BIOLOGIJA. 2008. Vol. 54. No. 4. P. 269–273
DOI: 10.2478/v10054-008-0055-z
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Magnetic cell sorting isolation of therapeutically effective BALB/c mouse bone marrow hematopoietic stem cell population

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Purified bone marrow cells are being successfully used to restore the injured tissue and may serve as an alternative to organ transplantation. From the therapeutic point of view, it is of importance to obtain individual immunophenotypically characterized hematopoietic cell populations which, after their predisposed differentiation performed *in vitro*, might be applied selectively. In this paper, we demonstrate, BALB/c mice using as a model, how a therapeutically effective bone marrow hematopoietic stem cell population can be easily isolated by magnetic cell sorting techniques. Hematopoietic progenitor stem cells were isolated from bone marrow mononuclear cell suspension by negative selection (differentiated cells were removed using nanoparticles labeled with antibodies against Lin^+). Hematopoietic stem cells of interest were isolated from Lin^- (negative fraction) using BD IMag™ anti-mouse CD117 particles (positive selection). Flow cytometrical analysis of these cells revealed that 42.59 ± 5.74% of cells expressed $CD38^+CD117^+$, 3.59 ± 0.84% expressed $CD34^-CD117^+$, and 60.94 ± 1.43% expressed $Sca1^+CD117^+$. The cell population was therapeutically highly effective when examined in a contact hypersensitivity experiment. We believe that the methodological approach described in this paper could be easily extended in regard to therapeutically important stem cells of other origins.

Key words: BALB/c mouse, bone marrow, magnetic cell sorting, flow cytometry

INTRODUCTION

Bone marrow is a rich source of stem cells [1]. Bone marrow cells are heterogeneous; their populations can differentiate to various cells and tissues. Hematopoietic stem cells isolated from the bone marrow of an adult individual differentiate to lymphoid, myeloid and erythroid cell lines [2]. Purified bone marrow cells are being successfully used to restore the injured tissue and may serve as a good alternative to organ transplantation [3, 4]. To optimize the use of bone marrow cells, it is advisable to obtain a progenitor population of these cells that could be purposely directed to restore the damaged tissue. The purification of stem cells is a rather complicated process because there is but a limited number of proteins expressed on the surface of these cells [5, 6]. These proteins could be found on the surface of other bone marrow cells, as well as their expression may vary not only within the different types of animals, but also during their development [7–10]. Thus, from the therapeutic point of view, it is of importance to obtain individual immunophenotypically characterized hematopoietic cell populations which might be applied selectively. With regard to the latter, we demonstrate here, BALB/c mice using as a model, how therapeutically effective bone marrow hematopoietic stem cell population can be easily isolated through purification on magnetic particles.

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MATERIALS AND METHODS

Animals

Female BALB/c mice (weighing 20–22 g) obtained from the Laboratory Animal Center of the Institute of Immunology of Vilnius University (Lithuania), were used. Animals were maintained in an environment of controlled temperature (20–22 °C). Food and water were provided *ad libitum*. All procedures were carried out in accordance with the guidelines of the European Union and were approved by Ethics Committee on Animal Experimentation of the Institute.

Isolation of mouse bone marrow cells

Mice, 6–8 weeks old, were sacrificed by cervical dislocation. The extracted bones were placed on a Petri dish containing Hanks' balanced salt solution (HBSS). Bone marrow was obtained by flushing with sterile HBSS through one of the femoral epiphyses, using a syringe needle (27-gauge). The bone marrow cells were collected in sterile HBSS and washed three times by centrifugation for 6 min at 300 g. The cells were characterized by flow cytometry.

Isolation of mononuclear cells

Mononuclear cells were obtained by separation of mouse bone marrow cells in the Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient. 3 ml aliquot of

Ficoll-Paque was placed into a plastic tube and the same amount of bone marrow cell suspension in HBSS was layered on the top. This was centrifuged for 40 min at 400 g. The cell fraction harvested from the interface between these two media was washed immediately and suspended in HBSS.

Magnetic cell sorting

Hematopoietic cells were purified using magnetic cell sorting (MACS) techniques with the BD IMag™ mouse hematopoietic progenitor enrichment set (composed of BD IMag™ Streptavidin Particles Plus – DM and biotin-conjugated monoclonal antibodies: 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), the BD IMag™ anti-mouse CD117 particles as well as the BD IMagnet™ (all from BD Biosciences, San Jose, CA, USA) applied as recommended by the manufacturer. Hematopoietic progenitor stem cells were isolated from mononuclear cell suspension by negative selection (differentiated cells were removed using nanoparticles labeled with antibodies against Lin^+). Hematopoietic stem cells of interest were isolated from Lin^- (negative fraction) using BD IMag™ anti-mouse CD117 particles (positive selection). Both positive and negative Lin fractions were evaluated for their therapeutic efficiency; the former was additionally characterized by flow cytometry.

Flow cytometrical analysis

For flow cytometrical analysis, the following monoclonal antibodies were used: rat anti-mouse CD117 conjugated with fluoresceinisothiocyanate (CD117-FITC) (Chemicon International, Temecula, CA, USA); rat anti-mouse CD38 conjugated with phycoerythrin (CD38-PE) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); CD34 conjugated with phycoerythrin (CD34-PE) (BD Biosciences, USA), and Sca1 conjugated with phycoerythrin (Sca1- PE) (Abcam, Cambridge, UK). Cell suspensions (2×10^5 per example) were labeled with antibody pairs: CD34-PE/CD117-FITC, CD38-PE/CD117-FITC, and Sca1-PE/CD117-FITC; 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 phosphate buffered saline (PBS). Cells were examined by FACS Callibur cytometry (Becton Dickinson, San Jose, CA, USA). Isotype-matched nonspecific antibodies, rat IgG2α -FITC (BD Biosciences, USA) and rat IgG2α -PE (Chemicon International, USA), were used for the control.

Evaluation of hematopoietic cell populations for their therapeutic efficiency

The therapeutic efficiency of hematopietic stem cell populations (Lin^-, Lin^+ and Lin^+ + Lin^-) was evaluated in a contact hypersensitivity experiment. The mice were randomly allocated into five groups (n = 10 in each). All the animals were sensitized by application of 30 µl of 0.5% 2,4-dinitrofluorbenzene (DNFB) (Sigma) on the shaved abdomen and 96 hours later were challenged with an injection of 10 µl of 0.3% DNFB into one of the hind paws. In all cases, the DNFB solution in acetone and olive oil mixture (4 : 1) was prepared just prior to application. 24 hours after the DNFB challenge, 1×10^6 of hematopoietic Lin^- cells/mouse, 1×10^6 of hematopoietic Lin^+ cells / mouse and 1×10^6 of hematopoietic Lin^+ + Lin^- cells / mouse suspended in 100 µl of HBSS were injected into the tail vein of the first second and third study group mice, respectively. Control mice received an injection of HBSS. The reference group was treated intraperitoneally with prednisolone (100 mg/kg of animal weight). 48 hours later, differences in the weight of the injected versus the uninjected paw were evaluated as the main indicator of the contact hypersensitivity response (paw edema).

Statistical analysis

A mean ± SEM was calculated for all representative experiments. Statistical significance among the mean values was determined using Student's t test. $P < 0.05$ was considered significant. All calculations were performed using Microsoft Excel (version 7.0) and SigmaPlot (version 9).

RESULTS AND DISCUSSION

Bone marrow contains several different cell populations, a small population of hematopoietic stem cells among them. As cell purification and identification is based on the presence of different cell surface markers, application of specific antibodies enables isolating even very small cell populations [11, 12]. These can be then multiplied by cultivating *in vitro*, and by providing an appropriate microenvironment their differentiation could be purposely targeted.

BALB/c mouse bone marrow cells were identified by flow cytometry (Fig. 1). The results showed that 27.73 ± 8.69% of cells expressed $CD38^+CD117^+$, 1.19 ± 0.05% expressed $CD34^-CD117^+$, and 21.94 ± 3.17% expressed $Sca1^+CD117^{+-}$ (Table). The surface markers under discussion are characteristic of murine hematopoietic stem cells [13, 14]. The CD117 is also called C-kit receptor which, by binding to the stem cell factor (SCF), promotes

Table. **Percentage of murine bone marrow cells, Lin-negative (Lin^-) fraction cells and Lin-negative CD117 positive (Lin^-CD117^+) fraction cells bearing CD34, CD38, CD117, and Sca1 surface markers**

Cell surface marker	Cell population		
	Bone marrow cells	Lin^-	Lin^-CD117^+
$CD38^+CD117^-$	7.17 ± 2.92	2.16 ± 0.57	1.78 ± 0.84
$CD38^+CD117^+$	27.73 ± 8.69	37.39 ± 7.54	42.59 ± 5.74
$CD38^-CD117^+$	35.13 ± 13.08	48.93 ± 8.46	50.52 ± 9.86
$CD38^-CD117^-$	29.97 ± 11.82	11.52 ± 1.74	5.11 ± 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	12.53 ± 2.36	16.29 ± 3.22
$Sca1^+CD117^+$	21.94 ± 3.17	63.23 ± 0.82	60.94 ± 5.43
$Sca1^-CD117^+$	2.84 ± 0.23	12.30 ± 2.80	9.42 ± 2.36
$Sca1^-CD117^-$	44.02 ± 3.86	11.94 ± 1.26	13.35 ± 2.34

Values are mean ± S. D., n = 6 experiments. CD – cluster of differentiation; Lin – lineage markers; Sca1 – stem cell antigen 1.

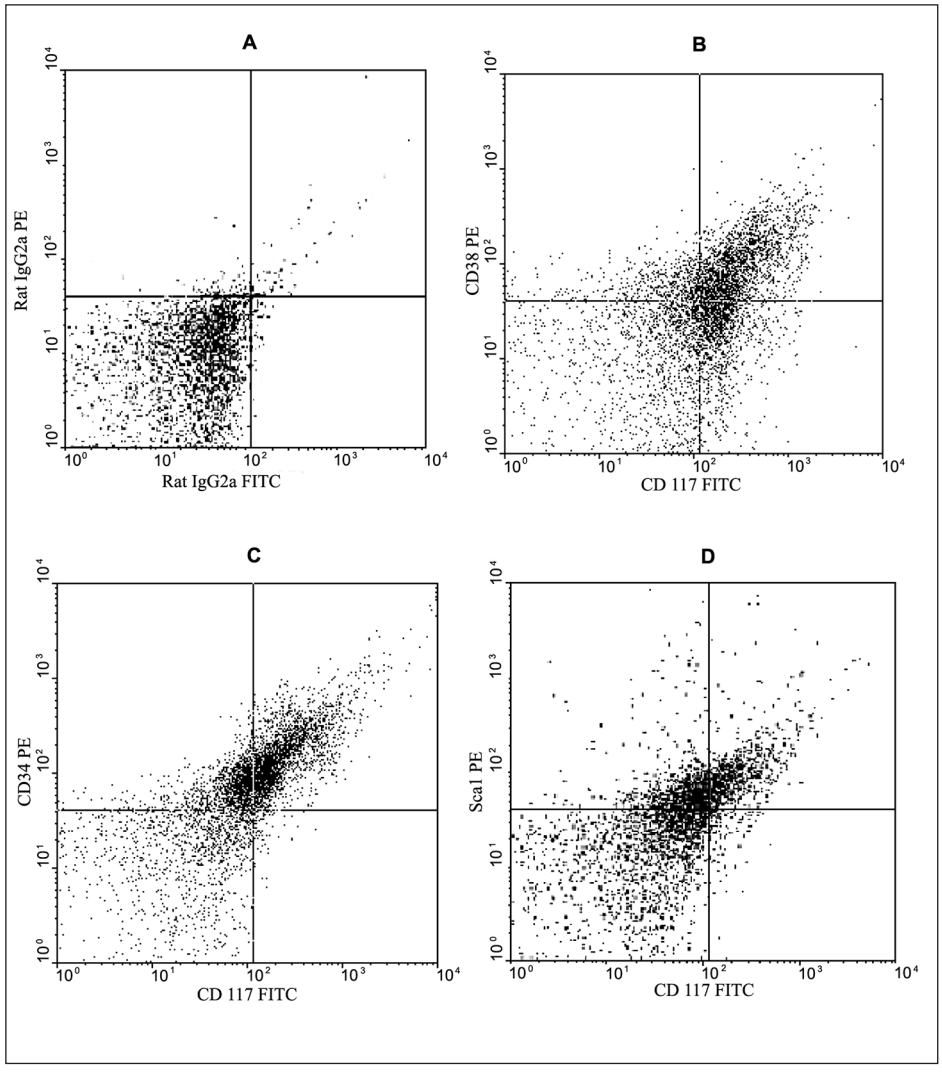

Fig. 1. Flow cytometric analysis of murine bone marrow cells. One representative of six similar experiments is presented. A – (isotypic control) rat IgG2a (PE) and rat IgG2a (FITC); B – expression of CD38 and CD117; C – expression of CD34 and CD117; D – expression of Sca1 and CD117

cell growth. This signal pathway plays an important role for cell survival, proliferation and differentiation [15].

Isolation of hematopoietic stem cells from bone marrow cell suspension was carried out by centrifugation in a density gradient with the subsequent magnetic cell fractionation, using antibodies against mouse hematopoietic progenitor cell antigens. A tube containing cells labeled with magnetic nanoparticles was placed into a strong constant BD™ IM magnet. A high-gradient magnetic field was induced to attract and hold the labeled cells on the walls of the tube. As the magnetic BD™ IM nanoparticles were produced from the biodegrading matrix, they could be easily analysed further by flow cytometry without any removal. 3.5 ± 1.5% of bone marrow cells were found in the negative fraction, while the rest 96.5 ± 1.5% was found in the positive one. Mature cells, such as T lymphocytes, B lymphocytes and monocytes, remained in the positive fraction which was removed; the negative fraction, composed mainly of hematopoietic stem cells, the so-called Lin^- population (Lin: CD3e; CD11b (Mac-1); CD45R / B220; Ly6G and Ly6C (GR-1) and TER119) was used in the further studies. It is well known that Lin^+ population expresses surface markers characteristic of mature cells; cells that express very small amounts of these markers or even do not express them at all (hematopoietic progenitor cells) are called Lin^- [10, 13]. However, not all hematopoietic stem cells bear the same cell surface maker combinations. There are cases when premature stem cells have no C-kit receptor [16]. Besides, there is no single surface marker exclusively common for hematopoietic stem cells; that is why a combination of markers is usually used.

The Lin^- population flow cytometry profile is presented in Fig. 2. 37.39 ± 7.54% of cells expressed $CD38^+$ $CD117^+$, 3.92 ± 0.43% expressed $CD34^-CD117^+$, and 63.23 ± 0.82% expressed $Sca1^+CD117^+$ (Table). Flow cytometry profile of hematopoietic stem cells isolated from Lin^- (negative fraction) using BD IMag™ anti-mouse CD117 particles (positive selection) is shown in Fig. 3. Of all cells, 42.59 ± 5.74% expressed $CD38^+CD117^+$, 3.59 ± 0.84% expressed $CD34^-CD117^+$, and 60.94 ± 1.43% expressed $Sca1^+CD117^+$ (Table).

The therapeutic efficiency of hematopoietic stem cell populations under investigation was evaluated in a BALB/c mouse contact hypersensitivity model, the model that has already been successfully applied when studying the anti-inflammatory activities of stem cells [17]. The results (Fig. 4) revealed a statistically significant ($P < 0.05$) edema inhibition by Lin^- cell population (the inhibition level was similar to that achieved with prednisolone).

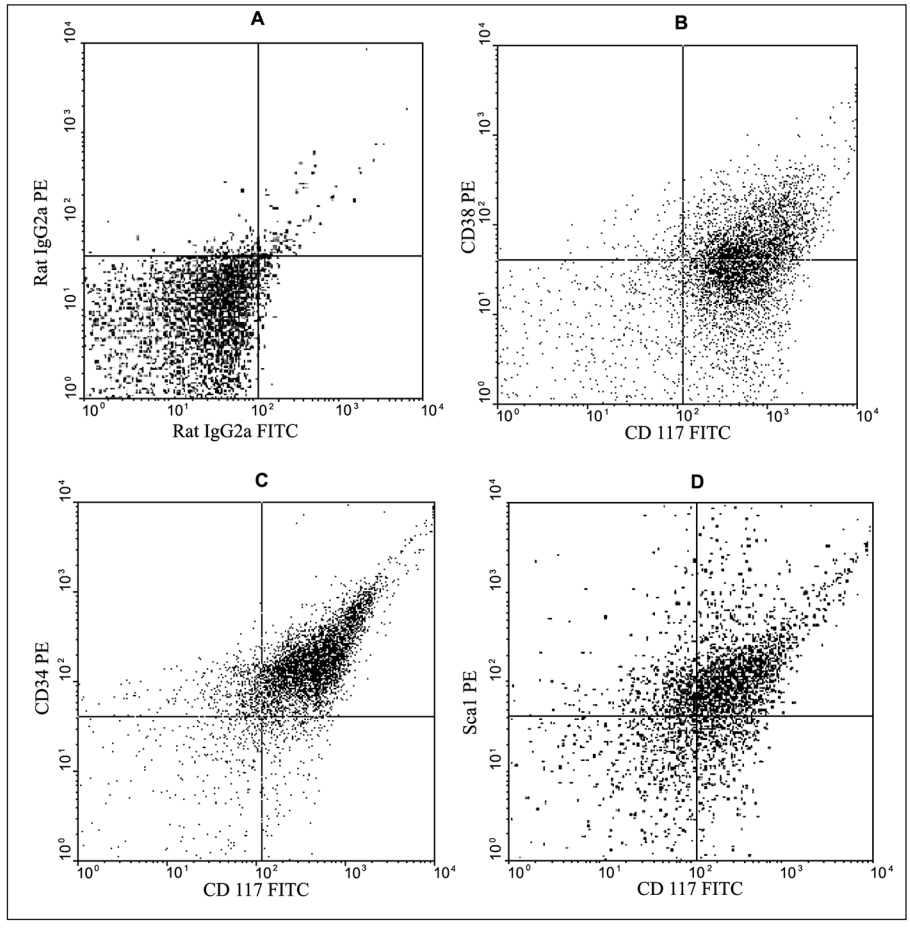

Fig. 2. Flow cytometric analysis of murine bone marrow Lin⁻ population. One representative of six similar experiments is presented. A – (isotypic control) rat IgG2a (PE) and rat IgG2a (FITC); B – expression of CD38 and CD117; C – expression of CD34 and CD117; D – expression of Sca1 and CD117

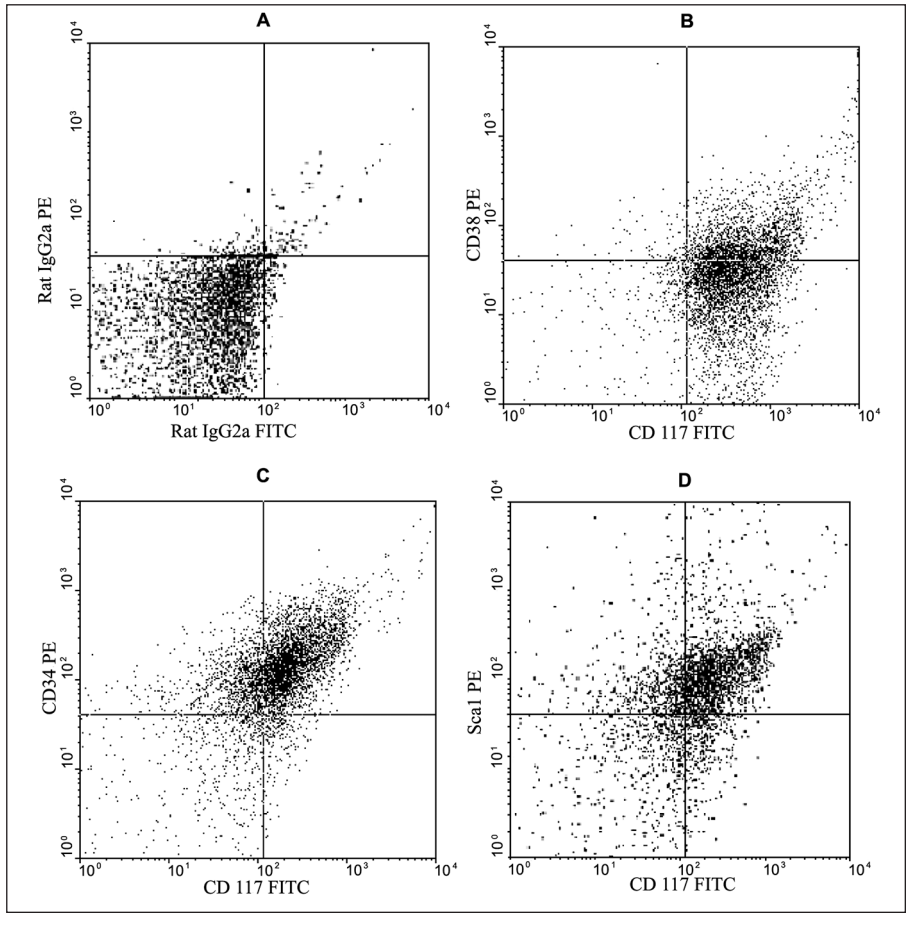

Fig. 3. Flow cytometric analysis of murine bone marrow Lin⁻ CD117-positive population. One representative of six similar experiments is presented. A – (isotypic control) rat IgG2a (PE) and rat IgG2a (FITC); B – expression of CD38 and CD117; C – expression of CD34 and CD117; D – expression of Sca1 and CD117

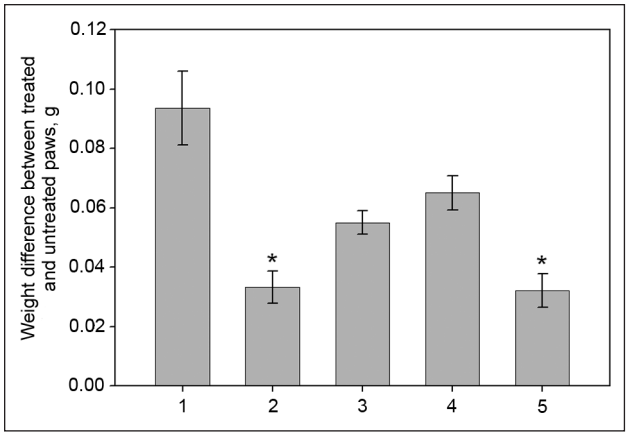

Fig. 4. Evaluation of therapeutic efficiency of hematopoietic stem cell populations (Lin^-, Lin^+, $Lin^- + Lin^+$) on paw edema (results of a contact hypersensitivity experiment). 1 – negative control – 100 µl of HBSS; 2 – positive control – prednisolone (100 mg/kg of animal weight); 3 – $Lin^- + Lin^+$ 1×10^6 cells / mouse; 4 – Lin^+ 1×10^6 cells / mouse; 5 – Lin^- 1×10^6 cells / mouse; n = 10 in each group; * result is significantly different ($P < 0.05$) as compared with the negative control

We believe that the methodological approach described in this paper could be easily extended in regard to therapeutically important stem cells of other origins.

FUNDING AND ACKNOWLEDGEMENTS

This work was supported by the Lithuanian State Science and Studies Foundation (Project "Cytotherapy 2"). The authors thank Dr. Almantas Šiaurys for assistance in performing flow cytometrical analyses.

Received 21 August 2008
Accepted 29 October 2008

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BALB/C PELIŲ KAULŲ ČIULPŲ HEMOPOETINIŲ KAMIENINIŲ LĄSTELIŲ POPULIACIJŲ MAGNETINIS ATSKYRIMAS IR JŲ TERAPINIO POVEIKIO ĮVERTINIMAS

Santrauka

Šiuo darbu mes atskleidėme, kaip taikant magnetinį ląstelių atskyrimą galima lengvai išskirti terapiškai veiksmingas hemopoetinių kamieninių ląstelių populiacijas. Hemopoetiniai kamieninių ląstelių pirmtakai buvo išskirti iš BALB/c pelių kaulų čiulpų vienbranduolių ląstelių suspensijos neigiamos selekcijos būdu panaudojant antikūnais žymėtas nanodaleles. Mus dominančios hemopoetinės kamieninės ląstelės buvo išskirtos iš Lin^- ląstelių frakcijos naudojant BD IMag™ daleles, žymėtas antikūnais prieš pelių CD117 (teigiama selekcija). Analizuojant šias ląsteles tėkmės citometrijos būdu buvo nustatyta, kad 42,59 ± 5,74% ląstelių ekspresuoja $CD38^+CD117^+$ žymenis, 3,59 ± 0,84% ekspresuoja $CD34^-CD117^+$ ir 60,94 ± 1,43% – $Sca1^+CD117^+$. Tiriant išskirtas hemopoetinių kamieninių ląstelių populiacijas kontaktinio hiperjautrumo modelyje *in vivo* nustatyta, kad efektyviausiu terapiniu poveikiu pasižymi Lin^- ląstelių populiacija. Manome, kad aprašyta metodologija gali būti lengvai pritaikyta ir kitoms terapiškai aktyvioms kamieninių ląstelių populiacijoms išskirti.