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In vivo over-expression of interleukin-10 increases resistance to focal brain ischemia in mice

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Abstract

Early studies showed that *the administration* of the anti-inflammatory cytokine interleukin-10 (IL10) protects against permanent middle cerebral artery occlusion (MCAO) in mice. In this study, transgenic mice expressing murine IL10 (IL10T) directed by the major histocompatibility complex Ea promoter were produced and used to explore the effect of chronically increased IL10 levels on MCAO-related molecular mechanisms. IL10 was over-expressed in astrocytes, microglia, and endothelial brain cells in IL10T compared with wild type mice. Four days following MCAO, IL10T mice showed a 40% reduction in infarct size which was associated to significantly reduced levels of active caspase 3 compared with wild type mice. Under basal conditions, anti-inflammatory factors such as nerve growth factor and GSH were up-regulated and the pro-inflammatory cytokine IL1β was down-regulated in the brain of IL10T animals. In addition, these mice displayed increased basal GSH levels in microglial and endothelial cells as well as a marked increase in manganese superoxide dismutase in endothelial lining blood vessels. Following ischemia, IL10T mice showed a marked reduction in pro-inflammatory cytokines, including tumor necrosis factor-α, interferon-γ, and IL1β. Our data indicate that constitutive IL10 over-expression is associated with a striking resistance to cerebral ischemia that may be attributed to changes in the basal redox properties of glial/endothelial cells.

Keywords: cytokines, glia, glutathione, interleukin-10, middle cerebral artery occlusion, nerve growth factor.

From a molecular viewpoint, the outcome of post-ischemic inflammatory processes mainly depends on the imbalance between the activation of pro-inflammatory cytokine cascade and the induction of anti-inflammatory cytokines and antioxidant mechanisms (Wang *et al.* 2007). In particular, it has been shown that the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ) leads to tissue damage because of the accumulation of secondary mediators such as reactive oxygen species (ROS) (Sawada *et al.* 1999). This effect can be counterbalanced by the activation of anti-inflammatory cytokines such as interleukin-10 (IL10) and antioxidant molecules such as GSH and manganese superoxide dismutase (MnSOD) (de Bilbao *et al.* 2004; Arsenijevic *et al.* 2006).

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Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase; GCS, γ-glutamylcysteine synthase; GFAP, glial fibrillary acidic protein; Glut1 and 4, glucose transporter 1 and 4; IFN, interferon-γ; IL10, interleukin-10; IL10T, transgenic mice expressing murine IL10; MCAO, middle cerebral artery occlusion; MCH, melanin-concentrating hormone; MDA, malondialdehyde; MHC, major histocompatibility complex; MnSOD, manganese superoxide dismutase; NeuN, neuronal nuclei; NGF, nerve growth factor; NPY, neuropeptide Y; ROS, reactive oxygen species; SOD, superoxide dismutase; Tg mice, transgenic mice; TNF, tumour necrosis factor-α; WT, wild type.

Cerebral ischemia triggers complex cellular processes involving not only the activation of resident glial cells but also the recruitment of inflammatory blood cells (Wang *et al.* 2007). The triad of endothelial cells, astrocytes, and microglia is thought to play an essential role in post-ischemic immune cell trafficking, yet the exact interactions between these cell types remain poorly understood. Under basal conditions, endothelial cells are known to interact directly with astrocytes and modifications of microvascular structure induced by ischemia are likely to affect their relationships. Microglia and astrocytes may in turn interact to regulate the endothelium transmigration (Fiala *et al.* 1998). During the acute post-ischemic period, neutrophils are the first inflammatory cells to be recruited within the lesioned area followed by monocytes/macrophages and lymphocytes (Wang *et al.* 2007). Endothelial cells may participate in this event by favoring leukocyte transmigration (Fiala *et al.* 1998). Microglial cells may promote post-ischemic brain damage directly via their transformation into phagocytes (Stoll *et al.* 1998) or indirectly through the release of ROS and cytotoxic cytokines like TNFα and IL1β (Saud *et al.* 2005). Importantly, they may also exert their function by participating in the recruitment of immune cells. However, glial cells are also the source of cytokines with anti-inflammatory properties. One of these, IL10, is a potent inhibitor of inflammation. It has been proposed that IL10 mediates neuroprotection by blocking caspase 3 activity (Bachis *et al.* 2001) and reducing pro-inflammatory cytokine production (Frenkel *et al.* 2003). Previous contributions have shown that both exogenous administration (Spera *et al.* 1998; Frenkel *et al.* 2003) and gene transfer (Ooboshi *et al.* 2005) of IL10 mediate neuroprotection after an ischemic insult. However, most of these studies were undertaken on *in vitro* models of inflammation (lipopolysaccharide, cytokine-stimulated cells) and those investigating the effect of IL10 on *in vivo* models of brain ischemia were based on post-ischemic exogenous administration of this cytokine (Spera *et al.* 1998; Ooboshi *et al.* 2005). To investigate the impact of chronic IL10 up-regulation on the morphological, biochemical, and behavioral post-ischemic changes following permanent middle cerebral artery occlusion (MCAO), we produced transgenic (Tg) mice over-expressing murine IL10 (IL10T) under the control of the major histocompatibility complex (MHC) Ea promoter. The MHC class I and II molecules have also shown to be up-regulated in the periinfarct area (Stoll *et al.* 1998) and may be regulated by MCAO-induced cytokines such as TNFα or IFNγ (O'Keefe *et al.* 1999). In particular, this promoter is induced in both glial and endothelial brain cells (Stoll *et al.* 1998; Girvin *et al.* 2002; Wang *et al.* 2007) but also professional antigen-presenting cells. Our data indicate a marked resistance of IL10-over-expressing Tg mice after MCAO that was mainly because of changes in cytokine production and oxidative stress regulation.

Material and methods

All procedures were approved by the Veterinary Office of the Canton of Zurich Health Directorate and the Veterinary Office of Geneva.

Mice and diets

Adult male mice (3–4 months) were used in these experiments. The IL10T and wild type (WT) co-littermates were established on a C57BL/6J genetic background. Mice were kept at room temperature (25°C) with a 12 : 12 h light/dark cycle. They were housed in individual cages for food intake and body weight studies and had *ad libitum* access to standard laboratory diet and water. Food intake was calculated using the weight of food pellets from the previous day and subtracting the weight of the remaining pellets (and spillage) of the following day (measured at 9.00 AM). For all feeding experiments, a minimum of 24 animals (12 WT and 12 IL10T) were used in each MCAO-treated group and their respective controls. Food intake and body weight were recorded for 3 days prior to MCAO, and then were assessed daily for 1 week. Preliminary tests have shown that there was no significant difference in body weight (22–25 g) and food intake between IL10T and WT mice.

Generation of interleukin-10 transgenic mice

Murine IL10 cDNA from position +63 (14 nucleotides upstream of ATG start codon) to +665 (55 nucleotides downstream from TAA stop codon) was placed under the control of the class II MHC Ea promoter sequence in a pDOI-5 plasmid (Kouskoff *et al.* 1993). The construct was microinjected into eggs from (C57BL/6 × DBA/2)F2 females and Tg founders were backcrossed to C57BL/6 mice, yielding C57BL/6.IL10 Tg positive or negative males and females. WT and homozygote IL10T mice used in this study were generated from hemizygously transgene-positive male and female mice which were from backcross-generation N_7 and N_8. The presence of the IL10 transgene in the offspring was detected by PCR on tail DNA using the primers 5′-TCAAACAAAGGACCAGCTGGACAACA-TACTG-3′ and 5′-CTGTCTAGGTCCTGGAGTCCAGCAGACT-CAA-3′, yielding one specific fragment of 420 bp, as confirmed by using molecular weight markers from Roche (Basel, Switzerland). Basal over-expression of IL10 protein was assessed by ELISA IL10 (GE Healthcare Europe, Otelfingen, Zurich, Switzerland) in brain, liver, lung, pancreas, and plasma in WT and IL10T mice (see Determination of brain cytokine levels section for methods).

Immunohistochemistry

Frozen brain coronal sections (12 μm) from non-operated WT and IL10T mice were processed for immunostaining using the following antibodies: rat anti-mouse IL10 (1/1000; Amersham), sheep anti-human MnSOD (1/100; Calbiochem, San Diego, CA, USA). To label astrocytes, microglia, endothelial cells, and neuronal cells, mouse polyclonal anti-glial fibrillary acidic protein (GFAP; 1/1200 – Chemicon International Inc., Temecula, CA, USA), rabbit polyclonal anti-Iba1 (1 μg/mL; kindly provided by Prof. Y. Imai, National Center of Neurology and Psychiatry, Tokyo, Japan), rat anti-mouse Cd54 (Von Willebrand factor, 1/5000; ebiosource, Vienna, Austria), and the mouse anti-neuronal nuclei monoclonal antibody (NeuN) (1/1000; Chemicon International Inc.) have been used, respectively. For double staining of cell types expressing IL10, the GFAP, anti-Iba1, anti-Cd54, and NeuN antibodies were detected

by using Nova Red kit (Vector kit; Vector laboratories, Burlingham, CA, USA) and IL10 antibody was revealed by Ni–diaminobenzidine staining. For double staining of cell types expressing GSH or MnSOD, the GFAP, anti-Iba1, anti-Cd54, and NeuN antibodies were detected using species specific secondary antibodies labeled with rhodamin (Vector Laboratories). MnSOD was detected using anti-sheep IgG FITC-labeled antibodies (Vector Laboratories) and GSH was detected by phthaldialdehyde (10 mM) (de Bilbao *et al.* 2004). Negative controls included deletion of the primary or secondary antibody. Photomicrographs of double immunofluorescent staining were constructed using the MetaMorph Imaging System (Visitron Systems, West Chester, PA, USA).

Induction of permanent focal cerebral ischemia and volume of the infarct

We performed permanent MCAO in IL10T (n = 6) and WT (n = 6) mice. After 4 days, quantification of the infarct area was performed for each animal as previously described (de Bilbao *et al.* 2004). In addition, cerebral vasculature anatomy was compared between the two genotypes in non-operated and operated mice. These methods are elaborated in Supporting information – Materials and methods.

Physiological parameters for MCAO study

Physiological parameters including arterial blood pressure, plasma glucose, and hematocrit were measured daily in IL10T and WT mice (n = 5) before MCAO and on days 1 and 4 post-injury (de Bilbao *et al.* 2004). Body temperature was measured prior to MCAO, during and following MCAO. All these procedures are further detailed in Supporting information – Materials and methods.

Western blot procedure for glucose transporters Glut1, Glut4, caspase 3, and G6PDH enzyme activity

Western blots on protein extracts from hemispheres of non-operated WT and IL10T mice were used (n = 4 for each groups). Membranes were incubated overnight with primary antibody [goat anti-glucose transporter 1 (Glut1) (sc1605 – 1/200) or goat anti-Glut4 (sc1608 – 1/200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-tubulin (1/25 000; Sigma, Buchs, Saint Gallen, Switzerland)]. Secondary antibodies LI-COR anti-mouse for tubulin (1/1000) and LI-COR anti-goat (1/1000) were used to detect bands. The signals were visualized with the use of the Odyssey Infrared Imaging System (Li-Cor Biosciences, Bad Homburg, Germany). Protein levels were expressed as the ratio against tubulin (Viswambharan *et al.* 2007). For caspase 3, ischemic hemispheres from lesioned WT and IL10T mice were used (n = 6 for each group). Membranes were incubated overnight with primary antibody [rabbit anti-caspase 3 (sc1605 – 1/1000; Santa Cruz Biotechnology) and mouse anti-tubulin (1/25 000; Sigma)]. Secondary antibodies LI-COR anti-mouse for tubulin (1/1000) and Alexa Fluor anti-rabbit for caspase 3 (1/1000) were used to detect bands. For complete methods, see Supporting information – Materials and methods.

The enzyme glucose-6-phosphate dehydrogenase (G6PDH) was also studied as it plays a key role in glucose metabolism and redox regulation via the pentose phosphate pathway (Spolarics 1998). Hemispheres from non-operated WT and IL10T mice were used (n = 4 for each groups). They were homogenized with polytron homogenizer in 0.2 M Tris buffer, pH 7.0, kept on ice. After centrifugation at 8500 *g*, the supernatant was collected and kept at −20°C prior to assay for G6PDH activity. Fifteen microliter of extract containing 1 mg/mL of protein as determined by Bradford (Bio-RAD, Reinbach, Basel, Switzerland) was added to 100 µL buffer containing 0.2 M Tris, pH 7.0, and $NADP^+$ (10 mg/mL). To start the reaction, 50 µL of glucose-6-phosphate (0.3 g in 1 mL) was added. The activity was determined by measuring the change in optical density at 340 nm during 15 min.

Lipid peroxidation in brain homogenates

Malondialdehyde (MDA) was assayed, as an indicator of endogenous lipid peroxidation, on days 1 and 4 after MCAO in WT and IL10T mice (n = 5 for each group). Non-operated mice were used as controls (n = 5 for each group). Biotech LPO-586 colorimetric assay kit and its protocol (Oxis, Portland, OR, USA) was used to determine MDA as described previously (de Bilbao *et al.* 2004; for further details, see Supporting information – Materials and methods).

Brain superoxide dismutase activity 1 day after MCAO

Superoxide dismutase activities in the ischemic hemispheres of IL10T and WT mice subjected to ischemia (1 day post-MCAO) and in the equivalent hemisphere in control non-operated animals (n = 6 mice per group) were determined using a biochemical assay (Ewing and Janero 1995; see Supporting information – Materials and methods for more details).

Brain GSH levels

Total reduced GSH was measured in ischemic hemispheres of operated animals 1 and 4 days after lesion (n = 4) and the equivalent hemisphere for control non-operated animals (n = 4), using a previously described method (de Bilbao *et al.* 2004; for complete description, see Supporting information – Materials and methods).

Determination of brain cytokine levels

We measured TNFα, IFNγ, IL1β, IL10, and nerve growth factor (NGF) in the brain ischemic hemispheres of WT and IL10T mice (n = 4 for each group). Non-operated mice were used as controls (n = 4 for each group). Cytokines were measured using immunoassay kits [for the four former (Amersham) and for NGF (Catalys, Wallisellen, Switzerland)] as described previously (Arsenijevic *et al.* 2006). See Supporting information – Materials and methods for more details.

RT-PCR for γ-glutamylcysteine synthase

Brain hemispheres (1 and 4 days post-MCAO, n = 4) were dissected from IL10T and WT mice and total RNA was isolated as described previously (Arsenijevic *et al.* 2006). Non-operated mice were used as controls (n = 4). The RNA was treated with Dnase and then reverse transcribed (Promega, Catalys, Wallisellen, Switzerland). Subsequently, a semiquantitative PCR (Invitrogen, Basel, Switzerland) was performed and the product was separated by gel electrophoresis containing ethidium bromide. The bands were then quantified using the Scion Image program (Scion Corporation, Frederick, MD, USA). The primers used were as follows: GCS (gamma-glutymlcysteine synthase) (sense 5′-ATCCTCCAGTT-CCTGCACAT-3′ and antisense 5′-TGTGAATCCAGGGCAGCC-TA-3′) (Li *et al.* 1996) and glyceraldehyde 3-phosphate dehydro-

genase, as reference control (sense 5′-TGAAGGTCGGTGTCA-ACGGATTTGGC-3′ and antisense 5′-CATGTAGGCCATGAGGT-CCCACCAC-3′) (Arsenijevic *et al.* 2006). All values for the semiquantitative RT-PCR expression of a gene were obtained after normalizing for glyceraldehyde 3-phosphate dehydrogenase.

Enzyme immunoassay for neuropeptide Y, orexin-A, and melanin-concentrating hormone

Hypothalami were dissected out in WT and IL10T mice 1 day after MCAO ($n = 5$), non-operated mice were used as controls ($n = 5$). Orexin-A, melanin-concentrating hormone (MCH), and neuropeptide Y (NPY) were quantified by enzyme immunoassay (Phoenix-Peptide, Belmont, CA, USA) (Gallmann *et al.* 2006). A more complete description of this method is given in Supporting information – Materials and methods.

Data analyses

All data are presented as mean ± SD. Statistical analyses were performed using Kruskal–Wallis one-way non-parametrical ANOVA. A value of $p < 0.05$ was considered as significant.

Results

PCR genotyping of IL10T mice and their WT co-littermates.

For all mice, PCR was used to detect Tg gene from tail biopsy (Fig. 1a).

Basal levels of IL10 protein are increased in peripheral and central tissues of IL10T mice

Compared with WT mice, IL10T animals displayed significant increases of IL10 in brain (56%, $p < 0.001$), liver (64%, $p < 0.001$), lung (12%, $p < 0.05$), pancreas (18%, $p < 0.01$), and plasma (200%, $p < 0.001$) tissues (Fig. 1b).

Basal immunohistochemical detection for IL10, MnSOD, and GSH in various brain cell types of IL10T and WT mice

In WT mice, IL10 immunostaining was only observed in cells surrounding the brain and lining ventricles (Fig. 2a, b, and c). In contrast, in IL10T mice immunostaining was also observed in brain astrocytes, microglia, and endothelial cells in all brain regions (including the cerebral cortex) (Fig. 2a, b, and c). Immunostaining was not observed in neuronal cells (Fig. 2b) or oligodendrocytes in both genotypes. MnSOD and GSH were detected in neurons and astrocytes of both IL10T and WT mice (data not shown). In addition, IL10T mice showed a specific labeling for GSH in microglia and endothelial cells and MnSOD in endothelial cells of blood vessels (Fig. 2d).

Physiological parameters before and after MCAO

WT and IL10T mice showed no pre- or post-operative differences in CBF (cerebral blood flow). Similar reductions were observed in both groups of mice after MCAO. Arterial pressure, plasma glucose, and hematocrit levels 1 day prior

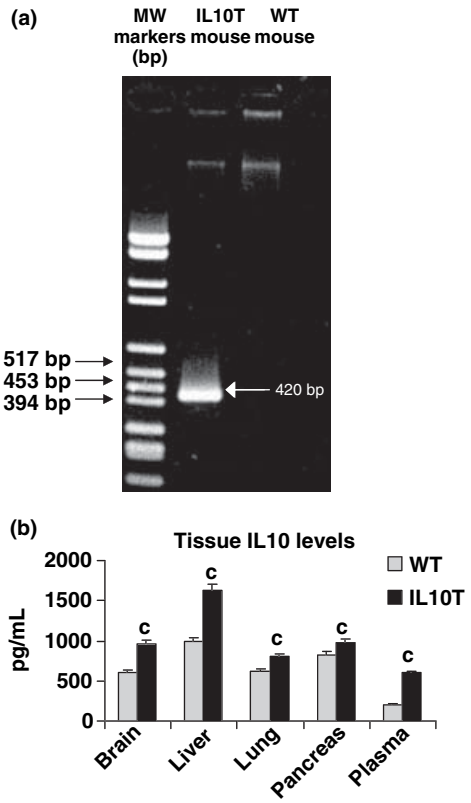

Fig. 1 (a) The presence of the IL10 transgene in the offspring was detected by PCR on tail DNA yielding one specific fragment of 420 bp after separation by gel electrophoresis (molecular weight; MW) (see text, for further details on method). (b) Basal IL10 protein levels in IL10T mice were significantly elevated in the four tissues analyzed compared with WT counterparts. Data are expressed in pg/mL. See text for details. For all groups, $n = 4$ animals; $^{c}p < 0.001$.

as well as on days 1 and 4 after MCAO were not significantly different between IL10T and WT mice (Table 1). After MCAO, both mice showed an absence of cerebral blood flow in the infarct area. Body temperature did not differ between mice 1 day prior as well as on days 1 and 4 after MCAO (Table 1). However, cytokines can modify body temperature following immunoactivation (Leon 2004) and despite the fact that we maintained body temperature by using a heating lamp post-surgery, it cannot be formally excluded that IL10 may affect body temperature once the lamp was removed. Therefore, body temperature changes may have a marginal impact on the outcome of ischemia.

Although no difference was observed in basal circulating glucose levels between IL10T and WT mice, we did observe that basal protein levels of the Glut1 and Glut4 were increased by 26% ($p < 0.001$) and 24% ($p < 0.01$), respectively, in the IL10T compared with WT brains (Table 1). Furthermore, we observed that basal G6PDH activity was increased by 2.8 times in the brain of IL10T mice compared with WT littermates ($p < 0.01$) (Table 1).

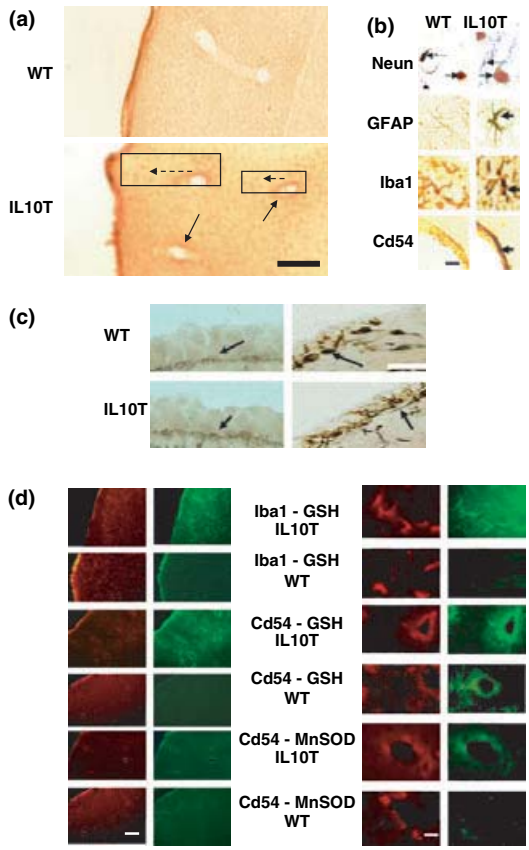

Fig. 2 (a) IL10 immunostaining in coronal sections of WT and IL10T mice. Distribution of IL10 stained cells was only observed in cells lining the brain in WT mice, whereas IL10T mice showed vast staining in endothelial cells (arrows) and non-vascular cells (dashed arrows) (see below); a selected area (thin lined rectangle) is shown magnified 1.5× in a thick line rectangle (scale bar, 30 μm) (b) NeuN positive neurons, GFAP positive astrocytes, Iba1 positive microglia, and Von Willenbrand factor/Cd54 positive endothelial cells double immunostaining for IL10 in WT and IL10T brains. Stained cells are found in the cortex and throughout the brain. Cell types were stained with Nova Red (orange-red) and IL10 with Ni–diaminobenzidine (black). Astrocytes, microglia, and endothelial cells were labeled for IL10 in IL10T brains only (thick arrow). In contrast, neurons were not positive for IL10 in both genotypes (thin arrow) (scale bar, 7 μm). (c) IL10 is detected in the epithelial cells that line ventricles (left) and the cells lining the brain (right) in both WT mice and IL10T mice (arrows) (scale bar, 7 μm). (d) Iba positive microglial cells (red) were double labeled for GSH (green) in IL10T mice. Von Willenbrand/Cd54 positive endothelial cells (red) were double labeled for GSH (green) or MnSOD (green) in IL10T brain blood vessels. Double labeling was not observed in WT mice. Left (scale bar, 30 μm) and right (scale bar, 7 μm for Iba1-GSH, 20 μm for Cd54-GSH and Cd54-MnSOD) panels correspond to low and high magnification, respectively.

Ischemic brain injury is reduced in IL10T mice

Brain weight and nuclei cytoarchitecture (cresyl violet staining) were comparable in IL10T and WT mice. Moreover, no difference was observed in vascular system (organization of the circle of Willis, position and diameters of the main cerebral arteries) between these mice. Four days after MCAO, infarct volume was decreased by 40% in IL10T mice (4.5 ± 0.5 mm^3) when compared with WT mice (7.5 ± 0.5 mm^3) ($p < 0.001$) (Fig. 3a and b), suggesting that IL10 over-expression may protect neocortical areas from ischemic damage. It is unlikely that this protective effect is because of enhanced neo-angiogenesis as the degree of revascularization assessed by endothelial cell distribution on 1 and 4 days post-MCAO was comparable in both mice strains (Fig. 3d).

Active caspase 3 protein levels in IL10T and WT mice

Ischemia resulted in the induction of active caspase 3 in both genotypes. However, active caspase 3 levels were reduced by 18% in IL10T mice compared with WT mice ($p < 0.001$; Fig. 3c). No significant differences in tubulin were detected between WT and IL10T mice that were lesioned or not.

Brain cytokine levels 1 day after MCAO

Basal brain levels of IL10 and NGF were higher in IL10T when compared with WT mice ($p < 0.001$ and $p < 0.01$, respectively) (Fig. 4a and b). One day post-MCAO, IL10 and NGF were induced in both genotypes ($p < 0.001$). However, post-MCAO levels of both cytokines were still higher in the IL10T compared with WT mice ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 4a and b). Pro-inflammatory cytokines (IFNγ, TNFα, and IL1β) were induced 1 day after ischemia in the brain of WT and, to a lesser extent, IL10T mice ($p < 0.001$) (Fig. 4c, d, and e). Importantly, basal levels of IL1β were reduced in IL10T compared with WT mice ($p < 0.001$) (Fig. 4e).

Brain oxidant/antioxidant status in mice after MCAO

One and four days after MCAO, brain MDA levels were significantly increased in WT and IL10T mice ($p < 0.001$); however, this increase was significantly more pronounced in WT mice ($p < 0.01$) (Fig. 5a). To explore possible differences in oxidative stress regulation between the two strains, we studied pre- and post-ischemic brain SOD activity and GSH antioxidant levels. Following MCAO, there was increased SOD activity in both WT and IL10T mice ($p < 0.001$) without significant differences between the two genotypes (Fig. 5b). Basal GSH levels were higher in IL10T compared with WT mice ($p < 0.001$). Following MCAO, GSH levels were significantly reduced in both groups ($p < 0.001$) (Fig. 5c), yet they were still higher 1 day post-MCAO in IL10T mice ($p < 0.001$). We also determined whether the increased GSH level in IL10T mice was associated with an elevated mRNA level of γ-glutamylcysteine synthase (GCS), which is the rate-limiting enzyme in GSH synthesis. Basal brain levels of GCS mRNA were higher in IL10T mice compared with WT mice (26% increase, $p < 0.01$) (Fig. 5d). Following

Table 1 Physiological parameters prior and after MCAO in IL10T and WT mice

Group and treatment	One day prior to MCAO	D1 post-MCAO	D4 post-MCAO
Hematocrit (%)			
WT	43 ± 4	45 ± 3	46 ± 4
IL10T	45 ± 2	43 ± 4	47 ± 4
Plasma glucose (mM)			
WT	7.1 ± 0.5	7.2 ± 0.4	7.4 ± 0.4
IL10T	7.4 ± 0.6	7.3 ± 0.4	7.5 ± 0.3
Brain G6PDH activity (mU/mg)			
WT	12.1 ± 0.3		
IL10T	34.3 ± 0.4**		
Brain glucose transporters			
Glut1 WT	0.573 ± 0.012		
Glut1 IL10T	0.706 ± 0.014***		
Glut4 WT	0.170 ± 0.003		
Glut4 IL10T	0217 ± 0.009**		
Body temperature (°C)			
WT	37.6 ± 0.4	37.6 ± 0.4	37.6 ± 0.4
IL10	37.1 ± 0.3	37.1 ± 0.3	37.1 ± 0.3
Arterial pressure (mmHg)			
WT	98 ± 4	101 ± 4	105 ± 4
IL10T	102 ± 4	104 ± 4	102 ± 4

MCAO, middle cerebral artery occlusion; IL10T, transgenic mice expressing murine IL10; IL10, interleukin-10; WT, wild type; G6PDH, glucose-6-phosphate dehydrogenase; Glut1 and 4, glucose transporter 1 and 4. Hematocrit, plasma glucose, body temperature, and arterial pressure were measured in WT and IL10T mice prior to ischemia, and then on days 1 (D1) and 4 (D4) after MCAO. No significant differences were observed between WT and IL10T mice. Basal brain G6PDH activity was significantly increased in IL10T mice compared with WT (**$p < 0.01$). Basal brain glucose transporters protein levels normalized to tubulin were elevated in IL10T mice compared with WT mice, Glut1 (**$p < 0.01$) and Glut4 (***$p < 0.001$). Data are mean ± SEM of five animals per group.

MCAO, WT and IL10T mice displayed a comparable increase in GCS mRNAs (63% and 44% increases, respectively, $p < 0.001$). On day 4 after ischemia, GCS mRNAs were further increased compared with day 1 levels.

Food intake responses of IL10T mice following MCAO

Interleukin-10T and WT mice showed no significant difference in basal food intake (Fig. 6a). WT mice showed acute hypophagia on the day of MCAO intervention. The hypophagic response on this day was blunted in IL10T mice (3.4 ± 0.08 vs. 2.4 ± 0.09 g/mouse in WT mice) ($p < 0.001$). IL10T mice rapidly entered in a hyperphagic state compared with WT (Fig. 6a). During the period of days 0–2 after MCAO, IL10T mice ate significantly more than WT mice ($p < 0.001$) (Fig. 6a). From days 3–5 post-MCAO, both IL10T and WT mice displayed a similar pattern of hyperphagia. After this time point, food intake returned to basal levels. In all animals, the body weight followed a parallel time course to that of food intake (data not shown). These results suggest that IL10 attenuates negative energy balance in response to central inflammation.

Implication of orexin-A, NPY, and MCH protein levels in resistance to anorexia in IL10T mice after MCAO

To explain the reduced anorexic effect of MCAO in IL10T compared with WT mice, we studied the hypothalamic protein levels of three orexigenic peptides in the brain, orexin-A, NPY, and MCH both under basal conditions and following MCAO (Fig. 6b, c, and d). Basal protein levels of orexin-A and NPY did not differ between IL10T and WT mice (Fig. 6b and c); however, MCH basal levels were increased by 31% in the IL10T compared with WT mice ($p < 0.001$). After MCAO, we observed that the three neuropeptide levels were increased in both groups of mice compared with non-operated controls (Fig. 6b, c, and d). There was a significant increase in orexin-A and NPY levels in IL10T ischemic mice compared with WT ischemic animals (32%, $p < 0.001$ and 23%, $p < 0.01$ for orexin-A and NPY) (Fig. 6b and c).

Discussion

The main finding of the present study is that the *in vivo* endogenous over-expression of IL10 markedly protected

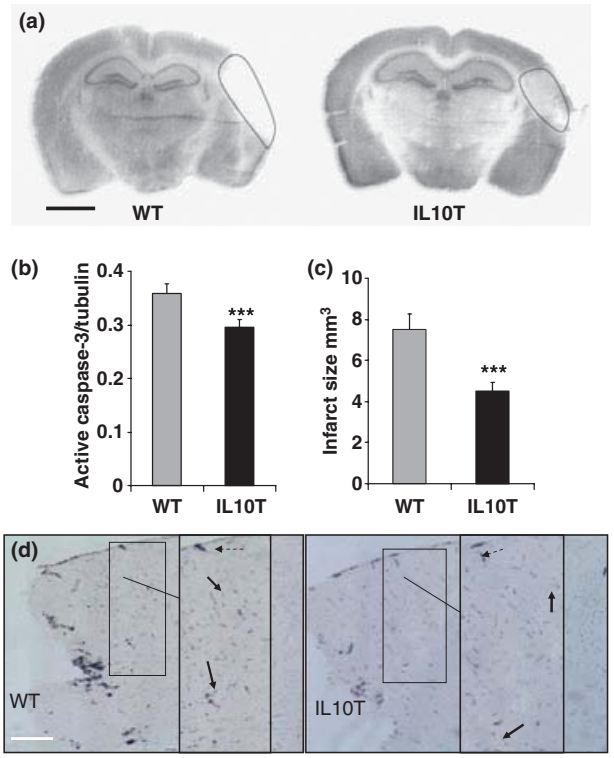

Fig. 3 Effect of IL10 over-expression on brain infarct size. (a) Representative coronal brain sections showing the ischemic infarct 4 days after MCAO in WT and IL10T mice. Brain sections were stained with cresyl violet. The surrounded areas denote the size of the ischemic area. The infarct size was found significantly decreased in IL10T mice ($p < 0.001$, $n_{IL10T} = 6$ and $n_{WT} = 6$; scale bar, 120 μm). Brain lesion size in mm^3 (b, ***$p < 0.001$) (each group included six mice). (c) On day 4 post-ischemia, induction of active caspase 3 protein levels is reduced in IL10T mice compared with WT mice (***$p < 0.01$, each group included six mice). (d) Endothelial cell immunostaining with Ni–diaminobenzidine on coronal brain sections 4 days after ischemia in WT and IL10T mice. Results indicate that no marked difference in revascularization occurred in the lesion area between the two genotypes (scale bar, 30 μm). We show by using a selected 1.5× magnification of an area (thin line rectangle) that the labeled cells are large blood vessels (dashed arrows) and capillaries (black arrow) (thick line rectangle).

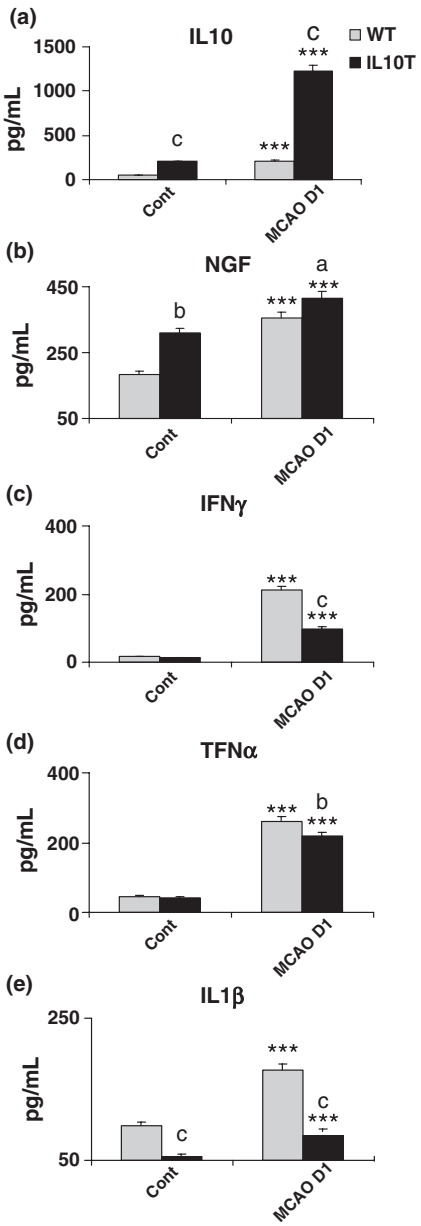

Fig. 4 Brain levels of IL10, NGF, IFNγ, and TNFα 1 day after MCAO in WT and IL10T mice. (a) IL10, (b) NGF, (c) IFNγ, and (d) TNFα were determined by immunoassay. Basal brain levels of IL10 (a) and NGF (b) were significantly higher in IL10T mice compared with WT animals. After their MCAO induction, both (a) IL10 and (b) NGF levels were significantly increased in IL10T compared with WT mice. (c) IFNγ levels were increased after MCAO in WT mice and to a significantly lesser extent in IL10T mice. (d) TNFα levels were increased to the same level after MCAO in WT and IL10T mice. (e) Basal IL1β levels were significantly higher in WT compared with IL10T mice. After their post-MCAO induction, IL1β levels remained significantly higher in WT mice. Each value is expressed as mean ± SEM ($n = 4$ mice per group) (***$p < 0.001$ indicate comparisons of mice groups with their respective controls; $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ indicate comparison between the genotypes with the same treatment). Data are expressed in pg/mL.

cortical tissue against cerebral ischemia. In line with a previous finding (Bachis *et al.* 2001), this neuroprotective effect was associated to the reduced activity of the pro-apoptotic protein caspase 3. A few previous contributions suggested that exogenous pre/post-ischemic administration of IL10 can provide neuroprotection following focal stroke (Spera *et al.* 1998; Frenkel *et al.* 2003; Ooboshi *et al.* 2005). However, these studies were based on the acute effect of IL10 administration and did not explore the cellular mechanisms involved in this neuroprotection. By using the Tg mice approach, we have been able to investigate the effect of basal IL10 Tg expression confined to brain glial cells (i.e.

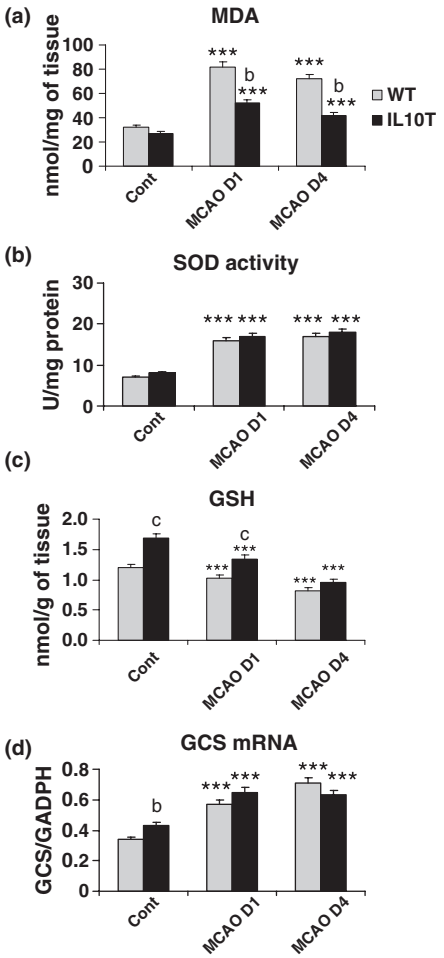

Fig. 5 Brain malondialdahyde (MDA) levels, superoxide dismutase (SOD) activity, and GSH levels on day 1 (D1) and day 4 (D4) after MCAO in WT and IL10T mice. (a) In both WT and IL10T mice, MCAO resulted in an increase in MDA; however, the increase in IL10T mice was less marked in IL10T compared with WT mice. (b) Basal SOD activity did not differ between WT and IL10T mice. MCAO increased SOD activity to similar levels in both genotypes. (c) Basal GSH was higher in IL10T than in WT mice. Although GSH levels were significantly decreased in both WT and IL10T mice 1 day after MCAO, they remained significantly higher in IL10T mice. (d) RT-PCR determination of brain γ-glutamylcysteine synthase (GCS) mRNA induction on day 1 after MCAO in WT and IL10T mice. Gene expression is reported as the ratio of the gene of interest to GADPH mRNA. Basal GCS was higher in IL10T than in WT mice. GCS mRNA was markedly induced following MCAO in both genotypes. Each value is expressed as mean ± SEM ($n = 5$ per group). ***Indicate statistical comparison of WT and IL10T mice with their respective non-treated controls (Cont), ^{b,c}indicate statistical comparison between WT and IL10T mice subjected to the same treatment (***$p < 0.001$, $^{b}p < 0.01$, and $^{c}p < 0.001$). Data are expressed in nmol/g of tissue and nmol/mg of tissue for MDA and GSH levels, respectively.

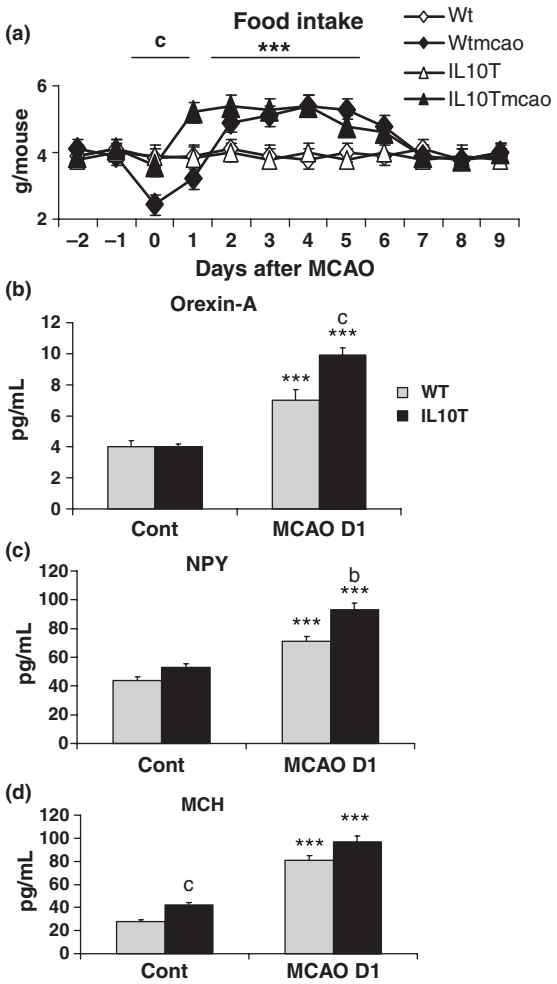

Fig. 6 Food intake responses (a) and hypothalamic protein levels of three orexigenic peptides (b, c, and d) in WT and IL10T mice following MCAO. (a) Over days 0–2, IL10T mice that had undergone ischemia (IL10Tmcao) ate significantly more than WT mice (Wtmcao). WT and IL10T mice showed similar hyperphagia on days 3–5 after MCAO when compared with non-operated mice (Wt and IL10T). Brain orexin-A (b), neuropeptide Y (NPY) (c), and melanin-concentrating hormone (MCH) (d) protein levels on day 1 after MCAO in wild type (WT) and IL10T mice. Basal MCH levels were significantly higher in IL10T compared with WT mice. MCAO resulted in significant increases in orexin-A (b) and NPY (c) in both genotypes. However, the levels of both hormones were significantly higher in IL10T compared with WT mice. (d) MCH levels were similarly increased after MCAO in WT and IL10T mice. Each value is expressed as mean ± SEM; ***$p < 0.001$ indicate comparisons of mice groups with their respective controls; $^{b}p < 0.01$, $^{c}p < 0.001$ indicate comparison WT and IL10T mice within a treatment group. Each group included 12 (a) and 5 (b, c, and d) mice. Data are expressed in g/mouse (a) and pg/mL for orexin-A, NPY, and MCH protein levels.

microglia, astrocytes, and endothelial cells) and analyze the chronic IL10 induced changes in inflammatory and redox regulation.

Our results provide possible molecular and cellular explanations for this IL10 mediated resistance to MCAO. Although, we cannot exclude that peripheral IL10 effects

may also participate in this phenomenon, the present study focuses on possible central mechanisms. Reduced brain lesion size in IL10 Tg mice was associated with a significant inhibition of the post-ischemic induction of central TNFα, IFNγ, and IL1β, three pro-inflammatory cytokines known to play a crucial role in the pathogenesis of ischemia (Arsenijevic *et al.* 2006). Consistent with these *in vivo* data, previous *in vitro* investigations also indicated that IL10 has the ability to inhibit the glial/lymphocyte production of these cytokines (Moore *et al.* 2001). Moreover, the inhibitory effects of IL10 on pro-inflammatory cytokine production have already been documented in various peripheral inflammatory models including endotoxemia, pancreatitis, or hepatitis (Moore *et al.* 2001). The fact that the protective effect of IL10 may be partly mediated by the relative inhibition of TNFα overproduction parallels previous *in vivo* findings showing that TNFα reduction can protect against ischemic insult (Yang *et al.* 1998). However and in contrast to these observations, neuronal injury after transient ischemia in mice genetically deficient in TNF receptors is known to be exacerbated (Bruce *et al.* 1996). Moreover, the attenuated brain infarction observed following post-ischemic IL10 gene transfer treatment in rats is associated with elevated levels of TNFα (Ooboshi *et al.* 2005). Methodological differences such as the model of cerebral ischemia used (global/focal and transient/permanent) or the post-ischemic interval may explain these discrepancies. In particular, a deleterious effect of TNFα on brain edema has been suggested (Bertorelli *et al.* 1998) and it has been proposed that the presence of TNFα may be neurotoxic or neuroprotective depending on the time points after brain injury (Scherbel *et al.* 1999). Alternatively, the beneficial/detrimental effect of TNFα may depend on threshold levels (Tracey 1995). Unlike TNFα, it is widely admitted that the cytokine IL1β exerts a potent pro-inflammatory effect. For instance, the intracerebroventricular administration of IL1β exacerbates brain damage caused by permanent focal ischemia in the rat (Loddick and Rothwell 1996). In the present experimental setting, the basal decrease in IL1β levels may participate to the increased post-MCAO resistance of IL10T mice. In contrast to pro-inflammatory cytokines, basal NGF levels were markedly elevated in brains of IL10T mice, indicating that IL10 neuroprotection after MCAO may be partly mediated by this anti-inflammatory cytokine (Guegan *et al.* 1999). To our knowledge, the present study is the first to suggest that IL10 can regulate NGF levels *in vivo*.

Another main finding of the present study concerns the effect of IL10 on antioxidant mechanisms associated with MCAO. The decrease in oxidative injury in IL10T mice is documented by the post-ischemic down-regulation of MDA contents. In addition, our data support the view that the anti-inflammatory effects of IL10 are mediated, at least in part, by the ability of IL10 to increase basal GSH levels. The basal increase in GSH could be explained by increased synthesis, decreased degradation or increase uptake of GSH from circulation. The increase in basal GCS mRNA expression in IL10T mice implies that an up-regulation in GSH synthesis may take place. In line with this possibility, a recent *in vitro* study evidenced that GCS gene expression could be up-regulated by IL10 in brain cells (Tu *et al.* 2007). Furthermore, IL10 treatment may prevent decreases in tissue GSH levels as has been shown in a model of renal ischemia-reperfusion injury (Köken *et al.* 2004). At the cellular level, the present work indicates that basal GSH is specifically detected in microglia and endothelial cells of IL10T but not WT brains. As microglia is a major source of ROS, the enhanced basal GSH levels may reduce ROS production following ischemia. Consistent with this protective effect during ischemia in IL10T animals, GSH has also been shown to modulate specific steps of the inflammatory process, including decreases in endothelial permeability and neutrophil–endothelial monolayer binding in response to ischemia–reperfusion (Kokura *et al.* 1999). Interestingly, brain SOD activity did not differ between WT and IL10T mice. However, our immunohistochemical study revealed that WT and IL10T genotypes displayed different MnSOD distribution. For instance, the mitochondrial MnSOD was only detected in IL10T brain endothelial cells. It has been previously suggested that this local MnSOD expression in endothelial cells could prevent the production of ROS. For instance, a protective role for MnSOD has been proposed in endothelial cells and blood–brain barrier following oxidative stress (Schroeter *et al.* 1999). The main objective of the present study was to analyze the molecular determinants of neuroprotection in IL10 Tg mice following brain ischemia so that we focused on antioxidant molecule expression in various types of brain cells as well as cytokines levels known to be produced by microglia, astrocytes, and macrophages. In this respect, morphological analyses including quantification of glial and infiltrating immune cells are warranted to complete our biochemical observations.

Among its plausible mechanisms of action, IL10 overexpression could improve the ischemic outcome by up-regulating glucose metabolism. Glucose is the primary energy source for the brain and, in stroke conditions, there is increased need for glucose because of rapid oxygen depletion. Therefore, brain regulators of intracellular glucose metabolism are important factors to consider in this context (Vannucci *et al.* 1997). We provide here evidence that the two main glucose transporters in the brain (i.e. Glut1 in the blood–brain barrier and Glut4 in neuronal cells) are up-regulated in IL10T mice suggesting that brain glucose uptake might be favored compared with WT mice. We also demonstrate that the G6PDH enzyme activity is up-regulated under basal condition in IL10T mice. As G6PDH is a key regulatory enzyme for the synthesis of NADPH which is involved in GSH production (Spolarics 1998; Wilmanski *et al.* 2005), one could postulate that the increased activity of

glucose transporters and G6PDH may contribute to the observed increase in brain GSH levels.

In the absence of major neurological symptoms in this ischemia model, we focused our analysis of post-ischemic behavioral changes on feeding behavior and showed that ischemic IL10T mice displayed transient hyperphagia that started earlier compared with WT. This finding is consistent with the attenuated post-ischemic induction of anorectic cytokines in the brain (TNFα and IFNγ) (Arsenijevic *et al.* 2006). In agreement with our previous observations in this field, the increased food intake in IL10T mice is associated with basal up-regulation of MCH levels and significant post-MCAO increases in NPY and orexin levels compared with WT mice (Arsenijevic *et al.* 2006). Additional studies are required to determine the role of these neuropeptides and neurotransmitters in altered food intake regulation following MCAO as they are known to influence both immune responses and neurodegeneration (Giuliani *et al.* 2006).

In conclusion, this study shows that IL10T mice were markedly resistant to permanent MCAO as assessed by reduced infarct size. This phenomenon was associated with significant changes in the pre- and post-ischemic regulation of pro- and anti-inflammatory cytokine levels as well as GSH metabolism changes. Importantly, we also documented that IL10 over-expression is associated with cell-specific antioxidant processes involving microglial GSH and endothelial MnSOD/GSH basal levels. Whether IL10 over-expression regulates the above-mentioned molecules directly or indirectly remains to be determined. Similarly, the exact functions of IL10 in the development of cellular/molecular inflammatory processes associated with cerebral ischemia require further clarification. However, these first data in a mouse model constitutively over-expressing IL10 imply that drugs targeting IL10 signaling pathways may prove beneficial in treating stroke by modulating microglia and endothelial cell GSH/MnSOD levels. Further studies including the conditioned expression paradigm are warranted to explore the relevance of IL10 over-expression as a possible molecular target for the treatment of stroke.

References

Arsenijevic D., de Bilbao F., Plamondon J., Paradis E., Vallet P., Richard D., Langhans W. and Giannakopoulos P. (2006) Increased infarct size and lack of hyperphagic response after focal cerebral ischemia in peroxisome proliferator-activated receptor β-deficient mice. *J. Cereb. Blood Flow Metab.* **26**, 433–445.

Bachis A., Colangelo A. M., Vicini S., Doe P. P., De Bernardi M. A., Brooker G. and Mocchetti I. (2001) Interleukin-10 prevents glutamate-mediated cerebellar granule cell death by blocking caspase-3-like activity. *J. Neurosci.* **21**, 3104–3112.

Bertorelli R., Adami M., Di Santo E. and Ghezzi P. (1998) MK 801 and dexamethasone reduce both tumor necrosis factor levels and infarct volume after focal cerebral ischemia in the rat brain. *Neurosci. Lett.* **246**, 41–44.

de Bilbao F., Arsenijevic D., Vallet P. *et al.* (2004) Resistance to cerebral ischemic injury in UCP2 knockout mice: evidence for a role of UCP2 as a regulator of mitochondrial glutathione levels. *J. Neurochem.* **89**, 1283–1292.

Bruce A. J., Boling W., Kindy M. S., Peschon J., Kraemer P. J., Carpenter M. K., Holtsberg F. W. and Mattson M. P. (1996) Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nat. Med.* **2**, 788–794.

Ewing J. F. and Janero D. R. (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Anal. Biochem.* **232**, 243–248.

Fiala M., Zhang L., Gan X. *et al.* (1998) Amyloid-beta induces chemokine secretion and monocyte migration across a human blood–brain barrier model. *Mol. Med.* **4**, 480–489.

Frenkel D., Huang Z., Maron R., Koldzic D. N., Hancock W. W., Moscowitz M. A. and Weiner H. L. (2003) Nasal vaccination with myelin oligodendrocyte glycoprotein reduces stroke size by inducing IL10 producing CD4+ T cells. *J. Immunol.* **171**, 6549–6555.

Gallmann E., Arsenijevic D., Williams G., Langhans W. and Spengler M. (2006) Effect of intraperitoneal CCK-8 on food intake and brain orexin-A after 48 h fasting in the rat. *Regul. Pept.* **133**, 139–146.

Girvin A. M., Gordon K. B., Welsh C. J., Clipstone N. A. and Miller S. D. (2002) Differential abilities of central nervous system resident endothelial cells and astrocytes to serve as inducible antigen-presenting cells. *Blood* **99**, 3692–3701.

Giuliani D., Mioni C., Altavilla D. *et al.* (2006) Both early and delayed treatment with melanocortin 4 receptor stimulating melanocortins produces neuroprotection in cerebral ischemia. *Endocrinology* **147**, 1126–1135.

Guegan C., Ceballos-Picot I., Chevalier E., Nicole A., Onteniente B. and Sola B. (1999) Reduction of ischemic damage in NGF-transgenic mice: correlation with enhancement of antioxidant enzyme activities. *Neurobiol. Dis.* **6**, 180–189.

Köken T., Serteser M., Kahraman A., Akbulut G. and Dilek O. N. (2004) Which is more effective in the prevention of renal ischemia-reperfusion-induced oxidative injury in the early period in mice: interleukin (IL)-10 or anti-IL-12? *Clin. Biochem.* **37**, 50–55.

Kokura S., Wolf R. E., Yoshikawa T., Granger D. N. and Aw T. Y. (1999) Molecular mechanisms of neutrophil–endothelial cell adhesion induced by redox imbalance. *Circ. Res.* **84**, 516–524.

Kouskoff V., Fehling H. J., Lemeur M., Benoist C. and Mathis D. (1993) Q vector driving the expression of foreign cDNAs in the MHC class II positive cells of transgenic mice. *J. Immunol. Methods* **166**, 287–291.

Leon L. R. (2004) Hypothermia in systemic inflammation: role of cytokines. *Front. Biosci.* **9**, 1877–1888.

Li S., Thompson S. A. and Woods J. S. (1996) Localization of gamma-glutamylcysteine synthase mRNA expression in mouse brain following methylmercury treatment using reverse transcription in situ PCR amplification. *Toxicol. Appl. Pharmacol.* **140**, 180–187.

Loddick S. A. and Rothwell N. J. (1996) Neuroprotective effects of human recombinant interleukin-1 receptor antagonist in focal

cerebral ischemia in the rat. *J. Cereb. Blood Flow Metab.* **16**, 932–940.

Moore K. W., de Waal Malefyt R., Coffman R. L. and O'Garra A. (2001) Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19**, 683–765.

O'Keefe G. M., Nguyen V. T. and Benveniste E. N. (1999) Class II transactivator and class II MHC gene expression in microglia: modulation by cytokines TGF-β, IL4, IL13 and IL10. *Eur. J. Immunol.* **29**, 1275–1285.

Ooboshi H., Ibayashi S., Shichita T. Y. *et al.* (2005) Postischemic gene transfer of interleukin-10 protects against both focal and global brain ischemia. *Circulation* **111**, 913–919.

Saud K., Herrera-Molina R. and von Bernhardi R. (2005) Pro- and anti-inflammatory cytokines regulate the ERK pathway: implications of the timing for activation of microglial cells. *Neurotox. Res.* **8**, 277–287.

Sawada M., Suzumura A., Hosoya H., Marunouchi T. and Nagatsu T. (1999) Interleukin-10 inhibits both production of cytokines and expression of cytokine receptors in microglia. *J. Neurochem.* **72**, 1466–1471.

Scherbel U., Raghupathi R., Nakamura M., Saatman K. E., Trojanowski J. Q., Neugebauer E., Marino M. W. and McIntosh T. K. (1999) Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury. *Proc. Natl Acad. Sci. USA* **96**, 8721–8726.

Schroeter M. L., Mertsch K., Giese H., Muller S., Sporbert A., Hickel B. and Blasig I. E. (1999) Astrocytes enhance radical defence in capillary endothelial cells constituting the blood–brain barrier. *FEBS Lett.* **449**, 241–244.

Spera A., Ellison J. A., Feuerstein G. Z. and Barone F. C. (1998) IL10 reduces brain injury following focal stroke. *Neurosci. Lett.* **251**, 189–192.

Spolarics Z. (1998) Endotoxemia, pentose cycle, and the oxidant/antioxidant balance in hepatic sinusoid. *J. Leukoc. Biol.* **63**, 534–541.

Stoll G., Jander S. and Schroeter M. (1998) Inflammation and glia responses in ischemic brain lesions. *Prog. Neurobiol.* **56**, 149–171.

Tracey K. J. (1995) TNF and Mae West or: death from too much of a good thing. *Lancet* **345**, 75–76.

Tu H., Rady P. L., Juelich T., Tyring S. K., Koldzic-Zinanovic N., Smith E. M. and Hughes T. K. (2007) Interleukin-10 regulated gene expression of hypothalamic – pituitary – adrenal axis origin. *Cell. Mol. Neurobiol.* **27**, 161–170.

Vannucci S. J., Maher F. and Simpson I. A. (1997) Glucose transporter proteins in brain: delivery of glucose to neurons and glia. *Glia* **21**, 2–21.

Viswambharan H., Carvas J. M., Antic V. *et al.* (2007) Mutation of the circadian clock gene Per2 alters vascular endothelial function. *Circulation* **115**, 2188–2195.

Wang Q., Tang X. N. and Yenari M. A. (2007) The inflammatory response in stroke. *J. Neuroimmunol.* **184**, 53–68.

Wilmanski J., Siddiqi M., Deitch E. A. and Spolarics Z. (2005) Augmented IL-10 production and redox dependent signalling pathways in glucose-6-phospahte dehydrogenase deficient mouse peritoneal macrophages. *J. Leukoc. Biol.* **78**, 85–94.

Yang G. Y., Gong C., Qin Z., Ye W., Mao Y. and Bertz A. L. (1998) Inhibition of TNFalpha attenuates infarct volume and ICAM-1 expression in ischemic mouse brain. *Neuroreport* **9**, 2131–2134.