Alternative Reprogramming of M1/M2 Phenotype of Mouse Peritoneal Macrophages *In Vitro* with Interferon-γ and Interleukin-4

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An important role in the development of the immune response is played by macrophages that acquire either anti-inflammatory M1 or anti-inflammatory M2 phenotype depending on their microenvironment. The possibility of targeted reprogramming of the initial M2 macrophage phenotype towards M1 phenotype and vice versa using macrophage reprogramming factors IFN-γ and IL-4, respectively, was demonstrated. We showed that macrophages of genetically different mouse strains did not practically differ by their reprogramming capacity. Our findings suggest that macrophage programming not only participates in the triggering of the immune response, but also can ensure plasticity of functional activity during the developing response.

Key Words: *macrophages; reprogramming; macrophage reprogramming factor; cytokines*

Inadequate inflammatory reaction is the key element of the pathogenesis of various diseases [1] and studying of the possibility of its correction is an important problem of modern medicine.

Macrophages play the central role in the development of inflammatory reaction in organs and tissues. The interaction of macrophages with intracellular microorganisms, bacteria and viruses, and/or with IFN-γ induces the formation of classical M1 phenotype [4]. M1 macrophages produce a variety of proinflammatory cytokines, IL-12, IL-18, IL-1β, IFN-γ, TNF-α, reactive oxygen species (ROS), and nitric oxide (NO) [7], possess pronounced phagocyte and bactericide properties, and are integrated in Th_1 response aimed at killing bacteria, viruses, and tumor cells [4]. Surface cell markers of M1 macrophages are MAPKO-receptor, CD80, CD86, TLR-2, TLR-4, FcγRIII (CD16), FcγRII (CD32), CD62, IL-1R1, CD127 [7]; their functional marker is enhanced production of NO due to activation of inducible NO synthase (iNOS) [11]; their morphological marker is round shape in cell culture [8].

The interaction of macrophages with extracellular parasites, fungi and helminthes and/or with IL-4 and IL-13 leads to the formation of alternative M2 phenotype [4]. M2 macrophages release many anti-inflammatory cytokines (IL-4, IL-10, IL-13, tumor growth factor-β [7]) and less proinflammatory cytokines, ROS, and NO in comparison with M1. M2 macrophages are integrated into Th_2 response aimed at killing extracellular parasites. M2 regulate inflammation, participate in remodeling and reparation of damaged tissues, and promote angiogenesis and tumor growth [7]. Surface cell markers of M2 macrophages are mannose receptor (MRC1, CD206), CD163, Fcε–RII (CD23, CD209), FIZZ1, ST2, SR-A and M60 receptors, CD184, TRAIL, ИЛ-1Rα [7], while their morphological marker is fibroblast-like flattened shape in cell culture.

The phenotype of resident macrophages can change. In some cases this phenotype reprogramming has adequate and therapeutic character and leads to reco-

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very, while in others it is inadequate and pathogenic phenomenon and promotes disease progress. This raised huge interest to methods of phenotype correction and macrophage reprogramming factors (MRF). It was demonstrated that IFN-γ released during the immune response by natural killers [3], macrophages [13], and Th_1-cells [13] promotes the formation of M1 phenotype [5], while IL-4 released by Th_2-cells [14], eosinophils, basophils [14], and macrophages [10] induces the formation of M2 phenotype [4].

These studies and other reports proved the possibility of programming native initial M0 phenotype to M1 or M2 using IFN-γ and IL-4, respectively. We hypothesized that the same MRF, *i.e.* IFN-γ (MRF1) and IL-4 (MRF2) not only programmed the phenotype of native M0 macrophages, but can also reprogram M1 into M2 and vice versa.

Here we verified this hypothesis on mouse peritoneal macrophages.

MATERIALS AND METHODS

The experiments was performed on cultured peritoneal macrophages from male C57Bl/6 and BALB/c mice. Macrophages of C57Bl/6 mice initially have M1 phenotype and macrophages of BALB/c have M2 phenotype [8]. The mice were anesthetized with chloral hydrate (32.5 g per 100 g body weight intraperitoneally). Peritoneal macrophages were isolated from peritoneal lavage fluid by centrifugation at 1000 rpm for 4 min at room temperature. The supernatant was discarded and the pellet was resuspended in serum-free RPMI-1640 medium. The cell suspension was adjusted to a required concentration, divided into 3 pools, and placed in wells of 48-well culture plates (0.5 mln. cells per well in 0.5 ml medium). After 1 hour, the medium was replaced with a fresh portion of RPMI-1640 with 10% serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and macrophage phenotype reprogramming procedure was started in 1 h.

Pool 1 was cultured with MRF1 for reprogramming to M1 phenotype, pool 2 served as the control, and pool 3 was cultured with MRF2 for reprogramming to M2 phenotype. IFN-γ (10 ng/ml) and IL-4 (20 ng/ml) served as MRF1 and MRF2, respectively. The three pools were cultured for 12 h for evaluation of functional activity or 24 h for evaluation of morphological differences between the pools.

For macrophage activation and evaluation of their functional activity after 12-h reprogramming, lipopolysaccharide (LPS, 500 ng/ml) was added for 24 h. After that, NO production by macrophages was measured spectrophotometrically by nitrite content in the culture medium using Griess reaction [11] and the content of iNOS was evaluated by Western-blot assay [12]. For morphological characteristics of the total pool, macrophages were cultured for 24 h and cell shape was analyzed and morphological index was calculated as the ratio of the number of macrophages with fibroblast-like shape (M2) to macrophages with round shape (M1) for 100 cells in 5 fields of view in the well.

RESULTS

After 24-h culturing, populations of fibroblast-like and round cells were obtained (Fig. 1).

The data on changes in functional NO-synthesizing activity of macrophages after reprogramming procedure are presented in Fig. 2. Culturing of mac-

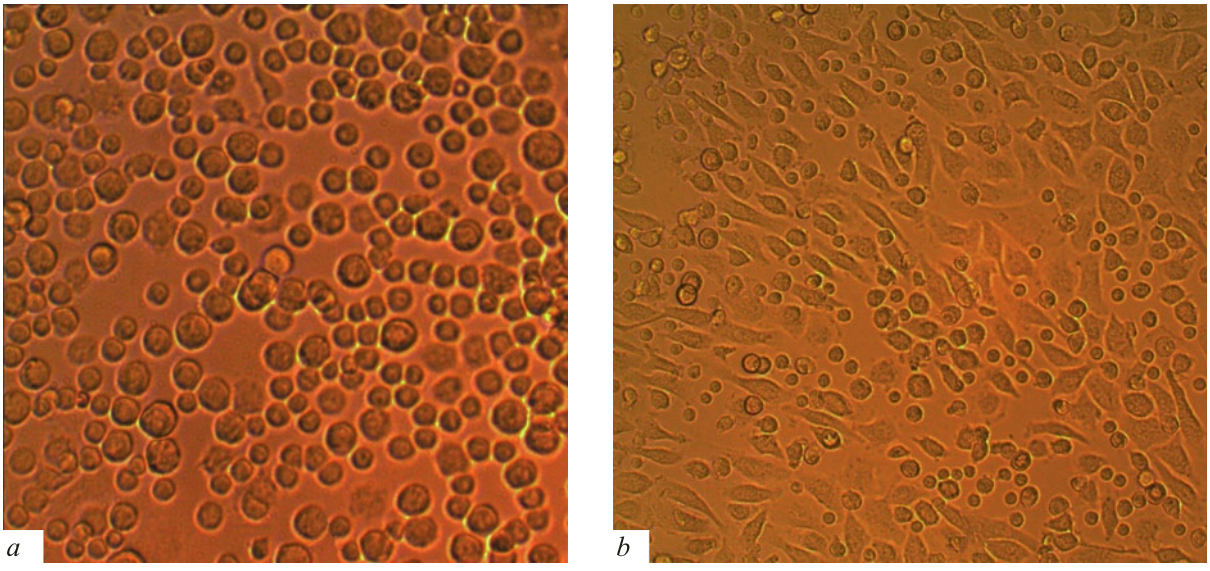

Fig. 1. Populations of cultured macrophages of primarily round (M1 phenotype, *a*) and flattened fibroblast-like (M2 phenotype, *b*) shape.

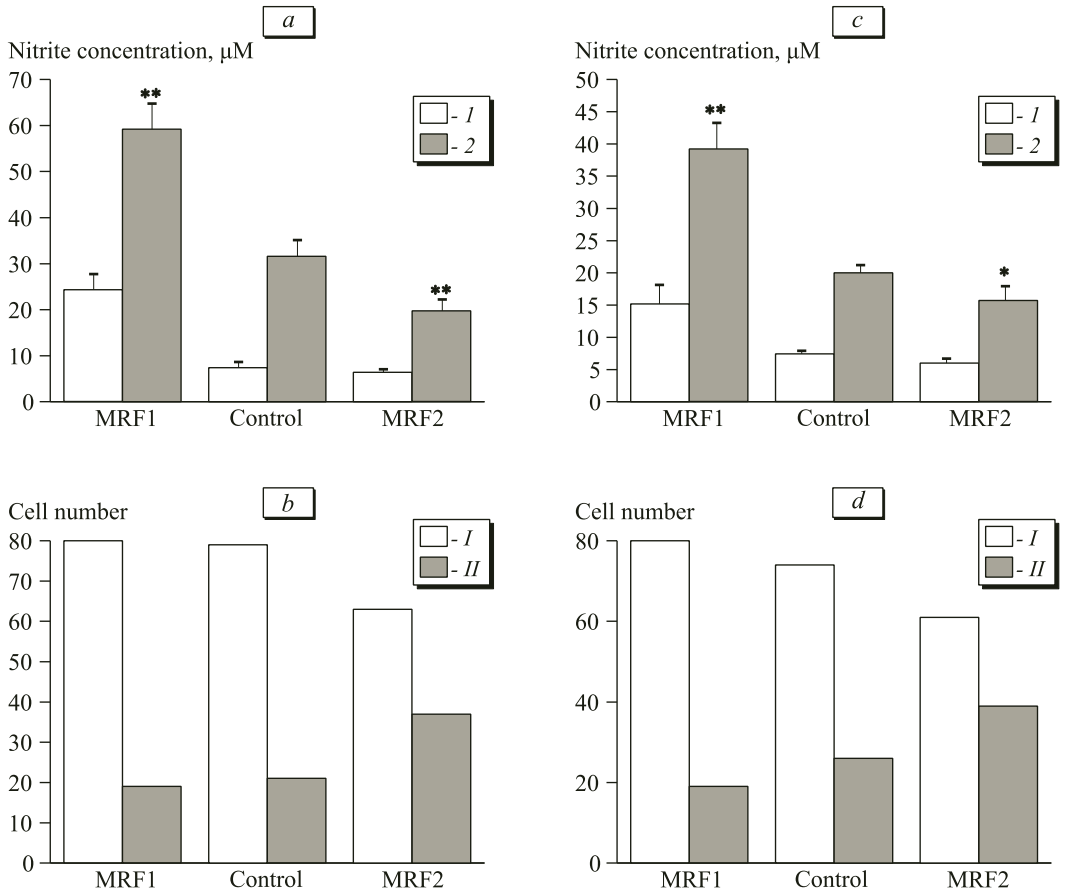

Fig. 2. Effect of MRF on NO production (*a*, *c*) and number of macrophages (*b*, *d*) of round (*I*) and flattened fibroblast-like (*II*) shape (per 100 cells). *a*, *b*: C57Bl/6 (M1 phenotype): *1*) without LPS, *2*) with LPS; *c*, *d*: BALB/c (M2 phenotype). **p*<0.05, ***p*<0.01 compared to the control.

rophages in the presence of IFN-γ increased the basal (non-stimulated) NO production by macrophages from both C57Bl/6 and BALB/c mice and considerably increased NO generation by macrophages from both mouse strains in response to LPS (Fig. 2). These changes attest to the formation of M1 phenotype.

Culturing of macrophages in the presence of IL-4 did not significantly change the basal NO production by macrophages from both C57Bl/6 and BALB/c mice, but reduced NO generation by macrophages from both strains in response to LPS (Fig. 2). These changes attest to the formation of M2 phenotype. Changes in NO production are determined by changes in iNOS content in macrophages during reprogramming. Indeed, addition of MRF1 increased, while MRF2 decreased iNOS content (Fig. 3).

These changes in functional activity of cells reflecting the process of macrophage reprogramming were accompanied by morphological changes. After 12 h of reprogramming, when different macrophage pools can be already divided into M1 and M2 phenotypes by functional criterion, no morphological changes were observed, but they appeared after 24-h culturing in the presence of MRF1 or MRF2 (Fig. 2). Culturing of macrophages from C57Bl/6 mice with MRF1 (10 ng/ml IFN-γ) did not increase the relative content of round cells. Macrophages of C57Bl/6 mice initially have M1 phenotype and further morphological reprogramming towards this phenotype is hardly possible. Therefore, morphological index remained practically unchanged. Culturing of macrophages from C57Bl/6 mice with MRF2 (20 ng/ml IL-4) increased the relative content of fibroblast-like cells from 21 to 37%. Since this shape is typical of M2 phenotype, the observed morphological changes confirm macrophage reprogramming to M2 phenotype. The morphological index (the ratio of M1 and M2 cells) increased from 0.26 to 0.59 (Fig. 4).

Addition of MRF to macrophages obtained from BALB/c mice induced more pronounced changes. Cul-

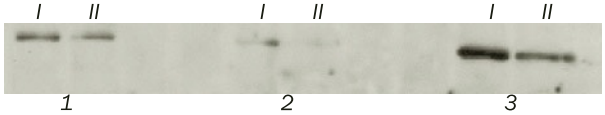

Fig. 3. Effect of MRF on iNOS content in LPS-stimulated macrophages from C57Bl/6 (*I*) and BALB/c (*II*) mice. *1*) control; *2*) MRF2 (IL-4, 20 ng/ml); *3*) MRF1 (IFN-γ, 10 ng/ml).

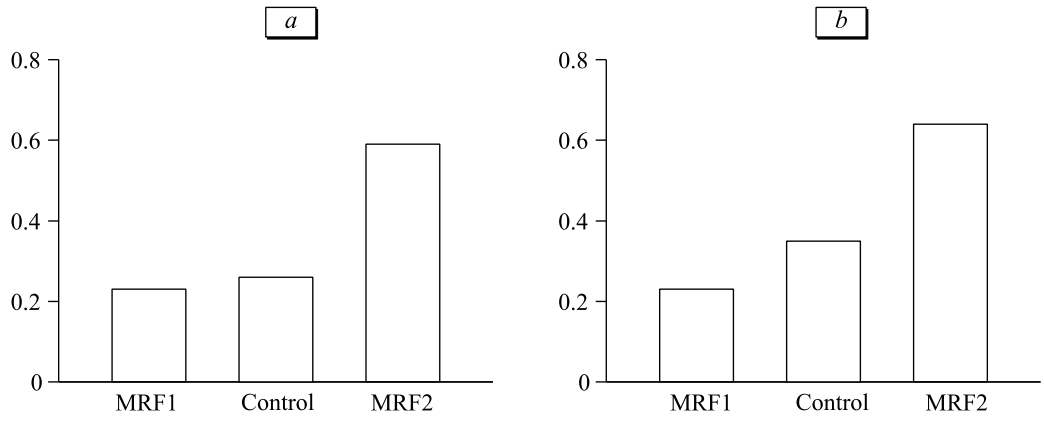

Fig. 4. Effect of MRF on morphological index of macrophages from C57Bl/6 (*a*) and BALB/c (*b*) mice.

turing of macrophages from BALB/c mice in the presence of MRF1 increased the relative content of round cells from 74 to 81% and decreased the morphological index from 0.35 to 0.23. These changes together with activation of NO production attest to macrophage reprogramming to M1 phenotype. Culturing of macrophages from BALB/c mice in the presence of MRF2 increased the relative content of fibroblast-like cells from 29 to 36%, which confirmed macrophage reprogramming to M2 phenotype; the morphological index increased from 0.35 to 0.64 (Fig. 4).

Thus, we reprogrammed the initial morphological and functional M2 phenotype of macrophages from BALB/c mice to M1 phenotype using IFN-γ (MRF1) and initial morphological and functional M1 phenotype of macrophages from C57Bl/6 mice to M2 phenotype using IL-4 (MRF2).

These results confirmed our hypothesis that macrophage phenotype can be purposefully reprogrammed towards M1 or M2 using the corresponding MRF. This finding is of great clinical importance, because macrophages under conditions of various pathologies are already programmed to a certain pathogenetic phenotype. For instance, M1 phenotype plays an important role in the pathogenesis of obstructive lung disease and sarcoidosis [9], while M2 phenotype is involved into pathogenesis of bronchial asthma [15]. Therefore, therapeutic correction should consist in reprogramming of the formed M1 phenotype to M2 or vice versa, rather that in programming of native M0 phenotype to M1 or M2.

It is also interesting that macrophages of two genetically different mouse strains C57Bl/6 (primarily M1 phenotype) and BALB/c (primarily M2 phenotype) practically did not differ by their reprogramming capacity: they were reprogrammed to M1 and M2 phenotypes with similar efficiency.

Our findings substantially supplement the knowledge on the role of environmental factors in the regulation of macrophage activity and suggest that macrophage programming not only participates in the triggering of the immune response, but also can ensure plasticity of functional activity during the developing response. Moreover, our findings suggest that MRF can be used as new immunological tools in the treatment of pathologies associated with inadequate inflammation.

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