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Nasal Vaccination with Myelin Oligodendrocyte Glycoprotein Reduces Stroke Size by Inducing IL-10-Producing CD4⁺ T Cells¹

Dan Frenkel,* Zhihong Huang,[‡] Ruth Maron,* Djordje N. Koldzic,* Wayne W. Hancock,[†] Michael A. Moskowitz,[‡] and Howard L. Weiner^{2*}

Inflammation plays an important role in ischemic stroke and in humans IL-10 may have a beneficial effect in stroke. We mucosally administered myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide to C57BL/6 mice before middle cerebral artery occlusion (MCAO) to induce an anti-inflammatory T cell response directed at CNS myelin. Nasal and oral administration of MOG_{35–55} peptide decreased ischemic infarct size at 24 and 72 h after MCAO surgery. Nasal MOG_{35–55} peptide was most efficacious and reduced infarct size by 70% at 24 h and by 50% at 72 h ($p \leq 0.0001$ vs control) and also improved behavior score. Immunohistochemistry demonstrated increased IL-10 and reduced IFN- γ in the area surrounding the ischemic infarct following nasal treatment. Nasal MOG did not reduce infarct size in IL-10-deficient mice. Adoptive transfer of CD4⁺ T cells to untreated mice from nasally tolerized mice before MCAO surgery decreased stroke size ($p < 0.001$ vs control), whereas, CD4⁺ T cells from nasally tolerized IL-10-deficient mice had no effect. Our results demonstrate that IL-10-secreting CD4⁺ T cells induced by nasal MOG reduce injury following stroke. In addition, we observed a dramatic reduction of CD11b⁺ cells in nasal MOG-treated animals. CD11b⁺ cells may contribute to secondary infarct expansion by enhancing NO synthesis that may be reduced by elevated IL-10 levels. Modulation of cerebral inflammation by nasal vaccination with myelin Ags that increase IL-10 in the brain may improve outcome after stroke and enhance mechanisms of recovery. *The Journal of Immunology*, 2004, 172: 6549–6555.

Ischemic stroke results from transient or permanent reduction in cerebral blood flow. It is one of the main causes of morbidity and mortality worldwide. The mortality from stroke is ~30%, 80–90% of stroke survivors exhibit motor weakness, and 40–50% experience sensory disturbances (1). In the center of the perfusion deficit, cerebral blood flow is typically 80% below normal levels (2). Ischemic tissue dies over minutes to many hours (2).

Inflammation is also initiated by ischemia at the blood-microvascular endothelial cell interface and contributes significantly to CNS damage. Polymorphonuclear leukocytes rapidly enter injured brain tissue (3) and white blood cells traverse the blood-brain barrier (BBB) 12–24 h after onset and may provide a source of oxygen-free radicals. Eventually, the infarcted zone is infiltrated with lymphocytes, polymorphonuclear cells, and macrophages (4). Neutrophils, important cellular components of the innate immune response, produce a number of potentially harmful substances including toxic oxygen metabolites, destructive enzymes, and proinflammatory cytokines with neurotoxic properties (5). Thus, the severity of postischemic injury can be affected by manipulation of the inflammatory response. Agents that limit white blood cell adhesion to endothelial receptors, such as Abs to P-selectin (6) and

ICAM-1 (7) reduce ischemic injury. However, in a multicenter acute clinical trial, anti-human ICAM-1 Ab increased mortality (3). Another immunological therapeutic approach is modulation of cytokine production.

Following ischemia, most cells in the brain, including endothelial cells, microglia, astrocytes, and neurons, can produce IL-1 β , IL-6, and TNF- α that can directly induce cell death as well as contribute to vessel wall injury, hemorrhage, edema, and tissue necrosis (8). There are immunoregulatory cytokines that can modulate immune processes and inhibit expression of inflammatory Th1-type responses as well as affect inflammation in general. Among them are IL-10 and TGF- β 1 (9). IL-10 is preferentially produced by Tr1-type regulatory T cells (10) and TGF- β is preferentially produced by Th3-type regulatory cells which suppress both Th1 and Th2 cells (9, 11). It has been reported that elderly patients with a history of stroke had significantly lower median IL-10 levels compared with elderly patients without stroke (12, 13). Moreover, TGF- β is expressed in brain following ischemia (14) and may play a role as an anti-inflammatory cytokine with neuroprotective properties. Mucosal (nasal or oral) administration of Ag preferentially induces regulatory T cells that secrete IL-10 and TGF- β at the anatomic site where the mucosally administered Ag is located, a phenomena termed bystander suppression. Becker et al. (15) previously demonstrated reduced infarct size in the rat after oral tolerance with myelin basic protein and suggested that TGF- β may play a role because there was increased TGF- β expression in the brain of orally tolerized animals. To investigate the role of T cells during the course of stroke, we studied mucosal tolerance in the mouse model of stroke. We mucosally administered myelin oligodendrocyte glycoprotein (MOG)³ 35–55 peptide in a mouse stroke model and demonstrate reduction of infarct size that is mediated by IL-10-dependent CD4⁺ T cells (15). This

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³ Abbreviations used in this paper: MOG, myelin oligodendrocyte glycoprotein; MCAO, middle cerebral artery occlusion; BBB, blood-brain barrier.

approach has applicability both as prophylactic therapy to reduce the severity of stroke in patients with transient ischemia and potentially as therapy during acute stroke.

Materials and Methods

Animals

Female C57BL/6 and C57BL/IL-10^{-/-} were purchased from The Jackson laboratory (Bar Harbor, ME). These mice were 8–10 wk of age and were housed in Harvard Medical School Animal Care Facilities according to the institutional guidelines. All protocols for animal experiments were approved by local committee review and were conducted according to the National Institutes of Health *Guide for Care and Use of Laboratory Animals*.

Oral and nasal MOG administration

Oral administration. Mice were fed with 250 μg of MOG_{35–55} (obtained from Dr. J. Elliot, Yale university, New Haven, CT) each day for 5 days.

Nasal administration. Mice were treated nasally with 25 μg MOG_{35–55} three times every other day. As a control protein, mice received OVA_{325–345} peptide (SynPep, Dublin, CA). For in vitro studies, mice were immunized in the footpad with 100 μg MOG_{35–55} mixed 1:1 with CFA 2 days following the last treatment. Mice were subject to middle cerebral artery occlusion (MCAO) surgery 2 days following the last treatment.

Cell culture of lymphocytes

For proliferation and cytokine assays, spleen cells from mice were pooled and cultured in 96-well plates at 5×10^5 and 10^6 cell/ml, respectively, in serum-free medium and X-VIVO 20 (BioWhittaker, Walkersville, MD). To measure cytokines, culture supernatants were collected at 24 h for IL-2 and IL-4; 40 h for IL-6, IL-10, and IFN- γ ; and at 72 h for TGF- β . For proliferation, cells were pulsed with thymidine at 72 h and radioactivity was determined 16 h later (16).

ELISA for cytokines

Quantitative ELISA for IL-2, IL-4, IL-10, and IFN- γ were performed using paired mAb specific for corresponding cytokines per the manufacturer's recommendations (BD PharMingen, San Diego, CA). TGF- β was determined as previously described (17).

Model of focal cerebral ischemia

Animals were anesthetized with 2% isoflurane in 70% N₂ and balanced O₂ by a face mask. Cerebral infarcts were produced by 2 h of MCAO followed by reperfusion as described previously (18). To do so, we introduced a silicone-coated 8–0 monofilament in the left internal carotid artery and advanced 9 mm from the carotid bifurcation so as to occlude the middle cerebral artery. After 2 h, the animals were briefly reanesthetized and the filament was withdrawn. In randomly selected animals, relative changes of regional cerebral blood flow was monitored by laser Doppler flowmetry (PF2B; Perimed, Stockholm, Sweden) as previously described (18, 19), and the left femoral artery was cannulated for arterial blood pressure and blood gas determination. Arterial blood samples were analyzed for pH, arterial oxygen pressure, and partial pressure of carbon dioxide by using a blood gas pH analyzer (Corning 178; Ciba-Corning Diagnostics, Medford, MA). Rectal temperature was maintained at 36.5–37.5°C with a temperature control unit (FHC, Brunswick, ME) during the monitoring period. In all experiments, animals underwent surgery, were scored, and the infarct volume was measured in blinded fashion.

Neurological deficits

Animals were tested for neurological deficits and scored as described elsewhere (20): 0, no observable neurological deficit (normal); 1, failure to extend right forepaw (mild); 2, circling to the contralateral side (moderate); and 3, loss of walking or righting reflex (severe). Animals were rated by a rater blinded to the treatment protocol either 24 or 72 h after reperfusion by an observer naive to the treatment group.

Determination of infarct size

After sacrifice, cerebral infarct sizes were determined on 2,3,5-triphenyl-tetrazolium chloride-stained 2-mm brain sections (24 h) or H&E-stained

20-mm cryostat sections (72 h) by means of an image analysis system (M4; Imaging Research, St. Catherine's, Ontario, Canada) and calculated according to the formula: percentage of (contralateral hemisphere volume – noninfarct volume in ipsilateral hemisphere)/contralateral hemisphere volume.

Histology and immunohistology

Histology was performed on animals sacrificed 24 or 72 h after ischemia. Brain sections (frozen tissue sections) from mice before and after MCAO surgery were fixed in 4% paraformaldehyde overnight followed by 4.5% sucrose for 4 h, then 20% sucrose for overnight at 4°C. Brains were frozen in the presence of OCT and stored until used at -70°C . The staining included immunological markers for T cells (CD3, CD4, and CD8), macrophages and neutrophils (CD11b), plus pro-inflammatory (IFN- γ) and anti-inflammatory (IL-4, IL-10, and TGF- β) cytokines as previously described. Sections were evaluated in a blinded manner, and controls included use of isotype-matched mAbs and a demonstration that preabsorption of anti-cytokine Abs with respective or irrelevant cytokines (5 $\mu\text{g}/\text{ml}$, 16 h, 4°C) either blocked or left unchanged the results of Ab staining, respectively (21).

Adoptive transfer of CD4⁺ T cells

Mice were nasally treated with 25 μg of MOG on days 1, 3, and 5. On day 7, they were immunized with MOG in CFA. Ten days after immunization, both lymph nodes and spleens were removed and stimulated in vitro with 40 μg of MOG_{35–55} in 24-well plates (1 ml in each well containing 5×10^6 cells) in T cell medium buffer (22) for 2 days. On the third day, cells were split into two wells and incubated for 1 more day with IL-2. On day 4, the cells were harvested and CD4⁺ T cells were purified by negative selection using a mouse CD4⁺ T cell column (catalogue no. MCD43; R&D Systems, Minneapolis, MN). Briefly, 10^6 CD4⁺T cells were injected in 0.2 of ml DMEM i.v. Mice were subjected to MCAO surgery 2 days after adoptive transfer of cells. For controls, cells from animals immunized with CFA were treated in an identical fashion.

Data analysis

All continuous and ordinal data are expressed as mean \pm SEM. Statistical analysis was performed by unpaired Student's *t* test or by one-way ANOVA followed with Duncan's test (infarct size) or by the Mann-Whitney *U* test (behavior score). Values of $p < 0.05$ were considered to be statistically significant.

Results

Cytokine profile following nasal or oral administration of MOG_{35–55} peptide

Before studies in the stroke model, we investigated the cytokine profile induced by mucosal administration in C57BL/6 mice fed 250 μg of MOG_{35–55} peptide five times on consecutive days or nasally administered 25 μg peptides three times every other day. Two days after the last treatment, mice were immunized with 100 μg MOG_{35–55} peptide in CFA and splenocytes were taken 10 days later for in vitro assays. As shown in Fig. 1, both nasally treated and fed mice showed a significant reduction in production of the proinflammatory cytokine IFN- γ compared with the control ($p \leq 0.0008$ and $p \leq 0.007$ respectively). Only nasally treated animals showed a significant elevation in the anti-inflammatory cytokine IL-10 ($p \leq 0.0001$ vs control) and a decrease in the total cell proliferation ($p < 0.02$). No active TGF- β was observed in either nasally or orally treated animals. Having shown the tolerogenic potential of mucosally administered MOG in C57BL/6 mice, we then proceeded to test mucosal MOG in the stroke model.

Mucosal tolerance to MOG_{35–55} peptide reduces tissue damage after stroke

Occlusion of the middle cerebral artery produces a localized infarct within the cerebral cortex and caudate putamen, which closely resembles the "at risk territory" in most embolic stroke patients (23). To investigate whether mucosal administration of MOG_{35–55} peptide affected tissue damage after ischemia insult, MOG_{35–55} peptide was administered by five repetitive gavages or

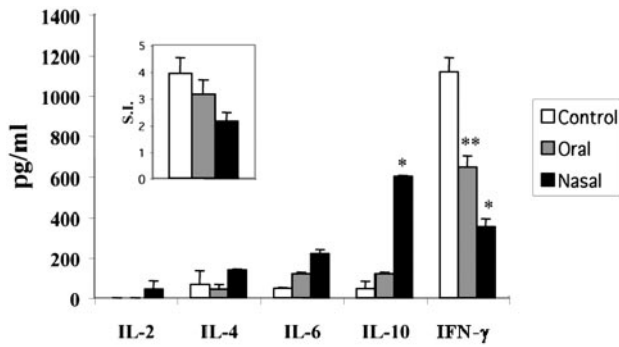


FIGURE 1. Cytokine profile of splenocytes from animals treated orally or nasally with MOG₃₅₋₅₅ peptide. C57BL/6 mice were tolerized with MOG₃₅₋₅₅ peptide through oral administration (250 μg every day for 5 days) or nasal administration (25 μg every other day for 5 days). Nasally PBS-treated mice served as a control group. Ten days after immunization, splenocytes were cultured at 0.5 × 10⁶ (for proliferation assay) or 1 × 10⁶ cells/well with 100 μg/ml MOG₃₅₋₅₅ peptide in 0.2 ml of medium. For proliferation, cells were pulsed with thymidine at 72 h and radioactivity was determined 16 h later (16). Cytokines were measured by ELISA at 24 h (IL-2 and IL-4), 40 h (IL-6, IL-10, and IFN-γ), and 72 h (TGF-β) and results are expressed as SEM. *, *p* ≤ 0.01, nasal vs oral/control for IL-10 and IFN-γ; **, *p* = 0.02 oral vs control for IFN-γ.

nasally administered three times over a 1-wk period (Fig. 2A). Control animals received OVA peptide given nasally. Two days after the last treatment, focal ischemia was induced by 2 h of MCAO using an 8–0 nylon filament. Twenty-four and 72 h after the MCAO, mice were sacrificed and the size of the cerebral ischemic infarct was measured on coronal sections by using 2% 2,3,5-triphenyltetrazolium chloride at 24 h or H&E staining at 72 h. Sections were analyzed for viable and dead tissue at five different levels that encompass the entire forebrain (18). Results for each group 24 h after surgery are shown in Fig. 2B. We found that nasal administration of MOG₃₅₋₅₅ peptide reduced ischemic infarct size by 70% at 24 h from 43 ± 4% to 13 ± 5% (*p* ≤ 0.0001) and by 50% after 72 h from 53 ± 3% to 27 ± 1%, (*p* ≤ 0.0001) as compared with control mice (Fig. 3A). We also investigated the functional impact of mucosal treatment on sensory motor testing at 24 and 72 h after MCAO as measured by evaluation of neurological deficits (18). The behavioral score improved by ~50% at both the 24-h (2.3 ± 0.3 to 1.2 ± 0.3, *p* ≤ 0.0001) and 72-h (2.2 ± 0.4 to 1.2 ± 0.3, *p* ≤ 0.0001) time points in nasally treated mice (Fig. 3B). With oral treatment, infarct volume also decreased at both 24 h (43 ± 4% to 33 ± 5%, *p* ≤ 0.02) and 72 h (53 ± 3% to 41 ± 3%, *p* ≤ 0.008), although not as much as with nasal treatment and there was no effect on the behavior score.

Immunohistochemistry of poststroke brain following mucosal treatment

Surrounding the area of the lethally damaged core of the ischemic infarct lies the penumbra, an area of constrained blood flow with partially preserved energy metabolism (2). Given time and without treatment, the penumbra progresses to infarction because of ongoing excitotoxicity, postischemic inflammation, and apoptosis (24).

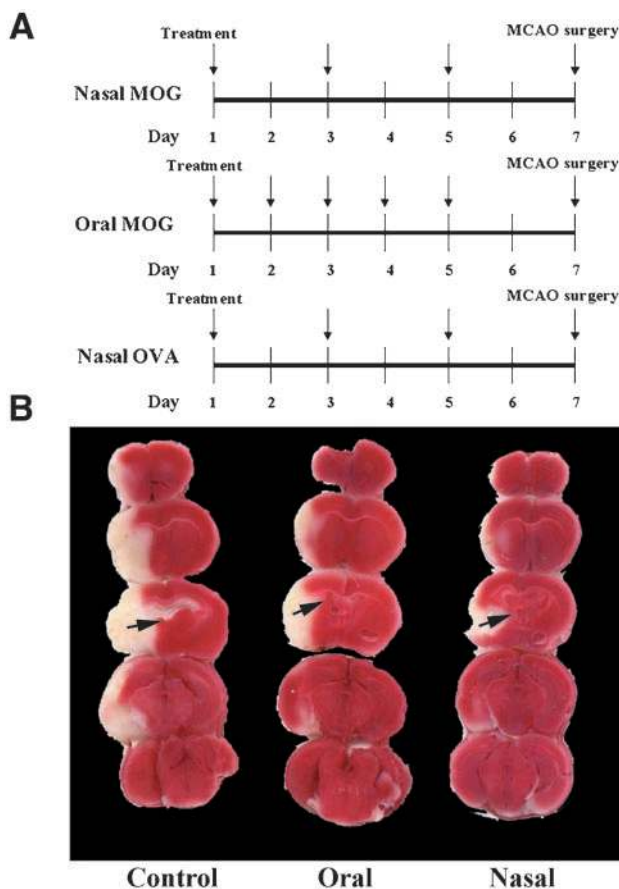


FIGURE 2. Cerebral ischemic infarct size following mucosal tolerance with MOG. *A*, Protocol of mucosal MOG peptide treatment before MCAO. *B*, At 24 h, brains were sectioned coronally (2 mm) and incubated in 2% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO) at room temperature for 30 min. The pale regions denote the infarcted tissue. Arrows indicate where tissue for immunochemistry was taken. The sections presented are from the entire brain of representative individual mice.

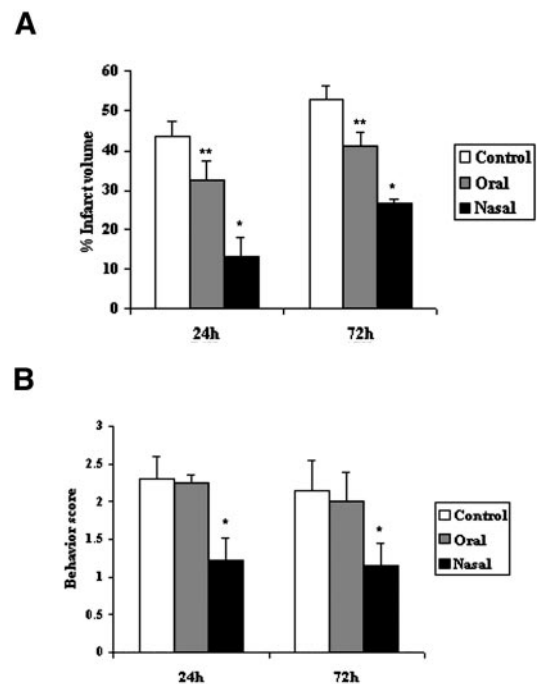


FIGURE 3. Ischemic infarct volume size and behavior score at 24 or 72 h after MCAO. *A*, Infarct volume (corrected for the presence of edema) at 24 h in oral (*n* = 12), nasal (*n* = 9), control (*n* = 12)-treated mice and at 72 h in oral (*n* = 9), nasal (*n* = 7), and control (*n* = 9) mice. Results are included from seven individual experiments at 24 h and eight individual experiments at 72 h. *B*, Behavior scores were determined at 24 or 72 h following MCAO surgery. *, *p* = 0.0001, nasal vs control for infarct volume/behavior; **, *p* < 0.02, oral vs control for infarct volume.

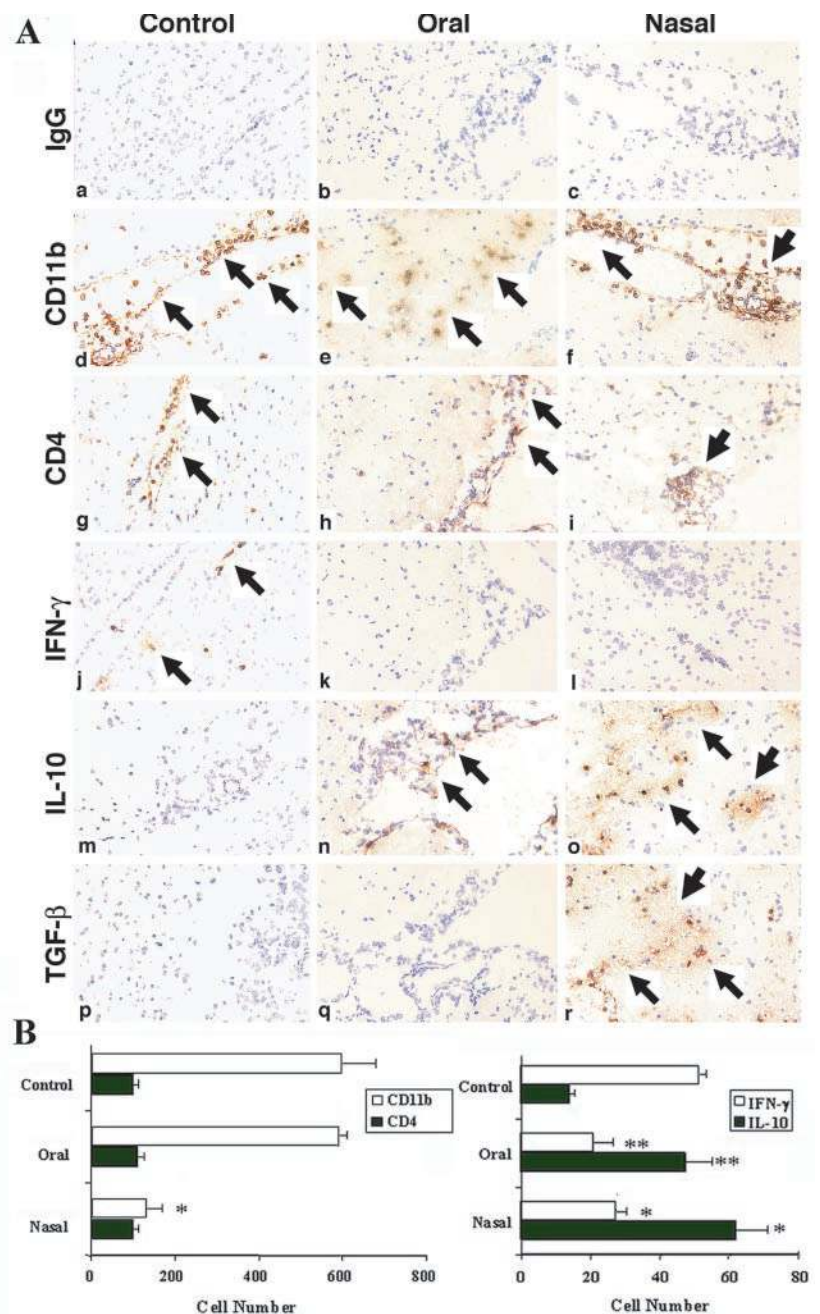
However, even in the most severe ischemic insult tissue destruction continues for hours to days following the insult (3, 4). Thus, it is evident that a prime goal of neuroprotection is to suppress ischemic inflammation within the penumbra. As shown in Fig. 4A, at 24 h IFN- γ , a proinflammatory cytokine, was not expressed within the penumbra in mice receiving nasal or oral therapy, compared with the control group. Both nasal and oral treatment groups showed the presence in the penumbra of the anti-inflammatory cytokine IL-10 in parallel with the presence of T cells, whereas only the nasal-treated group also showed TGF- β expression at 24 h. Neither IL-10 or TGF- β expression was seen in the brains of control mice. These data suggest a role for T cells and IL-10 in the reduction of ischemic infarct size poststroke following mucosal therapy. We also quantified CD4 and CD11b cells and cells expressing IFN- γ , IL-10, and TGF- β at 72 h. As shown in Fig. 4B, there was a reduction in the number of CD11b cells in the nasally treated group. No significant difference in CD4⁺ cells or in the

expression of TGF- β (data not shown) was observed. Cells expressing intracellular IL-10 were increased in both the nasally and orally treated groups ($p = 0.02$ and $p = 0.03$, respectively, vs control). In addition, there was a decrease in the number of cells expressing IFN- γ in both the nasally and orally treated groups ($p = 0.007$ and $p = 0.015$, respectively, vs control).

The neuroprotective effect of nasal MOG is absent in IL-10^{-/-} mice

To investigate the role of IL-10 in reduction of stroke size following nasal MOG, IL-10^{-/-} mice were subjected to MCAO. As shown in Fig. 5, there was no significant reduction in ischemic infarct volume or improvement in behavior score following nasal tolerization with MOG₃₅₋₅₅ peptide as compared with vehicle treatment in IL-10^{-/-} mice. A role for IL-10 was also observed in IL-10^{-/-} animals treated with a control peptide (OVA), as these mice showed a higher infarct volume 24 h after the surgery as

FIGURE 4. Immunohistochemistry of brain at 24 and 72 h after stroke. **A**, IL-10 and/or TGF- β expression within penumbra of MOG-treated mice at 24 h after stroke. The area of the penumbra where histochemistry was taken is shown by arrows in Fig. 2B. Serial sections of brains from control-, oral-, or nasal-treated mice 24 h after MCAO were labeled using (a–c) isotype control mAbs or with mAbs directed against (d–f) CD11b (g–i), CD4 (j–l), IFN- γ (m–o), or TGF- β (p–r). Results are representative of the blinded analysis of three samples/group (immunoperoxidase, hematoxylin counterstain; original magnifications, $\times 120$). **B**, Brain sections at -1.9 Bregma from MOG₃₅₋₅₅-treated mice and control (OVA) mice were stained with hematoxylin and with Abs against CD4, CD11b, IFN- γ , and IL-10 at 72 h after stroke. Quantification of cytokine expression in the brain was performed by counting the total number of cells staining in the area of the penumbra. Results are the SEM of three different brain sections from each group. *, Nasal vs control: for CD11b, $p < 0.001$; for IFN- γ , $p = 0.02$; for IL-10, $p = 0.007$. **, Oral vs control: for IFN- γ , $p = 0.015$; for IL-10 $p = 0.03$.



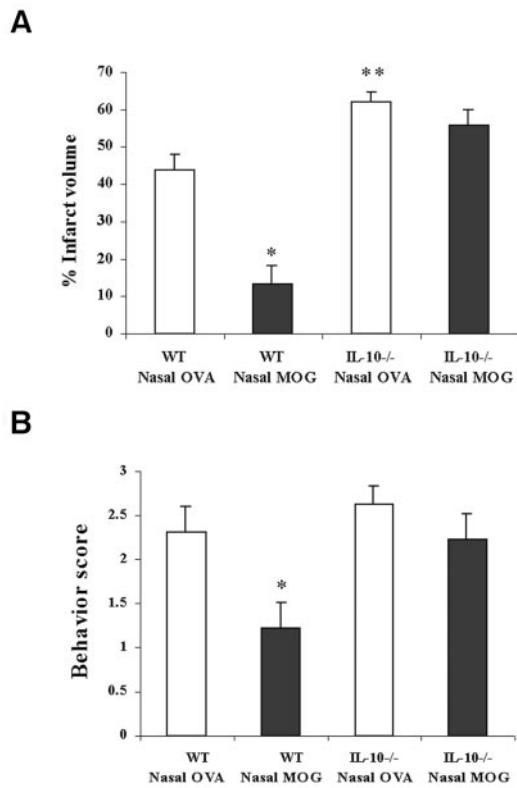


FIGURE 5. Effect of nasal MOG on stroke size in IL-10^{-/-} mice. Infarct volume (A) and behavior score (B) 24 h after MCAO surgery in wild-type (WT), nasal OVA ($n = 10$), and nasal MOG ($n = 9$) and treated normal mice and in IL-10^{-/-} nasal OVA ($n = 9$)- and nasal MOG ($n = 8$)-treated mice. *, $p = 0.001$, wild-type nasal MOG vs wild-type nasal OVA; **, $p = 0.03$, IL-10^{-/-} nasal OVA vs wild-type nasal OVA.

compared with wild-type mice ($p = 0.03$). Also consistent with these findings, we found increased in vitro production of IFN- γ in response to MOG in nasally treated IL-10^{-/-} animals (data not shown). These results clearly demonstrate a crucial role for IL-10 in reduction of ischemic infarct size by nasal MOG peptide.

Adoptive transfer of CD4⁺ T cells from MOG-tolerized mice to untreated mice reduces ischemic infarct size

Mucosal Ag induces tolerance by a number of mechanisms, including anergy, deletion, and active cellular regulation (25). Our results thus far suggested that nasal MOG was effective in the stroke model by inducing IL-10-secreting CD4⁺ T cells. To investigate the role of CD4⁺ cells in reduction of stroke size following nasal MOG, adoptive transfer experiments were performed. C57BL/6 mice were tolerized with MOG₃₅₋₅₅ peptide through nasal administration as described in Fig. 2A. To obtain sufficient cells for adoptive transfer, 2 days after the last nasal treatment, mice were immunized with 100 μ g of MOG₃₅₋₅₅ peptide in CFA in the hind footpad and draining lymph node, and spleen cells were taken 10 days later and stimulated in vitro with 40 μ g/ml MOG₃₅₋₅₅ peptide. After 4 days on in vitro culture, CD4⁺ T cells were purified by negative selection and adoptively transferred to untreated mice that were then subjected to MCAO surgery 48 h after CD4⁺ T cell transfer. Adoptive transfer of CD4⁺ T cells from nasal control-treated mice that were then immunized with CFA served as a control group. As shown in Fig. 6A, cerebral ischemic infarct size was reduced by 56% (from $70 \pm 3\%$ to $31 \pm 3\%$, $p < 0.0003$) in animals that received CD4⁺ T cells from MOG nasally treated mice, as compared with animals that

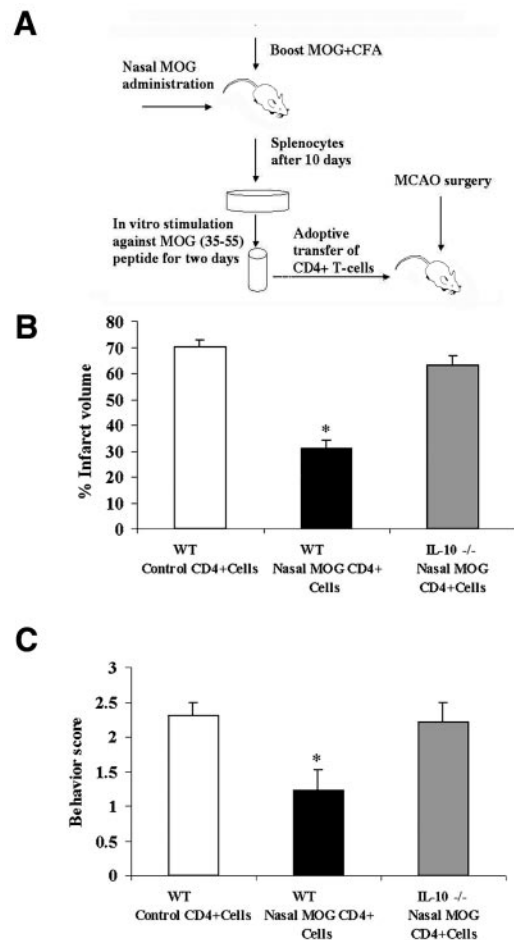


FIGURE 6. Adoptive transfer of CD4⁺ T cells from MOG nasally tolerized wild-type (WT) and IL-10^{-/-} mice. A, Scheme for adoptive transfer of CD4⁺ T cells from MOG₃₅₋₅₅-tolerized mice to naive mice before MCAO surgery. B, Infarct volume following adoptive transfer of CD4⁺ T cells from nasal MOG-treated wild-type ($n = 6$) or IL-10^{-/-} ($n = 10$) vs control animals ($n = 10$). C, Behavior score 24 h following MCAO surgery. *, $p < 0.003$, wild-type nasal MOG CD4⁺ T cells vs wild-type nasal MOG CD4⁺ T cells.

received CD4⁺ T cells from control mice immunized with CFA. To establish that IL-10 was also crucial in these adoptive transfer experiments, CD4⁺ T cells were adoptively transferred from nasally treated IL-10^{-/-} animals. As shown in Fig. 6B, no reduction of infarct size was observed when CD4⁺ T cells from nasal MOG-treated IL-10^{-/-} animals were transferred. Similar results were obtained when behavior score was measured (Fig. 6C). Thus, nasal MOG reduces stroke size via IL-10-dependent CD4⁺ T cells.

Discussion

Accumulated data demonstrate that inflammation plays an important role in the pathophysiology of ischemic stroke. Between the lethally damaged ischemic core and the normal brain lies the peri-infarct zone or penumbra, an area of constrained blood flow with partially preserved energy metabolism (2). Given time and without treatment, the penumbra progresses to infarction because of ongoing excitotoxicity, postischemic inflammation, and apoptosis. However, even in the most severe ischemic insult, the process of tissue destruction may not be completed for hours or days (3, 5, 15). This provides an opportunity for strategies of neuroprotection to salvage the ischemic inflammation within the penumbra.

The presence of recruited leukocytes at the site of inflammation is dependent upon the coordinated expression of adhesion molecules (ligands and receptors) on inflammatory cells and the activated capillary endothelium, respectively. Naive T cells that are not activated generally do not cross the BBB. Nonetheless, T cells readily cross the BBB if a T cell response against a CNS autoantigen is initiated in lymphoid organs. T cells are restimulated upon encounter with the target immunogen presented by local APCs. Thus, several types of CNS cells that may act as APCs are activated following stroke including microglia- and macrophage-like cells that express MHC molecules and produce proinflammatory cytokines such as TNF- α and IL-1 β , which may enhance the appearance of adhesion molecules (26). Following MCAO surgery, we observed macrophage-type cells (CD11b) by immunostaining of brain sections at 24 and 72 h (Fig. 4). These cells could act to enhance the destructive effect of infiltrating CD4⁺ T cells. Our results show that CD11b cells were decreased in nasal MOG-treated animals.

Oral or nasal Ag preferentially generates a Th2 (IL-4/IL-10)- or a Th3 (TGF- β)-type response and these cells have suppressive properties for Th1 and other immune cells. Because such regulatory T cells are triggered in an Ag-specific fashion but suppress via cytokine release in an Ag-nonspecific fashion, they mediate "bystander suppression" when they encounter the fed autoantigen at the target organ. Thus, mucosal tolerance can be used to treat inflammatory processes that are not autoimmune in nature via the secretion of cytokines such as TGF- β , IL-4, and IL-10 after Ag-specific triggering (25).

To address the role of tolerance and T regulatory cells in stroke, we tolerized C57BL/6 mice with the myelin autoantigen MOG₃₅₋₅₅. MOG is a target in the autoimmune model experimental autoimmune encephalomyelitis and is further expressed when there is CNS damage (27). We found that animals tolerized against MOG had enhanced expression of the anti-inflammatory cytokine IL-10 and reduced expression of the proinflammatory cytokine IFN- γ in the pnenbra. It has recently been shown that IFN- γ parallels inducible NO synthase activity during the course of stroke (3). The role of IL-10 as mediator of the ischemic infarct was established by investigation of IL-10^{-/-} mice.

IL-10 is an immunoregulatory cytokine that can modulate immune processes, inhibiting the expression of inflammatory Th1-type responses as well as affecting APC function. IL-10 has been shown to reduce inflammation in a variety of animal models including experimental autoimmune encephalomyelitis (22) and atherosclerosis (28). Within the brain, IL-10, may deactivate macrophage-like cells and astrocytes and thus limit their involvement in a secondary inflammatory process. Furthermore, IL-10 limits the role of glutamate cytotoxicity by inactivation of NF- κ B (29), a transcription factor that modulates inflammation and key regulatory proteins in cerebral ischemia (30). Moreover, IL-10 targets the interface between the CNS and periphery (BBB) by preventing adhesion and extravasation of leukocytes.

Becker et al. (15) previously demonstrated reduced infarct size in the rat after oral tolerance with myelin basic protein. Furthermore, recent experiments by Becker et al., (31) showed that adoptive transfer of splenocytes from nasal myelin basic protein-tolerized rats to naive rats reduces infarct size. This work suggested that TGF- β may play a role since there was increased TGF- β expression in the brain of orally tolerized animals. TGF- β may be increased following stroke independent of mucosal tolerization, is expressed in neuronal cells, and may play an important role in neuronal recovery (14). We found increased expression of TGF- β in nasally treated animals compared with controls at 24 h and increases in both treated and control animals at 72 h. Nevertheless,

in our studies IL-10 appears to be the crucial cytokine in decreasing stroke size.

Our results cannot be directly compared with Becker et al. (15, 31) who used a different Ag (myelin basic protein), a rat model, and did not perform adoptive transfer experiments of CD4⁺ T cells to establish the immunological mechanism responsible for the effect described. Becker et al.'s (15, 31) results are consistent, however, in suggesting that mucosal tolerance can be used to treat stroke by inducing an anti-inflammatory milieu in the CNS.

In our study, C57BL/6 mice were investigated, as it is a common background for transgenic mice. Nasal in addition to oral administration was chosen because the level of IL-10 has been shown to be increased following nasal tolerization (32, 33) and elderly patients with a history of stroke were reported to have significantly lower median IL-10 production levels at baseline compared with elderly patients without stroke (12, 13). We found that nasal administration of MOG₃₅₋₅₅ peptide was more effective than oral MOG and consistent with this, we found more IL-10 and less CD11b cells in nasally treated animals. It is known that nasal administration of proteins preferentially induce IL-10 responses as compared with oral administration (11).

Adoptive transfer of T cells is a classic method to demonstrate an active immunological role for both disease-inducing and disease-ameliorating T cells in animal disease models. Of note is that Schwartz and colleagues (34–36) in a series of studies have demonstrated a neuroprotective role for myelin-reactive T cells in models of optic nerve injury and spinal cord trauma. Our study is the first to demonstrate that CD4⁺ T cells expressing a regulatory T cell phenotype (IL-10) have a beneficial effect in stroke. Moreover, IL-10^{-/-} CD4⁺ T cells lack this activity, further establishing an IL-10-dependent cascade.

In addition to the IL-10 effects we observed, we also found a marked reduction of the CD11b⁺ cell number in mice nasally administered MOG compared with the wild type. CD11b is a marker for cells of the microglia/macrophage lineage. These cells increase the level of NO synthase (leading to NO synthesis) and thus may play a role in the evolving lesion and functionally contribute to secondary infarct expansion after ischemic injury (37). The reduction of CD11b⁺ cells, which may have been secondary to IL-10 production, may also have contributed to the beneficial effects of nasal MOG we observed.

Enhancing recovery from stroke and limiting ischemic damage are major goals to decrease stroke morbidity and mortality. Given that it has been suggested in humans that IL-10 may have a beneficial effect in stroke (13), our findings provide a novel immunological approach that is nontoxic and widely applicable for delivery of IL-10 to the site of ischemia to reduce stroke injury. Experiments are in progress to determine the effectiveness of this approach as therapy at the time of stroke. Nevertheless, based on our results, this approach may have applicability as prophylactic therapy to reduce the severity of stroke in high-risk patients with transient ischemia attacks.

References

1. Bogousslavsky, J., G. Van Melle, and F. Regli. 1988. The Lausanne Stroke Registry: analysis of 1,000 consecutive patients with first stroke. *Stroke* 19:1083.
2. Hossmann, K. A. 1994. Viability thresholds and the penumbra of focal ischemia. *Ann. Neurol.* 36:557.
3. del Zoppo, G. J., K. J. Becker, and J. M. Hallenbeck. 2001. Inflammation after stroke: is it harmful? *Arch. Neurol.* 58:669.
4. Koroshetz, W. J., and M. A. Moskowitz. 1996. Emerging treatments for stroke in humans. *Trends Pharmacol. Sci.* 17:227.
5. Li, H. L., N. Kostulas, Y. M. Huang, B. G. Xiao, P. van der Meide, V. Kostulas, V. Giedraitis, and H. Link. 2001. IL-17 and IFN- γ mRNA expression is increased in the brain and systemically after permanent middle cerebral artery occlusion in the rat. *J. Neuroimmunol.* 116:5.

6. Connolly, E. S., Jr., C. J. Winfree, C. J. Prestigiacomo, S. C. Kim, T. F. Choudhri, B. L. Hoh, Y. Naka, R. A. Solomon, and D. J. Pinsky. 1997. Exacerbation of cerebral injury in mice that express the P-selectin gene: identification of P-selectin blockade as a new target for the treatment of stroke. *Circ. Res.* 81:304.
7. Zhang, R. L., M. Chopp, Y. Li, C. Zaloga, N. Jiang, M. L. Jones, M. Miyasaka, and P. A. Ward. 1994. Anti-ICAM-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in the rat. *Neurology* 44:1747.
8. Hallenbeck, J. M. 2002. The many faces of tumor necrosis factor in stroke. *Nat. Med.* 8:1363.
9. Weiner, H. 2001. Induction and mechanism of action of transforming growth factor- β -secreting Th3 regulatory cells. *Immunol. Rev.* 182:207.
10. Groux, H. 2001. An overview of regulatory T cells. *Microbes Infect.* 3:883.
11. Weiner, H. L. 2001. The mucosal milieu creates tolerogenic dendritic cells and T(R)1 and T(H)3 regulatory cells. *Nat. Immunol.* 2:671.
12. Vila, N., J. Castillo, A. Davalos, A. Esteve, A. M. Planas, and A. Chamorro. 2003. Levels of anti-inflammatory cytokines and neurological worsening in acute ischemic stroke. *Stroke* 34:671.
13. van Exel, E., J. Gussekloo, A. J. de Craen, A. Bootsma-van der Wiel, M. Frolich, and R. G. Westendorp. 2002. Inflammation and stroke: the Leiden 85-Plus Study. *Stroke* 33:1135.
14. Krupinski, J., P. Kumar, S. Kumar, and J. Kaluza. 1996. Increased expression of TGF- β 1 in brain tissue after ischemic stroke in humans. *Stroke* 27:852.
15. Becker, K. J., R. M. McCarron, C. Ruetzler, O. Laban, E. Sternberg, K. C. Flanders, and J. M. Hallenbeck. 1997. Immunologic tolerance to myelin basic protein decreases stroke size after transient focal cerebral ischemia. *Proc. Natl. Acad. Sci. USA* 94:10873.
16. Maron, R., N. S. Melican, and H. L. Weiner. 1999. Regulatory Th2-type T cell lines against insulin and GAD peptides derived from orally- and nasally-treated NOD mice suppress diabetes. *J. Autoimmun.* 12:251.
17. Inobe, J., A. J. Slavin, Y. Komagata, Y. Chen, L. Liu, and H. L. Weiner. 1998. IL-4 is a differentiation factor for transforming growth factor- β secreting Th3 cells and oral administration of IL-4 enhances oral tolerance in experimental allergic encephalomyelitis. *Eur. J. Immunol.* 28:2780.
18. Huang, Z., P. L. Huang, N. Panahian, T. Dalkara, M. C. Fishman, and M. A. Moskowitz. 1994. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265:1883.
19. Huang, Z., P. L. Huang, J. Ma, W. Meng, C. Ayata, M. C. Fishman, and M. A. Moskowitz. 1996. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J. Cereb. Blood Flow Metab.* 16:981.
20. Endres, M., W. Z., Namura, S., Waerber, C., Moskowitz, M. A. 1997. Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J. Cereb. Blood Flow Metab.* 17:1143.
21. Khoury, S. J., W. W. Hancock, and H. L. Weiner. 1992. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis as associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor β , interleukin 4, and prostaglandin E expression in the brain. *J. Exp. Med.* 176:1355.
22. Bettelli, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel, and V. K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J. Immunol.* 161:3299.
23. Hunter, A. J., K. B. Mackay, and D. C. Rogers. 1998. To what extent have functional studies of ischaemia in animals been useful in the assessment of potential neuroprotective agents? *Trends Pharmacol. Sci.* 19:59.
24. Lo, E. H., T. Dalkara, and M. A. Moskowitz. 2003. Mechanisms, challenges and opportunities in stroke. *Nat. Rev. Neurosci.* 4:399.
25. Faria, A. M. C., and H. L. Weiner. 1999. Oral tolerance: mechanisms and therapeutic applications. *Adv. Immunol.* 73:153.
26. Emsley, H. C., and P. J. Tyrrell. 2002. Inflammation and infection in clinical stroke. *J. Cereb. Blood Flow Metab.* 22:1399.
27. Gregersen, R., T. Christensen, E. Lehrmann, N. H. Diemer, and B. Finsen. 2001. Focal cerebral ischemia induces increased myelin basic protein and growth-associated protein-43 gene transcription in peri-infarct areas in the rat brain. *Exp. Brain Res.* 138:384.
28. Maron, R., G. Sukhova, A. M. Faria, E. Hoffmann, F. Mach, P. Libby, and H. L. Weiner. 2002. Mucosal administration of heat shock protein-65 decreases atherosclerosis and inflammation in aortic arch of low-density lipoprotein receptor-deficient mice. *Circulation* 106:1708.
29. Bachis, A., A. M. Colangelo, S. Vicini, P. P. Doe, M. A. De Bernardi, G. Brooker, and I. Mocchi. 2001. Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity. *J. Neurosci.* 21:3104.
30. Schneider, A., A. Martin-Villalba, F. Weih, J. Vogel, T. Wirth, and M. Schwaminger. 1999. NF- κ B is activated and promotes cell death in focal cerebral ischemia. *Nat. Med.* 5:554.
31. Becker, K., D. Kindrick, R. McCarron, J. Hallenbeck, and R. Winn. 2003. Adoptive transfer of myelin basic protein-tolerized splenocytes to naive animals reduces infarct size: a role for lymphocytes in ischemic brain injury? *Stroke* 34:1809.
32. Hall, G., C. G. Houghton, J. U. Rahbek, J. R. Lamb, and E. R. Jarman. 2003. Suppression of allergen reactive Th2 mediated responses and pulmonary eosinophilia by intranasal administration of an immunodominant peptide is linked to IL-10 production. *Vaccine* 21:549.
33. Akbari, O., R. H. DeKruyff, and D. T. Umetsu. 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat. Immunol.* 2:725.
34. Yoles, E., E. Hauben, O. Palgi, E. Agranov, A. Gothilf, A. Cohen, V. Kuchroo, I. R. Cohen, H. Weiner, and M. Schwartz. 2001. Protective autoimmunity is a physiological response to CNS trauma. *J. Neurosci.* 21:3740.
35. Barouch, R., and M. Schwartz. 2002. Autoreactive T cells induce neurotrophin production by immune and neural cells in injured rat optic nerve: implications for protective autoimmunity. *FASEB J.* 16:1304.
36. Moalem, G., R. Leibowitz-Amit, E. Yoles, F. Mor, I. R. Cohen, and M. Schwartz. 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat. Med.* 5:49.
37. Stoll, G., S. Jander, and M. Schroeter. 2002. Detrimental and beneficial effects of injury-induced inflammation and cytokine expression in the nervous system. *Adv. Exp. Med. Biol.* 513:87.