Reduced Efficacy of Circulating Costimulatory Cells After Focal Cerebral Ischemia

Andreas Hug, MD*; Arthur Liesz, MD*; Bettina Muerle, MD; Wei Zhou; Julia Ehrenheim; Alexander Lorenz; Alexander Dalpke, MD; Roland Veltkamp, MD

Background and Purpose—Cerebral ischemia is ensued by a cellular immune depression syndrome. The postsischemic functional capacity of T lymphocytes is controversial, and interactions between leukocyte subsets are largely unknown. Understanding the immunologic interplay between antigen-presenting cells and lymphocytes as well as between distinct lymphocyte subsets after stroke might be of clinical/therapeutic significance because animal data argue for a cerebroprotective effect of, for example, CD4+/CD25+ regulatory T cells.

Methods—Ex vivo CD4+ T cell proliferation was analyzed in experimental and human stroke using fluorescence activated cell sorter analysis. To investigate suppressive effects of CD4+/CD25+ regulatory T cells as well as the influence of costimulatory cells on CD4+ T cell proliferation, subsets were magnetically sorted before proliferation assay setup.

Results—After stroke: (1) proliferation of mouse and human CD4+ T cells on T cell receptor stimulation was unaltered; (2) the suppressive effect of CD4+/CD25+ regulatory T cells in mouse and man was unaltered; and (3) efficacy of circulating costimulatory cells from stroke animals was reduced by a mean of 0.6 (SEM 0.1, P=0.001) CD4+ T cell division numbers compared with sham-treated animals.

Conclusions—Reduced costimulatory efficacy of circulating costimulatory cells in mice is an important feature of stroke-induced immunodepression. Understanding the interplay of costimulatory cells and responder T cells (eg, CD4+ T cells or CD4+CD25+ regulatory T cells) after stroke may offer new insights into the prevention of secondary inflammatory damage to the brain and help to guide new therapeutic strategies. (Stroke. 2011;42:3580-3586.)

Key Words: costimulation • ischemic stroke • T lymphocytes • Treg

Cerebral ischemia is followed by a cellular immune depression syndrome,1–4 whose pathophysiological relevance has yet to be fully determined. On the 1 hand, stroke-induced immune depression may prevent hosts from secondary inflammatory damage to the central nervous system.5 On the other hand, this immune depression renders animals and humans susceptible to severe infections.1,6,7 Infarct size is a major factor because it aggravates both cellular immune depression and susceptibility to infection in humans.8,9

The cellular and subcellular immunologic mechanisms of cerebral ischemia-induced immune depression are only partially understood. Particularly, the differential impact of specific circulating leukocyte subsets and their immunologic interplay early after acute cerebral ischemia have not been well characterized. In humans, a functional deactivation of circulating monocytes (eg, reduction of cell surface HLA-DR expression, reduced release of proinflammatory cytokines) was reported7 and this monocyte malfunction was demonstrated to be associated with a susceptibility to infections.7,8,10 Moreover, data derived from mice with large middle cerebral artery infarcts suggest a reduced ex vivo proliferation of splenocytes on polyclonal T cell stimulation.11 This observation, however, is challenged by human data, in which ex vivo proliferation of whole blood lymphocytes was unimpaired and phenotypes of circulating lymphocytes were skewed to a proinflammatory state.12 Even more complex, the immunologic interaction of antigen-presenting cells and T cells (T cell receptor stimulation, costimulation) as well as between distinct lymphocyte subsets (eg, detrimental CD4+ T cells13 and cerebroprotective CD4+CD25+ Treg14), which are differentially affected after brain ischemia,15,16 is unresolved.

Understanding the immunologic interplay between antigen-presenting cells and lymphocytes as well as between distinct lymphocyte subsets after brain ischemia might be of clinical/therapeutic significance because animal data argue for neurotoxic properties of proinflammatory lymphocytes13,17,18 in contrast to a cerebroprotective effect of, for
example, CD4+CD25+ Treg.14 In this respect, the endogenously driven relative increase of Treg in mice11,15 and humans16 might mediate cerebroprotection, however, on the cost of increased susceptibility to infection.

The purpose of the present study was to functionally characterize in ex vivo experiments the impact of focal cerebral ischemia in the mouse and humans (1) on proliferation of isolated CD4+ T cells; (2) on the suppressive capacity of isolated Treg on CD4+ T cells; and (3) on the potency of circulating costimulatory cells (CC) on CD4+ T cell proliferation.

Methods

Experimental Stroke Model

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 (Regierungspräsidium, Karlsruhe, Germany). Ninety-minute filament-induced middle cerebral artery occlusion (MCAO) was performed in 10- to 12-week-old male C57BL/6 mice as published previously.15 All functional ex vivo analyses were performed on spleen cells. Spleens were harvested on Day 3 after sham and MCAO treatment, respectively. Spleens of 2 MCAO animals were pooled because splenocyte yield was substantially lower compared with sham animals. Single cell suspensions were generated in phosphate-buffered saline by meshing the homogenate through a 40-mesh cell strainer (BD Biosciences). Infarct volumes were assessed by histology (see http://stroke.ahajournals.org/) as previously reported.19

Patient/Control Characterization

All study procedures were performed after obtaining informed consent according to a protocol approved by the independent local ethics committee of the Medical Faculty of the University Heidelberg. Twenty-two patients with acute ischemic stroke were enrolled. Blood was drawn on Day 3 after admission. Admission was defined as Day 0. Control individuals (n=10) were patients scheduled to undergo elective cataract surgery without a history of stroke, myocardial infarction, or peripheral artery disease. Infarct size was determined on a 24- to 36-hours follow-up CT or MRI scan as previously described.8 Based on unpublished data in which a National Institutes of Health Stroke Scale score of >8 discriminated well between the absence/presence of stroke-associated infections, we chose this cutoff to categorize patients into small and severe infarct cohorts.

Cell Separation

Peripheral blood mononuclear cells were isolated using density gradient centrifugation. CD4+ and CD4+CD25+ cells were separated using magnetic cell sorting according to the manufacturer’s instructions. After a T cell depletion step (Pan T Cell Isolation Kit II; Miltenyi Biotec), the CD4+CD25+ Regulatory T Cell Isolation Kit was used (Miltenyi Biotec). Purity of cells was routinely >90% for the CD4+ subset. Purity analyses for the CD4+CD25+ subset were done on a FACSCalibur after intracellular FoxP3 staining according to the manufacturer’s instructions (eBioscienes).

Proliferation/Suppression Assays

To track proliferation by fluorescence activated cell sorter analysis, CD4+ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE Cell Proliferation Kit; Invitrogen). A total of 1×10^6 CD4+ T cells/mL phosphate-buffered saline+5% fetal calf serum was treated with 3.6 μmol/L of CFSE for 5 minutes at room temperature. Culture medium for assays consisted of 1640 RPMI, 10% fetal calf serum, 100 μg/mL penicillin/streptomycin, 1 mmol/L Sodium-Pyruvat (all Invitrogen), and 0.05 mmol/L Mercapto-Ethanol (Roht). Cultures were performed in duplicate or triplicate on 96-well round-bottom plates (Greiner) in a total volume of 200 μL. For T cell receptor stimulation of human CD4+ T cells, species-specific anti-CD3 antibodies (eBioscience) were bound to the plates in 30 μL phosphate-buffered saline with indicated concentrations for 30 minutes at 37°C.

Human Cells

A total of 1×10^6 CD4+ T cells were used per well. Stimulation was performed using 0.1 μg/mL of plate-bound anti-CD3 and 0.1 μg soluble anti-CD28. For suppression assays, 0.25×10^5 Treg per well were added. Cells were cultured for 24 to 120 hours at 37°C, harvested, and directly analyzed by fluorescence activated cell sorter analysis.

Mouse Cells

A total of 5×10^5 CD4+ T cells were used per well. Additionally, 5×10^5 syngeneic spleen non-T cells (CC) were used per well after treatment with mitomycin C (briefly 5×10^7 AC in 1 mL phosphate-buffered saline for 20 minutes at 37°C with 50 μg/mL mitomycin C; Sigma-Aldrich). CD4+ T cells were stimulated with soluble species-specific anti-CD3 (eBioscience) at concentrations of 0.1 and 1.0 μg/mL. Cells were cultured for the indicated periods of time. For the suppression analyses, cells were cultured for 60 hours. Numbers of Treg were added to the cultures as indicated in Figures 3 and 4, and Supplemental Figure 1 (Treg ratio with respect to CD4+ T cells).

Analysis of Proliferation/Suppression

Fluorescence activated cell sorter analysis of human cells was performed on a FACSCalibur after CFSE staining without further labeling. Histograms of CFSE-fluorescence intensity were generated and the percentages of proliferated and unproliferated cells were calculated. Twenty-four-, 48-, 72-, 96-, and 120-hour proliferation fractions of CD4+ T cell cultures with and without CD4+CD25+ Treg cells were subtracted and the relative difference was used as estimation for suppression.

Mouse cells were analyzed using a BD LSR II. Analysis was done according to previously published protocols.20,21 Before flow cytometric analysis, cells were labeled with 5 μg/mL Hoechst 33258 (Sigma-Aldrich) and anti-CD4 Allophycocyanin (eBioscience).

Intracellular staining was done using anti-interferon-γ Allophycocyanin as T-helper 1- and anti-interleukin-4-PE as T-helper 2-cytokine markers, respectively, according to the instructions of the manufacturer (BD Biosciences). Cells were analyzed using BD LSR II.

Mean division numbers of mouse spleen CD4+ T cell proliferation assays were generated in 2 steps. First, a peak-fitting algorithm was applied to identify distinct CFSE peaks (Weasle-software; WEHI). Second, percentages of each peak were tabulated and fitted to a Gaussian curve. Hypothesis testing for categorical data were done using χ² analysis. For continuous data, t tests, analysis of variance, or Wilcoxon rank sum tests were applied as appropriate. An α level <0.05 was regarded statistically significant. A general linear model was fitted to analyze the independent effect of CC on CD4+ T cell proliferation in suppression assays. As a dependent variable, the mean division number of stimulated CD4+ T cells was chosen. The final model was parameterized with the variables: Treg ratios, anti-CD3 stimulus strength, and source of CC (sham or MCAO). Marginal means were used to estimate differences between CC groups. Another general linear model was fitted to estimate the effect of human stroke on proliferation of CD4+ T cells. In the final model, group status (levels: control, small stroke, severe stroke) and day of harvest (levels: 24, 48, 72, 96, 120 hours) were included as explanatory variables. Statistical analysis was done using SAS 9.2, JMP 8.0, and OriginLab 8.5 for Windows.

Results

Animal MCAO/Sham Data

The infarct volume was 38.8±1.1 (mean±SEM) mm³. Compared with sham animals, MCAO treatment led to a 50%
reduction of splenocyte counts on Day 3 after stroke (7.8±0.67×10^7 versus 4.3±0.84×10^7, *P=0.003*). Treg amounts per spleen were as follows: sham 1.5×10^6 compared to 0.7×10^6 in MCAO animals (*P<0.0001*). The cellular composition of isolated CC was comparable between sham and MCAO animals. This was also true for FoxP3-expressing non-T cells (Supplemental Table I).

**Patients With Acute Ischemic Stroke**

Patients were grouped according to their National Institutes of Health Stroke Scale score on admission (median for the small group: 6; interquartile range [IQR], 3–7; severe group: 15; IQR, 12–20; Table). Median infarct volumes were 15.5 (IQR, 1–30) and 72 (IQR, 32–178) for the small and severe stroke groups, respectively. The median time from stroke onset to blood sampling was 2.9 (IQR, 2.4–3.7) and 2.8 (IQR, 2.4–3.3) days, respectively. Routine blood analysis revealed higher serum C-reactive protein values and lower lymphocyte counts in the severe stroke cohort. After the isolation of CC at all analyzed time points with saturation of proliferation at approximately 60 hours of culture, Varying anti-CD3 dose (0.1, 1, and 10 μg/mL) revealed dose dependency (Figure 1A–B) and MCAO animals. This was also true for FoxP3-expressing non-T cells (Supplemental Table I). In analogy, no differences in the suppressive capacity of sham and MCAO-treated mice after in vitro stimulation with anti-CD3 (1 μg/mL), interferon-γ production was detectable in almost all CD4+ T cells as shown by intracellular fluorescence activated cell sorter analysis (Figure 1C–D). The T-helper 2 cytokine interleukin-4 was not detectable in the cultured CD4+ cells (Figure 1D).

**Ex Vivo Proliferation of Human CD4+ T Cells**

In analogy to the demonstrated animal data, ex vivo stimulation (0.1 μg/mL plate-bound anti-CD3 and 0.1 μg/mL soluble anti-CD28) of human CD4+ T cells revealed no differences in CD4+ T cell proliferation among control, small, and severe stroke groups (Figure 2A–B). In general linear model analysis adjusting for sequential time points of analysis (hours 24, 48, 72, 96, and 120), neither cohort exhibited an independent effect on the ex vivo proliferation fraction of CD4+ T cells (Figure 2B).

**The Suppressive Capacity of CD4+CD25+ Treg Cells**

Regardless of the T cell receptor signal strength (0.1 or 1.0 μg/mL anti-CD3) in mice, the suppressive capacity of spleen CD4+CD25+ Treg cells of sham or MCAO-treated animals on sham-treated CD4+ T cells cocultured with syngeneic sham CC did not differ (Figure 3A–B). Suppression was clearly dose-dependent as indicated by an increasing proliferation with decreasing ratios of CD4+CD25+ Treg/CD4+ T cells. Purity of sorted CD4+CD25+ Treg cells revealed no differences in CD4+CD25+ Treg cells of sham or MCAO-treated animals (Figure 2B).

### Table. Sample Characteristics (Patients)

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<th>Group</th>
<th>Control</th>
<th>Small</th>
<th>Severe</th>
<th>P</th>
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<td>6</td>
<td>16</td>
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<td>4</td>
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<td>2.0 (1.1–4.9)</td>
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<tr>
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<td>72 (32–178)</td>
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<td>CRP, mean±SEM</td>
<td>4.4±1.0</td>
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<td>68.7±13.5</td>
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<td>Leukocytes/mL, mean±SEM</td>
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<td>Purity of sorted CD4+CD25+ Treg, %, mean±SEM</td>
<td>91.5±2.99 (n = 3)</td>
<td>not available</td>
<td>89.0±3.18 (n = 3)</td>
<td>0.598</td>
</tr>
</tbody>
</table>

*IQR indicates interquartile range; NIHSSS, National Institutes of Health Stroke Scale score; CRP, C-reactive protein; Treg, regulatory T cells.

*Control vs severe and small vs severe.
†Control vs severe (Tukey post hoc test).
cells among control subjects, patients with small stroke, and patients with severe stroke could be observed (Figure 3C). Purity of sorted CD4⁺/CD25⁻ cells as analyzed by the intracellular FoxP3 expression was comparable between control and stroke individuals (Table).

Costimulatory Cells Seem to Play an Important Role for the Proliferation Response After Cerebral Ischemia

In mice, additional experiments were done using T cell-depleted syngeneic splenocytes as CC of 90 minutes MCAO and sham animals, respectively. Using 90-minute MCAO animal CC, the proliferation responses were weaker by 0.6±0.1 division numbers (general linear model analysis, \( P=0.001; R^2 \) for model fit=0.55; Figure 4A). Because a reduced proliferation of sham CD4⁺ T cells in coculture with MCAO CC was already obvious without the addition of CD4⁺CD25⁺ cells, CC seem to mediate effects through a CC:CD4⁺ T cell interaction. The CC effect was linearly distributed over all analyzed ratios of Treg:CD4⁺ T cells, indicating no additional effect of CC on CD4⁺CD25⁺ Treg (Figure 4B). Due to immunologic limitations (allogeneic response), similar experiments in humans were not feasible.

Due to the reduced costimulatory efficacy of CC from MCAO-treated animals, we analyzed the ex vivo major histocompatibility complex II surface expression and interleukin-12 secretion of sorted dendritic cells from mouse spleens on stimulation with CpG-oligodeoxynucleotide. Major histocompatibility complex II mean fluorescence intensity was lower in MCAO- compared with sham-treated animals: 1181 (IQR, 680–1386) and 1498 (IQR, 1437–1551), respectively. Interleukin-12 secretion was also reduced in MCAO-
treated animals: 1192 pg/mL (IQR, 284–2791) and 3040 pg/mL (IQR, 1854–4639), respectively (Figure 4C).

**Discussion**

Focal cerebral ischemia is ensued by a cellular immune depression syndrome with an absolute and relative decomposition of circulating leukocyte subsets. In this context, the role of specific leukocyte subsets and their immunologic interplay are not very well characterized.

In this report, we demonstrate that within the first 3 days after the onset of focal cerebral ischemia, (1) the proliferative response of mouse and human CD4+ T cells to T cell receptor stimulation is unaltered; (2) the suppressive effect of Treg in the mouse and humans is unchanged; and (3) the costimulatory effect of circulating CC on responding CD4+ T cells is reduced in mice.

With respect to the ex vivo proliferation of T lymphocytes, animal and human data are conflicting. Animal data argue for a markedly reduced capacity of T lymphocytes to proliferate, whereas human data argue for an unimpaired ex vivo proliferation. Our current findings corroborate the so far
published human data. One reason for the discrepancies might be stroke severity. Both in animals and humans, stroke severity was clearly shown to be associated with the extent of cellular immune depression and susceptibility to infection.1,8,15 The animal model used by Offner et al11 led to a massive lymphocytopenia, in which 4 days after experimental stroke, splenocyte counts decreased to only approximately 10% compared with sham-treated animals. In contrast, our experimental stroke model led to a 50% splenocyte count reduction compared with the sham control.

Another potential issue for the discrepant findings may be that proliferation in previous studies was measured only at 1 time point (ie, 96 hours after assay setup). In contrast, we monitored proliferation of mouse CD4+ T cells using the CFSE dilution method at several time points over a total period of 72 hours. In our assay, viability of cultured cells was monitored very closely (at least every 24 hours). After approximately 60 hours in culture, T cell proliferation was saturated. Therefore, it would not have been possible to measure any effect if we had measured proliferation after 60 hours of culture. Hence, reduced survival of assayed cells (eg, apoptosis, activation induced cell death) might have confounded previous studies. When stimulating cultured CD4+ T cells, features like apoptosis or activation-induced cell death should be accounted for, because several animal and human studies reported an increased activation status of circulating T lymphocytes after acute focal cerebral ischemia.16,22

Our current finding, of a decreased costimulatory efficacy of circulating CC after experimental stroke could be a further explanation for the reduced ex vivo proliferation of T lymphocytes in a previous report when unsorted splenocytes were stimulated with ConA or anti-CD3.11 Our proliferation assays of sorted sham-treated CD4+ responder T cells and either MCAO- or sham-treated CC accounted for this potential confounder.

Due to the fact of their cerebroprotective effect,14 we were also interested in the postischemic functional capacity of endogenous CD4+CD25+ Treg. We could clearly demonstrate that CD4+CD25+ Treg of mice and humans are fully functional after stroke with respect to their suppressive effect on ex vivo proliferating CD4+ T cells. The fact of a previously reported relative increase of CD4+CD25+ Treg after stroke11,15,16 and our observation of a weaker costimulatory effect of non-T cells might suggest that the net suppressive activity of endogenous peripheral CD4+CD25+ Treg is dominant after brain ischemia. Moreover, weaker stimuli have been shown to preferentially activate Treg over CD4+ effector T cells.23–25 Although this scenario is hypothetical, it is supported by 2 previous studies. Therin, sensitization to brain-specific autoantigens after stroke was associated with worse outcome26 and induction of tolerance against brain-specific peptides like myelin basic protein was associated with less severe infarctions,27 yet other Treg-independent mechanisms may also account for this.

Our study has several limitations: (1) so far, the reduced costimulatory efficacy was analyzed in mice only; (2) the specific mediating cell subset (eg, dendritic cells, monocytes, B cells) has yet to be determined; and (3) the reduced poststroke costimulation is representative for systemic (spleen) and not for brain-specific CC.

In conclusion, reduced costimulatory efficacy of circulating CC may be a key feature of stroke-induced immunodepression. Understanding the interplay between stimulating CC and responder T cells after stroke may offer new insights into the prevention of secondary inflammatory damage to the brain and help to guide new therapeutic strategies.

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Disclosures
None.

References


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Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2011/10/02/STROKEAHA.111.620948.DC1
Reduced Costimulatory Efficacy Of Circulating Costimulatory Cells After Focal Cerebral Ischemia

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Supplemental Methods

**Supplemental table 1 (S1)** Composition of sorted spleen cell fractions

**Supplemental figure 1 (S2)** Peripheral blood white cell counts in mice an man

**Supplemental figure 2 (S3)** FoxP3 expression of sorted human CD4+CD25 Treg
Supplemental Methods

*Cellular subset characterization of the mouse spleen CC fraction.*
Spleens from sham- (n=2) and MCAO-treated (n=2) mice were used. After magnetic cell sorting of single cell suspensions of mouse spleens with the use of a T Cell depletion kit (Pan T Cell Isolation Kit II, Miltenyi Biotec) the remaining magnetically labeled fraction of splenocytes (CC) was stained with directly fluorophore-labeled monoclonal anti-mouse antibodies (anti-B220, anti-CD11c, anti-Gr-1, anti-NK1.1) (all BD Biosciences). For this purpose 2x10^5 cells were stained with each antibody for 30 min in 100 µl staining buffer and washed once. For intracellular staining of Foxp3 an anti-mouse-Foxp3-PE antibody (eBioscience) was used according to the manufacturers’ instructions. Analysis of cell fractions was performed on a FACSCalibur.

*Proliferation/suppression assays of mouse cells.*
For proliferation analysis three independent experiments with multiple time points for anti-CD3 concentration 0,1 µg/ml (see figure 1A) and three independent experiments for anti-CD3-concentration 1,0 µg/ml (see figure 1B) were performed. For each single experiment one spleen of a sham-treated animal and 2 (pooled) spleens from MCAO-treated mice were used. For suppression analyses three independent experiments were performed. For each single experiment a Treg-titration series was performed. Each single experiment was done with the use of one spleen from a sham-animal and 2 (pooled) spleens from MCAO-treated mice. Hence, we sacrificed 9 mice for the analyses in figure 1A, 9 animals for analyses in figure 1B and 9 animals for analyses in figure 4B. Totally 27 animals were sacrificed for the proliferation and suppression analyses.

*Ex vivo functional analysis of dendritic cells from mouse spleens.*
Spleens were removed, disrupted gently, minced and mashed through a 100 µm cell strainer. Spleen cell suspensions of three mice were pooled and CD11c positive cells (dendritic cells) were sorted by MACS according to the recommendations of the manufacturer (Miltenyi). 7 independent experiments were performed. 1.5x10^5 sorted cells were stimulated with the 1000 nM TLR9 ligand CpG-Oligodesoxynucleotide #1668 (fully phosphothioate modified, TIB Molbiol, Berlin, Germany) overnight. Supernatants were removed and analyzed for secretion of IL12p40 by sandwich ELISA following the recommendation of the manufacturer (BD Biosciences). Furthermore 2x10^5 cells were stained for CD11c (PE) in combination with MHC-class II (FITC) (all from BD Biosciences) for 30 min in 100 µl of staining buffer, washed and analyzed by flow cytometry (BD FACS Canto). CD11c positive cells were analyzed for mean fluorescence intensity.
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<th>MCAO (%)</th>
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<td></td>
<td>n=2</td>
<td>n=2</td>
<td></td>
</tr>
<tr>
<td>CC</td>
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<td>86.2±1.5</td>
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Composition of sorted spleen cell fractions. CC: costimulatory cells. DC: Dendritic Cells
Supplemental Figure 1
A. Mice white blood cell counts 3 days after sham- or 90 min MCAO-treatment
B. Human white blood cell counts 3 days after stroke onset grouped into small and severe infarcts according to the admission NIHSSS
Supplemental Figure 2

A. Representative image for a FACS-analysis of FoxP3 expression in sorted CD4+CD25+ cells of a control individual

B. Representative image for a FACS-analysis of FoxP3 expression in sorted CD4+CD25+ cells of a stroke patient