Indirect differentiation of mouse embryonic stem cells into macrophage-like cells

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Abstract. Macrophages derived from pluripotent stem cells represent an attractive cellular system for the study of lentiviruses. The possibility of obtaining cells with a phenotype similar to macrophages in the process of differentiation of mouse embryonic stem cells (ESCs) in the presence of the conditioned medium (CS) from bone marrow-derived stromal cells or interleukin 3 (II-3) and macrophage colony-stimulating factor (M-CSF) was demonstrated. The results of flow cytofluorimetry showed that the replacement of CS with recombinant cytokines in induction media contributed to an increase in the number of cells positively stained antibodies (AB) against AG CD34 by 8% and CD45 by 20%. When seeding such cells in a methylcellulose medium supplemented with CS or a mixture of II-3 and M-CSF, the formation of colonies with different morphology, including monocyte/macrophage, was observed with an efficiency of 0.11% and 0.33%, respectively. The percentage of positively stained cells AB against F4/80 AG was 8±0.02%. The cells demonstrated the ability to non-specific phagocytosis of latex particles. Thus, it is shown that it is possible to obtain cells with a phenotype similar to macrophages from mouse ESCs during differentiation, and the advantage of using two recombinant cytokines for this purpose is established.

1. Introduction
The complexity of the pathogenesis and insufficient knowledge of retroviral infections requires the need to search for an adequate cell model for their in vitro study. Monocytes and macrophages are targets for many lentiviruses of animals. Tissue macrophages are highly specialized cells that are widely distributed in all body tissues and are a key component of the immune system. Understanding the regulatory mechanisms that drive the innate immune system can make lentivirus studies more effective. Obtaining in the culture of the cell population represented by monocytes or macrophages opens up new possibilities for studying the dependence of the replication of lentiviruses, in particular the equine infectious anemia virus (EIAV), on the degree of cellular differentiation. Indeed, most virulent strains of EIAV replicate only in cultures of equine diploid macrophages and cannot reproduce in other tissue cell cultures without prior adaptation [1]. In a macrophage culture, the virus retains infectivity comparable to its virulence in vivo in vivo.

For many years, macrophages derived from monocytes have been used for research. Monocytes were isolated from the peripheral blood of animals, and this required a large amount of it. The use of several
donors made the experimental observation not representative. Diploid cultures of equine macrophages are difficult to obtain and maintain in the culture for a long period of time due to their loss of ability to proliferate due to terminal specialization in vitro. In this regard, intensive studies were conducted to assess the sensitivity to EIAV of diploid cell cultures derived from other tissues and organs, which we mentioned earlier [2]. It has been shown that various strains of EIAV can replicate in equine dermal fibroblasts, kidney and endothelial cells. These cells were used to build the virus in vitro along with macrophages and demonstrated the ability of the EIAV to replicate in cells of non-macrophage origin. It is known that diploid cultures are characterized by short-term cultivation (up to 55 cellular doubling). Therefore, attempts have been made to adapt immortalized cell lines to the virus. As a result, it was shown that transplantable cell lines: mouse embryonic fibroblasts - FEA; dog thymus cells - Cf2Th; osteosarcoma of dogs - D17 and macrophages of dogs - DH82, capable of supporting replication of the EIAV in vitro. However, immortal cell lines, along with advantages, have many disadvantages. They, as a rule, with prolonged cultivation lose their tissue and even species identity. Perhaps, as a result of this, the virulence of the EIAV is weakened. The determinants of virulence can be lost or altered when viruses reproduce for a long time in other cells that differ from their target cells in vivo. Attempts have been made to create immortalized cultures of equine macrophages: EML-3C [3] and e-Cas [4]. However, it turned out that the permanent line of the e-Cas is mouse macrophages [5]. In this regard, obtaining macrophages during directed differentiation of mouse embryonic stem cells (ESCs) in vitro, including genetically transformed with equine genes, is of interest for veterinary medicine [6]. Earlier, we reported that ESCs have unique properties, one of which is the ability under certain conditions to form three-dimensional aggregates called embryonic bodies (EBs) resembling the early mouse embryonic development [7]. They are a unique model for studying the molecular mechanisms of cell differentiation, in particular, early hematopoiesis. ESCs originate from the embryoblast of early preimplantation embryos and in culture they remain similar to early embryos and contain a huge library of pre-synthesized mRNA genes for early embryogenesis and organogenesis. ESCs are thought to closely resemble the primitive ectoderm of early post-implantation embryos. They have high proliferative activity, are able to self-renew and maintain in undifferentiated form for a long period in vitro. A number of works appeared demonstrating the production of macrophages as a result of differentiation of ESCs [8, 9, 10, 11, 12]. Consequently, macrophages derived from pluripotent stem cells are an attractive alternative cellular system. However, the conditions for differentiation of ESCs into macrophages in culture are different and include the use of feeder layers represented by a monolayer of cells of various origins, indirect differentiation through the formation of EBs in culture, the addition of a mixture of cytokines and growth factors, as well as various combinations thereof.

The aim of the work was to optimize the conditions for differentiation of murine ESCs into cells with a macrophage-like phenotype and to evaluate the effect of the conditioned medium (CM) from bone marrow (BM) stromal cells and two recombinant interleukin 3 cytokines (IL-3) and macrophage-colony stimulating factor (M-CSF).

2. Materials and methods

The mouse ES cells of D3 line was used as an object (figure 1. A). For their cultivation, DMEM medium containing 4 g / l glucose (PanEco, Russia) was used, to which an additional 10% of the characterized blood serum of cow fetuses (FCS) from HyClone, USA was added, a solution of nonessential amino acids (1 ml of 100x solution per 100 ml of medium), streptomycin and penicillin at a final concentration of 50 μg / ml and 50 U / ml, respectively (PanEco, Russia). ESCs were cultured on a feeder layer represented by a monolayer of mouse embryonic fibroblasts (MEF) obtained from 18 days old mouse embryos whose mitosis was previously blocked by mitomycin C (OOO Sigma-Aldrich, Rus / Merck, Rus) at a final concentration of 10 μg / ml for 3 hours.
Murine stromal cells were isolated from BM, which was taken from the tibia using a biopsy needle (Jorgensen Labs, USA) after killing the mouse. Mouse stromal cells isolated from BMs were cultured in DMEM medium (PanEco, Russia) with a low glucose content (1 g / l), which contained 10% FCS (HyClone, USA) and the antibiotics mentioned above. A conditioned medium (CM) was obtained by collecting the medium in which mouse BM stromal cells were cultured in a complete monolayer, filtered and used for the experiment.

Differentiation was performed through the formation of EBs in culture according to the technique described by us earlier [7]. To do this, the day before the differentiation the colony of ESCs were purified from the feeder layer represented by MEF, collected without breaking them, and were sown with aggregates (100-200 μm in diameter) in low density of 10-20 aggregates per cm² in 24-well Aggre WellTM400 plates Stem Cell, Canada, blocking cell adhesion in DMEM medium, which contained 5% FCS and antibiotics at the concentration indicated above. The next day, 25% of CM from mouse BM-derived stromal cells or recombinant mouse IL-3 and M-CSF (OAO Sigma-Aldrich, Rus / Merck, Rus) in final concentration of 50 ng / ml were added in culture medium. On the 12th day of cultivation, EBs formed from ESCs were collected and treated with a 0.01% type I collagenase solution (40 min 37 °C) to obtain single cells. Part of the cells was selected for flow cytofluorimetry (Epics Elit Culer). For this, the cells were washed, counted, and aliquots of 2 × 10⁵ cells were incubated with primary Abs at a dilution of 1: 30 (PBS supplemented with 2% FCS) at 4 °C for 45 min in the dark. Used rat Abs against mouse CD34 (MEC 14.7) AG and mouse anti-CD45 mice labeled with phycocerythrin (Abcam).

The remainder of the cells was transferred using syringes and needles from StemCellTM, USA to semi-liquid medium MethoCultTM ES-CultTM M3120 from StemCell, Canada. The efficiency of obtaining clones was estimated as the ratio of the number of obtained clones to 10,000 seeded cells, multiplied by 100. For immunocytochemical analysis of obtained cells with phenotypes similar to macrophages, rat Abs against mouse F4 / 80 (CL: A3-1) AG from Abcam and rabbit Abs anti rat AG, labeled with peroxidase of the same company in a 1: 100 dilution were used. As a substrate for peroxidase, 3,3-diaminobenzidine tetrachloride was used (PanEco, Russia). Analysis of native and stained preparations was performed using an inverted phase contrast microscope (Carl Zeiss, Germany) with AxioVision Rel software. 4.8 (Carl Zeiss, Germany).

The phenotype of ESC-derived macrophages was evaluated by phagocytic function using nonspecific capture of latex particles (1.5 μm in size) from Dia-M, Russia. The day before the addition of particles, the cells were plated in 48-well plates at a concentration of 3 × 10⁵ cells / ml in RPMI1640 medium with 10% FCS, streptomycin and penicillin (final concentration of 50 μg / ml and 50 U / ml, respectively) and M-CSF (final concentration of 50 ng / ml). The next day, latex particles were added in a ratio of 1: 200, and cells were incubated with or without particles for 3 hours. The capture of latex particles by macrophage-like cells was evaluated in native specimens under the microscope indicated above. The phagocytic number (PhN) was calculated as the average number of latex particles in a cell with a phenotype similar to monocyte / macrophages. Phagocytic index (PhI) is the percentage of cells that entered phagocytosis of their total number. The results were threefold repeated and expressed by the arithmetic mean and its standard error. The significance of differences was assessed by Student t-test at P < 0.05.

3. Results
The depletion of the conditions for the cultivation of ESCs with their subsequent transfer to the suspension state promoted the formation of EBs (figure 1. B) on the 2nd day with high efficiency (99 ± 0.02%). At this stage, two experimental groups were created. In the first EBs, they were cultured for 12 days in suspension in a medium that contained 25% CM of the medium from mouse BM-derived stromal cells. In the second experimental group, instead of the CM, recombinant IL-3 and M-CSF were used. Differentiation conditions differed only at this stage. On day 12 of such cultivation, EBs were harvested
and treated with enzymes to produce single cells. Part of the cells was selected for immunophenotyping, and the other part was plated in semi-liquid methylcellulose medium (MT).

**Figure 1.** Differentiation of ESCs into cells with a macrophage-like phenotype in the presence of a conditioned medium (CM) from mouse BM-derived stromal cells and two recombinant murine cytokines II-3 and M-CSF: A - mouse ESC monolayer, B - formation of embryonic bodies (EBs), C, D - colonies of cells with a macrophage-like phenotype on day 14 of cultivation in MT medium with CM and cytokines, respectively, E - cell morphology, with a monotype/macrophase-like phenotype (Giemsa stain), F - F4/80 AG expression on cells with a phenotype similar to macrophages. Lenses: A, B, F -x20; C, D - x40; E - x63.

The results of flow cytometry, presented in figure 2, showed that the percentage of cells positively stained with ABs against CD34 (sialomucin) and CD45 (total leukocyte AG) was 37% and 5% in the first (figure 2 A) and 43% and 25 % in the second (figure 2 B) groups, respectively. A comparative analysis of the expression of hematopoietic marker genes showed an increase in the number of cells positively stained with anti-CD34 ABs by 8% and CD45 by 20% in a medium with cytokines compared to the conditions for differentiation of ESCs in the presence of CM from mouse BM-derived stromal cells.
Figure 2. Histograms of the expression of two AGs on the surface of cells isolated from EBs during differentiation towards hematopoiesis in the presence of a conditioned medium of stromal cells of mouse BM (A) and recombinant IL-3 and M-CSF (B). The gray color of the histogram is the control staining of cells with phycoerythrin (PE); histograms are white - staining of cells with specific ABs.

For differentiation, 25% CM or the same recombinant cytokines were added to the MT medium. The formation of colonies with different morphology, including mixed ones, was observed for 14 days. The analysis of the obtained results demonstrated the formation in MT of colonies with different morphology with an efficiency of 0.11% (11 ± 0.4 per 10000 cells) and 0.33% (33 ± 0.4 per 10000) in the first and second groups, respectively. Monocyte/macrophage colonies (figure 1. C, D) were separated from MT because it is a heat-labile semi-fluid medium. Morphological analysis (Giemsa dye) of the cell population showed cell morphology typical of monocytes and macrophages (figure 1. E). Monocyte-like cells included large mononuclear oval cells with a diameter of 20 μm with an eccentrically located polymorphic bean-shaped nucleus and a large amount of cytoplasm. Larger cells with a diameter of 20 to 50 μm and different shapes (round, irregular in shape, with invaginations) with an irregular contour of the granular cytoplasm were evaluated as cells with a macrophage-like phenotype. Due to the low frequency and loss of some clones during cloning at this stage, clones with macrophage-like cell morphology were combined. The results of our experiments showed that a large number of cells from the combined clones in which the cells had a macrophage-like morphology do not stain with anti-AG F4/80 ABs. The proportion of positively stained cells was 8 ± 0.02% (figure 1. F). Such low values could be due to the fact that the obtained clones were heterogeneous and other cell types could be present in them. Cells were cultured at this stage in RPMI 1640 medium (PanEco, Russia), to which 10% FCS, M-CSF (final concentration 50 ng/ml) and antibiotics: streptomycin and penicillin at a final concentration of 50 μg / ml and 50 U/ ml were added, respectively. Cells were subcultured in a monolayer at a concentration of 2.5 x10^5 cells / ml. The results of the capture of latex particles, obtained cells with a phenotype similar to macrophages, demonstrated the ability of these cells to nonspecific phagocytosis: the PhN was 1 latex particle per cell, and PhI was 5%.
4. Discussion
To date, methods are being developed to obtain macrophages in culture from ESCs [10, 13, 14, 15]. These methods include culturing ESCs on mouse stromal cells (OP9 cells) and / or purifying the progenitor cells from partially differentiated cultures at the stage of differentiation into monocytes. However, none of these protocols can be scaled due to the fact that the conditions for obtaining macrophage culture are not completely defined. The conditions for differentiation of ESCs into macrophages in the culture are different, and it was of interest to optimize them. The abcam.com website published a method for obtaining macrophages from mouse ESC lines of the E14 line, taken from Zhuang et al., [12]. This method suggests using a conditioned medium (15%) collected from under the murine fibrosarcoma L929 cells containing colony stimulating factor 1 (CGF-1), also known as M-CSF. The method allows obtaining from one Petri dish 12-24x10^6 macrophages within 10-20 days through repeated accumulation. Our use of a conditioned medium (25%) from mouse stromal cells of BM was not effective. The replacement of the conditioned medium with recombinant cytokines (IL-3 and M-CSF) made it possible to increase the efficiency of differentiation in the hematopoietic direction at different stages. The choice of recombinant cytokines was due to the fact that these cytokines have a pronounced stimulating effect on hematopoietic progenitor cells of a monocyte macrophage germ [16].

It was shown that cultured monocytes undergo a process of differentiation and maturation lasting from 5 to 10 days, which ultimately leads to the appearance of large macrophage-like cells. Of all the cytokines studied, only M-CSF, GM-CSF and IL-3 were able to maintain differentiation and long-term survival of cells with phenotypes similar to macrophages. An interesting fact was that the morphology of the obtained cells was very different and depended on what factors were used to induce it. Thus, in the presence of M-CSF, the obtained cells had an elongated fusiform shape, and the addition of IL-l or GM-CSF to the medium led to the formation of round cells with distinct nuclei [17]. In our case, detection of monocytes and macrophages in colonies indicates that these cell types are interconnected. Macrophages have the ability directly, i.e. without the participation of opsonins, bind bacteria and other foreign particles. As a result, it was shown that cells obtained from ESCs with macrophage phenotype possess phagocytic activity.

5. Conclusion
Thus, we optimized the conditions for macrophage differentiation from EBs derived from murine ESCs. New knowledge was obtained about the role of several factors into this differentiation, including recombinant cytokines (IL-3, M-CSF). As a result, it was shown that cells obtained from ESCs with macrophage phenotype possess phagocytic activity, which indicates their functional activity. Perhaps, in order to increase the efficiency of ESC differentiation in this direction, the introduction of other growth factors or a combination of these into the environment is required. The data obtained make it possible to study the biology of these cells in vitro and to carry out genetic manipulations with them in order to obtain cells with desired properties.

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Reference
[1] Leroux C, Cadore J-L and Montelaro R C 2004 Equine Infectious Anemia Virus (EIAV): what has HIV’s country cousin got to tell us? Vet Res 35 485-512 doi: 10.1051/vetres:2004020
[2] Savchenkova I P, Alekseyenkov S V and Yurov K P 2016 Mouse embryonic stem cells – a new cellular system for studying the equine infectious anemia virus in vitro and in vivo Problems
of Virology, Russian journal **61**(3) 107-111 doi:10.18821/0507-4088-2016-61-3-107-111

[3] Fidalgo-Carvalho I, Craig J K, Barnes S, Costa-Ramos C and Montelaro R C 2009 Characterization of an equine macrophage cell line: application to studies of EIAV infection *Vet Microbiol* **136**(1-2) 8-1 doi: 10.1016/j.vetmic.2008.10.010

[4] Werners A H, Bull S, Fink-Gremmels J and Bryant C E 2003 Generation and characterisation of an equine macrophage cell line (e-CAS cells) derived from equine bone marrow cells *Vet Immunol Immunopathol* **97**(1-2) 65-76 doi: 10.1016/j.vetimm.2003.08.012

[5] Evans E, Paillot R and López-Alvarez M R 2018 A comprehensive analysis of e-CAS cell line reveals they are mouse macrophages *Sci Rep* **29**(8) 8237 doi:10.1038/s41598-018-26512-3

[6] Savchenkova I P and Yurov K P 2017 Cellular receptor of equine infection anemia virus (EIAV) *Vet and Feed* **6** 6-9

[7] Savchenkova I P, Fleischmann Z M, Bulla J and Brem G 1996 The use of pluripotent mouse embryo stem cells for the production of chimeric animals *Tsitolozia* **38**(10) 1118-23

[8] Moore K J, Fabunmi R P, Andersson LP and Freeman MW 1998 In vitro-differentiated embryonic stem cell macrophages: a model system for studying atherosclerosis-associated macrophage functions *Arterioscler Thromb Vasc Biol* **18** 1647-52 doi: 10.1161/01.atv.18.10.1647

[9] Lindmark H, Rosengren B, Hurt-Camejo E and Bruder C E 2004 Gene expression profiling shows that macrophages derived from mouse embryonic stem cells is an improved in vitro model for studies of vascular disease *Exp Cell Res* **300** 335-44 doi: 10.1016/j.yexcr.2004.06.025

[10] Odegaard J I, Vats D, Zhang L, Ricardo-Gonzalez R, Smith K L, Sykes D B, Kamps M P and Chawla A 2007 Quantitative expansion of ES cell-derived myeloid progenitors capable of differentiating into macrophages *J. Leukoc Biol* **81** 711-9 doi: 10.1189/jlb.0104087

[11] Napoli I, Kierdorf K and Neumann H 2009 Microglial precursors derived from mouse embryonic stem cells *GLIA* **57** 1660-71 doi: 10.1002/glia.20878

[12] Zhuang L, Pound J D, Willems J J, Taylor A H, Forrester L M and Gregory C D 2012 Purepopulations of murine macrophages from cultured embryonic stem cells. Application to studies of chemotaxis and apoptotic cell clearance *J Immunol Methods* **30-385**(1-2) 1-14 doi: 10.1016/j.jim.2012.06.008

[13] Subramanian A, Guo B, Marsden M D, Galic Z, Kitchen S, Kacena A, Brown H J, Cheng G and Zack J A 2009 Macrophage differentiation from embryoid bodies derived from human embryonic stem cells *J. Stem Cells* **4**(1) 29-45

[14] Pittet M J, Nahrendorf M and Swirski F K 2014 The journey from stem cell to macrophage *Ann N Y Acad Sci* **1319**(1) 1-18 doi:10.1111/nyas.12393

[15] Yeung A T Y et al 2015 Conditional-ready mouse embryonic stem cell derived macrophages enable the study of essential genes in macrophage function *Scientific Reports* **5**: 8908 doi:10.1038/srep08908

[16] McGrath K E, Frame J M and Palis J 2015 Early hematopoiesis and macrophage development *Seminars in Immunology* **27** 379-87 doi.org/10.1016/j.smim.2016.03.013

[17] Young D A., Lowe L D and Clark S C 1990 Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture. Analysis of macrophage antibody-dependent cellular cytotoxicity *J. Immunol* **145** 607-15