A Role for Interferon-Gamma in Focal Cerebral Ischemia in Mice

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Abstract. The pro-inflammatory cytokine interferon-gamma (IFNγ) has traditionally been associated with inflammatory CNS disease and more recently with ischemia-induced pathology. Using a murine model of focal cerebral ischemia, we found no evidence for induction of IFNγ mRNA after permanent middle cerebral artery occlusion. In addition, we found that mice deficient in IFNγ or IFNγ receptors developed neocortical infarcts similar in size to those in wild type. In contrast, MBP promoter-IFNγ-transgenic mice consistently developed significantly larger infarcts than non-transgenic mice. Because IFNγ is a potent activator of microglia-macrophages, we investigated the involvement of microglial-macrophage-derived TNF in the larger infarcts. Numbers of TNF mRNA-expressing microglia-macrophages and levels of TNF mRNA and TNF in IFNγ-transgenic and non-transgenic mice were similar. Furthermore, the ischemic brain damage in IFNγ-transgenic mice was unaffected by recombinant soluble TNF receptor I. Taken together, the data argues against a role for IFNγ in cerebral ischemia under normal conditions. However, when present, IFNγ significantly exacerbates ischemia-induced brain damage by mechanisms that appear to be independent of TNF or synergistic neurotoxic interactions of IFNγ and TNF. Irrespective of the mechanism(s) involved, this enhancing effect of IFNγ on ischemia-induced neurotoxicity may need to be considered in diseases where immune IFNγ is involved, such as multiple sclerosis.

Key Words: Knockout mice; Middle cerebral artery occlusion; Microglia; Macrophages; Neurodegeneration; Transgenic mice; Tumor necrosis factor.

INTRODUCTION

Interferon-γ (IFNγ) is considered a key regulator of immune and inflammatory responses. IFNγ is absent from normal brain parenchyma. In inflammatory central nervous system (CNS) diseases, such as multiple sclerosis (MS) and infectious diseases, IFNγ is produced by infiltrating T-cells and NK-cells (1, 2), and its presence is associated with induction and aggravation of patholgy (3, 4). Findings of infiltrating T-cells and NK-cells (5, 6) and an increase in IFNγ mRNA by some, but not by other authors (7, 8) in rat models of focal cerebral ischemia, have raised the question whether IFNγ also plays a role in ischemia-induced neurotoxicity and inflammatory responses.

The effect of IFNγ in MS and experimental allergic encephalomyelitis (EAE) is associated with its multiple pro-inflammatory effects (9, 10). Thus it has been shown that IFNγ induces microgliosis and astrogliosis (11, 12), microglial-macrophage and astroglial synthesis of tumor necrosis factor (TNF) and interleukin-1beta (IL-1β) (12–16), and chemokines (17). IFNγ also induces the expression of inducible nitric oxide synthase, cyclooxygenase-2, and oxygen free radicals (18–21) all of which can potentiate ischemic brain damage (22, 23).

All nucleated cells including neurons have the potential to express IFNγ receptors (24). IFNγ induces major histocompatibility complex class I antigens on primary neurons (25, 26), and increases the excitability of neurons in cortical and cerebellar explants from cat and rat (27). Exposure of primary central and peripheral neurons to IFNγ has been shown both to promote (28) and suppress neuronal differentiation (29). IFNγ can also modify synaptic activity by perturbing the clustering of glutamate receptors of the α-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA) subtype (30), which like the N-methyl-D-aspartate glutamate receptors contribute to normal CNS functioning as well as to ischemia-induced neurotoxicity (31, 32). Interestingly, neurons upregulate their expression of the interferon induced transcription factor, interferon response factor-1 (IRF-1) (33), 2′-5′-oligoadenylate synthase (34), and interferon-inducible protein-10 (35) following cerebral ischemia. IRF-1 has been shown to contribute to ischemic brain damage (36).
In this study, we took advantage of a murine model of permanent focal cerebral ischemia, which leads to reproducible and quantifiable foci of neurodegeneration (37, 38). We used knock-out and transgenic mouse models (15, 39, 40) and real-time reverse transcription-polymerase chain reaction (RT-PCR) techniques to demonstrate that IFN-γ plays no role in cerebral ischemia under normal conditions, whereas transgenic expression of IFN-γ in CNS exacerbates neuronal sensitivity to cerebral ischemia. This interpretation of the infarct volumetric data was supported by the finding of a normal macro- and microvascular anatomy in the IFN-γ-transgenic (IFN-γ-Tg) mice. Furthermore, findings of comparable levels of TNF mRNA and TNF in IFN-γ-Tg and non-transgenic mice and of similar infarct volumes in IFN-γ-Tg mice subjected to anti-TNF-therapy suggested that the IFN-γ-enhanced ischemic pathology is independent of TNF. The results provide the first demonstration that the presence of IFN-γ exacerbates ischemic brain damage. This may have important implications for understanding the pathophysiology of inflammatory CNS disorders in which immune IFN-γ is involved.

**MATERIALS AND METHODS**

**Animals**

BALB/c-backcrossed IFN-γ-knock-out mice (IFN-γ-KO) (39) and age-matched littermate controls were originally obtained from Genentech (San Francisco, CA) and maintained as a colony at the Montreal Neurological Institute. C57BL/6-backcrossed mice deficient in IFN-γ receptors (IFN-γR-KO) were originally obtained from Dr. Michel Aguet (Institute of Molecular Biology Institute, Zurich, Switzerland) (40), and were further backcrossed to C57BL/6 mice at the animal facility at the Montreal Neurological Institute. All knockout mice were homozygous (IFN-γ−/− or IFN-γR−/−), and the controls were wildtype (WT) littersmates (IFN-γ+/+ or IFN-γR+/+). Adult, age-matched, IFN-γ-transgenic (IFN-γ-Tg) mice were homozygotes of the A519 line, backcrossed 6 times onto the SJL background (15). These mice express IFN-γ mRNA in their CNS under the control of a 1.3-kb myelin basic protein (MBP) promoter (15). Age-matched SJL mice were purchased from Bomholgaard A/S (Ry, Denmark) or Harlan-Sprague Dawley (Indianapolis, IN). SJL mice from these 2 vendors display the same sensitivity to focal cerebral ischemia (K.L. Lambertsen, unpublished observations). All mice used in the study were male, except the IFN-γ-Tg mice used in the real-time RT-PCR analysis. The IFN-γ-R-KO and their WT littersmates were a mixture of male and female mice. The SJL mice with EAE were female mice. The experimental mice were transferred to and housed in the animal facility, University of Southern Denmark, Odense, Denmark, and were given free access to food and water. Animal procedures were approved by the Danish Animal Health Care Committee (J. nr. 192000/561–272).

**Functional Cerebrovascular Anatomy**

The major cerebral arteries were examined in normal IFN-γ-Tg (n = 15) and normal SJL (n = 15) mice. Mice were anesthetized i.p. with an overdose of pentobarbital (7278631, Den Kgl. Veterinær- og Landbohøjskoles Apotek, Copenhagen, Denmark) and complete sternotomy was performed to expose the heart. Mice were perfused with 10 ml of 0.9% saline followed by 5 ml of a 50% solution of Quinck carbon black ink (Solv-X®, Parker) via the left cardiac ventricle to enhance visualization of the cerebral arteries. Following perfusion, mice were decapitated and the brains carefully removed and placed in 10% buffered formalin. The vessels of the circle of Willis and their branches were carefully examined and photographed under a dissecting microscope. The development of the left and right posterior communicating arteries was scored individually as described in Majid et al (41) as follows: 0, absent; 1, present but poorly developed (hypoplastic); and 2, well formed. A single posterior communicating artery development score was calculated for each animal by averaging the left and right scores.

**Induction of Focal Cerebral Ischemia**

Permanent occlusion of the distal part of the left middle cerebral artery (MCA) was performed according to the method previously described (37, 38). Mice were anesthetized by s.c. injection of 0.21 ml/10 g body weight of a 1:1:2 mixture of Hypnorm™ (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, Janssen-CILAG, Birkerød, Denmark), Stesolid™ (5 mg/ml Diazepanum, Dumex, Copenhagen, Denmark) and distilled H₂O. Mice were placed on a 37.5°C heating pad. A skin incision was made between the lateral part of the orbit and the external auditory meatus. The superior pole of the parotid gland was pushed downwards as was the upper part of the temporal muscle after partial resection. A burr-hole was drilled directly over the distal part of the MCA, dura mater was removed, and the MCA was coagulated by bipolar electrocoagulation applying forceps coupled to an electrosurgical unit (ICC50 from ERBE, Tubingen, Germany). The incision was closed with a 4-0 nylon-suture. 1 ml of physiological 0.9% saline was injected s.c. and the eyes coated in ointment (Öjensalve Neutral, SAD, Copenhagen, Denmark) to protect from drying. Mice were kept in a recovery room at 28°C for 20 h before return to the animal facility. In sham operated mice the electrocoagulator was applied in the CNS parenchyma just next to the MCA. Post-surgical pain treatment consisted of supplying the mouse s.c. with 0.15 ml Temgesic™ diluted 1:30 (stock: 0.3 mg Buprenorphinum, Reckitt & Colman, Hull, England) 3 times with an 8-hour interval starting immediately after the operation.

**Treatment with Recombinant Soluble TNF Receptor I**

Immediately after the induction of ischemia, 1 group of IFN-γ-Tg mice was injected i.v. with 1 mg/kg of recombinant soluble TNF receptor I (rSTNF-RI, 425-R1, R&D Systems, Oxon, UK). The control group of IFN-γ-KO mice received an i.v. injection of 20 μl 0.9% physiological saline per gram of mouse immediately following the induction of ischemia. Furthermore, as these mice also served as controls for an additional anti-inflammatory agent they received an i.p. injection of 0.5 ml phosphate buffered saline (PBS) twice daily for 5 days.

**Brain Tissue Processing and Determination of Infarct Volume**

**Infarct Volumetric Analysis:** IFN-γ-KO, IFN-γR-KO, and their respective WT mice were killed by cervical dislocation at day
1, and IFNγ-Tg and SJL mice at 1 or 5 days after MCA occlusion. Brains were removed and frozen in CO₂ snow. All brains were cut coronally into 30-μm-thick sections in a cryostat, and every sixth section was stained with toluidine blue for determination of infarct volume using the Computer Assisted Stereological Test Grid System (Olympus, Albertslund, Denmark) and the Cavalieri principle for volume estimation (37, 38).

Real-Time RT-PCR Analysis: IFNγ-Tg and SJL mice were decapitated by cervical dislocation. Survival times for the SJL mice were 30 min, 1, 2, 4, 6, 12 h, 1, 2, 5, or 10 days and for the IFNγ-Tg mice 1 h, 1 or 5 days. A number of unoperated IFNγ-Tg and SJL mice were included for control. The brains were removed, remaining meninges were removed, and the brainstem was transsected at the level of the superior colliculi. The cerebrum and the remaining part of the brainstem were divided into ipsi- and contralateral halves. Each half was placed in 10 volumes of RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany).

ICAM-1 Analysis: Unoperated IFNγ-Tg and SJL mice were deeply anesthetized and perfused through the left ventricle using 5 ml chilled 0.15 M Sørensen buffer (Sø-PB) (pH 7.4), followed by 20 ml chilled 4% paraformaldehyde (PFA) in 0.15 M Sø-PB, pH 7.4. The brains were carefully removed and post-fixed in 4% PFA for 1 h, immersed in 20% sucrose overnight, frozen using CO₂-snow, and stored at −40°C until serially sectioned into 16-μm-thick sections.

Induction of Experimental Allergic Encephalomyelitis

EAE was induced in female SJL mice (n = 3) by s.c. injections 2 weeks apart with 400 μg of MBP (Sigma-Aldrich) emulsified in Freund’s complete adjuvant containing 50 μg Mycobacterium tuberculosis H37RA (ICN, Aurora, OH) in combination with i.p. injections of 200 mg Pertussis toxin (PTX; Sigma-Aldrich, P7208) on day 0 and day 2. Unimmunized mice only receiving PTX on day 0 and day 2 served as controls (n = 3). The mice were monitored daily and scored clinically as follows: 0, no symptoms; 1, flaccid tail; 2, hindlimb paresis; 3, hindlimb paralysis; 4, complete fore/hind limb paralysis; 5, moribund. Mice were deeply anesthetized when they reached a clinical score of 3, perfused through the heart with ice-cold 0.15 M PBS, and the spinal cords collected for RNA isolation as described below.

Real-Time RT-PCR Analysis for IFNg mRNA and TNF mRNA

RNA Isolation: RNA was isolated in duplex from each sample using the RNeasy Protect Mini Kit from Qiagen according to the manufacturer’s directions.

Preparation of cDNA: cDNA synthesis was undertaken in duplicate for each RNA extract (i.e. 4 cDNA reactions per sample). One μg RNA was applied for each 20-μL cDNA reaction. For priming reverse transcription, random hexamers (d(N)₆, Roche) were used, and synthesis was driven by 200 U (1 μL) M-MLV RT (Invitrogen Life Technologies, Carlsbad, CA), all in accordance with the manufacturer’s instructions.

Real-time PCR: An iCycler (Bio-Rad, Herlev, Denmark) was used for PCR and real-time detection of the TaqMan probe generated fluorescent signals. The DNA sequences of the TaqMan systems applied for real-time PCR were designed to target exon-exon junctions of the respective genes in order to ensure cDNA specificity. Design was assisted by BeaconDesigner 2.0 (PREMIER Biosoft International, Palo Alto, CA) with melting temperature for PCR primer set at 58°C for IFNg and 60°C for TNF. Probes were set 10°C higher. For IFNg the sequence of the TaqMan probe was ACTACCTCTTCTGACGAGCGCA-AGGC-GAA, for the sense primer it was AAGCGTCA-TGAATCACCAGT, and for the antisense primer it was GTGGGTGTGTTGACCTCAAACCT. For TNF the sequence of the TaqMan probe was TGGCCCAAGACCTCACA-CTCACTCATC, for the sense primer it was TGGCCCTCCCTCCTCTC ATACGATC, and for the antisense primer it was TTTGCTGTTTGGACGAGGTG. Reactions were in a microtiter format. Each well of the microtiter plate contained 25 μL 1 × iQ Supermix (Bio-Rad) containing 5 μL of the diluted cDNA, 400 nM cDNA specific PCR primer, and 100 nM TaqMan probe. The 2-step PCR protocol consisted of 45 cycles of 95°C for 10 seconds and 60°C for 1 min for both cytokines. Temperature cycling was preceded by incubation for 3 min at 95°C to activate the inactivated Taq polymerase present in the iQ Supermix. For normalization of real-time data, hypoxanthine phosphoribosyltransferase 1 was used as an internal control gene. Normalized data was calculated for each RNA extract and the average of the 2 extracts was used for calculation of the relative gene expression. Gene expression levels were calculated relative to a baseline sample included on each real-time PCR plate. For standard curve construction, a 5-fold dilution series of spleen derived cDNA was applied.

Analysis of Microglial-Macrophage Reactions and TNF Synthesis

Immunohistochemistry: Immunohistochemistry was performed using a 3-step biotin-streptavidin-horseradish peroxidase technique with diaminobenzidine as a chromagen, or a 2-step procedure using an alkaline phosphatase (AP)-conjugated secondary antibody (42). Microglia-macrophages were visualized with a rat monoclonal antibody to murine macrophage-associated antigen-1 (Mac-1/CD11b) (MCA711, Serotec, Oxford, UK). The secondary antibody was a biotinylated sheep anti-rat antibody (RPNI002, Amersham, Buckinghamshire, UK). TNF was visualized using a polyclonal rabbit antibody directed against murine TNF (P-350, Endogen, Rockford, IL), and an AP-conjugated antibody to rabbit Ig (A-3812, Sigma-Aldrich). Control reactions for antibody specificity were performed on parallel sections by preabsorbing the antibody with recombinant murine TNF (T7539, Sigma-Aldrich), by incubating sections with an isotype-specific control antibody (IG-851125, Biosite, Täby, Sweden), rabbit serum, or with Tris buffered saline alone.

In Situ Hybridization: The in situ hybridizations were performed as previously described (42) using alkaline AP-labeled oligo DNA probes purchased from DNA Technology A/S (Aarhus, Denmark). The in situ hybridization of TNF mRNA was performed with a mixture of two 28’ mer DNA probes based on the primer sequences for real-time PCR detection of TNF message (12), that were complementary to bases 305–332 (5’-CTTTCTCATCCTTTGGGGACGGATCACC) and 570–597 (5’-GTAGTCGGGGACGCTTTGTCCCTTGA) of murine TNF cDNA (43). The specificity of the probes was confirmed.
by hybridizing with each probe alone, or a mixture of both probes, showing identical regional and cellular localization of the in situ signal, but with a stronger signal in sections hybridized with the probe mixture. Additionally, sections pretreated with ribonuclease “A” (27-0323-01, Pharmacia, Copenhagen, Denmark) prior to hybridization, or hybridized with a 100-fold excess of unlabeled TNF probe mixture or buffer alone, were devoid of signal. Ischemia-induced upregulation of oligodendrocyte MBP gene expression was visualized using a 30′ mer DNA probe (′-CTCTTCGCGCTGAGGACCCATATGGGT AGT), complementary to bases 302–331 of murine MBP cDNA (42).

**Combined In Situ Hybridization and Immunocytochemistry and Double Immunocytochemical Staining:** The cellular source(s) of TNF was determined by combining in situ visualization of TNF mRNA with immunohistochemical visualization of the astrocytic antigen glial fibrillary acidic protein (GFAP), and by double immunohistochemical staining for TNF and microglial-macrophage Mac-1 on 4% PFA fixed fresh-frozen sections as previously described (42).

**Scoring of Microglial-Macrophage Reactivity in IFN-γ-Tg and SJL Mice:** Comparison of the strength of the ischemia-induced microglial-macrophage Mac-1 reactivity was based on microscopic analysis of the Mac-1-stained sections, which were scored as follows: +, moderate microglial-macrophage reactivity in peri-infarct including the corpus callosum; ++, strong microglial-macrophage reactivity; and ++++, very strong microglial-macrophage reactivity.

**Quantification of TNF mRNA-Expressing Cells in IFN-γ-Tg and SJL Mice:** TNF mRNA-expressing cells were counted by an approximated (as 1/tsf = 1, see below) stereological counting technique, yielding an estimate of the total number of TNF mRNA-expressing cells within infarct and peri-infarct areas (45). The advantage of this method being that it is insensitive to edema-related changes in the volume of the ischemic cortex (46, 47). The average number of cells displaying a moderate to strong hybridization signal located in infarct and peri-infarct areas was obtained by counting every twelfth section, separated by 360 μm, from each animal using a ×100 objective and a 4,325-μm² frame area stepping 200 μm/200 μm in the XY-position using the Computer Assisted Stereological Test Grid System. The total number (N) of TNF mRNA-expressing cells in each animal was estimated using the formula: estimate of N = Q × (1/ssf) × (1/asf) × (1/tsf), where Q is the total number of counted cells, 1/ssf the sampling section fraction (1/ssf = 12), 1/asf the area sampling fraction (40,000/4,325), and 1/tsf is the thickness sampling fraction (1/tsf = 1), as described by West et al (47). The rostrocaudal distribution of TNF mRNA-expressing cells was analyzed by dividing the total number of TNF mRNA-expressing cells per section with the corresponding infarct area as measured on toluidine blue-stained sections.

**Western Blotting for TNF**

Western blotting for TNF was performed as previously described (38, 45). Mice were given an overdose of pentobarbital 24 hours after the induction of ischemia and perfused with ice-cold PBS (0.1 mol/L, pH 7.4) through the heart. The brains were removed, dissected into the ipsi- and contralateral cortex, and placed in RIPA buffer. The tissue was homogenized and a protein estimation was performed at 595 nm on a spectrophotometer (COBAS MIRA, Roche, Basel, Switzerland). Samples of 20 μg of protein from each mouse were loaded on a 10% Bis-Tris gel (NP0004, Novex, San Diego, CA) and electrophoresed in MES-SDS running buffer (NP0002, Novex) at 200 V for approximately 35 min. Proteins were transferred to a polyvinylidene difluoride membrane (IPUH00010, Millipore, Sundyberg, Sweden) at 30 V for 1 h and TNF was detected immunohistochemically as previously described (42) using an AP-conjugated secondary antibody.

**Analysis of Vascular Structures in IFN-γ-Tg and SJL Mice using ICAM-1**

Immunohistochemistry for the ICAM-1 cell adhesion molecule (CD54) was performed on PFA perfused brain sections from normal IFN-γ-Tg and normal SJL mice using the 3-step biotin-streptavidin-horseradish peroxidase technique with diaminobenzidine as a chromogen according to the described protocol (42). The primary antibody was a rat monoclonal antibody to murine ICAM-1 (CD54) (MCA818, Serotec) (1:200). The secondary antibody was a biotinylated sheep anti-rat antibody (RPN1002, Amersham). Control reactions for antibody specificity were performed on parallel sections by incubating sections with an isotype-specific control antibody (IG-851125, Biosite) or with Tris buffered saline alone.

**Analysis of Astroglial Reactions**

Normal and reactive astrocytes were visualized using a rabbit polyclonal antibody to bovine GFAP (1:1,800) (Z334, Dako A/S, Copenhagen, Denmark) and a 2-step procedure using a AP-conjugated secondary antibody (1:200) (A3812, Sigma, Denmark) as previously described (42). Control reactions were performed on parallel sections by incubating sections with rabbit serum or with Tris buffered saline alone.

**Visualization of Degenerating Nerve Fiber Tracts**

Sections were silver impregnated for visualization of degenerating axons using the method described by Jensen et al (12, 48).

**Data Analysis**

Volumetric data in text and figures are expressed as means ± SEM. Comparisons of mean infarct volumes, mean values for the total number of TNF mRNA-expressing cells, mean microglial-macrophage Mac-1 reactivity score, and mean posterior communicating artery scores were evaluated by the non-parametric Mann-Whitney test using the GraphPad Instat version 3.0a for Macintosh (GraphPad Software, San Diego, CA). Analysis of the relative change in IFN-γ mRNA gene expression in SJL mice was done using Kruskal-Wallis test (non-parametric ANOVA) using the GraphPad Instat 3.0a program. Correlation of Mac-1 reactions and infarct volume was done using non-parametric Spearman Rank Correlation analysis using the GraphPad Instat 3.0a program. Comparison of the relative change in TNF mRNA gene expression in IFN-γ-Tg mice and SJL mice was done using a 2-way ANOVA using the Graphpad Prism 4.0a program. Statistically significant differences for comparison of means were established at p < 0.05.
Fig. 1. Size of ischemic brain damage in IFNγ-KO, IFNγR-KO, and their respective WT mice. A-D: Toluídine blue staining demonstrating similar infarct volumes in IFNγ-KO (A) and BALB/c (B) mice and in IFNγR-KO (C) and C57BL/6 (D) mice 1 day after surgery. The pale area marked IF in (A) represents the infarct. Abbreviations: cc, corpus callosum; Ctx, cortex; Str, striatum. Scale bar = 2 mm.

RESULTS
Involvement of IFNγ in Ischemic Brain Injury under Normal Conditions

We used IFNγ- and IFNγR-deficient mice to determine if lack of IFNγ or its receptor influence infarct development. In all types of mice permanent occlusion of the MCA resulted in reproducible unilateral cortical infarcts within the frontotemporal and parietal cortices (Fig. 1). Mean infarct volumes in IFNγ-KO, IFNγR-KO, and their respective WT mice were compared 24 hours after surgery. Mean infarct volumes were comparable in IFNγ-KO and WT mice (IFNγ-KO: 32.4 mm³ ± 3.7 mm³, n = 6, versus BALB/c: 28.1 mm³ ± 2.2 mm³, n = 5) (p > 0.10) (Figs. 1, 2A). Similarly, IFNγR-KO and their WT mice showed comparable infarct volumes (IFNγR-KO: 11.5 mm³ ± 2.6 mm³, n = 12, versus C57BL/6: 8.4 mm³ ± 1.6 mm³, n = 12) (p > 0.5) (Figs. 1, 2A).

Using real-time RT-PCR we next investigated whether IFNγ mRNA is induced following MCA occlusion in normal SJL mice (Fig. 2B). We found no change in the relative IFNγ mRNA expression in SJL mice either at 30 min (n = 3), 1 h (n = 3), 2 h (n = 3), 4 h (n = 8), 6 h (n = 5), 12 h (n = 9), 1 day (n = 7), 2 days (n = 5), 5 days (n = 4), or 10 days (n = 5) after permanent MCA occlusion compared to control (n = 2) (Fig. 2B) or to sham operated mice decapitated at 12 hours or 5 days after induction of ischemia (data not shown). To validate our ability to detect changes in IFNγ mRNA expression, we included spinal cords from SJL mice with grade 3 EAE and PTX injected control mice. Induction of EAE resulted in a 122.1 ± 20.8 increase in the relative IFNγ mRNA gene expression (n = 3) compared to 0.8 ± 0.2 in control mice (n = 3) (Fig. 2B). In combination, the lack of induction of IFNγ mRNA in the ischemic brain of SJL mice and the lack of effect of IFNγ or IFNγR knock-out on the size of the ischemic injury strongly suggest that IFNγ plays no role in ischemic brain injury under normal conditions.

Functional Cerebrovascular Anatomy in IFNγ-Tg and non-Tg SJL Mice

In order to be able to draw valid conclusions from infarct volumetric analysis of IFNγ-Tg and non-Tg SJL mice we analyzed and compared the general growth characteristics and functional vascular anatomy in these mice. In line with previous studies (12, 15) we found that the A519 mice develop and breed normally and show no spontaneous pathology despite constitutive expression of IFNγ in their CNS.

Comparison of the cerebrovascular anatomy using carbon black showed that the circle of Willis was similar in SJL and IFNγ-Tg mice at the level of the anterior and middle cerebral arteries (Fig. 3A, B). In addition, the mean posterior communicating artery scores were identical between SJL mice and IFNγ-Tg mice (p > 0.75) (Fig. 3C). In detail, one of the posterior communicating arteries was hypoplastic in 1 SJL mouse out of 15 SJL mice and in 2 IFNγ-Tg mice out of 15 IFNγ-Tg mice. The rest of the mice had fully formed posterior communicating arteries, though in some SJL mice and in some IFNγ-Tg mice the posterior communicating arteries had a tendency to be thinner, but not hypoplastic, on one side. In such cases there were occasionally 2 rather than 1 artery. Inspection of the MCA revealed an atypical MCA in 1 IFNγ-Tg mouse and in 1 SJL mouse.

In addition, we immunohistochemically stained sections from normal SJL mice (Fig. 4A, C) and sections from normal IFNγ-Tg mice (Fig. 4B, D) for the adhesion molecule ICAM-1/CD54, which is expressed in low constitutive levels on the vascular endothelium and resting microglia in normal CNS. Endothelial and microglial ICAM-1 staining was similar in the 2 types of mice (compare Fig. 4A, C with Fig. 4B, D), suggesting that the blood-brain barrier and the microvascular function is similar in IFNγ-Tg mice and non-Tg SJL mice.

Volume of Ischemic Injury in IFNγ-Tg and non-Tg SJL Mice

We subjected IFNγ-Tg mice to permanent MCA occlusion to determine if constitutively expressed or ischemia-induced IFNγ in these mice might exacerbate ischemic brain injury (Fig. 5). Comparison of mean infarct volumes in IFNγ-Tg and SJL mice showed that IFNγ significantly exacerbated the cerebral infarct volumes both at day 1 (IFNγ-Tg: 31.0 mm³ ± 1.7 mm³, n = 12, versus SJL: 21.4 mm³ ± 2.7 mm³, n = 9) (p < 0.01), and at day 5 (IFNγ-Tg: 12.8 mm³ ± 1.0 mm³, n = 9, versus SJL: 8.8 mm³ ± 0.9 mm³, n = 13) (p < 0.01)
Fig. 2. Demonstration that IFNγ is not involved in ischemia-induced infarction under normal conditions but exacerbates infarction when present. A: Infarct volumes are comparable in IFNγ-KO and BALB/c mice (p > 0.10, 2-tailed Mann-Whitney test), and in IFNγR-KO and C57BL/6 mice (p > 0.50, 2-tailed Mann-Whitney test). B: Real-time RT-PCR analysis of IFNγ mRNA showed no induction of IFNγ mRNA gene expression from 30 min to 10 days after permanent occlusion of the MCA in normal SJL mice (Kruskal-Wallis non-parametric analysis of variance), whereas induction of experimental allergic encephalitis (EAE) resulted in a tremendous increase in the IFNγ mRNA expression compared to Pertussis toxin (PTX)-treated control mice. C: IFNγ-Tg mice develop significantly larger infarcts than SJL mice at 1 day and 5 days after MCA occlusion (*p < 0.01, 1-tailed Mann-Whitney test). D: Analysis of the rostrocaudal distribution of the infarct areas 1 day after MCA occlusion showed that gene-manipulated mice display similar rostrocaudal distribution of the infarct as their respective control mice. Results represent mean ± SEM.

(Figs. 2C, 5C–F). The considerably larger mean infarct volumes at day 1 compared to day 5 in both IFNγ-Tg and SJL mice (Figs. 2C, 5C–F) suggest the beginning of resorption of the infarcts by day 5.

The findings of increased infarct sizes in IFNγ-Tg mice were verified in a separate experiment carried out by a different individual. As before, IFNγ-Tg developed larger mean infarct volumes (25.5 mm³ ± 2.3 mm³, n = 14) than SJL mice (16.9 mm³ ± 1.3 mm³, n = 12) 1 day after permanent occlusion of the MCA (p < 0.01).

The similar rostrocaudal distribution of the infarcts in IFNγ-Tg and SJL mice confirmed that the IFNγ-transgene had no effect on the regional distribution of the MCA in SJL mice (Fig. 2D). Further, IFNγ-Tg and SJL mice showed the same regional distribution of infarcts as the C57BL/6 backcrossed IFNγR-KO and C57BL/6 mice, while infarct size and regional distribution were different in BALB/c-backcrossed IFNγ-KO mice (Fig. 2D). Taken together, the data suggests that IFNγ plays no role in ischemic brain damage under normal conditions. However, if present, IFNγ exacerbates the ischemic injury.

Transgenic IFNγ Does Not Influence Ischemia-Induced Microglial-Macrophage or Astroglial Reactivity

Since transgene-encoded IFNγ is known to result in increased lesion-induced microglial Mac-1 reactivity (12) we compared the lesion-induced microglial-macrophage response in IFNγ-Tg and SJL mice at 1 and 5 days (Fig. 6). One day after MCA occlusion there was no apparent difference in the microglia-macrophage reactions...
between IFNγ-Tg and SJL mice. Both types of mice displayed infiltrating round, macrophage-like cells located within the infarct and in peri-infarcted areas at day 1 (Fig. 6A, B) and a comparable, now massively upregulated, microglial-macrophage Mac-1 reactivity within and in the border zone of the infarct and in the corpus callosum in both hemispheres at day 5 (Fig. 6C, D). Comparison of the mean scores of the strength of the ischemia-induced microglial-macrophage Mac-1 reactivity showed similar reactivity in IFNγ-Tg and SJL mice both at day1 (p > 0.20, IFNγ-Tg: 2.3 ± 0.6 and SJL: 1.7 ± 1.0) and at day 5 (p > 0.60, IFNγ-Tg: 2.3 ± 0.7 and SJL: 2.1 ± 0.8). Correlation analysis showed that there was no correlation between the size of the infarct as visualized on toluidine blue sections and the degree of microglial reaction neither at day 1 (Spearman r = -0.05172, p = 0.84) or at day 5 (Spearman r = -0.1335, p = 0.60).

We also compared the lesion-induced astrocytic GFAP response in IFNγ-Tg and SJL mice at 1 and 5 days (data not shown). One day after MCA occlusion there was no apparent difference in the astrocytic response between IFNγ-Tg and SJL mice. Both types of mice displayed decreased astrocytic GFAP staining within the infarct and signs of astrocytic hypertrophy, as indicated by an increased GFAP immunoreactivity, in the peri-infarct. Five days after MCA occlusion, IFNγ-Tg and SJL mice again displayed comparable astroglial reactivity (data not shown). Only very few GFAP immunoreactive cells could be visualized within the infarct, whereas the peri-infarct and the surrounding cortex and the corpus callosum displayed strongly hypertrophied astrocytes with a pronounced upregulation in GFAP immunostaining.

The areas with increased microglial-macrophage and astroglial reactivity at day 5 (Fig. 6C, D) corresponded to areas with increased MBP mRNA expression in parallel in situ hybridized sections in both IFNγ-Tg and SJL mice (Fig. 6F, shown for the peri-infarct area only). In corpus callosum, areas with increased MBP mRNA expression and strong microglial-macrophage and astroglial reactivity corresponded to the location of silver impregnated degenerating fiber bundles visualized in parallel sections (data not shown).

Transgenic IFNγ Does Not Influence Ischemia-Induced Recruitment of TNF Synthesizing Microglia-Macrophages or TNF mRNA Expression

Because microglia-macrophages can be stimulated by IFNγ to produce TNF (13) and since TNF is known to exacerbate cerebral infarction (49–51), we analyzed the possible involvement of TNF in the IFNγ-enhanced cerebral infarction. One day after MCA occlusion, TNF mRNA-expressing and TNF-immunoreactive cells were located in the periphery of the infarct and in peri-infarct areas in both IFNγ-Tg and non-Tg SJL mice (Fig. 8A, B, shown for the SJL mouse only). As previously observed in genetically normal mice (38, 42, 45), these TNF mRNA-expressing and TNF-immunoreactive cells displayed a microglial-macrophage-like appearance (Fig. 8C, D), did not co-express the astroglial marker GFAP (Fig. 8E), and colocalized with Mac-1-positive microglia-macrophages in both IFNγ-Tg and non-Tg mice (Fig. 8F).

We compared the number of TNF mRNA-expressing cells in IFNγ-Tg and non-Tg SJL mice in the acute phase after injury (Fig. 9A). Cell count estimates showed that the total numbers of TNF mRNA-expressing cells in IFNγ-Tg (17,250 ± 1,730, n = 12) and SJL mice (16,450 ± 2,520, n = 9) (p > 0.50) were comparable (Fig. 9A). TNF mRNA-expressing cells were barely detectable in sham operated mice (IFNγ-Tg: 550 ± 220, n = 2, and SJL: 450 ± 510, n = 4) (Fig. 9A). Consistent with these data, real-time RT-PCR analysis of the relative levels of TNF mRNA in IFNγ-Tg and SJL mice revealed no difference between IFNγ-Tg and SJL mice at 1 h (n = 3–
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Fig. 4. Immunohistochemical staining for the cell adhesion molecule ICAM-1/CD54 in (A, C) normal IFNγ-Tg and (B, D) normal SJL mice showing similar endothelial (arrows in A and B) and microglial ICAM-1 expression in IFNγ-Tg and SJL mice. High magnification of ICAM-1-immunoreactive vessels (arrows in C and D) and microglial-like cells (arrowheads in C and D) in IFNγ-Tg (C) and SJL (D) mice. Scale bars: (A,B) = 500 μm; (C,D) = 30 μm.

6), 1 day (n = 8–11) and 5 days (n = 6–8) after permanent MCA occlusion (Fig. 9B). Similarly, Western blotting also revealed similar levels of TNF in IFNγ-Tg and non-Tg mice at day 1 (not shown).

The marginal increase in TNF message detected in hippocampus by semiquantitative RT-PCR in a previous study (12) was not detected by real-time RT-PCR analysis of whole brain from unoperated IFNγ-Tg and SJL mice (n = 2 in each group) in the present study (Fig. 9B).

Intervention Against TNF in IFNγ-Tg Mice

Finally, we performed an intervention study where we antagonized the potential effect of TNF in IFNγ-Tg mice using a dose of 1 mg/kg rsTNF-RI that was shown to be efficient in a rat model of permanent MCA occlusion by Dawson et al. (49). IFNγ-Tg mice were injected i.v. with rsTNF-RI or the vehicle solution immediately after MCA occlusion in order to antagonize the very early ischemia-induced increase in brain TNF levels (38, 52). Analysis showed that the mean infarct size in IFNγ-Tg mice treated with rsTNF-RI (11.2 mm³ ± 2.3 mm³, n = 6) was similar to that in placebo-treated mice (12.9 mm³ ± 1.3 mm³, n = 10) (Fig. 9C), demonstrating that this type and treatment schedule of anti-TNF therapy was ineffective in reducing the infarct volume in the IFNγ-Tg mice (p > 0.50).

DISCUSSION

The present study clearly demonstrated an increased degree of cerebral damage following permanent MCA occlusion in IFNγ-Tg mice compared to SJL mice. Since the functional vascular anatomy and the rostrocaudal distribution of the infarcts were similar in the IFNγ-Tg and SJL mice, the increased infarct size in the IFNγ-Tg mice does not appear to be related to differences in cerebral circulation and blood-brain barrier characteristics, but to effects of transgenic IFNγ exerted at the cellular level within the neural parenchyma. The finding that IFNγ, an immune mediator normally absent from CNS parenchyma but produced in the presence of activated T-cells and NK-cells (3, 4), enhances ischemia-induced neurotoxicity, may have implications for the understanding of the pathogenesis of inflammatory CNS-disease. Due to the pro-inflammatory effects of IFNγ, we anticipated that this effect would be due to secondary effects of IFNγ acting on micro- and astroglial cells and infiltrating leukocytes, rather than direct effects of IFNγ acting on injured or stressed peri-infarct neurons. This expectation was based on the finding that transgene-encoded IFNγ was previously shown to increase lesion-induced microglial reactivity and TNF expression and to increase autoimmune inflammation in the same line of mice (12, 15), and to induce inflammation, demyelination, and neuronal degeneration in other lines of mice (53, 54).
In contrast to what has been observed in axonal lesioning and EAE studies (12, 15), we found that IFNγ-Tg and SJL mice were similar in regard to i) numbers of TNF mRNA-expressing microglia-macrophages at day 1 after MCA occlusion, ii) levels of TNF mRNA and TNF at 1 hour, 1 and 5 days, and at 1 day, respectively, after MCA occlusion, and iii) microglial-macrophage as well as astroglial reactivity at 1 and 5 days after MCA occlusion. One possible explanation may be the acute nature and the very extensive damage of the brain and the blood-brain barrier, which are characteristic of the ischemic lesion. These distinguish ischemia from the injury that results from axonal lesioning (12) and from EAE (15). It may also be of relevance that IFNγ was expressed by oligodendrocytes and not by T- or NK-cells as in a physiological inflammatory reaction. Possibly, targeting IFNγ to astrocytes might have resulted in more pronounced effects, as has been suggested by the induction of inflammation and degeneration in mice with astrocyte-specific neuron-specific expression of transmembrane TNF (58, 59). However, the only IFNγ-Tgs that were and still are available are mice with MBP-promoter driven expression of IFNγ (15, 53, 54). The reason for choosing the A519 line of mice for this experiment was that these mice, unlike other lines of MBP-IFNγ-Tgs (53, 54), are phenotypically normal, with perfectly normal cytoarchitecture of their CNS, and devoid of spontaneous pathology (12, 15, the present study). This allowed us to evaluate the effects of IFNγ in ischemia, uncomplicated by effects of transgenic phenotype. Finally, it might be speculated that the increased neuronal sensitivity to ischemia in the MBP-IFNγ-Tg mice relates to a compromised trophic interaction between the oligodendrocytes/myelin and the neurons. It has been reported that oligodendrocytes engineered to overexpress MHC heavy chain underwent spontaneous degeneration in development (60), and it could be argued that IFNγ would induce analogous effects. However, taking together the presence of normal appearing MBP mRNA-expressing oligodendrocytes in unlesioned CNS, and the upregulation of oligodendroglial MBP mRNA expression in the peri-infarct at postsurgery day 5 in both IFNγ-Tg and SJL mice, it would seem unlikely that expression of the transgene per se was detrimental to oligodendrocytes in the A519 line of IFNγ-Tg mice.

Despite the comparable strength of the ischemia-induced microglial-macrophage TNF-response in the IFNγ-Tg and SJL mice, it was still surprising that neutralization of TNF, using doses of rsTNF-RI that were effective in other studies (49), had no effect on the size of the ischemic brain damage in the IFNγ-Tg mice. This was especially surprising since Dawson et al (49) used spontaneously hypertensive rats, which show the same cellular and spatio-temporal pattern of TNF synthesis following permanent occlusion of the MCA as that of SJL mice.

The demonstration of similar infarct volumes in IFNγ-KO and IFNγR-KO mice compared to their respective WT mice, and of almost complete absence of IFNγ mRNA in the CNS of SJL mice subjected to focal cerebral ischemia strongly suggests that T-cells, NK-cells, and IFNγ produced by these cells do not play a role in the expansion of the cerebral infarct under normal conditions. Since real-time RT-PCR is a very sensitive method the very small increases in IFNγ mRNA expression observed in a few mice at 2 h and 10 days after induction of ischemia may be explained by the occurrence of a single or a few IFNγ-producing cells within the ischemic hemisphere at this time. The extremely low level of IFNγ mRNA at 2 h and 10 days contrasts with the very high levels of IFNγ mRNA in the spinal cords of mice with EAE (55, the present study), where IFNγ-producing T-cells are known to be involved in the pathogenesis. An alternative source of the IFNγ mRNA at 2 h and 10 days might be neurons, as indicated in studies by Olsson et al (56) and Neumann et al (57) showing expression of IFNγ mRNA in cultivated motor and sensory neurons. However, the lack of effect of IFNγ knockout shows that these low levels of IFNγ were irrelevant to infarct development.

**Fig. 5.** Size of ischemic brain damage in IFNγ-Tg and SJL mice 1 and 5 days after MCA occlusion. A: Toluidine blue staining shows no cortical damage in unoperated SJL mouse, and B) minimal damage in SJL mouse 1 day after sham surgery. C–F: The size of the infarcts is larger in IFNγ-Tg than SJL mice both at day 1 (C, D) and at day 5 (E, F). Abbreviations: cc, corpus callosum; Ctx, cortex; IF, infarct; Str, striatum. Scale bar = 2 mm.
Fig. 6.  Microglial-macrophage reactions 1 and 5 days after permanent occlusion of the MCA in IFNγ-Tg and non-transgenic mice. A, B: Mac-1 staining for microglia-macrophages in an IFNγ-Tg (A) and a SJL (B) mouse 1 day after MCA occlusion showing activated microglia in peri-infarcted areas and infiltrating macrophages within the infarct and in the peri-infarct. Some of these macrophage-like cells were located in clusters related to the vasculature or meninges. C, D: Mac-1 staining for microglia-macrophages in an IFNγ-Tg (C) and a SJL (D) mouse 5 days after induction of ischemia. A mixture of activated, ramified microglia and brain macrophages was observed within the border zone of the infarct in both types of mice. Scale bar = 300 μm.

Fig. 7.  Oligodendrocyte MBP mRNA expression 1 day (A) and 5 days (B) following middle cerebral occlusion, shown for an IFNγ-Tg mouse. Oligodendrocytes markedly upregulate MBP gene expression (arrows) at 5 days in both IFNγ-Tg and SJL mice. Scale bar = 30 μm.

(52). Taken together, our data argue against TNF as a significant contributor to the enhanced cerebral damage in the IFNγ-Tg mice. Nevertheless, the possibility remains that subtle changes in soluble or membrane-bound TNF in the neural parenchyma within the supply area of the occluded MCA may have contributed to exacerbation of neuronal damage in the IFNγ-Tg mice. Likewise, transgene-encoded IFNγ might have modulated relative neuronal expression of the TNF-p55 and/or -p75 receptors and their downstream signaling cascades and neurotoxic functions.

Secondary effects of IFNγ might involve potentiation of neurotoxic or inhibition of neuroprotective mechanisms or mediators other than TNF. In addition to TNF, IL-1β and transforming growth factor beta 1 (TGFβ1) are also upregulated in microglia-macrophages within minutes to a few hours after induction of ischemia (22, 52, 61, 62). While IL-1β independently potentiates neurotoxic effects in cerebral ischemia (23, 63), TGFβ1 has mainly been ascribed a neuroprotective role (64). Other candidates, induced or amplified by IFNγ, are inducible nitric oxide synthase (15), cyclooxygenase-2 (21), and Fas/CD95 (65), as well as interferon-inducible protein-10, 2′-5′-oligoadenylate synthase, and IRF-1, all of which are upregulated and/or play a role in cerebral ischemia (19, 33–36, 66, 67). It is of interest that IRF-1
was first described as a transcription factor involved in interferon induced gene expression (68, 69), but is also induced by cytokines like TNF and IL-1β (70). IFNγ-mediated amplification of ischemia-induced signaling pathways may therefore have contributed to the increased neurotoxicity in the IFNγ-Tg mice.

There is evidence that ischemia sensitizes neurons to otherwise normal glutamatergic neurotransmission (32, 71). Despite the findings of similar astroglial responses in IFNγ-Tg mice and SJL mice 1 and 5 days after induction of ischemia, it is possible that effects of IFNγ might include effects mediated by astrocytes, which are responsible for metabolizing synaptically released glutamate, or microglia which can be induced to produce glutamate and glutamate analogues (72–75). In cerebral ischemia the sustained elevation of extracellular glutamate involves exocytosis of synaptic vesicles, reduced or reversed re-uptake of glutamate in presynaptic terminals, as
and rat allows speculation that IFNγ also may directly sensitize CNS neurons to ischemia, possibly by enhancing neuronal sensitivity to glutamate. Recent data demonstrating that IFNγ can modify synaptic activity by perturbing the clustering of glutamate receptors of the AMPA subtype (30) may also be of relevance. In combination, increased sensitivity of neurons to glutamate, reduced capacity of astrocytes to metabolize glutamate, as well as increased production of glutamate and other excitatory molecules by microglia (72, 73, 75) might contribute to the increased cerebral infarction in IFNγ-Tg mice. However, the molecular aspects of IFNγ on neurons need to be further investigated in order to draw conclusions about its exact mode of function.

The significance of the effects of IFNγ that we have observed may extend beyond ischemia to inflammatory CNS diseases, such as MS and EAE, which are characterized by infiltration with IFNγ-producing cells (1, 2, 78). These diseases are normally characterized by immune-mediated oligodendroglial degeneration and loss of myelin. However, it has become evident that not only oligodendrocytes and myelin but also axons and neurons undergo degeneration in MS (79). Treatment of rats with AMPA receptor antagonists reduced the degeneration of spinal cord oligodendrocytes and neurons in EAE, as well as neurological symptoms in the same animals (80). Our study therefore provides a novel and important perspective that IFNγ may sensitize CNS neurons in response to cerebral ischemia. In circumstances where IFNγ is expressed through T-cell inflammation in the CNS coincident with cerebral infarction, one would predict exacerbation of ischemic neurodegeneration.

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