hematology analyzer.

Proinflammatory cytokine concentrations

Il-1B, Il-2, Il-4, Il-6, Il-10, TNF-α and Il-17F concentrations were quantified using commercially available Flex™ Sets (Becton Dickinson) and flow cytometry technique, according to manufacturer's instructions. The results are given as a median (average of) three independent measurements.

Statistical analysis

All statistical calculations were performed using the StatSoft Inc. 2014 – STATISTICA version 12.0 (www.statsoft.com) and Microsoft Excel spreadsheet. Quantitative variables were characterized by the arithmetic mean, standard deviation, median, minimum and maximum (range), and 95%CI (confidence interval). Qualitative variables were displayed by number and percentage unless noted otherwise. For testing, if the quantitative variable was derived from the population with the normal distribution, the W Shapiro-Wilk test was selected. For testing the hypothesis of equal variances, the Leven's (Brown-Forsythe) test was used. Significance of differences between two groups (independent samples model) was tested by Student's t-test (in case of lack of homogeneity of variance – Welch t-test) or by U Mann-Whitney test (in case of not fulfilling the conditions to use the Student's t-test or for ordinal variables). The significance of differences between more

than two groups was verified using Kruskal-Wallis test. In the case of receiving statistically significant differences between groups, Dunn test was performed. Data were visualized using box and whiskers plot displays. The confidence interval (CI) of 95% was preconceived, p value < 0.05 was considered significant.

Results

Patient characteristics is summarized in Table 2. The median time from HCT was 17,4 (range 12 to 25) years. Twelve male and 8 female recipients received allo-HCT due to a variety of hematological disorders (Table 2). Eight (40%) recipients had a history of chronic GvHD. None of these recipients required active immunosuppressive treatment at the time of study enrollment. Infectious status was low in 12 recipients whereas the rest had high risk (8) infectious status according to our infectious risk stratification model (Table 1).

Table 2
Patients' characteristics

Patient no.	Diagnosis	Sex (R/D)	Time since allo-HCT (years)	Age at allo-HCT (years) R/D	Conditioning regimen	Chronic GvHD *	Infection risk status (low, high) **
1	CML	M/F	25	33/27	BuCy	-	Low
2	ALL	F/M	18	20/15	TBI	-	High
3	AML	M/M	15	23/25	BuCy	-	Low
4	AML	F/M	20	36/46	BuCy	Yes	Low
5	HES	M/F	19	32/33	BuCy	-	Low
6	CML	M/M	18	46/43	BuCy	-	Low
7	CML	F/F	17	22/10	BuCy	Yes	Low
8	PNH	M/M	18	27/20	BuCy	-	Low
9	CML	M/F	23	39/41	BuCy	Yes	High
10	AML	M/F	14	43/39	BuCy	Yes	High
11	AML	F/F	17	47/43	BuCy	Yes	High
12	CML	M/M	19	36/18	BuCy	-	High
13	ALL	F/M	24	28/24	BuCy	-	Low
14	AML	M/F	15	31/28	BuCy	-	Low
15	CML	M/M	20	44/43	BuCy	-	Low
16	MDS	F/F	12	42/43	BuCy	Yes	High
17	CML	F/M	17	38/43	BuCy	-	High
18	AML	F/M	12	38/38	BuCy	Yes	Low
19	CML	M/M	13	33/22	BuCy	Yes	High
20	AML	M/F	12	41/54	BuCy	-	Low

* History of chronic cGvHD

** Status assessment according to Table 1.

(CML – chronic myelogenous leukemia, ALL – acute lymphoblastic leukemia, AML – acute myelogenous leukemia, HES – hypereosinophilic syndrome, PNH – paroxysmal nocturnal hemoglobinuria, MDS – myelodysplastic syndrome, R – recipient, D – donor, MRD – matched related donor, MUD – matched unrelated donor, BuCy – busulfan & cyclophosphamide, TBI – total body irradiation)

Results

Pairwise (recipients vs donors) comparison of telomeric length

Median of telomeric length, expressed in kb per chromosome end) in $CD8^+$ lymphocytes was significantly greater in D (2,1kb [95%CI 1,8;2,7]) compared to R (1,7kb [95%CI 1,4;1,9]) (p = 0,02; n = 40). There were also similar tendencies in $CD4^+$ and $CD19^+$ lymphocyte subpopulations, respectively D – 2,2kb [95%CI 1,8;3,8], R – 1,6kb [95%CI 1,4;2,4] (p = 0,1; n = 40) and D –

2,3kb [95%CI 2,1;2,9], R- 2,1kb [95%CI 1,7;2,4] (p = 0,076; n = 40), although they have not reach statistical significance. We have not found differences in the $CD56^+$ population (D – 2kb [95%CI 1,8;2,3], R – 2kb [95%CI 1,5;2,3] (p = 0,53) (n = 40)) (Fig. 1.).

Also, we have not found any statistically significant differences nor any obvious trends in TL between recipients grouped according to infection status and GvHD status (Supplementary materials – Table 1. – Table 8.).

Proinflammatory cytokine concentrations

There were no statistically significant differences in proinflammatory cytokine concentrations i.e. Il-1β, IL-2, IL-4, IL-6, IL-10, TNF-α and IL-17 in any recipient-donor pair measured at a single time point after allo-HCT (Supplementary materials – Table 9.). Additionally, we have found no statistically significant differences in proinflammatory cytokine concentrations between recipients of allo-HCT grouped according to chronic GvHD history (Supplementary materials – Table 10.).

The analysis of proinflammatory cytokine concentrations in recipients of allo-HCT, grouped according to the infection status (low risk versus high risk) (see Table 1.) has shown statistically significant differences in concentrations of IL-4 and TNF-**α**. The median IL-4 concentration was higher in low-risk recipients – 0,07 pg/ml (95%CI 0,03;0,15) than in high-risk recipients – 0,00 pg/ml (95% CI 0,00; 0,02) (p = 0,003). Similarly, the median TNF **α** concentration was also higher in. low risk recipients – 0,36 pg/ml (95%CI -0,15;2,19) than in high risk recipients – 0,22 pg/ml (95%CI 0,03;0,39) (p = 0,0449) (Table 3.).

Table 3
Proinflammatory cytokine concentrations comparison between recipients of allo-HCT grouped according to infection risk status.

	Low risk (n = 12)	**High risk (n = 8)**	***P*-value U Mann-Whitney**
IL-2 (pg/ml)			0,1770
mean (SD)	0,22 (0,51)	0,03 (0,04)	
range	0,00–1,83	0,00–0,10	
median	0,09	0,01	
95%CI	[-0,10;0,55]	[0,00;0,06]	
IL-4 (pg/ml)			**0,0030**
mean (SD)	0,09 (0,09)	0,01 (0,01)	
range	0,00–0,27	0,00–0,03	
median	0,07	0,00	
95%CI	[0,03;0,15]	[0,00;0,02]	
IL-6 (pg/ml)			0,3348
mean (SD)	1,19 (1,50)	0,69 (0,20)	
range	0,38 – 5,42	0,48 – 1,10	
median	0,51	0,61	
95%CI	[0,23;2,14]	[0,52;0,86]	
IL-10 (pg/ml)			0,8471
mean (SD)	0,62 (0,86)	0,45 (0,27)	
range	0,00–3,20	0,11 – 0,87	
median	0,45	0,39	
95%CI	[0,08;1,17]	[0,23;0,67]	
TNF α (pg/ml)			**0,0449**
mean (SD)	1,02 (1,85)	0,21 (0,21)	
range	0,09 – 6,78	0,00–0,62	
median	0,36	0,22	
95%CI	[-0,15;2,19]	[0,03;0,39]	
IL-1B (pg/ml)			0,9692
mean (SD)	0,21 (0,52)	0,04 (0,08)	
range	0,00–1,73	0,00–0,22	
median	0,00	0,00	
95%CI	[-0,12;0,54]	[-0,03;0,11]	

	Low risk (n = 12)	High risk (n = 8)	*P*-value U Mann-Whitney
IL-17F (pg/ml)			0,9385[1]
mean (SD)	0,08 (0,28)	0,08 (0,24)	
range	0,00–0,98	0,00–0,67	
median	0,00	0,00	
95%CI	[-0,10;0,26]	[-0,11;0,28]	

Immunophenotype analysis

Median percentage of T CD4 + was significantly greater in D than in R: 44,3% (95%CI 37,2;48,3) and 40,1% (95%CI 31,9;40,8) respectively (p = 0,05; n = 34). In contrast $CD19^+$ percentage was greater in R than in D: mean 11,3% (95%CI 9,8;13,5) and 8,5% (95%CI 7,8;11,9) respectively (p = 0,03; n = 34). (Table 4) Moreover we observed difference trends in few others lymphocyte subpopulations (p value approaching 0.05, Table 4). Among the population of $CD4^+$ there was greater percentage of effector memory ($CD4^+$ EM) cells in R than D: 28,8% (95%CI 23,4;37,5) and 19,8% (95%CI 16,5;27,8) (p = 0,07; n = 34) respectively and lower percentage of $CD4^+$ naïve cells in R than D: 24,5% (95%CI 16,9;33,8) and 38% (95%CI 28,8;43,5) (p = 0,06 n = 34) respectively. Among the $CD8^+$ subpopulation there was greater percentage of $CD8^+$ expressing eomesodermin ($CD8^+$ Eomes) in R – 39,4% (95%CI 29,7;47,7) than D – 31,5% (95%CI 24,2;36,7) (p = 0,07; n = 34). Among the CD19 + population there was greater percentage of B1 lymphocytes in D – 21,7% (95%CI 17;27,5) than R – 17,2% (95%CI 12,8;24,3) (p = 0,08; n = 34) and greater percentage of B2 lymphocytes in R – 81,6% (95%CI 74,4;86,4) than D – 77% (95%CI 71,1;82) (p = 0,07; n = 34)) (Table 4.).

Table 4
Immunophenotypic differences between recipients and donors of allo-HCT

	R (n = 17)	D (n = 17)	*P*-value
$CD4^+$			**0.05**
mean (SD)	36,4 (8,4)	42,8 (10,4)	
range	19,9–49,5	16,8–58,4	
median	40,1	44,3	
95%CI	[31,9;40,8]	[37,2;48,3]	
$CD4^+$ Effector Memory			0.07
mean (SD)	30,4 (13,2)	22,2 (10,7)	
range	13,0–59,0	9,0–54,1	
median	28,8	19,8	
95%CI	[23,4;37,5]	[16,5;27,8]	
$CD4^+$ Naive			0.06
mean (SD)	25,3 (15,9)	36,1 (13,8)	
range	4,6–55,3	2,4–52,9	
median	24,5	38,0	
95%CI	[16,9;33,8]	[28,8;43,5]	
$CD8^+$ Eomes			0.07
mean (SD)	38,7 (16,3)	30,4 (11,2)	
range	1,3–66,9	11,1–49,5	
median	39,4	31,5	
95%CI	[29,7;47,7]	[24,2;36,7]	
$CD19^+$			**0.03**
mean (SD)	11,7 (3,4)	9,8 (3,9)	
range	7,4–20,0	5,9–19,5	
median	11,3	8,5	
95%CI	[9,8;13,5]	[7,8;11,9]	
B1			0.08
mean (SD)	18,5 (10,8)	22,2 (9,8)	
range	2,6–49,8	5,7–47,4	
median	17,2	21,7	
95%CI	[12,8;24,3]	[17,0;27,5]	

	R (n = 17)	D (n = 17)	*P*-value
B2			0.07
mean (SD)	80,4 (11,2)	76,5 (10,2)	
range	48,1–97,2	50,4–93,7	
median	81,6	77,0	
95%CI	[74,4;86,4]	[71,1;82,0]	

$CD4^+/CD8^+$ ratio

Median $CD4^+/CD8^+$ ratio was higher in donors than in recipients of allo-HCT – 2,1 (95%CI 1,3;2,1) and 1,5 (95%CI 1,8;2,6) respectively (p = 0,0396) (n = 38) (Table 5.).

Table 5

$CD4^+/CD8^+$ ratio in recipients of allo-HCT and their donors.

	R (n = 19)	D (n = 19)	*P*-value (U Mann-Whitney)
$CD4^+$ to $CD8^+$			**0,0396**
mean (SD)	1,7 (0,9)	2,2 (0,9)	
range	0,7 – 4,6	1,0–4,6	
median	1,5	2,1	
95%CI	[1,3;2,1]	[1,8;2,6]	
[1]U Mann-Whitney			

Analysis of the recipients of allo-HCT depending on the GvHD status

Immunophenotype analysis according to GvHD and infectious status

Differences in immunophenotype were tested in recipients divided into two groups: with and without history of cGvHD history. We have found significant differences in the percentage of T $Helios^-$ with the expression of eomesodermin (T $Helios^-$ $Eomes^+$), B1 with the expression of PD1 and B2 with the expression of PD1 – in all aforementioned the higher percentage was found in cGvHD group p-value was 0,0,227, 0,0147 and 0,0448 respectively (n = 34). In contrast, a higher percentage in the group without cGvHD was found in the population of $CD19^+$ cells with the expression of PD1. (Table 6.).

Table 6
Immunophenotype comparison between recipients of allo-HCT grouped according to chronic GvH disease history.

Parameter	cGvHD	Without cGvHD	*P*-value
Treg Helios- Eomes			**0,0227**
mean (SD)	4.1 (1.3)	8.7 (4.8)	
range	2.4–5.4	4.2–19.1	
median	4.6	7.2	
95%CI	[2.7;5.5]	[5.2;12.1]	
B1 PD1			**0,0147**
mean (SD)	4.0 (2.7)	10.4 (5.5)	
range	0.2–8.7	3.6–18.7	
median	3.7	9.7	
95%CI	[1.2;6.9]	[6.4;14.3]	
B2 PD1			**0,0448**
mean (SD)	0.7 (0.7)	1.8 (1.8)	
range	0.1–2.1	0.6–6.2	
median	0.5	1.1	
CD19 PD1			**0,0147**
mean (SD)	1.2 (0.9)	3.3 (2.3)	
range	0.2–2.9	1.2–8.9	
median	0.9	3.0	
95%CI	[0.2;2.2]	[1.6;4.9]	

Analysis of the recipients of allo-HCT depending on the infection status

Immunophenotype analysis

Differences in immunophenotype were also tested in recipients divided again into two groups: low risk and high risk of infection. We have found significant differences in the percentage of NK cells ($CD56^+$), which was higher in low risk recipients' group (p = 0,0344). Furthermore, among the NK cells population we have found differences in the NK cells with the expression of perforin (NK Perforin) and CD28. NK Perforin percentage was higher in low risk recipients group (p = 0,0079) and NK $CD28^+$ percentage was higher in high risk patients group. There was also a difference in the percentage of NK dim cells – it was higher in low risk recipients group (p = 0,0344) (Table 7.).

Table 7
Immunophenotype comparison between recipients of allo-HCT grouped according to infection risk status.

Parameter	Low risk	High Risk	*P*-value
%NK Perforin^{+}			**0,0079**
mean (SD)	86.4 (29.8)	57.9 (44.0)	
range	2.2–99.8	1.4–92.3	
median	95.2	82.0	
95%CI	[65.1;107.7]	[11.7;104.1]	
%NK CD28^{+}			**0,0344**
mean (SD)	6.5 (8.7)	14.3 (9.5)	
range	1.8–30.8	3.6–27.5	
median	3.8	11.1	
95%CI	[0.3;12.7]	[4.4;24.3]	
%NK CD56^{dim}			**0,0433**
mean (SD)	18.5 (12.6)	6.5 (5.8)	
range	0.1–45.9	0.1–15.4	
median	20.0	6.9	
95%CI	[9.5;27.5]	[0.4;12.6]	
%NK			**0,0448**
mean (SD)	22.1 (13.0)	10.5 (3.1)	
range	9.3–52.0	7.1–15.3	
median	22.3	9.6	
95%CI	[12.8;31.4]	[6.6;14.3]	

Discussion

In our work, we assumed that studying long-term surviving donor-recipient allo-HCT pairs would allow us to find differences between the donors' transplanted cells exposed to immense proliferative and environmental stress which accelerated their ageing and the donor cells that remained intact in the donor and were ageing naturally. Such a scenario limits the number of major factors affecting the differences in ageing between donors and recipients' lymphocyte populations to just two: allogeneic transplantation itself and the diffrent host's microenvironments. We have tested TL in four main lymphocyte subpopulations and found that the telomeres were significantly shorter (0,4kb) in the T CD8^{+} lymphocyte subpopulation of the recipients. The similar tendencies have been found for T CD4^{+} and B (CD19^{+}) lymphocytes – telomeres were shorter in recipients by 0,6kb (p = 0,1) and 0,2kb (p = 0,076) respectively. The strong difference between recipients and donors in CD8^{+} population may result from faster reconstitution of CD8^{+} lymphocytes population in the recipient compared to CD4^{+} population after allo-HCT (12)(13)(14). Moreover, the increased proliferation of CD8^{+} corresponds well with the inverted CD4^{+}/CD8^{+} ratio in recipients of allo-HCT which is observed at least in the first 2 years after transplantation (14)(15). In the literature few reports on the telomere shortening in allo-HCT recipients refer to different cell populations and use different

methods and not surprisingly conclude with contradictory results. According to Mathioudakis et al. statistically significant telomeric shortening assessed with terminal restriction fragment (TRF) method and Flow-FISH method with an average difference in mean telomere of ~ 0,94kb, (although with considerable variability among individuals) was found in granulocytes of long-term (over 20 years from transplantation) allo-HCT recipients (16). The authors concluded that recipients of allo-HCT are “older” compared to their respective donors by 15–20 replicative years (17) (18) S. Boettcher et al. studying 42 recipient-donor pairs with quantitative PCR technique similar to that used in our study, have also found significantly shorter telomeres in recipients than in donors in granulocyte population although they have expressed the difference in years of premature ageing of hematopoietic system (~ 20 years) when compared to donors (17). Wynn et al. has shown less pronounced telomere shortening i.e. 0,6kb and 0,5kb for neutrophils and lymphocytes respectively (18), although the patients underwent allo-HCT as teenagers from adult donors and the median time from Tx was 7 years. Interestingly, on average it was not greater than shortening observed early after transplantation. Their results seem to be similar to ours taking into account that we have tested lymphocyte subpopulations separately whereas Wynn et al. tested all lymphocytes together. In contrast, de Pauw et al., who performed the analysis with terminal restriction fragment (TRF) technique in 10 recipient-donor pairs, did not find any statistically significant difference in the mean telomeric length between donors and recipients (19). The lack of any difference nor any trend for the difference in TL in NK cells ($CD56^+$) is difficult to explain. Our observation might be partially explained by the fact that NK cells are the first to proliferate during the reconstitution period and may reach the normal values even within a month after allo - HCT (20) (21). This could lead to relatively small proliferative stress and in consequence, would not be reflected by lack of significant telomeric length shortening. Moreover, it is unlikely that increased endogenous telomerase activity is responsible for this observation because of the low telomerase activity in aged NK cells (22).

We have found differences in the median percentage of $CD4^+$ lymphocytes – it was higher in donors (44,3%) than in recipients (40,1%). Among $CD4^+$ population there were also similar tendencies in $CD4^+$ naïve cells and $CD4^+$ EM (Effector Memory) cells. $CD4^+$ naïve cells accounted for 24,5% in recipients and 38% in donors (p = 0,06). On the other hand $CD4^+$ EM comprised of 28,8% in recipients and 19,8% in donors (p = 0,07). Interestingly, such changes are typical for physiological ageing. Physiologically, the decrement of naïve cells during the ageing process is caused mainly due to thymic involution, as well as expansion of memory cells (27). In the allo-HCT long-term survivors, the mechanism could be similar as thymus suffers considerable injury after conditioning (28). Though the decrement of the percentage of naïve cells is not limited to $CD4^+$ naïve cells, we did not find differences nor any trends in $CD8^+$ naïve cells. Moreover, with age the percentage of differentiated $CD4^+$ and $CD8^+$ memory and central memory cells increases (29). Though we have found such tendency in $CD4^+$ EM, strangely there were no trends in $CD8^+$ EM. The increased proliferation of $CD8^+$ lymphocytes was already mentioned above. We did not find differences or trends in $CD8^+$ percentages with the exception found in the subpopulation expressing Eomesodermin ($CD8^+$ $Eomes^+$). In recipients it was greater than in donors – 39,4% and 31,5% respectively (p = 0,07). Eomesodermin is a transcription factor expression of which in $CD4^+$ and $CD8^+$ seems to be essential for development of effector memory cells (30) and therefore increased percentage of $CD4^+$ and $CD8^+$ with expression of this transcription factor may be one of the indicators of aged immune system.

In our study, we have found a significant decrease in $CD4^+/CD8^+$ ratio in recipients (1, 5) compared to their donors (2, 1) who retained normal $CD4^+/CD8^+$ ratio (23) (Table 5.). Interestingly, in physiological ageing, inverted $CD4^+/CD8^+$ is common. It affects about 16% of people between 60–94 years of age (24) and is one of the features of immunosenescence (25)(26). Our observation may suggest that decreased $CD4^+/CD8^+$ ratio in allo-HCT recipients is a sign of T cell exhaustion and/or accelareted ageing induced by allo-HCT.

We have found that B-cell percentage of the total lymphocyte population significantly differs between recipients and donors – 11,3% and 8,5% respectively (p = 0,03). In physiological ageing, we observe a decrement of both percentage and absolute count of $CD19^+$ cells (31)(32). Strangely, we have found an increased percentage of B-lymphocytes in recipients compared to their donors. This might result from the increased incidence of autoimmune diseases in all-HCT recipients compared to

their respective donors as an example of “alloimmunization” (33). We also observed some interesting trends in the percentages of B1 and B2 lymphocytes. Recipients tended to show lower percentage of B1 lymphocytes – 17,2% compared to donors 21,7% (p = 0,08) and greater percentage of B2 lymphocytes 81,6% in recipients compared to 77% in donors (p = 0,07). In physiological ageing, the proportion of B1 cells which produce antibodies without antigen stimulation and are the part of innate immunity (34) decreases with age which may be connected with increased incidence of infections in older age (35). As a consequence, the proportion of B2 cells which make up the majority of B-cells is increased though the absolute count decreases (32). It seems that changes in B-cells in recipients of allo-HCT tend to mimic those observed in physiological ageing process.

Our findings do not suggest that inflammageing increases in allo-HCT recipients - we have not found any statistically significant differences nor any trends in proinflammatory cytokines concentrations between recipients and donors. This suggests that the allo-HCT per se does not influence the inflammatory response induced most likely by chronic antigen stimulation throughout the life of an individual (7).

Chronic GvHD and infectious risk status influence

We were not able to confirm our initial hypothesis of greater telomeric shortening in individuals with a history of chronic GvHD, and with high infectious risk status. This observation supports Mathioudakis et al. suggestion that demand for increased proliferation of hematopoietic stem cells stabilizes early after the period of initial post-transplant acceleration (16) and maybe limited only to the reconstitution period and is not affected by other post-transplant complications.

Immunophenotypic differences between recipients stratified according to infection risk status revealed that in recipients with low risk status there were higher percentages of NK cells (p = 0,0344). Among NK cells there were also higher percentage of NK ^{dim} population (p = 0,0344) and NK with the expression of perforin NK Perforin (p = 0,0079) in recipients with low risk status. In physiological ageing process there is an increase in NK cells percentage and among them most pronounced in NK^{dim} population (36)(37). Interestingly, the perforin (that is an effector of the cytotoxic activity of those cells) expression declines with age (37). It would suggest that about 60% of recipients with lower incidence of infections (Table 1.) present both features of the aged innate immune system (NK cells specifically) and increased cytotoxic activity (increased Perforin expression) which results in decreased incidence of infections. We did not find any differences or even trends in Treg or NK cells populations though in physiological ageing process the number of Tregs decreases (38) and NK cells, especially dim population increases (36). However, our sample could have been too small to identify them.

Interestingly, we have found that the concentration of some proinflammatory cytokines – Il-4 and TNF α differed between recipients of allo-HCT, depending on the infection status. That would correspond with the phenomenon of slightly increased proinflammatory cytokines concentration understood as ageing adaptation mechanism that is “state of preparation for fighting the infection by innate immunity”. The concentrations were higher in the low-risk patients and lower in high risk patients. It may be speculated that such findings would indicate that the patients with higher proinflammatory cytokines concentrations have lower risk of developing infections. (6)(7). Along with increased concentrations of Il-4 and TNF α, changes in NK cells in low risk status recipients may imply the crucial role of the innate immune system in protection against infections in recipients of allo-HCT.

To our surprise, the history of GVHD did not affect any studied outcomes It may be due to multiple factors – history of pharmacological immune suppression, resolution of all GVHD symptoms at the time of entering our study, the presence of age-related diseases, and finally small sample size.

Conclusions

To conclude, our findings seem to support our initial hypothesis of increased ageing of the recipients’ lymphocytes (mainly CD8 subset) compared to their respective donor cells reflected by the difference in the telomere length. However, quantitative

changes in the immunophenotype of the transplanted cells in the donors are consistent with the natural ageing process. No difference in cytokine concentrations suggests that inflammageing does not increase in allo-HCT recipients. However, a history of lower infection numbers in HCT recipients seems to be associated with an increased concentration of proinflammatory cytokines and with an increased percentage of NK cells. The history of GVHD does not affect the rate of ageing. Therefore, the observed differences between transplanted and not transplanted most likely result from the huge proliferative stress in the early period after allo-HCT and to some extent the difference between host and recipients' microenvironments which is the only other variable that may influence the identical cells originating from donor hematopoiesis.

Declarations

Ethics approval and consent to participate

Each participant gave informed consent to participate in the study; the study was approved by the Ethic Committee at the Medical University of Gdańsk – NKBBN/394-594/2019 and NKBBN/394-45/2020.

Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article are included within the article and supplementary material.

Competing interests

The authors declared no conflicts of interest.

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Author's contribution

All authors revised the manuscript. The authors read and approved the final manuscript. M.C.C. and J.M.Z. wrote the manuscript. M.C.C., P.T., J.M.W., M.D., J.M.Z. were responsible for study design. M.C.C., A.P., A.S., J.M.Z., E.Z., M.B., M.D., A.H. have taken part in patient's recruitment and clinical data acquisition. M.C.C., I.O., J.M.W., J.M.Z. and M.M. performed the laboratory and clinical data analysis. M.C., J.S., M.M., M.Z., J.M.W. and P.T. performed the laboratory work.

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Figures

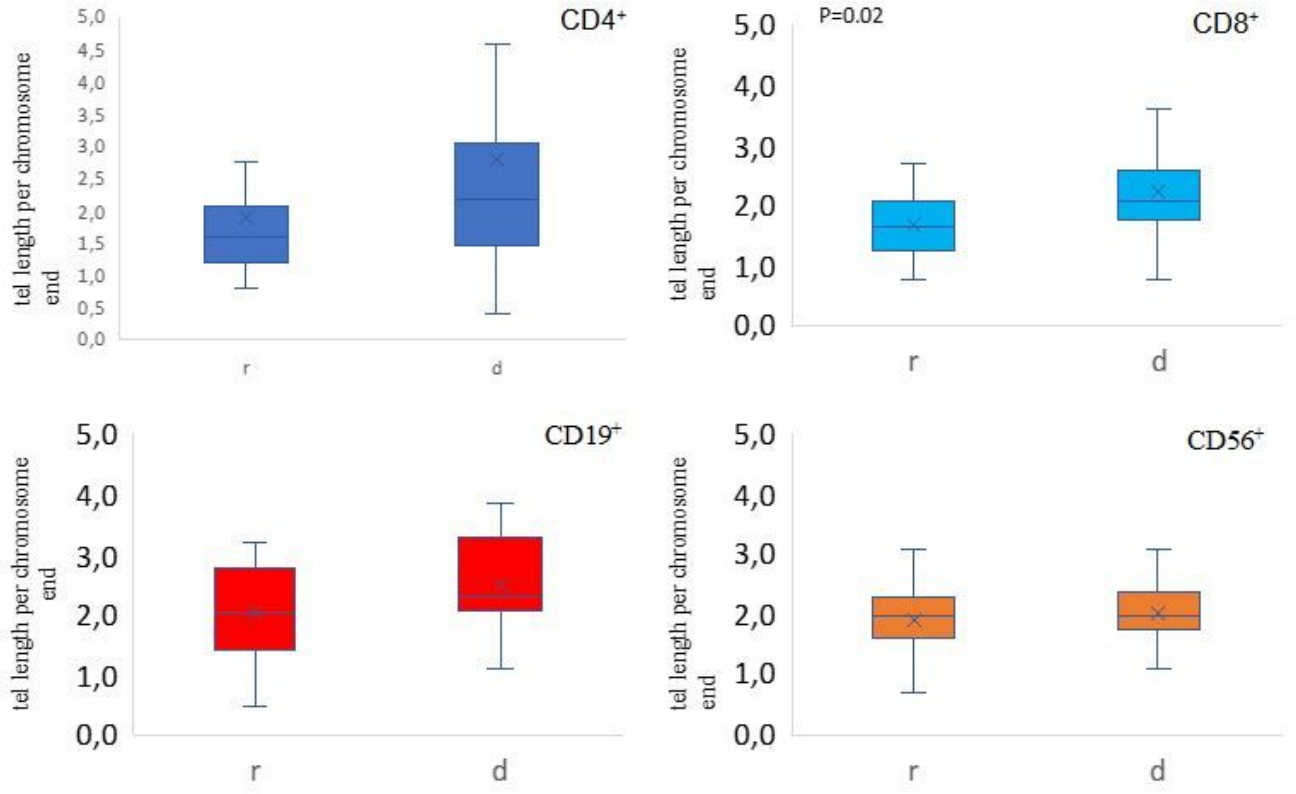

Figure 1

Box plots of mean telomeric length (median kb) in recipients (R) of allo-HCT and their donors (D) in main lymphocyte subpopulations CD4$^+$, CD8$^+$, CD19$^+$ and CD56$^+$. The box and whiskers plots are corresponding to median, 25th and 75th quartile and outlayers. Means are marked as X.

Supplementary Files

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