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Evaluation of proinflammatory and immunosuppressive cytokines in blood and bone marrow of healthy hematopoietic stem cell donors☆

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ABSTRACT

Introduction: Cytokine composition of bone marrow microenvironment in comparison to blood is poorly explored. The goal of this study was to investigate the levels of cytokines present in peripheral blood and bone marrow of healthy hematopoietic stem cells donors. The data obtained on this subject with addition to cytometric analysis can provide new insight into the hematopoietic stem cells microenvironment.

Methodology: Study consisted of cytokine concentration analysis performed by ELISA tests of peripheral blood of healthy peripheral blood stem cells donors and bone marrow of healthy bone marrow donors. Additionally we have tested the expression of CD47 and CD274 proteins on the surface of hematopoietic stem cells by the flow cytometry analysis.

Results: The results has shown different composition of analyzed cytokines (IL-1 β, IL-2, IL-4, IL-6, IL-10, IL-17A, TGF-β1, IFN-γ and TNF-α) present in bone marrow and blood of stem cells donors. The hematopoietic stem cells in peripheral blood are subjected to higher levels of proinflammatory cytokines whilst the lower level of those cytokines in bone marrow with a very high level of TGF-β1 which possibly creates a more immunosuppressive environment. The IL-10 level was significantly higher in peripheral blood of PBSC donors after the administration of mobilizing factor (G-CSF). The percentage of CD47 + HSCs was significantly higher in bone marrow compared to peripheral blood of mobilized donors.

1. Introduction

The hematopoietic stem cells (HSCs) transplantation is an important tool in the treatment of hematological diseases often being the sole curative option for patients with leukemia, lymphoma or myeloma [1]. HSCs reside in bone marrow, but they are capable of migrating out of their niche and coming back [2]. HSCs for transplantation may be collected either directly from bone marrow cavities or from peripheral blood with the use of leukapheresis. The latter requires preceding stimulation with granulocyte - colony stimulating factor (G-CSF) which causes the release of HSCs from bone marrow to peripheral blood. Those two types of material are successfully used for transplantation. It is known that the source of stem cells may have an impact on outcome [3]. The reconstitution of hematopoiesis is usually more rapid after peripheral blood stem cells (PBSCs) transplantation. On the other hand the PBSCs transplantation may lead to a higher risk of chronic graft versus host disease (cGVHD) [4]. It can be explained with different cellular composition (both in percentage and absolute volume) of transplantation material or with different properties of cells being transplanted. HSCs properties can be regulated by a variety of factors including the surrounding microenvironment, cell to cell signaling and interaction, cytokine and chemokine influence and many others. The microenvironment of the HSCs, including cytokine composition can have an impact on the state and properties of the cells and therefore on the properties of the transplantation material.

It is believed, that HSCs in bone marrow are actively protected from toxic or infectious factors. This protective role of HSCs niche was studied by Fujisaki et al. They observed prolonged survival of allogeneic HSCs in the bone marrow of mice after HSC transplantation without immunosuppression [5]. Fujisaki et al. hypothesized that the HSC niche

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; G-CSF, granulocyte - colony stimulating factor; PBSCs, peripheral blood stem cells; GVHD, graft-versus-host disease; cGVHD, chronic graft-versus-host disease; IL, interleukin; Tregs, T regulatory lymphocytes; PD-L1, Programmed death ligand 1; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α; IFN-γ, interferon γ

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within the bone marrow is an immune-privileged site [5,6]. Currently it is considered, that immune privileged places include the eyes, brain, placenta and testis [7,8]. These sites are able to tolerate the presence of antigen (like allogeneic graft), without the initiation of immune response. This phenomenon is an active process, related to the higher frequency of Tregs, increased concentration of immunosuppressive cytokines like TGF-β1, IL-4, IL-10, IL-12 and decreased level of proinflammatory cytokines (IL-1β, IL-6) [9]. It provides protection for the more valuable cells like HSCs. Despite of protection offered by microenvironment, HSCs themselves are able to inhibit attack of the immune system. Fujisaki et al. showed, that HSCs secrete IL-10, which is critical for Treg-mediated immunosuppression [5].

HSCs are also capable of direct interaction with the immune system cells, by altered expression of surface molecules [10]. CD47 is a protein, known as a “don't-eat-me” signal, because it inhibits host cell phagocytosis by binding the signal regulatory protein on macrophage surface [11]. CD274 (also called B7-H1or PD-L1 - *programmed death ligand 1*) downregulates several T lymphocytes functions such as proliferation and production of effector cytokines [12]. It promotes T-cell apoptosis and plays a potential role in inhibiting T cell immune response [13]. Probably the elevated expression of CD47 and CD274 on the surface of HSCs could be another evolutionary adaptation, which prevents potential damage of HSCs by immune system.

Protection mechanisms of HSCs in bone marrow are still under investigation and we believe it could be an important aspect of HSCs transplantation. According to our best knowledge, this is first study of differences in protection mechanisms between HSCs collected by two different ways: peripheral blood stem cells (PBSCs) collected by leukapheresis procedure and HSCs collected directly from bone marrow cavities. The main aim of this study was to compare concentration of selected pro- and anti-inflammatory cytokines and also the expression of immunosuppressive CD47 and CD274 proteins on HCSs from PBSCs and bone marrow of healthy donors.

2. Material and methods

2.1. Sampling methodology

All samples were obtained from healthy donors of HSCs. We have collected samples from two groups of donors: PBSCs donors and bone marrow donors. All of the donors are thoroughly examined for any past and present medical conditions and also for any form of taken medication. All of the donors were in full health and were not taking any form of medication.

PBSCs donors prior to leukapheresis were stimulated with G-CSF (*granulocyte colony stimulating factor*) 10 μg/kg/day for 4 consecutive days. Whole blood samples were collected two times from each donor. First sample was taken before the G-CSF stimulation, the second sample was taken on the day of leukapheresis (before the procedure).

Bone marrow samples were collected at the start of the bone marrow donation. The first syringe (approx. 2 ml) was taken for the analysis to minimize the possible dilution caused by peripheral blood.

All samples were collected into EDTA vacutainer tubes (Becton-Dickinson, New Jersey, USA). They were used to evaluate cytokine concentration by ELISA test and immunosuppressive protein expression on HSCs by FACS analysis.

A characteristic of groups of donors is shown in Tables 1 and 2.

Permission for studies was obtained from the local Bioethical Committee (Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice, Poland).

2.2. Determination of cytokines concentration

The levels of IL-1 β, IL-2, IL-4, IL-6, IL-10, IL-17A, TGF-β1, IFN-γ and TNF-α were analyzed. All cytokines concentrations were measured using ELISA kits (eBioscience, San Diego, USA) according to the manufacturer’s manual (to increase assay sensitivity, the samples were incubated with capture antibody overnight – 16–18 h). After collection samples of blood and bone marrow were centrifuged at 500 $\times g$ for 20 min. Next, aliquots of plasma samples, were divided into portions and immediately stored in liquid nitrogen until the time of analysis. Synergy 2 Multi-Mode Microplate Readers (BioTek, Winooski, USA) was used for the analysis.

Table 1
Samples collected for cytokine concentration analysis.

Type of Donor	PBSCs donors	Bone marrow donors	p
Material	Whole blood sample taken before G-CSF stimulation and on the day of leukapheresis	Bone marrow sample taken at the start of the donation	
Number of samples	28	23	
Median age of donors (range)	28 (20–47)	23 (19–40)	0.33
Sex male/female	22/6	12/11	0.11

Table 2
Samples collected for flow cytometry analysis.

Type of Donor	PBSCs donors	Bone marrow donors	p
Material	Whole blood sample taken on the day of leukapheresis	Bone marrow sample taken at the start of the donation	
Number of samples	16	18	
Median age of donors (range)	27.5 (20–37)	25 (20–45)	0.34
Sex Male/Female	9/7	13/5	0.29

2.3. Flow cytometry analyses

The flow cytometry analysis of CD47 and CD274 expression on HSCs was performed immediately after PBSCs or bone marrow collection. Whole blood cells were incubated for 20 min., at room temperature, with appropriate antibodies. To remove erythrocytes, the cells were incubated with BD Pharm Lyse™ lysing buffer (BD Biosciences, San Jose, CA, USA). After washing in Cell Wash Buffer (BD Biosciences, San Jose, CA, USA), the cells were suspended in Cell Wash Buffer and analyzed using fluorocytometer FACS Canto (BD Biosciences, San Jose, CA, USA).

The subpopulations of HSCs ($CD45^+CD34^+$ phenotype) showing high expression of CD274 or CD47 markers were identified using CD34-PE, CD45-PerCP, CD47-Alexa Fluor 647, CD274-FITC antibodies (all antibodies obtained from BD Biosciences, San Jose, CA, USA).

2.4. Statistical analysis

U Mann–Whitney test was used to evaluate differences between concentration of cytokines in the whole blood and bone marrow samples and also to compare expression of CD47 and CD274 on HSCs. Wilcoxon test was used to evaluate differences between concentration of cytokines in peripheral blood of donors prior to and past the mobilization. Patients’ characteristics were compared using the U Mann–Whitney test.