ASSESSING FUNCTIONAL ACTIVITY OF MICROGLIA AND MACROPHAGES IN BARRIER-ASSOCIATED BRAIN AREAS OF SPONTANEOUSLY HYPERTENSIVE RATS

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The cerebrovascular disorder associated with arterial hypertension results in neuroinflammation, in which microglia and macrophages of the brain are actively involved. The study aimed to assess functional activity and immunophenotype of microglia and macrophages in the areas of brain barriers in spontaneously hypertensive rats (SHR). Specimens of the brain of male Wistar rats and SHR (age 3–4 months, n = 10) were used. The study involved the use of immunohistochemistry analysis and confocal laser microscopy. The presence of M2 activation (CD206) and phagocytic activity (CD68) markers in the population of microglia and macrophages was assessed. It was shown that the CD206 protein was present in perivascular cells, the counts of which were considerably increased in SHR (40.69 ± 4.87 cells per 1 mm^2 vs. 28.73 ± 1.39 in Wistar rats; t-test, p = 0.0007). The quantitative analysis conducted allowed us to identify the upward trend of the share of phagocytic cells in the brain of SHR compared to Wistar rats. No changes in the CD68 protein distribution were found in SHR, therefore, activation of microglia and macrophages is not accompanied by the phagocytic activity increase. The findings suggest alternative activation of brain macrophages in neuroinflammation caused by arterial hypertension.

Keywords: neuroinflammation, microglia, macrophages, spontaneously hypertensive rats, immunohistochemistry

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ОЦЕНКА ФУНКЦИОНАЛЬНОЙ АКТИВНОСТИ МИКРОГЛИИ И МАКРОФАГОВ В АССОЦИИРОВАННЫХ С БАРЬЕРАМИ ОБЛАСТЯХ ГОЛОВНОГО МОЗГА СПОНТАННО-ГИПЕРТЕНЗИВНЫХ КРЫС

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Нарушение мозгового кровотока при артериальной гипертензии приводит к развитию нейровоспаления, активными участниками которого являются микроглия и макрофаги головного мозга. Целью работы было изучение функциональной активности и иммунофенотипа микроглии и макрофагов в области барьеров головного мозга спонтанно-гипертензивных крыс (SHR). Использовали материал головного мозга крыс-самцов Вистар и SHR (возраст 3–4 месяца, n = 10). Работа выполнена с применением методов иммуногистохимического анализа и конфокальной лазерной микроскопии. Оценивали наличие маркера М2 активации (CD206) и фагоцитарной активности (CD68) в популяции микроглии и макрофагов. Показано, что белок CD206 присутствует в периваскулярных клетках, число которых значительно увеличено у крыс SHR (40,69 ± 4,87 клеток на 1 мм^2 против 28,73 ± 1,39 у крыс Вистар; t-test, p = 0,0007). Проведенный количественный анализ позволил выявить тенденцию увеличения доли фагоцитирующих клеток в головном мозге у крыс SHR по сравнению с крысами Вистар. Изменений в распределении белка CD68 у крыс SHR не выявлено, следовательно, активация микроглии и макрофагов не сопровождается усилением фагоцитарной активности. Полученные результаты свидетельствуют об альтернативной активации макрофагов головного мозга при нейровоспалении, вызванном артериальной гипертензией.

Ключевые слова: нейровоспаление, микроглия, макрофаги, спонтанно-гипертензивные крысы, иммуногистохимия

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Macrophages of the brain represent a multicomponent diverse cell population. These include microglia of the brain's nervous tissue, macrophages of the meninges, perivascular areas, and choroid plexus, as well as the monocyte-derived macrophages migrating from the vascular bed into the nervous tissue in various disorders [1]. Such population diversity, regional specificity, as well as high plasticity of microglia and macrophages determine the versatility of their morphological and functional characteristics. In the last decade, numerous studies focused on the origin and morphofunctional heterogeneity of

these immune system cells were conducted. Thus, several border-assosiated microglia and macrophage subtypes under physiological conditions and in neuroinflammation associated with various CNS disorders were described [2–4].

In particular, microglia is involved in regulation of blood flow through cerebral blood vessels and preservation of the blood-brain barrier (BBB) integrity. It has been shown that when inflammation is triggered microglia phagocytizes the astrocyte terminal processes, disrupting the BBB integrity [5], while selective microgliocyte elimination results in microvascular dysfunction [6]. Perivascular macrophages are capable of presenting exogenous antigens by MHC class II. Therefore, these represent T-cell infiltration sites in autoimmune disorders, Alzheimer's and Parkinson's diseases [7]. Epiplexus macrophages of the brain's choroid plexus, or Kolmer cells, limit the amount of peripheral blood molecules, as well as pathogens and lymphocytes, entering the cerebrospinal fluid under the conditions of invasion and tissue damage [8]. Perivascular and epiplexus macrophages generate more reactive oxygen species in response to the increase in blood or cerebrospinal fluid levels of certain compounds, which can aggravate the disease [9]. In contrast, some data demonstrate that perivascular and meningeal macrophages contribute to β-amyloid clearance in Alzheimer's disease or cerebral amyloid angiopathy [10]. Taken together, the data suggest that the contribution of macrophages to neuroinflammation depends on numerous factors, which opens large-scale prospects for modulation of their activity, and, consequently, the use of this approach for therapeutic purposes.

However, it is difficult to clearly delineate microglia and macrophage subtypes in the CNS due to limited specificity of most marker proteins, such as CD11b, F4/80, CX3CR1, CD45, and Iba-1 [11]. Immunophenotypic characteristics of epiplexus macrophages match the characteristics of other mononuclear phagocytes comprising the MHC II, CD11b, CD68, and Iba-1 marker proteins [12]. In addition, when culturing cells *ex vivo*, microgliocytes lose their characteristic features (morphological traits, genetic and epigenetic markers), and become virtually indistinguishable from macrophages [13]. In this regard, immunophenotyping of the CNS microglia and macrophages in vivo seems possible only in conjunction with morphological assessment.

Several experimental models simulating pathological features of certain diseases are currently used to study manifestations of neuroinflammation [14]. Thus, arterial hypertension, which is considered a serious health problem for people worldwide [15] and can be a neuroinflammation pathology model, is associated with impaired inflammation regulation. Spontaneusly hypertensive rats (SHR) are used for standardized hypertension. The research shows that in SHR the cerebral blood flow impairment associated with chronic hypertension leads to inflammation, which can result in neurodegeneration [16]. The results of the study focused on the brain immune system characteristics in arterial hypertension show that microglia of SHR shows morphological signs of activation [17–19]. Preliminary data were also obtained that made it possible to predict the type of polarization of activated microglial cells in SHR [20].

Disruption of the blood-brain and blood-cerebrospinal fluid barriers [16, 21] resulting from the decreased elasticity of cerebral blood vessels caused by remodeling of their cellular layers is a typical manifestation of arterial hypertension reported for SHR. Thus, the response of microglia and macrophages to systemic and local pro-inflammatory stimuli turns out to be most pronounced in the zones of the blood-brain, blood-cerebrospinal fluid, and cerebrospinal fluid-brain barriers. Nevertheless, morphofunctional state of the brain's immune cells in the zones of barriers in this disorder is still poorly understood.

The study aimed to assess functional activity and immunophenotype of microglia and macrophages in the subependymal zone of the lateral and third ventricles, as well as in the choroid plexus of the SHR brain.

METHODS

Brain specimens of male Wistar rats and SHR with the body weight of 250–300 g (age 3–4 months, n = 10) fixed in zinc-ethanol-formaldehyde and paraffin-embedded in accordance with the standard method were used for immunophenotyping of microglia and macrophages in normotensive and hypertensive animals. When forming groups of animals with arterial hypertension, SHR with the average systolic blood pressure equal to or above 200 mHg were selected. Blood pressure of SHR was measured before biomaterial collection using the Systola noninvasive blood pressure measurement system (Neurobotics, Russia).

Identification of microglia and macrophages in brain sections was performed using antibodies against the Iba-1 protein (ab5076, Abcam, UK and ET-1705-78, Huabio, China), CD68 lysosomal glycoprotein (GB113109, Servicebio, China), and CD206 mannose receptor (HA722892, Huabio, China).

Reagents from the UltraVision Quanto Detection System HRP (TL-060-QHL, Fisher Scientific, USA), anti-Goat HRP-DAB Cell & Tissue Staining Kit (CTS008, R&D Systems, USA), Mouse and Rabbit Specific HRP/DAB IHC Detection Kit (ab236466, Abcam, UK) were used as secondary reagents. The 3'3-diaminobenzidine chromogen from the Stable DAB/Plus kit (Diagnostic BioSystems, USA) was used for the monoenzyme reaction product visualization. For immunofluorescence, the sections incubated in secondary antibodies were treated with the Cy2 fluorochrome-conjugated streptavidin (016-220-084, Jackson ImmunoResearch, USA) and the solution of the Cy3 fluorochrome-conjugated goat anti-horseradish peroxidase antibody (123-165-021, Jackson ImmunoResearch, USA).

The resulting preparations were assessed using the Leica DM750 microscope (Germany) and the LSM800 confocal laser microscope equipped with the Airyscan system. Immunomorphological analysis of microglia and macrophages was performed in the barrier zones adjacent to brain centers: ependyma of the lateral and third ventricles (in the area of striatum and mediobasal hypothalamus, respectively), pia mater, lateral ventricular choroid plexus. The expert, who performed morphological and quantitative assessment of the preparations obtained, was blinded in terms of information about the test objects.

Quantification involved enumeration of cells in three fields of view for each preparation using the ×10 and ×20 lens magnification, then standardization based on the scale length of 1 mm^2 was applied (the number of values used to calculate the mean for each case was 6). The percentage of cells containing two markers (Iba-1 and CD68) was calculated by dividing the Iba-1^+/$CD68^+$ cell counts by the Iba-1^+ cell counts. To analyze the images, the ImageJ2 program in the FIJI extension was used (https://imagej.net/software/fiji/). The Coloc2 (https://imagej.net/plugins/coloc-2) and Colocalization Finder (http://rsb.info.nih.gov/ij/plugins/colocalization-finder.html) were used to assess co-localization of the studied markers. Pearson's correlation coefficients were determined for various brain areas of Wistar rats and SHR: subependymal zone of the lateral and

Table 1. Systolic blood pressure in SHR

Rat No.	Systolic blood pressure, mmHg			
	First measurement	Second measurement	Third measurement	Mean
1	211	201	229	214
2	215	203	207	208
3	200	222	235	219
4	251	235	240	242
5	208	209	210	209

third ventricles, choroid plexus. Statistical processing was performed in GraphPad Prism 8 (GraphPad Software, USA). The samples were tested for normality using the Shapiro–Wilk test for small samples. The distribution was considered normal at $p > 0.05$. One-way and two-way ANOVA was used to compare the data, with subsequent comparison of the group using the post-hoc Tukey's test and the Kruskal–Wallis test involving the use of the post-hoc Dunn's test. The data were provided as the mean ± standard deviation. The differences were considered significant at $p < 0.05$.

RESULTS

The results of blood pressure measurement in the SHR selected for the study are provided in Table 1.

Immunohistochemical staining of CD206 in brain sections of Wistar and SHR revealed perivascular cells (in the nervous tissue of the brain and the choroid plexus) and cells of the pia mater (Fig. 1). No cells staining positive for CD206 were reported in the nervous tissue of the brain. The average $CD206^+$ cell counts per 1 mm^2 higher in rats with genetically determined arterial hypertension (*t*-test, $p = 0.0007$), these were 40.69 ± 4.87 cells (vs. 28.73 ± 1.39 in Wistar rats).

The use of double immunohistochemical staining made it possible to identify microglia and macrophages ($Iba\text{-}1^+$) containing the CD68 protein (Fig. 2). These are found in all the studied barrier zones (subependymal zone of the lateral and third ventricles, choroid plexus) and have specific morphological features. Near the ependyma of the lateral and third ventricles these represent typical dendritic subependymal microgliocytes of the basket-like and spindle-like morphotype. The $CD68^+$ granule distribution in the cytoplasm of these cells corresponds to general ideas about the localization of lysosomes. Cells of the choroid plexus are characterized by the sparsely-branched or spindle-like morphotype, and their $CD68^+$ granules are also distributed in the areas of lysosome localization. We should also mention the cells associated with neither ependyma, nor choroid plexus, but distributed freely in the ventricular lumen. Such cells were usually found in the preparations from SHR. The CD68 protein granules in the intraventricular cells occupied almost the entire cytoplasm, concealing the nucleus (Fig. 2a).

The differences in the quantitative distribution of $Iba\text{-}1^+$/$CD68^+$ microgliocytes and macrophages in various barrier zones of the brain turn out to be region-specific, which is confirmed by the analysis of variance ($F = 117.4$, $p < 0.0001$). Thus, it has been shown that the highest percentage of

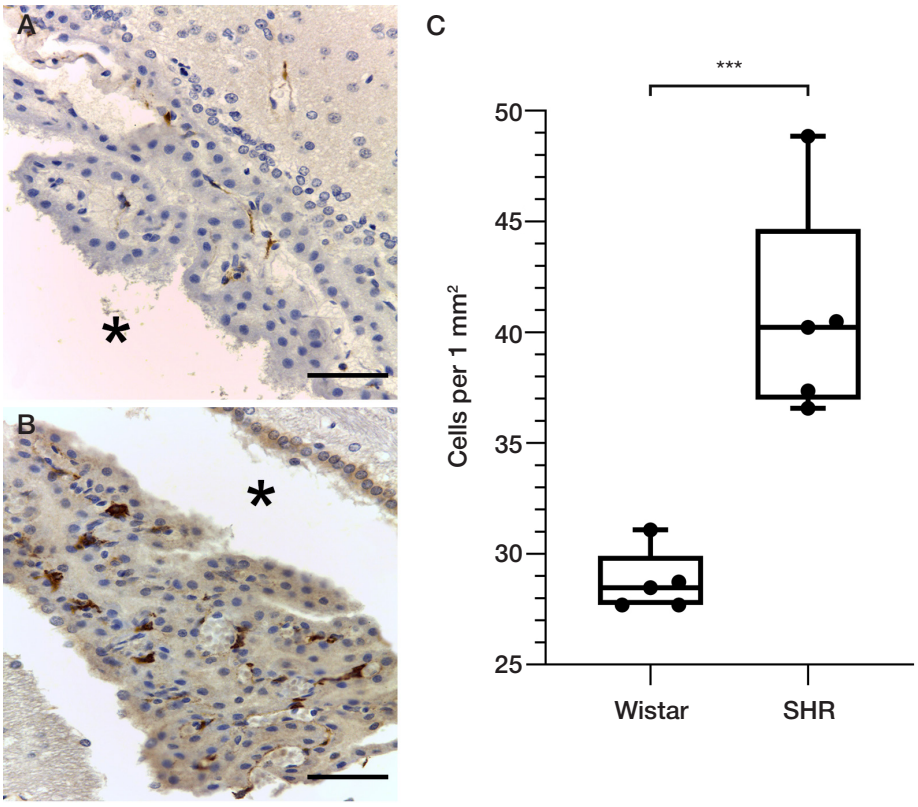

Fig. 1. CD206 in the choroid plexus of the brain of Wistar rats **(A)**, SHR **(B)**. Immunohistochemical staining of CD206 with hematoxylin staining of nuclei. The lateral ventricular cavity is marked by an asterisk. **C**. Difference between $CD206^+$ counts in rats of different lineages, *p*-value < 0.01. The line inside the box plot represents the median (Me). The scale bar is 50 μm

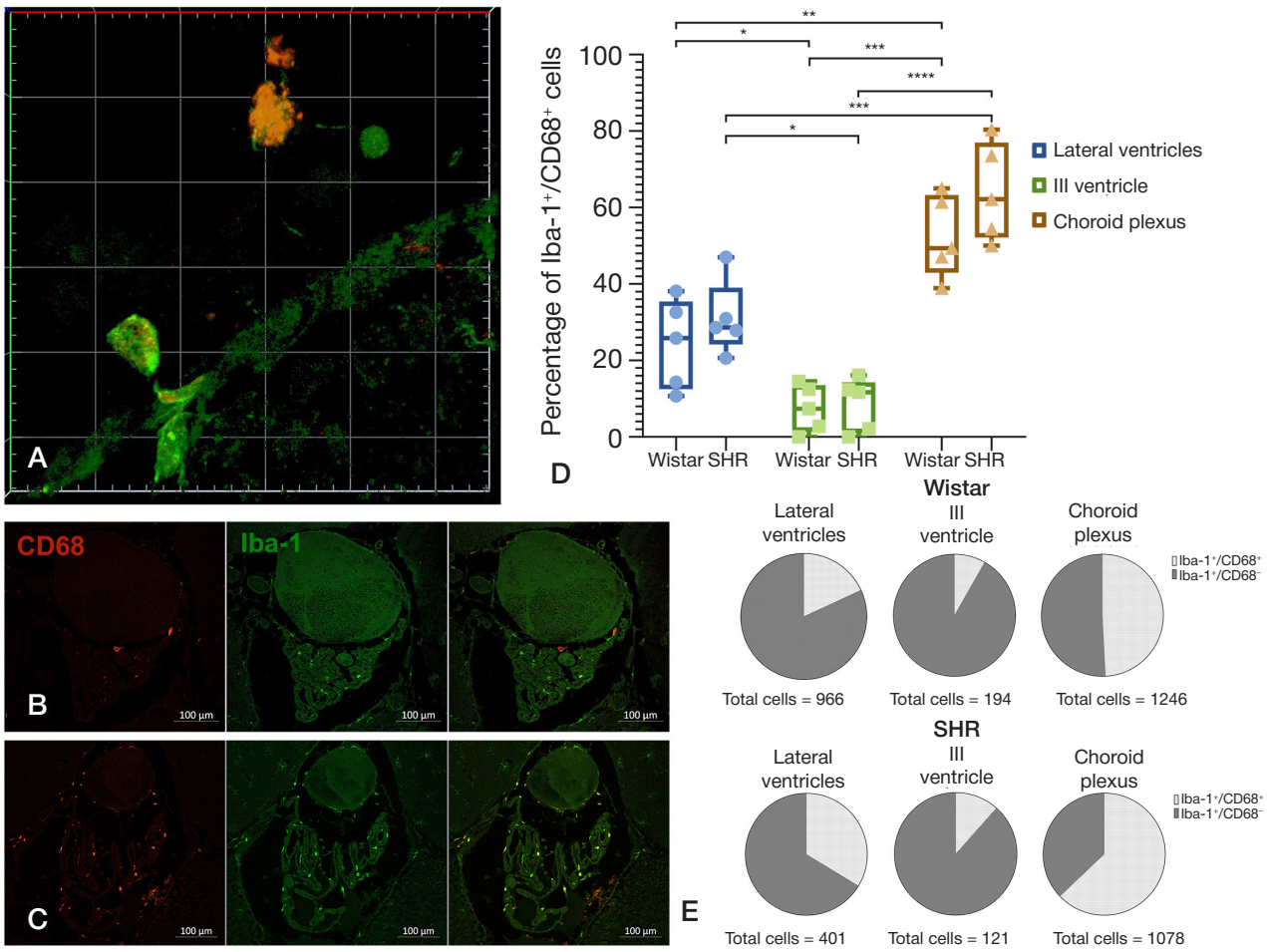

Fig. 2. Quantitative analysis results. Double immunohistochemical CD68 (*red channel*) and Iba-1 (*green channel*) stain. **A**–**C**. Overall view of subependymal microglia of the lateral **(A)** and third (**B**, **C**) ventricles. **A**. Subependymal and intraventricular microglia of SHR, 3D reconstruction of a series of optic slices, measuring grid cell size 10 × 10 μm, Wistar rats **(B)**, SHR **(C)**. **D**, **E**. Comparison results for the percentage of the cells staining positive for two markers, box plot **(D)** and pie chart **(E)**. *P*-value: * — < 0.05; ** — < 0.01; *** — < 0.001. The line inside the box plot represents the median (Me)

Iba-1$^+$/CD68$^+$ cells is observed in the choroid plexus. In the subependymal zone of the lateral and third ventricles, the content of double-immunopositive cells is not so high (Table 2). Quantification revealed no significant differences between the groups of normotensive and hypertensive rats ($F = 2.19$, $p = 0.16$). However, the data of the SHR sample are slightly displaced relative to the Wistar rat sample (Fig. 2D, E) toward the increase in the percentage of the cells staining positive for two markers.

The analysis of co-localization (Fig. 3) yielded the average correlation values for each area in both groups (Wistar rats and SHR; Table 3). In all the cases, the correlation criterion accepts the values that are enough for co-localization to be considered non-random (the criterion values are different from zero). However, no apparent displacement of values associated with arterial hypertension was reported (two-way ANOVA, $F = 0.56$, $p = 0.48$). Furthermore, there were also no significant differences in the level of correlation of the Iba-1 and CD68 revealed when analyzing different areas of interest (two-way ANOVA, $F = 3.45$, $p = 0.06$).

DISCUSSION

Macrophages play a special role in maintaining tissue homeostasis, neutralization of pathogens and aberrant cells, as well as initiation and modulation of adaptive immunity due to their phagocytic activity, capacity for efferocytosis and antigen presentation. The decades of research focused on physiological and cytochemical features of macrophages of various organs in inflammation made it possible to reveal two major functional states designated as M1 and M2 polarization types [22]. Later the scientific community gradually moved away from the rigid dichotomy concept and concluded that the macrophage functional states represented a wide range of phenotypes, from the pro-inflammatory M1 to the reparative M2 [23, 24]. Further division into subtypes made it possible to more accurately characterize the role of these cell populations in inflammation and develop individual therapeutic strategies for treatment of various disorders [25].

It is assumed that the brain's microglia shows similar polarization features during activation [26], which enables

Table 2. Percentage of Iba-1$^+$/CD68$^+$ cells in various brain areas of Wistar rats and SHR. The data are provided as the mean ± standard deviation

Area	Wistar	SHR
Subependymal zone of the lateral ventricles	24.31 ± 11.68	30.99 ± 9.727
Subependymal zone of the III ventricle	7.435 ± 6.178	8.442 ± 6.987
Choroid plexus	52.34 ± 10.72	64.09 ± 12.75

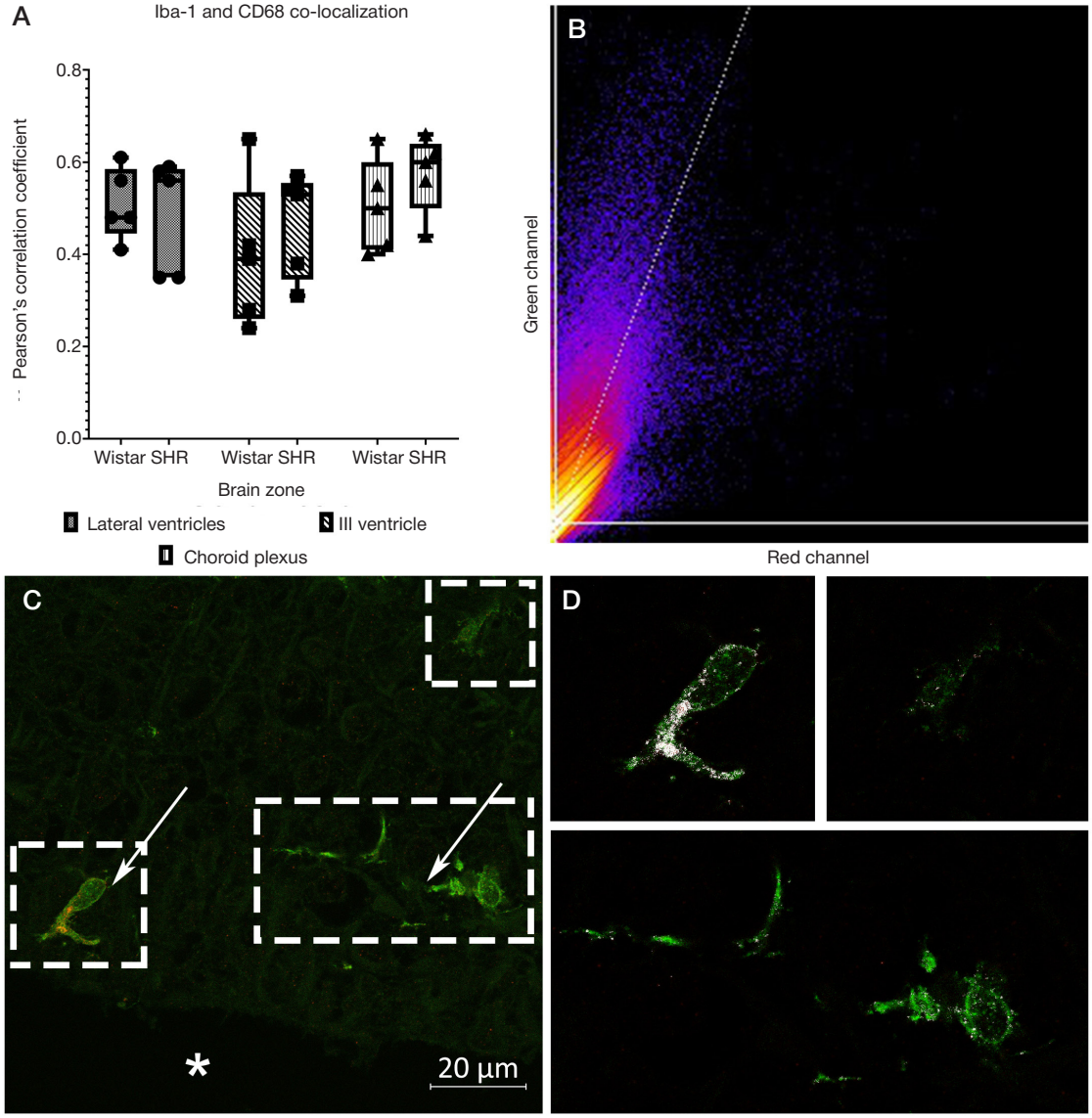

Fig. 3. Co-localization analysis results. Double immunohistochemical Iba-1 (*green channel*) and CD68 (*red channel*) stain. **A**. Pearson's correlation coefficients in various brain areas of Wistar rats and SHR. **B**. Correlation diagram of two channels. **C**. Microglia of the third ventricular floor. **D**. Co-localization of Iba-1 and CD68. Sites of marker co-localization are highlighted in *white* in Fig. **D**. Arrows point at microglia, *frames* show the areas presented in Fig. **D**, the ventricular cavity is marked by an *asterisk*. The line inside the box plot represents the median (Me)

the use of the existing paradigm for comparative studies and extrapolation of the results obtained for various organs. However, microgliocytes differ from other macrophages in both histogenesis and a number of structural characteristics [2, 27]. In this case, it is necessary to use a differentiated approach to assessment of microglia and macrophages considering their features.

The earlier reported study showed that microglia of the rats with genetically determined arterial hypertension could show increased phagocytic activity [11]. In this study, a hypothesis was tested about the possibility of M2a polarization of the brain's microglia in SHR.

It is well known that the activated M2a macrophages are capable of endocytosis, these stimulate cellular growth and tissue regeneration [25]. In laboratory rodents, the most typical M2a subtype marker proteins include CD206, Fizz1, Ym1/2, and arginase 1 [28]. The CD206 mannose receptor was selected for assessment of functional activity of the brain's immune cells based on the characteristics of the proposed markers [29] and the results of the screening immunohistochemistry testing involving the use of various antibodies.

The immunohistochemistry staining results obtained and subsequent quantification show a significant increase in $CD206^+$ cell number in SHR, which may indicate a shift towards M2a polarization type. This allows us to provide further directions in the field of immunophenotyping of microglia and macrophages of the SHR CNS, for example, by multiplex immunohistochemistry involving the use of the broad antibody panel against proteins specific for the M2a phenotype.

Table 3. Pearson's correlation coefficient in various brain areas of Wistar and SHR rats. The data are provided as the mean ± standard deviation

Area	Wistar	SHR
Subependymal zone of the lateral ventricles	0.51 ± 0.08	0.49 ± 0.12
Subependymal zone of the III ventricle	0.4 ± 0.16	0.47 ± 0.11
Choroid plexus	0.5 ± 0.1	0.58 ± 0.08

Identification of the role of macrophages in arterial hypertension attracts the researchers' attention. It is noted that SHR have the increased levels of intestinal CD11b$^+$ cells inhibiting the release of pro-inflammatory cytokines. However, the SHR levels of pro-inflammatory cytokines are also increased compared to the control group [30]. In contrast, it is noted that M1 macrophages predominate in the abdominal cavity by month 4, but the difference in the quantity of the M1- and M2-polarized cells becomes smaller as early as by month 6 [31]. The authors assume that macrophages of different organs will also acquire different immunophenotypes, i.e. M1 can predominate in one organ and M2 can predominate in another one. This can be due to the fact that tissue-resident macrophages can attract the immune cells arriving to the tissues from different sources, which, in turn, are influenced by the region-specific microenvironment. Apparently, in arterial hypertension, the nervous tissue microenvironment reinforces the need for at least CD206 expression increase and at most M2a polarization of the brain's macrophages.

However, localization of those is limited to perivascular spaces, pia mater, and the choroid plexus. The cells staining positive for CD206 specifically in the brain's nervous tissue were reported in none of the studied cases.

The lack of CD206 marker in dendritic microglia and the fact of finding CD206 in typical macrophages only can indicate differences in the expression profiles of activation markers in microgliocytes and macrophages. Nevertheless, the results of certain studies are likely to be in conflict with this assumption. In one of the recent reports focused on functional heterogeneity of the CNS glial cells, the authors admit the possibility of existence of the CD206$^+$ microglia population [13]. The original research results obtained by flow cytometry show that some human P2Y12$^+$ cells can have low CD206 protein levels [32]. Another study reported simultaneous CD32 and CD206 expression in microgliocytes under combined exposure to electromagnetic field and the TNFα neuroinflammation inducer [33]. The authors interpret the emergence of such a combination of markers in favor of tissue restoration in response to damaging effects. In rodents, the emergence of CD206$^+$ microglia was reported in spinal cord injuries [34] and during early postnatal development [35]. Considering the fact that the above studies involved cell cultures and the use of flow cytometry, these data are not strictly comparable with immunohistochemistry assessment results.

The presence of CD206 in the rat brain's microglia detected by immunohistochemistry was, for example, reported in the paper focused on the effect of quercetin on activation of the brain's immune cells [36]. Co-localization of Iba-1 and CD206 proteins is clearly visible in the brain sections acquired after the exposure to quercetin having anti-inflammatory and antioxidant effects and presumably contributing to type M2 microglia activation. However, in ischemic/reperfusion damage, the microglial response to antiCD206 antibodies was rather weak, and there was no immunohistochemical response in the control group. In contrast, in mice with the Alzheimer's disease-associated neuroinflammation [37], no CD206 and Iba-1 co-localization was revealed. In this regard, the authors conclude that the CD206$^+$ macrophages and Iba-1$^+$ microglial cells represent different populations. Furthermore, the use of antibodies against marker proteins of macrophages (such as CD206 and Iba-1) also does not give us all information about the origin of the cells found in brain sections. That is why it seems difficult to perfectly distinguish microglia, microglia-like cells, and macrophages that infiltrate the brain. Thus, the hypothesis about the possibility or impossibility of the CD206 mannose receptor expression by microglia in rats with various nervous system states needs further verification.

The quantitative analysis results obtained in this study allowed us to determine readiness for phagocytosis of microglia cells and macrophages based on the presence of functional lysosomes that are detectable due to the presence of macrosialin (CD68), the transmembrane glycoprotein of lysosomes and phagosomes [38].

When assessing co-localization of the Iba-1 and CD68 proteins, it was hypothesized that the Iba-1$^+$/CD68$^+$ microglia reported in the vicinity of brain barriers in SHR was in active state. The Pearson's correlation coefficient was selected to compare Iba-1 and CD68 protein co-localization levels due to the ease of interpretation of values. Its values vary from –1 to 1, where "–1" indicates a complete negative, "1" a complete positive, and "0" a random correlation [39]. In both Wistar rats and SHR, co-localization of proteins in all the studied zones was non-random (the spread in mean values was 0.4–0.6). However, the quantification performed suggests the lack of significant differences in Iba-1 and CD68 protein co-localization levels.

One more methodological approach involved quantitative assessment of the percentage of cells staining positive for two markers relative to the general population of Iba-1$^+$ cells in the choroid plexus, as well as near the ependyma of the lateral and third ventricles. It was noted that the largest percentage of Iba-1$^+$/CD68$^+$ cells was typical for the brain's choroid plexus, where a specific macrophage population (Kolmer cells) was localized [40]. The nature of these cells is still a matter of debate, but their function is associated with active phagocytosis. The least percentage of double immunopositive cells is reported for the subependymal zone of the third ventricle in the area of hypothalamus, which is likely to result from the presence of specific glial cells (tanycytes) in the ependyma. Tanycytes ensure bidirectional transport of bioactive molecules between cerebrospinal fluid and blood [41]. The subependymal zone of the lateral ventricles, which does not have such a lining, occupies an intermediate position.

Despite the fact that the analysis of variance revealed no significant differences between the percentage of cells staining positive for Iba-1 and CD68 in different rat strain, the analysis of descriptive statistics shows that the data of the SHR sample are displaced relative to the Wistar rat sample towards the increase in the counts of such cells. In particular, the median, as well as minimum and maximum values can be higher (Fig. 2). The findings suggest heterogeneity of the studied SHR group, regardless of the selection performed based on blood pressure. Larger samples should be used for further research.

The data obtained suggest that rats with arterial hypertension can show activation of phagocytic activity of microglia and macrophages in the zones of the blood-brain, blood-cerebrospinal fluid, and cerebrospinal fluid-brain barriers. Furthermore, considering the co-localization analysis results, it is necessary to emphasize that it is probably the number, but not the functional activity level of cells that changes.

CONCLUSIONS

Neuroinflammation caused by arterial hypertension in SHR results in polarization shift towards M2 variant in macrophages of the pia mater, choroid plexus, and perivascular spaces. The population affiliation of the brain's CD206$^+$ cells remains unclear and should be clarified through further research using double immunohistochemical labeling. Activation of microglia and macrophages in SHR is apparently not accompanied by the

increase in the phagocytic activity of these cells. The identified trend towards the increase in the percentage of Iba-1$^+$/CD68$^+$ cells in the SHR brain compared to the Wistar rat brain can result from intrapopulation differences; it can also be a sign of the increase in the percentage of active phagocytes. Further research is required to test these hypetheses.

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