

CNS Antigen-Specific Neuroinflammation Attenuates Ischemic Stroke With Involvement of Polarized Myeloid Cells

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Abstract

Background and Objectives

Experimental studies indicate shared molecular pathomechanisms in cerebral hypoxia-ischemia and autoimmune neuroinflammation. This has led to clinical studies investigating the effects of immunomodulatory therapies approved in multiple sclerosis on inflammatory damage in stroke. So far, mutual and combined interactions of autoimmune, CNS antigen-specific inflammatory reactions and cerebral ischemia have not been investigated so far.

Methods

Active MOG₃₅₋₅₅ experimental autoimmune encephalomyelitis (EAE) was induced in male C57Bl/6J mice. During different phases of EAE, transient middle cerebral artery occlusion (tMCAO, 60 minutes) was induced. Brain tissue was analyzed for infarct size and immune cell infiltration. Multiplex gene expression analysis was performed for 186 genes associated with neuroinflammation and hypoxic-ischemic damage.

Results

Mice with severe EAE disease showed a substantial reduction in infarct size after tMCAO. Histopathologic analysis showed less infiltration of CD45⁺ hematopoietic cells in the infarct core of severely diseased acute EAE mice; this was accompanied by an accumulation of Arginase1-positive/Iba1-positive cells. Gene expression analysis indicated an involvement of myeloid cell-driven anti-inflammatory mechanisms in the attenuation of ischemic injury in severely diseased mice exposed to tMCAO in the acute EAE phase.

Discussion

CNS autoantigen-specific autoimmunity has a protective influence on primary tissue damage after experimental stroke, indicating a very early involvement of CNS antigen-specific, myeloid cell-associated anti-inflammatory immune mechanisms that mitigate ischemic injury in the acute EAE phase.

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Glossary

BBB = blood-brain barrier; **CFA** = complete Freund adjuvant; **EAE** = experimental autoimmune encephalomyelitis; **MS** = multiple sclerosis; **I/R** = ischemia/reperfusion; **ROI** = regions of interest; **tMCAO** = transient middle cerebral artery occlusion.

Multiple sclerosis (MS) is a chronic demyelinating disease characterized by glial cell activation and immune cell infiltration into the CNS leading to successive neurodegeneration. Although histopathologic neuroinflammatory changes predominate, characteristics common to cerebral ischemia, such as vascular impairment and a sustained hypoxia-like tissue injury, have been described in acute MS lesions.^{1,2} Similarly, immunologic changes increasingly receive attention as potential therapeutic targets in the ischemic brain. A rapid decrease in oxygen supply followed by an immediate death of neurons in the lesion core triggers inflammatory processes, among which the activation of microglia, the innate immune cells of the brain, and the infiltration of blood-derived leukocytes are noteworthy.^{3,4} Furthermore, studies on human ischemic stroke biopsies indicate an age-dependent and comorbidity-dependent activation of microglia with a potential influence on neurodegeneration after stroke.⁵

Thus, several lines of experimental evidence point to similar molecular pathomechanisms active in cerebral hypoxia-ischemia and autoimmune neuroinflammation, albeit different in quality and extent. This has led to investigations using immunomodulatory MS therapeutics aiming at secondary stroke-associated inflammatory damage in experimental and clinical studies.⁶⁻⁹ Similarly, several approaches focus on interfering with hypoxic mechanisms during neuroinflammation and effects on the microvasculature.¹⁰⁻¹³ So far, potential interactions during simultaneous autoimmune neuroinflammation and cerebral ischemia have not been investigated yet. The question is clinically relevant because due to disease-modifying therapies, an increased number of patients with MS are approaching disease categories at risk for stroke events.

Here, we aimed at investigating the effect of an existing CNS autoantigen-specific inflammatory reaction during experimental autoimmune encephalomyelitis (EAE) on subsequent experimental cerebral ischemia during transient middle cerebral artery occlusion (tMCAO). Paradoxically, higher severity of the neuroinflammatory disease correlated with smaller infarct size. Multiplex gene expression analyses indicate a potential involvement of polarized myeloid cells in this protective effect of CNS autoantigen-specific neuroinflammation.

Methods

Standard Protocol Approvals and Registrations

Animal experiments were performed as approved by the Veterinary Office of the Canton Bern (Permission Nos.: BE 67/17 and BE101/16) and the North Rhine-Westphalian authorities for animal experimentation (Landesamt für Umwelt, Natur und Verbraucherschutz, Recklinghausen,

Permission No.: G1361/13, Az.:84-02.04.2013.A192) in accordance with the E.U. guidelines (Directive 2010/63/EU) for the care and use of laboratory animals and reported based on Animal Research: Reporting In Vivo Experiments (ARRIVE guidelines).

Animals, Experimental Planning, and Randomization

Male wild-type C57Bl/6J mice (Envigo Research Models and Services, the Netherlands) were kept in a 12 hours:12 hours light/dark cycle in cages of 5 animals with food and water ad libitum. Experiments were strictly randomized and analyzed in a blinded manner. Statistical planning assumed an α -error of 5% and 1-statistical power (α β -error) of 20%.

Experimental Autoimmune Encephalomyelitis

Active EAE was induced in 8–10-week-old mice as described previously.^{14,15} Briefly, myelin oligodendrocyte peptide 35–55 (MOG₃₅₋₅₅) (Institute of Medical Immunology, Charité, Berlin, Germany) emulsified in complete Freund adjuvant (CFA) was injected subcutaneously, followed by the intraperitoneal injection of pertussis toxin at days 0 and 2. Sham-immunized control mice received CFA alone without MOG peptide (subsequently referred to as CFA mice). Neurologic deficits were assessed daily using a 10-point EAE score: 0 = no clinical signs; 1 = reduced tone of tail; 2 = limp tail, impaired righting; 3 = absent righting; 4 = gait ataxia; 5 = mild hind limb paraparesis; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia; 8 = tetraparesis; 9 = moribund; and 10 = death.^{14,15} The acute phase of EAE was defined as 1–4 days after first clinical symptoms and the chronic phase of EAE as remission of symptoms with a stable score for at least 2–3 days. The scores given in the figures are maximum disease scores detected in the acute EAE phase.

Transient Focal Cerebral Ischemia

tMCAO was performed on isoflurane-anesthetized mice during different phases of EAE (acute and chronic EAE) and CFA mice using the intraluminal filament model.¹⁶⁻¹⁸ Briefly, the left common and external carotid arteries were ligated, and a silicon-coated microfilament (Doccol Corporation, Sharon, MA) was introduced to occlude the MCA for 60 minutes. The body temperature was maintained at 37°C, and the cerebral blood flow was measured by laser Doppler flow. Appropriate analgesia was ensured by subcutaneous injection of buprenorphine (0.1 mg/kg body weight). Neurologic deficits after tMCAO were evaluated using a modified Bederson score¹⁹⁻²¹: 0 = no deficits; 1 = failure to extend the ipsilateral and/or contralateral paw; 2 = circling to the paretic side; 3 = falling to the paretic side. After 3 or 24 hours of reperfusion, mice were perfused with PBS and CNS and spleens were fresh-frozen for further analysis.

Histologic Analysis

Histologic analysis was performed on 20 μm coronal cryostat sections collected from the core of the middle cerebral artery (+1.1 to +0.7 mm from bregma). Sections were fixed for 10 minutes with 4% paraformaldehyde at RT or acetone for 10 minutes at -20°C .

For analysis of the infarct extent, 2 sections per animal were stained with cresyl violet, digitalized with a Panoramic Digital Slice Scanner (3DHISTEC, Hungary), and regions of interest (ROI) were drawn manually using image analysis software (ImageJ; NIH, Bethesda, MD). The extent of infarction was quantified as follows:

$$\text{Infarct size} = \text{area}_{(\text{contralateral hemisphere})} - \text{area}_{(\text{intact ipsilateral hemisphere})}$$
$$\text{Edema size} = \text{area}_{(\text{ipsilateral hemisphere})} - \text{area}_{(\text{contralateral hemisphere})}$$

For hematopoietic cells, sections were stained using a biotinylated rat α -CD45 antibody (5 $\mu\text{g}/\text{mL}$; Becton Dickinson, Germany), detected using an ABC peroxidase kit (Vectastain, Vector, Germany), and counterstained with Mayer hemalum solution (VWR International, Germany). For immunofluorescence, sections were stained for CD3⁺ T cells (rat α -CD3, 10 $\mu\text{g}/\text{mL}$; AbD Serotec, Germany), Iba1⁺ myeloid cells (rabbit α -Iba1, 2.5 $\mu\text{g}/\text{mL}$; Wako Chemicals Europe, Germany), GFAP⁺ astrocytes (rat α -GFAP, 10 $\mu\text{g}/\text{mL}$; Thermo Fisher Scientific, Germany), and Arginase1⁺ myeloid cells and astrocytes (goat α -Arg1 Alexa Fluor 488, 5 $\mu\text{g}/\text{mL}$; Santa Cruz Biotechnology, Germany). Staining was detected using goat α -rabbit Alexa Fluor 488 or 555 or goat α -rat Alexa Fluor 488 secondary antibodies. Sections were digitalized with a Panoramic digital slice scanner (3DHISTEC, Hungary), and the number of positive cells in cortex and striatum of the MCA territory (ROI) was determined in a blinded manner. Interhemispheric differences were calculated as a ratio of the number of positive cells inside the ROI ipsilateral and the number of positive cells inside the ROI contralateral to MCAO.

Gene Expression Analysis

RNA was isolated from fresh-frozen brain tissue containing the ipsilateral or contralateral MCA territory (bregma 0.7–2 mm) using a TRIZOL and RNeasy Kit Mini (Qiagen, Germany). One hundred micrograms of total RNA were used for NanoString nCounter XT Codeset Gene Expression Assay (NanoString Technologies, Seattle, WA) using a customer codeset targeting 186 candidate genes and 6 internal control genes (*Aars*, *Ccdc127*, *Cnot10*, *Csnk2a2*, *Fam104a*, *Lars*). The assay and the standard quality control of the raw data were performed according to the manufacturer's protocol. For visualization, the data were normalized using the Bioconductor package NanoStringDiff v. 1.12.0.

Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA) or SPSS (IBM Corp., Armonk, NY). Respective statistical tests are indicated in figure legends. Briefly, 2-group comparisons

were established using the Mann-Whitney *U* test for independent groups and the Wilcoxon signed rank test for dependent groups. A Kruskal-Wallis test (analysis of variance) with Dunn's post hoc analysis was performed for multiple comparisons. Spearman ρ was used for correlation analysis.

Gene expression data were analyzed using limma v. 3.38.3 (Gordon Smyth, Walter and Eliza Hall Institute of Medical Research, Parkville Victoria, Australia) in R v. 3.5.2. Specifically, hemisphere, immunization, and disease score were collapsed into a single factor with 5 levels. Linear models were run, separately for the acute and chronic phases of EAE, on the voom-normalized counts including this combined factor and individual as a random effect. *p* Values were false discovery rate-adjusted using the procedure of Benjamini and Hochberg. Probability levels (*p* value) are indicated as **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Data Availability

The complete data sets of this article will be made available to any qualified researcher on reasonable request.

Results

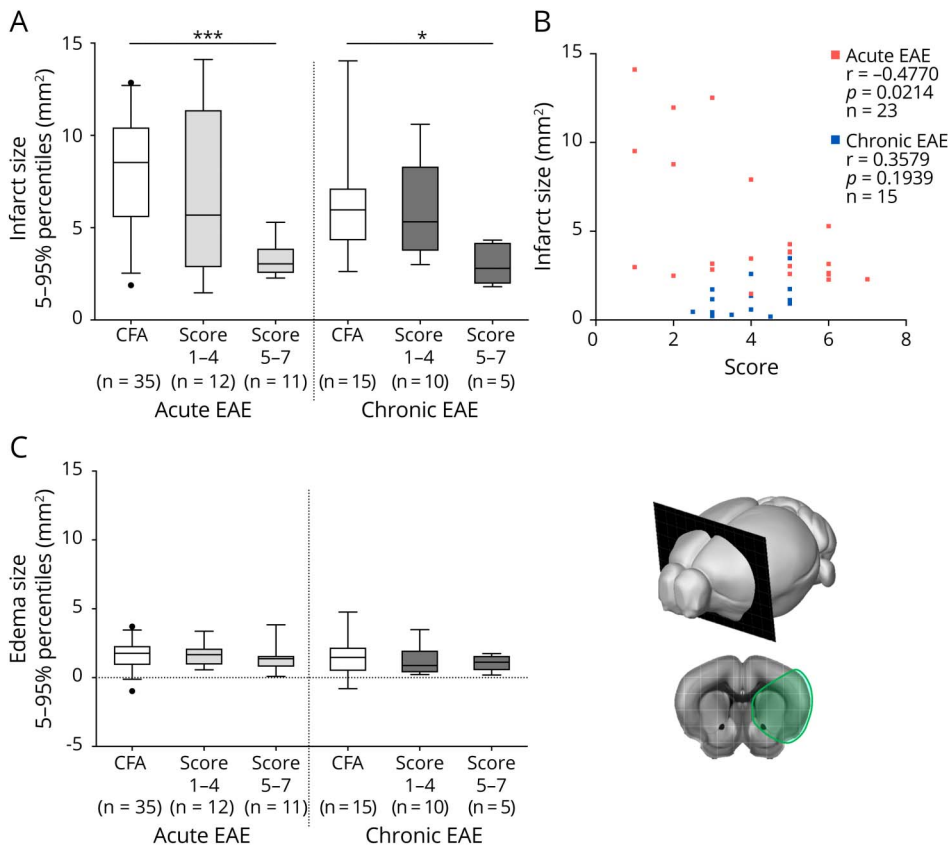
Decreased Ischemic Injury in Mice With Severe EAE Score

To elucidate whether a pre-existing neuroinflammation influences ischemia/reperfusion (I/R) injury, we first examined the infarct size of animals subjected to 60-minute tMCAO during EAE. After 24 hours of reperfusion, infarcts were smaller in both mice with acute and chronic EAE compared with control mice receiving CFA without the CNS autoantigen only (acute EAE: 1.6-fold decrease; chronic EAE: 1.4-fold decrease vs CFA). In a subgroup analysis further subdividing animals according to EAE disease severity (mild-to-moderate, score 1–4; severe, score 5–7), this effect was driven by severely diseased animals (Figure 1, A and B). This held true for both acute and chronic phases of EAE (Figure 1A). For mild-to-moderate EAE, changes in infarct size did not reach statistical significance. Edema size was not affected in any of the groups (Figure 1C). In animals killed at 3 hours after tMCAO, acute EAE reduced edema but not infarct size (eFigure 1, links.lww.com/NXI/A717).

Altered Immune Cell Accumulation After tMCAO in EAE Animals

To investigate the effect of EAE or EAE along with I/R on immune cell infiltration into the brain, we first examined the expression of the general leukocyte marker CD45⁺. The absolute number of CD45⁺ in the nonischemic contralateral brain hemisphere was moderately increased in acute and chronic mild EAE, when compared with the nonischemic hemisphere of CFA-immunized mice (Figure 2A). Furthermore, we observed a significant increase of CD45⁺ cells in the ischemic hemisphere of both CFA-immunized mice and EAE mice subjected to stroke (Figure 2A). To specifically assess

Figure 1 EAE Severity Has a Beneficial Effect on Extent of Infarction After 24 Hours of Reperfusion



(A) Infarct size of CFA-immunized and MOG₃₅₋₅₅-immunized mice 24 hours after tMCAO for 60 minutes induced in different phases (acute and chronic) of EAE. Box plot with 5%–95% percentiles. Kruskal-Wallis test with Dunn's post hoc test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (B) Correlation of infarct size and EAE severity. Only MOG₃₅₋₅₅-immunized mice. Spearman's rank correlation coefficient. (C) Edema size of CFA-immunized and MOG₃₅₋₅₅-immunized mice 24 hours after tMCAO for 60 minutes induced in different phases (acute and chronic) of EAE. Box plot with 5%–95% percentiles. Kruskal-Wallis test: $p = n.s.$ Schematic drawing of the mouse brain illustrates localization of infarct and edema size measurement. CFA = complete Freund adjuvant; EAE = experimental autoimmune encephalomyelitis; tMCAO = transient middle cerebral artery occlusion.

alterations associated with I/R, we analyzed the ratio of CD45⁺ cells between ischemic and nonischemic hemispheres. This ratio was markedly reduced in all EAE animals undergoing I/R in the acute EAE phase (Figure 2B) compared with CFA-immunized mice.

Next, we analyzed the expression of Iba1 as a marker of myeloid cells in brain sections. Mild acute EAE led to a significant increase of Iba1⁺ cells in nonischemic contralateral brain tissue, which was not observed in severely diseased mice (Figure 2C). As expected after I/R, there was an accumulation of Iba1⁺ cells in the ischemic tissue in CFA-immunized mice, as well as in severely diseased EAE mice, whereas there was no further increase of Iba1⁺ cells in mildly diseased EAE mice. This resulted in a lower interhemispheric ratio in mildly diseased compared with severely diseased EAE mice (Figure 2D). By contrast, mice subjected to I/R in the chronic EAE phase showed only a nonsignificant increase of Iba1⁺ cells in both the nonischemic and ischemic hemispheres compared with CFA-immunized mice.

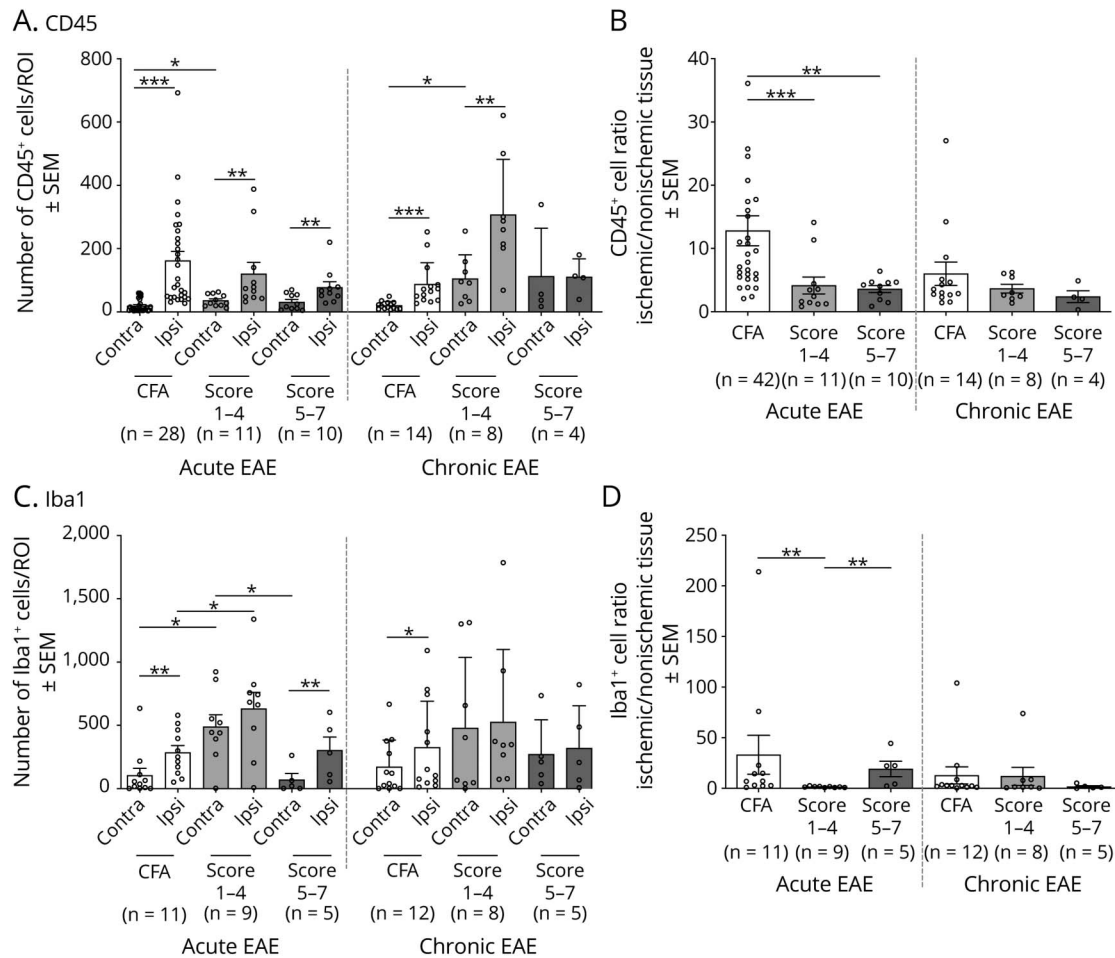
When analyzing CD3⁺ T cells in brain sections, we could not detect any statistically significant changes upon EAE in the nonischemic contralateral hemisphere (eFigure 2, links.lww.com/NXI/A717). CD3⁺ cells moderately increase upon I/R. Overall, the cell numbers and interhemispheric differences

were low. In summary, we observe EAE-mediated alterations of immune cell accumulation into the ischemic brain, which may contribute to attenuated I/R injury.

Postischemic Increase of Iba1⁺ Myeloid Cells Is Associated With Anti-inflammatory Differentiation in EAE Animals

To gain further insight into the molecular mechanisms active in the combined animal model of neuroinflammation and cerebral ischemia, we performed a multiplex gene expression analysis of the infarcted tissue and corresponding contralateral regions. We prepared a codeset targeting 186 genes that are associated with neuroimmunologic and hypoxic mechanisms. Of these genes, 31 were altered upon EAE and I/R (Figure 3A). Principal component analysis revealed close proximity of the different groups (CFA vs acute EAE vs chronic EAE, Figure 3B). Further analysis showed an EAE-induced upregulation of genes associated with antigen presentation and inflammatory processes in both contralateral and ipsilateral hemispheres of ischemic animals exposed to acute or chronic EAE compared with CFA mice (data not shown). Again, subgroup analysis considering the severity of EAE revealed 18 differentially regulated genes in the infarct core of severely diseased EAE mice during acute EAE compared with CFA mice (Figure 3C). Here, downregulated genes were associated with cell response to stress and with

Figure 2 CD45⁺ and Iba1⁺ Cells in the Striatum and Surrounding Cortex 24 Hours After tMCAO in Different Phases of EAE



Absolute number of (A) CD45⁺ or (C) Iba1⁺ cells in the infarct core and surrounding cortex 24 hours after tMCAO (ipsi, ipsilateral) or the corresponding regions in the nonischemic hemisphere (contra, contralateral) in different phases of EAE and cell ratios (ischemic/nonischemic hemisphere) of (B) CD45⁺ or (D) Iba1⁺ cells. *p* Values of Wilcoxon rank sum test comparing absolute numbers ipsilateral vs contralateral for every group. **p* < 0.05; ***p* < 0.01; ****p* < 0.001. *p* Values of Kruskal-Wallis with Dunn's post hoc for multiple testing between all groups: **p* < 0.05; ***p* < 0.01; ****p* < 0.001. EAE = experimental autoimmune encephalomyelitis; tMCAO = transient middle cerebral artery occlusion.

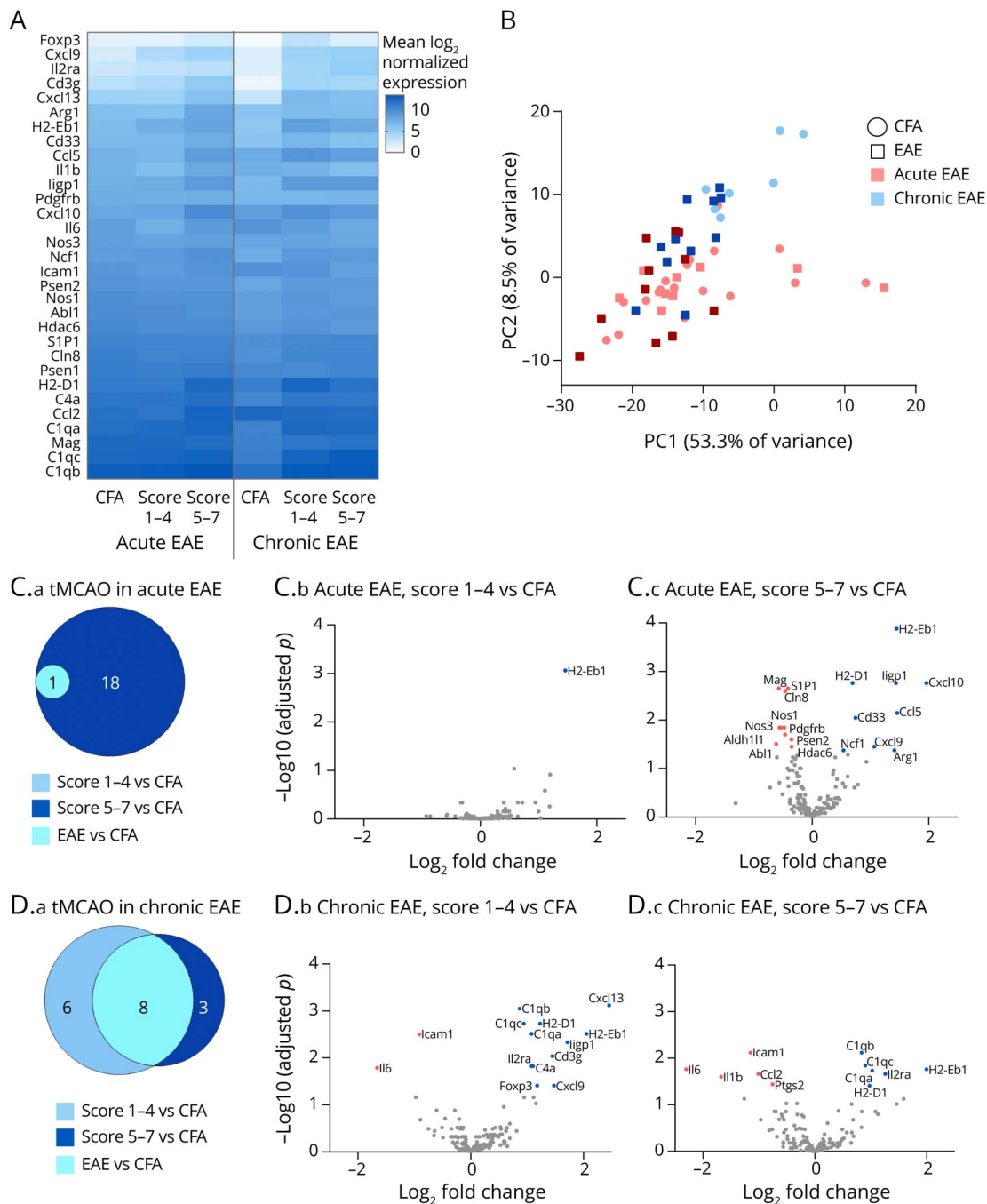
energy metabolism (e.g., *Nos1*, *Nos3*, *Hdac6*). Upregulated genes were associated with immune cell activation and migration (e.g., *H2-Eb1*, *H2-D1*, *Cxcl-10*, *Ccl5*) and included *Arg1* (coding for Arginase1; 1.4-fold [log₂], adj. *p* = 0.042), as a potential marker for an anti-inflammatory phenotype of microglia and macrophages (eFigure 3, links.lww.com/NXI/A717). By contrast, upregulated genes in the infarct core during chronic EAE were associated with cell adhesion and complement activation (e.g., *C1qa*, *C1qb*, *C1qc*, *C4a*; Figure 3D). An involvement of potential anti-inflammatory mechanisms was further supported histologically with an Arginase1-specific staining where severely diseased (score 5–7) EAE animals showed a 17.1-fold increase of Arginase1+ cells in mice exposed to I/R in the acute phase but not in the chronic phase (Figure 4B). In addition, Iba1/Arginase1 double staining indicated that only in the acute phase, the number of Iba1+/Arginase1+ myeloid cells was increased in severely diseased EAE animals and that most of the Arginase1+ cells were also Iba1+ (Figure 4, C and D). In

brains from chronically diseased animals, however, around 50% of cells positive for Arginase1 were also positive for Iba1, irrespective of disease severity (Figure 4B). To elucidate whether part of the Arginase1+/Iba1- cells are astrocytes, we performed Arginase1/GFAP double staining. However, the number of Arginase1/GFAP double-positive cells in any of the groups was negligible (less than 5% of Arginase1+ cells were also positive for GFAP; eFigure 3B).

Discussion

By exposing mice with active EAE as a widely used model for CNS antigen-specific autoimmunity to tMCAO, which is a clinically relevant model for ischemia/reperfusion (I/R) injury, we show that animals severely affected by EAE develop less injury upon I/R than control mice immunized with adjuvant only. Our data suggest that the attenuation of ischemic injury in acute EAE might be mediated by an EAE-associated increase of anti-inflammatory Arginase1 expressing myeloid cells, which is

Figure 3 Expression Profiles in the Infarct Core After tMCAO in Acute and Chronic Phases of EAE



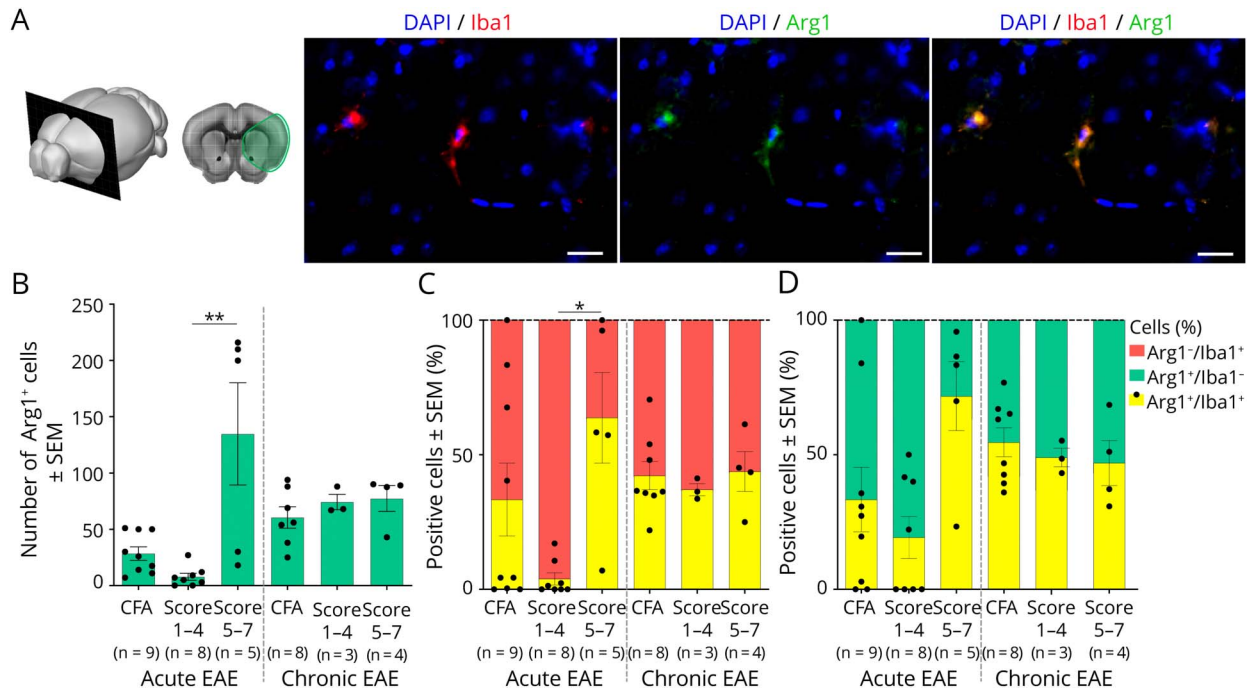
(A) Heat map of regulated genes in at least 540, one of the examined groups compared with the corresponding CFA group. (B) Principal component analysis. Circles or squares represent different immunization schemes (CFA or EAE-diseased), colors represent different phases of EAE (acute [red] or chronic [blue] EAE), lighter squares represent animals with EAE score 1–4, darker squares represent animals with EAE score 5–7. (C.a–c) Venn diagram of regulated genes 24 hours after tMCAO induced in the acute EAE and corresponding volcano plots. (D.a–c) Venn diagram of regulated genes 24 hours after tMCAO induced in the chronic EAE and corresponding volcano plots. CFA = complete Freund adjuvant; EAE = experimental autoimmune encephalomyelitis; tMCAO = transient middle cerebral artery occlusion.

supported by gene and protein expression profiles. MS is characterized by increased infiltration of peripheral immune cells over the impaired blood-brain barrier (BBB) into the spinal cord and brain tissue, leading to an activation of glial cells and subsequent demyelination.^{22,23} Similarly, upon ischemia tight junctions between endothelial cells as part of the BBB start

to degrade, leading to BBB breakdown.²⁴ Accordingly, an increased infiltration of different immune cells into the brain causing an inflammatory response is observed after stroke.²⁵

Directly after an ischemic insult, rapid ionic failure induces the formation of an irreversibly injured infarct core surrounded by

Figure 4 Accumulation of Arginase1⁺ and Iba1⁺ Cells in the Infarct Core and Surrounding Cortex 24 Hours tMCAO in Different Phases of EAE



(A) Exemplanary histologic stainings of Iba1+/Arginase1 (Arg1)+ cells in the infarct core and surrounding cortex (ROI indicated in the figure) Scale bar: 20 μm. (B) Number of Arginase1+ cells. Kruskal-Wallis test: ***p* = 0.001. (C) Amount of Arginase1+ cells compared with the total amount of Iba1+ myeloid cells. Kruskal-Wallis test: **p* = 0.020. (D) Amount of Iba1+ myeloid cells compared with the total amount of Arginase1+ cells. Kruskal-Wallis test: *p* = 0.023. Dunn's multiple comparisons test: **p* < 0.05; ***p* < 0.01. CFA = complete Freund adjuvant; EAE = experimental autoimmune encephalomyelitis; tMCAO = transient middle cerebral artery occlusion.

an adjacent penumbra with still salvageable tissue.²⁶⁻²⁸ Simultaneously, edema formation starts minutes after stroke and lasts for several days. During early cytotoxic edema, anoxic processes lead to increased osmolarity and subsequent water influx in neurons and especially in astrocytes through aquaporin-4 (reviewed in reference 29). After 4–6 hours, vasogenic edema characterized by reduced BBB integrity allows for serum protein leakage and immune cell infiltration into the brain parenchyma.^{30,31} Accordingly, after a reperfusion time of 3 hours, we were not able to detect differences in immune cell infiltration and infarct size, but in edema formation. Diseased EAE animals showed a decrease in edema size indicating a very early protective involvement of CNS autoimmunity. Although EAE has been described to predominantly affect the spinal cord, mitochondrial dysfunction and disturbed tissue homeostasis have also been observed in the brain parenchyma³²⁻³⁴ and might play a beneficial role in the observed effects on edema formation.

Mononuclear phagocytes including infiltrating monocyte-derived macrophages and CNS-resident microglia have been described to play a distinct role in the course of EAE because they are the predominant cells in lesions and their depletion prevents EAE progression.³⁵⁻³⁷ Whereas microglia activation takes place even before the development of clinical symptoms, CNS-infiltrating phagocytes seem to be necessary to induce severe symptoms.³⁷ Early microglia activation has also been

described in stroke, especially at the edge of the lesion with a time-dependent change of their morphology and pronounced proliferation in the lesion core within 24 hours of reperfusion.²⁷ This early activation leads to secretion of proinflammatory cytokines, antigen presentation through major histocompatibility complex II, and subsequent recruitment of blood-derived macrophages, thus infiltrating to a later time point and peaking after several days of reperfusion.^{5,38,39}

In the combined EAE-stroke model, we show an increased activation of myeloid cells in both the CFA and seriously diseased EAE animals already 24 hours after tMCAO. However, this was linked to protective effects in the infarct areas of seriously diseased EAE animals only and accompanied by a distinct upregulation of major histocompatibility complex II genes. As these mechanisms normally take place several days after I/R injury,³⁹ it might well be that due to the already existing EAE, these processes had already been initiated⁴⁰ and thus lead to protective mechanisms at this early time point after tMCAO. Thus, myeloid cells seem to not only contribute to the resolution of the prevailing EAE-induced inflammation but could also have a beneficial effect on an additional exogenous insult, as induced by tMCAO.

This assumption is supported by the results of the gene expression analysis and immunostainings showing an upregulation

of Arginase1 in severely diseased animals. Arginase1 is considered as signature enzyme for an anti-inflammatory activation state of macrophages and competes with NOS for L-arginine in the urea cycle. Whereas NOS induces reactive oxygen species production and thus is linked with tissue damage, Arginase1 induces tissue regeneration by catalyzing L-arginine to L-ornithine and urea.⁴⁰ Although this classic concept of macrophage activation is still under debate and it is still unclear to which extent it plays a role in vivo,⁴¹ the contribution of phagocytic cells on CNS tissue damage seems to depend not only on their origin but also on their functional activation state, on their location, and especially on the phase of the insult. Although in EAE, CNS-infiltrating mononuclear phagocytes present with a proinflammatory polarization status in the acute phase, they change their phenotype to an intermediate state before they predominantly display an anti-inflammatory state with Arginase1 expression in later phases of especially severe EAE in the spinal cord.⁴² Similarly, an intermediate activation state of mononuclear phagocytes showing proinflammatory and anti-inflammatory markers in the lesion core and adjacent tissue could be detected in human and experimental stroke studies; before in late stages, no blood-derived macrophages but predominantly homeostatic microglia are present.^{3,43,44} Together with previous observations that Arginase1 is expressed only several days after I/R when regeneration processes are initiated, this is in line with our data from brain tissue, suggesting that immediate Arginase1 expression and thereby the polarization of myeloid cells toward an anti-inflammatory phenotype in the brain could play a role in tissue protection against a later I/R injury. The pre-existing inflammation in the CNS seems to influence the polarization of mononuclear phagocytes in the acute phase after stroke. Our data show that accumulation of Arginase1+ cells in acute EAE is transient. If the attenuation of I/R injury in chronic EAE is mediated by Arginase1+, myeloid cells reprogrammed in the acute EAE phase or if another (yet unknown) factor influences I/R injury could not be clarified in this work. A very early involvement of neutrophils has been described in both the pathogenesis of EAE and tissue-damaging processes after cerebral ischemia.⁴⁵⁻⁴⁷ Whereas in some pre-clinical models, a profound infiltration into the lesions has been observed after ischemic stroke,⁴⁷⁻⁴⁹ in other studies, neutrophils were largely confined to the neurovascular unit and the meninges.^{e1} In this study using a model of acute and chronic EAE, we did not detect significant neutrophilic infiltration in the brain parenchyma after 24 hours of reperfusion. In addition, gene expression analysis did not show a specific regulation of neutrophils in any of the groups. Another important player in the pathogenesis of EAE are T cells, mainly infiltrating the spinal cord and to a lesser extent the brain.^{33,e2} In line with the latter findings, we did not detect an increase in T cells in the contralateral hemisphere of EAE animals compared with CFA mice. In the ischemic hemisphere, T cell numbers were low. In ischemic stroke, T cell infiltration evolves slowly, with a peak after 48 hours or later.^{e3,e4} Although EAE animals showed less T cell infiltration in the ipsilateral hemisphere than CFA animals, a biological effect in this setting seems to be unlikely because of the low absolute numbers. Thus, the molecular mechanisms that

underlie the observed protective effect of EAE on subsequent I/R injury rather seem to be independent from T cell-driven processes, as described in the concept of protective autoimmunity, postulating the induction of neuroprotective processes by CNS antigen-specific T cells.^{e5,e6} Still, a prolongation of reperfusion times might give further insights into a potential and additional influence of infiltrating T cell subpopulations in the combined model but has not been focus of this study.

A shortcoming of our study is the lack to further distinguish between CNS-resident microglia and macrophages using cell-specific markers (e.g., Tmem119/Ly6Chigh). After our initial characterization of the profound effect of CNS autoantigen-specific autoimmunity and associated immune cell changes, further elucidation of subpopulations will be focused for future investigations.

Although Arginase1 expression was mainly driven by myeloid cells in our model as shown by histologic analysis, additional cell types are involved. Despite negligible amounts of Arginase1/GFAP double-positive cells in our brain sections, we cannot exclude a relevant functional effect of astrocytes. In a photothrombotic stroke model, increased Arginase1 expression was demonstrated upon ischemia not only in macrophages but also in astrocytes accompanied by an upregulation of BDNF.^{e7} Furthermore, after MCAO, astrocytes predominantly display a protective phenotype inducing the expression of neurotrophic factors and cytokines.^{e8} In our study, we did not detect any regulation of the included astrocytic markers (*Egfr*, *Cd14*, *Tgm1*, *S100a10*, *Lcn2*, *Ptgs2*, or *S1pr3*) making a prominent role of astrocytes in Arginase1 expression in this study unlikely. Further experiments should focus on cell-specific transcriptome analyses in the ischemic area instead of infarcted striatum and cortex tissue as in this study, preferably with nontargeted expression analyses.

In conclusion, the results of this study indicate a very early involvement of immune mechanisms in the combined model of EAE and I/R. Acute CNS autoimmunity seems to have a protective influence on I/R-induced tissue damage with the involvement of potential M2-promoting factors in brains of severely affected EAE mice. This effect seems to be linked to an active CNS involvement of an antigen-specific inflammatory reaction. Further studies are needed to reveal cell type-specific analyses of alterations in mRNA expression in the brain and in peripheral tissues and how these contribute to a protection of brain tissue from ischemia. A better understanding of the interactions between CNS autoimmunity and cerebral ischemia is of particular relevance regarding increasing prevalence of vascular comorbidities with MS and similar risk factors for MS and ischemic stroke.^{e9-e11}

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Disclosure

K. Guse is a former employee of Biogen, not related to this study. N. Hagemann declares no disclosures relevant to the

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Name	Location	Contribution
Kirsten Guse, PhD	Inselspital, University of Bern, Switzerland and Ruhr-University, Bochum, Germany	Study design and conceptualization; methodology, data acquisition, data analysis and interpretation; drafting the manuscript for intellectual content
Nina Hagemann, PhD	University of Duisburg-Essen, Germany	Study design and conceptualization; methodology, data acquisition, data analysis and interpretation; drafting the manuscript for intellectual content
Lisa Thiele, PhD	Inselspital, University of Bern, Switzerland	Conceptualization, methodology, review of manuscript
Jana Remlinger, MSc	Inselspital, University of Bern, Switzerland	Methodology, data acquisition, review of manuscript
Anke Salmen, MD	Inselspital, University of Bern, Switzerland	Data analysis, review of manuscript
Robert Hoepner, MD	Inselspital, University of Bern, Switzerland	Data analysis, review of manuscript

Appendix (continued)

Name	Location	Contribution
Irene Keller, PhD	Interfaculty Bioinformatics Unit, Department for BioMedical Research and Swiss Institute of Bioinformatics, University of Bern, Switzerland	Data analysis, review of manuscript
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Denis Grandgirard, PhD	Institute for Infectious Diseases, University of Bern, Switzerland	Conceptualization, methodology, review of manuscript
Stephen L. Leib, MD	Institute for Infectious Diseases, University of Bern, Switzerland	Methodology, conceptualization, review of manuscript
Erik Vassella, PhD	Institute of Pathology, University of Bern, Switzerland	Methodology, conceptualization, review of manuscript
Giuseppe Locatelli, PhD	Theodor Kocher Institute, University of Bern, Switzerland	Conceptualization, methodology, review of manuscript
Dirk M. Hermann, MD	University of Duisburg-Essen, Germany	Study design and conceptualization, supervision, resources, review, and editing
Andrew Chan, MD	Inselspital, University of Bern, Switzerland	Study design and conceptualization, supervision, resources, review, and editing

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