Temporal and Spatial Dynamics of Cerebral Immune Cell Accumulation in Stroke

Mathias Gelderblom, MD; Frank Leypoldt, MD; Karin Steinbach; Doerthe Behrens; Chi-Un Choe, MD; Dominic A. Siler; Thiruma V. Arumugam; Ellen Orthey; Christian Gerloff, MD; Eva Tolosa, PhD; Tim Magnus, MD

Background and Purpose—Ischemic stroke leads to significant morbidity and mortality in the Western world. Early reperfusion strategies remain the treatment of choice but can initiate and augment an inflammatory response causing secondary brain damage. The understanding of postischemic inflammation is very limited. The objectives of this study were to define the temporal and spatial infiltration of immune cell populations and their activation patterns in a murine cerebral ischemia–reperfusion injury model.

Methods—Transient middle cerebral artery occlusion was induced for 1 hour followed by 12-hour to 7-day reperfusion in C57/Bl6 mice. Immunohistochemistry and flow cytometry were used to quantify the infiltrating immune cell subsets.

Results—Accumulation of microglia and infiltration of the ischemic hemisphere by macrophages, lymphocytes, and dendritic cells (DCs) preceded the neutrophilic influx. DCs were found to increase 20-fold and constituted a substantial proportion of infiltrating cells. DCs exhibited a significant upregulation of major histocompatibility complex II and major histocompatibility complex II high-expressing DCs were found 100 times more abundant than in sham conditions. Upregulation of the costimulatory molecule CD80 was observed in DCs and microglial cells but did not further increase in major histocompatibility complex II high-expressing DCs. No lymphocyte activation was observed. Additionally, regulatory immune cells (natural killer T-cells, CD4−/CD8− T lymphocytes) cumulated in the ischemic hemisphere.

Conclusion—This study provides a detailed analysis of the temporal dynamics of immune cell accumulation in a rodent stroke model. The peculiar activation pattern and massive increase of antigen-presenting cells in temporal conjunction with regulatory cells might provide additional insight into poststroke immune regulation. (Stroke. 2009;40:1849-1857.)

Key Words: dendritic cell ■ flow cytometry ■ ischemia–reperfusion injury ■ microglia ■ middle cerebral artery occlusion

Ischemic stroke is a devastating disease representing the third leading cause of death in the Western world and the leading cause of disability in adults. Cerebral ischemia mostly results from occlusion of major brain arteries. Stroke is envisioned to be a multiphasic process.1 This concept emphasizes a secondary progression of ischemic brain injury associated with an intense inflammatory response. Within the first hours, a rapid activation of resident microglia and production of proinflammatory cytokines, including tumor necrosis factor-α and interleukin-1β, takes place.2 Neutrophils and monocytes/macrophages infiltrate and accumulate in microvessels and ischemic cerebral parenchyma.3

The contention that inflammation is a cause of secondary brain damage rather than merely a consequence of brain injury is supported by several studies. Immunodeficient mice experience a reduction of infarct size and improved outcome after transient middle cerebral artery occlusion.4,5 Pretreatment with leukocyte adhesion-blocking antibodies6 or genetic deficiencies in adhesion molecules (intercellular adhesion molecule-1, CD11b/CD18, P-selectin)7,8 leads to smaller cerebral infarcts in this model. Nevertheless, it is important to note that the action of the immune system is not uniform and that activation of immune cells may also be neuroprotective and supportive for regeneration.9 Moreover, at the level of extracerebral organs, acute ischemic stroke is known to induce immunodepression and enhanced susceptibility to infections such as pneumonia.10

The reasons for these double-edged immune effects in the brain remain to be elucidated and a comprehensive characterization of the migration and phenotype of immune cells in an ischemia–reperfusion stroke model is missing. Understanding dynamics of postischemic inflammation is a prerequisite for therapeutic intervention in this fragile system to prevent harmful side effects. We used an established murine...
model of transient focal cerebral ischemia to analyze the temporal and spatial distribution and the activation profile of the infiltrating cells and microglia.

Materials and Methods

Animal Experiments

All experiments were done in accordance with the institutional guidelines and approved by the local ethics committee. C57/BL6 mice (20 to 25 g, 12 weeks; TVH, University Medical Center Hamburg-Eppendorf) were anesthetized (isoflurane 1% to 2% v/v oxygen) and underwent anesthesia (buprenorphine 0.03 mg/kg body weight intraperitoneally every 12 hours for 24 hours). Temporary middle cerebral artery occlusion was achieved by using the intraluminal filament method (6-0 nylon) as previously described for 1 hour.11 In the sham group, arteries were visualized but not ligated. Mice were euthanized using CO2. Most mice were monitored using trans temporal laser Doppler and every mouse was scored on a scale from 0 to 5 after reawakening and before being euthanized.12 Only mice with a score >1 were included. Of 78 mice, 12 mice were excluded due to lack of neurological deficit, and 10 mice died before analysis (inclusion 72%, mortality 13%).

Immunohistochemistry

Animals were euthanized 3 days postreperfusion, and brains were immediately transferred to −80°C. Ten-micron slices were cut on a cryostat and stained according to standard protocols (Vectastain-ABC-kits; Vector Laboratories; Table).

Fluorescence-Activated Cell Sorter Analysis

Animals were euthanized and perfused with phosphate-buffered saline. Brains were dissected, cerebella removed, and hemispheres divided into left ischemic (ipsilesional) or right nonischemic (contralesional). Three to 4 hemispheres were pooled, incubated for 30 minutes at 37°C (1 mg/mL collagenase, 0.1 mg/mL DNAse I in DMEM), and pressed through a cell strainer (40 μm; BD Biosciences). Next, cells were incubated with standard erythrocyte lysis buffer on ice, separated from myelin and debris by Percoll gradient (GE Healthcare; 1095 g/mL and 1030 g/mL), centrifugation, and incubated with appropriate antibody cocktails (30 minutes, room temperature; Table) in fluorescence-activated cell sorter buffer (0.5% bovine serum albumin, 0.02% sodium azide in phosphate-buffered saline). For TrueCount (Becton Dickinson) fluorescence bead absolute quantification, 10% of cells were quantified according to the protocol on the basis of CD45-positive events. For Foxp3 staining, 5 hemispheres were pooled and freshly prepared spleen cells were used for controls; staining was done according to the protocol with the Mouse-Regulatory-T-cell-Staining-Kit (eBioscience).

Cells were analyzed by blinded evaluators using a LSR II (BD Biosciences) and FACS Diva software (BD Biosciences). Up to 2 000 000 forward light scatter events per tube were acquired. Means and SE of means of independent experiments were calculated by one-way analysis of variance with Tukey-Kramer post hoc test (Prism; Graph Pad). Three independent experiments for sham, 12 hours, Days 1 and 3, and 2 independent experiments for Day 7 reperfusion conditions (3 to 4 mice per experiment) were included. Antigen-presenting cell activation was analyzed by subtracting median fluorescence intensity of macrophages, microglia, or dendritic cells (DCs; separated as described previously) stained with CD40, CD80, and MHCII antibodies from median fluorescence intensity of unstained cells (3 independent experiments, 3 to 4 mice each).

Results

Influx of Inflammatory Cells

On immunohistochemical sections, we observed loss of astrogliosis 3 days after reperfusion (Figure 1A) and widespread neutrophil infiltration into the ischemic hemisphere (Figure 1B) and to a much lesser degree into the contralesional hemisphere (not shown). Iba1-positive microglia/macrophages (Figure 1C) were numerous found in close proximity of the infarct. Considerable numbers of CD11c-positive cells (Figure 1D), likely reflecting DCs, were almost exclusively located in the ischemic hemisphere close to the penumbra.

Quantification and Temporal Distribution of Different Subclasses of Inflammatory Cells

Using flow cytometric analysis, cells were gated for CD45 high expression and further subdivided by side scatter. The former were denoted neutrophils; from the latter natural killer (NK) cells were separated by NK1.1 staining and the remainder analyzed for CD11b and CD11c expression. CD11b+/CD11c− were considered lymphocytes, CD11b+/CD11c− macrophages, CD11b+/CD11c+ nonmyeloid DCs, CD11b+/CD11c− myeloid DCs, and CD45intermediate+CD11b+ microglia. NKS were further subdivided into CD3+ NKT and CD3− NK cells. Lymphocytes were subdivided into CD3− B-cells and CD3+ T-cells and the latter analyzed for CD4 and CD8 expression (see supplemental Figure I, available online at http://stroke.ahajournals.org).

Neutrophils, Macrophages, and Microglia

Neutrophilic granulocytes appeared in small amounts after 12 and 24 hours and virtually overwhelmed (65 000 cells) the ischemic hemisphere by Day 3 postreperfusion (Figure 2B). A noticeable decline followed by Day 7. Surprisingly, they were found significantly less often than macrophages and microglia until Day 3 (Figure 2I). The latter 2 started to appear already at 12 hours after ischemia and were significantly increased after 24 hours. There were roughly equal numbers of neutrophils and microglia in the infarcted hemisphere at the peak of inflammation on Days 3 and 7 (Figure 2A, B, H). On Day 3, the majority of the remaining cells were macrophages (63%). We did not observe a significant increase of neutrophils, macrophages, or microglia in the contralesional hemisphere (Figure 2A, B, H).

As expected, in sham-operated animals, microglial cells (Figure 2A) were the most abundant immune cell subtype (27 000 cells/hemisphere, 80%; Figure 2H). They increased very early (60 000 cells/hemisphere on Day 1, P<0.05; Figure 2A) but were eventually (by Day 3) outnumbered by blood-derived immune cells (microglia 34%, neutrophils 39%, macrophages, dendritic cells, NK cells, and lymphocytes 27%; Figure 2H).

Lymphocytes

With 3000 cells/hemisphere on Day 3, lymphocytes made up a much smaller proportion of infiltrating cells (Figure 2D). Lymphocytes consisted of approximately equal amounts of T- and B-lymphocytes. T-lymphocytes (P<0.01; Figure 2D) and especially CD4+ T-helper cells (P<0.001; Figure 2E) were significantly increased on Day 3 postreperfusion in the infarcted hemisphere. T-lymphocyte subset analysis (Figure 2E) indicated significant infiltration of CD4+/CD8− lymphocytes (P<0.05) already at Days 1 and 3 postreperfusion and of CD4+ cells on Day 3. By Day 3, every sixth lymphocyte found ipsilaterally had a CD4+/CD8− phenotype. Again, no significant increase of lymphocytes contralesionally was observed.
### Table. Antibodies Cocktails Used for Flow Cytometry and Immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen Isotype Supplier</th>
<th>Suppliers</th>
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<tbody>
<tr>
<td>CD11c ArHam IgG(N418)</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD3 Rabbit IgG polyclonal</td>
<td>DAKO</td>
</tr>
<tr>
<td>Ly6G Rat IgG2b(LIMP-R14)</td>
<td>Hycult</td>
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<td>Iba1 Rabbit IgG polyclonal</td>
<td>Wako</td>
</tr>
<tr>
<td>ArHam IgG AP-</td>
<td>Jackson</td>
</tr>
<tr>
<td>Rabbit IgG AP-</td>
<td>Jackson</td>
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<tr>
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<td>Jackson</td>
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<tr>
<td>Mouse IgG AP-</td>
<td>Jackson</td>
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<tr>
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<td>NK1.1-PE-Cy7 Ms IgG2a,k</td>
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<tr>
<td>CD8-PaBlue Rat IgG2a,k</td>
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GFAP indicates glial fibrillary acidic protein; APC, antigen-presenting cell.
Dendritic Cells
Somewhat surprisingly, a strong and significant increase in cell numbers was observed in DCs (Figure 2F). Compared with sham-operated animals, there was a 20-fold increase on Day 3 (600 versus 13,000 cells/hemisphere; \( P < 0.001 \); Figure 2F), which was still 12-fold on Day 7 (7,000 cells/hemisphere). The largest fraction was myeloid DCs (Figure 2F). The increase was even more pronounced if only MHCII high-expressing DCs were analyzed, resulting in a highly significant 100-fold increase compared with sham conditions (Figure 3C).

NK Cells
The quantification of NK cells (NK, CD45\(^+/SSC^{low}/\) NK1.1\(^+\)) showed a significant increase in cell numbers of immune regulatory NKT cells at Day 3 (\( P < 0.05 \); Figure 2G) but no significant changes for NK cells (Figure 2G).

Activation Markers on Antigen-Presenting Cells
Although absolute numbers of immune effector cells reflect the magnitude of inflammation, activation levels illustrate the intensity. Therefore, we quantified the expression of the costimulatory molecules CD40, CD80, and major histocompatibility complex (MHC) Class II molecules on antigen-presenting cells.

A statistically significant increase in median fluorescence intensity of MHC II was observed ipsilesionally on DCs at Day 3 (\( P < 0.05 \); Figure 3A–F), reflecting an increase in median MHC II molecules per cell. Our data also indicated a rapid increase in numbers of MHC II-positive DCs in the ischemic hemisphere (Figure 3C). Neither macrophages nor microglia showed a similar increase in MHC Class II expression, although macrophages did show some MHC II upregulation not reaching significance (Figure 3A, F). Interestingly, although no increase in numbers of DCs was seen contralaterally (Figure 2F), we saw a strong but not significant trend toward higher MHC II expression on these cells (not shown).

We also observed a reproducible but nonsignificant increase of CD80 expression on DCs and macrophages (Figure 3A, D) in the ischemic hemisphere. Unexpectedly, MHC II high-expressing DCs did not upregulate costimulatory CD80 (Figure 3B) compared with MHC II\(^{low}\) DCs. The activation marker CD40 was not significantly different between sham and ischemic conditions (Figure 3A, E). Of note, microglia did not either upregulate significant amounts of MHC II nor costimulatory molecules, although we saw a trend toward higher CD80 expression (Figure 3A, D–F).

T-Cell Activation and FoxP3 Expression
To characterize the corresponding T-cell activation, we used CD25 and CD69 on CD3-positive CD4\(^+\) or CD8\(^+\) cells. No significant upregulation of these markers was found (data not shown). This could be due to only transient expression of these early activation markers. We found less than 5% FoxP3-positive T-cells (CD4\(^+\) 4.2%, CD8\(^+\) 1.3%, CD4\(^+\)/CD8\(^−\) 2.2%; Figure 4). In splenic T-lymphocytes, we found a higher percentage of CD4\(^+\) FoxP3\(^+\) cells (21.0%) and CD8\(^+\) FoxP3\(^+\) lymphocytes (9.4%) but no CD4\(^−\)/CD8\(^−\) FoxP3\(^+\) cells (Figure 4).

Discussion
In the last decade, the interest in the inflammatory response after cerebral ischemia has risen exponentially. The extent of neuronal damage seems to correlate with the degree of innate immune activity and numerous studies have demonstrated the critical role of the cellular and humoral immune system in postischemic brain injury.\(^1\) In experimental animal models in mice and rat, the crucial functions of invading immune cells and proinflammatory cytokines are well accepted.\(^4,13\) Also, imaging studies in human stroke with ultrasmall superpara-
Figure 2. Temporal and quantitative characterization of postischemic inflammation by whole brain multicolor flow cytometry for sham conditions 12 hours, 1, 3, and 7 days postreperfusion. A–G, Infiltrating cells per animal and hemisphere. A, Microglia; (B) neutrophils; (C) macrophages; (D) lymphocytes (black, T-cells; white, B-cells); (E) T-lymphocytes (black, CD4+/CD8−; gray, CD4−/CD8+; white, CD4+/CD8+). F, DCs (white, myeloid; black, nonmyeloid). G, NK cells (white, NK; black, NKT). H, Mean relative composition of infiltrating immune cells, Microglia (white), neutrophils (gray), and remaining immune cells (macrophages, DCs, lymphocytes, and NK cells; black). I, Comparison of neutrophil and macrophage infiltration into the ipsilesional hemisphere. *P<0.05; **P<0.01; ***P<0.001. Error bars=SEM.
magnetic iron oxide-enhanced MRI and histological studies of human stroke lesions showed evidence for an inflammatory response in the ischemic region. Nevertheless, an exact quantification and characterization of the invading cell populations and their temporal distribution is lacking.

In accordance with previous reports, we found the majority of immune cells in the ischemic hemisphere 3 days after reperfusion to be neutrophils (Figures 2H and 5A). However, in our model, the peak of the neutrophil influx occurred later than previously reported. Rather, it appears that the influx of neutrophils into the parenchyma is preceded by an accumulation of microglial cells and to a lesser extent macrophages, lymphocytes, and DCs (Figures 2 and 5A–B). The early accumulation of these immune cells and activation of large amounts of DCs could be critical for the subsequent massive accumulation of neutrophils. Activated immune cells in the postischemic area are producing proinflammatory cytokines, including interleukin-1, interleukin-6 and tumor necrosis factor-α. These cytokines are involved in the upregulation of cell adhesion molecules, including intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin, thereby promoting neutrophil accumulation and migration into the brain parenchyma.

The importance of neutrophil infiltration during stroke has been demonstrated in several species. It was shown that neutrophils are involved in rat focal ischemia and that there is a dramatic accumulation of neutrophils in infarcted tissue during reperfusion. This finding was further supported by a number of other studies in rat models of ischemic stroke in which neutrophil infiltration into the ischemic brain was implicated in posts ischemic brain injury. Studies in human brain after ischemic stroke confirmed that neutrophils intensively accumulate in the regions of cerebral infarction. Their accumulation correlates with the severity of brain tissue damage and poor neurological outcome. However, especially in rats but also in mice, several studies did not show a significant improvement after depletion of neutrophils.

**Figure 3.** Antigen-presenting cell (APC) activation patterns. A, Expression of MHC II, CD40, and CD80 on DCs, macrophages, and microglia. Histograms of fluorescence intensities for sham (light) and 3 days postreperfusion conditions (dark) of ipsilesional APCs in the brain are shown. B, CD80 expression of DCs in sham mice (light), MHC II high-expressing DCs (dark), and MHC II low-expressing DCs (dark) in stroked brains (ipsilesional Day 3). C, Accumulation of MHC II high-expressing DCs in the ipsilesional hemisphere. D–F, Time course of MHC II (D), CD40 (E), or CD80 (F) expression on DCs, macrophages, and microglia in the ipsilesional hemisphere. Median autofluorescence subtracted from median fluorescence (AMFI). Error bars = SEM. NS indicates not significant; *P < 0.05; ***P < 0.001.
Some of these variable results might be explained by the late arrival of neutrophils in the infarct area.

The massive increase and proliferation of microglia or macrophages in the border zone of the ischemic area has been reported previously. The function of macrophages and microglial cells in the postischemic response is still under debate. On one side, these cell populations produce toxic molecules (eg, nitric oxide, oxygen radicals, arachidonic acid derivatives) and proinflammatory cytokines that potentially damage neurons, oligodendrocytes, and extracellular matrix. On the other side, immune cells phagocyte debris and microglial cells are capable of producing neurotrophins as well as transforming growth factor-β1 and plasminogen, which are involved in tissue repair. In addition, macrophages and microglial cells may act as antigen-presenting cells and are potentially involved in the restimulation of T-cells in the ischemic zone. Interestingly, we did not observe a significant upregulation of MHC II and costimulatory molecules on macrophages and microglial cells.

The detrimental role of both CD4+ and CD8+ T-cells has recently been described in an experimental stroke model in mice. Supporting the crucial function in ischemia/reperfusion injury, T-cells can also be detected after cerebral ischemia in rats and even humans. In accordance, we demonstrated a significant increase in T-lymphocytes 3 days postreperfusion. Remarkably, not only CD4+ and CD8+ lymphocytes, but also CD4+/CD8− lymphocytes showed an increase in the ischemic hemisphere of which a small per-
percentage was FoxP3-positive. Double-negative T-cells have been suggested as regulatory cells with important roles in the maintenance of immunologic self-tolerance and in down-regulating various immune responses. Overall, the function of regulatory immune cells in stroke is not understood. Nevertheless, several recent studies have shown that preischemic T-cell tolerization is beneficial in stroke suggesting crucial functions of immune regulatory T-cells in the postischemic immune response.

In parallel to the increase in lymphocytes, we observed an increase in NKT cells in the ischemic hemisphere, whereas the number of NK cells did not increase. NKT cells are a subset of T-cells that coexpress an aβ-T-cell receptor but also express a variety of molecular markers associated with NK cells such as NK1.1. Several reports have suggested that NKT cells may serve as regulatory cells in autoimmune diseases, including multiple sclerosis. However, NKT cells can also augment inflammatory conditions, and their crucial function in postischemic tissue is underlined by their importance in renal ischemia reperfusion injury.

Surprisingly, DCs showed one of the largest increases in cell numbers. The accumulation in ischemic hemispheres started already on Day 1 and reached its maximum on Day 3 after reperfusion. High expression of CD11b indicated a myeloid origin. DCs are key elements in the control of immune activation or immune tolerance. We could show strong and sustained upregulation of MHC II and only mild and not significant upregulation of CD80 on the DCs. These costimulatory molecules play a vital part in activating lymphocytes after encounter of a pathogen by an antigen-presenting cell. In contrast, lack of these molecules while presenting MHC II bound antigen leads to anergy and clonal deletion of T-lymphocytes and activates regulatory T-cells. Although all DCs in the ischemic hemisphere showed similar levels of CD80 expression, there appeared to be 2 different populations of DCs based on MHC II expression. Whether these 2 different states are important for immune regulation in postischemic inflammation remains to be elucidated. However, the lack of a concomitant upregulation of costimulatory molecules on DCs with a high expression of MHC II in the ischemic brain might offer an explanation for the missing coincidence of stroke and a long-lasting and detrimental local autoimmune response in the postischemic area. This hypothesis might further be supported by the observed failure in T-cell activation in our study. In addition, some of the newly described cell populations such as NKT cells and CD4−/CD8− T-cells may have additional regulatory properties.

In summary, we provide a precise analysis of the spatial and temporal infiltration of immune cells in an ischemia reperfusion model (Figure 5). Few others have either concentrated on permanent ischemia models or have not performed an extensive subdifferentiation of immune cells. We show that the infiltration of the penumbra by macrophages, lymphocytes, and DCs precedes the subsequent influx of neutrophils into the ischemic hemisphere. Moreover, we demonstrate a massive influx of immune competent DCs upregulating MHC II, which is not paralleled by equally upregulated costimulatory molecules. Furthermore, we find regulatory immune cells accumulating in the ischemic hemisphere, including FoxP3 lymphocytes, NKT cells, and CD4−/CD8− T-cells.

For a better understanding of the inflammatory response after cerebral ischemia, further characterization of the functions and mechanisms of inflammatory immune cells, but also of regulatory immune cells, is needed. In the clinical setting, an improved knowledge of these mechanisms could be used to develop new therapies in stroke.

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Disclosures
None.

References
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