The Spectrum of Systemic Immune Alterations After Murine Focal Ischemia
Immunodepression Versus Immunomodulation

Arthur Liesz, MS; Sebastien Hagmann, MD; Carolin Zschoche, MS; Johanna Adamek, MS; Wei Zhou, MD; Li Sun, MD; Andreas Hug, MD; Markus Zorn, MSc; Alexander Dalpke, MD; Peter Nawroth, MD; Roland Veltkamp, MD

Background and Purpose—Therapeutic modification of the postischemic immune processes is a key target of current experimental stroke research. For successful translation into the clinical setting, experimental studies must account for the impact of different strokes on the immune system including susceptibility to infection. Herein, we characterize the impact of 3 ischemia models on systemic immunological and microbiological parameters.

Methods—In C57Bl/6 mice (n=235), the middle cerebral artery was occluded (MCAO) either permanently by distal coagulation or transiently by an intraluminal filament for 30 minutes or 90 minutes. Differential leukocyte counts were performed in blood and lymphatic organs. Lymphocyte subpopulations and apoptotic cells were characterized by flow cytometry. Blood cytokine concentrations were measured by ELISA. Microbiological cultures were grown from blood and lung samples.

Results—Only extensive infarcts induced leukopenia 24 hours, 3 days and 7 days after MCAO and decreased lymphocyte counts in spleen, lymph nodes and thymus. In contrast, small infarcts led to no significant changes in differential blood count or reduction of overall cell counts in lymphatic organs. Splenic lymphocyte apoptosis and blood cytokine production was significantly increased after extensive lesions compared to mild ischemia. Hypothermia and weight loss occurred only in mice with large infarcts which also suffered from pneumonia and sepsis. In contrast to infarct size, location and side of the infarct did not affect physiological parameters and immune cell alterations.

Conclusions—Postischemic systemic immunomodulation and infectious complications differ substantially among stroke models. Translational studies of immunomodulatory therapies for stroke must account for this heterogeneity. (Stroke. 2009;40:2849-2858.)

Key Words: brain ischemia ▪ immune response ▪ leukocytes ▪ cytokines ▪ bacterial infection

Ischemic stroke activates multiple inflammatory cascades1–4 and triggers substantial alterations of the systemic immune system.5–9 Various leukocyte subsets invade the postischemic brain and exert mainly neurotoxic functions. Also, cerebral expression of inflammatory cytokines is induced and contributes to secondary tissue loss6,10,11; these processes have come into the focus of translational cerebrovascular research as they play an important, mainly detrimental role in the pathophysiology of ischemic stroke, and may potentially be amenable to protective therapies. Another clinically important facet of cerebral ischemia is a stroke-induced immunodeficiency syndrome encompassing innate and adaptive immune cells which predisposes patients to pneumonia and sepsis.5,8 On the other hand, stroke-induced alterations of the systemic immune system may represent an adaptive cerebroprotective response of the immune system limiting cerebral and systemic inflammation.9 Indeed, we recently showed that regulatory T cells (T_\text{Reg}) function as endogenous key counterregulators interfering with various detrimental inflammatory reactions after brain ischemia.12 Notably, the protective effectiveness of T_\text{Reg} varied among different experimental stroke models.12

In view of the heterogeneity of clinical stroke on the one hand and the variability and complexity of brain-immune system interactions in experimental ischemia on the other hand, detailed characterization of the differential effects of different stroke models on immunological parameters should be considered a prerequisite before translation of immunomodulatory therapeutic strategies into the clinical setting. In particular, size and location of the ischemic infarct can be
expected to be important variables determining the extent and pathophysiological consequences of poststroke immunomodulation. To date, however, experimental ischemia studies examining the systemic immune system after stroke were mainly performed after prolonged filament-induced middle cerebral artery occlusion (MCAO).6,13,14 This murine model results in extensive subcortical and cortical infarcts in the MCA territory and is associated with severe neurobehavioral deficits, frequent severe infectious complications, and a high mortality.8 Whether similar changes of the systemic immune system and an increased susceptibility to infections are present after more circumscribed infarcts is presently unknown.

The purpose of the present study was to characterize the differential impact of different focal ischemia models on systemic immune cells, their main cytokines and the occurrence of bacterial infections.

Materials and Methods

Animals

The study was conducted in accordance with national guidelines for the use of experimental animals, and the protocols were approved by the institutional and governmental committees for animal care and use (Regierungspraesidium Karlsruhe, Germany). In all experiments sexually mature male mice (C57BL/6, Charles River Laboratories, 10 to 12 weeks of age, n=235) were used.

Coagulation Model

Small cortical infarcts were induced by irreversible transtemporal MCA coagulation distal of the lenticulostriate arteries. Mice were anesthetized with 1.0% to 2.0% halothane in O2/N2O enriched air. After a 1 cm skin incision between left eye and ear, a burr hole was drilled through the temporal skull. The dura mater was removed and the MCA permanently occluded using a bipolar electrocoagulation forceps (ERBOTOM, Erbe, Germany). For laser doppler measurements the probe (P403, Perimed, Sweden) was placed 3 mm lateral and 6 mm posterior to the bregma and relative perfusion units were obtained (Perflux 4001, Permed, Sweden). During the operation, temperature was kept at 37°C using a feedback controlled heating pad. The skin lesion was stitched and the mouse placed in a cage under an infrared heating lamp. Sham operation was performed identically as described above, including reanesthesia 90 minutes after sham surgery, except for only brief introduction of the filament into the external carotid artery. The total anesthesia time was similar in all 3 stroke models (approx. 20 minutes).

Assessment of Infarct Volume

The infarct volume was determined on cryosections stained with the high-contrast silver staining technique.15 In brief, mice were deeply anesthetized with tribromoethanol and perfused transcardially with saline. Brains were removed, immediately frozen and 20 µm thick coronal cryosections were cut every 400 µm. After staining, sections were scanned at 600 dpi, and the infarct areas measured using a public domain image analysis program (Scion Image). The total infarct volume was obtained by integrating measured areas and distance between sections. Correction for brain edema was applied by subtraction of the ipsilateral minus contralateral hemisphere volume from the directly measured infarct volume.

Microbiological Analysis

Sterile blood samples were taken 5 days after MCAO by cardiac puncture after thoracotomy. Lobes of lungs were collected, minced and homogenized under sterile conditions. 100 µL of all specimens were serially diluted in PBS and plated onto blood agar plates (Becton Dickinson) and MacConkey agar plates (Biomerieux). Blood samples were additionally tested for growth of anaerobic bacteria. After 24 and 48 hours of incubation at 37°C, agar plates were analyzed for growth of colonies by a technician blinded to treatment groups.

Leukocyte Count in Blood and Organs

Blood samples were collected from naïve mice and at 24 hours, 3 days and 7 days after MCAO by puncturing the peribital venous sinus in deep anesthesia, and blood was transferred into standard EDTA test tube. Samples were analyzed in the Core Laboratory Facilities of the University Hospital Heidelberg for complete blood counts. Spleen, lymph node and thymus were collected after sacrificing the mouse and processed to single cell suspensions. After lysis of erythrocytes the leukocytes were counted in a Neubauer hemocytometer.

Flow Cytometry

500 µL of venous blood was drawn at same time points as for complete blood counts from the peribital sinus into heparinized tubes and leukocytes isolated by Ficol-Hypaque gradient. Lymphatic organs were collected after transcardial perfusion with heparinized saline by sterile sampling and processed to single-cell suspensions. Cells were stained for anti-mouse CD3, CD4, CD8, CD25, B220 and Foxp3 and the appropriate isotype control following the manufacturer’s protocols (eBioscience). Apoptotic cells were detected using a commercial Annexin V detection kit with Propidium iodide (BD Pharmingen). Flow cytometry was performed on a Beckton Dickinson FACScan and analyzed by CellQuest Pro software.

Cytokine ELISA

For measurements of blood cytokine concentrations, blood samples were drawn, clotted and centrifuged for serum collection and immediately frozen until analysis. Commercial kits were used for determining the protein concentration of TNF-α, INF-γ, TGF-β (all from R&D Systems) and IL-10 (eBioscience).

Statistical Analysis

Data in boxplots (Figures 1, 2, 4 and 5) are expressed as median, interquartile range (Q1, Q3) and 95% CIs (whiskers). Treatment groups were significantly differing for individual parameters if the 95% confidence limits of the estimates did not overlap. Values in
Results

Modeling Stroke Severity

As expected, size and location of ischemic lesions differed substantially among the 3 models of experimental brain ischemia. Mean infarct volume in the Coagulation-MCAO

Figures 3 and 6 are expressed as mean±standard deviation (SD). Values in supplemental Figures I and II (available online at http://stroke.ahajournals.org) are presented as mean values. Comparison of mean values was performed by ANOVA for multiple comparisons with post hoc Tukey test using SPSS analysis software. A probability value <0.05 was considered statistically significant.
Figure 2. Differential blood cell counts and leukocyte counts in lymphatic organs. Ninety minutes Filament-MCAO induced reduction of (a) blood leukocytes depends mainly on the marked decrease in (b) lymphocyte number. (c) Neutrophils remained rather stable after 90 minutes Filament-MCAO and (d) monocyte numbers were increased after Filament-Sham, but not MCAO. Additionally, total leukocyte counts were determined per (e) spleen, (f) one mesenteric lymph node, and (g) thymus (n=5 per group).
model was 15.5 ± 2.8 mm$^3$ and infarcts were limited to the cortex. In the 30 minutes Filament-MCAO model, mean lesion size was 14.2 ± 3.1 mm$^3$ and encompassed cortical and subcortical regions (Figure 1a). The mortality rate of 90 minutes Filament-MCAO mice was significantly higher (25%) than of Coagulation-MCAO and 30 minutes Filament-MCAO mice (each less than 5%; Figure 1b). Rectal temperature and body weight were used as general parameters reflecting physical activity, food intake and potential infectious illness after operation (Figure 1c,d). For both markers, a profound reduction was observed from 24 hour until 7 days after 90 minutes Filament-MCAO compared to mice with small infarcts or after respective sham surgery. In contrast, both parameters were not significantly altered after Coagulation-MCAO and 30 minutes Filament-MCAO. Previous studies reported a stroke induced spontaneous hyperthermia in rats by ischemic lesion to the hypothalamic region.\textsuperscript{16} However, the hypothalamic region was not infarcted in any of the mouse stroke models used for this study.

**Bacterial Infections After Brain Ischemia**

We further analyzed the microbiological status of mice 5 days after the respective MCAO operation. Lung tissue and blood were processed for analysis of bacterial growth, because pneumonia and bacteremia were the common infections in previous experimental stroke experiments.\textsuperscript{8} Mice with pure cortical infarcts in the coagulation model (n=6) had no bacterial growth in blood samples and bacterial growth in 50% of lung tissue cultures with a maximum of 200 CFU/mL which consisted of mainly nonpathogenic bacteria (\textit{Acidovorax, Micrococcus, Staphylococcus}). In contrast, 60% of the 90 minutes Filament-MCAO mice (n=6) had bacterial growth in blood cultures and 100% had growth in lung homogenates with high bacterial loads of up to 800 CFU/mL (\textit{Streptococcus, Staphylococcus, E. coli and Enterococcus}).
Blood Cell Count and Leukocytes in Lymphatic Organs

Cell counts of leukocytes and their subpopulations in nonoperated (WT) mice were within the published range of normal values for C57BL/6 mice. Leukocyte as well as lymphocyte numbers were reduced at all measured time points after 90 minutes Filament-MCAO compared to all other procedures (Figure 2a and 2b and supplemental Figure I). Absolute number of polymorphonuclear neutrophils was increased after 30 minutes Filament-MCAO and also higher after

Figure 4. Lymphocyte subpopulations in blood and lymphatic organs. Blood, lymph nodes and spleens were analyzed by flow cytometry for \textsuperscript{T}\textsuperscript{Helper} cells (CD3\textsuperscript{+}CD4\textsuperscript{+}), \textsuperscript{T}\textsuperscript{Cytotoxic} cells (CD3\textsuperscript{+}CD8\textsuperscript{+}), B cells (B220\textsuperscript{+}) and \textsuperscript{T}\textsuperscript{Reg} cells (CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+}). Values are displayed as median, interquartile range (box) and 95% CI (whiskers) of absolute cell numbers per \(\mu\)L of whole blood or per one organ, respectively (n=5 per group).
Coagulation-MCAO compared to 90 minutes Filament-MCAO 3 days and 7 days after ischemia (Figure 2c and supplemental Figure I). In contrast, monocyte counts did not significantly differ between the 3 MCAO models (Figure 2d and supplemental Figure I).

Previous studies using the 90 minutes Filament-MCAO model showed decreased numbers of viable splenocytes and thymocytes 22 hours and 4 days after MCAO.7 We were able to reproduce these findings for the later time points and detected significantly lower numbers of white blood cells 3 days and 7 days after 90 minutes Filament-MCAO in all lymphatic organs (Figure 2e to 2g and supplemental Figure I). In contrast, 30 minutes Filament-MCAO did not cause a significant WBC count reduction in any organ compared to sham treatment.

Brain Ischemia Induces Apoptosis in Lymphatic Organs

We analyzed the percentage of apoptotic lymphocytes in spleen, lymph node and thymus 24 hours after the respective operation. Apoptotic cells were differentiated by flow cytometry in early-apoptotic cells (Annexin V+/PI-) and late-apoptotic/necrotic cells (Annexin V+/PI+) (Figure 3a). Among the lymphatic organs, splenic lymphocytes showed the best association with lesion size (Figure 3a,b): the ratio of Annexin V+ apoptotic cells was significantly increased more than 4-fold by 30 minutes and 90 minutes Filament-MCAO compared to sham treatment (P<0.01, n=5 per group), whereas Coagulation-MCAO did not significantly induce apoptotic cell death. Additionally, 90 minutes Filament MCAO also significantly increased the proportion of already late-apoptotic cells (Annexin V+/PI+) compared to all other groups. Lymphocyte apoptosis in lymph nodes was not affected in any model (Figure 3c). In contrast, the surgical procedures caused a substantial increase of apoptotic cells in the thymus regardless of the operational protocol compared to WT mice (Figure 3d), but with no significant difference among the MCAO models.

Lymphocyte Subpopulations are Differentially Affected in Moderate Ischemia

We compared absolute numbers of T_{Helper} cells (CD3+CD4+), T_{Cytotoxic} cells (CD3+CD8+), B cells (B220+), T_{Reg} cells (CD4+CD25+Foxp3+) and the percentage of T_{Reg} cells within the CD4+ population of blood, spleen and lymph node (Figure 4 and supplemental Figure IIa). T and B cells were significantly reduced 3 days and 7 days after 90 minutes Filament-MCAO, corresponding to the observed global leukocyte depression in
this model (compare Figure 2). In contrast, experiments with mice undergoing Coagulation-MCAO or 30 minutes Filament-MCAO revealed a more differentiated cellular immune response (Figure 4 and supplemental Figure IIa) which was generally very similar for these 2 models inducing smaller lesions. After small infarcts, single data points differed significantly for CD4^+ T_Helper and CD8^+ T_Cytotoxic cells. In contrast, B cell numbers remained constant in blood and were decreased after Coagulation-MCAO in the secondary lymphatic organs.

T_Reg cells are key regulators of the postischemic immunomodulation.12 Their cell count in blood remained stable after Coagulation-MCAO in contrast to simultaneously decreasing CD4^+ and CD8^+ counts (Figure 4, fourth row). Thirty minutes Filament-MCAO did also not significantly affect the number of circulating T_Reg cells. Interestingly, T_Reg counts were significantly reduced after 90 minutes Filament-MCAO but had already recovered 7 days after MCAO when the other lymphocyte subpopulations were still diminished. Consequently, the proportion of T_Reg within the circulating CD4^+ population was significantly increased in all models (Figure 4, lowermost row).

In the thymus (Figure 5 and supplemental Figure 2b), cell numbers of immature lymphocytes (CD4^+CD8^+) were compared to those of mature CD3^+CD4^+ and CD3^+CD8^+ lymphocytes and CD4^+CD25^+Foxp3^+ T_Reg cells. Intriguingly, CD3^+CD4^+ cells were decreased by 30 minutes and 90 minutes Filament-MCAO at day 3 and 7, respectively, whereas CD3^+CD8^+ were not affected by any experimental model. In contrast, immature thymocytes were significantly diminished in the thymus at day 7 in all 3 models compared to sham treatment and naïve mice. Ninety minutes Filament-MCAO even vastly depleted this cell population to only 0.1% of the original cell count.

**Immunological Alterations Are Independent of the Side of Ischemia**

The effect of MCAO and sham surgery of the left or right hemisphere (n=7 per group) was examined in the Coagulation-MCAO and 90 minutes Filament-MCAO models (data not shown). In both models no significant difference was found between animals undergoing right or left hemispheric ischemia regarding daily body temperature and weight within the first week after ischemia, and total cell counts of spleen, lymph nodes and thymus 3 days after operation. Also, CD4^+, CD8^+, T_Reg, and B cells did not differ in lymphatic organs between left and right hemispheric ischemia in both models.

**Modulation of Serum Cytokine Levels After Stroke**

We analyzed serum cytokine concentrations to determine the impact of the different stroke models on lymphocyte function and systemic inflammatory activation (Figure 6). The proinflammatory cytokines TNF-α and IFN-γ and the antiinflammatory cytokines IL-10 and TGF-β have previously been shown to play an important role after cerebral ischemia.2,6,12,18 While IFN-γ (Figure 6a) was significantly elevated only at 24 hours after 90 minutes Filament-MCAO, TNF-α (Figure 6b) concentration was still significantly increased 3 and 7 days after 90 minutes Filament-MCAO compared to both other MCAO models. Intriguingly, an even more pronounced difference between models was found for concentrations of antiinflammatory cytokines. Significantly
higher IL-10 concentrations were measured 24 hours and 7 days after 30 minutes and 90 minutes Filament-MCAO, respectively (Figure 6c). Also, 90 minutes Filament-MCAO increased TGF-β expression significantly at all measured time points (Figure 6d).

Discussion

The major new findings of the present study are that only extensive infarcts induce a profound leukocyte depression and infectious complications. An increase in systemic cytokine release was most distinctive after large ischemic lesions. In contrast, after circumscribed subcortical or cortical ischemia, lymphocyte subpopulations are differentially affected resulting in an increased proportion of T_{reg} in blood. Overall, postischemic immune system alterations depend on infarct size rather than location.

Several studies using prolonged filament-induced MCAO have provided important insights into the profound immunological alterations occurring after extensive cerebral infarction. Similar to our present study, the systemic immune system was therein globally affected including innate and adaptive immune cells. However, because most human strokes do not affect the entire but only parts of the MCA territory, the therapeutic potential of neuroimmunological therapies would be more adequately evaluated in experimental ischemia models inflicting only moderate brain damage. In accordance with this concept, endogenous immunomodulatory mechanisms can be highly protective in moderate but ineffective in large experimental stroke models. Therefore, detailed characterization of immune system alterations in a spectrum of ischemia models is essential.

A clinically important facet of postischemic systemic immune alterations is suppression of the host defense against bacteria. As demonstrated in the present study, systemic and pulmonary infections develop only after extensive infarctions but not moderate lesions. Data from our and other studies suggest that ischemia-induced lymphopenia and immune cell dysfunction causes the increased susceptibility to infections. However, other factors including the compromised general condition of mice after extensive infarcts including substantial weight loss, hypothermia and swelling disturbances may contribute to the increased rate of infectious complications.

Although prolonged Filament MCAO caused lymphocytopenia, our data reveals also a strong increase of both pro and antiinflammatory blood cytokine production after extensive brain infarction, confirming a recent report of marked peripheral immune system activation after 90 minutes Filament-MCAO. The integrated pathophysiological consequences of these opposing phenomena after large infarcts—decreased cellular immune function and lymphocytopenia versus the massive increase of blood cytokines—remain to be elucidated. Intriguingly, smaller subcortical or cortical lesions induced rather specific than global alterations of systemic lymphocytes. For example, effector T cells were susceptible to cerebral ischemic stress, whereas T_{reg} cells were relatively resistant. Whether this modified numeric balance between different lymphocyte subpopulations translates into a shift of immune system function is currently unclear. As endogenous T_{reg} cells play an important cerebroprotective role after ischemia, this T lymphocyte shift may represent a biologically meaningful adaptation of the immune system. Finally, some studies suggest that infarct location and hemispheric laterality, respectively, as well as transient versus permanent ischemia may have a different effect on the immune system. However, our findings clearly demonstrate that infarct size is the primary factor determining postischemic systemic immune system alterations, whereas neither infarct side and location nor ischemia model have a major impact.

In view of the vast differences of immunological consequences between extensive and smaller ischemic lesions, our results underscore that immunological data measured in extensive ischemia models might hardly be applied to studies inducing only small lesions. Furthermore, prolonged MCA occlusion might be a suitable model for analyzing ischemia-induced immunosuppression and subsequent infections but less adequate for studying specific postischemic immunopathways and immunological interactions. Thus, our findings are of considerable relevance for understanding the spectrum of the complex postischemic immune system alterations and for appropriate modeling in translational stroke research.

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Disclosures

None.

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