VILNIUS UNIVERSITY
STATE RESEARCH INSTITUTE
CENTRE FOR INNOVATIVE MEDICINE

Vytautas Kašėta

INVESTIGATION OF BONE MARROW HEMATOPOIETIC STEM CELL MIGRATION DURING INFLAMMATION IN BALB/c MICE

Summary of doctoral thesis
Biomedical sciences, biology (01B);
Immunology, serology, transplantation (B 500)

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This study was carried out in 2006-2010 at the Institute of Immunology of Vilnius University and, after reorganization, at the State Research Institute Centre for Innovative Medicine

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The dissertation is available at the Library of State Research Institute Centre for Innovative Medicine and Vilnius University Library.

VILNIAUS UNIVERSITETAS
VALSTYBINIS MOKSLINIŲ TYRIMŲ INSTITUTAS
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Vytautas Kašėta

BALB/c PELIŲ KAULŲ ČIULPŲ HEMOPOETINIŲ KAMIENINIŲ LĄSTELIŲ MIGRACIJOS TYRIMAI UŽDEGIMO METU

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Introduction

Stem cell (SC) research, currently widely performed in the world, expands the current knowledge about the possibility to use these cells for the purpose of therapeutic and regenerative medicine. Recently, more and more evidence, showing the positive impact of SC to restoring the process and function of affected organs, is appearing in literature. It is supposed that each functionally inefficient organ could be subjected to cell therapy. The use of embryonic SC for therapy is unacceptable because of a variety of technical, political and ethical reasons; therefore, adult hematopoietic stem cells (HSC) can be used as an alternative. Because of their proliferative potential and the availability, HSC, as confirmed by the research data, are particularly suitable for this purpose. It was found that from the HSC can be developed not only blood cells, but also several types of nonhematopoietic tissue cells. However, many questions about the behavior and function of the transplanted stem cells *in vivo* remain to be answered. The bone marrow is the main source of HSC in adults, and those cells are already used for transplantation. The HSC is one of the best studied types of adult stem cells.

Though, the HSC population in the body is very small, but they still manage to produce millions of mature blood cells and their precursors every day. The number of HSC increases in case of tissue injury, organ damage, heavy blood loss or during inflammation and other cases. However, isolation of the hematopoietic cells is complex enough, because of the undefined surface markers. Only a few of these proteins, expressed on the cell surface, are known. The same markers may be expressed by other bone marrow cells and their expression may vary among different animal species as well as during their development. It was found that bone marrow cells are heterogeneous. To get pure enough fractions of HSC (i.e. for transplantation) the methods with few different parameters should be used. According to the appropriate marker combinations, it is possible to identify and isolate hematopoietic cell populations and later use them to restore the damaged tissue (for various breach reconstructions). However, there is no consensus, which population of cells should be used for the therapy in different disorders. Determination of the surface markers and isolation of hematopoietic stem cell populations, as well as their migration studies *in vivo*, are important for the further HSC selective use in therapy.

It is known that migration is an integral feature of hematopoietic cells, which makes them leave the bone marrow, move into the tissue and/or return back to the bone marrow. This process is known as a physiological hematopoietic cell migration. It was found that stem cells can migrate to the damaged tissue, but the mechanisms of cell migration are not yet clear. Whereas the pathological processes, in which the use of cell therapy is expected, are usually accompanied by inflammation. Therefore, it is particularly important to clarify how the stem cells affect the inflammation and how their migration is affected by inflammation. Data on, how the SC affect the inflammation process is lacking, and the migration research results, obtained in different organ injury models (liver, kidney, etc.), are controversial. It is believed that physiological and transplanted cell migration processes are regulated by the general mechanisms; however, there is a lack of a consistent kinetic evaluation of migration, and the separate studies contradict to each other. It is not clear so far how long it takes for a stem cell to migrate to the sites of injury, where it is localized and in which environment they fall after transplantation. It is not known how many transplanted HSC migrate to the place of injury, how many of them fall into other organs and changes in levels of transplanted cells during inflammation. A transplanted hematopoietic stem cell migration research during inflammation, carried out in this work, contributes to clarification of stem cell migration patterns in case of pathological processes. This is particularly important in ensuring a safe and effective stem cell application in clinical practice.

The aim of dissertation work

To investigate the anti-inflammatory effect of murine bone marrow hematopoietic stem cells and their migration in the BALB/c mouse contact hypersensitivity model *in vivo*.

The objectives of this work were as followed:

1. Isolation and identification of the BALB/c mouse bone marrow hematopoietic stem cell population;
2. Identification of the anti-inflammatory effect of isolated populations in the BALB/c mouse contact hypersensitivity model *in vivo*;

3. Evaluation of the migration kinetics of the transplanted BALB/c mouse hematopoietic stem cells;
4. Quantifying the migration of the hematopoietic stem cells to the injury sites and other organs.

Scientific novelty

In this study, BALB/c adult bone marrow hematopoietic stem cell populations were isolated and identified. The studies of isolated populations showed that lin^- HSC population markers (CD117 and Sca-1) expression is characteristic for hematopoietic stem cells. For the first time anti-inflammatory effect of the hematopoietic stem cells was evaluated for the BALB/c mouse contact hypersensitivity model *in vivo*. It was found that isolated lin^- HSC, lin^+, lin^- HSC with lin^+ hematopoietic cell populations had a significant anti-inflammatory effect and inhibited the edema. The studies showed that the most efficient (up to 66%) inhibition of foot edema was obtained when using the HSC population. For the first time the HSC population migration kinetics in the BALB/c mouse contact hypersensitivity model *in vivo* was investigated. We determined that cells of this population can be found in mice paw edema and liver after just one hour. A little bit later they are detected in the spleen.

For the first time we did the HSC population quantitative migration kinetic studies in the BALB/c mouse contact hypersensitivity model *in vivo*. We found that in case of foot inflammation there is a secondary migration of the transplanted HSC migrating from the bone marrow to the spleen hematopoietic niche. We have shown that these cells selectively migrate into the inflammation areas of the foot edema. Transplanted cells quantity in the samples of foot edema, as compared with the untreated foot, was more than 1000 times higher.

The defensive statements

- Mouse bone marrow HSC have an effective anti-inflammatory effect and inhibit a foot edema.
- BALB/c mouse bone marrow-derived HSC population selectively migrate to the inflammatory sites in the affected foot.

- Inflammation affects the weaker transplanted HSC migration into the lung, liver, spleen and bone marrow.

Materials and methods

Laboratory Animals. BALB/c mice (weighing 22–24 g), obtained from the Laboratory Animal Center of State Research Institute Centre for Innovative Medicine (Lithuania), were used. Animals were maintained in an environment of controlled temperature (23±1^{o}C). Food and water were provided ad libitum. All procedures were approved by the Lithuanian Ethics Committee on the Use of Laboratory Animals under State Veterinary Service (No. 0160/ 2007).

Contact hypersensitivity model. Contact hypersensitivity reaction was induced with 2,4-dinitroluorbenzene (DNFB) by Hiltz (1990) with some modifications. All animals were sensitized by application of 25 µl of 0.5% DNFB onto the shaved abdomen. After 3 days, mice were challenged with an injection of 10 µl of 0.1% DNFB into the paw. The DNFB solution was prepared just prior to application of the acetone-olive oil mixture (4:1). Cell transplantation was performed 24 h after the DNFB challenge. A total of 10^6 cells/mouse in 100 µl PBS were intravenously injected. The control group mice received 100 µl injections of phosphate buffered saline (PBS). The reference group was treated intraperitoneally with prednisolone (100 mg/kg of animal weight). Differences in the weight of the injected versus the uninjected paw were evaluated after the treatment.

Isolation of mouse bone marrow cells. Isolation was performed as described by Joupperi (2007) with some modifications. BALB/c mice, 7 weeks old, were sacrificed by cervical dislocation. Their femurs and tibias were removed aseptically and placed on a Petri dish containing PBS. Bone marrow was obtained by flushing with sterile PBS through one of the femoral epiphyses, using a syringe needle (27-gauge). The bone marrow cells were collected in sterile PBS and washed three times by centrifugation for 6 min at 300×g.

Isolation of low-density cell fraction. Isolation was performed as described by Joupperi (2007) with some modifications. The low-density cell fraction was obtained by separation of mouse bone marrow cells in a Percol (GE Healthcare BioSciences AB, 1.087 g/ml) density gradient. A 5 ml of prepared Percol^{TM} (1.077-1.081-1.087 g/ml) was placed into a plastic tube and the same amount of bone marrow cell suspension in RPMI-1640 was layered on the top. This was centrifuged for 30 min at 1200×g. The cell fraction, harvested from the interface between these two media, was washed immediately and suspended in PBS.

Preparation of lineage-negative cell fraction. Cells were purified using magnetic cell sorting (MACS) techniques with the BD IMagTM mouse hematopoietic progenitor enrichment set (composed of BD IMag™ Streptavidin Particles Plus – DM and biotin-conjugated monoclonal antibodies against hematopoietic lineages: anti-mouse CD3e, clone 145-2C1; anti-mouse CD11b, clone M1/70; anti-mouse CD45R/B220, clone RA3-6B2; anti-mouse Ly-6G and Ly-6C (Gr-1), clone RB6-8C5; anti-mouse TER-119, clone TER-119) applied as recommended by the manufacturer. Hematopoietic progenitor stem cells were isolated from the mononuclear cell suspension by negative selection (differentiated cells were removed using nanoparticles labeled with antibodies against lin^{+}).

Flow cytometrical analysis. The procedure was performed as described by Suzuki (2001). For flow cytometrical analysis, the following monoclonal rat anti-mouse antibodies were used: c-kit (CD117) conjugated with luoresceinisothiocyanate (FITC)(CD117-FITC) (Chemicon International); Sca-1 conjugated with phycoerythrin (PE)(Sca1-PE) (Abcam, Cambridge); CD38-PE (Santa Cruz Biotechnology); CD34-PE (Santa Cruz Biotechnology). Cell suspensions ($2x10^5$ per sample) were labeled with antibody pairs: PE/FITC; the appropriate amounts of antibodies were added to the cell suspension and the mixture was incubated at 4°C for 30 min in the dark. The cells were pelletted and washed twice with PBS. Then cells were analyzed by FACS Callibur cytometry (Becton Dickinson). Isotype-matched nonspeciic antibodies rat IgG2α-FITC (BD Biosciences,) and rat IgG2α-PE (Chemicon International) were used for the control.
Cell labeling with fluorescent dye. lin^- cells were labeled with PKH67, a green fluorescent probe (Sigma) procedure performed as recommended by the manufacturer. 2×10^7 cells were suspended in 1 ml of the comercial diluent (C), and stained rapidly by mixing with an appropriate amount of freshly prepared 4 μM working PKH67 solution. Staining was stopped by adding 2 ml of FCS. Cells were collected by centrifugation (400xg, 10 minutes, 4 °C), resuspended in RPMI-1640 supplemented with 10% FCS, and washed twice with RPMI. After final washing, cells were suspended in PBS. The average recovery of this procedure was 78% viable cells, as assessed by the trypan blue exclusion test. Cell staining was verified by the fluorescence microscopy.
Histological study. Following an appropriate time after cell transplantation, all mice groups were sacrificed for histological study, and spleen, liver, untreated paw and paw edema specimens of the recipient mice were collected and cryopreserved. Sections (4-5 micrometers thick) were cut using a cryotome, and every 10th section was used for examination. Histological sections were prepared in cooperation with the National Center of Pathology. The presence of injected stained cells in sections was evaluated by the fluorescent microscopy with a Nikon TE2000U instrument. Unchallenged mice paws served as a histological control.
Cytokine determination. Following an appropriate time after cell transplantation mouse blood was collected. Concentrations of cytokines IL-10 and TNF-α were quantitatively determined in mouse sera using ELISA kits for IL-10 and TNF-α assay (Amersham Biosciences), respectively, according to the manufacturer's instructions. Optical density on 450 nm wavelength was measured by the Sunrise (Tecan) plate reader.
Investigation of fluorescently-labeled cell migration.
The first part of the experiments. DNFB challenged mice were randomly allocated into groups (n = 5 in each). Experimental BALB/c mice were placed into two groups, according to transplanted cell labeling. A total of 10^6 cells/mouse of lin^- HSC cells in 100 μl PBS were injected into the tail vein of the study group mice. Following an appropriate time after cell transplantation, mouse spleen, liver, untreated paw and paw edema specimens of the recipient mice were collected and cryopreserved. Preparations were evaluated by a histological study. This experiment was performed in triplicate.
The second part of the experiments. DNFB challenged mice were randomly allocated into groups (n = 5 in each). Experimental BALB/c mice were placed into 7 groups, according to histological specimen collection time (1, 4, 8, 12, 24, 48, 72 hours after cell transplantation). A total of 10^6 cells/mouse of lin^- HSC cells in 100 μl PBS were injected into the tail vein of the study group mice. Following an appropriate time after cell transplantation, mouse spleen, liver, untreated paw and paw edema specimens of the

recipient mice were collected and cryopreserved. Preparations were evaluated by a histological study. This experiment was performed in triplicate.

Real-time qPCR study of migration. DNFB challenged mice were randomly allocated into groups (n = 5 in each). Experimental BALB/c mice were placed into 7 groups, according to the biopsy specimen collection time (1, 4, 8, 12, 24, 48, 72 hours after cell transplantation). A total of 10^6 cells/mouse of lin^- HSC colected from male mice in 100 µl PBS were injected into the tail vein of the female mice study group. Following an appropriate time after cell transplantation, mouse bone marrow, spleen, liver, lung, kidney, untreated paw and paw edema specimens of the recipient mice were collected and cryopreserved. Preparations were evaluated by the real-time PCR analysis. This experiment was performed in triplicate.

Biopsy preparation for DNA extraction.

Mouse bone marrow samples. Bone marrow cells were obtained by flushing with 500µl sterile TE buffer through one of the femoral epiphyses, using a syringe needle (27-gauge). 200µl cell suspension was used for furher DNA extraction.

Mouse tissue samples. Spleen, liver, lung, kidney, untreated paw and paw edema specimens were prepared using the tissue homogenizer - TissueLyser2 (Invitrogen). 40-50 mg of tissue were placed in a special test tube with a 4.5 mm diameter steel ball and 400µl TE buffer. Shaked for 2 min at 30 Hz. 200µl cell suspension was further used for DNA extraction.

DNA extraction. Total DNA isolated from cell suspensions using „Genomic DNA purification kit“ (Fermentas), according to the manufacturer's instruction. DNA concentration was determined by measuring the absorbance (A) at 260 nm wavelength. DNA purity was defined by calculating the A^{260} and A^{280} absorbance ratio. DNA purity is considered sufficient if the A^{260}:A^{280}> 1.5. The measurements were performed using Synergy 2 (Biotek) plate reader.

Real-time PCR. The reaction was performed using the Rotor-Gene 6000 (Corbet Research) rotary analyzer. Used primers and probes were designed by Wang (2002), to detect the mouse TSPY (mouse testis-specific Y-encoded protein)(forward:5`-GAG AAC CAC CTT GGT GAT TCT CT- 3`; reverse: 5` -TCC TTG GGC TCT TCA TTA TTC TTA AC- 3`) and mouse β-aktin (forward 5`-ACG GCC AGG TCA TCA CTA TTG- 3`; reverse: 5` -CAA GAA GGA AGG CTG GAA AAG A- 3`) genes. TSPY pseudogene quantities were determined in the samples, this gene is only detected in the mouse male cells (Y chromosome). TSPY gene amplification was normalized by mouse β-aktin (detected in all mouse cells) amplification. Specific FAM (carboxy-fluorescein) probes were used to quantify the specific gene products. TSPY (5`-/56-FAM/-TCC TGG ATC-/ZEN/-AGA GTG GCT TAC CCA GG-/3IABkFQ/-3`) and β-aktin (5`-/56-FAM/-CAA CGA GCG-/ZEN/-GTT CCG ATG CCC T-/3IABkFQ/-3). The reaction with FAM-labeled probes was performed using "Absolute Blue qPCR mix (Thermo Scientific). 100 ng of DNA sample, 200 µM of primers and 100 µM probe were used per 15 µl of the reaction volume. An initial 15 min denaturation step at 95 °C followed by 40 cycles of 0.25 min denaturation at 95 °C, for 1 min annealing at 60 °C and 0.5 min elongation at 72 °C. In each reaction group, different concentrations of the positive control (BALB/c male DNA from 0.0001 to 200 ng), negative control (PCR reagents without DNA, PCR reagents with BALB/c female DNA) and calibration samples (various concentration combinations of BALB/c male and female DNA, per 100 ng of the total DNA ratio from 1/10 to 1/100, 000) were used. Test samples were duplicated

and two independent tests were performed. The quantity of the product was determined in each cycle by measuring the specific probe fluorescence (excitation 470 ± 10 nm detection 510± 5nm). The product size was verified by agarose gel analysis.The quantitative data normalization was performed according to the comparative quantification method (Wilhelm et al., 2003; Skern et al., 2005). For normalization of DNA levels, the housekeeping gene β-actin was used. Based on the calibrator and standard curve data, the sample product was normalized and the relative concentration was calculated.

Statistics. Results are shown as mean ± standard deviation. Statistical significance among the mean values was determined using two-sided Student's t-test. $P < 0.05$ was considered significant. All calculations were performed using SigmaPlot (version 11).

Results and discussion

Isolation and identification of cell populations. In this study the total population of the BALB/c mouse bone marrow cells was isolated. From the total cell population by the density gradient centrifugation (1.087g/ml) and magnetic cell separation, lin^- hematopoietic stem cell population was obtained. For magnetic cell separation, the antibodies against murine hematopoietic cell lineage antigens were used.

The isolated cells were identified by the flow cytometry. The results showed that 27.39 ± 7.54% of lin^- cells expressed CD38 and CD117, while 73.92 ± 11.79% expressed CD117 and CD34. Sca-1 were expresed on 83.27 ± 2.92% of lin^- population cells, CD117 - 81.69 ± 3.22%. Sca-1 and CD117 were found on 73.36 ± 4.96% of cells (*Table 1*).

From the lin^- cell population by a positive selection using specific antibody coated nanoparticles, the $CD117^+lin^-$ population was isolated. The identification of surface markers showed that 32.59 ± 5.74% of cells expressed CD38 and CD117, 68.52 ± 9.85% - CD34 and CD117, while 60.94 ± 1.43% - Sca1 and CD117 (*Table 1*). In lin^-CD117^+ population, we found a decrease in the expression of haematopoietic stem cell specific markers. It should be noted that after the lin^-CD117^+ fraction separation less than 0.1% of the total bone marrow cells are recovered.

The high expression of CD34 marker of lin^- HSC and lin^-CD117^+ populations (77 and 72%) suggest an activated state of HSC in the isolated populations. Our data shows that more than 70% of lin^- HSC population did not express CD38, which means that HSC in this population are activated. The major part (more than 85%) of lin^- HSC population cells bearing CD34 confirms that. Meanwhile, the $CD38^-$and $CD34^+$ cell

percentage of lin^-CD117^+ population (55% and 70%, respectively) only partly confirm the HSC being in an activated state.

Obtaining hematopoietic stem and progenitor cell populations and the establishment of surface markers are important for the successful use of them in a clinical practice.

Table 1. The percentage of murine hematopoietic cell populations bearing CD34, CD38, CD117 and Sca1 surface markers.

Surface marker	**Hematopoietic cell population**		
	Bone marrow	lin^-	lin^-CD117^+
$CD38^+CD117^-$	7.17±2.92	2.16±0.57	11.78±0.84
$CD38^+CD117^+$	27.73±8.69	27.39±7.54	32.59±5.74
$CD38^-CD117^+$	**35.13±13.08**	**48.93±8.46**	**40.52±9.86**
$CD38^-CD117^-$	29.97±11.82	21.52±1.74	15.11±1.68
$CD34^+CD117^-$	30.50±9.41	13.79±7.53	18.57±7.45
$CD34^+CD117^+$	**40.43±15.74**	**73.10±11.79**	**68.52±9.85**
$CD34^-CD117^+$	1.19±0.05	3.92±0.43	3.59±0.84
$CD34^-CD117^-$	27.88±6.38	9.19±4.69	9.32±10.23
$Sca1^+CD117^-$	31.20±0.93	9.91±3.64	16.29±3.22
$Sca1^+CD117^+$	21.94±3.17	**73.36±4.96**	**60.94±5.43**
$Sca1^-CD117^+$	2.84±0.23	8.33±2.00	9.42±2.36
$Sca1^-CD117^-$	**44.02±3.86**	8.46±1.43	13.35±2.34

CD – cluster of differentiation; Sca-1 – stem cell antigen; lin – lineage markers;1. Values are means ± SD; n = 7 experiments.

The experiments showed that the HSC specific markers, CD117 and Sca-1, bearing cells comprised about 22% in the total bone marrow cell population. Meanwhile, the isolated lin^- HSC population had more than 3 times (73%) bigger expression of these markers.

In summary, isolated mouse bone marrow lin^- HSC population expressed a high percentage of HSC specific markers (CD117 and Sca-1) bearing cells and a lack of hematopoietic lineage markers. Comparing the data of our study with the results of other authors, we can say that the isolated from mouse bone marrow lin^- HSC population, is assigned to the KLS population, which consists of a long and short-term, haematopoietic stem cells and early hematopoietic progenitors (Okada et al., 1992; Challen et al., 2009). High expression of CD34 markers (more than 85%) and CD38 negative percentage of cells (more than 70%) in the separated lin^- HSC population indicate that the majority of these HSC is activated, i.e. belong to the short-term HSC.

Thus, the majority of our isolated BALB/c mouse bone marrow lin^- HSC population consists of the short-term HSC, the rest - a long-term HSC and early hematopoietic progenitors. For the further studies lin^- HSC population was used.

The in vivo anti-inflammatory effect of isolated hematopoietic cell populations. As the diseases, in which the cell-based therapies are planned to be applied, are usually accompanied by inflammation, we believe that the research of the anti-inflammatory effect of stem cells is important and valuable. However, we did not succeed in finding the data on the research of systemic anti-inflammatory effect of different hematopoietic cell populations. In this study, the anti-inflammatory properties of isolated and identified hematopoietic cell populations in the BALB/c mouse contact hypersensitivity model *in vivo* were examined.

We investigated the anti-inflammatory effect of the total population of bone marrow, lin^- and lin^+ cells as detected by the weight difference between the DNFB affected and healthy feet.

The anti-inflammatory effects of total bone marrow cell population. The results obtained with the different total bone marrow cell population doses ($2x10^6$, 10^6, 10^5 cells / mouse) 48 hours after transplantation showed that this population did not produce the anti-inflammatory effect (not found notable differences in the feet weights). We believe that anti-inflammatory effect was not revealed because of a relatively small amount of stem cells transplanted in the total bone marrow cell population. Therefore, for the further studies, lin^- HSC population was used.

The anti-inflammatory effect of lin^- HSC population at different concentrations. The anti-inflammatory effect of different concentrations of lin^- HSC population was evaluated in

BALB/c mouse contact hypersensitivity model. We found that after 48 hours, all investigated cell concentrations ($5x10^6$, 10^6, 10^5 cells/mouse) of this population, effectively and significantly (as compared with the negative control) inhibited the paw edema *(Fig. 1.)*. The most effective concentration of lin^- HSC population was at 10^6 cells/mouse; it inhibited paw edema 1.28 times better than the reference substance – prednisolone. Therefore, in the further studies of the anti-inflammatory effect and migration, the concentration of 10^6 cells/mouse was used.

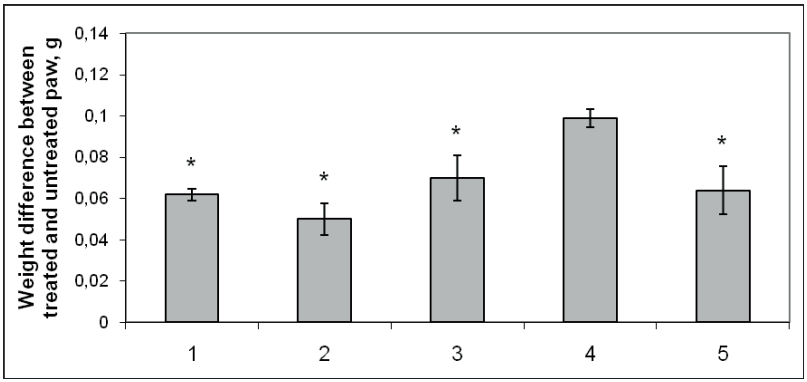

Fig. 1. **The effect of different doses of murine lin^- HSC population on paw edema in contact hypersensitivity model (48 h after cell injection).** 1) $5x10^6$ cells/mouse; 2) 10^6 cells/mouse; 3) 10^5 cells/mouse; 4) Negative control - 100µl PBS; 5) Reference substance - prednizolone (100 mg/kg of animal weight); n = 10 in each group. * Data are significantly different from the negative contol (*P<0.05). Data are presented as mean ± SD.

The anti-inflammatory effect of lin^- HSC and lin^+ cell populations. The anti-inflammatory effect of different cell populations, lin^- HSC, lin^+ and lin^- with lin^+ (1:10 ratio) was evaluated in BALB/c mouse contact hypersensitivity model. We found that all tested cell populations efficiently and statistically significantly inhibited the paw edema (*Fig. 2*). The foot edema decreased from 1.4 to almost 3 times when compared with the negative control. The lin^- HSC population was the most efficient in reducing the paw edema up to 66%.

We noticed that the lin^+ population also inhibited the foot edema, it was by 31% less as compared to the negative control. However, the lin^+ population enriched with 10% of lin^- HSC, inhibited foot edema by 18% more efficiently. Meanwhile, lin^- HSC

population alone, was twice more efective at the same dose. Our data, obtained with the different haematopoietic bone marrow cell populations suggest that the efficiency of anti-inflammatory effect of these populations is directly related to the relative content of lin^- HSC.

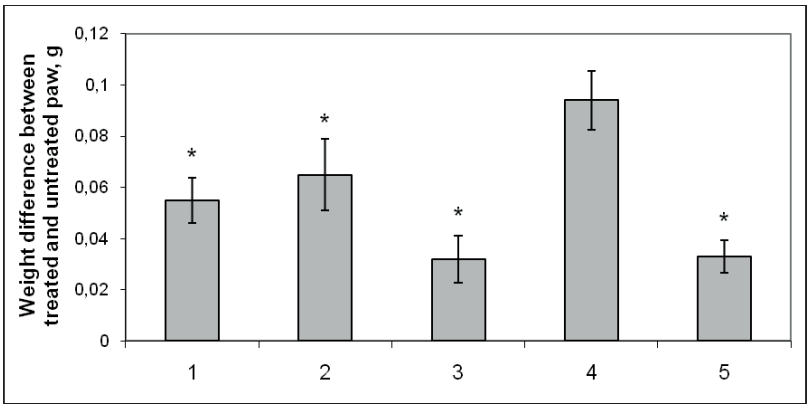

Fig. 2. **Effect of 10^6 cells/mouse concentration of different murine lin^- HSC, lin^+ and lin^- with lin^+ (1:10 ratio) populations on paw edema in contact hypersensitivity model (48 h after cell injection).** 1) lin^- HSC + lin^+; 2) lin^+; 3) lin^- HSC; 4) Negative control - 100µl PBS; 5) Reference substance - prednizolone (100 mg/kg of animal weight); n = 10 in each group. * Data are significantly different from the negative contol (*P<0.05). Data are presented as mean ± SD.

It seems that a circulating HSC are able to reduce inflammation by modulating the immune response. That could explain the anti-inflammatory effect of lin^- HSC population on foot edema. On the other hand, the circulating HSC may be a source for the local production of the immune effector cells and, thus to participate in inhibition of inflammation. It also explains the anti-inflammatory effect of lin^- HSC population on foot edema.

The lin^+ cell population also had the anti-inflammatory effect on foot edema in the mouse contact hypersensitivity model. Although, much weaker as compared with the lin^- HSC population, but anyway, transplantation of more differentiated hematopoietic cells inhibited foot edema. It could be that more differentiated cells of lin^+ population accessed the affected foot and might have increased the amount of the immune effector

cells involved in the inhibition of inflammation. The heterogeneity and proliferative properties of lin^+ population explain the weaker effect on foot edema.

The effect of lin^- HSC and lin^+ cell populations on cytokine levels in mouse blood sera. It is known that activation of cytokines, chemokines and proteolytic enzyme production occurs during inflammation of the damaged tissue. Pro-inflammatory cytokine TNF-α enhances, whereas anti-inflammatory cytokine IL-10 reduces the inflammation by inhibition of pro-inflammatory cytokine production. Since the levels of inflammatory cytokines (TNF-α and IL-10) are important indicators of inflammation, we evaluated the influence of different hematopoietic cell populations on the production of these cytokines in the contact hypersensitivity model. Our data indicate that all tested hematopoietic cell populations, lin^- HSC, lin^+ and lin^- with lin^+ (1:10 ratio), decrease TNF-α levels in the mouse serum from 1.2 to 3.5 times as compared to the negative control. (*Fig. 3*).

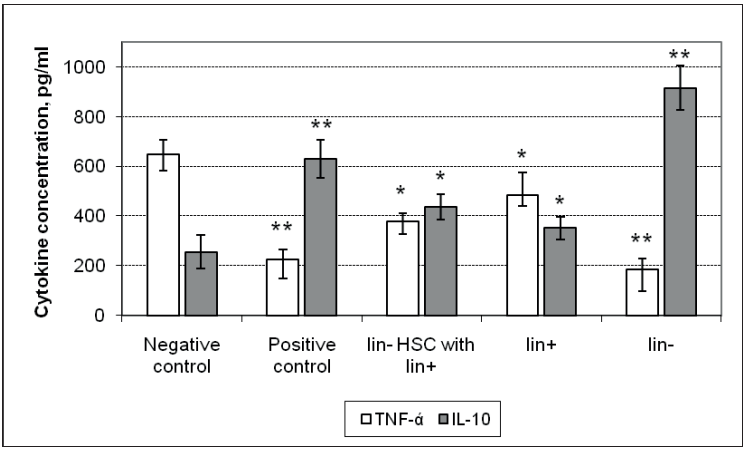

Fig 3. **Effects of different murine populations, lin^- HSC, lin^+ and lin^- with lin^+ (1:10 ratio), on pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokine levels in mouse sera in the contact hypersensitivity model (48 h after 10^6 cells/mouse injection).** n = 10 in each group. Data are presented as mean ± SD. Data marked with an asterisk are significantly different from the negative control (*P<0.01; **P<0.001). **a)** Negative control - 100µl PBS; **b)** Reference substance - prednizolone (100 mg/kg of animal weight).

Meanwhile, the IL-10 levels were from 1.3 and 3.6 times higher. The cytokine levels change after the transplantation of different hematopoietic cell populations. It indicates that inflammatory processes are inhibited. After transplantation (10^6 cells/mouse) of different hematopoietic cell populations, lin^- HSC, lin^+ and lin^- with lin^+ (1:10 ratio) in the mouse contact hypersensitivity model, the changes in cytokine levels, after 48 hours are proportional to the inhibition efficiency of foot edema at the same conditions. This confirms the anti-inflammatory properties of the lin^- HSC, lin^+ and lin^- with lin^+ (1:10 ratio) populations.

We found that the smallest changes in cytokine levels, as well as the weakest infibition of foot edema were found in the mouse after the transplantation of lin^+ cell population. The most significant changes of the cytokines were observed after the lin^- HSC transplantation. Compared with the control TNF-α concentration was 3.5 times lower, and IL-10 – 3.6 times higher. This population has shown to express the most efficient foot edema inhibition.

The anti-inflammatory effect of systematically transplanted HSC may be associated with indirect effects on inflammation, however, a direct participation of HSC in inflammation is also possible. It may be, that in case of injury ,the circulating HSC have a dual effect on inflammation, modulate the immune response and serve as a source for the local production of effector immune cells. In this way, they reduce inflammation and stimulate tissue regeneration.

In the further study, we investigated the migration of lin^- HSC in BALB/c mouse contact hypersensitivity model to identify the potential nature of the anti-inflammatory effect in this population.

Evaluation of transplanted lin$^-$ hematopoietic stem cell migration. Migration of hematopoietic stem cells to the damaged peripheral tissues and their possible differentiation are still poorly understood. It is believed that the HSC can differentiate into myeloid or tissue-specific cells in site of inflammation. The studies on transplanted cell migration and their kinetics are not uniform. Scientists disagree as to migration targets of the HSC in case of injury, and whether this migration is specific to the site of injury or not. The time, needed to migrate, is also at issue.

Evaluation of the HSC migration. The aim of our experiments was to evaluate the migration sites of lin^- HSC in case of injury. Migration was investigated in BALB/c

mouse contact hypersensitivity model. Membranes of transplanted cells were labeled with the PKH67 fluorescent dye. Following 48 hours after transplantation of the 10^6 lin^- HSC cells to evaluate the migration sites, the foot edema, healthy feet, liver and spleen tissue histological analysis were performed.

The labeled lin^- HSC were found in foot edema 48 hours after transplantation; meanwhile, in the histological preparations of healthy feet, these cells were not detected. In the spleen and liver, migrated labeled cells were found at the same time. It does not contradict with the data of other studies about stem cell distribution following 48 hours after the transplantation. Our results showed the presence of transplanted lin^- HSC in spleen and liver as well as in the affected feet, after 48 hours of transplantation. Spleen and liver can be considered as intermediate organs of migration, from where the migration of HSC is progressing further. The labeled cells in healtly feet were not found in 48 hours after transplantation, this suggests that migration into the affected foot is not accidental and lin^- HSC selectively migrate to the site of injury. In liver injury model , it was proved that the HSC not only selectively migrated to the injury site, but also participated in the regeneration of liver *(Dalakas et al., 2005)*. Our data indicate that lin^- HSC have anti-inflammatory properties and reduce the foot edema. However, the time for the transplanted cell to reach the injury site and other healthly organs is not clear.

HSC migration kinetics. To find out the migration kinetics, histology analysis of foot edema, healthy feet, liver and spleen was performed 1, 4, 8, 12, 24, 48 and 72 hours after the labeled cells (10^6 lin^- HSC) transplantation. The experiment was accomplished in BALB/c mouse contact hypersensitivity model (*Fig. 4*).

The analysis has shown that already after 1 hour, labeled lin^- HSC are found in the foot edema and liver. While labeled cells in the spleen are found only in 4 hours after the transplantation (*Table 2*). Our data show that in the liver transplanted cells are found up to 48 hours, meanwhile in the spleen and feet edema they are detected even after 72 hours. In all our investigated time points, labeled lin^- HSC in healthy feet were not found.

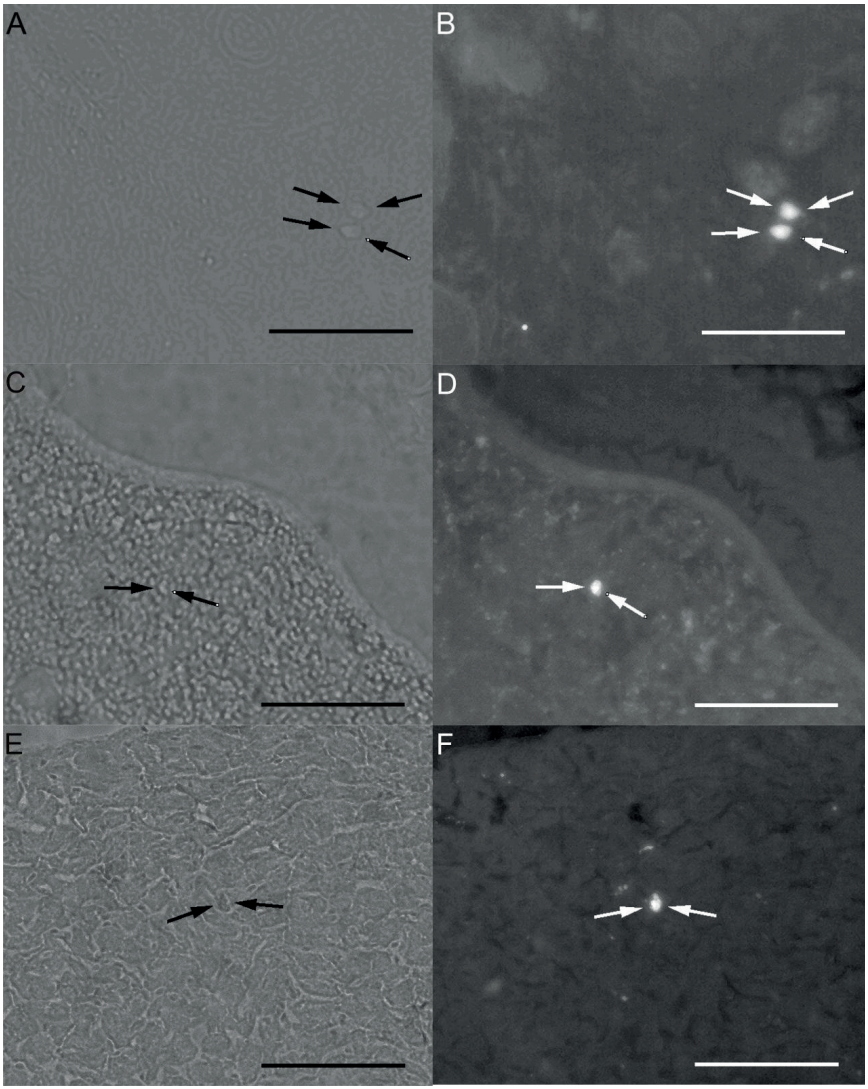

Fig. 4. **Histological preparations of BALB/c mouse organs.** Histological preparations of paw edema (A, B), spleen (C, D) and liver (E, F), where migrated fluorescent stained cells were found. After 1 h these cells were found in paw edema and liver, and after 4 h in the spleen. Fluorescence and bright field optical images are presented in the right and left panels, respectively; original magnification x20. The arrows indicate the fluorescent donor cells. Scale bar is 100 μm

Table 2. **Evaluation of the presence of migrated donor cells in histological preparations of untreated paw, paw edema, spleen and liver during various time intervals following the cell transplantation.**

Hours / Preparation	1	4	8	12	24	48	72
Paw edema	+	+	+	+	+	+	+
Spleen	-	+	+	+	+	+	+
Liver	+	+	+	+	+	+	-
Untreated paw	-	-	-	-	-	-	-

(+) - positive; (-) – negative. This experiment was performed in triplicate

Thus, transplanted lin$^-$ HSC in BALB/c mouse contact hypersensitivity model first migrated into the mouse foot edema and participated in inflammation, also they were found in the liver and later in the spleen. Whereas, lin$^-$ HSC were not detected in the liver after 72 hours. It suggests that the liver can be the intermediate organ for the migration of these cells. Although, transplanted cells were found in the spleen only after 4 hours, however, they remained here up to 72 hours. It contradicts the fact that spleen could serve as an intermediate organ of HSC migration.

The data of other authors indicate that hematopoietic stress can trigger the HSC migration to the spleen ectopic HSC niches *(Jaiswal and Weissman, 2009)*. Thus, inflammation in the mice feet might have influenced lin$^-$ HSC migration to the spleen ectopic niches, and in this case spleen is not an intermediate migration organ, but serves as a migration target of the HSC. To confirm this, the additional tests are needed. As in healthy foot transplanted cells were not found in all our investigated time points, we can state that lin$^-$ HSC selectively migrated to the injury site of paw edema. Our studies also have shown that lin$^-$ HSC quickly migrated to the foot edema in the contact hypersensitivity model: after 1 hour. The cells were found at the site of injury and remained there up to 72 hours. This supports the suggestion that tranplanted HSC can directly participate in the inflammation inhibition and tissue reconstruction.

Quantitative evaluation of transplanted hematopoietic stem cell lin$^-$ population migration kinetics. In this part of study, the migration kinetics of transplanted male lin- HSC was investigated in the female BALB/c mouse contact hypersensitivity model. Migration into the inflammatory site and healtly organs was established. Quantitative

analysis was carried out with the real-time polymerase chain reaction method. Spleen, kidney, bone marrow, lung, liver, damaged and healthly foot tissue samples were collected for analysis. The TSPY (target gene) and β-actin (housekeeping gene) specific amplification was determined. After performing the data processing, we compared the results with the standard curve data.

Migration of HSC to the spleen. The analysis of spleen samples has shown that transplanted lin- HSC after 4 hours are found in female recipient spleens, where they remain up to 72 hours (*Fig. 5*).

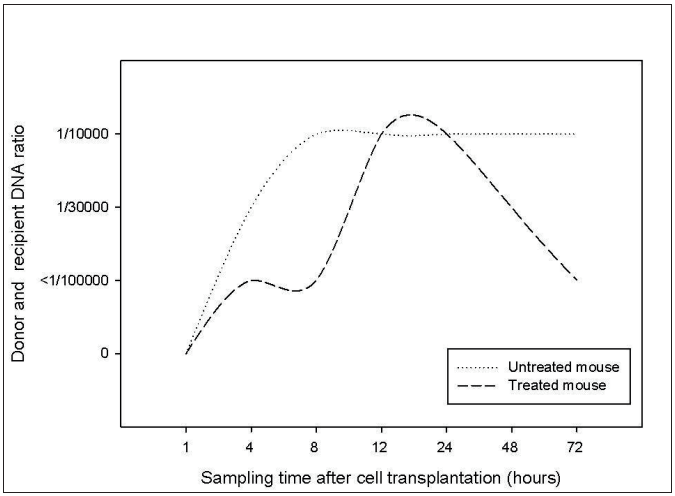

Fig. 5. **The quantitative evaluation of the transplanted lin- HSC migration kinetics to the spleen in BALB/c mouse contact hypersensitivity model.**

The levels of transplanted donor cells in the healthy and affected mouse spleen are different. Four hours after transplantation, in mice with feet edema only a trace (<1:100 000) of donor cells in the spleen was detected. Meanwhile, in healthy mice, the amount of donor cells was more than 3 times higher – 1:30 000. After 8 hours, donor origin cells were found in the spleen of healthy mice at the ratio of 1:10 000 and remained in the organ up to 72 hours. In case of inflammation, the same amount (1:10 000) of donor cells was found only after 12 hours following the transplantation. However, after 48 hours the amount of donor type cells in the spleen was found to be

decreased 3 times (1:30 000) and after 72 hours only trace amounts (<1:100 000) were found.

It seems that inflammation of the foot has affected the transplanted lin- HSC migration to the spleen at different time points. According to the data of other authors, under stress conditions the HSC can migrate from bone marrow into the ectopic niches of spleen. Our data suggest that the subsequent HSC migration to the spleen in case of inflammation is related with the absence of free ectopic hematopoietic niches in the spleen. Twelve hours after transplantation, some niches became vacant, thus enabling a secondary cell migration into the spleen. After another 12 hours, the amount of donor origin cells in the spleen began to decline. Such a decrease may be related to the niche replacement or the exit of transplanted cells to the circulation.

Migration of HSC to the kidney. The analysis of kidney samples revealed that transplanted lin^- HSC after 1 hour were found in the affected and healthy mouse kidney at same quantities, while after 4 hours only their traces were detected. The same amount remained up to 72 hours (*Fig. 6*). The differences between quantities of the donor origin cell in the kidneys of healthy and affected mice were not observed.

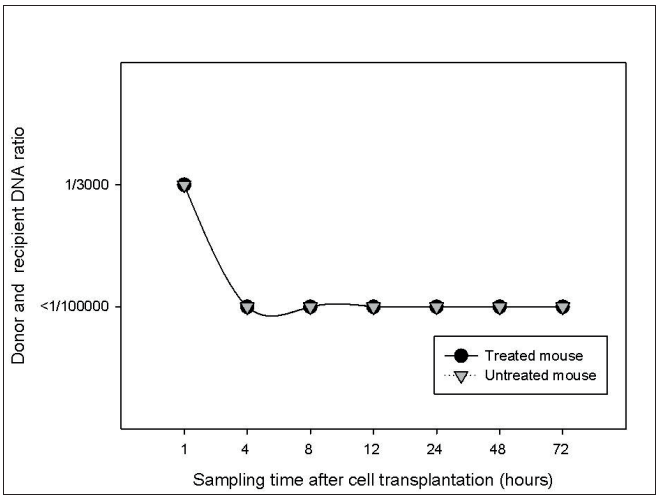

Fig. 6. **The quantitative evaluation of the transplanted lin^- HSC migration kinetics to the kidney in BALB/c mouse contact hypersensitivity model.**

Migration of HSC to the bone marrow. One hour following lin$^-$ HSC transplantation, large quantities (1:300) of donor type cells in the recipient mice bone marrow were found. Transplanted cells, detected in the bone marrow, remained there up to 72 hours (*Fig. 7*). The transplanted cell quantity in affected and healthy mouse bone marrow after 1 hour was the same. However, after 4 hours in the affected mouse bone marrow, the donor type cell quantity decreased more than 3 times (to 1:1000). In the healthy mouse bone marrow, the same amount remained for 12 hours. Following 12 hours after the transplantation, the number of donor origin cells in affected and healthy mice bone marrow was found to be more than 3 times decreased (to 1:3000 and 1:1000, respectively). In healthy mice, the same amount remained for 72 hours. Meanwhile, in the affected mouse bone marrow 48 hours after transplantation a threefold increase of donor type cells (1:1000) was found, and the same quantity remained up to 72 hours.

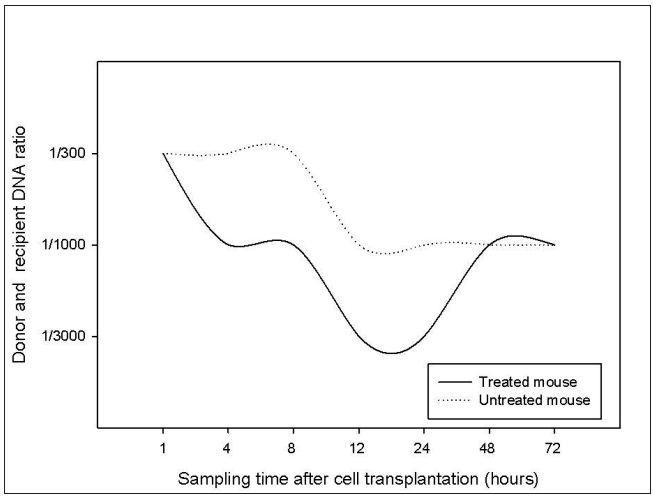

Fig. 7. **The quantitative evaluation of the transplanted lin$^-$ HSC migration kinetics to the bone marrow in BALB/c mouse contact hypersensitivity model.**

We believe that the quantity variation of donor origin cells is related with the exit of the HSC to circulation and their re-homing into the bone marrow. The data of other authors suggest that after the entrance from the bone marrow to circulation, HSC can migrate to various tissues (spleen, liver, kidneys, lungs, etc.). In the absence of the

anchoring signals, the HSC from the tissue pass into the lymph , then back into the bloodstream and return home to the bone marrow or repeat their journey through the body *(Massberg et al., 2007)*. In our study, we found that the quantity of transplanted lin$^-$ HSC cells decreased in the bone marrow of the affected mice after 12 hours and increased after 48 hours *(Fig. 7)*. It corresponds to the increase of donor origin cells after 12 hours and decrease after 48 hours in the spleen of the affected mice *(Fig. 5)*. The data found in the literature state that cytotoxic factors or cytokines increase the HSC amount in peripheral blood and spleen. The HSC amount in the bone marrow increases before *en masse* migration to the spleen and/or liver. Such mobilization and increase of HSC occur during the infections or other stressful situations of the organism, e.g., during inflammation when large quantities of the effector cells should be to quickly generated.

Our data indicate that in contact hypersensitivity model, the transplanted lin$^-$ HSC migrate to the bone marrow, then, following 12 hours after transplantation, occur the inflammation caused a secondary tranplanted cell migration to the spleen ectopic hematopoietic niches. However, 48 hours passed, the donor origin cells leave these niches in the spleen. At the same time, the increase in the quantity of donor origin cells in the bone marrow allows to suggest that cells fom the spleen return back into the bone marrow. We believe that donor origin cells leave the ectopic spleen niches because of the weakening inflammation. Returning back to the circulation, these cells migrate to their usual route, i.e. to the bone marrow.

Migration of the *HSC to the lungs*. The analysis of lung samples revealed that transplanted lin- HSC after 1 hour were found in the lungs of female recipients, where their traces remained up to 72 hours *(Fig. 8)*. After 1 hour of transplantation, the donor origin cells were found in a relatively large quantity (1:10 000) in the lungs. However, after 12 hours in the affected mice, and 24 hours in healtly mice, the donor origin cells in the lung were found only in trace amounts (<1:100 000), which remained up to 72 hours.

The donor cell levels in the lungs of affected and healtly mice following 1 hour of transplantation were the same. However, after 4 hours the amount of donor cells was found to be 3 times less in the lugs of affected mice. In the lungs of affected mice donor cell quantity also decreased after 8 and 12 hours *(Fig. 8)*. The data of other investigators suggest that after the entrance into the circulation, the HSC can migrate to various tissues (spleen, liver, kidneys, lungs, etc.), and in the absence of the anchoring signals

they can return back into the bloodstream *(Massberg et al., 2007*). Our data show that one hour after transplantation lin- HSC are found in the lungs of treated and healthy mice in the same quantities, but in the lungs of affected mice the donor origin cell quantity after 4 hours was 3 times less and after 8 hours - 10 times less than in healthy mice. This shows that inflammation causes a faster lin- HSC exit from the lungs. Following 12 hours after transplantation in affected mice, and 24 hours in healtly mice, the amount of donor origin cells in the lung was found only in trace (<1:100 000). Longer retention of HSC in the lungs was not identified, suggesting that in case of inflammation the lungs are an intermediate migration organ.

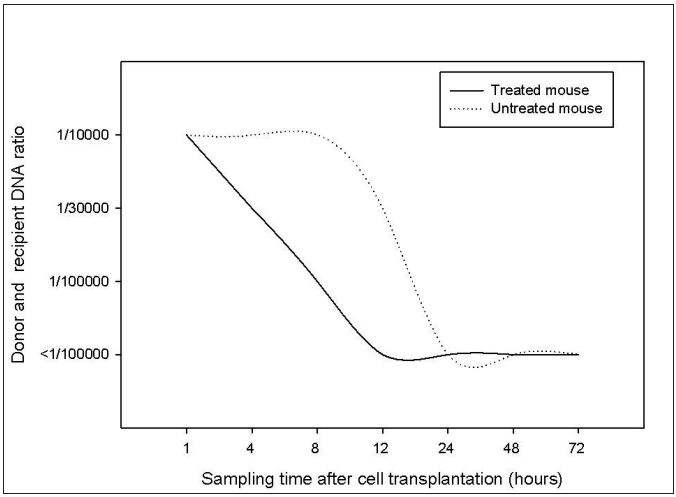

Fig. 8. **The quantitative evaluation of the transplanted lin⁻ HSC migration kinetics to the lungs in BALB/c mouse contact hypersensitivity model**

Migration of the HSC to the liver. Our study has shown that lin-HSC an hour after transplantation are found in the recipient female mouse liver, where their traces remain up to 72 hours *(Fig. 9)*. 24 hours later, the transplantation donor origin cells in the liver are found only in traces. The donor origin cell quantity diferences in the liver of affected and healthy mice were detected only 4 hours after transplantation *(Fig. 9)*. At this time point, in affected mice liver the amount of donor type cells was 3 times more. In all other time points investigated the difference in quantities was not found. This suggests that

inflammation had no effect on the exit of the transplanted lin$^-$ HSC from liver kinetics. 24 hours after transplantation, the donor origin cells were found in the liver only in trace amounts, that shows that the liver is an intermediate migration organ in the contact hypersensitivity model.

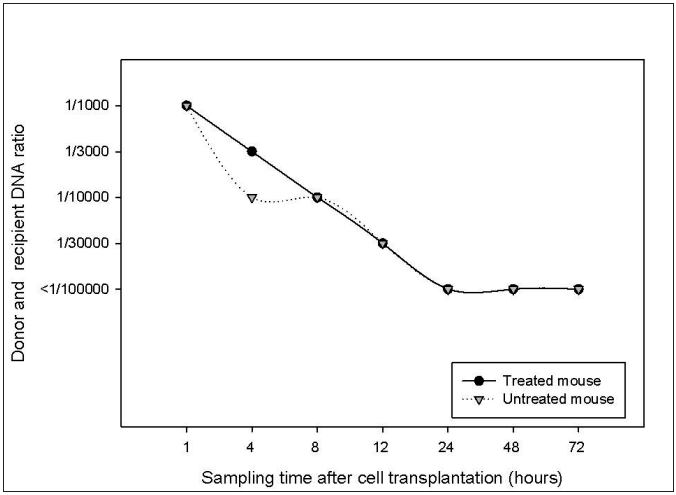

Fig. 9. **The quantitative evaluation of the transplanted lin$^-$ HSC migration kinetics to the liver in BALB/c mouse contact hypersensitivity model.**

Migration of the HSC to the injured foot. The analysis of foot edema samples shows that lin- HSC an hour after transplantation are found in the recipient mice affected foot, where remain up to 72 hours *(Fig. 10)*. In all tested time points in the untreated feet of afftected and healthly mice, the donor origin cells were found only at trace amounts(<1:100 000). Following one hour after transplantation, lin- HSC in foot edema were detected in large quantities (1:100). The quantity of these cells was found to be more than 1000 times higher in comparison with the untreated foot. This confirms that transplanted lin$^-$ HSC selectively migrate into the site of inflammation.

Our data shows that from 1 to 48 hours after transplantation, the quantity of donor origin cells gradually decreases in the affected foot and then remains at the same level up to 72 hours. Following 48 hours after transplantation, a 100-fold decrease (from 1:100 to 1:10000) of donor type cells in foot edema was observed, but still it remained more than

10 times higher than that of the healthy foot. We believe that the gradual decrease of donor origin cells in the site of injury was influenced by the inflammation inhibition. Our results show that lin$^-$ HSC possess an anti-inflamatory effect and effectively inhibit foot edema. Following 72 hours after transplantation, the amount of donor type cells in foot edema remained more than 10 times higher than in the healthy foot. It suggests that injured nonhematopoietic tissue in the foot edema is the final migration target of the transplanted lin$^-$ HSC.

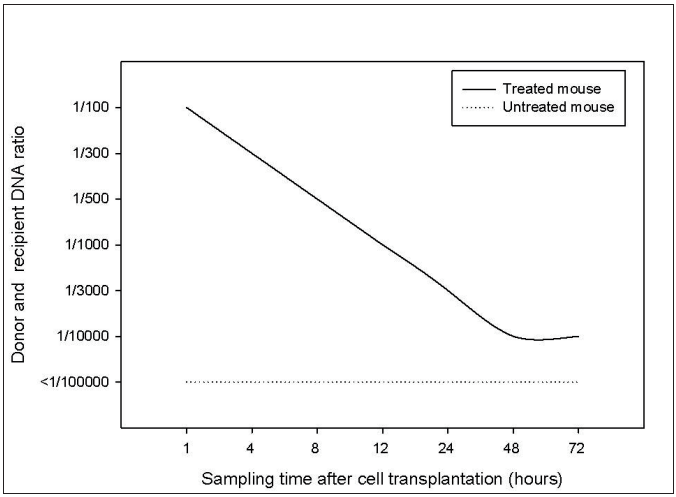

Fig. 10. **The quantitative evaluation of the transplanted lin$^-$ HSC migration kinetics to the hind limb feet in BALB/c mouse contact hypersensitivity model.**

In summary, we noticed that the transplanted HSC, after one hour of introduction, were found at the sites of inflammation (affected foot). In healthy feet at all tested time intervals, they were found only in traces. Following one hour after lin- HSC transplantation, the cell amount in the samples of foot edema was more than 1000 times higher than that in the healthy foot. This confirms the selective migration of transplanted HSC into the inflammatory focus.

Besides, following one hour after transplantation, the lin$^-$ HSC were found in remarkable amounts in the kidneys, lungs, liver and bone marrow, and later, - in spleen. These cells in kidney remained up to 4 hours, in lungs and liver for up to 12 hours and in

the spleen and bone marrow for up to 72 hours. With inflammation, the decrease of transplanted lin^- HSC cell counts from the bone marrow after 12 hours and an increase after 48 hours, comply with the increase of these cells in the spleen after 12 hours and decrease - after 48 hours. This indicates that in the contact hypersensitivity model the transplanted lin^- HSC migrate to the bone marrow, and after 12 hours of transplantation the inflammation caused secondary migration in ectopic spleen of HSC niches takes place. After 48 hours, the donor cells leave the niche and at that time, the detected increase in their amount in the bone marrow, suggests their "homing" from the spleen to the bone marrow. In the contact hypersensitivity reaction model, the transplanted lin^- HSC in the lungs, liver, spleen and bone marrow in different time intervals were found to be decreased up to 30 times. The inflammation influenced a weaker HSC migration to these organs. This was determined by the migration of lin^- HSC to the site of injury on foot, stimulated by the chemoatractants. Cell migration to the healthy foot was not monitored.

Conclusions

1. The hematopoietic cell populations isolated and identified from BALB/c mouse bone marrow. It was found that the lin^- population expressed a phenotype $lin^-CD117^+Sca\text{-}1^+$, characteristic of HSC.
2. Isolated from the bone marrow hematopoietic cell populations have an anti-inflammatory effect in BALB/c mouse contact hypersensitivity model in vivo. The HSC population was most efficient (up to 66%) in inhibition of edema.
3. The anti-inflammatory effect of the transplanted HSC are displayed by modulating pro-and anti-inflammatory cytokine levels in the blood; an increase in the number of IL-10 and a decrease in the amount of TNF-α by more than 3 times.
4. The hematopoietic stem cells selectively migrate to the inflammatory site. In the paw edema, one hour after the transplantation, the HSC amount was up to 1000 times higher than in a healthy foot.
5. Changes in the transplanted HSC levels over time in mouse bone marrow and spleen suggest that inflammation induced a secondary hematopoietic stem cell migration.
6. Stem cell migration quantitative studies have shown that inflammation leads to 30-fold weaker migration of the HSC to the lungs, liver, bone marrow and spleen.

List of publications

1. Biziulevičienė G, **Kašėta V**, Ramanauskaitė G, Vaitkuvienė A. Investigation of unmatured hematopoietic cell migration in a mouse model. *Central European Journal of Biology*, 2010, 5(5):585–589.
2. Ramanauskaitė G, **Kašėta V**, Vaitkuvienė A, Biziulevičienė G. Skin regeneration with bone marrow-derived cell populations. *International Immunopharmacology*, 2010, 10(12):1548–1551.
3. Vaitkuvienė A, **Kašėta V**, Ramanauskaitė G, Biziulevičienė G. Cytotoxicity of the pharmaceutical and cosmetic gel-forming polymers, preservatives and glycerol on primary murine cell cultures. *Acta Medica Lituanica*, 2009, 16(3–4):92–97.
4. **Kašėta V**, Biziulevičienė G, Ramanauskaitė G, Vaitkuvienė A, Biziulevičius GA. Magnetic cell sorting isolation of therapeutically effective BALB/c mouse bone marrow hematopoietic stem cell population. *Biologija*, 2008, 54(4):269–273.

Participation at conferences

- Annual Conference of the German Society of Immunology, Leipzig, Germany. „Skin regeneration with bone marrow-derived cell populations“, 2010 September 22–25th.
- Conference of the Lithuanian Society for Immunology and Lithuanian Academy of Sciences, Vilnius, Lithuania. "Stem cell niches and migration”, 2009 October 1st "
- Conference of the Lithuanian Society of Oncology**,** Vilnius, Lithuania. "Stem cell migration” 2009 May 19th.

Reziumė

Šiuo metu pasaulyje plačiai atliekami kamieninių ląstelių tyrimai suteikia bei praplečia žinias apie šių ląstelių panaudojimo terapijoje bei regeneracinėje medicinoje perspektyvas. Pastaruoju metu daugėja duomenų, įrodančių teigiamą kamieninių ląstelių poveikį ne tik pažeistų organų, bet ir jų funkcijų atstatymui. Keliama prielaida, jog kiekvienam funkciškai neveikliam organui galėtų būti taikoma ląstelinė terapija. Nepaisant to, tebelieka daugybė klausimų, į kuriuos dar reikia atsakyti. Patologiniai procesai, kuriems numatoma taikyti ląstelių terapiją, paprastai yra lydimi uždegiminių reakcijų, todėl ypač svarbu išsiaiškinti kamieninių ląstelių migraciją uždegimo metu bei jų poveikį uždegimui. Pagrindinis hemopoetinių kamieninių ląstelių šaltinis yra kaulų čiulpai. Iš jų išskirtos kamieninės ląstelės šiuo metu jau naudojamos transplantacijai, įvairių audinių pažeidimų atstatymui. Nustatyta, kad kaulų čiulpų ląstelės yra heterogeniškos, atskiros jų populiacijos gali diferencijuotis į specializuotas skirtingų audinių ląsteles, tačiau nėra bendro sutarimo – kuri populiacija tinkamiausia ląstelinei terapijai vieno ar kito pažeidimo atveju. HKL populiacija organizme yra labai nedidelė, šių ląstelių išskyrimas yra pakankamai sudėtingas, nes žinomi tik keli šių ląstelių paviršiuje ekspresuojami baltymai. Be to, tuos pačius baltymus gali ekspresuoti ir kitos kaulų čiulpų ląstelės. Tam tikromis aplinkybėmis pagal Sca-1 ir CD117 žymenis galima identifikuoti ir išskirti hemopoetinių ląstelių populiacijas bei vėliau tikslingai jas panaudoti vieno ar kito pažeidimo atstatymui. Norint gauti kuo grynesnes frakcijas, tinkamas naudoti, pvz., transplantacijoje, tarpusavyje turi būti derinami keliais parametrais besiskiriantys metodai. Taigi paviršiaus žymenų nustatymas bei hemopoetinių kamieninių ląstelių ir pirmtakų populiacijų gavimas, kaip ir jų migracijos tyrimai, yra svarbūs tolesniam jų selektyviam pritaikymui terapijoje.

Darbo metu iš bendros BALB/c pelių kaulų čiulpų ląstelių populiacijos centrifugavimo tankio gradiente (1.087g/ml) ir magnetinio ląstelių frakcionavimo, panaudojant antikūnus prieš pelių hemopoetinių ląstelių pirmtakų antigenus, metodais išskyrėme hemopoetinių kamieninių ląstelių populiacijas. Atskirta pelių kaulų čiulpų lin^- HKL populiacija pasižymėjo aukščiausia pelių kaulų čiulpų HKL būdingų žymenų raiška bei neturėjo hemopoetinių linijinių žymenų. Ši populiacija buvo $lin^-CD117^+Sca\text{-}1^+$ fenotipo. Daugumą lin^- HKL populiacijos sudarė trumpalaikės HKL, likusiąją dalį - ilgalaikės HKL ir ankstyvieji hemopoetiniai pirmtakai.

Kadangi susirgimai, kuriems numatoma taikyti ląstelių terapiją, paprastai yra lydimi uždegimo – kamieninių ląstelių priešuždegiminio poveikio tyrimai yra svarbūs ir tikslingi. Mes ištyrėme atskirtų ir identifikuotų ląstelių populiacijų priešuždegiminį poveikį BALB/c pelių kontaktinio hiperjautrumo modelyje *in vivo*. Rezultatai, gauti tiriant bendrą kaulų čiulpų ląstelių populiaciją, parodė, kad ji priešuždegiminiu poveikiu nepasižymi. O visos tirtosios lin^- HKL populiacijos ($5x10^6$; 10^6; 10^5 ląstelių/pelei koncentracijomis) efektyviai slopino edemą. Efektyviausiai edemą inhibavo 10^6 ląstelių/pelei dozė, kuri 1,28 karto geriau nei prednizolonas slopino edemą. Tokia ląstelių koncentracija buvo naudota visuose vėlesniuose tyrimuose. Ištyrę išskirtų lin^- HKL, lin^+, lin^- HKL kartu su lin^+(1:10) ląstelių populiacijų priešuždegiminį poveikį nustatėme, kad efektyviausiai (net iki 66%) edemą inhibavo lin^- HKL populiacija. Gali būti, kad cirkuliuojančios HKL, esant pažeidimui, pasižymi dvejopu poveikiu: moduliuoja imuninį atsaką ir tarnauja šaltiniu vietinei efektorinių imuninių ląstelių gamybai pažeidimo vietoje. Tokiu būdu slopinamas uždegimas ir skatinama audinio regeneracija. Mūsų tyrimų duomenys, gauti tiriant skirtingas kaulų čiulpų hemopoetines populiacijas, parodė, kad šių populiacijų priešuždegiminio poveikio efektyvumas yra tiesiogiai susijęs su santykiniu populiacijoje esančių HKL kiekiu. O citokinų koncentracijos pokyčiai pelių kraujo serume, įvedus skirtingas hemopoetinių ląstelių populiacijas, yra proporcingi pėdos edemos inhibicijai. Mažiausi citokinų kiekių pokyčiai, kaip ir poveikis pėdos edemai, nustatyti įvedus lin^+ populiacijos ląsteles, o žymiausi – lin^- HKL. Šiuo atveju TNF-α koncentracija sumažėjo 3.5 karto, o IL-10 padidėjo 3.6 kartais, lyginant su kontrole.

Taip pat ištyrėme transplantuotų lin^- HKL migraciją BALB/c pelių kontaktinio hiperjautrumo reakcijos modelyje bei kiekybiškai įvertinome jų migraciją į uždegimo židinį pėdoje ir nepažeistus organus, praėjus 1, 4, 8, 12, 24, 48 ir 72 val. po fluorescentiškai žymėtų lin^- HKL (10^6) įvedimo. Atlikome histologinę analizę, kiekybiniam vertinimui taikėme realaus laiko polimerazinės grandininės reakcijos metodą. Pastebėjome, kad transplantuotos lin^- HKL, praėjus 1 val. po jų įvedimo, yra aptinkamos uždegimo vietoje (pažeistoje pėdoje). Sveikose pėdose visais tirtais laiko intervalais jų aptikome tik pėdsakus. Praėjus 1 val. po lin^- HKL transplantacijos, šių ląstelių kiekis pėdos edemos mėginiuose buvo daugiau nei 1000 kartų didesnis negu sveikoje pėdoje. Tai patvirtina selektyvią transplantuotų lin^- HKL migraciją į uždegimo

židinį. Be to, praėjus 1 val. po transplantacijos, lin$^-$ HKL žymesniais kiekiais buvo aptinkamos inkstuose, plaučiuose, kepenyse bei kaulų čiulpuose, o kiek vėliau ir blužnyje. Šios ląstelės inkstuose išliko iki 4 val., plaučiuose ir kepenyse iki 12 val., o blužnyje ir kaulų čiulpuose iki 72 val. Esant uždegimui, transplantuotų lin$^-$ HKL ląstelių kiekio sumažėjimas pelių kaulų čiulpuose po 12 val. ir padidėjimas po 48 val. atitinka šių ląstelių kiekio blužnyje padidėjimą po 12 val. ir sumažėjimą po 48 val. Tai rodo, kad kontaktinio hiperjautrumo modelyje vyksta transplantuotų lin$^-$ HKL migracija į kaulų čiulpus, o praėjus 12 val. po transplantacijos, prasideda uždegimo įtakojama antrinė šių ląstelių migracija į blužnies ektopines HKL nišas. Po 48 val. ląstelės šias nišas palieka, o šiuo metu stebimas donorinės kilmės ląstelių kiekio padidėjimas kaulų čiulpuose leidžia manyti, kad vyksta iš blužnies išėjusių ląstelių „homingas“ į kaulų čiulpus.

Kontaktinio hiperjautrumo reakcijos modelyje transplantuotų lin$^-$ HKL plaučiuose, kepenyse, kaulų čiulpuose ir blužnyje įvairiais laiko intervalais buvo aptinkama iki 30 kartų mažiau. Uždegimas įtakojo silpnesnę HKL migraciją į šiuos organus. Tai apsprendė transplantuotų lin$^-$ HKL migraciją į pažeidimo vietą pėdoje skatinantis chemoatraktantų poveikis. Ląstelių migracija į sveiką pėdą nebuvo stebima.

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