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

DOCTORAL THESIS

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TABLE OF CONTENTS

KEYWORDS

- Ageing
- Telomeric shortening
- Immunosenescence
- Inflammageing
- Proinflammatory cytokines
- Graft versus host disease
- Immunophenotype
- Allogeneic hematopoietic cells transplantation
- Proliferative stress
- Adaptive immunity
- Innate immunity

ABBREVIATIONS

5-FU 5-fluorouracil

aGvHD Acute graft versus host disease

ALC Absolute lymphocyte count

Allo-HCT Allogeneic hematopoietic cells transplantation

AML Acute myeloid leukemia

ANC Absolute neutrophil count

APC Antigen-presenting cell

ARD Age-related disease

BuCy Busulfan & Cyclophosphamide

cGvHD Chronic graft versus host disease

CM Central Memory

CML Chronic myelogenous leukemia

CMV Cytomegalovirus

D Donors

DAMP Danger-associated molecular pattern

EDTA Ethylenediaminetetraacetic acid

EM Effector memory

Eomes Eomesodermin

GvHD Graft versus host disease

GvL Graft versus leukemia

HCT Hematopoietic cells transplantation

HES Hypereosinophilic syndrome

HSC Hematopoietic stem cells

HSCT Hematopoietic stem cells transplantation

Il – Interleukin

MDS Myelodysplastic syndrome

MiHA Minor histocompatibility antigens

MRD Matched-related donor

MUD Matched-unrelated donor

NK cells Natural killer cells

PAMP Pathogen-associated molecular pattern

PBMC Peripheral blood mononuclear cells

PD-1 Programmed death receptor 1

PNH Paroxysmal nocturnal hemoglobinuria

qPCR Qualitative polymerase chain reaction

R Recipients

ROS Reactive oxygen species

SASP Senescence-associated secretory phenotype

TBI Total body irradiation

TCR T-cell receptor

TEMRA Terminally differentiated effector memory cells re-expressing CD45RA

TL Telomeric length

TLR Toll-like receptor

TNF- α Tumor necrosis factor α

Treg Regulatory T-cells

Tx Transplantation

SUMMARY

Ageing is a biological phenomenon common for almost all living organisms which leads to gradual deterioration of the function of the cells and ends with entering cellular senescence or apoptosis. It applies also to the immune system, and its consequence may be the impaired cellular and humoral response against pathogens and autoimmune diseases. The allo-HCT procedure leads to immense proliferative stress on the recipient's hematopoietic cells, lymphocytes included. Theoretically, it may lead to accelerated ageing of the immune cells of the allo-HCT long-term survivors. The main purpose of this doctoral dissertation was to answer the question of whether the recipients of allo-HCT present with molecular features of ageing compared to their respective donors, namely features of immunosenescence and inflammageing. The population studied consisted of 20 recipient-donor pairs at least 10 years after transplantation. The molecular features of ageing tested were: telomeric length of main lymphocyte subpopulations ($TCD4^+$, $TCD8^+$, B lymphocytes and NK cells), immunophenotype, and proinflammatory cytokines concentrations (Il-1β, Il-2, Il-4, Il-6, Il-10, TNF-α and Il-17F). The aforementioned parameters were then correlated with chronic GvHD occurrence and infection risk status (assessed based on number of infections in the anamnesis). We have found significant telomeric shortening but only in $TCD8^+$ lymphocyte subpopulation, and some features of ageing-resembling immunophenotype in recipients of allo-HCT compared to their donors. The age, gender, infection risk status, and chronic GvHD seem to have no impact on the telomeric length of the recipients. No differences were found in proinflammatory cytokines concentrations, neither between recipients and donors nor between recipients grouped according to infection risk status and cGvHD in the anamnesis. Therefore, we did not observe inflammageing-resembling phenomenon in recipients of allo-HCT. Moreover, low infection risk recipients had a higher percentage of NK cells when compared with high infection risk recipients, which highlights the

crucial role of the innate immune system in protection against infections in this group of patients. The observed differences between recipient's and donor's lymphocytes most likely result from the immense proliferative stress in the early period after allo-HCT and, to some extent, the difference between the donor's and recipient's marrow microenvironment, which is the only other variable that may influence the identical cells originating from the same progenitors obtained from the donor. The demand for increased proliferation of hematopoietic progenitors seems to stabilize after initial acceleration in the early period after transplantation.

STRESZCZENIE

Starzenie jest procesem wspólnym dla niemal wszystkich organizmów żywych, prowadzącym do stopniowego pogarszania funkcji komórek, a ostatecznie ich samobójczej śmierci na drodze apoptozy lub przejścia w stan senescencji. Zjawisko to dotyczy również układu immunologicznego, a jego konsekwencją może być upośledzona odpowiedź komórkowa i humoralna oraz choroby autoimmunologiczne. Po allogenicznym przeszczepieniu krwiotwórczych komórek progenitorowych (allo-HCT), komórki hematopoetyczne dawcy (w tym limfocyty) w organizmie biorcy poddane są nasilonemu stresowi proliferacyjnemu. Teoretycznie, może to skutkować przyspieszonym starzeniem komórek układu immunologicznego długoletnich biorców allo-HCT. Głównym celem pracy doktorskiej była próba odpowiedzi na pytanie – czy biorcy allo-HCT w porównaniu z ich dawcami prezentują molekularne wykładniki starzenia układu immunologicznego, mianowicie cechy „inflammageingu" oraz immunosenescencji. Populacja badana liczyła 20 par biorca-dawca, po co najmniej 10 latach od procedury allo-HCT. U wszystkich pacjentów zbadano molekularne wykładniki procesu starzenia, tj. długość telomerów głównych subpopulacji limfocytów ($TCD4^+$, $TCD8^+$, limfocytów B oraz komórek NK), immunofenotyp oraz stężenia cytokin prozapalnych (Il-1β, Il-2, Il-4, Il-6, Il-10, TNF-α i Il-17F). Następnie powyższe parametry zostały skorelowane z występowaniem przewlekłej choroby GvH oraz ryzykiem infekcji (ocenionym na podstawie liczby przebytych infekcji).

Stwierdziliśmy krótsze telomery, jednak wyłącznie w subpopulacji limfocytów $TCD8^+$ oraz pewne wykładniki fenotypu przypominającego fenotyp senescentny u biorców allo-HCT w porównaniu z ich dawcami. Wiek, płeć, ryzyko infekcji oraz występowanie GvHD nie miały wpływu na długość telomerów u biorców. Nie znaleźliśmy również różnic w stężeniach cytokin prozapalnych, zarówno pomiędzy dawcami i biorcami, jak i pomiędzy biorcami podzielonymi pod względem ryzyka infekcyjnego oraz

występowania GvHD. Biorąc pod uwagę powyższe, nie stwierdziliśmy cech inflammageingu u biorców allo-HCT. Ponadto, biorcy o niskim ryzyku infekcyjnym mieli wyższy odsetek komórek NK w porównaniu z biorcami wysokiego ryzyka, co podkreśla kluczową rolę wrodzonej odpowiedzi immunologicznej w zwalczaniu infekcji w tej grupie chorych. Obserwowane różnice pomiędzy limfocytami biorców i dawców allo-HCT najprawdopodobniej wynikają z wpływu nasilonego stresu proliferacyjnego we wczesnym okresie po transplantacji i w pewnym stopniu z różnic pomiędzy mikrośrodowiskiem szpiku biorców i dawców – jedynym innym czynnikiem mogącym wpływać na identyczne komórki biorców i dawców, będące potomstwem komórek progenitorowych, pochodzących od dawcy. Zapotrzebowanie na nasiloną proliferację krwiotwórczych komórek progenitorowych wydaje się stabilizować po przejściowej akceleracji we wczesnym okresie potransplantacyjnym.

LIST OF WORKS CONCERNING DOCTORAL THESIS

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INTRODUCTION

Ageing is a universal biological phenomenon that affects almost all cells in the majority of living organisms. However, no universal definition of ageing exists due to its complexity. It can be described as a highly heterogeneous process that affects all tissues and systems, leading to a gradual loss of function. In the context of cellular ageing, it is characterized by dysregulation of the mitochondria, following increased reactive oxygen species (ROS) production, DNA damage, and telomeric shortening. Nowadays, there is a growing tendency to perceive ageing not only as a detrimental process but also as a constant adaptation to changing internal environment of the organism ("adaptage theory") (1). The notion of "adaptage theory" was developed by prof. Tamas Fulop and encompasses all age-associated changes of the immune system which serve as an adaptation to changing internal conditions of the organism in contrast to the traditional conception of those changes, perceived as mainly detrimental (2)(1).

To better understand that concept, we need to go back to the first studies that originated the field of ageing on a molecular level. Since the 1960's we know that cells divide until they reach the so-called Hayflick limit which is a certain, finite number of cellular divisions, before entering senescence. It is due to telomeric shortening, occurring with each cellular division (3)(4). After reaching the critical length of telomeres, a cell enters the senescent phase or undergoes apoptosis (5). The most pronounced telomeric shortening throughout life happens in lymphocytes. At birth, telomeric length for lymphocytes is ~ 11 kb and decreases to ~ 4 kb at 100 years of age. With ageing, telomeric shortening gradually decelerates. The average annual rate of telomeric shortening for human lymphocytes is 1190 bp in the first year of life, then 126 bp/year in childhood and 43 bp/year through the rest of adult life (6).

When telomeres shorten to a certain length, measured in base pairs (bp), further divisions are impossible without damaging the cell's coding DNA sequence. Therefore, reaching the Hayflick limit is considered parallel with entering cellular senescence or apoptosis (7). One of the well-established explanations of this phenomenon may be impaired repair of telomeric DNA, due to high demand on the repair machinery, caused by damage to DNA by ROS, according to A.M. Olovnikov (8) who proposed the "theory of marginotomy" which postulates shortening of the replica in comparison to the DNA template. It has directly led to the discovery of telomeres. Those two studies gave a molecular basis for discovering the cellular senescence phenomenon.

The ageing of the immune system consists of two, mutually interconnected phenomena, namely - immunosenescence and inflammageing. The immunosenescence is a plain decline in many immune parameters, predominantly concerning the adaptive immunity. Immunophenotypic changes associated with ageing 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 (9)(10). $CD28^-$ T-cells are characterized by reduced replicative lifespan, decreased proliferative capacity, and reduced response for antigen stimulation while exhibiting increased cytotoxic activity (11). Inflammageing is a chronic, sterile, non-infectious, low-grade inflammation found in the elderly (12). It is caused by the accumulation of proinflammatory factors and the change of the cell's (T-cells included) phenotype to proinflammatory one which occurs with ageing (13). We observe moderate rise in proinflammatory cytokines concentrations (e.g. Il-β, Il-2, Il-4, Il-6, Il-10, TNF-α, Il-17). Both inflammageing and immunosenescence play a major role in the development of age-related diseases (14), however, recent findings suggest that they may also serve as an adaptation process in the course of life of an individual. Moreover, it remains unclear whether quantitative and qualitative changes in the immune cells result from the ageing process or are an adaptation to life-long exposure to pathogens (15). Until recently, it was

assumed that ageing leads to age-related diseases (ARD's), such as cardiovascular and neurodegenerative diseases. Their occurrence correlated with age-related immune system changes (immunosenescence). Vaccine response in the elderly remains adequate when compared with young subjects (16) as well as response for immune checkpoint inhibitors, even in old age (17). Therefore, age-related changes in the immune system reflect rather its adaptation (12) to the pressure of environmental factors.

Almost all aforementioned changes in the immune parameters seem to have one common denominator which is the proliferative stress. It can be simply described as an increased demand for cellular replication due to the need to fight pathogens, autoimmune processes, wound healing, growth, replacement of senescent cells and finally regeneration of hematopoiesis in case of allogeneic hematopoietic cell transplantation (allo-HCT).

The introduction of allogeneic hematopoietic cell transplantation (allo-HCT) as a standard method of treatment for several malignant and non-malignant hematological diseases has created an excellent platform to study human immunology and cellular senescence. The key aspect of allogeneic hematopoietic cell transplantation (allo-HCT) is the restoration of the whole hematopoiesis in the recipient from the relatively small (2–5 × 10^6/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's system (18). The immune part of the hematopoietic system is particularly exposed to the proliferative stress since it is also stimulated by the differences between recipient's and donor's minor histocompatibility antigens (MiHAs), leading to the graft versus host reaction, which is clinically manifested as graft versus host disease (19). Partially, in patients with malignant diseases, this reaction is responsible for HCT's success in eradicating the residual malignant cells (graft-versus-leukemia reaction). However, it may also lead to undesirable complications such as graft-versus-host disease (GvHD), resembling autoimmune diseases affecting several host organs. To prevent and control

symptoms of graft versus host reaction, immunosuppressive agents disrupting lymphocyte proliferation (such as methotrexate and calcineurin inhibitors) are routinely administered after transplantation. Donor's T lymphocytes play a key role in GvHD, however, B-lymphocytes are also important for the development of GvHD, especially chronic (20)(21). Involved donor's lymphocytes undergo an additional extensive proliferation which may contribute to the accelerated telomeric shortening. Moreover, recipients of allo-HCT are susceptible to infectious complications that cause additional proliferative stress to immune cells (22).

Consequently, we have hypothesized that progeny of the donor HSC in the recipients of allo-HCT undergoes accelerated ageing.

In this dissertation we aimed at answering whether long-term recipients of allo-HCT are older on molecular level than their respective matched-related donors. To answer that question, we compared the magnitude of telomeric shortening of the transplanted donor cells to the same cells that remained intact in the donor, immunophenotypic changes of respective lymphocyte subpopulations between donors and their respective recipients and proinflammatory cytokine profile of the same population of patients, i.e. long-term recipients of allo-HCT and their respective donors, to determine whether allo-HCT has led to ageing-resembling changes. Moreover, we compared the telomeric length, immunophenotype and proinflammatory cytokines concentrations of the recipients grouped according to their infection and cGvHD status.

The dissertation includes also a review article describing current state of knowledge about the ageing of the immune system and its two main features, namely – inflammageing and immunosenescence and the impact of proliferative stress on both.

OBJECTIVES

The biology of allo-HCT and its impact on the human's immune system is still not quite well understood. Due to the immense proliferative stress that is the transplantation procedure, the transplanted donor's cells in the recipient undergo a considerable number of cellular divisions which must affect their phenotype. Therefore, we aimed at comparative characterization of the molecular and phenotypical changes of the immune cells (specifically in lymphocyte subpopulations) of both recipients and donors of allo-HCT which originated from the same progenitor cells, to determine whether recipients' lymphocytes present with features of ageing in comparison to their respective donors. The main goals of the doctoral dissertation were:

1. Comparison of telomeric length of four main lymphocyte subpopulations ($TCD4^+$, $TCD8^+$, B lymphocytes and NK cells) of long-term recipients and donors of allo-HCT.

2. Comparison of immunophenotype of long-term recipients and donors of allo-HCT.

3. Comparison of proinflammatory cytokines concentrations of long-term recipients and donors of allo-HCT.

4. Assessment whether aforementioned parameters differ between recipients grouped according to cGvHD and infection risk status.

SCIENTIFIC CONFERENCES AND AWARDS

1. 09-12.09.2020 Society of Hematologic Malignancies 2020 Annual Meeting, Houston, Texas, “Ageing of long-term allogeneic hematopoietic cells recipients compared to their donors”, e-poster

2. 09-12.09.2020 Society of Hematologic Malignancies 2020 Annual Meeting, Houston, Texas, Travel Grant funded by Young Investigator Program

3. 15-17.06.2022 European Hematology Association Hybrid Congress, “Ageing of long-term allogeneic hematopoietic cells recipients compared to their donors”, abstract published in the HemoSphere Supplement and EHA Library

RESULTS

The doctoral dissertation consists of two original articles, presenting the results obtained during the study and one review article. The original articles describe the changes in telomeric length, immunophenotype, and proinflammatory cytokines concentrations in recipients of allo-HCT compared to their donors and changes in molecular features of ageing and the differences in those parameters between recipients when grouped according to their infection risk status and cGvHD status. The review article summarizes the current state of knowledge of the impact of proliferative stress, both physiological and iatrogenic, on the ageing of the immune system, reflected by immunosenescence and inflammageing.

Article 1

Article entitled "Ageing-resembling phenotype of long-term allogeneic hematopoietic cells recipients compared to their donors", published in Immunity & Ageing BMC, presents the results of a comparative analysis of telomeric length in main lymphocyte subpopulations and immunophenotype of recipients and donors of allo-HCT and the differences between the recipients when grouped according to cGvHD status (GvHD (+) and GvHD (-)) and infection risk status (low-risk and high-risk recipients). The telomeric length comparable determination was performed with qPCR technique on four main lymphocyte subpopulations ($TCD4^+$, $TCD8^+$, B cells, and NK cells), isolated by immunomagnetic separation technique. The immunophenotype was assessed by flow cytometry.

We have found that recipients present with some features of ageing compared to their respective donors, that is, shortened telomeres, but only in $CD8^+$ lymphocytes. We have checked the influence of gender and age of the donors on the mean telomere length in

recipients and have found no correlation. In the $CD8^+$ cells population the age of donors was inversely correlated with mean telomere length of the donors. Also, the number of $CD34^+$ cells infused was inversely correlated with the mean telomeric length of the $CD8^+$ cells of the recipients. We have also found that recipient's lymphocytes have ageing resembling phenotype, that is, lesser percentages of $TCD4^+$ and naïve $TCD4^+$ lymphocytes as well as B1 lymphocytes and greater percentages of $TCD4^+$ effector memory cells, $CD8^+$ Eomes lymphocytes, $CD19^+$ cells, and B2 lymphocytes in comparison to their respective donors. Median $CD4^+/CD8^+$ ratio was higher in donors than in recipients of allo-HCT. 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 the low infection risk recipients.

The results obtained in the article suggest that the recipient's lymphocytes present with some features of ageing when compared to the donor's lymphocytes (shortened telomeres in $CD8^+$ lymphocytes and quantitative changes in lymphocyte subpopulations).

The history of lower infection numbers in allo-HCT recipients seems to be associated with increased percentage of NK cells which underlines the role of innate immune response as a primary defensive mechanism against pathogens in this group of patients. The history of GvHD does not affect the rate of ageing. Therefore, the observed differences between the recipients' and donors' lymphocytes most likely result from the immense proliferative stress in the early period after allo-HCT and, to some extent, the difference between the donors' and recipients' microenvironment which is the only other variable that may influence the identical cells, originating from the same progenitors obtained from the donor.

Article 2

Article entitled “Long-term allogeneic hematopoietic cells transplantation survivors’ proinflammatory cytokine profile compared to their respective donors and immunophenotype differences depending on GvHD history and infection status,” published in Acta Haematologica Polonica, Via Medica, presents the results of proinflammatory cytokines profile analysis of long-term recipients of allo-HCT when compared to their respective donors, immunophenotype and proinflammatory cytokine concentrations differences between recipients grouped according to their infection risk and GvHD status. The proinflammatory cytokines concentrations (Il-1β, Il-4, Il-6, Il-10, TNF-α and Il-17F) of recipients and donors of allo-HCT were assessed using commercially available FlexSets with flow cytometry technique as well as immunophenotype assessment. We have not found any significant differences in proinflammatory cytokines concentrations, neither between recipients and donors in general, nor between recipients grouped according to infection risk status. Therefore, our data does not confirm our initial hypothesis that allo-HCT leads to inflammageing-resembling process in recipients of allo-HCT. The lack of differences in long-term recipients of allo-HCT, when grouped according to cGVHD history, may suggest that the immune system tends to stabilize within years after transplantation.

Article 3

Review article entitled “Impact of proliferative stress on both adaptive and innate immune response “, published in Journal of Transfusion Medicine, Via Medica, summarized the current state of knowledge about the impact of proliferative stress, both physiological and iatrogenic, on innate and adaptive immune response, influencing two major features of the ageing of the immune system, namely - inflammageing and immunosenescence.

CONCLUSIONS

1. We have partially confirmed our initial hypothesis on greater telomeric shortening in recipients of allo-HCT compared to their respective donors in $TCD8^+$, $TCD4^+$ and B lymphocyte subpopulations. Although, only in $TCD8^+$ have our results reached statistical significance. No difference was found in NK cells. The strong difference between recipients and donors in $CD8^+$ subpopulation may result from faster reconstitution of $CD8^+$ lymphocytes in the early period after transplantation. The lack of differences in telomeric length between recipients and donors in NK cells may be due to the fact that they are the first cells to proliferate during the hematopoietic reconstitution period and may reach the normal values within a month after allo-HCT. The age, gender, infection risk status and chronic GvHD seem to have no impact on the telomeric length of the recipients. Therefore, we conclude that the increased telomeric shortening in recipients of allo-HCT occurs due to the increased demand for hematopoietic cells proliferation in the reconstitution period and stabilizes early after initial acceleration after transplantation and is not affected by post-transplant complications.
2. The immunophenotype of recipients of allo-HCT compared to their respective related donors has some similarities with immunophenotype of physiologically aged individuals and, therefore may be referred to as ageing-resembling. The differences included the increased percentage of $TCD4^+$ Effector Memory cells, $TCD8^+$ Eomes cells, $CD19^+$ cells and B2 lymphocytes, and decreased percentage of $TCD4^+$ lymphocytes, $TCD4^+$ naïve cells and B1 lymphocytes. Moreover, we have found that even after more than a decade after transplantation, the recipients of allo-HCT have decreased $CD4^+/CD8^+$ ratio which is commonly found in the elderly. We have found that chronic GvHD does not significantly impact the phenotype of the recipients when compared to their respective donors. Although, when grouped according to infection

risk status, we have found that recipients with low infection risk status had higher percentages of NK cells when compared to high infection risk recipients. It may imply the pivotal role of the innate immune system in protection against infections in recipients of allo-HCT.

3. Surprisingly, we have found no differences in proinflammatory cytokines concentrations between long-term survivors of allo-HCT and their respective matched-related donors. Furthermore, there were no differences in proinflammatory cytokines concentrations between recipients grouped according to infection risk status. It contradicts our initial hypothesis that allo-HCT induces an inflammageing-resembling process and implies that transplantation does not lead to chronic low-grade, sterile inflammation. Strangely, the proinflammatory cytokines concentrations of the recipients who had a history of GvHD, which consist of chronic inflammation, also did not differ from the recipient who did not develop GvHD. This suggests that the allo-HCT *per se* does not influence the inflammatory response, induced most likely by chronic antigenic stimulation throughout the life of an individual.

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Immunity & Ageing

RESEARCH Open Access

Ageing-resembling phenotype 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 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 2,1 kb and 1,7 kb 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). Lower infection risk recipients had also a significantly greater percentage of NK cells (22,3%) than high-risk patients (9,3%) p = 0,04.

Conclusion Our data do not support the initial hypothesis of accelerated aging in the long term all-HCT recipients with the exception of the recipients lymphocytes (mainly $CD8^+$) which present some molecular features, characteristic for physiological ageing (telomeric shortening, immunophenotype) when compared to their respective donors. However, a history of lower infection numbers in HCT recipients seems to be associated with increased percentage of NK cells. The history of GVHD seems not to affect the rate of ageing. Therefore, it is safe to conclude that the observed subtle differences between recipients' and donors' cells result mainly from the proliferative stress in the early period after allo-HCT and the difference between hosts' and recipients' microenvironments.

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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 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 ~11 kb and decreases to ~4 kb at the age of 100 years. With ageing, telomeric shortening gradually decelerates. The average annual rate of telomeric shortening for human lymphocytes is 1190 bp in the first year of life, than 126 bp/year in childhood and 43 bp/year through the rest of adult life [2].

Immunophenotypic changes associated with ageing 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].

The key aspect of allogeneic hematopoietic cells transplantation (allo-HCT) is the restoration of the whole hematopoiesis in the recipient from the relatively small 2–5×106/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. [6]. The immune part of the hematopoietic system is particularly exposed to the proliferative stress since it is also stimulated by the differences between recipient's and donor's minor histocompatibility antigens (MiHAs) leading to the graft versus host reaction which clinically is manifested as graft versus host disease (GvHD) [7]. Moreover, recipients of allo-HCT are susceptible to infectious complications that cause additional proliferative stress to immune cells [8]. Consequently, we have hypothesized that progeny of the donor HSC in the recipients of allo-HCT undergoes accelerated ageing, which may be responsible for those 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 between donors and their respective recipients.

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

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.

Peripheral blood mononuclear cells (PBMC) and lymphocyte isolation

PBMC was 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, 9] Isolated lymphocyte subpopulations were pelleted by centrifugation and stored at -80ºC 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 100 bp 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 2×10^5 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.

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 those 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).

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 (2,1 kb [95%CI 1,8;2,7]) compared to R (1,7 kb [95%CI 1,4;1,9]) (p=0,02; n=40). There were also similar tendencies in CD4$^+$ and CD19$^+$ lymphocyte subpopulations, respectively D – 2,2 kb [95%CI 1,8;3,8], R – 1,6 kb [95%CI 1,4;2,4] (p=0,1; n=40) and D – 2,3 kb [95%CI 2,1;2,9], R- 2,1 kb [95%CI 1,7;2,4] (p=0,076; n=40), although they have not reached statistical significance. We have not found differences in the CD56$^+$ population (D – 2 kb [95%CI 1,8;2,3], R – 2 kb [95%CI 1,5;2,3] (p=0,53) (n=40)) (Fig. 1.). We have checked the influence of gender and age of the donors on the mean telomere length in recipients and have found no correlation. In the CD8$^+$ cells population the age of donors was inversely correlated with mean telomere length of the donors (Correlation coefficient −0.59; p=0.007; Spearman). Also, the number of CD34$^+$ cells infused was inversely correlated with mean telomeric length of the CD8$^+$ cells of

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) **	Number of $CD34^+$cells infused (x 10^6/kg)
1	CML	M/F	25	33/27	BuCy	-	Low	-
2	ALL	F/M	18	20/15	TBI	-	High	7,78
3	AML	M/M	15	23/25	BuCy	-	Low	6,3
4	AML	F/M	20	36/46	BuCy	Yes	Low	11,6
5	HES	M/F	19	32/33	BuCy	-	Low	1,56
6	CML	M/M	18	46/43	BuCy	-	Low	5,6
7	CML	F/F	17	22/10	BuCy	Yes	Low	-
8	PNH	M/M	18	27/20	BuCy	-	Low	1,31
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	3,4
13	ALL	F/M	24	28/24	BuCy	-	Low	-
14	AML	M/F	15	31/28	BuCy	-	Low	3,9
15	CML	M/M	20	44/43	BuCy	-	Low	8
16	MDS	F/F	12	42/43	BuCy	Yes	High	1,94
17	CML	F/M	17	38/43	BuCy	-	High	1,35
18	AML	F/M	12	38/38	BuCy	Yes	Low	6,06
19	CML	M/M	13	33/22	BuCy	Yes	High	-
20	AML	M/F	12	41/54	BuCy	-	Low	0,88

* 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)

recipients (Correlation coefficient −0.55; p=0.05; Spearman) (Table Suppl 9–18).

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 3) Moreover we observed difference trends in few others lymphocyte subpopulations (p value approaching 0.05, Table 3). 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 3).

$CD4^+/CD8^+$ 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 4).

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 5).

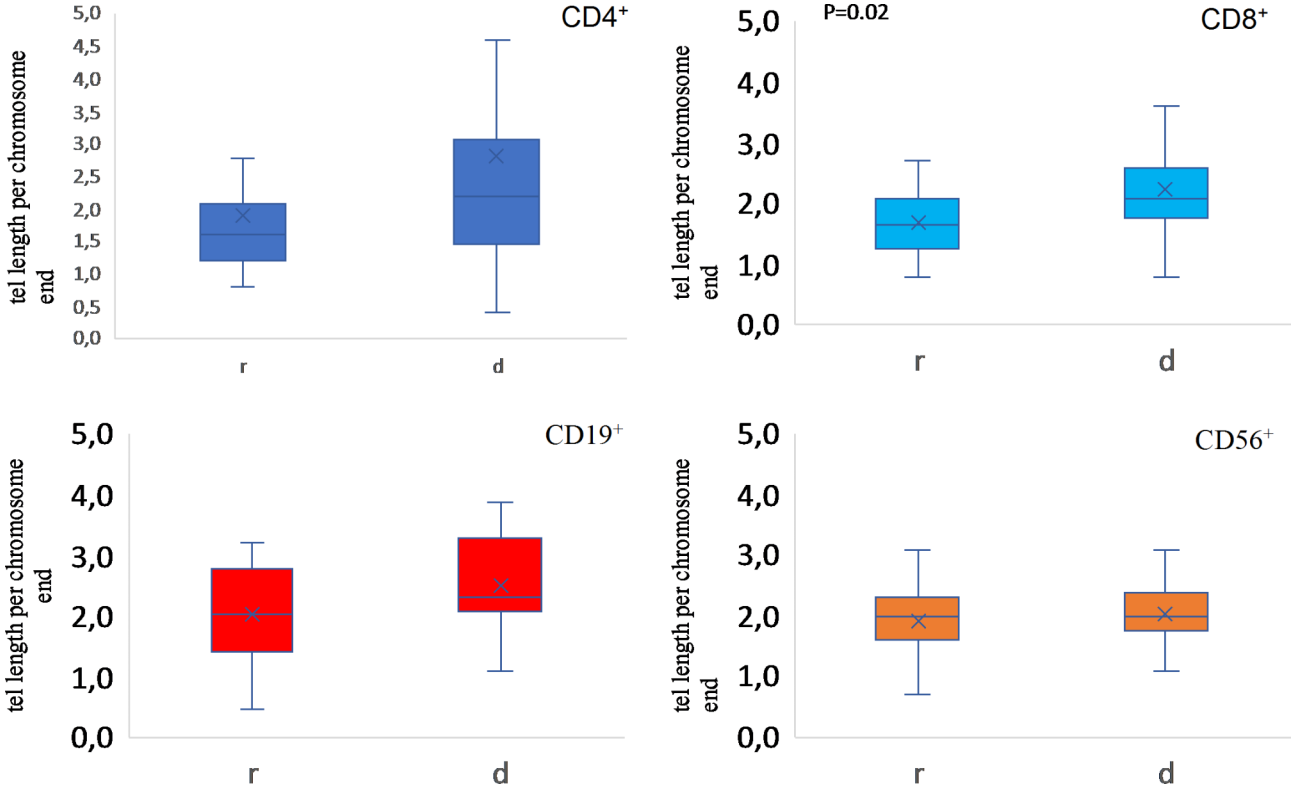

Fig. 1 Box plots of mean telomeric length (median kb) in recipients (R) of allo-HCT and their donors (D) in main lymphocyte subpopulations $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$. The box and whiskers plots are corresponding to median, 25th and 75th quartile and outlayers. Means are marked as X

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 different host's microenvironments. We have tested TL in four main lymphocyte subpopulations and found that the telomeres were significantly shorter (0,4 kb) 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 0,6 kb (p=0,1) and 0,2 kb (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 [10–12]. 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 [12, 13]. 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 [14, 15]. This could lead to relatively small proliferative stress and in consequence, would 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 [16]. The telomere length did not differ between recipients and donors respectively depending on the age of the recipients or gender. We also have not found differences in the mean telomere length of the recipients in any lymphocyte subpopulation tested depending on the donor's age (Table Suppl 17.). Interestingly, the analysis has shown inverse correlation of mean telomere length of donors and age of donors but only in $CD8^+$ lymphocyte subpopulation. Those findings seem to confirm crucial impact of allo-HCT on telomeric shortening since the inverse correlation of age of donor and donor's mean telomeric length was not observed in recipient's mean telomeric length in the same lymphocyte subpopulation tested (CD 8^+). Strangely, the mean telomere length of the recipients was also inversely correlated with the number of cells transplanted and also only

Table 3 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]	
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]	

Table 4 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

in CD8$^+$ lymphocyte subpopulation (Tables 16 and 17 in Supplementary Material). However, interpretation of this result is challenging since stem cells only consist of some percentage (different in each donor) of CD34$^+$ cells. The other conceivable factor (not studied in the work) that may influence the results may be the telomerase activity of the stem cells.

Table 5 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]	

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 [17]. In the allo-HCT long-term survivors, the mechanism could be similar as thymus suffers considerable injury after conditioning [18]. 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 [19]. 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 [20] 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 [1, 2] who retained normal $CD4^+/CD8^+$ ratio [21] (Table 4). Interestingly, in physiological ageing, inverted $CD4^+/CD8^+$ is common. It affects about 16% of people between 60 and 94 years of age [22] and is one of the features of immunosenescence [23, 24]. 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 [25, 26]. 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" [27]. 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 [28] decreases with age which may be connected with increased incidence of infections in older age [29]. As a consequence, the proportion of B2 cells which make up the majority of B-cells is increased though the absolute count decreases [26]. It seems that changes in B-cells in recipients of allo-HCT tend to mimic those observed in physiological ageing process.

Infectious risk status influence

We were not able to confirm our initial hypothesis of greater telomeric shortening in individuals 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 [30] 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 [31, 32]. Interestingly, the perforin (that is an effector of the cytotoxic activity of those cells) expression declines with age [32]. 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 [33] and NK cells, especially dim population increases [31]. However, our sample could have been too small to identify them. Interestingly, changes in NK cells in low-risk status recipients may imply the pivotal 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.

There are some limitations of our study- the most important is a bias regarding donor-recipient pair selection based on the long-term survival of the whole pair. An additional important limitation is the lack of information on the aging status of the hematopoietic system of the donors at the time of donation which obviously is not accessible anymore.

Conclusion

To conclude, our findings would suggest that recipients' lymphocytes seem to have some features of physiological ageing when compared to their respective donors which is reflected by the difference in the telomere length (mainly CD8 subset) and immunophenotypic quantitative changes of transplanted cells, characteristic for ageing. However, a history of lower infection numbers in HCT recipients seems to be associated 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 cells 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.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12979-022-00308-6.

Supplementary Material 1

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Author 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. M.M. and K.R-D. 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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Data availability
The dataset supporting the conclusions of this article are included within the article and supplementary material.

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.

Competing interests
The authors declared no conflicts of interest.

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Publisher's note

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ORIGINAL RESEARCH ARTICLE

Long-term allogeneic hematopoietic cells transplantation survivors' proinflammatory cytokine profiles compared to their respective donors and immunophenotype differences depending on GvHD history and infection status

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Abstract

Introduction: In the course of allogeneic hematopoietic cell transplantation (allo-HCT) the donor's hematopoietic progenitor cells are exposed to immense proliferative stress to reconstitute in the recipient the functional hematopoiesis. Moreover, recipients who develop infections or chronic graft-versus-host disease (GvHD) are subjected to further proliferative stress, especially in the lymphocyte subset. We hypothesized that allo-HCT may induce changes in proinflammatory cytokines profile and immunophenotype in the allo-HCT recipients, especially in patients with a chronic GvHD history.

Material and methods: We compared the cytokine profile [interleukin (IL)-6, IL-10, and tumor necrosis factor α (TNF-α)] between long-term allo-HCT recipients and their respective donors and we analyzed cytokine profiles and the immunophenotype of lymphocytes in long-term recipients grouped according to their infection and GvHD history.

Results: We found no differences in the proinflammatory cytokines between allo-HCT recipients and their respective donors, or between recipients grouped according to their infectious risk status. Immunophenotyping of recipients grouped according to their GvHD status revealed an increased percentage of B-cell presenting programmed death-1 in recipients without a history of GvHD.

Conclusions: A lack of differences in proinflammatory cytokines concentrations between recipients and donors of allo-HCT would suggest that allo-HCT does not induce acceleration of the 'inflammaging'-resembling phenomenon.

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No differences in the cytokine profile and immunophenotype between recipients grouped according to infectious risk status suggest that infectious risk is not reflected by the immunophenotype and cytokine profile. Furthermore, the lack of significant differences in immunophenotype of the recipients grouped according to a history of GvHD may suggest that in long-term survivors the immune system tends to stabilize with time.

Key words: GvHD, cytokines, allo-HCT, immunophenotype

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Introduction

The introduction of allogeneic hematopoietic cell transplantation (allo-HCT) as a standard method of treatment for several malignant and non-malignant hematological diseases has created an excellent platform upon which to study human immunology and cell senescence. Since only a small percentage of the donor stem cells pool is collected and infused into the donor to engraft and reconstitute hematopoiesis, the cells are exposed to immense proliferative stress.

However, successful allo-HCT requires also that two important immunological barriers be overcome: host versus graft and graft versus host. Graft-versus-host reaction results from the exposure of lymphoid donor cells to the recipient antigens which induce donor lymphocyte activation and proliferation. Partially in patients with malignant diseases, this reaction is responsible for HCT's success in eradicating the residual malignant cells (graft-versus-leukemia reaction). However, it may also lead to undesirable complications such as graft-versus-host disease (GvHD) resembling autoimmune diseases affecting several host organs. To prevent and control symptoms of graft versus host reaction, immunosuppressive agents disrupting lymphocyte proliferation (such as methotrexate and calcineurin inhibitors) are routinely administered after transplantation. A key role in GvHD is played by donor T cell lymphocytes but also B-lymphocytes [1, 2]. Involved donor lymphocytes undergo an additional intensive proliferation which may contribute to the accelerated telomere shortening in donor lymphocytes.

All of the above lead to the immense proliferative activity of the cells, including lymphocytes in allo-HCT recipients. We hypothesized that this could lead not only to accelerated telomeric shortening but also to immunophenotypic changes characteristic of natural aging. Healthy human ageing process includes in its characteristics the phenomenon of 'inflammaging'. It may be defined as chronic, low-grade inflammation, without the presence of infection. In biochemical evaluation it presents with increased concentrations of proinflammatory cytokines due to antigenic stimulation over a lifespan of an individual [2].

It is also well known that the concentration of some proinflammatory cytokines [such as tumor necrosis factor α (TNF-α), interleukin (IL)-6] increases, whereas others decrease (such as IL-10) during the course of chronic GvHD [3–5].

We reported recently our observations regarding the changes in immunophenotype and shortened telomeres in CD8+ lymphocyte subpopulation in long-term allo-HCT recipients compared to their respective donors [6]. Here, we present data on the proinflammatory cytokine profile of the same population of patients, i.e. long-term recipients of allo-HCT and their respective donors, to determine whether allo-HCT led to the changes in the proinflammatory cytokines. Moreover, we compared the immunophenotype of the recipients grouped according to their infection and cGvHD status.

Material and methods

The content of the materials and methods section were adapted from Czarnogórski et al. 2022 [6].

Patients

The study consist of 20 allo-HCT recipient-donor pairs. The span from the transplantation was more than 12 years ago. The study was conducted at University Clinical Center, Medical University of Gdansk. From all participants full venous blood sample was collected (50 mL).

GvHD and infectious status assessment

Patients were stratified according to their history of chronic GvHD status (yes vs. no) and infectious complications according to an infection risk status score developed for the purpose of this study [6].

Peripheral blood mononuclear cells and lymphocyte isolation

Perpheral blood mononuclear cells collection was performed from full venous blood with Ficoll-Hypaque centrifugation technique. Following lymphocyte isolation was performed by immunomagnetic separation. The lymphocyte subpopulations were TCD4+, TCD8+, B-lymphocytes and natural killers (NK) cells. The quality of collected material was assessed according to validated protocols [7, 8].

Proinflammatory cytokine concentrations

Proinflammatory cytokines concentrations (IL-1B, IL-2, IL-4, IL-6, IL-10, TNF-α and IL-17F) were assessed with flow cytometry. The results which did not reach the reference were excluded from the study.

Immunophenotyping

Immunophenotyping was performed according to protocol used by Czarnogórski et al. [6].

Statistical analysis

The statistical analysis was performed by STATISTICA 12.0 and with Microsoft Exel, detailed analysis was described according to Czarnogórski et al. [6]. The W Shapiro-Wilk test, and the Leven's (Brown-Forsythe) test were used. The significance of differences between the two groups (independent samples model) was tested by Student's *t*-test or by U Mann-Whitney. The significance of differences between more than two groups was verified using the Kruskal-Wallis test. In the case of receiving statistically significant differences between groups, the Dunn test was performed. A *p* value <0.05 was considered significant.

Results

Patient characteristics

The time from Tx to full venous blood cytometric analysis was at least 12 years with range 12–25 years (median 17.4 years). The population studied consisted of 12 males and 8 females. The prevalence of chronic graft versus host disease among recipients was 40%. Infection risk status was assessed according to Czarnogórski et al. [6]: 12 low risk recipients and 8 high risk recipients.

Proinflammatory cytokine concentrations

Surprisingly, we have found no statistically significant differences in the concentrations of the cytokines: TNF-α, IL-6, IL-10 (Table I). The results of assessment of IL-17F, IL-1β, IL-4, IL-2 concentrations were out of range, therefore they could not be included into analysis.

Neither we have found any differences between recipients when grouped according to infection risk status (Table II).

Immunophenotype of allo-HCT recpients grouped according to chronic GvHD history

The analysis of immunophenotype of the allo-HCT recipients grouped according to cGvHD history showed no significant differences (*see* Supplementary Table 1), with the exception of a few parameters such as Treg Helios-Eomes+, B1 PD1+, B2 PD1+ and C19 PD1+. Lymphocytes B in recipients of allo-HCT who did not develop cGvHD had greater expression of PD-1 (Table III).

Table I. Recipients and donors of hematopoietic cell transplantation — cytokines concentrations

Parameter	R	D	*p* value
IL-6 [ng/L]:	N = 20	N = 20	0.5792*
• avr (standard deviation)	0.99 (1.17)	1.61 (2.37)	
• range	0.38–5.42	0.07–9.53	
• median	0.58	0.72	
• 95% CI	0.44–1.54	0.50–2.72	
IL-10 [ng/L]:	N = 19	N = 18	0.5333*
• avr (standard deviation)	0.58 (0.69)	0.72 (0.71)	
• range	0.01–3.20	0.15–3.04	
• median	0.42	0.52	
• 95% CI	0.25–0.91	0.36–1.07	
TNF-α [ng/L]:	N = 18	N = 19	0.3234*
• avr (standard deviation)	0.77 (1.53)	0.83 (1.91)	
• range	0.01–6.78	0.02–8.51	
• median	0.33	0.22	
• 95% CI	0.01–1.54	–0.09–1.75	

*U Mann-Whitney test; IL — interleukin; CI — confidence interval; TNF-α — tumor necrosis factor α

Table II. Recipients grouped according to infection risk status — cytokines concentrations

Parameter	Low risk	Intermediate/ /high risk	*p* value
IL-6 [ng/L]:	N = 12	N = 8	0.3159*
• avr (standard deviation)	1.19 (1.49)	0.69 (0.20)	
• range	0.38–5.42	0.48–1.10	
• median	0.52	0.61	
• 95% CI	0.24–2.14	0.52–0.86	
IL-10 [ng/L]:	N = 11	N = 8	0.9671*
• avr (standard deviation)	0.68 (0.88)	0.45 (0.27)	
• range	0.01–3.20	0.11–0.87	
• median	0.48	0.39	
• 95% CI	0.09–1.27	0.23–0.67	
TNF-α [ng/L]:	N = 12	N = 6	0.1898*
• avr (standard deviation)	1.02 (1.85)	0.28 (0.20)	
• range	0.09–6.78	0.01–0.62	
• median	0.37	0.28	
• 95% CI	–0.15–2.19	0.07–0.49	

*U Mann-Whitney test; IL — interleukin; CI — confidence interval; TNF-α — tumor necrosis factor α

Table III. Recipients grouped according to chronic graft-versus--host disease (cGvHD) status — immunophenotype

Parameter	cGvHD	Without cGvHD	*p* value
Treg Helios–Eomes:			**0.0227**
• avr (standard deviation)	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**
• avr (standard deviation)	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**
• avr (standard deviation)	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**
• avr (standard deviation)	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	

SD — standard deviation; CI — confidence interval

Discussion

In this study, we tried to answer the question of whether allo-HCT accelerates the aging of the hematopoietic system by determining the differences in cytokine profile between long-term allo-HCT survivors and their respective donors of allo-HCT.

Studying donor-recipient pairs creates a unique model in which donor cells remaining in the donor could be compared to the donor cells infused into respective recipients. We were particularly interested in the features of postulated 'inflammaging'. We also compared the same cytokine profile of the recipients when grouped according to infectious status (low vs intermediate/high) (*see* Czarnogórski et al. [6]). We hypothesized that allo-HCT recipients should have higher concentrations of proinflammatory cytokines as a robust indicator of aging. We also hypothesized that low-risk recipients according to their infection status would have increased concentrations of the same cytokines as an adaptation for fighting the infections.

Physiologically, the proinflammatory cytokine profile of older people is characterized by increased concentrations of the aforementioned cytokines (IL-1B, IL-2, IL-4, TNF-α, IL-6, IL-10, IL-15, IL-17, IL-18). These concentrations however do not exceed the upper reference range. Hence, inflammaging is defined as the process of chronic, sterile, low-grade inflammation.

There is no data on inflammaging in a population of allo-HCT survivors compared to their respective recipients serving as controls. We did not find any statistically significant differences in IL-6, IL-10 and TNF-α concentrations, either between main groups (recipients vs. donors) nor between recipients grouped according to infection risk status. Our data did not confirm our initial hypothesis that allo-HCT accelerates the inflammaging-resembling process.

We also did not find any differences between low and intermediate/high risk recipients stratified by their infection status, which could imply that infectious risk is not directly connected to the efficacy of one's innate immune response. It would imply that allogeneic hematopoietic cells transplantation by itself does not impact the inflammaging [9]. However, the issue remains controversial since chronic low-grade inflammation (inflammaging) is a well-established risk factor for developing neoplasia [10, 11] which could be debatable in the population of our allo-HCT survivors since they were diagnosed with hematological malignancies in their 20 s and 30 s. On the other hand, there is ample data on the reduction of relapse risk after allo-HCT in patients who developed chronic GvHD that is in fact a chronic inflammation [12]. Moreover, it is difficult to differentiate if heightened concentrations of proinflammatory cytokines after allo-HCT result from chronic GvHD [13] or possibly are an adaptation for fighting the infection. There is some data correlating the occurrence of inflammaging and immune exhaustion in some hematological malignancies, such as plasmocytic myeloma [14]. Thus, it is challenging to determine whether the inflammaging features are due to older age or to the neoplasia itself.

Surprisingly, the incidence of chronic GvHD also did not impact any studied parameters, especially immunophenotype with the exception of B-cells expressing PD-1 which serves as the programmed death ligand-1 (PD-L1) receptor and plays a role in modulating immune response [15]. We also found no differences in T-cells expressing PD-1. An increased percentage of B-cells presenting PD-1 in recipients without chronic GvHD in anamnesis is difficult to interpret. Those differences in receptor expression in antigen-presenting cells (APCs) such as B-cells seem to be insignificant or accidental. The lack of differences in long-term (12 years+ from Tx) recipients of allo-HCT when grouped according to cGvHD history may suggest that the immune system tends to stabilize in the years following Tx. Many factors might explain such notion, that is immune suppression used, history of chronic degenerative diseases, GvHD resolution and small number of participants. Our study has

several limitations. Firstly, it was performed in long-term survivors who were able to fight infections successfully and whose cGvHD status became stable. Secondly, the results are affected by the small population (20 pairs) and unfortunately the results of some cytokines assays were out of range, which might be related to laboratory errors. Unfortunately, we were unable to repeat tests with out-of-range results due to sample destruction during an electricity outage. Nevertheless, our results may suggest that allo-HCT does not accelerate the aging of the hematopoietic system despite a clear reduction of telomere shortening in specific cell populations and some immunophenotypic differences reported by us [6].

Authors' contributions

All authors revised manuscript and read and approved final manuscript. MCC and JMZ wrote manuscript. MCC, PT, JMW, MD, JMZ were responsible for study design. MCC, AP, AS, JMZ, EZ, MB, MD, AH took part in patient recruitment and clinical data acquisition. MCC, IO, JMW, JMZ, MM and KRD performed laboratory and clinical data analysis. MC, JS, MM, MZ, JMW and PT performed laboratory work.

Conflict of interest

The authors declare no conflict of interest.

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Ethics

This study was approved by the Ethics Committee at the Medical University of Gdańsk — NKBBN/394-594/2019 and NKBBN/394-45/2020. Each participant signed an informed consent form.

Supplementary Table 1. Comparative characteristics of allogeneic hematopoietic cell transplantation recipients immunophenotype when grouped according to graft-versus-host disease status

Parameter	*p* value	Parameter	*p* value	Parameter	*p* value
B1	0.1193	NK CD39	0.5508	B1 PD1	0.0147
B2	0.0973	NK CD56 dim	0.2548	B2	0.2123
CD19	0.6511	NK CD56 high	0.2548	B2 Fas	0.9567
CD3	0.9599	NK Eomes	0.9567	B2 PD1	0.0448
DNT	0.4808	NK Perforin	0.7042	CD19	0.9567
NK	0.7595	NKT like	1.00	CD19 Fas	0.9567
NK CD56 dim	0.9512	Q1	0.5508	CD19 PD1	0.0147
NK CD56 high	0.4624	Q1 CD39	0.8708	CD4 CD27+CD28–	0.3290
NKT like	0.0662	Q1 Eomes	0.3566	CD4 CD27+CD28+	0.2123
T CD4	0.7250	Q1 IL10	0.5508	CD4 CD27–CD28–	0.4808
T CD8	0.9567	Q1 Perforin	0.0827	CD4 CD27–CD28+	0.7042
B1	0.0927	Q2	0.9567	CD4 CD28	0.3566
B1 CD39	0.4159	Q2 CD39	0.3028	CD4 CD57	0.3028
B1 Eomes	0.3566	Q2 Eomes	0.7863	CD4 FasL	0.8283
B1 IL10	0.1752	Q2 IL10	0.1585	CD4 PD-1	0.6255
B2	0.0927	Q2 Perforin	0.1752	CD8 CD27+CD28–	0.7683
B2 CD39	0.7449	Q3	0.3290	CD8 CD27+CD28+	0.1949
B2 Eomes	0.0577	Q3 CD39	0.7042	CD8 CD27–CD28–	0.6800
B2 IL10	0.6255	Q3 Eomes	0.2123	CD8 CD27–CD28+	0.5959
CD19	0.4808	Q3 IL10	0.1931	CD8 CD28	0.7683
CD19 CD39	0.4159	Q3 Perforin	0.8708	CD8 CD57	0.6800
CD19 Eomes	0.0448	RTE	0.1158	CD8 PD-1	0.3165
CD19 IL10	0.4477	T CD4	0.7863	DNT	0.3566
CD3	0.6255	T CD8	0.7863	Memory B	0.0735
CD4 CD39	0.4808	Treg FoxP3	0.9567	NK	0.2123
CD4 CM	0.3028	Treg FoxP3 CD39	0.6255	NK CD27	0.7449

→

Supplementary Table 1 (cont.). Comparative characteristics of allogeneic hematopoietic cell transplantation recipients immunophenotype when grouped according to graft-versus-host disease status

Parameter	*p* value	Parameter	*p* value	Parameter	*p* value
CD4 EM	0.7042	Treg FoxP3 Eomes	0.9136	NK CD28	0.8708
CD4 Eomes	1.00	Treg FoxP3 IL10	0.0735	NK CD56 dim	0.2123
CD4 IL10	0.5508	Treg FoxP3 Perforin	0.2548	NK CD56 high	0.5508
CD4 Naive	0.9567	Treg Helios–	0.6255	NK CD57	0.3566
CD4 Perforin	0.0577	Treg Helios– CD39	0.3566	NK PD-1	0.6255
CD4 Temra	0.7863	Treg Helios– Eomes	0.0227	NKT like	0.9567
CD8 CD39	0.8137	Treg Helios– IL10	0.1752	Q1	0.8708
CD8 CM	0.3768	Treg Helios– Perforin	0.2548	Q1 CD27	0.9567
CD8 EM	0.0875	Treg Helios+	0.7042	Q1 CD28	0.1431
CD8 Eomes	0.2159	Treg Helios+ CD39	0.7042	Q1 CD57	0.2123
CD8 Naive	0.2629	Treg Helios+ Eomes	0.0577	Q1 FasL	0.7863
CD8 Perforin	0.3768	Treg Helios+ IL10	0.0927	Q1 PD-1	0.3566
CD8 Temra	0.953	Treg Helios+ Perforin	0.6255	Q2	0.9567
DNT	0.4159	B1	0.2123	Q2 CD27	0.7449
NK	0.2123	B1 Fas	0.8708	Q2 CD28	0.5508
Q2 CD57	0.0735	CD3	0.9567	Treg FoxP3 CXCR5	0.1431
Q2 FasL	0.4159	CD4 CD152	0.6255	Treg FoxP3 TIGIT	0.7863
Q2 PD-1	0.9567	CD4 CXCR4	0.7042	Treg Helios–	0.4808
Q3	0.1158	CD4 CXCR5	0.1431	Treg Helios– CCR5	0.5508
Q3 CD27	0.3566	CD4 TIGIT	0.4477	Treg Helios– CD152	0.7042
Q3 CD28	0.7449	CD8 CXCR4	0.7683	Treg Helios– CXCR4	0.4159
Q3 CD57	0.0057	CD8 CXCR5	0.1116	Treg Helios– CXCR5	0.6255
Q3 FasL	0.1431	CD8 TIGIT	0.5169	Treg Helios– TIGIT	0.7042
Q3 PD-1	0.0577	DNT	0.4159	Treg Helios+	0.5508
T CD4	0.7042	NK	0.2123	Treg Helios+ CCR5	0.8708
T CD8	0.6255	NK CCR5	0.4477	Treg Helios+ CD152	0.7042
Treg FoxP3	0.9567	NK CD56 dim	0.2123	Treg Helios+ CXCR4	0.6255
Treg FoxP3 CD27	0.1037	NK CD56 high	0.4159	Treg Helios+ CXCR5	0.4808
Treg FoxP3 CD28	0.7042	NK CXCR4	0.2548	Treg Helios+ TIGIT	0.8708
Treg FoxP3 CD57	0.0735	NK CXCR5	0.3566		
Treg FoxP3 FasL	0.1931	NK TIGIT	0.4808		
Treg FoxP3 PD-1	0.4808	NKT like	0.9567		
Treg Helios–	0.0735	Q1	0.5508		
Treg Helios– CD27	0.9567	Q1 CCR5	0.4159		
Treg Helios– CD28	0.3028	Q1 CD152	0.5876		
Treg Helios– CD57	0.0577	Q1 CXCR4	0.8708		
Treg Helios– FasL	0.2781	Q1 CXCR5	0.1431		
Treg Helios– PD-1	0.4808	Q1 TIGIT	0.3028		
Treg Helios+	0.7863	Q2	0.8708		
Treg Helios+ CD27	0.1585	Q2 CCR5	0.9567		
Treg Helios+ CD28	0.8708	Q2 CD152	0.3028		
Treg Helios+ CD57	0.0079	Q2 CXCR4	0.4477		

→

Supplementary Table 1 (cont.). Comparative characteristics of allogeneic hematopoietic cell transplantation recipients immunophenotype when grouped according to graft-versus-host disease status

Parameter	*p* value	Parameter	*p* value	Parameter	*p* value
Treg Helios+ FasL	0.3028	Q2 CXCR5	0.9567		
Treg Helios+ PD-1	0.7863	Q2 TIGIT	0.2548		
B1	0.0927	Q3	0.4159		
B1 CCR5	0.3566	Q3 CCR5	0.7863		
B1 CD152	0.0735	Q3 CD152	0.6255		
B1 CXCR5	0.2548	Q3 CXCR4	0.7042		
B2	0.0577	Q3 CXCR5	0.4808		
B2 CCR5	0.7449	Q3 TIGIT	0.6644		
B2 CD152	0.3028	T CD4	0.8708		
B2 CXCR5	0.4477	T CD8	0.8708		
CD19	0.4159	Treg FoxP3	0.9567		
CD19 CCR5	0.4159	Treg FoxP3 CCR5	0.7042		
CD19 CD152	0.1752	Treg FoxP3 CD152	0.7042		
CD19 CXCR5	0.7042	Treg FoxP3 CXCR4	1.00		

*U Mann-Whitney

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Impact of proliferative stress on both adaptive and innate immune response

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Summary

Human ageing is by far one of the most complex biological phenomena which affects all cells and tissues, leading to gradual loss of function, decrement in proliferative activity, and impaired cellular response. One of the key mechanisms of cellular ageing is proliferative stress which results in telomeric attrition, DNA damage, and deposition of senescence-associated proteins. Allogeneic hematopoietic cells transplantation (allo-HCT) serves as a good model for cellular ageing. Here we review the ageing of the immune system and the impact of proliferative stress on both innate and adaptive immune response, reflected by immunosenescence and inflammageing phenomena, in the context of iatrogenic proliferative stress induced by allo-HCT.

Key words: immunosenescence, inflammageing, allo-HCT, proliferative stress

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Introduction

Ageing is a universal biological phenomenon that affects almost all cells in most living organisms. However, no universal definition of ageing exists due to its complexity. It can be described as a highly heterogeneous process that affects all tissues and systems, leading to a gradual loss of function. In the context of cellular ageing, it is characterized by dysregulation of the mitochondria, following increased reactive oxygen species (ROS) production, DNA damage, and telomeric shortening. Nowadays, there is a growing tendency to perceive ageing not only as a detrimental process but also as a constant adaptation to changing internal environment of the organism ("adaptage theory") [1]. The notion of "adaptage theory" was developed by prof. Tamas Fulop and encompasses all age-associated changes of the immune system which serves as an adaptation to changing internal conditions of the organism in contrast to traditional conception of those changes, perceived as mainly detrimental [1, 2].

To better understand that concept we need to go back to the first studies that originated the field of ageing on a molecular level. Since the 1960's we know that cells divide until they reach so-called Hayflick limit, which is a certain, finite number of cellular divisions, before entering senescence. It is due to telomeric shortening occurring with each cellular division [3, 4]. When telomeres shorten to a certain length, measured in base pairs (bp), further divisions are impossible without damaging the cell's coding DNA. Reaching the Hayflick limit is therefore considered parallel with entering cellular senescence or apoptosis [5]. One of the

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well-established explanations of this phenomenon may be impaired repair of telomeric DNA, due to high demand on the repair machinery, caused by damage to DNA by ROS, according to Olovnikov [6] who proposed the "theory of marginotomy" which postulates shortening of the replica in comparison to the DNA template. It has directly led to the discovery of telomeres. Those two studies gave a molecular basis for the discovery of the cellular senescence phenomenon.

In this review, we would like to focus on the ageing of one of the crucial regulatory systems, namely the immune system. A thorough understanding of the ageing of the immune system is crucial to adjust the treatment for aged individuals in the future and further development of personalized treatment that takes into consideration not only genomics but also the immune profile. However, the main purpose of our review is not to find the potential therapeutic molecular targets but to better understand how proliferative stress, which is the common denominator of many stressors, influences the immune system, leading to ageing and age-related changes.

The ageing of the immune system consists of two phenomena, mutually connected, namely immunosenescence and inflammageing. The immunosenescence is a plain decline in many immune parameters predominantly concerning the adaptive immunity, among others, the number of TCD4$^+$, expression of CD28, and naïve TCD4$^+$ cells, whereas inflammageing is a chronic, sterile, non-infectious, low-grade inflammation found in the elderly [7]. It is caused by the accumulation of proinflammatory factors and the change of the cell's (T-cells included) phenotype to proinflammatory one, which occurs with ageing [8]. Both inflammageing and immunosenescence play major role in the development of age-related diseases [9], however, recent findings suggest that they may also serve as an adaptation process in the course of life of an individual. Moreover, it remains unclear whether quantitative and qualitative changes in the immune cells are the result of the ageing process or an adaptation to life-long exposure to pathogens [10]. Until recently, it was assumed that ageing leads to age-related diseases (ARD's), such as cardiovascular and neurodegenerative diseases. Their occurrence was correlated with age-related changes in the immune system (immunosenescence).

Vaccine response in the elderly remains adequate when compared with young subjects [11] as well as response for immune checkpoint inhibitors even in old age [12]. Therefore, age-related changes in the immune system reflect rather its adaptation [7] to the pressure of environmental factors.

Almost all aforementioned changes in the immune parameters seem to have one common denominator, which is the proliferative stress. It can be simply described as the increased demand for cellular replication due to the need to fight pathogens, autoimmune processes, wound healing, growth, replacement of senescent cells and regeneration of hematopoiesis in case of allogeneic hematopoietic cell transplantation (allo-HCT).

The allo-HCT creates an immense demand for cellular replication since a very small population of hematopoietic progenitors must reconstitute functional hematopoiesis in the transplant recipient. It implicates immense proliferative stress to hematopoietic cells in general and specifically to lymphocytes. Therefore, in theory, it must lead to telomeric shortening and should increase senescence.

Innate immune response and inflammageing

Inflammageing is considered to be the physiological response to antigenic stress over the lifespan of an individual and might be considered beneficial as long as it remains balanced by anti-inflammatory mechanisms (such as lipoxin A4, prostanoids, adenosine, nitric oxide and annexin) as shown in some recent studies [13, 14]. Low-grade proinflammatory state is not only commonly found in centenarians but also correlates strongly with longevity as shown by Arrai et al. [15], Witkowski et al. [16] and Fulop et al. [17]. It is suggested that it is epigenetically regulated [18]. The consequence of chronic low-grade inflammation is a decrease in the function of the innate immune system called immune paralysis [19], which leads to increased protection against self-inflicted damage (e.g. autoimmune diseases) at the expense of decreased protection against PAMPs (pathogen-associated molecular patterns) and DAMPs (danger-associated molecular patterns).

With ageing the need for more economical energy expenditure increases, which is reflected by changes in the innate immune system, which gradually becomes more important than senescing adaptive immunity. In aged individuals, this can be reflected by the phenotype shift from macrophages M1 (proinflammatory) to M2, which promotes angiogenesis and cancer growth [20]. A gradual decrement in antigen presenting cells (APC's) in aged individuals is observed, which in addition are

characterized by impaired antigen presentation and TCD4$^+$ activation [21]. There is evidence that those innate immunity cells, even in the quiescent state, are able to produce proinflammatory cytokines, which would contribute to increased inflammageing, portraying the mutual interplay between innate and adaptive immune systems. Moreover, it would indirectly account for significant basal activation of APC's in older individuals [22]. There is also some data on the impact of the innate response on adaptive immune response with ageing which could be well exemplified by the down-regulation of CD28 expression in CD4$^+$T cells, which results in decreased clonal expansion of those cells [23].

Therefore, inflammageing may be interpreted not only as increased concentrations of proinflammatory cytokines (Il-1, Il-4, Il-6, TNF-α, Il-17F and others) but as a complex interplay between proinflammatory and anti-inflammatory proteins and qualitative and quantitative changes in innate immune cells phenotype.

Adaptive immune system and immunosenescence

Immunosenescence is a decline in many immune parameters of aged individuals when compared to young healthy subjects. It is considered detrimental due to the accumulation of proinflammatory factors as well as the development of inflammageing [2]. However, from the evolutionary perspective, those changes can be considered adaptive (among others, increment in central memory and effector memory T-cells counts and increased percentage of T cytotoxic cells). The most important changes in adaptive immunity occurring with ageing are decrement in the proportion of naïve TCD8$^+$ and TCD4$^+$ due to thymic involution, loss of CD28 antigen, and an increase of the number of T central memory (Tcm) and T effector memory (Tem) expressing either CD8 or CD4 antigens [24, 25]. Especially terminally differentiated effector memory (TEMRA) CD8$^+$ T cells increase in number and percentage. That shift is commonly explained as a result of chronic antigenic stimulation throughout the lifespan of an individual, with the pivotal role of CMV infection [26]. Inverted CD4$^+$/CD8$^+$ ratio is also a common finding in the elderly [27, 28].

Ageing of the immune system is associated with gradual involution of the thymus [29, 30]. Thymic involution is an evolutionary adaptation since its high metabolic activity is energy-consuming. However, it leads to a decrease in the production of naïve T cells [31] and, therefore, a decrease in TCR repertoire. Although, decrease in TCR repertoire is also affected by clonal selection of the T-cells. Recent findings, however, are contradictory [32]. It has been postulated that aged organism is well adapted to fight mainly known pathogens which it had encountered over the years, whereas the demand for new pathogens recognition is scarce. Furthermore, an increased percentage of Tcm and Tem cells serve the purpose of ameliorating antipathogenic response [33].

As mentioned above, with ageing, the percentage of TCD8$^+$ cells increases (though their count decreases). Those cells play a pivotal role in direct response against pathogens by elimination of virally-infected and cancer cells in the elderly. Surprisingly, recent data suggest that the increment of naïve TCD8$^+$ percentage is not associated with prolonged lifespan [34].

Another factor that influences the immunophenotype of aged individuals is cytomegalovirus (CMV) infection. CMV is not only detrimental, as it was thought to be the main cause of age-related immune changes, but according to novel studies, it may be the main stimulatory factor that sustains immune response for e.g. vaccination [35]. However, it has been proven that CMV infection does not influence the longevity of the aged individuals [36].

With ageing considerable change in the secretory phenotype of the T-cells occurs. It is characterized by the secretion of pro-inflammatory molecules, which stimulates inflammageing [37]. Senescent T-cell presenting with the above mentioned SASP (senescence-associated secretory phenotype) [38] could also be detrimental due to their decreased ability to proliferate as well as impaired response to antigen stimulation [39]. TCD8$^+$ CMV-specific memory cells, which were previously considered to be inactive, have the SASP and contribute to the development of inflammageing [40] which underlines beforementioned parallel of immunosenescence and inflammageing.

Hematopoietic stem-cell transplantation as a model for studying senescence of the immune system

Chronic stress (like serial bone marrow transplantations (BMT's)) may lead to the decline of function of hematopoietic stem cells (HSC's) and lead to their exhaustion in humans [41]. In the murine model, the usage of chemotherapy like 5-fluorouracil (5-FU) promotes quiescent HSC's proliferation resembling that found in the ageing

process [42]. Proliferative stress may also be triggered by infections (bacterial, viral or fungal) through stimulation of Toll-like receptors (TLR) on HSC's or respective receptors for certain proinflammatory cytokines [43] but this is rather acute stress and usually is promptly resolved and does not lead to HSC exhaustion [44]. One of the probable reasons that acute stress may be resolved with little loss/damage of HSC's, may be the innate immune system's dominant role in response to acute stimuli [45].

Therefore, HSCT, which induces prolonged proliferative stress, might be a good model for studying hematopoietic cell senescence. Transplanted HSC's undergo extensive proliferative stress for a span of a few months after transplantation (Tx) [46]. In addition, transplant recipients underwent chronic GvHD and experienced increased incidence of infections, when compared to healthy population, due to immune suppression after transplantation. It is a well-established fact that allo-HCT leads to telomeric shortening in recipients compared to their donors and that this phenomenon persists even after decades after transplantation, as proven in humans by Mathioudakis et al., de Pauw et al. and Wynn et al. [47–49] and in canines by Zaucha et al. [50]. In turn, telomeric attrition results in a similar phenotype to that occuring in the cellular senescence [51]. We recently asked a question whether the senescence of the immune system in allo-HCT recipients is increased compared to the senescence of their respective family donors. We have compared the immune parameters such as telomeric length in main lymphocyte subsets, immunophenotype, and proinflammatory cytokines concentrations between recipients and donors of allo-HCT after more than a decade after transplantation. Our results were not clearly conclusive to support the hypothesis of faster senescence of the immune system in transplant recipients. We found shorter telomeres in recipients but only in $TCD8^+$ subpopulation and subtle changes in the numbers of certain immune cells — $TCD8^+$, B-cells, and $TCD4^+/TCD8^+$ ratio [52]. All those changes resembled an ageing immune phenotype but do not clearly indicate that the immune system of allo-HCT recipients ages faster compared to their respective donors [52].

Summary

Successful ageing is complex and still not a well-understood phenomenon. Ageing of the immune system includes two mutually interconnected phenomena: inflammageing and immunosenescence. The common denominator of the ageing of the immune system is chronic proliferative stress which results in the shortening of telomeres, qualitative and quantitative changes in the immune cells, and shift to the proinflammatory phenotype of the immune cells. The allo-HCT was thought to be an excellent platform for studying the ageing of the immune system. However, our recently published findings indicate the presence of only few quantitative changes in lymphocyte subpopulations in long-term allo-HCT survivors when compared to their donors, which resemble those found in aged individuals. This indirectly indicates that the elasticity of the immune system exposed to the immense proliferative stress at the time of allo-HCT is big enough to prevent the significant and clinically relevant acceleration of the immune system's ageing.

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