

## Article

# Morphofunctional changes in brain and peripheral blood in aged Wistar rats due to AlCl<sub>3</sub> exposure

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**Abstract:** The purpose of the study: To examine morphofunctional changes of brain and peripheral blood in aged Wistar rats to observe adaptive reactions as a response on AlCl<sub>3</sub> exposure.

**Methods:** The work was performed on male Wistar rats, 24 months of age. Animals consumed a solution of AlCl<sub>3</sub> 100 mg/kg per day for 60 days. Morphological changes of neurons and microglia, mRNA expression levels of pro- and anti-inflammatory cytokines, microglia activation markers, amyloid-related and hypoxia-related proteins, as well as monocyte and lymphocyte subpopulations in peripheral blood, were examined.

**Results:** AlCl<sub>3</sub>-treated old rats showed the increasing of hyperchromic neurons in 2 out of 3 examined regions of the hippocampus; morphological features of microglia cells' dystrophy; the upregulation of pro-inflammatory cytokine Il-18 and the downregulation of anti-inflammatory cytokine Il-10, as well as App, Bace1, and Hif-1a; the decreasing of percentage of B-cells, general CD3+ lymphocyte population, including CD4+ T-helpers and CD8+ cytotoxic cells, but the increasing of CD4+/CD8+ ratio in peripheral blood.

**Conclusion:** AlCl<sub>3</sub>-treated aged rats demonstrated systemic maladaptation to AlCl<sub>3</sub> impact. Unlike adult rodents, aged ones have the background of inflammaging, as well as elderly people. The exposure of AlCl<sub>3</sub> could potentially be a replacement of integral cellular and molecular processes accompanying age-related diseases, presenting in most elderly people, as an enhancer of inflammaging and hypoxia. These conditions make this model of neurodegeneration a reliable one to explore the initial mechanisms of such detrimental process, as well as prove aged rats more suitable subjects to perform future researches in this field.

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## 1. Introduction

The stable growing of world's population as well as average lifespan, especially in developed countries, leads to the increasing of age-related diseases' incidence and prevalence, including ones leading to dementia. It is the most prevalent cause of disability globally [1] and its most common cause is neurodegenerative diseases such as Alzheimer's disease (AD). There were more than 55 million patients with dementia worldwide in 2019 [1], and this number will triple in next 30 years. It is a great burden for not only patients, their families, and healthcare workers. It also demands an annual global cost of just over 1 trillion USD on treatment and social support [1]. Among other reasons, this amount of costs is due the absence of any effective treatment for dementia. Existing and approved drugs can only faintly and briefly slow down the symptoms.

The development of new effective treatment approaches is hampered by insufficient data concerning the initial mechanisms of AD pathogenesis. Recent studies showed that it is quite complex process not limited by amyloid deposits alone [2], [3], and 3rd part clinical trial of Lecanemab, an amyloid-antibody based drug, showed questionable efficacy [4]. The role of inflammaging, or chronic age-related low-grade systemic inflammation is one of the most perspective and intensively studying hypotheses of neurodegeneration's initiation so far. It represents a manifestation of senescence-associated secretory phenotype (SASP), which is expressed by senescent cells of aged organisms [5]. Inflammaging is one of risk factors of other age-related diseases development



as well, including advanced stage of atherosclerosis, type 2 diabetes mellitus, metabolic syndrome, etc. At the same time, these very pathological conditions as well as some others, like major depressive disorder [6], may contribute in enhancing of its pro-inflammatory background.

Sporadic form of AD, also known as late onset AD (LOAD), begins to manifest with mild cognitive impairment in people of age >60-65 years [7] and belongs to the group of age-related pathologies. However, its modeling is still being conducted mostly on adult rodents, and lots of studies are performed on various lines of transgenic mice, which pathological processes do not exactly correspond with ones leading to neurodegeneration on humans. Among vast variations of AD animal models there are one based on exposure of aluminum compounds. Al<sup>3+</sup> ions are capable of increasing the production of reactive oxygen species (ROS). They involve in mitochondrial and DNA damage as well as promoting of pro-inflammatory mediators production and hypoxic condition establishing [8], [9]. All these events are typical for aging as well, which means that experiments on old animals can provide more relevant data due to the cellular senescence presence. Hence, the purpose of this work was to study morphofunctional changes in brain and peripheral blood in aged Wistar rats to observe their adaptive reactions on AlCl<sub>3</sub> exposure.

## 2. Materials and Methods

### 2.1. Animals and neurodegeneration modelling

The work was performed on aged male Wistar rats 24 months old (n=20). Animals were divided randomly on 2 groups, 10 animals each. Animals were kept in plastic cages (60 x 38 x 18.5 cm) in social groups of 5 animals each with free access to food and water. The temperature in the vivarium room was maintained within 18-22°C, and air humidity was 50-65%. The study was approved by the Bioethical Commission of the Avtsyn Research Institute of Human Morphology of "Petrovsky National Research Centre of Surgery" (Protocol №36 (12) March 28, 2022). All experimental work involving animals was carried out according to directive 2010/63/EU of the European Parliament and of the Council of the EU on the protection of animals used for scientific purposes (Strasbourg, September 22, 2010).

Rats of the experimental group consumed aluminum chloride (AlCl<sub>3</sub>) in dosage of 100 mg/kg per day for 60 days with drinking water, as described before [10]. Animals of the control group consumed regular drinking water.

### 2.2. Samples obtaining and histological preparations

On the 61st day of the experiment, samples of peripheral blood were obtained under Zoletil (Vibrac Sante Animale) anaesthesia, then animals were euthanized by overdose (15 mg/kg) of Zoletil. The whole brains were fixed in 10% buffered formalin (BioVitrum, Russia) for 24 hours, then dissected at the level of 6.0 mm posterior relative to bregma (each sample of 5 mm thick) [11]. After that, the specimens were dehydrated with ethanol of ascending concentration, cleared with xylene, infiltrated with a histological wax, and embedded in paraffin blocks for further slicing (5 µm thick).

### 2.3. Morphological study

For morphological study, histological sections of brains were stained according to Nissl's method. The absolute number of neurons in the standard area of the visual field (25000 µm<sup>2</sup>) and the relative number of hyperchromic and morphologically altered neurons were evaluated on these sections in zones CA1, CA3, and the dentate gyrus of the hippocampus. The pictures were captured with the Leica microscope (DM 2500 Leica Microsystems) on magnification x400.

### 2.4. Immunohistochemical study

For ICH-P study, frontal histological sections of brains (6.0 mm posterior relative to bregma) were prepared as previously described [9]. Then they were stained with rabbit primary antibodies Iba1 (1:100; P4C288Ra01, Cloud Clone) and secondary HRP Donkey-anti-Rabbit antibody (1:500; 416035, Novex Life Technologies) with additional hematoxylin staining. The pictures were captured with the Leica microscope (DM 2500 Leica Microsystems) on magnification x1600.

### 2.5. qPCR-RT study

The expression mRNA was assayed by real-time qPCR in tissue fragments of the prefrontal cortex, preserved in IntactRNA solution (Eurogen, Russia) and stored on -20C until studied. The performed analysis included the detection and evaluation of expression levels of pro-inflammatory cytokines (Il-6, Il-18, and Tnf-α), anti-inflammatory cytokines (Il-10 and Tgf-β), microglia M1 (iNos) and M2 (Cd163+) activation markers, amyloid-related proteins (App and Bace1) and hy-



poxia marker Hif-1a. The levels of all aforementioned mRNA expression relative to GAPDH expression level as a reference were determined using a qPCRmix-HS SYBR (Eurogen, Russia) containing fluorescent intercalating dye SYBR Green I. Amplification with detection and digital analysis of fluorescence in real time was carried out on DT-96 Real-Time PCR Cycler (DNA-Technology JSC, Moscow, Russia) in a standard mode at 95°C for 5 minutes followed by 95°C for 15 seconds, 62°C for 10 seconds + reading and 72°C for 20 seconds × 45.

**Table 1.** Used primers' sequences (all picked up by on-line soft Primer-BLAST precisely for rat specie).

Primer	Forward sequence	Reverse sequence
GAPDH	GCGAGATCCCCTAACATCA	CCCTTCCACGATGCCAAAGT
IL-18	GACAAAAGAAACCCGCCTG	ACATCCTTCCATCCTTCACAG
TNF- $\alpha$	CCACCACGCTCTTCTGTCTA	GCTACGGGCTTGCTACTCG
IL-10	GCCCAGAAATCAAGGAGCAT	TGAGTGTACAGTAGGCTTCTA
TGF- $\beta$	CCGCAACAACGCAATCTATG	AGCCCTGTATTCCGTCTCCTT
iNOS	CGCTGGTTTGAACTTCTCAG	GGCAAGCCATGTCTGTGAC
CD163	TCTTGTGGACTCTGAAGCGA	TCTTAAATGCCAACCCGAGG
APP	TGGATGATCTCCAACCGTG	CGTCGACAGGCTCAACTTC
BACE1	GGGCAGTAGTAATTTTGCAGT	TTCGGAGGTCTCGGTATGT
HIF-1a	TCACAGTCGGACAACCTCAC	TGCTGCAGTAACGTTCCAATTC

## 2.6. Flow cytometry

The relative numbers of lymphocytes various subpopulations and monocyte were counted using flow cytometry (Beckman Coulter, USA) in peripheral blood. The following antibodies (eBioscience, USA) were used for immune phenotypic analysis: anti-rat CD3-PE for total T-lymphocyte population, anti-rat CD4-FITC for CD3+CD4+ T-helpers, anti-rat CD8-PE-Cy5 for CD3+CD8+ for T-cytotoxic cells, anti-rat CD45R-FITC for CD45R+ B-cells, and anti-rat CD43-PE for CD43+ monocyte. Erythrocytes were lysed with the OptiLyse C solution (eBioscience, USA).

## 2.7. Statistical analysis.

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 Mann-Whitney test was used to establish the reliability of differences between groups by median. With  $p < 0.05$ , it was considered as statistically significant.

## 3. Results

### 3.1. The percentage of altered hyperchromic neurons

The absolute numbers of neurons in zones CA1, CA3 and the dentate gyrus of the hippocampus were almost the same in both groups, whereas the relative numbers of altered hyperchromic neurons differed significantly. Rats that consumed AlCl<sub>3</sub> have shown 2.1-fold number of hyperchromic neurons in CA3 hippocampal zone and 1.7-fold one in the dentate gyrus compared with control group animals (Fig. 1a,b).



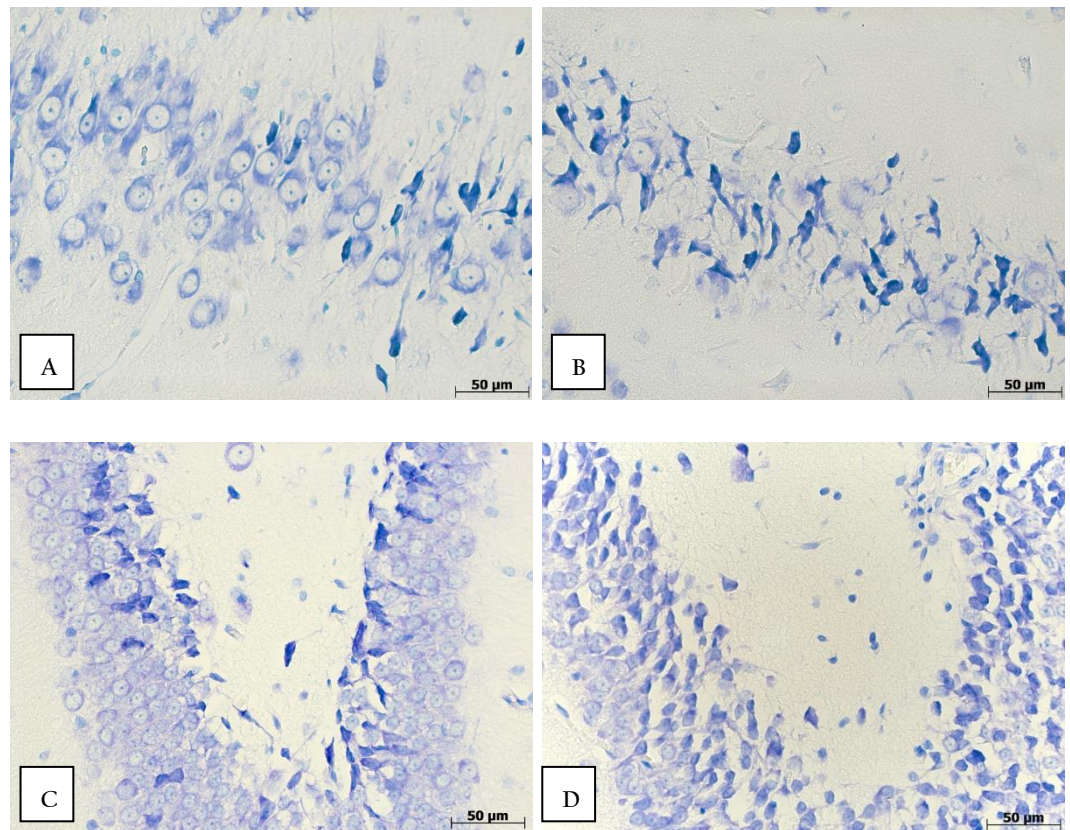


Figure 1a. CA3 zone(A, B) and the dentate gyrus (C, D) of the hippocampus in rats of control (A, C) and experimental (B, D) groups. Nissl's staining, x400.

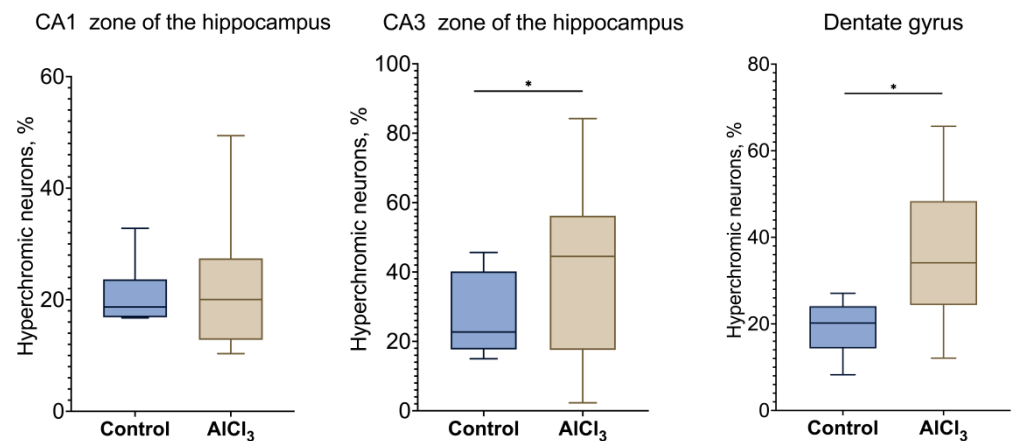


Figure 1b. The percentage of hyperchromic neurons in zones CA1, CA3, and the dentate gyrus of the hippocampus in rats of control and experimental groups.

The data displayed as: line - median, box - 25-75 quartiles, whiskers - non-outlier range;

\* -  $p < 0.05$ . The Mann-Whitney test comparisons.

### 3.2. Morphological features of microglia

Identified by ICH staining with anti-Iba1 antibody, in rats of control group microglia cells had an increased size ( $>30 \mu\text{m}$  with a reference of  $15-30 \mu\text{m}$ ) and spheroidal swelling, hypertrophic, beaded, and tortuous processes. At the same time, there were microglia of an increased size concurrently with beaded, tortuous, and fragmented, but not thickened processes in group of AlCl<sub>3</sub>-consumed rats (Fig. 2).



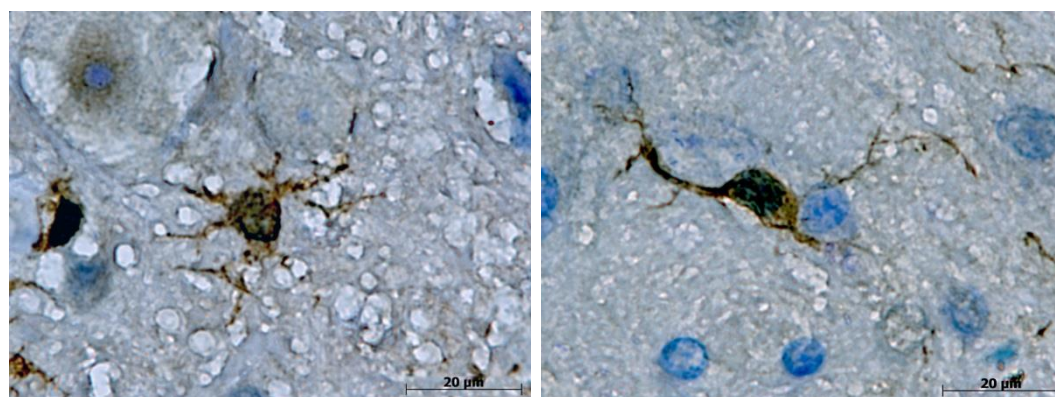


Figure 2. Morphological characteristics of microglia cells with thin and short processes in both Adult-C (A) and Adult-A $\beta$ 13 (B) groups, enlarged microglia with spheroidal swelling, hypertrophic, beaded, and tortuous processes in Old-C rats (C), and microglia of an increased size with beaded, tortuous, and fragmented, but not thickened processes in Old-A $\beta$ 13 rats (D). Iba-1 antibody + HRP secondary antibody IHC and hematoxylin staining, x1600.

### 3.3. qPCR-RT examination of the prefrontal cortex

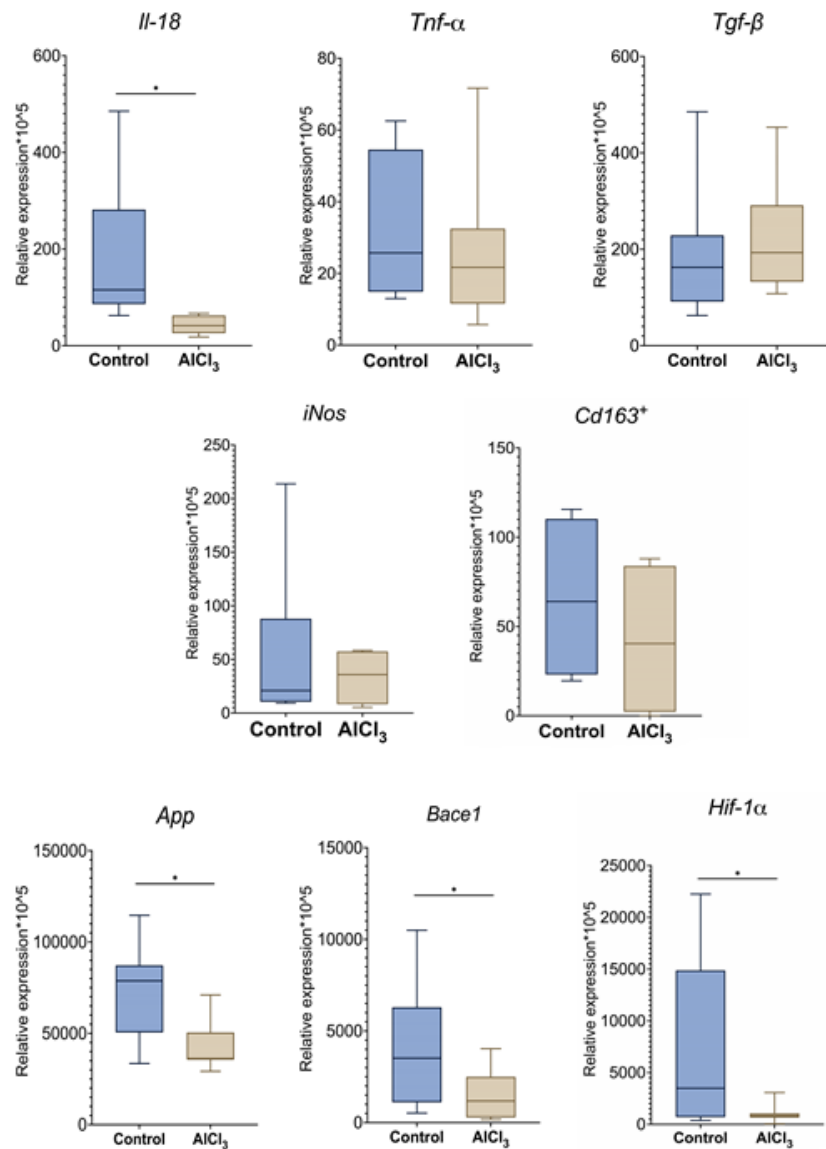
The result of qPCR-RT of prefrontal cortex tissue fragments demonstrated the statistically significant difference between groups due to Il-18 expression level – it was in 2.8 times less in experimental group rats than in control ones. However, no reliable distinctions were detected in pro-inflammatory cytokine Tnf- $\alpha$  expression levels, as well as in anti-inflammatory Tgf- $\beta$  ones (Fig. 3). The expression of anti-inflammatory cytokine Il-10 was not detected at all.

The levels of expression of M1 activated microglia marker iNos and M2 activated microglia marker Cd163 were also similar in both groups despite the statistically insignificant tendencies of upregulation of iNos expression and downregulation of Cd163+ one in rats of experimental groups relative to control rodents (Fig. 3).

At the same time, amyloid precursor protein (App) expression was 1.8 times less in rats of experimental group than in control one. Like App, Beta-site APP-cleaving enzyme 1 (Bace1) expression changed in similar way and was downregulated in 2.4 times in rats consumed A $\beta$ 13 compared with control group animals. Also, hypoxia-inducible factor 1-alpha (Hif-1a), which displays a presence of a hypoxic condition always appearing alongside inflammation, was downregulated in 4.2 times in experimental group animals in comparison with control ones (Fig. 3).







**Figure 3.** mRNA expression levels of pro-inflammatory cytokines Il-18 and Tnf- $\alpha$  and anti-inflammatory cytokine Tgf- $\beta$ , microglia activation markers iNos (M1) and Cd163<sup>+</sup> (M2), App, Bace1, and Hif-1 $\alpha$  in the prefrontal cortex in rats of control and experimental groups.

The data displayed as: line – median, box – 25-75 quartiles, whiskers – non-outlier range;

\* - p<0.05. The Mann-Whitney test comparisons.

### 3.4. Flow cytometry immune phenotypic analysis

The immune phenotypic analysis of lymphocyte subpopulations and monocyte was performed to estimate the impact of AlCl<sub>3</sub> on the relative numbers of various immune cells in peripheral blood. Flow cytometry data demonstrated statistically significant differences in the percentage of general CD3<sup>+</sup> lymphocyte population as well as CD45R<sup>+</sup> B-cells, but not CD43<sup>+</sup> monocyte between the observed groups. As for lymphocyte subpopulations, both CD3<sup>+</sup>CD4<sup>+</sup> T-helpers and CD3<sup>+</sup>CD8<sup>+</sup> T-cytotoxic cells decreased in AlCl<sub>3</sub>-treated rats comparing with control ones, whereas CD4<sup>+</sup>/CD8<sup>+</sup> ratio displayed that the proportion of CD4<sup>+</sup> T lymphocyte increased in experimental group rats (Fig. 4).



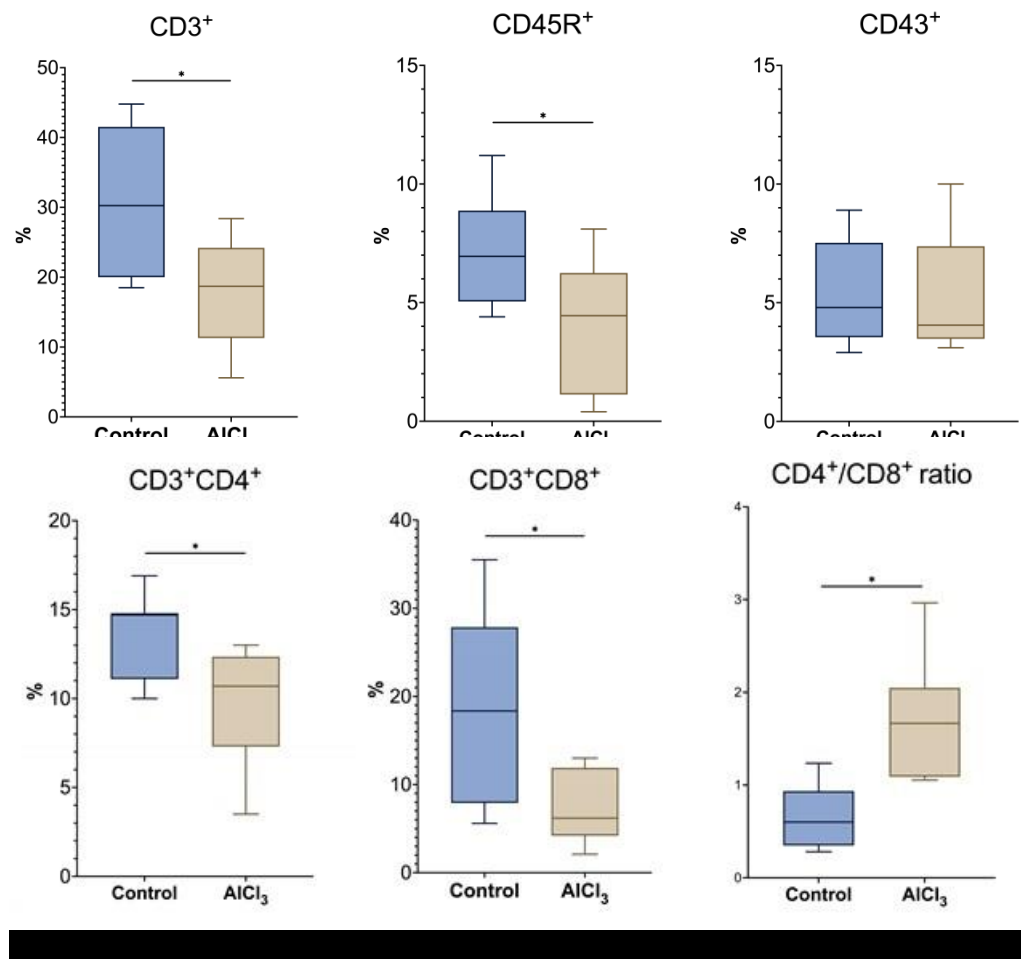


Figure 4. The percentage of general CD3<sup>+</sup> T-lymphocyte population, CD45R<sup>+</sup> B-cells, and CD43<sup>+</sup> monocyte, CD3<sup>+</sup>CD4<sup>+</sup> T-helpers, CD3<sup>+</sup>CD8<sup>+</sup> T-cytotoxic cells, and CD4<sup>+</sup>/CD8<sup>+</sup> ratio in peripheral blood in rats of control and experimental groups.

The data displayed as: line – median, box – 25-75 quartiles, whiskers – non-outlier range;

\* - p<0.05. The Mann-Whitney test comparisons.

#### 4. Discussion

The main hallmark of aging is cellular senescence manifesting in the increasing number of SASP-expressed cells. According to our data [15], aged Wistar rats of control group displayed it the same way as it appeared in humans [5]. It makes aged rats a more reliable subject to perform neurodegeneration modelling due to the presence of pro-inflammatory background of inflammaging, which is absent in adult rodents, including transgenic ones. AICl<sub>3</sub>-based models of AD are described widely in literature, including 100 mg/kg dosage giving orally [10], [12], [13], [14], and all these researches were conducted on adult rats and mice. In our previous study, we determined several pivotal morphofunctional distinctions in microglia and immune cells of peripheral blood between groups of adult and old Wistar rats without any external exposure [15]. Now the purpose was to determine and evaluate the response of old rodents on AICl<sub>3</sub>.

We detected a notable increasing of the relative number of hyperchromic neurons in CA3 zone and the dentate gyrus of the hippocampus in aged AICl<sub>3</sub>-treated rats relative to control ones. We counted mostly morphologically altered neurons of more intense dying, as well as lesser size and polygonal shape, shrunken, and wrinkled. Apparently, neurons with such morphological alterations have ceased to function due to their extinction of interaction with other ones [16], [17]. The reason of more pronounced neuronal damage observed in CA3 zone and the dentate gyrus in comparison with CA1 zone could be probably explained by their different susceptibility to hypoxia. CA1 neurons are presumably more vulnerable to hypoxia and oxygen-glucose deprivation than CA3 and DG [18] probably due to their higher activity [19]. CA1 zone's prime function is the maintaining of short-term memories, whereas CA3 is mostly involved in establishing of spatial and



contextual memory [18]. The data concerning the tolerance to hypoxia changing with aging are controversial, although it might increase in advanced age as a result of adaptation processes [20]. However, in case of this particular study, AlCl<sub>3</sub> more likely had a direct toxic impact on neurons throughout mitochondrial damage rather than aggravating hypoxic condition.

Unlike aged rats of control group, microglia cells displayed signs of dystrophy in old AlCl<sub>3</sub>-treated rats instead of activation and/or hypertrophy features. Similar findings were described by Shahidehpour et al. in elder humans, precisely patients with neurodegenerative diseases [21]. Highly likely, it is evidence of their maladaptation due to AlCl<sub>3</sub> harmful impact and self-maintaining resources critical shortage in the same way as it occurred in neurons. Such cells are no longer capable of producing enough amounts of export proteins, which are vital for proper functioning, such as immune surveillance and synapse clearance.

The data from experiments conducted on adult AlCl<sub>3</sub>-treated rats demonstrated upregulation of pro-inflammatory mediator Tnf- $\alpha$  [22], [23] It was also detected in healthy aged rats in comparison with healthy adult ones, whereas the expression level of anti-inflammatory cytokine Tgf- $\beta$  did the opposite [15]. Meanwhile, we observed no differences in the expression levels of Tnf- $\alpha$  and Tgf- $\beta$ , as well as in ones of M1 microglia activation marker iNos and M2 microglia activation marker Cd163+ between experimental and control groups. It could probably be explained by dystrophic state of microglia cells, which are both reached the upregulation limits and unable to adapt to malevolent environmental conditions any longer. At the same time, the pronounced downregulation of Il-18 might be caused by neuronal hibernation and death, since they are another source of this mediator [24], [25].

Our data confirms that App and Bace1 expression levels and, therefore, these proteins' subsequent biosynthesis decreased greatly in AlCl<sub>3</sub>-treated rats relative to control group. It probably displays the advanced stage of neurodegenerative detrimental processes, when neurons are no longer capable of producing APP to form new synapses or maintain deteriorating ones. Therefore, BACE1, which is an enzyme involved in its metabolism, is no longer needed either.

Currently, the data concerning the variability of Hif-1 $\alpha$  during the ontogenesis and its impact on age-related genes expression are scarce and controversial. It probably depends on individual hypoxia tolerance and could be both up- or downregulated with aging [20]. However, significant downregulation of Hif-1 $\alpha$  in AlCl<sub>3</sub>-treated rats compared with control ones is likely caused by dystrophy and death of neuron and glial cells as a result of maladaptation to AlCl<sub>3</sub> toxic effect.

We observed a statistically significant decline of general CD3+T-lymphocyte population including CD3+CD4+ T-helpers and CD3+CD8+ T-cytotoxic cells in AlCl<sub>3</sub>-treated rats comparing with control ones. Previously, no difference in there percentage was determined in Wistar rats due to aging alone [15], whereas Zhuang et al. observed its decrease under AlCl<sub>3</sub> exposure [26]. However, previous reports [8],[26], [27] observed a decline of CD4+/CD8+ ratio as well, while our data displayed that the proportion of CD4+ T-helpers were higher in AlCl<sub>3</sub>-treated rats relative to control group. Such discrepancy probably might have occurred because our experiment was performed on aged rats, whilst previous ones were conducted on adult rodents. Hence, differences in T-lymphocyte population's reactions could vary greatly with aging, but future investigations are warranted.

Unlike relative number of CD3+T-lymphocyte, the decrease of CD45R+ B-cells and CD43+ monocyte percentage was detected in aged humans and rats relative to adult ones [15], [28], [29]. The cause of CD45R+ B-lymphocyte percentage was abated is probably the same as in case of general CD3+T-lymphocyte population, and it is the direct toxic effect of AlCl<sub>3</sub>. As for CD43+ monocyte, they probably might as express higher resistance to AlCl<sub>3</sub> impact; at the same time, they could also decrease both their percentage in peripheral blood and the rate of tissue migration due to inauspicious conditions caused by AlCl<sub>3</sub>.

## 5. Conclusions

In regard with all reported data, AlCl<sub>3</sub>-treated aged rats demonstrated systemic maladaptation to external exposure of AlCl<sub>3</sub>. With its capability of oxidative stress enhancing, AlCl<sub>3</sub> leads to progressive shortage of cell resources and subsequent decline of their functions, already compromised by cellular senescence itself. Unlike adult rodents, aged ones have the background of inflammaging, as elderly people do. Besides, most elderly people have some non-infectious chronic diseases including age-related ones, such as atherosclerosis, obesity, type 2 diabetes mellitus, etc., whereas rodents do not have them. The exposure of AlCl<sub>3</sub> could potentially be a replacement of integral cellular and molecular processes accompanying these diseases as an enhancer of inflammaging and hypoxia. These conditions make this model of neurodegeneration a reliable one to explore the initial mechanisms of such detrimental process, as well as prove aged rats more suitable subjects to perform future researches in this field.

**Author Contributions:** Conceptualization A.S. and A.K.; methodology, A.K.; software, A.S., E.M. and I.T.; validation, A.S., E.M. and A.K.; formal analysis, A.S. and A.K.; investigation, A.S., E.M., I.T. and A.K.; resources, A.K.; data curation, A.S., E.M. and A.K.; writing—original draft preparation, A.S. and A.K.; writing—review





and editing, A.S., E.M., I.T. and A.K.; visualization, A.S. and I.T.; supervision, A.K.; project administration, A.K. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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