Bone Marrow Cell Transplantation Time-Dependently Abolishes Efficacy of Granulocyte Colony-Stimulating Factor After Stroke in Hypertensive Rats

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Background and Purpose—We aimed to determine a possible synergistic effect of granulocyte colony-stimulating factor (G-CSF) and bone marrow–derived mononuclear cells (BM MNC) after stroke in spontaneously hypertensive rats.

Methods—Male spontaneously hypertensive rats were subjected to middle cerebral artery occlusion and randomly assigned to daily injection of 50 μg/kg G-CSF for 5 days starting 1 hour after stroke (groups 1, 2, and 3) with additional intravenous transplantation of 1.5×10E7 BM MNC per kilogram at 6 hours (group 2) or 48 hours (group 3) after stroke, or control treatment (group 4). Circulating leukocyte counts and functional deficits, infarct volume, and brain edema were repeatedly assessed in the first week and first month.

Results—G-CSF treatment led to a significant neutrophilia, to a reversal of postischemic depression of circulating leukocytes, and to a significantly improved functional recovery without affecting the infarct volume or brain edema. BM MNC cotransplantation was neutral after 6 hours, but reversed the functional effect of G-CSF after 48 hours. Short-term investigation of combined G-CSF and BM MNC treatment at 48 hours indicated splenic accumulation of granulocytes and transplanted cells, accompanied by a significant rise of granulocytes in the circulation and the ischemic brain.

Conclusions—G-CSF improved functional recovery in spontaneously hypertensive rats, but this effect was abolished by cotransplantation of BM MNC after 48 hours. In the spleen, transplanted cells may hinder the clearance of granulocytes that were massively increased by G-CSF. Increased circulation and infiltration of granulocytes into the ischemic brain may be detrimental for stroke outcome. (Stroke. 2014;45:2431-2437.)

Key Words: cell- and tissue-based therapy ■ granulocyte colony-stimulating factor ■ immune system ■ stroke

Stroke remains a major challenge of modern medicine, belonging to the most frequent causes of death and permanent disability, whereas causal treatment is not applicable to most patients. Unfortunately, many promising drug candidates got lost in translation, with the hematopoietic hormone granulocyte colony-stimulating factor (G-CSF) being one of the most recent examples.1 However, this result strikingly contrasts with the convincing preclinical data for G-CSF and may be owed to the fact that therapeutic effect sizes decline with increasing study population heterogeneity and complexity. Hence, enhancing the therapeutic effect, for instance, by synergistic combination2 of G-CSF with another favorable approach may afford its clinical use.

Evidence exists that the mobilization of bone marrow (BM) cells, either as endogenous response,3 or by G-CSF treatment4 is protective after stroke and significantly improves neurological outcome. However, the latter process requires a successive degradation of cell-arresting stromal cell-derived factor 1 within the BM5 and thus begins delayed after stroke. By contrast, intravenously transplanted BM-derived mononuclear cells (BM MNC) rapidly increase cerebral blood flow and reduced tissue damage after acute and chronic brain ischemia.6,7 We, therefore, hypothesized that the delayed mobilization by G-CSF could be compensated by early cotransplantation of BM MNC, which is also an established preclinical stroke therapy.8

To test this hypothesis, we applied a classical G-CSF regime extended by cotransplantation of syngeneic BM MNC at 6 or 48 hours after experimental stroke. The study was planned in line with recommendations for good preclinical stroke research,9 including the use of comorbid animals and long-term monitoring.
of functional and imaging end points. Surprisingly, our study showed that combining G-CSF and BM MNC at 48 hours had significant adverse effects on the neurological outcome. Further mechanistic experiments indicated an interaction of granulocytes, which were massively mobilized by G-CSF, and transplanted BM MNC in the spleen. The consecutive spliover of granulocytes may finally explain the unfavorable outcome. Although our experiments failed to provide a novel preclinical approach for stroke, it emphasizes the importance of the immune system for the development and translation of stroke therapies.

Materials and Methods
A detailed description of materials and methods is provided in the online-only Data Supplement.

Experimental Stroke
Animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by legal authorities (TVV 12/11). Spontaneously hypertensive rats (SHR; Charles River, Sulztitz, Germany; 12 weeks of age) were anesthetized with ketamine/xylazine and subjected to permanent right middle cerebral artery occlusion (pMCAO).

Experimental Set Up and Treatment
Animals were randomly assigned to following experimental groups: (1) G-CSF monotherapy (G-CSF; n=18) with daily intraperitoneal injection of 50 μg/kg body weight G-CSF for 5 days starting 1 hour after pMCAO; (2) G-CSF plus intravenous transplantation of 1.5x10⁷ BM MNC per kilogram body weight at 6 hours after pMCAO (G-CSF+BM MNC 6 hours; n=18); (3) G-CSF plus intravenous transplantation of 1.5x10⁷ BM MNC per kilogram body weight at 48 hours after pMCAO (G-CSF+BM MNC 48 hours; n=18); (4) control group (control; n=18) receiving phosphate buffered saline instead of G-CSF and BM MNC. Stroke induction, treatments, and analyses were performed by investigators blinded to group allocation.

Study End Points
Infarct volume and space-occupying effect were determined by magnetic resonance imaging at days 1, 3, 7, and 30. Neurological deficits were assessed by the adhesive removal test at days 2, 7, 14, 21, and 28. Circulating leukocytes were identified by specific antigens (Table I in the online-only Data Supplement) within the first week after stroke using flow cytometry. Cytokine levels were repeatedly measured by multiparametric enzyme-linked immunosorbent assay. At day 30 after stroke, expressions of plasticity and inflammation-related genes were assessed by quantitative real-time polymerase chain reaction. T cells were visualized by immunofluorescence staining and counted within the infarct border and the entire ischemic hemisphere.

Biodistribution and Short-Term Effects of Cell Transplantation
In a subgroup analysis, animals were randomly assigned to groups 1, 3, and 4 (n=3 per group). Distribution of PKH26-labeled BM MNC and immune cell frequencies were determined in the blood, BM, and single cell lysates of the spleen and brain at 52 hours after stroke by flow cytometry. Splenic gene expression of pro- and anti-inflammatory cytokines was investigated by real-time polymerase chain reaction.

Results
Study Enrollment
All animals except for 1 control (1.4%) survived pMCAO. One animal of G-CSF+BM MNC 6 hours group was excluded because of partial infarction. Two animals (one in control and one in G-CSF+BM MNC 6 hours) died during day 3 magnetic resonance imaging. Further animals were excluded solely from magnetic resonance imaging because of technical problems (Table II in the online-only Data Supplement). Finally, 1 animal with G-CSF monotherapy was excluded from behavioral tests because of lack of compliance.

Characterization of Cell Graft
Previously isolated and cryopreserved syngeneic BM MNC grafts were thawed and characterized by flow cytometry. We observed only minor variation of the main leukocyte populations among the different cohorts (n=8). Mature B cells compromised the major part of the graft (48.2±6.5%), followed by myeloid CD11b+ cells (22.2±3.0%), T cells (4.9±0.5%), and remaining uncharacterized leukocytes (CD45only: 27.2±3.7%). 7.1±1.3% of BM MNC expressed the CXC chemokine receptor-4 (CXCR4), whereas we did not found detectable CXCR4 expression by CD34+ cells. Hematopoietic progenitor cells analyzed by coexpression of CD45 and CD34 accounted for 5.0±1.5% of all BM MNC with a frequency of 2.0±0.3% for granulocyte/monocyte progenitors determined by colony-forming unit–granulocyte/macrophage. Analysis of programmed cell death among the graft revealed 23.5±3.3% early apoptotic cells, 6.7±2.5% late apoptotic cells, and 1.9±1.0% necrotic cells.

Peripheral Leukocyte Counts
The impact of G-CSF administration and additional BM MNC transplantation on peripheral leukocyte counts was analyzed 6, 48, 52, 96, and 144 hours after pMCAO. Expectably, G-CSF treatment caused a distinct increase of peripheral leukocyte counts, reaching its peak at 48 hours with a successive normalization until 144 hours after stroke. However, additional administration of BM MNC after either 6 or 48 hours did not impact overall leukocyte counts (Figure 1). The increase of leukocytes was predominantly driven by granulocytes and, to a much lesser extent, by monocytes, T cells, B cells, and natural killer cells (Figure 1). Four hours after transplantation of BM MNC at 48 hours, we observed a statistically significant increase of granulocyte counts in the G-CSF+BM MNC group compared with the G-CSF monotherapy (Figure 1). When comparing leukocyte counts of stroke and naive animals, we observed a considerable decrease of T and B cells, monocytes and natural killer cells 48 hours after stroke. This observation is consistent with recent studies and corresponds to the phenomenon of post-stroke immunodepression.12 Although the suppression of T cells, monocytes, and natural killer cells was limited to 48 hours, we found a persistent depression of B cells (Figure 1). Interestingly, at 48 hours after stroke, poststroke cellular immunodepression was largely reversed by G-CSF treatment, independent of a concomitant cell therapy. Moreover, G-CSF provoked a significant downregulation of CXCR4 on peripheral blood mononuclear cells at 48 hours (Figure 1). CXCR4 expression completely recovered in groups with G-CSF monotherapy or additional early (6 hours) BM MNC administration, but remained suppressed in the G-CSF+BM MNC 48 hours group. Finally, differentiation of T cells into T helper cells, cytotoxic T cells, CD4/CD8 double-positive, as well as CD4/CD8 double-negative T cells (data not
shown) and regulatory T cells (Figure 1) revealed no statistically significant differences among the treatment groups.

Serum Cytokines

Serum concentrations of interleukin (IL)-4, IL-10, IL-17A, monocyte chemotactic protein-1, and macrophage inflammatory protein-1α changed significantly as a function of time ($P<0.05$), whereas interferon-γ levels remained stable ≤144 hours after stroke (Figure I in the online-only Data Supplement). At 96 hours, the serum level of macrophage inflammatory protein-1α was slightly but statistically significant reduced by G-CSF+BM MNC 48 hours. Besides that, none of the treatments had significant influence on cytokine levels (Figure I in the online-only Data Supplement).

Infarct Volume and Brain Edema

At day 1 after stroke, the mean edema-corrected infarct volume (%HLVe) amounted to 15.7±4.5% of the hemisphere without any differences between the experimental groups. Although the infarct volume remained constant during the first week after stroke, we observed a 30% increase of the infarct volume to 19.2±3.7% at day 30 (Figure 2). G-CSF monotherapy or combination therapy had no detectable impact on the infarct volume at any time point. The space-occupying effect of the ischemic lesion (%HSE) was maximally pronounced at day 1 after stroke and successively declined during the first week (Figure 2). At day 30, we measured negative values for %HSE that likely correspond to loss of hemispheric brain tissue and is consistent with the aforementioned increase of infarct volume at the same time point. Neither the early edema–related space-occupying effect nor the delayed brain atrophy was influenced by one of the treatments (Figure 2).

Functional Outcome After Stroke

We next used the adhesive removal test to monitor the clinical course within 30 days after stroke. To clearly define a hypothesis (treatment improves or impairs functional improvement), we a priori decided to perform a summarized analysis of the functional development by means of an area under the curve. At the baseline prior to stroke induction, rats needed ≈5 seconds to notice and remove the sticky tape from their contralateral forelimb. As expected, we observed a substantial increase of the tape removal time after stroke (to 91±37 seconds in the pMCAO-only control), which subsequently declined by ≈50% within 7 days to reach a constant level reflecting the permanent neurological deficit (Figure 3A). The area under the curve analysis revealed a statistically significant decrease of the tape removal time in G-CSF and a strong trend in the G-CSF+BM MNC 6 hours group ($P=0.05$). By contrast, the latter effect was
abolished in the G-CSF+BM MNC 48 hours group (Figure 3A). Body weights of all animals completely attained presurgery levels within the following days after stroke. Interestingly, after 30 days, we observed a significant increase of body weight in animals receiving BM MNC at 6 or 48 hours (Figure 3B).

Expression of Plasticity-Related Genes and Chronic Inflammation

Next, we investigated the expression of genes that are involved in neural regeneration and delayed inflammation in the ischemic hemisphere. Thirty days after stroke, the expression of Brevican, Neurocan, Gap-43 (Figure 3C), Versican, MARCKS, and Slit1 (Table III in the online-only Data Supplement) was not affected by any treatment. The growth factors insulin-like growth factor-1 (Figure 3C) and brain-derived neurotrophic factor (Table III in the online-only Data Supplement) showed a 1.5- to 2-fold increase in the ischemic hemisphere, but were not altered by G-CSF or G-CSF+BM MNC. Similarly, expression levels of IL-1β and CD45 were increased in the ischemic hemisphere 30 days after stroke, but did not differ among the experimental groups (Figure 3C). Immunohistochemical investigation revealed numerous T cells in the ischemic lesion 30 days after pMCAO, most of them located in the cortical infarct border. Neither G-CSF monotherapy nor cotransplantation of BM MNC altered T-cell counts in the ischemic brain (Figure 3D).

Short-Term Effects of Delayed Combination Treatment

Finally, we tried to elucidate the mechanisms behind the antagonizing effect of late BM MNC transplantation on the protective effect of G-CSF. Therefore, BM MNC were stained by the lipophilic membrane dye PKH26 to track short-term biodistribution. Labeling efficiency was 99.3±0.08% (n=3), and the dye had no impact on composition or vitality of the graft (data not shown). Four hours after intravenous transplantation of labeled BM MNC, we could identify PKH26+ cells in all samples investigated (blood, spleen, BM, and brain tissue; Table). The composition of recovered PKH26+ cells varied from the original graft, indicating differences in biodistribution or survival among the BM MNC subpopulations (Figure II in the online-only Data Supplement). Gene expression analysis of markers associated with inflammation and neural plasticity at day 30 after treatment with G-CSF, G-CSF+BM MNC 6 hours or G-CSF+BM MNC 48 hours had no impact on the expression of analyzed genes (n=6 samples per group). D, T-cell counts in the ischemic lesion border (open bars) and the entire ipsilateral hemisphere (striped bars) were not altered by G-CSF or cell transplantation. The representative illustration of the infarct border zone showed a massive activation of ionized calcium-binding adapter molecule-1 (Iba1)-positive microglia/macrophages and abundance of extravascular T cells (vessels were stained with solanum tuberosum lectin [STL]). By contrast, T cells remote from the ischemic lesion were exclusively found within vessels. pMCAO indicates permanent right middle cerebral artery occlusion. *P<0.05, ***P<0.001 determined by 1-way ANOVA; n=16 to 17 animals per group; bars: 20 μm. 

Figure 3. A, Assessment of neurological deficits using the adhesive removal test (ART). Experimental stroke caused a significant increase of ART time in all experimental groups, which was less pronounced after granulocyte colony-stimulating factor (G-CSF) and G-CSF plus bone marrow-derived mononuclear cells (BM MNC) 6 hours treatment. BM MNC transplantation at 48 hours was performed 12 hours before the ART at day 2. The area under the curve (AUC) analysis revealed a significantly improved functional recovery after G-CSF monotherapy and a strong trend by G-CSF+BM MNC 6 hours; †P<0.05. B, BM MNC therapy after 48 hours caused a significant increase of the body weight at day 30. C, Gene expression analysis of markers associated with inflammation and neural plasticity at day 30 after stroke. Treatment with G-CSF, G-CSF+BM MNC 6 hours or G-CSF+BM MNC 48 hours had no impact on the expression of analyzed genes (n=6 samples per group). D, T-cell counts in the ischemic lesion border (open bars) and the entire ipsilateral hemisphere (striped bars) were not altered by G-CSF or cell transplantation. The representative illustration of the infarct border zone showed a massive activation of ionized calcium-binding adapter molecule-1 (Iba1)-positive microglia/macrophages and abundance of extravascular T cells (vessels were stained with solanum tuberosum lectin [STL]). By contrast, T cells remote from the ischemic lesion were exclusively found within vessels. pMCAO indicates permanent right middle cerebral artery occlusion. *P<0.05, ***P<0.001 determined by 1-way ANOVA; n=16 to 17 animals per group; bars: 20 μm.
we found most of the splenic granulocytes being vital. A small fraction of necrotic granulocytes was also increased by BM MNC when compared with the G-CSF group (Figure 4C). Finally, we quantified immune cells within the ischemic (Figure 4D) and contralateral hemisphere (Figure III in the online-only Data Supplement) 52 hours after experimental stroke. Transplantation of BM MNC after 48 hours caused a significant increase of vital granulocytes in the ischemic hemisphere when compared with the G-CSF or control group (Figure 4D). Interestingly, almost half of all granulocytes in the ischemic brain were early apoptotic; however, neither G-CSF monotherapy nor BM MNC cotransplantation had an effect on the extent of this fraction (Figure 4D).

**Discussion**

In this study, we aimed to investigate the potential synergistic effect of 2 successfully tested experimental treatments for stroke: the repeated injection of G-CSF combined with transplantation of BM MNC. Our study yielded 3 major findings: first, we observed that G-CSF, neither alone nor in combination with BM MNC, had any impact on our primary end point, the infarct volume. Second, we found that a monotherapy with G-CSF and the cotransplantation of BM MNC after 6 hours significantly improved functional recovery. Surprisingly, this effect was entirely abolished when BM MNC were given after 48 hours. Finally, we found that G-CSF with and without auxiliary cell therapy significantly altered systemic immune responses to stroke.

The neuroprotective effect of G-CSF has been discovered and mechanistically defined in a landmark study by Schneider et al,13 who showed that G-CSF provides strong antiapoptotic effects in neurons exposed to ischemia. Many studies

<table>
<thead>
<tr>
<th>PKH26+ Cells (Mean±SD)</th>
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<tr>
<td>Graft (in 0.8 mL), body weight–adapted</td>
</tr>
<tr>
<td>Blood (in 1 mL)</td>
</tr>
<tr>
<td>Spleen</td>
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<tr>
<td>Bone marrow (1 femur bone)</td>
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<tr>
<td>Brain: stroke hemisphere</td>
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<td>Brain: contralateral hemisphere</td>
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**Table. Total Amount of PKH26+ Cells 4 Hours After Transplantation**

Figure 4. Short-term effects of the combined treatment with granulocyte colony-stimulating factor (G-CSF) and bone marrow–derived mononuclear cells (BM MNC) at 48 hours after stroke. **A**, Biodistribution analysis 4 hours after cell transplantation revealed a reduced proportion of B cells in the blood, spleen, bone marrow (BM), and brain. In the circulation, primarily CD11b+ monocytes were positive for the cell marker PKH26, whereas T cells and CD45-only cells were enriched in the spleen and BM. Almost all PKH26+ cells in the brain were CD11b+. Immunofluorescence staining of spleen sections revealed an accumulation of PKH26+ cells in the marginal zone around the lymphatic follicles. **B**, Gene expression analysis of the spleen showed a significant increase of monocyte chemotactic protein-1 (MCP-1) and interleukin (IL)-10 after BM MNC transplantation. By contrast, IL-1β mRNA was increased by G-CSF, but not further affected by cell therapy. **C**, Flow cytometric quantification of major leukocyte subpopulations in the spleen showed a distinct increase of granulocytes after G-CSF treatment, further increased by BM MNC transplantation. The vast majority of splenic granulocytes were vital, reflecting the group differences of total granulocytes. Necrotic granulocyte counts were doubled by G-CSF and quintupled by G-CSF+BM MNC 48 hours. **D**, Major leukocyte subpopulation in the ischemic brain did not differ between the experimental groups; however, vital granulocytes were significantly increased after BM MNC transplantation. Iba1 indicates ionized calcium-binding adapter molecule-1; and PMN, polymorphonuclear cells (granulocytes). †P<0.05 vs G-CSF, *P<0.05, **P<0.01, ***P<0.001, ‡P=0.06 determined by 1-way ANOVA; n=3 animals per group; bar: 20 μm.
reproduced this finding and 2 meta-analyses consistently confirmed a significant impact of G-CSF on the infarct volume, the most frequently used surrogate for neuroprotection.\textsuperscript{2,3} Interestingly, the same meta-analysis revealed, in contrast to one recent study,\textsuperscript{14} that the infarct volume was not influenced by G-CSF treatment in permanent stroke,\textsuperscript{2} likely owing to the fact that the neuroprotective time window is shorter in permanent stroke (3 hours) compared with the classical ischemia/reperfusion models (up to 12 hours).\textsuperscript{15} In SHR, neuroprotection is even limited to the first 60 minutes after MCAO.\textsuperscript{16} We hence reasoned that the lack of influence of G-CSF treatment and cell transplantation on the ischemic lesion is likely caused by the absence of reperfusion and the comorbidities present in SHR. Conversely, the functional effect of G-CSF observed in our study is likely mediated by mechanism beyond neuroprotection. This is highly relevant for translational stroke research,\textsuperscript{11,15} but it should also be noted that inbred SHR may be compromised by genetic aberrations that could bias the value of our experiment.

G-CSF has an impact on various pathophysiological aspects relevant for stroke outcome: it promotes endogenous neurogenesis and angiogenesis, modulates immune responses, reduces brain edema, and enhances structural and functional regeneration capacities of the central nervous system.\textsuperscript{17} These mechanisms could explain the significant functional improvement after G-CSF treatment that was prevalent in most of the published preclinical experiments\textsuperscript{2,3} being corroborated by ours. Angiogenesis and neurogenesis were not investigated here. However, we examined the expression of various growth-promoting and growth-inhibiting genes during the late stage of poststroke axonal sprouting\textsuperscript{18} (day 30) but found no differences between any treatment regime and control. Furthermore, and again in contrast to another study using a reperfusion model of stroke,\textsuperscript{19} we found the brain edema was also not influenced by G-CSF.

With respect to the early functional improvement observed in our study, we suggest acute immunologic interactions as one key mechanism for the efficacy of G-CSF and its antagonization by late BM MNC transplantation. Two days after stroke, we found a significant decline of circulating CXCR4\textsuperscript+ mononuclear cells in all treatment groups, likely as a consequence of chemokine receptor downregulation by G-CSF.\textsuperscript{20} Recent studies showed that early CXCR4 antagonism significantly improved outcome after stroke by regulating the infiltration of leukocytes into the ischemic brain.\textsuperscript{21} In this manner, G-CSF may dampen central nervous system inflammation and damage, and in fact, we found a strong trend toward less macrophages/dendritic cells infiltrating the ischemic lesion in G-CSF--treated animals.

Interestingly, we found a recovery of depressed spleen weights and circulating leukocyte counts in all groups receiving G-CSF. Postischemic immunodepression is a consequence of an overactivated cholinergic anti-inflammatory pathway finally contributing to morbidity after stroke.\textsuperscript{32} We found no evidence for infections, such as increased mortality or decreased body weights in our study, but the restoration of immunocompetence by G-CSF may have protected the brain from chronic central nervous system inflammation.\textsuperscript{22} In fact, we found ongoing immune responses including an accumulation of T cells at the infarct border at day 30 after stroke, but T-cell counts and brain atrophy were not influenced by G-CSF treatment or accompanying cell therapy.

One surprising finding of our study was that transplantation of BM MNC after 48 hours completely neutralized the beneficial effect of G-CSF. Early transplantation of BM MNC after transient stroke in SHR have had no positive or negative impact on functional outcome\textsuperscript{23}; however, it is possible that late BM MNC transplantation is detrimental after stroke and simply deleted the protective effects of the accompanying G-CSF therapy. This notion remains hypothetical because we did not investigate the exclusive effect of late BM MNC transplantation here. Nevertheless, adverse effects of BM-derived cells have been described in animal models of diabetes mellitus and arteriosclerosis,\textsuperscript{24,25} suggesting a compromised cell graft under translationally relevant conditions of hyperglycemia and hypertension.\textsuperscript{26,27}

We found almost 30% of the BM MNC being apoptotic before transplantation, a finding that may reflect hypertension-related oxidative stress in the BM.\textsuperscript{26,27} In steady state, circulating apoptotic cells were trapped by specialized splenic macrophages in the marginal zone between red and white pulp.\textsuperscript{14} This endogenous scavenging system carefully down-regulates immune-stimulatory functions and helps to maintain self-tolerance.\textsuperscript{29} It is thus tempting to speculate that the intravenous transplantation of numerous (pre)apoptotic BM MNC may induce endogenous anti-inflammatory programs,\textsuperscript{30,31} and in fact, we found an accumulation of BM MNC in the splenic marginal zones together with increased expression of anti-inflammatory mediators.

Intriguingly, this assumed universal mechanism for intravenously administered cells may also explain the interaction effect between late BM MNC and G-CSF found in our study. Circulating granulocytes have a short half-life and are continuously cleared in the splenic marginal zone. Mice deficient for marginal zone macrophages develop increased granulocyte counts and IL-1β levels.\textsuperscript{32} Accordingly, we found that treatment with G-CSF caused a 10-fold increase of splenic granulocytes and 2-fold increase of IL-1β expression, indicating an overload of the granulocyte clearance system. The simultaneous engagement of marginal zone macrophages with transplanted BM MNC and massively increased granulocytes may further overload this scavenging system, as it has been shown after the application of polystyrene particles.\textsuperscript{33} This would explain the significant increase of vital granulocytes in the blood, spleen, and brain of animals that received BM MNC at the peak point of G-CSF--induced neutrophilia. Higher neutrophil counts in the brain could finally explain the negative functional effect either by disturbing the microcirculation or by releasing toxic mediators.\textsuperscript{34}

**Conclusions**

In our study, we could confirm the beneficial effect of G-CSF on long-term functional recovery, but not on neuroprotection after stroke in hypertensive rats. The cotreatment with BM MNC was without effect after 6 hours, but reversed the protective effect of G-CSF after 48 hours, probably by overloading the splenic scavenging system by the apoptotic fraction of BM MNC and massively increased granulocytes.
Acknowledgments
We thank Isabelle Glocke, Sebastian Baasch, Christin Kellner, Isabell Schulz, and Natalia Shurawel for excellent technical support, and Karoline Möller and Dr Gesa Weise for helpful discussions.

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Disclosures
Dr Schäbitz is an inventor on a patent claiming the use of granulocyte-colony-stimulating factor for the treatment of stroke. The other authors report no conflicts.

References
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SUPPLEMENTAL MATERIAL

Bone marrow cell transplantation time-dependently abolishes efficacy of G-CSF after stroke in hypertensive rats

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Supplemental methods

Experimental stroke

Animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and approved by the appropriate regional authorities (reference number TVV 12/11). A total of 81 spontaneously hypertensive rats (SHR; Charles River, Sulzfeld, Germany) at the age of 12 weeks were anesthetized with ketamine hydrochloride (100mg/kg) and xylazine (10mg/kg) given as an intraperitoneal injection. During the surgical procedure, body core temperature was constantly measured and maintained at 37.5°C. Experimental stroke was induced by permanent occlusion of the right middle cerebral artery (pMCAO) as described previously. Briefly, the right temporal scull bone and dura mater were opened, and the subjacent middle cerebral artery was permanently occluded by thermocoagulation. Health status including the body weight was monitored daily within the first week after stroke and weekly thereafter. The exclusion criteria were defined as follows: i) weight loss of more than 20% body weight during week 1; ii) absence of a cortical brain lesion typical for distal MCAO in the MR sequence; iii) incomplete injection of G-CSF, cell suspension or vehicle solution.

Experimental group allocation

In the first study, we defined the reduction of the infarct volume (%HLVe, see below) by at least 30% as primary endpoint. Accordingly, the sample sizes were a priori calculated for an expected difference of the mean of 30% with α=0.05 and β=0.8 for a one-way ANOVA (Sigma Plot version 11.0). The expected standard deviation (σ=3.2 %HLVe) was obtained from a previous study (day 1 and day 3 after pMCAO from SHRs). SHRs were randomly assigned (balanced randomization by lot) to one of the following experimental groups: (i) G-CSF monotherapy group (henceforth G-CSF, n=18) with a daily intraperitoneal injection of 50 µg/kg Neupogen (rhG-CSF; Amgen GmbH, Thousand Oaks, USA) for 5 days starting 1h after pMCAO; (ii) combination group with early cell transplantation (henceforth G-CSF+BM MNC 6h, n=18) with a G-CSF schedule according to (i) and an additional transplantation of 1.5x10E7 BM MNCs per kg bodyweight at 6h after pMCAO; (iii) combination group with late cell transplantation (henceforth G-CSF+BM MNC 48h, n=18) with a G-CSF schedule according to (i) and an additional transplantation of 1.5x10E7 BM MNCs per kg bodyweight at 48h after pMCAO; (iv) control group (henceforth control, n=18) receiving phosphate buffered saline (PBS) instead of G-CSF and BM MNCs. The administrations in all treatment groups were completely controlled by PBS injections, meaning, for instance, that the G-CSF group received PBS injections at 6h and 48h after stroke.

In the second study, we investigated the distribution of PKH26-labeled BM MNC and the short-term immunological responses to late transplantation of BM MNC (48h). Nine animals were randomly assigned to the following experimental groups (in accordance with the first study): (i) G-CSF, n=3; (iii) G-CSF+BM MNC 48h, n=3; (iv) control group, n=3.
BM MNC isolation and transplantation
Syngeneic rat bone marrow mononuclear cells (BM MNC) were enriched by magnetic depletion of granulocytes as described previously and cryo-preserved until further use. At the day of cell transplantation, BM MNCs were re-thawed in Dulbecco's modified Eagle's medium containing 4.5g/L glucose (PAA Laboratories, Cölbe, Germany) and 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany), washed twice in medium. Vital cell numbers were determined by the trypan blue exclusion method using a hemocytometer. Cellular composition of cell grafts was characterized by flow cytometry for B cells (CD45R+), T cells (CD3+) and myeloid cells (CD11b+ and RP1-; for details please refer to supplemental table I). CXCR4+ BM MNC were identified by polyclonal rabbit anti-rat CXCR4 (Abcam, Cambridge, UK) which was secondly labeled with donkey anti-rabbit PE (ebioscience, San Diego, USA). Hematopoietic progenitors expressing CD34 (polyclonal goat anti-rat; R&D Systems, Minneapolis, USA) were secondly conjugated with donkey anti-goat PE-Cy™5 (Santa Cruz Biotechnology, Dallas, USA) and differentiated in bipotent hematopoietic progenitors using granulocyte-macrophage colony forming unit assay (CFU-GM) as described elsewhere. For the analysis of the biodistribution, BM MNC were labeled with the red fluorescent dye PKH26 according to the manufacturer’s instructions (Sigma Aldrich, St. Louis, USA). Prior to transplantation, 1.5x10E7 BM MNCs per kg bodyweight were resuspended in 800µl PBS. Cell suspension was slowly administered via the tail vein. The application of G-CSF, BM MNCs or vehicle solution was performed by an investigator blinded to the group allocation 12h prior to functional testing and MR imaging.

Analysis of peripheral blood leukocytes and cytokine levels
Peripheral blood and serum samples were collected at 6h, 48h, 52h, 96h and 144h after pMCAO. Anticoagulated blood samples were harvested in 0.2M EDTA and stored in EDTA-monovettes for hematological analysis. Absolute leukocyte counts were determined by an animal blood cell counter (scil Vet abc, SCIL animal care company GmbH, Viernheim, Germany). Leukocyte subsets were identified and categorized according to their antigen expression (for details please refer to supplemental table I) using multichannel flow cytometry. Natural killer (NK) cells were identified by biotinylated anti-CD161a and secondly conjugated with streptavidin Horizon™V500 (BD Pharmingen, Heidelberg, Germany). Purified CXCR4 was secondly labeled with donkey anti–rabbit PE as mentioned above (ebioscience, San Diego, USA). For this, 50µL of blood were diluted in 50µL PBS and incubated with a mixture of monoclonal antibodies (supplemental table I) for 20min at 4°C. Erythrocytes were lysed by short-term incubation (30 seconds) with distilled water followed by two washing steps with PBS containing 3% fetal calf serum (PBS/3% FCS). Remaining leukocytes were resuspended in 300µL PBS/3% FCS. Flow cytometric acquisition and analysis was performed using a FACS Canto II equipped with FACS Diva software (BD Biosciences, Heidelberg, Germany). For the quantification of circulating cytokine levels, serum (n=6 per group and day, randomly assigned by lot) was separated from 300µl coagulated blood samples by centrifugation at 5000 rpm for 5min and stored in aliquots at -80°C. Cytokine secretion levels of IFNγ, IL-4, IL-10, IL-17A, MCP-1 and MIP-1α were analyzed by multiparametric ELISA (Multimetrix GmbH, Regensburg, Germany).

Biodistribution study and flow cytometric analysis of spleen and brain tissue
Animals from the second study were sacrificed exactly 52h after stroke and transcardially perfused with 200mL of ice-cold PBS. Spleens, femur bones and brains were removed. Spleens were weighed and segmented into three parts for (i) histological analyses, (ii) for gene expression analysis and (iii) for flow cytometric analyses.
For flow cytometry, spleens were mechanically dissected using razor blades and further dissociated through 100µm and 40µm cell strainers using glass pestles. Bone marrow was harvested from one femur bone by repeated flushing with PBS. Total cell counts and viability were determined by trypan blue exclusion in a hemocytometer. Total leukocyte counts were defined by counting in Turk’s solution. Single cell suspensions of the ischemic and contralateral brain hemispheres were isolated by mechanical dissection and enzymatic digestion as described previously\(^4\). Immune cells were separated by density gradient centrifugation on discontinuous Percoll (GE Healthcare, München, Germany) gradients composed of four sequent layers (80%/38%/21% Percoll covered with cell culture medium). Cells accumulating in between 80%/38% Percoll were harvested and washed repeatedly. Total counts of brain leukocytes were determined by additional Trucount Tube measurements (BD Biosciences). Frequencies of PKH26+ cells and of major leukocyte populations were assessed by flow cytometry. In total, 1x10E6 isolated spleen or bone marrow cells and 2x10E5 brain cells were incubated with a specific FC-blocking reagent (purified anti-rat CD32; BD Bioscience) for 10min at 4°C and labeled with a mixture of monoclonal antibodies (supplemental table I). After incubation for 20min at 4°C, cells were washed and incubated with annexin V PE-Cy™7 (ebioscience, San Diego, USA) in 100 µL annexin V binding buffer for 15min at room temperature. After washing, cells were stained with 7-AAD viability staining solution (ebioscience). Flow cytometric acquisition and analysis was performed by an investigator blinded to the group allocation using a 3-laser FACS Canto II equipped with FACS Diva software (BD Biosciences).

**Determination of infarct volume**

The infarct volume and space occupying effect of the lesion were determined in vivo by means of magnetic resonance imaging (MRI) at day 1, 3, 7 and 30. Image sequences were acquired in clinical MR scanner (1.5 T Gyroscan Intera human whole-body spectrometer equipped with a 47mm loop RF-Coil, Philips). Briefly, animals were anesthetized as described above. T2-weighted sequences (T2-TSE) consisting of 20 transverse slices (matrix: 224x224; field of view: 50mm; slice thickness: 1mm) were acquired. Hemispheric lesion volume corrected for edema (%HLVe) and hemispheric space occupying effect (%HSE) of the lesion were calculated by a blinded investigator as described previously.\(^5\)

**Assessment of functional recovery**

Neurological deficits were repeatedly assessed by means of the adhesive removal test (ART) as described previously.\(^6\) Animals were allowed to adapt to the ART conditions for 3 days. The baseline data was ascertained one day before pMCAO. Following pMCAO, ART was performed at day 2, 7, 14, 21 and 28 by an investigator blinded to group allocation. The time needed to remove the adhesive tape was measured in technical triplicates that were averaged for one individual at each day. For the statistical analysis, time series data was summarized as individual area under the curve (AUC).

**Quantitative RT-PCR**

Animals were sacrificed and transcardially perfused with 200mL of ice-cold PBS. Spleen segments, ipsilateral and contralateral brain tissue were manually dissociated by razor blades. Next, total RNA of 100mg tissue was extracted by homogenization in 1mL Trizol using a ULTRA-TURRAX® (Ika, Staufen, Germany) and further purified by RNeasy Mini Kit (Qiagen, Hilden, Germany). Single-strand cDNA copies were generated from 1µg of total purified RNA by using random primers (Promega, Mannheim, Germany) and Superscript III reverse transcriptase (Invitrogen/Life Technologies, Darmstadt, Germany) according to manufacturer’s instructions. Quantification of mRNA expression was performed and monitored using an ABI 7900 real-time PCR system (Applied Biosystems, Darmstadt,
(Germany) applying the following conditions: initial denaturation at 95°C for 10min, followed by 50 cycles at 95°C for 15s and 55°C for 1min. All qRT-PCR reactions were conducted in a total volume of 15µL with addition of Power SYBR Green I PCR Master Mix (Applied Biosystems, Darmstadt, Germany) and gene specific QuantiTect® Primer Assay (supplemental table III for brain and supplemental table IV for spleen; Qiagen, Hilden, Germany). Data was analyzed using the relative standard curve method, normalized on the average cycle threshold of the housekeeping genes B2m (QT00176295, NM_012512), Rpl13a (QT00425873, NM_173340), Rpl22 (QT00385119, NM_031104) and Yhwaz (QT02382184, NM_013011) and normalized to the control group (spleen tissue) or to the contralateral hemisphere (brain tissue).

**Histological analysis**

30 days after stroke, animals were sacrificed and transcardially perfused with 200mL of PBS and 200mL of formalin solution (4%). Removed brains were vitrified in sucrose solution (30%) and cryoconserved at -80°C. Frozen brains were cut into 20µm coronal sections and mounted on coated slides. Brain sections (n=4 animals per group, randomly assigned by lot) were incubated with 5% goat serum and 0.3% Trition-X-100 for 30 minutes, followed by polyclonal rabbit anti-Iba1 (Ionized calcium binding adaptor molecule 1; 1:200; Wako Chemicals, Neuss, Germany), monoclonal mouse anti-15-16A1 (1:500; Hycult Biotech, Beutelsbach, Germany; for T cells) and biotinylated solanum tuberosum lectin (STL; 1:300; Linaris, Dossenheim, Germany) for 24h at 4°C. Sections were then incubated with goat anti-rabbit IgG (1:200, Invitrogen), goat anti-mouse IgG (1:200, Invitrogen) or streptavidin (Dianova) conjugated with either Alexa Fluor® 488, 546 or Cy5 for 1h at room temperature. Sections were counterstained with DAPI (2.5µg/mL, Sigma). Fluorescence images were acquired using a Zeiss LSM710 confocal laser-scanning microscope (Objective: Plan-Apochromat 63x / 1.40 oil). T cells were counted in 4 brain regions (Bregma anteroposterior +1.5mm, 1.0mm, 0.5mm and 0.0mm) in technical duplicates using a Stereo Investigator system (MBF Bioscience). Briefly, two regions of interest (ROI) were defined as “ipsilateral hemisphere” and “infarct border”, the latter define as a 100µm deep band adjacent to the border of the pseudocyst. The area of the ROIs, and the T cell count within the ROIs were determined by an investigator blinded to the group allocation. Finally, T cell counts were summarized for each biological replicate and indicated as cells per area.

Spleen segments were fixed in formalin solution (4%) for 48h, vitrified in sucrose solution (30%) and cryoconserved at -80°C. Frozen spleens were cut into 20µm coronal sections and mounted on coated slides. Sections were incubated with 5% goat serum and 0.3% Trition-X-100 for 30 minutes, followed by primary incubation with polyclonal rabbit anti-Iba1 for 24h at 4°C and secondary labeling with Alexa Fluor® 488 conjugated goat anti-rabbit IgG for 1h at room temperature. Sections were counterstained with DAPI (2.5µg/mL, Sigma).

**Statistical analysis**

Peripheral leukocyte counts, summarized ART data, brain histology were analyzed by one-way ANOVA followed by Bonferroni’s post-hoc test. Time series of serum cytokine levels and MR investigations were analyzed by repeated measures two-way ANOVA followed by Bonferroni’s post-hoc test. Immune cell distribution in spleen and brain tissue was analyzed by one-way ANOVA followed by Newman-Keuls test. A p-value of less than 0.05 was considered statistically significant. All data were displayed as mean ± standard deviation (SD). Data analysis was performed by Graph Pad Prism (version 5.03).
## Supplemental Tables

### Supplemental Table I

**Anti-rat monoclonal antibodies used for flow cytometry**

<table>
<thead>
<tr>
<th>Panel Identification</th>
<th>Antigen</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>B cells</th>
<th>T cells</th>
<th>NK cells</th>
<th>Mo*</th>
<th>Ma#</th>
<th>PMN†</th>
<th>CD45 only</th>
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<td></td>
<td>CD11b</td>
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<td>MRC OX-42</td>
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<td>+</td>
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<tr>
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<td>Biotin</td>
<td>Okt 78</td>
<td>BD Bioscience</td>
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<td>1F4</td>
<td>BD Bioscience</td>
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<td>W3/25</td>
<td>Biolegend</td>
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<tr>
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<td>APC-Cy™7</td>
<td>OX-1</td>
<td>BD Bioscience</td>
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</table>

*Monocytes; #Macrophages; †Polymorphonuclear cells (Granulocytes) *not analyzed

### Supplemental Table II

**Animals excluded from MR investigation**

<table>
<thead>
<tr>
<th>Day</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>30</th>
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<tr>
<td>Control</td>
<td>1</td>
<td>4</td>
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<td>2</td>
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<td>G-CSF</td>
<td>0</td>
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<td>1</td>
<td>1</td>
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<td>GCSF+BM MNC 6h</td>
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<td>2</td>
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<td>1</td>
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<td>GCSF+BM MNC 48h</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
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### Supplemental Table III

**Relative mRNA expression within the ischemic hemisphere 30 days after stroke**

<table>
<thead>
<tr>
<th>QuantiTect primer assay</th>
<th>Transcript</th>
<th>Control</th>
<th>G-CSF</th>
<th>+BM MNC 6h</th>
<th>+BM MNC 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>NM_012513</td>
<td>1.61</td>
<td>1.15</td>
<td>1.29</td>
<td>0.73</td>
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<tr>
<td>Brevican</td>
<td>NM_012916</td>
<td>0.97</td>
<td>0.06</td>
<td>0.96</td>
<td>0.10</td>
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<td>CD45</td>
<td>NM_001109887</td>
<td>2.11</td>
<td>0.49</td>
<td>2.21</td>
<td>0.39</td>
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<tr>
<td>Gap-43</td>
<td>NM_017195</td>
<td>0.83</td>
<td>0.11</td>
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<td>IGF-1</td>
<td>NM_001082479</td>
<td>1.28</td>
<td>0.64</td>
<td>1.67</td>
<td>0.73</td>
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<tr>
<td>IL-1β</td>
<td>NM_031512</td>
<td>1.27</td>
<td>0.40</td>
<td>1.42</td>
<td>0.23</td>
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<tr>
<td>MARCKS</td>
<td>XM_001060954</td>
<td>0.99</td>
<td>0.24</td>
<td>1.20</td>
<td>0.29</td>
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<td>Neurocan</td>
<td>NM_031653</td>
<td>1.14</td>
<td>0.26</td>
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<td>Slit1</td>
<td>NM_022953</td>
<td>0.86</td>
<td>0.09</td>
<td>1.02</td>
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<td>Versican</td>
<td>XM_001058160</td>
<td>1.07</td>
<td>0.22</td>
<td>1.10</td>
<td>0.13</td>
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*mean; *standard deviation; n=6 per group
Supplemental Table IV
Relative mRNA expression within the spleen 52h after stroke

<table>
<thead>
<tr>
<th>QuantiTect primer assay</th>
<th>Transcript</th>
<th>Control M ± SD</th>
<th>G-CSF M ± SD</th>
<th>+BM MNC 48h M ± SD</th>
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</thead>
<tbody>
<tr>
<td>CCL6  QT01573523</td>
<td>NM_001004202</td>
<td>1.00 ± 0.13</td>
<td>1.70 ± 0.17</td>
<td>1.60 ± 0.20</td>
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<tr>
<td>CXCL2  QT00184891</td>
<td>NM_053647</td>
<td>1.00 ± 0.56</td>
<td>1.70 ± 0.31</td>
<td>2.50 ± 0.49</td>
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<tr>
<td>CXCL5  QT00392777</td>
<td>NM_022214</td>
<td>1.00 ± 0.07</td>
<td>0.84 ± 0.10</td>
<td>1.10 ± 0.18</td>
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<tr>
<td>HMGB1  QT00368410</td>
<td>NM_012963</td>
<td>1.00 ± 0.04</td>
<td>0.92 ± 0.05</td>
<td>0.94 ± 0.05</td>
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<tr>
<td>IL-1β  QT00181657</td>
<td>NM_031512</td>
<td>1.00 ± 0.05</td>
<td>2.00 ± 0.30</td>
<td>2.10 ± 0.17</td>
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<td>IL-6   QT00182896</td>
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<td>IL-10  QT00177618</td>
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<td>1.00 ± 0.19</td>
<td>1.10 ± 0.09</td>
<td>1.70 ± 0.19</td>
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<tr>
<td>MCP-1  QT00183253</td>
<td>NM_031530</td>
<td>1.00 ± 0.15</td>
<td>1.35 ± 0.03</td>
<td>2.10 ± 0.21</td>
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<tr>
<td>TNFα   QT00178717</td>
<td>NM_012675</td>
<td>1.00 ± 0.11</td>
<td>0.79 ± 0.22</td>
<td>0.95 ± 0.20</td>
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</table>

*mean; †standard deviation; n=3 per group
Supplemental Figure I
Serum protein levels of inflammatory cytokines 48, 96 and 144 hours after stroke onset. Serum levels of anti-inflammatory (Interleukin (IL) -4, IL-10) and pro-inflammatory (IL-17A, interferon (IFN) γ and macrophage inflammatory protein (MIP)-1α) cytokines showed a moderate increase from 48h to 96h. The chemotactic molecule monocyte chemotactic protein (MCP)-1 was detectable at relatively high serum levels (around 200 pg/mL serum) but without any relevant kinetics during the observation period. At 96h, the serum level of MIP-1α was slightly, but statistically significant reduced by G-CSF+BM MNC 48h. Apart from that, none of the treatment regimes had a significant influence on the cytokine levels. *p<0.05 compared to control group determined by repeated measurement two-way ANOVA; n=14-17 animals per group.
Supplemental Figure II
Laser scanning microscopy of the splenic marginal zone. A, a maximum intensity projection of a 15µm thick confocal stack showed various ionized calcium binding adapter molecule 1 (Iba1)+ macrophages. B, the detail is displayed as orthographic projection. PKH26+ signals could be clearly localized within the cytoplasm of the macrophage. Bar: 10µm
Supplemental Figure III
Flow cytometric analysis of major leukocyte populations in the contralateral hemisphere 52h after stroke. G-CSF treatment caused a significant increase of granulocytes that was not further influenced by BM MNC transplantation at 48h. *p<0.05 determined by one-way ANOVA; n=3 animals per group.
Reference List


