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Pro-inflammatory interferon gamma signaling is directly associated with stroke induced neurodegeneration

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Abstract

The delayed immune response to stroke is responsible for the increased neural injury that continues to occur after the initial ischemic event. This delayed immune response has been linked to the spleen, as splenectomy prior to middle cerebral artery occlusion (MCAO) is neuroprotective. Interferon gamma (IFN γ) is linked to the splenic response, which enhances neural injury following MCAO. IFN γ activates the expression of the inflammatory chemokine interferon-inducible protein 10 (IP-10). This study was designed to determine the role of IFN γ signaling in the inflammatory response following MCAO. Expression of IP-10 increased in the brain and the spleen following MCAO. Splenectomy inhibited the increase of IP-10 in the brain post-MCAO, while recombinant IFN γ administration to splenectomized rats returned IP-10 levels in the brain to levels found in rats after MCAO only. Systemic administration of an IFN γ neutralizing antibody to MCAO-treated rats reduced infarct volume and IP-10 levels in the brain. T cell infiltration was reduced in the MCAO-damaged brains of IFN γ antibody-treated animals relative to those that received isotype control antibodies. Additionally, inhibiting IFN γ signaling with splenectomy or an IFN γ neutralizing antibody blocked the induction of IP-10 expression and decreased neurodegeneration following MCAO. Targeting this pro-inflammatory pathway following stroke could be a promising stroke therapeutic.

Keywords

Spleen; IP-10; Monocytes; Chemokines; Lymphocytes

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Conflict of Interest

The authors declare that they have no conflict of interest.

Introduction

The inflammatory response after stroke has been well documented in enhancing delayed neurodegeneration after ischemic injury (Leonardo and Pennypacker 2011). Downstream signaling of the pro-inflammatory cytokine interferon gamma (IFN γ) induces the expression of the chemokine interferon-inducible protein 10 (IP-10), also known as CXCL10. IP-10 is a pro-inflammatory chemokine that selectively drives the propagation of the Th₁ response by interacting with the CXCR3 receptor (Loetscher et al. 2001). Microglia/macrophages produce IP-10 in response to IFN γ stimulation (Luster 2002), and IP-10 induces chemotaxis of Th₁ cells to the site of injury. In addition, IP-10 can prevent the activation of Th₂ cells by competitive antagonism of the CCR3 receptor (Loetscher et al. 2001). These interactions of IP-10 with CXCR3 and CCR3 create a pro-inflammatory feed forward mechanism that effectively recruits more IFN γ producing cells to the site of injury, leading to more IP-10 production through increased IFN γ signaling.

Investigations of IP-10 in experimental mouse models of stroke have shown that IP-10 mRNA is upregulated early in the brain at 6 and 22 h following MCAO. In the spleen, IP-10 mRNA levels are increased at 22 h post-MCAO (Offner et al. 2006; Hurn et al. 2007). Although IP-10 transcript has been measured at selected time points following stroke, IP-10 protein levels have not been quantified in the brain or spleen following MCAO or at longer time points after MCAO. Further investigation into the role of IP-10 following brain ischemia is necessary to elucidate the IFN γ /T cell response in stroke.

The contribution of the peripheral immune response to ischemic stroke injury has been directly linked to the spleen, and IFN γ signaling appears to play a key role in stroke-induced neurodegeneration. The spleen is a large reservoir for immune cells and splenectomy prior to middle cerebral artery occlusion (MCAO) is neuroprotective in rats (Ajmo et al. 2008) and mice (Jin et al. 2013). IFN γ is a pro-inflammatory cytokine that relays the splenic response to MCAO. IFN γ levels increase in the spleen 24 h post-MCAO (Seifert et al. 2012b), and are upregulated in the injured brain at 72 h post-MCAO in rats (Seifert et al. 2012b) and mice (Jin et al. 2013). Interestingly, splenectomy prior to MCAO blocks post-stroke IFN γ elevations in the brain (Seifert et al. 2012b; Jin et al. 2013), and administration of recombinant IFN γ to splenectomized rats negates the protective effects of splenectomy. These data demonstrate that spleen-derived IFN γ plays a detrimental role following stroke.

Since splenocytes have also been tracked *in vivo* and were found to migrate to the injured brain after MCAO (Seifert et al. 2012a), we have been investigating the downstream effects of IFN γ signaling that may be responsible for promoting degenerative injury following stroke. Previous studies have demonstrated increased levels of IFN γ , the main inducer of IP-10 synthesis, in the brain (Seifert et al. 2012b; Jin et al. 2013) and the spleen (Seifert et al. 2012b) post-MCAO. In this study, we assessed the time course of IP-10 expression in the spleen and brain after MCAO to determine whether IP-10 is a critical mediator of the peripheral immune response following stroke. Splenectomy and/or administration of an IFN γ neutralizing antibody were utilized to inhibit IFN γ signaling. Results showed that blocking IFN γ signaling effectively reduced IP-10 expression and decreased neural injury following stroke. T cell recruitment to the brain was also investigated, as IP-10 is a

chemoattractant for Th₁ cells, and decreased IP-10 protein expression was associated with diminished T cell infiltration into the infarct. These data indicate that IFN γ /IP-10 is a major inflammatory signal pathway in the immune response to stroke.

Materials and Methods

Animal Care

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals with a protocol approved by the Institutional Animal Care and Use Committee at the University of South Florida. Male Sprague-Dawley rats (300–350g) were used for the following experiments. All rats were purchased from Harlan Labs (Indianapolis, IN), maintained on a 12 h light/dark cycle (6 am – 6 pm) and given access to food and water *ad libitum*.

Laser Doppler Blood Flow Measurement

Laser Doppler was used to monitor blood perfusion (Moor Instruments Ltd, Devon, England). A hole was drilled 1 mm posterior and 4 mm lateral from Bregma, and a guide screw was placed. The laser doppler probe was inserted into the guide screw, and the tip of the probe was placed against the surface of the brain. Rats that did not show 60% reduction in perfusion during MCAO were excluded from this study (Ajmo et al. 2006; Ajmo et al. 2008; Hall et al. 2009). Sham operated rats had the guide screw and laser doppler probe placed and blood flow was monitored to ensure that there was not a drop in cerebral blood flow during the sham procedure.

Splenectomy

Splenectomy was performed two weeks prior to MCAO as previously described (Seifert et al. 2012b). Briefly, rats were anesthetized with 5% isoflurane and maintained on 2–3%. The abdominal cavity was opened on the anatomical left. The splenic blood vessels were ligated and the spleen was removed. The incision was then closed with sutures, first closing the abdominal wall and then the skin incision. Sham operations were also performed where the spleen was exteriorized and then reinserted into the cavity. Animals were allowed to wake in fresh cages.

Permanent Middle Cerebral Artery Occlusion

MCAO surgery was performed using the intraluminal method originally described by Longa *et al.* (Longa et al. 1989) and previously reported (Ajmo et al. 2006; Ajmo et al. 2008; Hall et al. 2009). Briefly, rats were anesthetized with 5% isoflurane and maintained on 2–3%. Blunt dissection was performed to isolate the common carotid artery, the internal carotid artery (ICA), and the external carotid artery (ECA). The ECA was ligated and cut. Then a 40 mm monofilament was introduced into the ECA, fed distally into the ICA, and advanced to the origin of the MCA. The filament was tied off on the ECA to produce a permanent occlusion. The incision was then sutured closed and the rat was allowed to wake in a fresh cage.

Antibody Treatment Injections

A goat anti-rat polyclonal IFN γ neutralizing antibody (R&D Systems, Minneapolis, MN) and a goat IgG isotype antibody (R&D Systems) were reconstituted with phosphate buffered saline (PBS) to a concentration of 100 μ g/ml. Animals in the antibody treatment study were randomly assigned to one of three treatment groups: IFN γ neutralizing antibody, IgG isotype control or the PBS control. Beginning at 24 h post-MCAO animals were administered either 5 μ g (0.05 ml) of a goat anti-rat IFN γ neutralizing antibody, a goat IgG isotype control, or an equivalent amount of PBS via an intraperitoneal (i.p.) injection. Treatment was administered at 24, 48, and 72 h post-MCAO.

Recombinant IFN γ Administration

Rat recombinant IFN γ (rIFN γ) was administered as previously described (Seifert et al. 2012b). The lowest dosage of rIFN γ (ProSpec, Rehovot, Israel) that elicited a physiologic response in naïve rats was previously determined. The dosage of 20 μ g (in 0.21 ml sterile ddH $_2$ O) was injected intravenously (i.v.), via the tail vein, at 48 and 72 h post-MCAO to determine the effects of IFN γ on neural injury in splenectomized and sham-splenectomized rats.

Tissue Extraction and Sectioning

The animals were euthanatized with a ketamine/xylazine mix, 75 mg/kg and 7.5 mg/kg respectively, i.p. at 24, 48, 72 or 96 h post-MCAO for the time course experiment and at 96 h post-MCAO for the antibody treatment and rIFN γ experiments. Anesthetized animals were then perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in phosphate buffer (PB). The spleen and thymus were removed prior to perfusion. Spleens were weighed immediately following removal and were subsequently snap frozen and stored in the -80°C with the thymi. The brains were harvested, post fixed in 4% paraformaldehyde, and immersed in 20% followed by 30% sucrose in PBS. Brains were frozen and sliced into 30 μ m sections using a cryostat. Coronal sections were taken at six points from 1.7 to -3.3 mm from Bregma. Sections were either thaw mounted on glass slides or placed in Walter's Anti-freeze cryopreservative and stored at -20°C .

Fluoro-Jade Staining

Brain sections mounted on glass slides were stained with Fluoro-Jade, which labels degenerating neurons. This method was adapted from that originally developed by Schmued *et al.* (Schmued et al. 1997) and has been described by Duckworth *et al.* (Duckworth et al. 2005). Slides were dried at room temperature overnight, placed in 100% ethanol for 3 min, 70% ethanol for 1 min, and then ddH $_2$ O for 1 min. Slides were oxidized using a 0.06% KMnO $_4$ solution for 15 min followed by three 1 min rinses with ddH $_2$ O. Slides were stained in a 0.001% solution of Fluoro-Jade (Histochem, Jefferson, AR) in 0.1% acetic acid in the dark for 30 min. Slides then were rinsed 4 times with ddH $_2$ O for 3 min each time, allowed to dry at 45°C for 20 min, cleared twice with xylene and then cover slipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA).

Infarct Quantification

Fluoro-Jade stained tissue was digitally photographed with Zeiss Axioskop2 (Carl Zeiss Inc, Thornwood, NY) microscope controlled by Openlab software (Improvision, Waltham, MA) at a magnification of 1x. The area of neurodegeneration was measured using the NIH ImageJ software. The area of the contralateral hemisphere was also measured and used to compensate for possible edema in the ipsilateral hemisphere. Infarct volumes were then calculated by the total area of ipsilateral staining divided by the total contralateral area for a given animal. Infarct quantification was done for all animals.

Immunohistochemistry in the Brain

The slides were dried at 37°C for 1 h then rinsed with PBS pH 7.4. Slides were placed in permeabilization buffer containing 10% serum, 3% 1M lysine, and 0.3% Triton X-100 in PBS for 1 h at room temperature. Next, sections were incubated overnight at 4°C in a primary antibody solution (PBS with 2% serum and 0.3% Triton X-100) in a humidified chamber. Slides were subsequently rinsed with PBS and incubated with a secondary antibody solution (PBS, 2% serum, 0.3% Triton X-100) for 1 h. Slides were washed thoroughly with PBS and dried for 1 h at 45°C. Once dry, the slides were rinsed twice with xylene and cover slipped using DPX (Electron Microscopy Sciences). Slides were protected from light during these steps. As a negative control, sections were processed as above without the primary antibody.

Double label immunohistochemistry for IP-10 and immune cell surface markers was achieved by incubating the slides with primary antibodies, followed by incubation with secondary antibodies conjugated to 594nm or 488nm fluorophores.

The following primary antibodies were used: rabbit anti-rat IP-10 (1:5,000; abcam; Cambridge, MA), mouse anti-rat CD3 for T cells (1:2,000; BD Biosciences, San Jose, CA), and mouse anti-rat CD11b for microglia/macrophages (1:3,000; Serotec, Raleigh, NC). Alexa-Fluor® 488 goat anti-rabbit (1:300; Life Technologies, Grand Island, NY) secondary was used for all IP-10 staining. Alexa-Fluor® 594 goat anti-mouse (1:300; Life Technologies) secondary was used in conjunction the immune cell surface markers for double staining with IP-10. Alexa-Fluor® 488 rabbit anti-mouse (1:300; Life Technologies) secondary was used when only staining for CD3 T cell.

IP-10 Immunohistochemistry in the Spleen

Spleens were fixed in 4% paraformaldehyde overnight. The spleens were then placed in a solution of 20% glycerol and 2% dimethyl sulfoxide (DMSO) and embedded in a gelatin matrix using MultiBrain Technology© (NeuroScience Associates, Knoxville, TN). The block of spleens was rapidly frozen in isopentane with crushed dry ice (-70°C). Using a microtome the block was sliced into 25 µm sections. Six consecutive sections were taken and collected in Antigen Preservation solution (50% ethylene glycol, 49% PBS pH 7.0, 1% polyvinyl pyrrolidone). The spleen sections were stained free floating in Tris-buffered saline (TBS) solutions. Endogenous peroxide activity was extinguished by treatment with 3% hydrogen peroxide for 15 min. After washing with TBS sections were incubated for 30 min in permeabilization buffer (TBS with 0.3% TritonX-100 and 10% serum). Following

permeabilization, slides were incubated overnight at room temperature with primary antibody in TBS with 2% serum. The sections were rinsed with TBS and incubated in secondary biotinylated antibody in TBS with 2% serum for 1 h. After being rinsed with TBS, sections were incubated with an avidin/biotin/horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 1 h. Staining was visualized with 3,3'-Diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, St. Louis, MO). The sections were then mounted on gelatinized slides, dried, dehydrated, cleared with xylene, and cover slipped with PermOUNT (Fischer Scientific, Pittsburgh, PA). The primary antibody used was rabbit anti-rat IP-10 (1:500; abcam) and the secondary antibody was biotinylated goat anti-rabbit (Vector Laboratories). The IP-10 antibody's specificity was determined by Western blotting using recombinant IP-10.

Immunohistochemistry Quantification

IP-10 stained brain tissue sections were digitally photographed with Zeiss Axioskop2 microscope controlled by Openlab software at a 10x magnification. Three images from each of 4 Bregma points (1.7 mm to -1.3 mm) were taken for a total of twelve images per brain. The area selected for quantification was the striatum of the ipsilateral hemisphere for all animals. The images were analyzed for percent of immunostaining per area with ImageJ software. These twelve values were then averaged for each brain. Percent immunostaining was used for the brain sections since the IP-10 staining was diffuse which didn't lend itself to an "intensity" measure as noted below for the more prevalent staining of the spleen.

CD3 stained brain tissue sections were digitally photographed with Zeiss Axioskop2 microscope controlled by Openlab software at a 10x magnification. Images from Bregma point +0.7 mm were taken of the peri-infract region of the striatum near the lateral ventricle of the ipsilateral hemisphere. The images were analyzed for percent of immunostaining per area with ImageJ software.

Splenic IP-10 images were taken with a Nikon 90i microscope using a 20x objective and NIS Elements BR 4.00.07 software at a high resolution. The images were processed and analyzed with Photoshop CS5 (Adobe Systems Inc., San Jose, CA). The intensity of the staining was measured in the histogram for the entire image and the amount of staining per image was analyzed. Six sections per spleen were analyzed for each rat.

Confocal Imaging

Tissue sections that were double labeled were viewed on the Leica SP2 confocal microscope (Leica Microsystems, Buffalo Grove, IL). Images were taken at a magnification of 63x. Each fluorophore was scanned sequentially and then the two images were merged.

Statistical Analysis

All data are expressed as group mean $\pm$ SEM. Significance of the data was determined by ANOVA with Fischer's Least Significant Difference post hoc test for all analysis. A value of $p < 0.05$ was considered significant. All sections were blinded prior being analyzed by an investigator.

Results

IP-10 levels are elevated in the brain following MCAO

To determine if IP-10 protein is expressed in the brain following MCAO, its protein expression in the striatum of the ipsilateral hemisphere was characterized over time. To quantify IP-10 levels, immunohistochemistry for IP-10 was performed on brain sections from animals euthanized at 48, 72, and 96 h following MCAO and naïve (Figs 1b–e) or sham operated rats. IP-10 protein levels were significantly increased at 72 h and remained elevated at 96 h ($p < 0.01$) compared to sham operated rats 96 h after surgery (Fig 1a).

Splenic IP-10 levels increase after MCAO and remain elevated

In the spleen, IP-10 protein levels were quantified using immunohistochemistry. Spleens from naïve or sham rats euthanized at 96 h and animals euthanized 24, 48, 72, and 96 h post-MCAO (Figs 2b–g) were used to perform immunohistochemistry to determine IP-10 protein expression. IP-10 levels were significantly elevated at 24 h and remained elevated out to 96 h following MCAO compared to naïve spleens ($p < 0.0007$). The sham operated animals had increased levels of IP-10 but this did not reach statistical significance (Fig 2a).

IP-10 levels in the brain are reduced by splenectomy and restored by rIFN γ administration

Splenectomy prior to MCAO is neuroprotective and reduces the amount of IFN γ in the brain (Seifert et al. 2012b; Jin et al. 2013). IP-10 expression is induced by IFN γ ; therefore, brains of splenectomized rats that underwent MCAO were stained for IP-10 protein expression. Splenectomy significantly reduces the amount of IP-10 in the infarct area of rats post-MCAO. Whereas, administration of rIFN γ to splenectomized rats increases IP-10 levels to those not significantly different from sham splenectomized rats post-MCAO ($p = 0.05$; Fig 3a). However, splenic IP-10 expression was unaffected by administration of rIFN γ (Fig 3b).

IP-10 is produced in monocytic cells in the brain following MCAO

IP-10 expression is induced by IFN γ in cells of monocytic origin. Double staining with IP-10 and CD11b, a marker for monocytes, was performed on the brains from animals 96 h post-MCAO. Expression of IP-10 and CD11b were co-localized in the infarcted area of the ipsilateral hemisphere (Fig 4).

IFN γ neutralizing antibody administration decreases infarct following MCAO

To inhibit the pro-inflammatory IFN γ signaling pathway that increases IP-10 transcription following MCAO, an IFN γ neutralizing antibody was administered 24, 48, and 72 h post-MCAO. Infarct volumes, as measured by Fluoro-Jade staining, at 96 h post-MCAO were significantly decreased in the IFN γ neutralizing antibody group compared to the vehicle and isotype control groups ($p < 0.007$; Fig 5d). Representative micrographs from Fluoro-Jade stained brains show decreased staining in the IFN γ neutralizing antibody treated group (Fig 5c) compared to the staining in the PBS (Fig 5a) and IgG isotype control (Fig 5b) groups.

IFN γ neutralizing antibody decreased IP-10 in the brain

IP-10 protein expression was quantified in the striatum of the ipsilateral hemisphere from rats treated with PBS (Fig 6a), control IgG (Fig 6b), or IFN γ antibody (Fig 6c). IP-10 immunoreactivity was significantly decreased in the IFN γ antibody treatment group compared to the IgG isotype and PBS control groups ($p < 0.009$; Fig 6d).

Splenic IP-10 is not altered by antibody administration

IP-10 levels were measured in the spleen of animals treated with PBS, IgG isotype, or IFN γ neutralizing antibody. Representative micrographs show IP-10 staining in the spleens of PBS treated animals (Fig 7a) and in the groups of animals that received IgG (Fig 7b) or IFN γ neutralizing antibody (Fig 7c). Quantification showed that splenic IP-10 staining intensity was not significantly increased in either group that received antibody compared to the vehicle-treated group (Fig 7d).

The amount of CD3 immunoreactivity decreases in the brains of IFN γ neutralizing antibody treated animals

Brain sections from animals that underwent MCAO and then administered an IFN γ neutralizing antibody, an IgG isotype antibody, or PBS were stained using an anti CD3 antibody to visualize the presence of T cells. At 96 h post-MCAO the amount of CD3 immunostaining in the ipsilateral striatum was significantly decreased in the IFN γ antibody treated group compared to the IgG and PBS control groups ($p < 0.04$; Fig 8d). Representative micrographs of the striatum show reduced CD3 staining in the brains of IFN γ antibody (Fig 8c) treated animals compared to the CD3 staining in the IgG (Fig 8b) and vehicle (Fig 8a) groups.

Discussion

IP-10 levels are induced in the MCAO-injured hemisphere where its expression significantly increases at 72 h and remains elevated at 96 h post-MCAO. These results are consistent with a delayed response and up regulation of protein compared to an earlier increase in IP-10's mRNA levels. Studies looking at mRNA levels of IP-10 in the brain following MCAO found increased mRNA as early as 6 h post-MCAO (Offner et al. 2006). As expected, IP-10 within the infarct is expressed in cells of the monocytic lineage (CD11b⁺), which are most likely microglia. IFN γ is known to activate cells of monocytic origin to produce IP-10 (Boehm et al. 1997). Splenectomy decreases IFN γ levels in the brain (Seifert et al. 2012b), subsequently prevents the increase in IP-10 in the brain following MCAO. However, we have previously shown that administration of rIFN γ after MCAO reversed the protective effects of splenectomy (Seifert et al. 2012b) and additionally this treatment increased the expression of IP-10 protein in the injured brain to levels not significantly different from those from sham splenectomized rats. Additionally, sham splenectomized rats that received rIFN γ after MCAO did not further elevate levels of IP-10 in the spleen or the brain, showing that the endogenous IFN γ response elicited by the spleen to stroke is a maximal response.

The spleen reacts to bodily injuries by eliciting an inflammatory response that further exacerbates cellular damage. Splenectomy is protective in a variety of ischemic injuries in

other organs including the liver, (Okuaki et al. 1996) kidney, (Jiang et al. 2007) intestines, (Savas et al. 2003) and heart (Leuschner et al. 2010). Additionally, removal of the spleen is neuroprotective in several types of brain injuries including ischemic stroke, (Ajmo et al. 2008; Jin et al. 2013) intracerebral hemorrhage, (Lee et al. 2008) and traumatic brain injury (Li et al. 2011; Das et al. 2011; Walker et al. 2010). Radiation of the spleen following MCAO also reduces infarct volume (Ostrowski et al. 2012). The elimination of splenocytes by any means results in protection from ischemic tissue injuries. These studies reveal that splenocytes probably via the expression of inflammatory cytokines are universally detrimental to ischemic injuries in mouse and rat injury models.

In the spleen the levels of IFN γ spike at 24 h post-MCAO (Seifert et al. 2012b) while mRNA levels of IP-10 in the spleen 22 h following stroke in mice (Offner et al. 2006; Hurn et al. 2007). We show that the increase in IFN γ is followed by a prolonged increase in the expression of the pro-inflammatory chemokine IP-10 that begins at 24 h and remains elevated at least out to 96 h post-MCAO. IFN γ despite a very short half-life elicits protracted effects. An IFN γ -induced feed forward inflammatory cycle is initiated when the protracted expression of IP-10 secreted from macrophage/microglia recruits inflammatory T cells to the infarct and they express and secrete more IFN γ , leading to further activation and expression of IP-10 in macrophages/microglia ultimately resulting in delayed neural injury. Since IFN γ is not directly neurotoxic (Seifert et al. 2012b), its downstream activation of macrophages/microglia appears to be the mechanism by which this inflammatory cytokine can indirectly be responsible for delayed neurodegeneration.

Splenectomy not only reduces infarct volume but neuroinflammation as demonstrated by decrease in levels of IFN γ , IP-10, CD3 T cell immunoreactivity, and CD11b microglial activation in the infarct (Seifert et al. 2012b; Jin et al. 2013). Removal of the spleen blunts the IFN γ mediated immune response to the brain following stroke, which includes IP-10 expression, and recruitment and activation of T cells. The importance of IFN γ in the splenic response is clearly demonstrated in that the administration of IFN γ reverses the neuroprotective effects provided by splenectomy. Using a neutralizing antibody directed against IFN γ administered after MCAO could provide further evidence that IFN γ is the neurodegenerative mediator originating from the spleen.

Systemic administration of an IFN γ neutralizing antibody significantly decreases infarct volume when compared to vehicle controls. These results are consistent with previous observations where IFN γ was blocked (Yilmaz et al. 2006; Liesz et al. 2009; Liesz et al. 2011). Studies using IFN $\gamma^{-/-}$ mice (Yilmaz et al. 2006) or inhibiting IFN γ with a neutralizing antibody injected directly into the brain three days following MCAO (Liesz et al. 2009) both decreased infarct volume. Additionally, using an antibody directed at CD49d (very late antigen 4, VLA4) prevented immune cells from entering the brain following MCAO, leading to decreased infarct volume. This treatment precluded the cells producing IFN γ from entering the injured brain (Liesz et al. 2011). These studies did not investigate the downstream effects of IFN γ inhibition on the expression of chemokines like IP-10. Our study shows that striatal IP-10 levels in the brain are significantly decreased with IFN γ neutralization. The amount of CD3 immunoreactivity is significantly reduced in the striatum of these rats as well. This is expected as IFN γ is known to induce IP-10 production (Boehm

et al. 1997) and IP-10 is a strong chemoattractant for pro-inflammatory IFN γ producing T cells (Groom and Luster 2011). The infarct volumes for the IFN γ neutralizing antibody were significantly reduced compared to the IgG and vehicle controls. Moreover, a potentially more effective approach would be to use these antibodies in conjunction with pharmaceuticals that block IFN γ or its downstream intracellular signal transduction.

The increase in IP-10 expression in the spleen, and later the brain, is an important indicator of a pro-inflammatory pro-degeneration splenic response to ischemic stroke. IFN γ signaling induces the expression of IP-10. Inhibiting IFN γ signaling results in a reduction in infarct volume, IP-10 protein expression in the brain, and T cell recruitment to injured brain area leading to decreased infarct size. Selectively targeting the IFN γ signaling pathway is a potential treatment for stroke.

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Fig 1.

Quantification of IP-10 levels in the brain post-MCAO. Immunohistochemical quantification of striatal IP-10 protein levels in the brains of naïve, sham, 48, 72, and 96 h post-MCAO (n= 4, 3, 3, 4, and 4 respectively) demonstrate IP-10 levels are significantly elevated at 72 and 96 h post-MCAO compared to naïve brains (*p<0.01; a). Sham animals had a survival time of 96 h. Representative micrographs of IP-10 stained brains from naïve (b), 48 h (c), 72 h (d), and 96 h (e) post-MCAO. Scale bar = 100μm. Arrows indicate areas of staining. Boxes in the brain graphic indicated the three regions used for quantification.

Fig 2.

Quantification of IP-10 levels in the spleen post-MCAO. Immunohistochemical quantification of IP-10 protein levels in the spleens of naïve, sham, 24, 48, 72, and 96 h post-MCAO (n= 4, 3, 7, 3, 3, and 7 respectively) demonstrate IP-10 levels are significantly elevated at 24 h and remain elevated out to 96 h post-MCAO compared to naïve spleens (*p<0.0007; a). Representative micrographs of IP-10 stained spleens from naïve (b), 96 h sham (c), 24 h (d), 48 h (e), 72 h (f), and 96 h (g) post-MCAO. Scale bar = 120µm.

Fig 3.

Splenectomy and rIFN γ administration affects IP-10 in the brain and not the spleen. Rats that underwent splenectomy two weeks prior to MCAO had significantly reduced levels of IP-10 in the brain at 96 h post-MCAO (* p 0.05) and IP-10 levels were not significantly different from sham splenectomized rats when splenectomized rats were administered rIFN γ following MCAO (a). Administration of rIFN γ to sham splenectomized rats did not cause a significant increase in IP-10 levels in the spleen post-MCAO (b). Treatment groups: sham-splenectomy-vehicle (SS-V, $n=3$ brains and 5 spleens), sham-splenectomy-rIFN γ (SS-IFN γ , $n=3$ brains and 6 spleens), splenectomy-vehicle (S-V, $n=3$), and splenectomy-rIFN γ (S-IFN γ , $n=4$).

Fig 4.

IP-10 is produced in monocytic cells in the brain following MCAO. Confocal micrograph shows amoeboid CD11b positive cells (red) with intracellular IP-10 (green) in the striatum of the ipsilateral hemisphere 96 h following MCAO. Scale bar = 75 μ m. Arrows indicate areas of co-localization.

Fig 5.

IFN γ neutralizing antibody administration following MCAO decreases infarct volume. Administration of an IFN γ at 24, 48, and 72 h post-MCAO (n=3) significantly decreased infarct volume, as measured by Fluoro-Jade staining, at 96 h when compared to the vehicle (n=4) and isotype (n=5) control groups (*p<0.007; d). Representative micrographs of Fluoro-Jade stained brains from PBS vehicle (a), IgG isotype (b), and IFN γ antibody (c) treatment groups 96 h post-MCAO. Scale bars = 2 mm.

Fig 6.

Quantification of IP-10 levels in the brain post-MCAO with administration of an IFN γ neutralizing antibody. Immunohistochemical quantification of striatal IP-10 protein levels in the brains of vehicle (n=3), IgG Isotype (n=4), and IFN γ antibody (n=4) 96 h post-MCAO demonstrate IP-10 levels are significantly decreased in the IFN γ antibody treated group compared to the vehicle treated group (*p<0.009; d). Representative micrographs of IP-10 stained brains from PBS vehicle (a), IgG isotype (b), and IFN γ antibody (c) treatment groups 96 h post-MCAO. Scale bars = 100 μ m. Arrows indicate areas of staining. Boxes in the brain graphic indicated the three regions used for quantification.

Fig 7.

Quantification of IP-10 levels in the spleen post-MCAO with administration of an IFN γ neutralizing antibody. Immunohistochemical quantification of IP-10 protein levels in the spleens of vehicle (n=4), IgG Isotype (n=7), and IFN γ antibody (n=7) 96 h post-MCAO demonstrate IP-10 levels are elevated in the IFN γ antibody and the IgG groups compared to the vehicle treated group (d). Representative micrographs of IP-10 stained spleens from the PBS vehicle (a), IgG isotype antibody (b), and IFN γ antibody (c) treatment groups 96 h post-MCAO. Scale bars = 120 μ m.

Fig 8.

CD3 immunoreactivity decreased in IFN γ antibody treated brains. The amount of T cells in the brain following MCAO is decreased in the IFN γ antibody treated group (n=3) compared to controls (*p<0.04; n= 4 each;d). Images are from the striatum of the ipsilateral hemisphere. There is decreased CD3 (T cell) immunoreactivity in the brains of IFN γ antibody treated animals (c) compared to the amount of staining in the two control groups PBS vehicle (a) and isotype (b). Scale bars = 100 μ m. Arrows indicate areas of staining.