EXTENDED REPORT

Amelioration of experimental arthritis by stroke-induced immunosuppression is independent of T_{reg} cell function

Ingo M Irmler,1 Mieczyslaw Gajda,2 Thomas Kamradt1

ABSTRACT

Objectives Clinical evidence suggests that neurological lesions can protect from arthritis. Acute cerebral ischaemia induces severe immunosuppression, resulting in enhanced susceptibility to infections. We aimed to determine if stroke-induced immunosuppression can ameliorate arthritis and to delineate the immunological mechanisms involved.

Methods Unilateral cerebral ischaemia was induced in mice by occlusion of one middle cerebral artery (MCAO) at different time points after induction of G6Pi-induced arthritis in mice. Clinical and histological signs of arthritis were assessed. Regulatory T cells were specifically depleted by injection of diphtheria toxin into transgenic DEREG mice. Immunological correlates of MCAO were determined by flow cytometry and serological methods.

Results MCAO reduced the clinical and histological signs of arthritis significantly. To be effective, stroke had to be induced during the induction phase or the early clinical stage of arthritis. MCAO induced a global loss of leucocytes. Despite the reduced absolute number of lymphocytes, the functional differentiation of T helper cells into Th1/17 cells and the production of autoantibodies were unimpaired. Depletion experiments showed that regulatory T cells were dispensable for the protective effect of MCAO.

Conclusions MCAO ameliorates arthritis. The correlate of protection from arthritis is not the reduction of a particular pathogenic leucocyte subset or the preferential expansion or emergence of a protective cell population but the global reduction of leukocytes during arthritis.

INTRODUCTION

The immune system and the nervous system communicate continually.1 Neurotransmitters modulate innate and adaptive immune responses while cytokines modulate body temperature, mood and sleep. Acute cerebral ischaemia induces severe immunosuppression,2–6 resulting in enhanced susceptibility to respiratory and urinary tract infections, which are the leading cause of death in stroke patients.4 6–8 Stroke-induced tissue destruction and disruption of the blood–brain barrier also result in the release of CNS autoantigens and their presentation to T-lymphocytes under proinflammatory immunological conditions.4 6–9 It has, therefore, been suggested that stroke-induced immunosuppression protects from stroke-induced autoimmune responses against CNS autoantigens.4 Autoimmune responses are essential in the pathogenesis of rheumatic diseases such as rheumatoid arthritis and spondyloarthropathies.10 11 Clinical evidence suggests that ischaemic, traumatic or postinfectious damage to the nervous system can protect from or ameliorate arthritis.12–17 However, the underlying mechanisms have remained enigmatic.

The aims of this study were to examine if stroke-induced immunosuppression can ameliorate arthritis and to delineate the immunological mechanisms involved.

MATERIALS AND METHODS

Mice and induction of glucose-6-phosphate isomerase-induced arthritis

DBA/1 and DBA/1 DEREG mice were bred and underwent experiments at the animal facility of the University Hospital Jena. All animal studies were approved by the appropriate authorities (reg. No. 02-006/08). Arthritis was induced by subcutaneous immunisation with 200 µg of recombinant human G6Pi in complete Freund’s adjuvant (Sigma-Aldrich, Taufkirchen, Germany) and evaluated clinically as described.18 Depletion of regulatory T cells (T_{reg}) was performed in DBA/1 DEREG mice by intraperitoneal application of 0.5 µg of diphtheria toxin and 24 h before and 4 and 5 d after G6Pi immunisation. This resulted in reliable transient depletion of T_{reg} cells (data not shown). For experiments analysing the time point of middle cerebral artery occlusion (MCAO) performance on arthritis severity, DBA/1 or non-depleted DBA/1 DEREG mice were used.

Histopathological assessment of glucose-6-phosphate isomerase-induced arthritis

Haematoxylin-eosin-stained sections of fixed and decalcified joints were evaluated by a pathologist. Inflammation and cartilage and bone destruction were graded as described before.19

Middle cerebral artery occlusion

MCAO was performed under isoflurane anaesthesia. A nylon monofilament coated with silicone (Heraeus Kulzer, Hanau, Germany) was introduced into the common carotid artery and moved to the origin of the middle cerebral artery (MCA) to block perfusion. The filament was left in this position for 60 min. In sham-operated animals, it was immediately withdrawn 4–5 mm to prevent brain ischaemia. After MCAO, mice were kept in heated cages, and 3 d before surgery Enrofloxacin (25 mg/mL; Bayer HealthCare, Leverkusen, Germany) was added to the drinking water.
Enzyme-linked immunosorbent assay (ELISA)
Single-cell suspensions were prepared from spleens and lymph nodes (LN) (inguinal, axillary, paraaortic) and cultured in medium alone or with 25 μg/mL huG6PI or 2 μg plate-bound anti-CD3 and 2 μg anti-CD28. Supernatants were harvested and concentrations of IFN-γ, TNF-α, IL-4, IL-5, IL-6 and IL-17 were measured by sandwich ELISAs (eBiosciences, Frankfurt, Germany) using recombinant cytokines (Peptotech, Hamburg, Germany) as standard. G6PI-specific antibody serum concentrations were determined as previously described.21

Proliferation assay
Single-cell suspensions from draining LN were prepared 9 d after immunisation and cultured with medium alone or 25 μg/mL G6PI for 72 h. [3H]-thymidine (0.5 μCi/well; Hartmann Analytic) per well was added for the last 18 h. Cells were harvested and incorporation of radio labelled thymidine was measured using a microplate scintillation luminescence counter (Canberra-Packard, Rüsselsheim, Germany). The stimulation index (SI) was calculated as described.22

Flow cytometric analysis of cell populations and cytokine production
Single-cell suspensions from spleen and draining LN were prepared 9 d after immunisation and cultured with anti-CD16/32 (2.4G2) and rat IgG to prevent unspecific binding, followed by fluorochrome-conjugated antibodies directed against B220 (RA3-6B2), CD3, CD4, CD8, Gr-1, CD11b (all from eBioscience) or CD11c (M1/70).

Analysis of G6PI-specific cytokine-producing T cells was performed after culturing for 6 h with 25 μg/mL G6PI or medium alone as described.23–25 Data were acquired using a LSRII flow cytometer (Becton Dickinson, Heidelberg, Germany) and analysed using FlowJo V8.1.1 software (Tree Star, Ashland, Oregon, USA). Gates for CD154 were set using unstimulated control samples.

Statistical analysis
Statistical differences between groups were evaluated using non-parametric Mann–Whitney U test. Statistical significance was accepted for p values of less than 0.05 (**p<0.05; ***p<0.01; ****p<0.001). All calculations were performed using the SPSS software V16.0 (SPSS Inc, Chicago, Illinois, USA). Data are presented as arithmetic mean and SE of the mean.

RESULTS
MCAO-induced ischaemia
Temporary occlusion of the middle cerebral artery (MCAO) induced substantial necrosis in the parietal lobe supplied by this vessel. Ischaemia was observable macroscopically in the left hemisphere, whereas the contralateral hemisphere was not affected (figure 1A). Histological sections revealed disbandment of cortical structures in the ischaemic brain areas (figure 1B) and extensive necrosis (figure 1C).

MCAO ameliorates arthritis
To evaluate the effects of stroke-induced immunosuppression at different stages of arthritis pathogenesis, stroke was induced at various time points after G6PI immunisation. MCAO 3 days before G6PI immunisation (d-3) had no effect on incidence or severity of arthritis (figure 2A). MCAO 3 days after immunisation with antigen in CFA resulted in an unacceptably high mortality rate (data not shown). In contrast, MCAO 6 days after immunisation (d6) ameliorated the course of arthritis significantly (figure 2B). MCAO 10 days after G6PI immunisation, when clinical signs of arthritis were already clearly visible, also reduced arthritis severity (figure 2C). Stroke induction at later time points (d12, d15) had only minor effects on arthritis (data not shown). Thus, stroke ameliorates the clinical course of arthritis when MCAO is performed in the induction phase or during the early clinical stage of G6PI-induced arthritis.

Mice that had undergone MCAO or sham surgery 6 days after G6PI immunisation were examined histopathologically 16 d after G6PI immunisation. Paws from animals with experimental stroke had diminished signs of acute and chronic inflammation. They also had less cartilage and bone destruction compared with animals of the sham group, confirming the clinical scoring (figure 2D).

MCAO induces a profound and prolonged loss of leucocytes
To determine the immunological correlates of protection, we examined the effect of stroke on the number of leucocytes within the secondary lymphoid organs. At day 16 after G6PI immunisation (10 d after MCAO), when arthritis severity was maximal in control mice, we found significantly reduced cell numbers in LN and spleens of MCAO mice (figure 3A). Flow cytometric analyses revealed that the reduction of cell numbers was not caused by the loss of a particular cell population. The numbers of B220+ B cells, CD3+CD4+ T helper cells, CD3−CD8+ cytotoxic T cells, as well as macrophages (CD11b+), dendritic cells (CD11c+) and neutrophil granulocytes (Gr-1+), were all reduced significantly by 45–75% (figure 3B). Thus, amelioration of arthritis was associated with a massive and prolonged global loss of leucocytes. Adoptive transfer of 107 unseparated cells from LN and spleens from G6PI-immunised mice 2 days after MCAO did not reconstitute disease development (data not shown).

MCAO does not affect autoantibody production
Autoantibodies against G6PI are necessary but not sufficient for induction of G6PI-induced arthritis.21 26 27 Sixteen days after G6PI immunisation, titres of G6PI-specific IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies were slightly but not significantly reduced in the MCAO group (figure 4A). Therefore, stroke-induced dampening of the humoral immune response does not explain the reduced arthritis severity in MCAO mice.

Cytokine production is similar in MCAO and control mice
Sixteen days after G6PI immunisation (10 d after MCAO), proliferation in response to G6PI was similar in cells from the draining LN of MCAO or sham-operated mice (figure 4B). Concentrations of IL-6, IL-17, IFN-γ and TNF-α in the supernatants of LN cells were similar in both groups in response to stimulation with G6PI or anti-CD3/CD28 (figure 4C). Concentrations of IL-4 and IL-5 were near the detection limit in both groups.

Functional differentiation of G6PI-specific Th cells is unimpaired after MCAO
TNF-α, IL-17 and IFN-γ are key cytokines in the pathogenesis of G6PI-induced arthritis.21 28 29 We analysed the G6PI-specific Th cell cytokine production 10 d after immunisation in mice that had undergone MCAO or sham surgery at d6 after G6PI immunisation. The number of LN and spleen cells did not differ between MCAO and sham animals at this time point (figure 5A). We used polychromatic flow cytometry to analyse the functional differentiation of G6PI-specific Th cells 10 d after immunisation. We found no difference in the numbers of
total CD4\(^+\) Th cells or G6PI-specific CD4\(^+\)CD154\(^+\) cells in MCAO and sham mice (figure 5B). The numbers of IFN-\(\gamma\), TNF-\(\alpha\) or IL-17-producing G6PI-specific T cells were also similar in control and MCAO mice (figure 5C). Thus, differentiation towards a Th1 or Th17 phenotype was unimpaired following stroke.

**T\(_{\text{reg}}\) cells are dispensable for stroke-induced immunosuppression.**

CD4\(^+\)CD25\(^+\)FoxP3\(^+\) regulatory T cells have been shown to be relevant for the prevention and down-modulation of experimental arthritis.\(^{25} 31–34\) An increased frequency of T\(_{\text{reg}}\) cells was reported 4 days after experimental stroke.\(^{35}\) Moreover, T\(_{\text{reg}}\) cells have been claimed as major modulators of postischaemic inflammatory brain damage.\(^{36}\) We employed DEREG mice, in which Foxp3\(^+\) regulatory T cells can be depleted by injecting diphtheria toxin,\(^{19}\) to examine the relevance of T\(_{\text{reg}}\) cells for the MCAO-induced amelioration of arthritis. Depletion of regulatory T cells resulted in enhanced severity and chronicity of G6PI-induced arthritis (figure 6A), confirming our earlier findings.\(^{23}\) MCAO reduced clinical arthritis in T\(_{\text{reg}}\)-cell-depleted DEREG mice significantly compared with sham-operated mice. Arthritis severity was significantly reduced in both DBA/1 DEREG mice and non-transgenic littermates upon MCAO without a difference between these groups. T\(_{\text{reg}}\) cells and other cells produce the anti-inflammatory cytokine TGF-\(\beta\). Serum concentrations of TGF-\(\beta\) can be modulated by the sympathetic nervous system (SNS).\(^{37}\) We, therefore, examined TGF-\(\beta\) serum concentrations 16 and 25 d after GPI immunisation. There was no difference between mice that had undergone MCAO 6 d after G6PI immunisation and sham-operated mice (data not shown). Thus, stroke-induced immunosuppression protects from arthritis even in the absence of T\(_{\text{reg}}\) cells and independently of increased TGF-\(\beta\) production.

**MCAO does not induce increased numbers of regulatory T cells in arthritic mice**

We used green fluorescent protein (GFP) expression by T\(_{\text{reg}}\) cells in the DEREG mouse strain to determine the frequency of T\(_{\text{reg}}\) cells during the course of experimental arthritis. There was no difference between MCAO and sham-operated animals. Approximately 5% of CD4\(^+\) T cells were GFP\(^+\) T\(_{\text{reg}}\) before...
day 15. When acute arthritis was already resolving 25 d postim-
munisation, the frequency of Treg cells was 9.9% in the MCAO
group and 12.7% in the sham group, respectively (figure 6B).
Therefore, the resolution of arthritis was associated with an
increase in Treg cells, but this increase was not further enhanced
by MCAO.

**DISCUSSION**

Stroke-induced immunosuppression is a major pathogenic factor
predisposing patients for morbidity and mortality through sec-
ondary infections, particularly pneumonia.2-4 Anecdotal clinical
evidence has suggested that stroke or denervation can
protect from arthritis.12-17 We show here that stroke-induced
immunosuppression modulates the pathogenic immune
responses that induce autoimmune arthritis.

The main ex vivo correlate of stroke-induced protection from
arthritis was a global reduction in leucocyte numbers. Reduced
lymphocyte numbers in secondary lymphatic organs3,6-7 35 39
and the peripheral blood35 are characteristics of stroke in
murine models. Several studies have demonstrated that this
lymphocyte loss was due to apoptosis.3,7,35 Offner et al35 reported a reduction in splenic cell yield already 22 h after
stroke. This differs from our findings: we did not find
dramatically reduced cell numbers in spleen and LN 4 d after
MCAO; instead we found a massive reduction in leucocyte
numbers 10 d after MCAO. Moreover, we found an approxi-
mately twofold reduction in splenic cell numbers, whereas
Offner et al35 reported an approximately fivefold reduction.
These differences are explicable by several factors. First, we
induced MCAO 6 d after immunisation with G6PI in CFA, that
is, during an ongoing immune response when T-lymphocytes
and B-lymphocytes proliferated in the draining LN and spleen.
In contrast, Offner et al analysed unimmunised mice after
MCAO. The ongoing activation and proliferation is likely to
fluence both the cells’ susceptibility to stroke-induced apop-
tosis and the time needed to replenish the lymphocyte pool.
Second, we studied DBA/1 mice, whereas Offner et al used
C57BL/6 mice. Mouse strains differ in their susceptibility to
stroke-induced immunosuppression.41 In addition to global
lymphocyte numbers, we also analysed the antigen-specific
immune responses to G6PI, the autoantigen inciting the arthri-
ogenic immune responses in G6PI-induced arthritis.18-30 There
are several novel aspects in the current study of stroke-induced
immunosuppression. B cell numbers were significantly reduced
in mice in which arthritis was ameliorated after MCAO. In con-
trast, G6PI-specific antibody titres were not reduced in these

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![Figure 2](image-url)  
**Figure 2** Middle cerebral artery occlusion (MCAO) ameliorates arthritis. (A) MCAO three days before G6PI immunisation (d-3) did not affect arthritis in MCAO (open triangles) and sham (filled circles) groups (n=9 per group; one of two experiments with similar results is shown). (B and C) MCAO 6 d (B) or 10 d (C) after immunisation decreased arthritis severity (n=8–9 per group; for MCAO d6, one of six experiments with similar results is shown). (D) Histopathological scoring of MCAO d6 animals 16 d after immunisation revealed decreased inflammation and joint destruction (n=9 per group). (*p<0.05).
animals, reflecting the fact that most of the antibody production had occurred prior to the stroke-induced B cell loss and confirming our earlier findings that antibodies are not sufficient to induce G6PI-induced arthritis.18 21

Studies on stroke patients reported reduced numbers and function of lymphocytes in the blood, whereas granulocyte numbers were increased, unaltered or not reported.26–83 8

The published data on the influence of stroke on T-cell cytokine production is contradictory. One study reported an initial hyperinflammatory reaction characterised by an increased production of proinflammatory cytokines preceding the stroke-induced immunosuppression in mice.42 A Th1/Th2 shift following clinical41 or experimental59 stroke was reported, whereas another study found unimpaired production of TNF-α and IL-6 in T cells from stroke patients.44 One possible explanation for these contradictory findings is that global ELISAs were used in those studies. Using flow cytometry, antigen-specific Th cells can be identified by their expression of CD154 upon a brief ex vivo stimulation with their cognate antigen.21 23–25 29 45–47 Ten days after immunisation with G6PI, we found similar numbers of G6PI-specific CD4+CD154+...
Th cells in the draining LN from control mice and mice that had undergone MCAO. Moreover, the number of cytokine-producing CD4\(^+\)CD154\(^+\) G6PI-specific Th cells was similar in MCAO mice and controls. Therefore, the proliferation of antigen-specific Th cells and the acquisition of Th cell effector functions are unaltered after MCAO.

CD4\(^+\)CD25\(^+\)FoxP3\(^+\) regulatory T cells maintain immune homeostasis by suppressing immune responses to self and non-self antigens. A increased frequency of T\(_{reg}\) cells has been reported in patients for up to 3 weeks after stroke. An increased frequency of T\(_{reg}\) cells has been reported in patients for up to 3 weeks after stroke. A increased frequency of T\(_{reg}\) cells has been reported in patients for up to 3 weeks after stroke. A increased frequency of T\(_{reg}\) cells has been reported in patients for up to 3 weeks after stroke. A increased frequency of T\(_{reg}\) cells has been reported in patients for up to 3 weeks after stroke.  

Due to the massive loss of lymphocytes, absolute T\(_{reg}\) cell numbers were nevertheless strongly decreased in MCAO mice in that study. In contrast to these findings, we did not find an increase in T\(_{reg}\) cells in MCAO mice. One important difference between our study and the earlier study is that we examined the consequences of MCAO in mice with an ongoing autoimmune response. We found increased T\(_{reg}\) cell frequencies during the remission phase of arthritis both in MCAO and control mice. This increased T\(_{reg}\) cell frequency was not further enhanced by MCAO. Whether T\(_{reg}\) cells contribute to the systemic stroke-induced immunosuppression has not been...
analysed to date. Using a genetic model of Treg cell depletion, we found that Treg cells were dispensable for the arthritis-ameliorating effect of experimental stroke.

How does stroke mediate these immunomodulatory effects? Two immediate candidates are the hypothalamic–pituitary–adrenal axis and the SNS, both of which are known to be immunosuppressive and activated in response to stroke. An elegant experimental study showed that both SNS-blockade with Propranolol or application of the glucocorticoid receptor antagonist RU486 prevented the apoptosis-induced decrease in lymphocyte counts. Yet, only Propranolol but not RU486 prevented bacteraemia and pneumonia and improved survival in MCAO mice. Thus, activation of the SNS seems to be a major mechanism for stroke-induced immunosuppression. Confirming

Figure 5  Unperturbed expansion and functional differentiation of G6PI-specific Th cells at the onset of clinical arthritis. (A) The number of spleen and lymph node cells 10 d after G6PI-immunisation was not different in middle cerebral artery occlusion (MCAO) and sham mice. (B) The number of CD4+ Th cells and G6PI-specific CD4+CD154+ Th cells was unaltered in MCAO mice as compared with sham controls. (C) Expression of IL-17, TNF-α and IFN-γ in G6PI-specific CD4+CD154+ T cells was similar in both groups.
and extending these findings, we have found that chemical sympathectomy with 6-hydroxydopamine (6-OHDA) prevents G6PI-induced arthritis (Müller et al., manuscript in preparation).

CONCLUSION

Experimental stroke causes a global loss of leucocytes and ameliorates arthritis. The correlate of protection from arthritis is not the reduction of a particular, pathogenic leucocyte subset or the preferential expansion or emergence of a protective cell population but the global reduction of leucocytes during arthritis.

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Contributors TK conceived of the concept, ll performed experiments and analysed data. MG did and interpreted histopathological studies. IMI and TK designed the studies, interpreted data and drafted the manuscript. All authors read and approved the final manuscript.

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Competing interests None.

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