

# Morphological evidence of the beneficial role of immune system cells in a rat model of surgical brain injury

Małgorzata Frontczak-Baniewicz<sup>1\*</sup>, Dorota Sulejczak<sup>2\*</sup>, Jarosław Andrychowski<sup>3,4</sup>, Magdalena Gewartowska<sup>5</sup>, Milena Laure-Kamionowska<sup>6</sup>, Wojciech Kozłowski<sup>7</sup>

<sup>1</sup>Electron Microscopy Platform, Mossakowski Medical Research Centre, Polish Academy of Sciences, <sup>2</sup>Department of Experimental Pharmacology, Mossakowski Medical Research Centre, Polish Academy of Sciences, <sup>3</sup>Department of Neurosurgery, Medical University of Warsaw Second Faculty of Medicine, Bielanski Hospital, <sup>4</sup>Department of Neurosurgery, Mossakowski Medical Research Centre, Polish Academy of Sciences, <sup>5</sup>Department of Human Epigenetics, Mossakowski Medical Research Centre, Polish Academy of Sciences, <sup>6</sup>Department of Experimental and Clinical Neuropathology, Mossakowski Medical Research Centre, Polish Academy of Sciences, <sup>7</sup>Department of Pathomorphology, Military Institute of Medicine, Warsaw, Poland

\*These authors contributed equally to this work.

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## Abstract

*The blood-brain barrier prevents infiltration of peripheral immunocompetent cells into the CNS under physiological conditions. Following brain trauma there is reported a rapid and massive immunological response. Our earlier data indicated that surgical brain injury causes breaking of brain parenchyma integrity and results in cell changes and death, astrogliosis and disruption of blood vessels. The aim of the present studies was to investigate and characterize immunocompetent cells entering brain damaged parenchyma in the early period following the injury in a rat model of surgical damage. In the investigations we used light and electron microscopy techniques. Four days following the lesion many monocytes and macrophages were detected in the injured parenchyma. We also found many activated microglial cells with phagosomes within the cytoplasm. The phagocytes digest the cellular debris and clean up the parenchyma. The data suggest the beneficial role of immunocompetent cells following surgical injury.*

**Key words:** surgical brain injury, brain repair, macrophages, microglia, monocytes, ultrastructure.

## Introduction

The blood brain barrier (BBB) is impermeable to cells of the immune system under physiological condition, but it becomes leaky when breached following injury [18]. Traumatic brain injury (TBI) induces several pathological features, including the disruption of the BBB with entry of systemic inflammatory cells and circulating blood proteins into the brain

parenchyma [4,8,9,12,28,33]. Peripherally derived mononuclear phagocytes, glial cells which produce and secrete cytokines, may all contribute to CNS repair. The surviving resident brain cells (for example microglia) are not typical immune cells, but contribute to the security of the brain through the expression of the innate immune response, promoting the clearance of neurotoxic proteins and apoptotic cells

## Communicating author:

Dorota Sulejczak, Department of Experimental Pharmacology, Mossakowski Medical Research Centre, Polish Academy of Sciences, 5 Pawinskiego St., 02-106 Warsaw, Poland, phone: +48 22 608 65 81, fax: +48 22 608 65 90, e-mail: dsulejczak@imdik.pan.pl

from the brain parenchyma. This stimulates both tissue repair and the fast restoration of tissue homeostasis [12,19,22,26]. It is known that TBI rapidly leads to activation of microglia, macrophages, and neutrophils, and to local release of inflammatory cytokines [11,14,23,24,34]. However, following the initial trauma, immune system cells can expand brain damage [23]. It follows that these cells possess the potential for both benefit and harm. While inflammatory mechanisms may be required for wound sterilization, the response can extend neuronal cell death and impair recovery.

Monocytes, a type of white blood cell, play multiple roles in immune function. In response to inflammatory signals monocytes can move quickly to sites of infection in the tissues and divide or differentiate into macrophages and dendritic cells. Monocytes can also replace resident macrophages and dendritic cells under normal states.

Macrophages, phagocytes derived from monocytes, mesenchymal stem cells and glial cells can move by amoeboid movement [20]. Macrophages are present in all living tissues and act in non-specific innate immunity as well as initiate specific adaptive immunity. Their role is to phagocytose and digest cellular debris and pathogens. They also stimulate lymphocytes and other immune cells to respond to pathogens. Macrophages also attack external substances, microbes and cancer cells through destruction and ingestion. They participate in regeneration.

Macrophages have previously been studied in models of CNS injury including experimental autoimmune encephalitis (EAE), ischaemic stroke, and spinal cord injury as well as TBI, and still it is not clear whether macrophages are overall harmful or beneficial to the brain. A negative role for macrophages has been found in most neuroimmunological studies [1,6,7,15,17,22,25]. However, the inflammatory response is also important for clearing necrotic debris and for wound repair [5]. Shechter *et al.* proved in a model of spinal cord injury that these cells suppress inflammation and were critical for recovery [27] and Weber *et al.* confirmed the thesis in EAE [32]. These differing roles for macrophages may reflect different functional states of macrophage activation.

Microglia, small cells resident in the central nervous system, may act as phagocytes in regions of tissue damage or inflammation. They have several forms and slender elongated processes. Microglial cells are activated following injury/disease and migrate by amoeboid movement into the damaged region. They

act as macrophages, clearing cellular debris and dead neurons.

Our experimental rat model of surgical brain injury (SBI) imitates well the respective human neurosurgery situations which take place during life-saving procedures (e.g. clipping of an aneurysm or removal of a brain tumour) and are connected with injury of the neighbouring region of brain parenchyma. In our previous studies we characterized the phenomena taking place in the cerebral cortex after SBI, with particular consideration of cell ultrastructure and immunophenotypes as well as long-term consequences of this procedure. SBI causes massive neuronal death and intense micro- and astrogliosis with increased immunoreactivity for glial fibrillary acidic protein (GFAP) and morphological changes in astrocytes characterized by hypertrophy of cell bodies and *clasmotodendrosis*. The gliosis was detected in the region surrounding the wound. We also detected that injury results in BBB breakdown [8].

The aim of the present study was to characterize the cells entering the injured brain cortical region.

## Material and methods

All applied procedures involving animals were authorized by the 4<sup>th</sup> Local Animal Experimentation Ethics Committee and were compatible with European Union regulations concerning the care and use of laboratory animals. Animals were kept in precisely specified and controlled conditions: in 60-70% relative humidity and temperature of 20°C in a 12 h/12 h light/dark cycle (lights on at 7 a.m.), and were fed on standard food for laboratory rodents (Ssniff M-Z, ssniff Spezialdiäten GmbH, Soest, Germany) and purified tap water *ad libitum*. Experiments were carried out on adult Wistar male rats, weighing at the beginning of the experiment 200-250 g. Animals were divided into three experimental groups:

- 1) control, intact animals (sacrificed after 4 days of the experiment; 7 animals),
- 2) animals that received a surgical injury of the cerebral cortex (sacrificed at 4 days following the lesion; 7 animals),
- 3) sham-operated animals (the full procedure of skull trepanation was conducted) (sacrificed at 4 days following the lesion; 7 animals).

Neurosurgical damage of the cerebral cortex was performed according to the procedure described previously [8]. Briefly, the operation of surgical inju-

ry of the cerebral cortex was performed under deep anaesthesia (20 mg/kg ketamine hydrochloride). After the skin was incised the frontal bone was trepanned 2 mm laterally from the bregma and 2 mm anteriorly to the coronal suture and the meninges were incised. The operated cortex (a 2 × 2 × 2 mm region of the somatosensory cortex) was hemisected with a small scalpel and the wound was closed. The skin wound was sutured. After the operation the animals were placed in standard animal house conditions under the care of an experimenter.

Past above specified survival times the animals were perfused and material collected from their brains was evaluated using techniques of light and electron microscopy (see below).

All animals were deeply anaesthetized using Nembutal (80 mg/kg b.w., i.p.) and perfused by the ascending aorta, initially applying 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer pH 7.4 (PBS), and afterwards with 'regular' ice-cold fixative (with 4% formaldehyde in 0.1 M phosphate buffer pH 7.4 for light microscopy or with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for transmission electron microscopy). Following the perfusion, the brains were taken away from the skulls to be immersed for 2 h in the same fixing agent. Afterwards, the brains were saturated with sucrose through immersing in 10, 20 and 30% (w/v) sucrose solutions in PBS and cut on 40 µm-thick free-floating coronal sections applying a cryostat (CM 1850 UV, Leica, Germany). In the next step the sections were prepared for immunohistochemical (IHC) studies.

Free-floating brain sections were pre-incubated with 3% normal goat serum solution in PBS supplemented with 0.2% Triton X-100 (PBS + T). Subsequently, the sections were incubated for 1 h at 37°C with PBS + T containing 1% normal goat serum and the following primary antibodies (Abs): 1) murine monoclonal Ab against macrophage (Chemicon, USA, dil. 1 : 1000); 2) murine monoclonal Ab against CD14 (Santa Cruz, USA, dil. 1 : 400) and 3) murine monoclonal Ab against monocyte/macrophage ED1 (Santa Cruz, USA, dil. 1 : 400). The reaction was terminated by washing the sections with PBS + T. Next, the sections were incubated for 1 h at 37°C with the respective secondary Abs. The secondary Abs used were as follows: 1) goat antimouse antibody conjugated with Alexa Fluor 594 or 488 (Invitrogen, USA, dil. 1 : 100) or 2) goat anti-mouse antibody conjugated with HRP (Bio-Rad, USA, cat. no. 170-5047, dil.

1 : 1000; and the reaction was visualized using Vector SG kit or DAB as a chromogen). Finally, the sections were rinsed with PBS + T, mounted on silanized glass slides (Sigma) and covered with Vectashield mounting medium for fluorescence microscopy (Vector Labs Inc., Burlingame, CA, USA).

Immunostaining was detected with a model Optiphot-2 Nikon fluorescent microscope (Japan) equipped with the appropriate filters, and recorded with a model DS-L1 Nikon camera (Japan). Specificity of the immunostaining was verified by performing a control ("blank") staining procedure with primary antibodies omitted in the incubation mixture. No staining was observed in these control sections.

Immunolabelled sections were in the next stage of the experiment counterstained with the chromosomal dye bisbenzimidazole (Hoechst staining). Sections were washed in PBS and incubated in a 1 µg/ml solution of Hoechst stain (bisbenzimidazole dye, No 33258, B-2883, Sigma; prepared in PBS) for 2 min at room temperature. The stain was then drained off and cover slipped with PBS for visualization.

In order to visualize the morphological structure of injured and control brain tissues representative samples of the lesioned area of brain were fixed with 10% buffered formalin and embedded in paraffin. Paraffin sections (4 micrometers thickness) were used for routine hematoxylin and eosin (H&E) staining. Stained samples were examined with an Olympus light microscope.

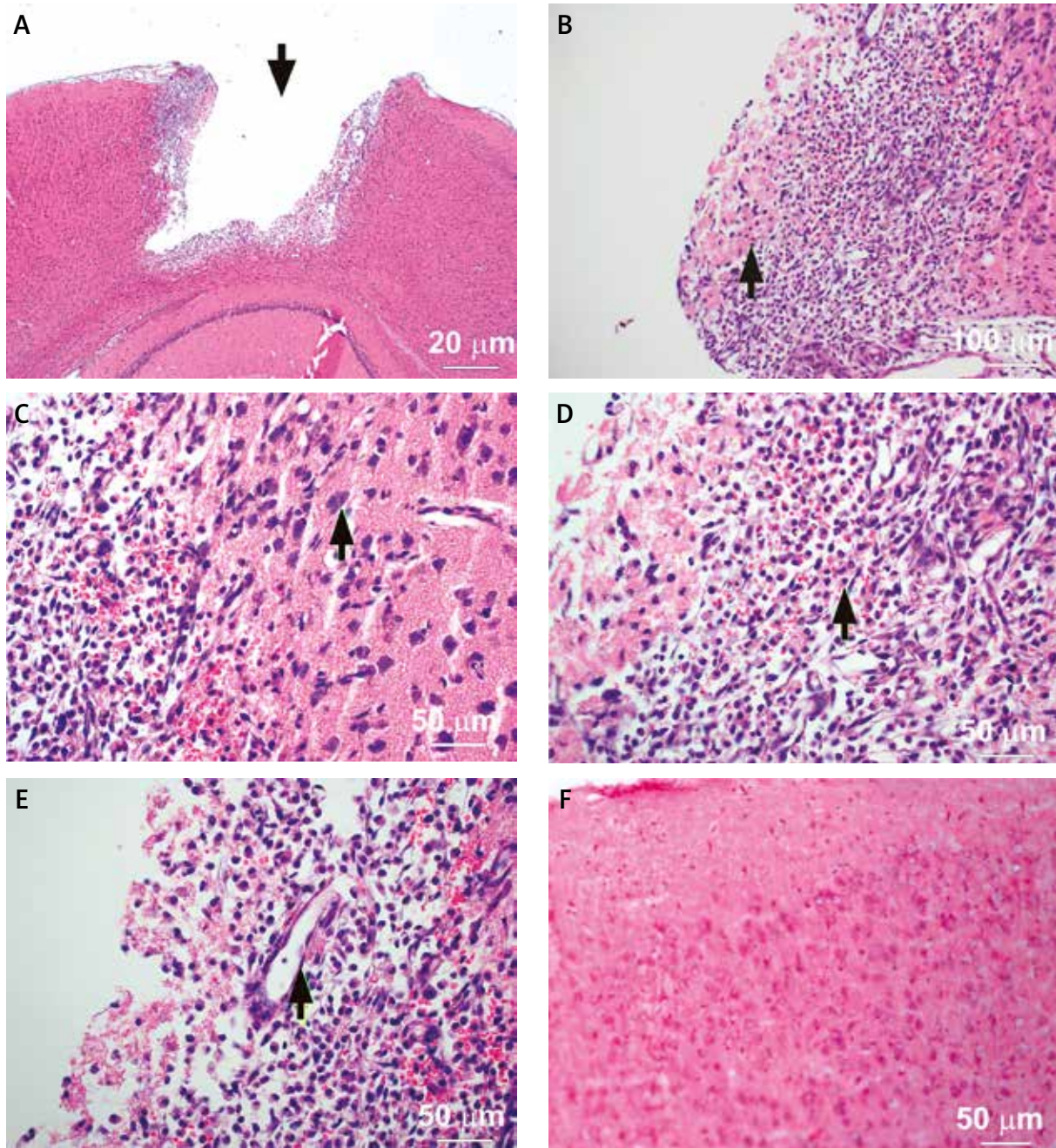
Tissue specimens for electron microscopy analyses were collected from rats anaesthetized and perfused as above. The tissue specimens for electron microscopy analyses were collected from the cerebral cortex bordering the surgical cortical injury in the operated rats and from the related cortex part in the control rats. The specimens were handled with the typical ice-cold fixative (see above) post-fixed in 1% (w/v) OsO<sub>4</sub> solution in deionized water, dehydrated in an ethanol gradient, and finally encased in epoxy resin (Epon 812). In the first step the semithin sections were analysed and next the ultrathin (60 nm) sections were prepared as described earlier [9]. Material was examined in a transmission electron microscope (JEM-1200EX, Jeol, Japan).

## Results

Macroscopic evaluation of the brain after surgical injury revealed its primary cavity post-opera-

tion. In the histological examination (staining H&E, Fig. 1) the peripheral region of the cavity contains granular repair tissues (Fig. 1A). From the site of the

cavity there are macrophages with granular eosinophil cytoplasm (Fig. 1B). Surroundings of the granular repair tissue in brain tissues contain numer-



**Fig. 1.** Hematoxylin and eosin (H&E) staining of brain derived from operated (A-E) and control (F) rats. **A)** Primary cavity (arrow) and surrounding granular tissues repair of brain after surgical injury (4 days after surgical injury). **B)** Macrophage (arrow) with granular eosinophil cytoplasm of the granular repair tissues. **C)** Damaged astrocytes in the brain (arrow) from surroundings of the granular repair tissues. **D)** Mononuclear cells (arrow; monocyte/macrophage and microglia) of the granular repair tissues. **E)** Erythrocytes around ruptured small blood vessels and small blood vessel (arrow) of the granular repair tissues.

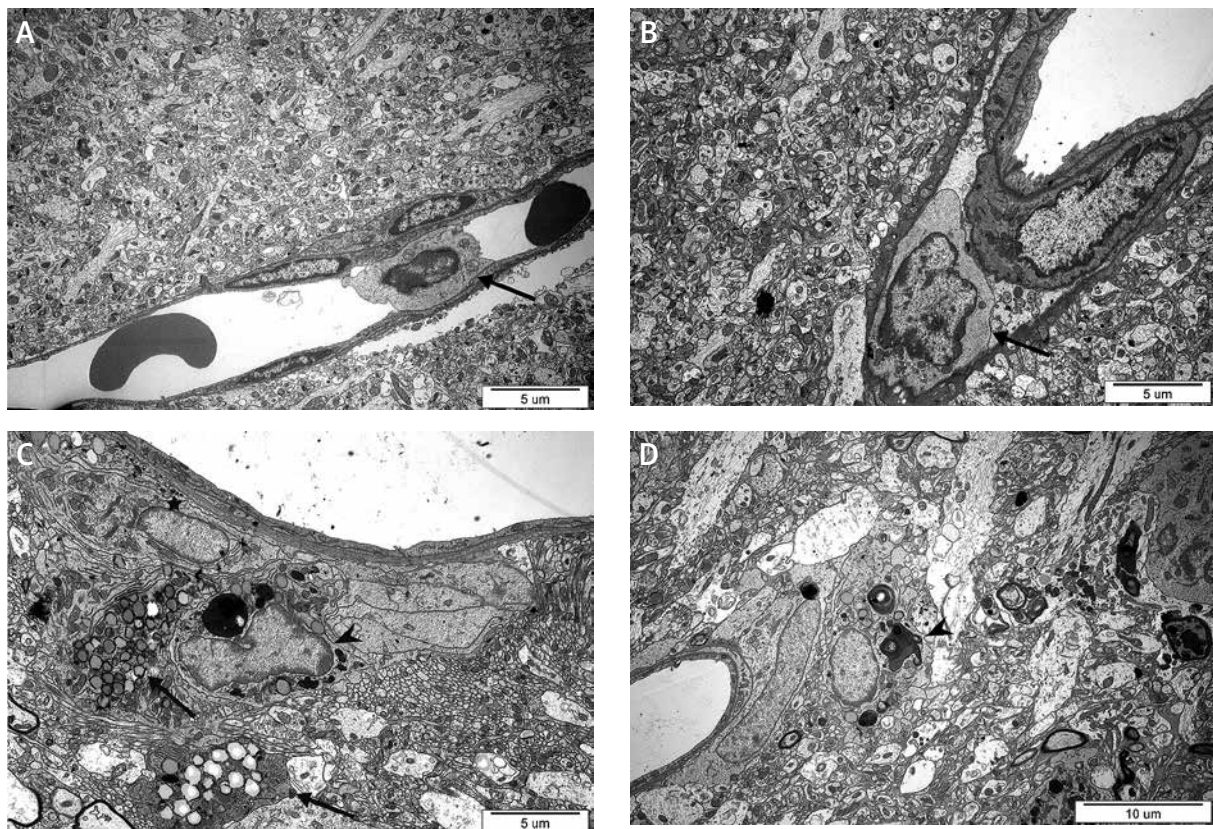


ous hypertrophic astrocytes (Fig. 1C). Peripherally, around small blood vessels the mononuclear cells (monocytes/macrophages and microglia were determined by immunocytochemistry) were crowded (Fig. 1D). Among the cellular part of the granular repair tissues there are sparse granulocytes. In peripheral regions of the tissues ruptured small blood vessels and extravasations of erythrocytes were identified (Fig. 1E). Typical histological features of the non-damaged brains in both control groups (control and sham-operated animals) were observed.

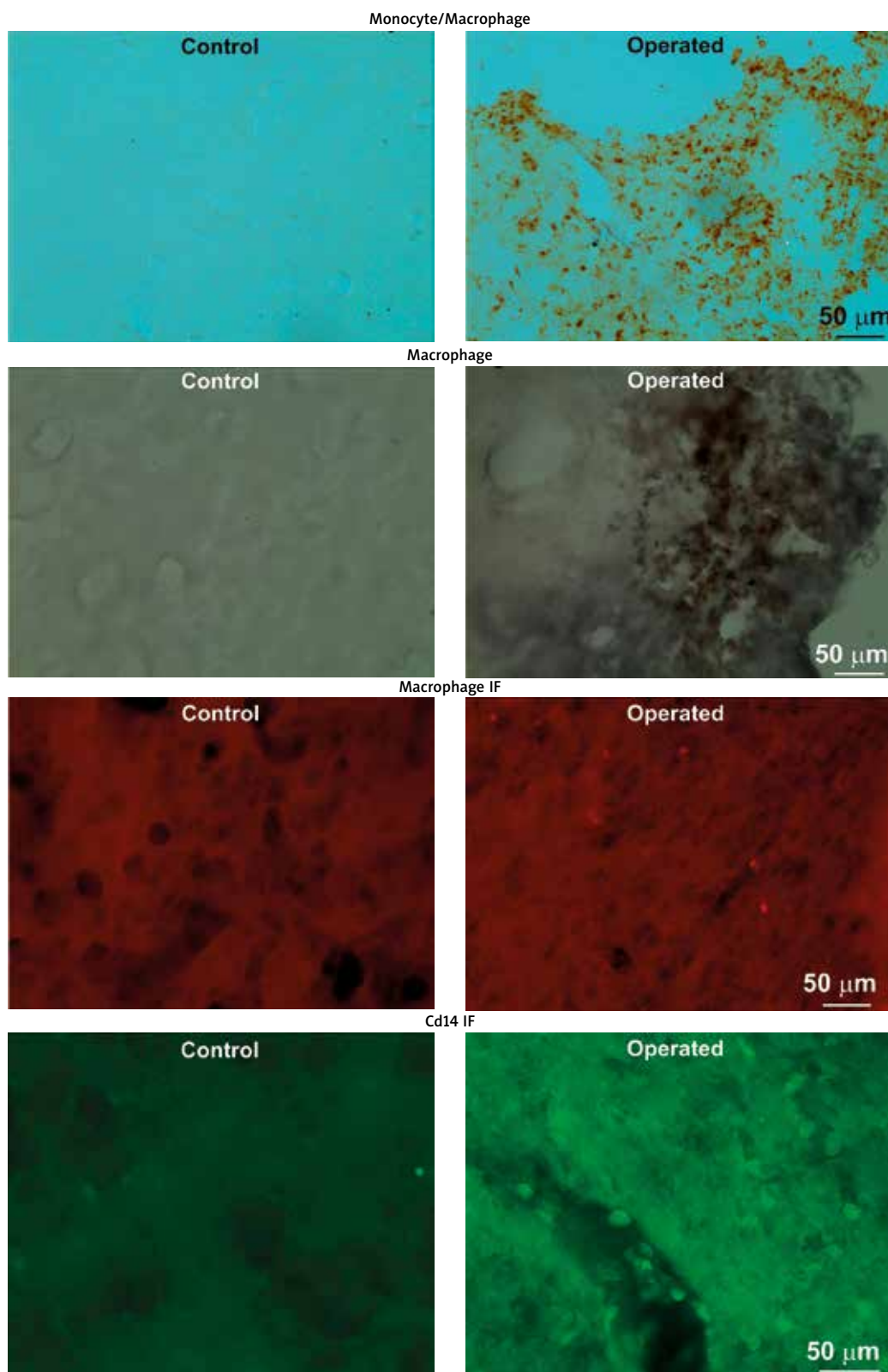
In the perilesional area four days following the SBI, the ultrastructural studies revealed the presence of a few monocytes (Figs. 2A-B) with the characteristic irregular shape of nuclei and the presence of lysosomes in the cytoplasm. In this region numerous macrophages were also seen. The characteristic feature of macrophages was the accumulation of huge lysosomes and phagosomes within the cyto-

plasm (Fig. 2C). Light microscopic analyses showed many cells stained positively for antibodies against macrophages and monocytes/macrophages (Fig. 3) in the same region. Numerous macrophages were found close to the blood vessels. Individual cells were detected in the vessel walls. In the parenchyma of control animals no or only single macrophages (one cell per observation field) were seen. In the material derived from the control sham-operated animals we did not observe macrophages (Fig. 3).

In the control material microglial cells possessed proper morphological features. Within the area adjacent to the lesion apart from the macrophages we also detected activated microglial cells (Figs. 2C-D). The cells showed morphological heterogeneity: some of them were ameboid, whereas others had irregular shapes. They are round or elongated and possess a regular or irregular nucleus situated in the centre or on the periphery of the cell body. In



**Fig. 2.** Electronograms of lesioned cerebral cortex; 4 days post-lesion. Upper panel reveals monocyte (arrow) within the lumen of the capillary vessel (**electronogram A**) and within the capillary vessel wall (**electronogram B**). Bottom panel shows macrophages (arrows) and active hypertrophic microglial cells (arrowheads) in the perivascular space (**electronogram C and D**). The asterisk points to the resting resident microglial cell without phagocytic properties.



**Fig. 3.** Immunohistochemical examination of monocytes and macrophages within control and lesioned cerebral cortex. Within cortical sections derived from control animals (left panel) we did not find either monocytes or macrophages. In the injured cortex many monocytes/macrophages were detected (right panel). Numerous macrophages were also found in the close vicinity of the capillary vessel. Some of them were localized in the vessel lumen.

cytoplasm are dispersed slightly distended channels of rough endoplasmic reticulum. Activated microglial cells (presenting hypertrophy of cell bodies and processes) become macrophage cells with numerous phagosomes and vacuoles in the cytoplasm. Many of them were filled with amorphous or granular electron-dense material (Figs. 2C-D).

## Discussion

SBI causes massive cell degeneration and death within perilesional areas [8,29]. Our earlier results proved that the cells died by apoptosis but also by necrosis. SBI leads to damage of vessel integrity. The injury of blood vessel endothelium also initiates *erythrodiapedesis* and migration of immune system cells into the perilesional area.

Our present studies are concentrate on the infiltration of brain parenchyma by immunocompetent cells. We detected different populations of immunocompetent cells within the perilesional region of injured brain. Many monocytes/macrophages were observed in this region at the fourth day following the operation. These cells were concentrated around the wound and often were found in close proximity to the blood vessels. Similar observations have also been reported in another model of brain injury [10].

The induction of immune system cells requires signal transduction via receptors such as CD14, the key receptor of the innate immune system [2]. It is the receptor for the endotoxin of Gram-negative bacteria – lipopolysaccharide. The CD14 protein is expressed in myeloid cells, including macrophages and activated microglial cells. Following brain injuries the presence of CD14 is observed on activated parenchymal microglia/macrophages and infiltrating monocytes [2]. Our results are in agreement with these findings. We detected the expression of CD14 on the vessel walls as well as in the lumen of blood vessels and on the cells localized in the brain parenchyma.

It is known that the immune system activation caused by brain injury involves not only the entry of macrophages but also activated resident brain microglia [30]. Our data indicate the massive activation of microglial cells following SBI. Many of these cells become phagocytes.

According to the present knowledge, microglia are not typical immune cells, but play a helper role in the security of the CNS as scavengers. Microglia clean up the brain parenchyma from damaged cells, infectious factors and cellular debris. Many authors

report that the activation of microglial cells is a mark of various pathological situations, such as infection and inflammation, neurodegenerative disorders, ischaemia and different brain injuries [30]. Our electron-microscopic data show that the migration of monocytes and the presence of microglial cells in the injured cortex is observed up to 12 months following the injury.

Phagocytosis is a significant mechanism to control post-injury neuronal death via apoptosis as well as necrosis. The clearance of degenerated cells and cellular debris from brain parenchyma can avoid delayed additional injury [30].

Recent literature data show the presence of intense cross-talk between the nervous and the immune system [13,18,30]. The elements of both systems express and secrete a range of cytokines (for the immune system) and neurotrophic factors (for the CNS) that control growth as well as cell differentiation. For example, microglia can regulate synaptic formation and plasticity [30].

However, long-lasting activation of glial cells causes neuronal damage that finally results in neurodegeneration and neuroinflammation. These processes may be kept in check for the restoration of homeostasis in the CNS. Neurons may inactivate immunocompetent cells by several mechanisms dependent on cell-cell contact or the secretion of soluble factors such as neurotrophins and neurotransmitters [3,16,31]. The balance between the induction and inhibition of several types of cells allows brain parenchyma remodelling and rebuilding following brain injury.

## Conclusions

Our present and earlier data show the relationship between elements of the CNS and immune system cells. We found that SBI causes rapid and massive neuronal degeneration, gliosis and infiltration of the injured region by immunocompetent cells. The processes are accompanied by the activation of microglia. Phagocytes (monocyte and microglial origin) clean up cellular debris from the damaged region and help to restore CNS homeostasis and brain repair/remodelling.

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