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MORPHOLOGY AND PATHOMORPHOLOGY

Morphofunctional Characteristics of Lung Macrophages in Rats with Acute Respiratory Distress Syndrome

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A comprehensive morphofunctional study of the lungs and alveolar macrophages was carried out in Sprague-Dawley rats with acute respiratory distress syndrome (n=10) induced by intratracheal administration of *E. coli* LPS 0111:B4 in a dose of 15 mg/kg. On the first day after LPS administration, bronchopneumonia was observed in the lungs, the number of macrophages of the bone marrow origin and the number of M1 macrophages with the proinflammatory phenotype in the bronchoalveolar lavage increased, the expression of proinflammatory cytokines increased and the expression of anti-inflammatory cytokines decreased, which was accompanied by an increase in LPS and C-reactive protein in the blood serum. The revealed changes correspond to the development of acute respiratory distress syndrome in humans, and the decrease in the number of macrophages in the lungs and their predominant polarization to the M1-proinflammatory phenotype substantiate the use of cell therapy with reprogrammed M2 macrophages.

Key Words: *inflammation; lungs; alveolar macrophages; bronchoalveolar lavage; pro- and anti-inflammatory cytokines*

Acute lung injury and its more severe form, acute respiratory distress syndrome (ARDS), is one of the causes of high mortality from pneumonia, including those caused by SARS-CoV-2 [1]. ARDS develops in 42% of patients with pneumonia due to COVID-19 and in 61-81% of patients requiring intensive care [2]. At the same time, the mechanisms that cause lung damage are not fully understood [3].

An important role in the pathogenesis of ARDS is played by numerous mediators, in particular, cytokines, oxidants, and other molecules, as well as their complex interactions. There is now increasing evidence that macrophages, including resident alveolar macrophages (AM) and macrophages recruited from the blood, determine the pathogenesis of ARDS [4]. In ARDS, resident AM phagocytize apoptotically dying cells and participate in alveolar repair [5], but overproduction of proinflammatory cytokines observed in this pathology stimulates migration of recruited macrophages into the lungs, their differentiation into M1 macrophages, and initiation of inflammation [6].

LPS, known also as endotoxin, is a component of the cell wall of Gram-negative bacteria that is widely used for *in vivo* modeling of human ARDS [7]. The administration of LPS to laboratory animals causes the development of symptoms detected during ARDS in humans: impaired microcirculation in the lungs, migration of neutrophils to the interalveolar septa, intra-alveolar edema, pronounced inflammatory reactions in the lungs, in particular, an increase in the

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content of proinflammatory cytokines in the bronchoalveolar lavage (BAL), impaired gas exchange, and difficult breathing, resulting in high mortality rates [8]. The cascade of proinflammatory responses is triggered by LPS binding to TLR4 on the surface of the alveolar epithelium, which leads to transcription of NF-κB and further production of cytokines TNFα, IL-1, IL-6, and chemokines, including monocyte chemoattractant protein 1 (MCP-1) that activates migration of mononuclear leukocytes from the blood into the interalveolar septa [9,10]. Overproduction of proinflammatory cytokines is often the cause of high mortality rates from ARDS, and the problem of finding effective approaches to the treatment of this syndrome remains relevant. To develop approaches to more effective treatment of ARDS using reprogrammed macrophages, it is necessary to assess the morphofunctional state of macrophages in the lungs on the first day after intratracheal administration of LPS.

The aim of the study was comprehensive morphofunctional study of the lungs and alveolar macrophages in rats with ARDS induced by intratracheal administration of LPS.

MATERIALS AND METHODS

The study was performed on adult (n=19) male Sprague-Dawley rats (body weight 200-220 g) obtained from Pushchino breeding nursery (M. M. Shemyakin and Yu. A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences). The study was performed in compliance with Directive 2010/63/EU of the European Parliament and of the Council (September 22, 2010; On the Protection of Animals Used for Scientific Purposes) and was approved by the Bioethical Commission of the A. P. Avtsyn Research Institute of Human Morphology (Protocol No. 21; March 21, 2019). The animals were kept in plastic cages (460×300×160 mm) with stainless steel lattice lids with a stern recess and had free access to water and food. The temperature in the vivarium room was maintained at 23±3°C, and air humidity was 35-65%.

In the experimental group (n=10), ARDS was simulated by intratracheal injection of *E. coli* LPS 0111:B4 (Sigma) in a dose of 15 mg/kg and a volume of 0.125 ml. The control group (n=9) was injected intratracheally with 0.9% NaCl in the same volume. The animals were euthanized by overdose of Zoletil (15 mg/kg; Vibrac Sante Animale) in 24 h. Samples of peripheral blood were obtained before sacrifice and stored at -70°C until used for no longer than 2 months. In the blood serum, the content of C-reactive protein (CRP) and LPS was assessed by ELISA using the corresponding commercial kits (Cloud-Clone Corp.).

Right lungs were obtained from 5 experimental and 5 control animals, fixed in Carnoy's fluid, dehydrated in ethanol (96 and 70%), and embedded in Histomix. Step-staged slices (5-μm thick) were prepared and stained with hematoxylin and eosin. In histological sections, morphological changes in the lungs were analyzed under a microscope with a Leica DFC290 camera. For immunohistochemical study, histological sections of lungs were stained with rabbit primary antibodies to CD68 (1:200, Affinity Bioscience) and secondary anti-rabbit antibodies labeled with Alexa Fluor 488 (Life Technologies).

To obtain BAL samples, 4 control and 5 experimental animals were injected intratracheally with 5 ml of DMEM (PanEco) under Zoletil anesthesia. Immediately after the injection, the culture medium was aspirated once. The return volume ranged from 35 to 70%. The absolute cell number in 1 ml of BAL was measured on Luna II automatic cell counter (Logos Biosystems) and then adjusted to 2×10^5/ml. Differential cell count (per 200 cells) was performed in BAL smears stained according to Romanowsky–Giemsa, and the data were expressed in %.

The evaluation of the absolute and relative numbers of the main macrophage subpopulations in BAL was performed by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences) using anti-rat antibodies to monocyte marker leukosialin (CD43 Antibody, PE), pan-macrophage marker (CD68 Antibody, PE- 70), M1-macrophage marker (CD86 Antibody, Vio Bright FITC), monocyte and granulocyte receptor (CD11b/c Antibody, FITC) (all from Miltenyi Biotec), M2-macrophage marker (CD163 Monoclonal Antibody (TNKUPJ), PE, eBioscience; Invitrogen), and marker of antigen-presenting cells (MHC II-PE; BD Biosciences).

One day after intratracheal administration of LPS, a fragment of the right lung of 5 control and 5 experimental rats was placed in an IntactRNA fixative (Eurogen). The expression mRNA was assayed by real-time qPCR in tissue fragments of the right lungs. The performed analysis included the detection and evaluation of expression levels of proinflammatory (*Il-1β*, *Il-6,* and *Tnfα*) and anti-inflammatory (*Il-10*, *Tgfβ)* cytokines, and arginase-1 (*Arg1*), *Mmp2*, and *Mmp9*, as well as *Timp2* and *Timp1*, relative to GAPDH expression level as a reference. The fragments of the lungs were homogenized, and total RNA was isolated from the obtained cell suspension using the RNeasy Plus Mini Kit (Qiagen). The synthesis of cDNA from the matrix of the obtained total RNA was carried out using a ready-made MMLV RT kit (Eurogen). The resulting cDNA was reacted with qPCRmix-HS SYBR reagent kits containing SYBR Green I fluorescent intercalating dye (Eurogen). All the primers sequences were chosen using Primer-BLAST online tool.

The results were analyzed by Statistica 8.0 software (StatSoft, Inc.). The normality of data distribution was checked by using the Kolmogorov–Smirnov test. The data were expressed as Me (25%-75%). As the data were not distributed normally, the nonparametric Mann–Whitney test was used to establish significance of differences between the groups. The differences were significant at $p<0.05$.

RESULTS

One day after the intratracheal administration of LPS, the levels of CRP and LPS in the blood serum of Sprague-Dawley rats increased (Fig. 1).

No pathological changes were detected in the lungs of control animals: bronchial lumen was free, bronchial epithelium had clear-cut contouring cilia, the lamina propria was represented by the fibrous connective tissue with single diffusely scattered lymphocytes. There are numerous lymphoid nodules in the bronchial wall, some of them with light centers. In the lumen of the alveoli and alveolar ducts, the epithelial lining is not disturbed; they contain single macrophages (Fig. 2).

Bronchopneumonia foci were detected in the lungs of rats 1 day after the administration of LPS. Neutrophils, single macrophages, lymphocytes, and cells of desquamated epithelium are present in the lumens of the main and bronchi of the II-III order. The bronchial walls were moderately and diffusely infiltrated with lymphocytes, macrophages, and neutrophils. The lumens of the respiratory bronchioles, alveolar ducts, and alveoli adjacent to the bronchi were filled with macrophages, neutrophils, and single lymphocytes. Some interalveolar septa were thickened with inflammatory infiltration, but most septa were poorly distinguished. In the lungs outside the zones of bronchopneumonia, no inflammatory changes in the walls of the bronchi were found, the interalveolar septa were focally thickened and infiltrated with neutrophils and single lymphocytes. Marked hyperplasia of broncho-associated lymphoid tissue and lymphoid nodules with wide light centers were seen. In the adventitia of the pulmonary veins, muff-like lymphocyte accumulations were revealed (Fig. 2).

In ARDS, the number of $CD68^+$ macrophages in the lungs was reduced in comparison with the control (Table 1; Fig. 2). In BAL smears from control rats, lymphocytes, neutrophils, and macrophages were found. Administration of LPS led to an increase in the number of neutrophils in BAL and a decrease in the relative number of macrophages (Table 1). It has been previously shown that the number of neutrophils and monocytes/macrophages in infected lung tissues increased in patients with severe or fatal SARS-CoV or MERS-CoV infection [1].

Analysis of macrophage subpopulations in BAL showed that in rats with ARDS, the relative number of $CD43^+CD11^+$ monocytes of bone marrow origin increased, and the proportion of $CD86^+$ proinflammatory M1 macrophages and the number of cells expressing MHCII among macrophages increased (Table 2).

The pathogenesis of ARDS includes several phases: exudative phase, rehabilitation (or proliferative) phase, and fibrous (or fibroproliferative) phase [11]. At each stage, macrophages play an important role, so they can be a potential target for the treatment of ARDS, including in COVID-19. For instance, resident AM can play a protective role at the early stages of SARS-CoV-2 infection [12]. In ARDS, peripheral blood monocytes migrate to the lungs, where they differentiate into M1 macrophages [6]. It has been shown that patients infected with SARS-CoV-2 experience excessive activation of monocytes/macrophages with the development of a “cytokine storm” and subsequent acute lung injury leading to ARDS

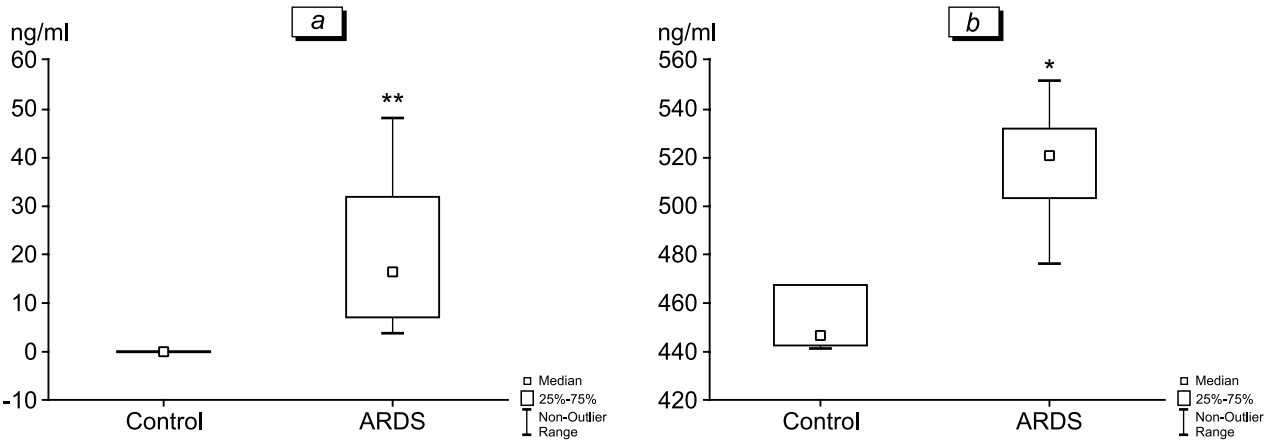

Fig. 1. Serum levels of CRP (*a*) and LPS (*b*) in Sprague-Dawley rats on the first day of ARDS development. *p=0.03, **p=0.005 in comparison with control (Mann—Whitney test).

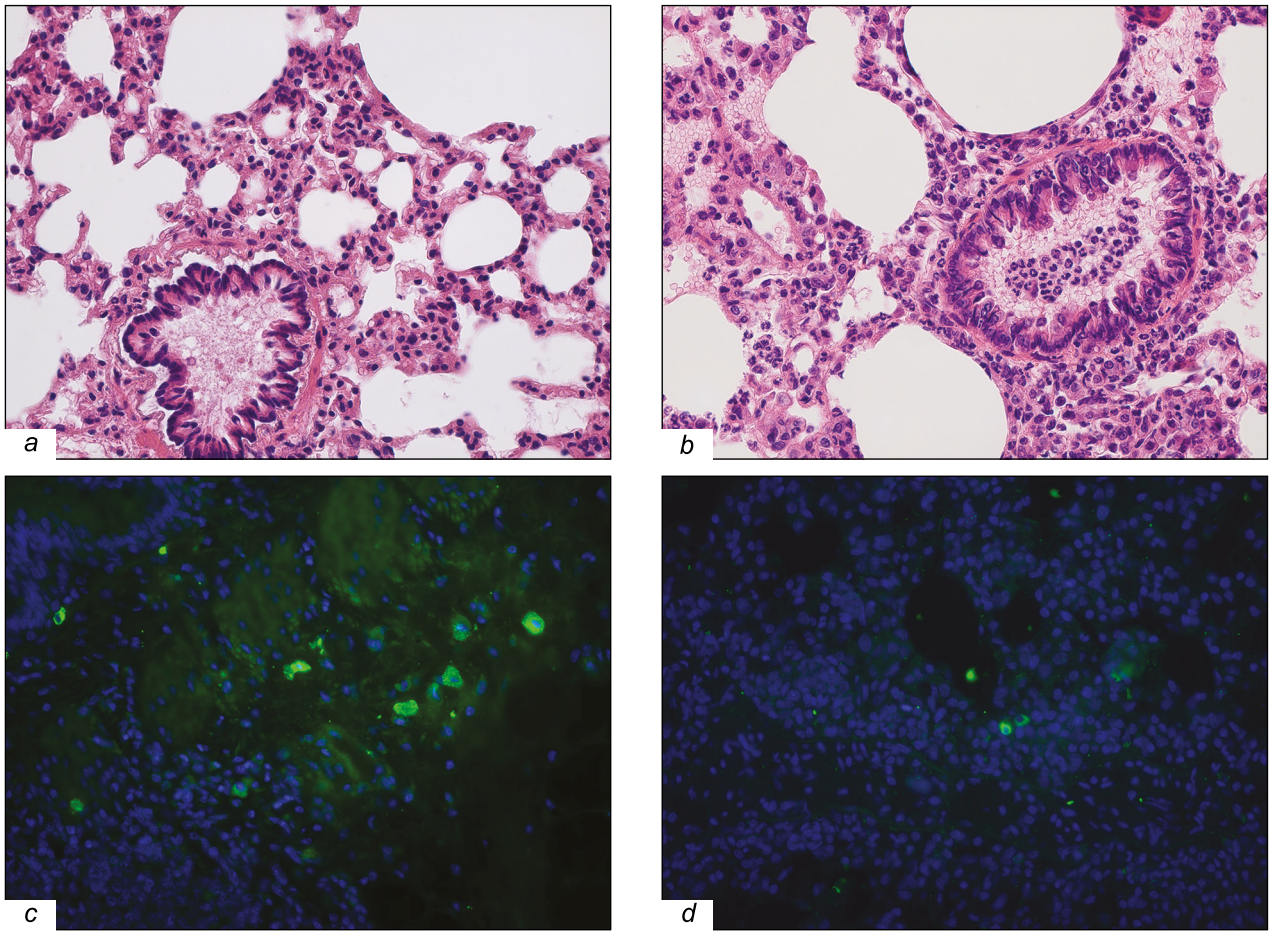

Fig. 2. Morphological characteristics of the lungs in the control (*a*, *c*) and 1 day after LPS injection (*b*, *d*). Staining with hematoxylin and eosin (*a*, *b*) and immunohistochemical staining with antibodies to CD68 (green glow) (*c*, *d*), ×400. *a*) The lumen of the bronchus is filled with eosinophilic masses, the interalveolar septa are thin. *b*) The lumen of the bronchus is filled with eosinophilic masses with neutrophils and lymphocytes; the interalveolar septa are thickened and contain neutrophils, erythrocyte masses are in the lumen of the alveoli. *c*) $CD68^+$ macrophages in the lungs. *d*) A decrease in the number of $CD68^+$ macrophages in comparison with the control.

[13]. Detailed cytometric analysis of peripheral blood samples from 28 patients infected with SARS-CoV-2 revealed significant changes in the morphological and functional characteristics of monocytes. Intermediate and non-classical monocytes predominated in the blood of patients, they were significantly larger in size and secreted large amounts of IL-6, IL-10, and TNFα, which directly indicates the development of a reaction of the M1 proinflammatory type.

Thus, both pro- and anti-inflammatory AM are involved in the development of ARDS, and cell therapy with reprogrammed M2 type macrophages can

TABLE 1. Absolute Number of $CD68^+$ Macrophages in Lungs and Relative Number of Macrophages, Lymphocytes, and Neutrophils in BAL of Sprague-Dawley Rats with ARDS (Me (25%-75%))

Parameter	Control	ARDS
Lungs		
Number of $CD68^+$ macrophages per 1 cm^2	123 (106-206)	51 (32-88)*
BAL		
Macrophages, %	37 (36-37)	11.5 (7.8-17.0)**
Neutrophils, %	32 (28-34)	58 (54-69)**
Lymphocytes, %	29 (28-30)	25 (16.5-35)

Note. *p=0.04, **p=0.01 in comparison with the control. For immunohistochemical examination of the lungs, 5 rats were taken from each group, for BAL study, 4 control and 5 experimental rats were used.

TABLE 2. Relative Content (%) of Monocytes, Major Macrophage Subpopulations, and MHC II Cells in BAL of Sprague-Dawley Rats with ARDS (Me (25%-75%))

Marker	Control (*n*=4)	ARDS (*n*=5)
$CD43^+$	16.9 (1.0-38.0)	82.2 (49.3-83.7)*
$CD11^+$	15.1 (8.8-28.1)	77.4 (39.1-83.8)*
$CD43^+CD11^+$	12.8 (6.6-25.8)	77.2 (36.9-80.6)*
$CD43^+CD11^-$	4.1 (1.8-7.1)	1.2 (0.5-1.4)
MHC II^+	16.0 (12.7-19.0)	34.6 (22.1-51.4)*
$CD68^+$	0.13 (0.06-0.02)	0.15 (0.12-0.39)
$CD86^+$	0.2 (0.1-0.4)	0.82 (0.8-2.0)*
$CD163^+$	0.09 (0.06-0.10)	1.3 (0.8-2.0)

Note. **p*=0.03 in comparison with the control (Mann—Whitney test).

be one of the strategies for treating inflammatory processes in the lungs. Moreover, a shift in macrophage polarization from the proinflammatory to the anti-inflammatory phenotype can help to reduce lung damage in ARDS.

Numerous mediators (cytokines, oxidants, other molecules and their complex interactions) play an important role in the pathogenesis of ARDS. Compared with the control group, the expression of proinflammatory cytokines IL-1β, IL-6 and TNFα and anti-inflammatory IL-10 increased in the lungs of experimental rats on the first day after LPS injection, while expression of TGF-β and the M2 macrophage marker Arg1 decreased (Table 3).

An increase in the expression level of proinflammatory cytokines IL-1β, IL-6, and TNFα contributes to the development of inflammation in the lungs and activation of macrophages according to the M1 proinflammatory type [14]. The increase in the number of M1 macrophages in BAL revealed by us can be due to the microenvironment and the high level of production of proinflammatory cytokines. Along with the increase in the expression of proinflammatory cytokines, 1 day after intratracheal administration of LPS, a decrease in the level of expression of anti-inflammatory TFG-β and the M2-macrophage marker Arg1 was found.

Arg1 is a cytoplasmic enzyme highly expressed by macrophages and dendritic cells in response to Th2 cytokines IL-4 and IL-13 [15]. TGF-β, like Arg1, is a marker of M2 macrophages [16], therefore, the decrease in the expression level of Arg1 and TGF-β in the lungs may reflect the polarization of macrophages, mainly towards the M1 phenotype.

Thus, 1 day after intratracheal administration of LPS, bronchopneumonia was observed in the lungs of Sprague-Dawley rats, the number of macrophages of the bone marrow origin and the number of M1 macrophages with a proinflammatory phenotype in BAL increased, the expression of proinflammatory cytokines increased, while the expression of anti-inflammatory cytokines decreased, which was accompanied by elevation of LPS and CRP concentrations in blood serum. The identified changes correspond to the development of ARDS in humans, in particular, caused by SARS-CoV-2, so this model can be used to develop new approaches to the treatment of ARDS, including COVID-19.

The work was carried out within the framework of the State Assignment “Development of Cell Therapy for Acute Respiratory Distress Syndrome (ARDS) with Genetically Modified M2 Macrophages” (state registration No. 123030700103-6).

TABLE 3. The Level of mRNA Expression of Proinflammatory and Anti-Inflammatory Cytokines, as well as MMP and TIMP in the Lungs of Sprague-Dawley Rats with ARDS (Me (25%-75%))

Cytokine mRNA, 10^{-5}	Control (*n*=5)	ARDS (*n*=5)
	Proinflammatory cytokines	
Il-1β	31.6 (29.5-54.5)	312.9 (247.4-344.9)***
Il-6	19.6 (5.7-20.9)	64.7 (41.5-71.7)**
Tnfα	2.8 (2.1-3.7)	35.0 (11.3-50.9)*
	MMP and TIMP	
Mmp-2	4.9 (1.8-59.1)	13.0 (4.1-16)
Timp-2	11.0 (3.9-29.6)	0.6 (0.01-17)
Mmp-9	7.0 (0-21.2)	13.0 (2.1-22.4)
Timp-1	696.6 (13-782.9)	710.3 (141.9-1028.8)
	Anti-inflammatory cytokines	
Il-10	0.0 (0-0)	9.2 (5.9-15.8)*
Arg1	61.0 (7.3-1949.8)	9.2 (3.4-27.7)*
Tgfβ	3192.4 (1437.6-4.997.05)	260.9 (52-1925.9)*

Note. **p*<0.05, ***p*<0.01, ****p*<0.005 in comparison with the control (Mann—Whitney test).

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