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Intracerebral interleukin-10 injection modulates post-ischemic neuroinflammation: An experimental microarray study

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HIGHLIGHTS

- Intracerebral IL-10 administration is neuroprotective after ischemic stroke.
- Whole-genome microarray analysis revealed IL-10 impact on cerebral gene regulation.
- IL-10 mainly downregulates pro-inflammatory immune pathways after stroke.

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ABSTRACT

Stroke induces a profound neuroinflammatory reaction that leads to secondary cerebral tissue injury. Interleukin-10 (IL-10) is a key anti-inflammatory cytokine that is endogenously produced by immune cells and limits this inflammatory reaction. Previously, therapeutic administration of IL-10 has been shown to be neuroprotective in experimental stroke. However, the signaling pathways affected by this approach are largely unknown. The aim of this study was to verify the neuroprotective effects of IL-10 in an experimental mouse stroke model and to analyze the pathways modulated by this approach. Therefore, we performed a whole genome microarray analysis comparing the cerebral gene expression profile at two time points after cortical stroke in IL-10-treated and control C57Bl/6J mice. We administered IL-10 locally by intracerebroventricular injection. We were able to validate a reduction of infarct volume by IL-10 administration and characterized the kinetics of endogenous cerebral IL-10 expression after stroke. The microarray analysis revealed that IL-10 treatment effectively downregulated pro-inflammatory signaling cascades which were upregulated by the ischemic lesion in the acute phase after the stroke. This is the first study characterizing the global gene regulation profile of IL-10 immunotherapy for ischemic stroke. Our results emphasize the key role of IL-10 as a neuroprotective cytokine and suggest several novel downstream pathways for further investigation to better understand the mechanisms of post-stroke neuroinflammation.

1. Introduction

Acute brain injury in ischemic or hemorrhagic stroke induces a profound neuroinflammatory immune response [6]. Key features of this neuroinflammatory reaction are the invasion of peripheral immune cells across the damaged blood–brain barrier, the activation of resident immune cells (microglia) and the production of inflammatory humoral mediators such as cytokines and chemokines [13]. Various immunotherapeutic paradigms were tested in previous studies targeting the secondary inflammatory reaction after brain injury in order to improve stroke outcome. Besides blocking the cellular immune cell infiltration to the damaged brain [1,18,30], exploiting endogenous regulatory and immunosuppressive pathways have been intensively investigated [13,15,19].

A key anti-inflammatory cytokine produced by regulatory T cells, B cells as well as microglial/monocytic cells is interleukin-10 (IL-10). Several reports have demonstrated the neuroprotective

Abbreviations: Treg, regulatory T cells; IL-10, interleukin-10; BSA, bovine serum albumin; aCSF, artificial cerebrospinal fluid.

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properties of IL-10 administration in experimental stroke models [19,24,26,27]. Yet, under physiological conditions – and even more pronounced after brain injury – not only the cellular source and kinetics of IL-10 production are highly diverse but also its receptors and numerous signaling pathways [7]. The IL-10 receptor is constitutively expressed on all hematopoietic cells but can also be induced upon stimulation on several non-hematopoietic cell lines including astrocytes, microglia and even neurons [14,23].

Despite the well documented and consistent finding that therapeutical, intracerebral application of IL-10 reduces infarct lesions, the affected pathways and signaling cascades of this treatment approach are largely elusive. Due to the multifunctional action of IL-10 it might be possible that its effect is not even mainly directed to inflammatory pathways but rather to neuronal metabolism, plasticity or astrocytic activity.

The aim of this study was therefore to comprehensively analyze genes and pathways that are regulated by the intracerebral IL-10 administration using a whole-genome microarray approach.

2. Materials and methods

2.1. Animals

The study was conducted in accordance with national guidelines for the use of experimental animals, and the protocols were approved by the governmental committees (Regierungspraesidium Karlsruhe, Germany). We used age-matched, male mice (C57BL/6J, 10–12 weeks, 23–25 g body weight, Charles River Laboratories).

2.2. Experimental stroke model

Experimental brain ischemia was induced by permanent transcranial coagulation of the middle cerebral artery distal of the lenticulostriatal arteries and infarct volumetry by cresyl violet staining as previously described in detail [20]. Briefly, after a transtemporal trepanation the distal middle cerebral artery (MCA) was occluded by electrocoagulation. The overall mortality in the coagulation model was less than 5%. Sham treatment was performed like the MCA operation (MCAO) but without coagulation of the MCA. 20 μm sections at 400 μm distance sections were stained using a standard cresyl-violet staining protocol. The Swanson method [28] was applied for indirect infarct measurement. The total infarct volume was determined by integrating measured areas and distances between sections.

2.3. Intracerebroventricular IL-10 injection

Five minutes after induction of MCA occlusion, IL-10 was administered intracerebroventricularly. After a midline incision of the skin, we used a 10 μl Hamilton syringe for i.c.v. injection of 100 ng control vehicle [bovine serum albumin (BSA) in 2 μl artificial cerebrospinal fluid (aCSF) consisting of mM: 126 NaCl; 2.5 KCl; 1.2 NaH_2PO_4; 1.3 $MgCl_2$ and 2.4 $CaCl_2$ at pH 7.4], or 100 ng IL-10 [recombinant mouse protein (rmIL-10, LabGEN) in 2 μl aCSF] at the following coordinates: 0.9 mm lateral, 0.1 mm posterior, and 3.1 mm deep relative to the bregma.

2.4. Probe sampling for microarray analysis

Anesthetized mice were transcardially perfused at the indicated time points with sterile saline at 4 °C. The brains were quickly removed and immediately frozen in liquid nitrogen. Then the brains were again thawed in DEPC-treated water containing RNAse inhibitor (1 μ/μl, Promega), the contralateral hemisphere and cerebellum were removed and a 2 mm thick brain slice was cut at the level of the bregma. From this brain slice we removed the basal ganglia and the infarcted, cortical tissue so that only the cortical brain tissue surrounding the infarct core remained. Total RNA was isolated by the RNeasy kit by the manufacturer's protocol (Qiagen).

2.5. Expression profiling

Sentrix MouseWG-6v3 Whole Genome Expression BeadChips (Illumina, San Diego, CA) were used. To synthesize first and second strand cDNA and amplify biotinylated cRNA from the total RNA, an Illumina Totalprep RNA Amplification Kit was used as per manufacturer's instructions. Hybridization to the BeadChip was performed according to the manufacturer's instructions without modification. A maximum of 10 μl cRNA was mixed with a 20 μl GEX-HYB hybridization solution. Preheated 30 μl assay sample was dispensed onto the large sample port of each array and incubated for 18 h at 58 °C. Following hybridization, the samples were washed according to the protocol and scanned with a BeadArray Reader (Illumina, San Diego, CA). Raw data were exported to microarray-analysis software Chipster that allows quality assessment and visualization of expression data. After quantil normalization and filtering by standard deviation, significantly differentially expressed genes were calculated by two-group *t*-test. The resulting *p*-values were adjusted to multiple testing according to Benjamini–Hochberg [2]. $P < 0.001$ was considered statistically significant for comparison between treatment groups.

2.6. RT-PCR

RNA was isolated from each cerebral hemisphere using the RNeasy kit (Qiagen) taken from mice that were operated in a separate, additional experiment. Reverse transcription was performed with the High Capacity Complementary DNA Archive Kit and real-time polymerase chain reaction with SYBR-Green assays on a LightCycler 480 system (Roche). Primers for RT-PCR shown in Figs. 1 and 3 (IL-10, CXCL9, Igtp, PD-L1, Gapdh) were purchased as ready-to-use primer sets for each gene (Qiagen). All assays were run in duplicates. Each gene was quantified relative to the expression of the housekeeping gene encoding for Gapdh according to the relative standard curve method.

2.7. Statistical analysis

All values in bar graphs are expressed as mean ± standard deviation (SD). We analyzed infarct volumes by two-tailed Student's *t*-test after validating the normal distribution of these data sets (D'Agostino–Pearson test). Expression values of RT-PCR results were compared by the Mann–Whitney test (Fig. 3c) or the Kruskal–Wallis test with posthoc Dunn's multiple comparison test for data in Fig. 1c. All statistical analyses were performed using Graphpad Prism 6 software.

3. Results

3.1. IL-10 is neuroprotective after experimental brain ischemia

We analyzed the impact of intracerebroventricular (i.c.v.) IL-10 administration on the resulting infarct volume 7 days after MCAO induction (Fig. 1a and b). We observed a significant reduction of infarct volumes in the IL-10 i.c.v. treated group compared to mice receiving control (BSA in aCSF) treatment (Fig. 1b). Then, we investigated the kinetics of endogenous IL-10 production by analyzing IL-10 mRNA in ipsi- and contralateral hemispheres at several time points within the first week after MCAO. This revealed a significant increase of IL-10 expression only after 3 days compared to baseline

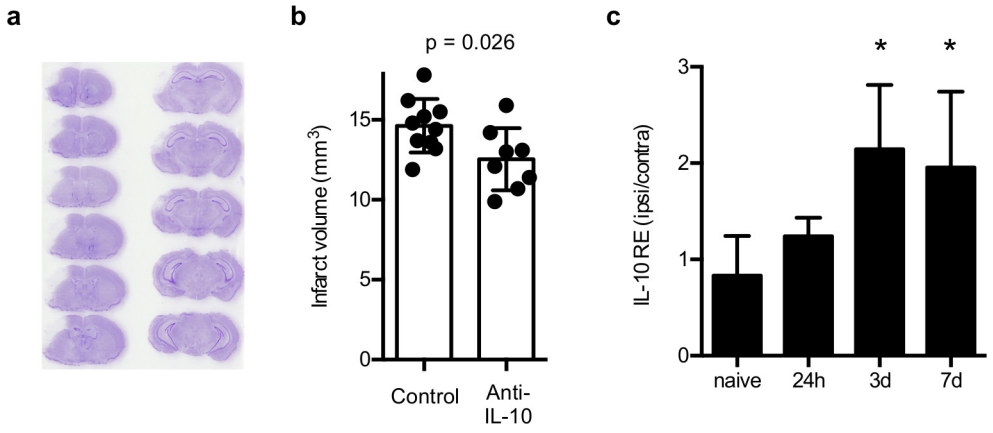

Fig. 1. Interleukin-10 treatment reduces infarct volume after experimental brain ischemia and is upregulated in the delayed phase after stroke. (a) Representative coronal brain sections stained by cresyl violet of a brain 7 days after infarct induction in the coagulation-MCAO model as used for infarct volumetry. (b) Results of infarct volumetry 7 days after MCAO. IL-10 i.c.v. treatment significantly reduces the resulting infarct volume ($n = 10$ per group, Student's t-test). Error bars depict SD. (c) IL-10 mRNA expression in ipsilateral hemispheres normalized to expression in the contralateral hemisphere (RE) of naïve mice and at indicated time points after MCAO ($n = 5$ per group). Bars depict mean + SD. RE: relative expression. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

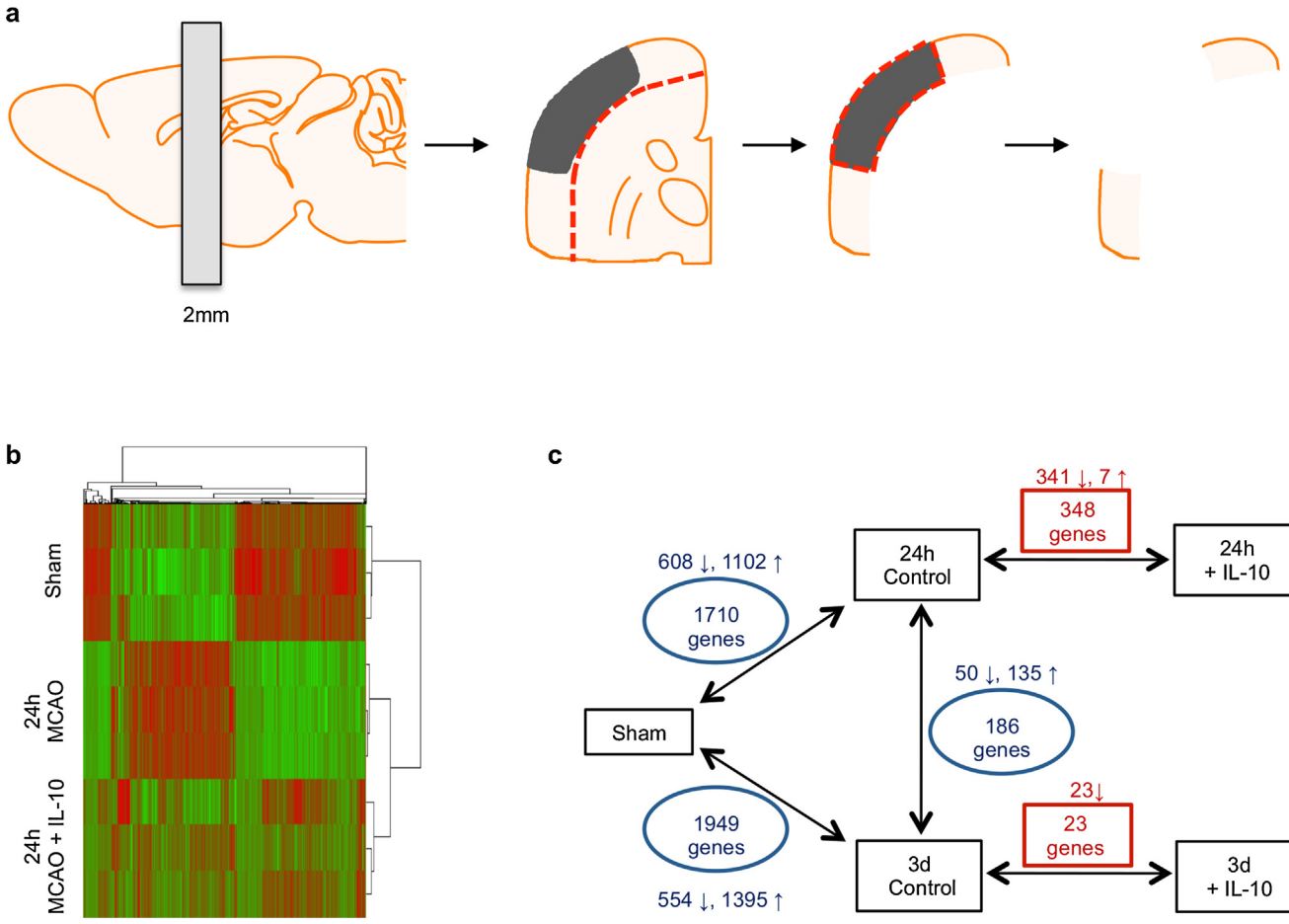

Fig. 2. Whole-genome microarray analysis of gene regulation after experimental stroke and IL-10 treatment. (a) Schematic overview depicting the process of isolating the peri-ischemic, cortical tissue surrounding the ischemic core from a 2 mm tissue section at the center of the longitudinal infarct expansion. (b) Hierarchical heat map (without technical replica) for whole genome analysis from brain homogenates of sham treated animals and at 24 h after brain ischemia in control- and IL-10-treated animals. (c) Schematic overview of group comparisons and number of regulated genes between treatment groups for microarray analysis. Blue boxes represent comparisons between Sham- and MCAO-operated animals, red boxes between Control- (MCAO + BSA i.c.v.) and IL-10-treated (MCAO + IL-10 i.c.v.) animals at the respective time points after stroke. ↑: upregulated genes, ↓: downregulated genes between groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

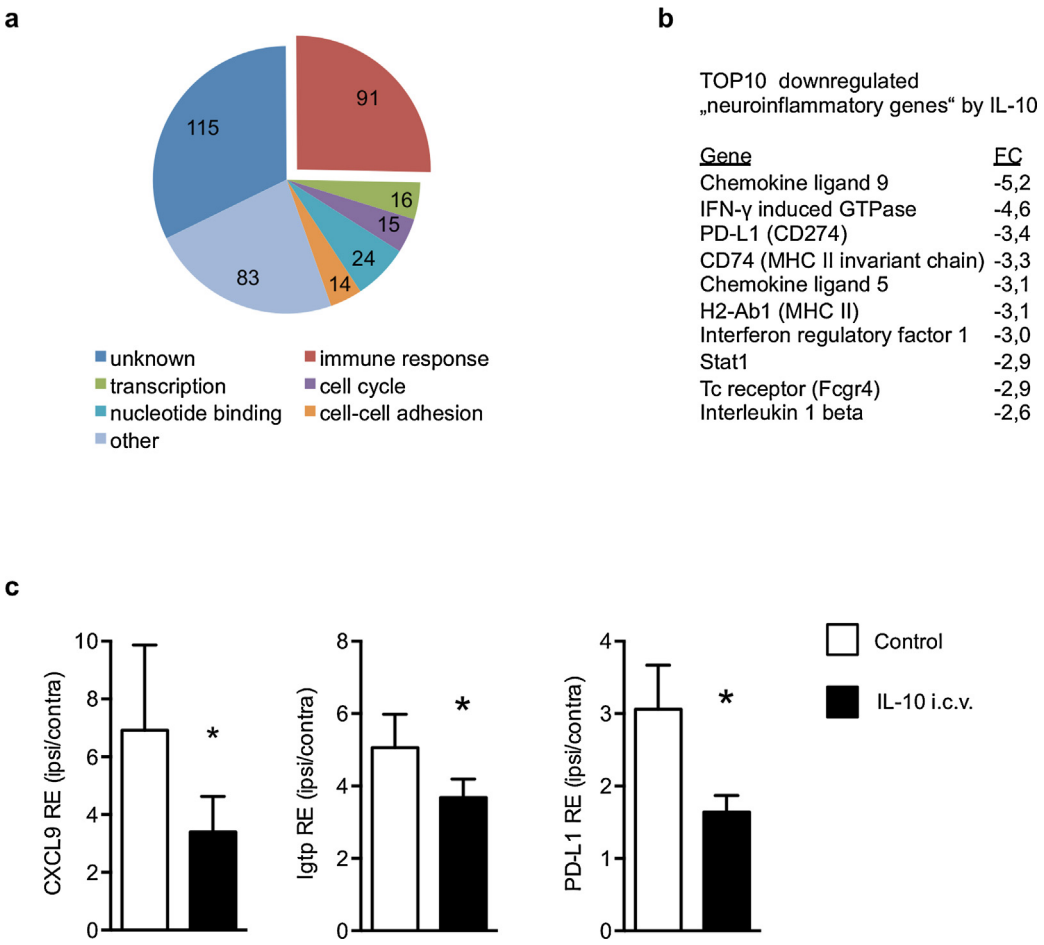

Fig. 3. IL-10 treatment modulates the neuroinflammatory response after stroke. (a) Analysis of the association of regulated genes between treatment groups 24 h after MCAO to the gene ontology subgroup coding and (b) excerpt of the most strongly downregulated genes involved in post-ischemic neuroinflammation by IL-10-treatment. (c) Differential expression of key inflammatory genes most highly regulated by IL-10 (IL-10 i.c.v.) compared to vehicle injection (BSA, control) was verified by RT-PCR of ipsilateral hemisphere samples at 24 h after brain ischemia.

levels in naïve mice. Upregulation was sustained for at least 7 days after MCAO (Fig. 1c).

3.2. Interleukin-10 has a major impact on gene regulation in the ischemic brain

We performed whole genome microarray analyses of brains from sham-operated controls and mice 24 h and 3 days after MCAO. Ischemic animals received intracerebroventricular injection of either control protein (BSA) or IL-10 (100 ng) in aCSF. Then we specifically isolated the cortical areas surrounding the infarct core at the center of the stroke expansion in order to minimize biological variability and bias by the infarcted tissue itself (Fig. 2a). Ischemia induced a profound induction of genes most of which were upregulated already at 24 h after MCAO (1710 genes) (Fig. 2b and c). Only additional 186 genes were upregulated at 3 days compared to the 24 h timepoint (Fig. 2b and c). I.c.v. IL-10 treatment resulted in a highly significant ($p < 0.001$) regulation of 347 genes compared to control animals at 24 h after MCAO. The main effect was downregulation of gene expression, and nearly all of the regulated genes (341 out of 347) represented genes upregulated by ischemia (Fig. 2c and Supplementary Table 1 for a complete list of regulated genes). Of the seven genes that were upregulated by IL10, only one (Hcrtr1) has a known function (i.e. encoding the hypocretin (orexin) receptor 1). We detected only minor further gene regulation at 3 days after MCAO between control- and IL-10-treated animals (Fig. 2c).

Supplementary Table 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.07.003.

3.3. IL-10 mainly downregulates inflammatory genes in the acute phase after stroke

Intriguingly, the majority of the genes downregulated by IL-10 at 24 h after MCAO were either directly associated with immune responses by the gene ontology coding or had crucial roles in immune-pathways coded in subgroups like “transcription” (e.g. STAT1 and Interferon regulatory factor), “cell cycle” (e.g. TNF-alpha induced protein), or “cell–cell adhesion” (e.g. VCAM-1, ICAM-1, L-selectin) (Fig. 3a and Table 1 for listing of gene ontology subgroup analysis). Many of these significantly regulated neuroinflammatory genes have previously been shown to be neurotoxic in brain ischemia [9,21] (Fig. 3b). In an individual experiment, we further validated the expression of some of the key neuroinflammatory genes regulated by IL-10 using RT-PCR of the indicated genes (Fig. 3c), confirming the results obtained by the microarray analysis.

4. Discussion

This study investigated for the first time the differential impact of intracerebral IL-10 overexpression on signaling pathways by investigating gene expression using whole-genome microarray analysis. The main finding of this report is that the neuroprotective cytokine IL-10 is a very potent regulator of gene expression in

Table 1
Gene ontology subgroup analyses of significantly (p<0.001) regulated genes by IL-10 administration.

Gene ontology subgroup coding	GO term	Nr. of regulated genes	Important known genes in neuroinflammation
0: unknown		**115**	
1: Immune response	**6955**	**91**	
1.1 Cytokine activity	5125	17	CCXL 2,5,7, 9, 10, 12; IL-12; IL-12R; granulin
1.2 Nucleotide binding	166/6955	12	Guanylate binding protein 3, Fc receptor, IFN regulatory factor 8
1.3 Complement activation	6958	6	C2, C3, C4b
1.4 Antigen presentation via MHC I	2474	11	MHC I, locus 1-23
1.5 Antigen presentation via MHC II	2504/19886/19884	11	MHC II, IFN-gamma inducible protein 30
1.6 Regulation of T cell cytotoxicity	1916	7	IL-15, MHCII k, TAP2
1.7 Hydrolase activity	16787/6955	4	Immunity-related GTPase family M member 1
1.8 Ig-mediated immune response	16064	3	Irf7
1.9 Interleukin-1 receptor binding	5149	3	IL-1RA, IL-1b
1,11 Others		17	CD86, CD40, MyD88, T-cell specific GTPase
2: Transcription	**6350**	**16**	
2.1 CD8-positive, T cell differentiation	43374	5	Irf1
2.2 Cytokine-mediated signaling	19221/6350	4	Stat1
2.3 Type 1 interferon biosynthesis	45351	1	Irf9
2.4 Cellular response to hypoxia	71456	2	
2.5 Other		4	Stat2, Trim30, Parp14,
3: Cell cycle	**7049**	**15**	
3.1 Apoptosis	6915	7	Nod1, DAXX
3.2 Cell proliferation	8285	5	Schlafen 1 (cell cycle arrest in T cells), CD274
3.3 Cell differentiation	30154	3	TNF-alpha-induced protein 2
4: Nucleotide binding	**166**	**24**	
5: Cell-cell adhesion	**7155**	**14**	VCAM, ICAM, L-Selektin, TGFbi, Itgb1, CD74
9: Other		**83**	
9.1 Endoplasmic reticulum	5783	3	CD74 (= MHC, invariant peptide)
9.2 Proteinase activity	8233/4175/6508	12	Secretory leukocyte peptidase inhibitor
9.3 DNA repair	6281	2	
9.4 Catalytic activity	3824	5	
9.5 Metal ion binding	46872	8	
9.7 Lysozyme activity	3786/5764	6	Lysozyme 1 and 2
9.8 Hydrolase activity	16787	3	
9.9 GTPase activity	3924	2	
9,11 Receptor activity	4872	15	Fc receptor, IL-10R, CD86
9,12 Norepinephrine metabolism	42415	3	Ly-6e
9,13 Other		24	
Total		**358**	

the acute phase after brain ischemia and that it is intriguingly specific and effective in the reduction of pro-inflammatory pathways after the brain lesion.

IL-10 is a key anti-inflammatory cytokine with potent inhibitory effects on innate as well as adaptive immune cells [10,23]. Besides this main immunological function, IL-10 can mediate multiple effects on diverse biological functions such as lipid metabolism or cytoskeleton remodeling [22]. The results of the microarray analysis demonstrated the potency of IL-10 on post-stroke gene regulation within the acute phase after the brain injury where IL-10 downregulated more than 300 genes which were previously upregulated by the brain ischemia. Strikingly, the vast majority of the (known) genes that were downregulated by IL-10 were directly or indirectly associated with an inflammatory pathway, confirming the key role of IL-10 in orchestrating the post-ischemic neuroinflammatory reaction [12,17,19,27]. In contrast, the only upregulated gene with known function (orexin receptor 1) is an important receptor within the hypocretin pathway that was previously shown to be neuroprotective in cerebral ischemia potentially by inhibiting neuroinflammation [29].

We and others have previously demonstrated that IL-10 is a central cytokine of the post-stroke immune reaction which is mainly produced by regulatory T cells invading the ischemic brain after stroke. Additionally, IL-10 is produced by resident microglial cells, invading monocytes and regulatory B cells in the ischemic brain [3,12,19,24]. These cells fulfill the role of inhibiting an otherwise overshooting immune reaction after the brain injury that might lead to an increased inflammatory tissue damage as it can be observed after depletion of these regulatory mechanisms [17,24]. However, endogenous IL-10 production in significant amounts occurs only delayed after brain injury and correlates with the invasion of peripheral regulatory cells to the brain approximately three days after the ischemic injury [11,19]. This might explain the consistently reported beneficial effect of IL-10 administration shortly after stroke induction over the physiological delayed expression of IL-10.

Instead of the clinically more feasible systemic administration of IL-10, the present experiments investigated the effect of local IL-10 administration because we focused on the immediate CNS effects of IL-10. Previous reports found conflicting results concerning the efficacy of systemically administered IL-10, in contrast to the well reproducible neuroprotection of i.c.v. IL-10 injections in experimental stroke [5,8,17,26,27]. Furthermore, systemic IL-10 administration might have diverse additional effects on peripheral immune cells and non-immune pathways that might have indirectly affected the readout of our microarray analysis.

Although the neuroinflammatory response after ischemic brain injury consists of an highly complex interplay of humoral and cellular immune pathways including resident immune cells, endothelial activation, leukocyte invasion, the complement system, cytokine production and many more [6,13], IL-10 was not only specific to downregulate immune-related genes but also demonstrated a predominance for the regulation of genes associated to microglial cells and the Interferon-gamma pathway. Several of the most highly regulated genes by IL-10 can be recognized as microglial activation markers (e.g. MHC II, chemokine expression, IL-1β). Indeed, previous reports have demonstrated that IL-10 overexpression is able to reduce the post-stroke activation and expansion of microglial cells [17,24]. Furthermore, several of the highly regulated genes are directly associated to IFN-γ production or its

downstream signaling pathway (e.g. Igtp, irf-1, Stat-1). This is also in accordance with the well-documented importance of IFN-γ as an neurotoxic pro-inflammatory cytokine mainly produced by brain-invading lymphocytes. IL-10 functionally inactivates lymphocytes and inhibits their invasion into the injured brain [3,8,17,19,24].

Another highly regulated gene by IL-10 administration was PD-L1, which was recently reported in several studies as a crucial mediator of inflammatory tissue damage after brain ischemia [4,16,25]. IL-10 significantly downregulated PD-L1 expression in our microarray analysis which was also verified by RT-PCR of whole brain hemispheres after experimental stroke. Interestingly, PD-L1 seems to play a role in the neuroprotective pathway of regulatory T cells after stroke [16] while at the same time it might by a mediator of inflammatory tissue damage inflicted by other immune cell populations [4,25].

The results of this study pave the way to further experiments in order to better understand the potent effects of IL-10 as a key neuroprotective and anti-inflammatory cytokine in acute ischemic brain injuries. However, this study was based on microarray analysis and selective confirmation by PCR only. Therefore, analysis on protein level will be required. Subsequent studies will also be necessary to delineate the exact molecular pathways under the control of IL-10 in specific cell populations, like microglial cells after brain injury. Also, alternative administration routes ensuring an effective and specific IL-10 administration to the injured brain while avoiding unspecific systemic effects are needed to lead the promising approach of IL-10 stroke therapy to a translational direction.

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