

Regulatory CD8⁺CD122⁺ T-cells predominate in CNS after treatment of experimental stroke in male mice with IL-10-secreting B-cells

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Abstract Clinical stroke induces inflammatory processes leading to cerebral and splenic injury and profound peripheral immunosuppression. IL-10 expression is elevated during major CNS diseases and limits inflammation in the brain. Recent evidence demonstrated that transfer of IL-10⁺ B-cells reduced infarct volume in male C57BL/6J (wild-type, WT) recipient mice when given 24 h prior to or 4 h after middle cerebral artery occlusion (MCAO). The purpose of this study was to determine if passively transferred IL-10⁺ B-cells can exert therapeutic and immunoregulatory effects when injected 24 h after MCAO induction in B-cell-sufficient male WT mice. The results demonstrated that IL-10⁺ B-cell treated mice had significantly reduced infarct volumes in the ipsilateral cortex and hemisphere and improved neurological deficits vs. Vehicle-treated

control mice after 60 min occlusion and 96 h of reperfusion. The MCAO-protected B-cell recipient mice had less splenic atrophy and reduced numbers of activated, inflammatory T-cells, decreased infiltration of T-cells and a less inflammatory milieu in the ischemic hemispheres compared with Vehicle-treated control mice. These immunoregulatory changes occurred in concert with the predominant appearance of IL-10-secreting CD8⁺CD122⁺ Treg cells in both the spleen and the MCAO-affected brain hemisphere. This study for the first time demonstrates a major neuroprotective role for IL-10⁺ B-cells in treating MCAO in male WT mice at a time point well beyond the ~4 h tPA treatment window, leading to the generation of a dominant IL-10⁺CD8⁺CD122⁺ Treg population associated with spleen preservation and reduced CNS inflammation.

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Abbreviations

CNS	Central nervous system
MCAO	Middle cerebral artery occlusion
WT	wild-type Green Fluorescent Protein (GFP)
TNF- α	Tumor necrosis factor α
INF- γ	Interferon γ
CD	Cluster of Differentiation
MHC II	Major Histocompatibility Complex II
RPMI	Roswell Park Memorial Institute
IL	Interleukin
PBS	Phosphate-buffered saline
DNase I	Deoxyribonuclease I
FACS	Fluorescence Activated Cell Sorter
PI	propidium iodide.

Introduction

Stroke still remains the fourth leading cause of death and one of the major causes of disability in the United States (Minino et al. 2011). Although treatment with recombinant tPA is the only approved therapy for acute stroke, less than 5 % of the stroke patients benefit from this intervention (Fang et al. 2010) due to a very limited therapeutic window and a probable risk of hemorrhagic transformation. It is therefore paramount to understand the endogenous mechanisms that are activated in response to acute stroke, so that novel therapies that take advantage of this understanding can be developed for the treatment of this devastating condition. Inflammation plays a critical role in the pathogenesis of ischemic stroke and other forms of ischemic brain injuries. Key features of this neuroinflammatory reaction are the invasion of peripheral immune cells across the damaged blood–brain barrier, the activation of resident innate immune cells (microglia) and the production of inflammatory humoral mediators such as cytokines and chemokines (Iadecola and Anrather 2011). Various immunotherapeutic strategies have been tested for dampening the secondary inflammatory reaction after brain injury in order to improve stroke outcome. Besides blocking the cellular immune infiltration to the ischemic, damaged brain (Becker et al. 2001; Liesz et al. 2011; Zhou et al. 2013), exploiting endogenous regulatory and immunosuppressive pathways has been intensively investigated (Iadecola and Anrather 2011; Li et al. 2013; Liesz et al. 2013). However, attempts to translate these approaches into clinical practice will benefit from a better understanding of the complexities of molecular mechanisms responsible for these immune responses.

Interleukin (IL)-10 is one of the key anti-inflammatory cytokines produced by the various regulatory immune subpopulations, including B- and T-cells and monocytes/microglia. The neuroprotective properties of IL-10 administration in experimental stroke models have already been demonstrated (Spera et al. 1998; Strle et al. 2001). However, little is known about the cellular source and kinetics of IL-10 production after induction of ischemic stroke. Recent experimental data demonstrate the complexities of modulating the immune response after stroke and suggest that immune cells can have both beneficial and detrimental effects. Given that the experimental model influences the time course and number of infiltrating immune cells as well as the degree of microglial activation (Zhou et al. 2013), it becomes paramount to know the timing and duration of interventions aimed at modulating inflammation. Hence, one of the rapidly evolving areas of stroke research involves defining the molecular and cellular bases for the augmented tissue injury and inflammation associated with transient cerebral ischemia.

Our laboratory has been actively investigating these post-ischemic inflammatory mechanisms and the interaction of

activated immune cells with ischemic brain tissue. Notably, we have demonstrated that B-cells are critical for governing infarct size and that MCAO-induced changes can be prevented in B-cell-deficient ($\mu\text{MT}^{-/-}$) mice after transfer of highly purified IL-10⁺ B-cells, but not IL-10-deficient B-cells. These studies clearly implicated IL-10-secreting B-cells as a major regulatory cell type in ischemic stroke (Offner and Hurn 2012; Ren et al. 2011). The critical role played by IL-10⁺ B-cells was confirmed with the demonstration that B-cell-deficient mice replenished with IL-10-rich B-cells 24 h prior to MCAO had a significant decrease in infarct volumes (Bodhankar et al. 2013; Chen et al. 2012). The transferred IL-10⁺ B-cells also mitigated pro-inflammatory responses both in the brain and the periphery. Moreover, our most recent study demonstrated the immunoregulatory role of IL-10-enriched B-cells in reducing the infarct size in *B-cell-sufficient* mice. IL-10-rich B-cells were efficient in limiting infarct volumes when given prophylactically (24 h before) or therapeutically (4 h after) MCAO-induction (Bodhankar et al. 2014). The primary purpose of the present study was to determine whether IL-10-rich B-cells can still elicit immunoregulation upon treating B-cell-sufficient mice at a more amenable, therapeutically relevant time-point as late as 24 h after the induction of MCAO. Our results very clearly demonstrate that treatment with IL-10⁺ B-cells significantly reduces infarct volume and partially reverses splenic atrophy when administered 24 h after the onset of stroke. The protection provided could be attributed largely to the dominant changes in the CD8⁺CD122⁺ regulatory T (Treg) populations in male WT mice treated with IL-10⁺ B-cells compared to Vehicle, leading to a decreased pro-inflammatory milieu both in the periphery and the MCAO-affected hemisphere of the brain. Our studies are the first to demonstrate a major immunoregulatory role for IL-10⁺ B-cells in rendering protection in male WT mice as late as 24 h post-stroke and in generating another highly potent regulatory subset (CD8⁺CD122⁺ Treg cells), which in turn appears to play a dominant role in attenuating pro-inflammatory reactions generated upon reperfusion-based cerebral injury.

Materials and methods

Animals

Male C57BL/6J (wild-type, WT) mice 8 to 12 weeks of age (Jackson Laboratory, Sacramento, CA, USA) and weighing 20 to 25 g were used as recipients for adoptive transfers and induction of transient focal cerebral ischemia. All WT mice were housed at the Oregon Health and Science University. Male IL10-GFP reporter mice (on a C57BL/6J background) were used at 8 to 10 weeks of age as donors for adoptive transfers. These mice were bred and housed in the Animal

Resource Facility at the Portland Veterans Affairs Medical Center in accordance with institutional guidelines. The IL10-GFP reporter mice have a floxed neomycin-IRES eGFP cassette (Madan et al. 2009) inserted between the endogenous stop site and the poly (A) site of the *Il10* gene to help track IL-10 producing cells in vivo. The mice, designated as Vert-X, are homozygous, develop normally and are viable and fertile without any obvious phenotype. All experimental protocols were approved by Oregon Health and Science University and Portland Veteran Affairs Medical Center Animal Care and Use Committees.

Cell sorting and adoptive transfer of B-cells

Male IL-10-GFP reporter mice served as donors of B-cells. Splenic CD19⁺ B-cells were purified using paramagnetic bead-conjugated antibodies (Abs) from the CD19 cell isolation kit and subsequently separated by AutoMACS (Miltenyi Biotec, Auburn, CA). The negative fraction of the cells thus separated were CD19⁺ B-cells with a purity of $\geq 92\%$. CD19⁺ B-cells were suspended in RPMI 1640 medium with 2 % Fetal Bovine Serum (FBS) and cultured in the presence of 1 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS, *E. coli* strain K12). After 48 h of culture, B-cells were harvested from culture plates, washed free of LPS and viable cells were counted using a hemocytometer with the trypan blue exclusion method. Five million purified IL-10-GFP⁺ B-cells from the donor mice were suspended in 100 μL RPMI 1640 medium and were transferred intravenously (i.v.) via tail vein injection into recipient WT mice (experimental group) 24 h after MCAO while the Vehicle control group received 100 μL RPMI 1640 medium.

Middle cerebral artery occlusion (MCAO) model

Transient focal cerebral ischemia was induced in male WT mice for 1 h by reversible right MCAO under isoflurane anesthesia followed by 96 h of reperfusion as previously described (Bodhankar et al. 2014). The individual performing all MCAO surgeries was blinded to treatment group. Head and body temperature were controlled at 36.5 ± 1.0 °C during surgery, MCAO and early reperfusion with warm water pads and a heating lamp. Occlusion and reperfusion were verified in each mouse by laser Doppler flowmetry (LDF) (Model DRT4, Moor Instruments, Inc., Wilmington, DE, USA). Occlusion was achieved by introducing a 6–0 nylon monofilament (ETHICON, Inc., Somerville, NJ, USA) with a silicone-coated (Xantopren comfort light, Heraeus, Germany) tip through an external carotid artery stump distal to the internal carotid artery to the origin of the middle cerebral artery. Adequacy of MCAO was confirmed by monitoring cortical blood flow at the onset of the occlusion with an LDF probe affixed to the skull. Animals were excluded if intra-ischemic LDF was greater than 25 % pre-ischemic baseline. After

occlusion was initiated, the incision was closed with 6–0 surgical sutures (ETHICON, Inc., Somerville, NJ, USA). Each mouse was then awakened during occlusion and placed in a separate cage with a warm water pad and heating lamp. At the end of the 1 h ischemic period, mice were briefly re-anesthetized with isoflurane, the LDF probe was repositioned over the same site on the skull, the occluding filament was withdrawn for reperfusion and the incision was closed with 6–0 surgical sutures (ETHICON, Inc., Somerville, NJ, USA). Each mouse was then awakened and recovered in a separate cage with a warm water pad.

Neurological deficit scores

Neurological deficit scores were determined at 1, 24, 48, 72, and 96 h of reperfusion to confirm ischemia and the presence of ischemic injury using a 0 to 4 point scale as follows: 0, no neurological dysfunction; 1, failure to extend left forelimb fully when lifted by tail; 2, circling to the contralateral side; 3, falling to the left; and 4, no spontaneous movement or in a comatose state (Chen et al. 2012). Any animal without a deficit at 1 h of reperfusion was excluded from the study.

Infarct volume analysis

Individual performing infarct volume analysis was blinded to treatment group. Mice were euthanized and brains collected at 96 h of reperfusion for 2,3,5-triphenyltetrazolium chloride histology and then digital image analysis of infarct volume as previously described (Chen et al. 2012). Images were analyzed using Sigma Scan Pro 5.0 Software (Systat, Inc., Point Richmond, CA). To control for edema, regional infarct volume (cortex, striatum, and hemisphere) was determined by subtraction of the ipsilateral non-infarcted regional volume from the contralateral regional volume. This value was then divided by the contralateral regional volume and multiplied by 100 to yield regional infarct volume as a percent of the contralateral region.

Isolation of leukocytes from spleen and brain

Spleens from individual control and B-cell recipient WT mice were removed and a single-cell suspension was prepared by passing the tissue through a 100 μm nylon mesh (BD Falcon, Bedford, MA). The cells were washed using RPMI 1640. Red cells were lysed using $\times x$ red cell lysis buffer (eBioscience, Inc., San Diego, CA) and incubated for 3 min. Cells were then washed twice with RPMI 1640, counted and resuspended in stimulation medium (RPMI containing 10 % FBS, 1 % sodium pyruvate, 1 % L-glutamine and 0.4 % β -Mercaptoethanol). The brain was divided into the ischemic (right) and nonischemic (left) hemispheres, dissociated mechanically through a 100 μm nylon mesh screen, resuspended

in 80 % Percoll (GE Healthcare, Pittsburgh, PA) overlaid with 40 % Percoll and subjected to density gradient centrifugation for 30 min at 1600 rpm, according to a method described previously (Campanella et al. 2002). Inflammatory cells were removed from the interphase for further analysis. Cells were then washed twice with RPMI 1640, counted and resuspended in stimulation medium. Cells from individual brain hemispheres were evaluated by flow cytometry.

Analysis of cell populations by fluorescence-activated cell sorting (FACS)

All antibodies were purchased from BD Biosciences (San Jose, CA) or eBioscience, Inc. (San Diego, CA) as published (Offner et al. 2006b, c). Four-color (FITC, PE, APC and PI/PerCP/PECy7) fluorescence flow cytometry analyses were performed to determine the phenotypes of splenocytes and brain leukocytes as previously published (Offner et al. 2006a). Single-cell suspensions were washed with staining medium (PBS containing 0.1 % NaN₃ and 0.5 % bovine serum albumin, Sigma, Illinois) and incubated with combinations of the following monoclonal antibodies: CD4 (GK1.5, BD Pharmingen), CD8a (53–6.7, BD Pharmingen), CD11b (M1/70, eBioscience), CD45 (Ly-5, BD Pharmingen), CD19 (1D3, BD Pharmingen), CD1d (1B1, BD Pharmingen), CD122 (TM-β1 BD Pharmingen), MHCII (2G9, BD Pharmingen), CD69 (H1.2 F3, BD Pharmingen) for 10 min at 4 °C. One mL of staining buffer was added to wash the cells. Propidium iodide (PI) was added to identify dead cells whenever only 3 channels on the FACSCalibur were used for detection of fluorescent antibody staining. The FoxP3 staining kit was used according to the manufacturer's protocol (eBioscience) as previously described (Zhang et al. 2010). FACS data acquisition was performed on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and data were analyzed using FCS express software (De Novo Software, Los Angeles, CA).

Intracellular staining

Intracellular staining was visualized using a published immunofluorescence protocol (Subramanian et al. 2011). Briefly, 2×10^6 cells/mL were resuspended in complete medium (RPMI 1640 containing 10 % fetal calf serum, 1 mM/L pyruvate, 200 μg/mL penicillin, 200 U/mL streptomycin, 4 mM/L L-glutamine, and 5×10^{-5} mol/L 2-β-mercaptoethanol) with PMA (50 ng/mL), ionomycin (500 ng/mL), and Brefeldin A (10 μg/mL; Sigma-Aldrich) for 4 h. For intracellular IL-10 detection, a modification was followed for the immunofluorescence staining protocol (Yanaba et al. 2008). Briefly, isolated leukocytes or purified cells were resuspended (2×10^6 cells/mL) in complete medium and cultured with LPS (10 μg/mL) in addition to PMA (50 ng/mL), ionomycin (500 ng/mL), and Brefeldin A (10 μg/mL)

(all reagents from Sigma-Aldrich) for 4 h. Fc receptors were blocked with anti-FcR mAb (2.3G2; BD Pharmingen) before cell-surface staining and fixed and permeabilized with the Fixation/Permeabilization buffer (eBioscience) according to the manufacturer's instructions. Permeabilized cells were washed with $1 \times$ Permeabilization Buffer (eBioscience) and were stained with tumor necrosis factor-α (MP6-XT22, BD Pharmingen), IL-17A (TC11-18H10, BD Pharmingen), Interferon-γ (XMG1.2, eBioscience) and/or APC-conjugated anti-IL-10 mAb (JES5-16E3, eBioscience). Isotype matched mAb served as negative controls to demonstrate specificity and to establish background TNF-α, IL-17, IFN-γ and IL-10-staining levels.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from brains of control and IL-10⁺ B-cell recipient WT mice using the RNeasy mini kit protocol (Qiagen, Valencia, CA, USA) and converted into cDNA using oligo-dT, random hexamers and Superscript RT II (Invitrogen, Grand Island, NY, USA). Quantitative real time PCR was performed using TaqMan Gene Expression Array FAST plates for mouse immune response on a StepOnePlus Real Time PCR System. cDNA from mice from each group (control vs. treated) was pooled for the gene array analysis. GAPDH housekeeping gene was used as an endogenous control. Fold change in the expression of genes was calculated using the formula $2^{-\Delta\Delta C_t}$. Reverse transcription-PCR was performed using TaqMan PCR master mix and pre-designed Taqman primers for IL-4, IL-10, CXCL10 (IP-10), CXCR3, ICAM-1, IL-33 and MMP-9 primers (Applied Biosystems, Foster City, CA, USA). mRNA was quantified in reactions conducted on the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and data represented as relative units compared with the GAPDH reference gene.

Statistical analysis

Infarct volume data are presented as mean±SEM. Differences in regional infarct volumes were determined with Student's *t*-test. Functional outcomes for neurological deficit scores were analyzed by Mann Whitney Rank Sum test. Statistical significance was $p < 0.05$. Statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA). Statistical significance for the differences between percentages of cellular subtypes by FACS analysis for splenocytes was analyzed with Student's *t*-test. Statistical significance for data obtained by FACS analysis for brain leukocytes was analyzed by one-way analysis of variance (ANOVA) followed by a post *hoc* Tukey's test. The criterion for statistical significance was $p \leq 0.05$. All values are reported

as mean±SEM. Significant differences are denoted as * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Results

Adoptive transfer of IL-10⁺ B-cells, 24 h after MCAO, reduces infarct volume in male WT mice

As shown in Fig. 1a, WT mice that received IL-10⁺ B-cells ($n=11$) 24 h after MCAO exhibited significantly reduced cortical ($p=0.025$) and total hemisphere ($p=0.043$) infarct volumes after 1 h MCAO followed by 96 h of reperfusion compared to no-cell transferred Vehicle (RPMI) controls ($n=11$). No differences were seen in striatal ($p=0.753$) infarct volumes between mice that received IL-10⁺ B-cells ($n=11$) 24 h after MCAO compared to Vehicle (RPMI)-treated controls ($n=11$). Representative cerebral sections from WT mice treated with RPMI or IL-10⁺ B-cells are shown in Fig. 1b.

Adoptive transfer of IL-10⁺ B-cells, 24 h after MCAO, improves neurological deficit scores 96 h after MCAO

Median neurological deficit scores evaluated for mice in both the infarct and immunology treatment groups 96 h after MCAO showed a significant improvement ($p=0.0235$) in mice receiving adoptively transferred IL-10⁺ B-cells ($n=30$) vs. Vehicle controls ($n=28$) treated 24 h after

MCAO (Table 1), although no significant differences were observed at earlier time points (Table 1).

Mortality and exclusions

Overall mortality from MCAO for infarct volume and immunology studies was 12 mice out of a total of 71 mice, with mortality ranging from 4 to 8 mice within the experimental groups. Overall number of mice excluded due to intra-ischemic LDF greater than 25 % pre-ischemic baseline was 1 mouse out of a total of 71 mice, with exclusions ranging from 0 to 1 mouse within the experimental groups.

Treatment with IL-10⁺ B-cells, 24 h after the induction of MCAO, ameliorates splenic atrophy

Our lab has repeatedly established the phenomenon of stroke-induced splenic atrophy (Offner et al. 2006a), a process that could be partially prevented by transfer of IL-10⁺ B-cells 24 h before MCAO (Bodhankar et al. 2013, 2014). The question was whether splenic atrophy could also be impacted by treatment of mice with IL-10⁺ B-cells given 24 h after MCAO. As expected and previously demonstrated (Bodhankar et al. 2013; Offner et al. 2006b), MCAO resulted in large reductions in spleen cell numbers in Vehicle-treated mice. The spleen counts in mice receiving Vehicle averaged ~20 million cells after 1 h of MCAO and 96 h of reperfusion (Fig. 2a), a ~70 % reduction compared to spleens from naïve mice. In contrast, viable spleen cell counts in the recipients of IL-10⁺ B-cells

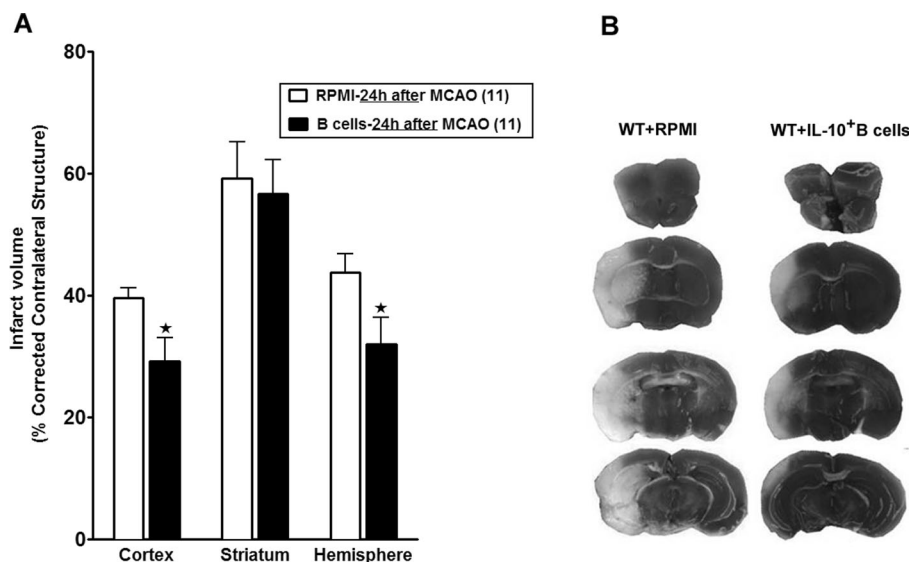


Fig. 1 Adoptive transfer of IL10⁺ B-cells, 24 h after MCAO, reduces infarct volume in male WT mice. **a** Intravenous transfer of 5 million IL10⁺ B-cells given 24 h after surgery to induce middle cerebral artery occlusion (MCAO) reduced infarct volume in C57BL/6 J (wild-type, WT) mice 96 h following 1 h of MCAO compared to intravenous transfer

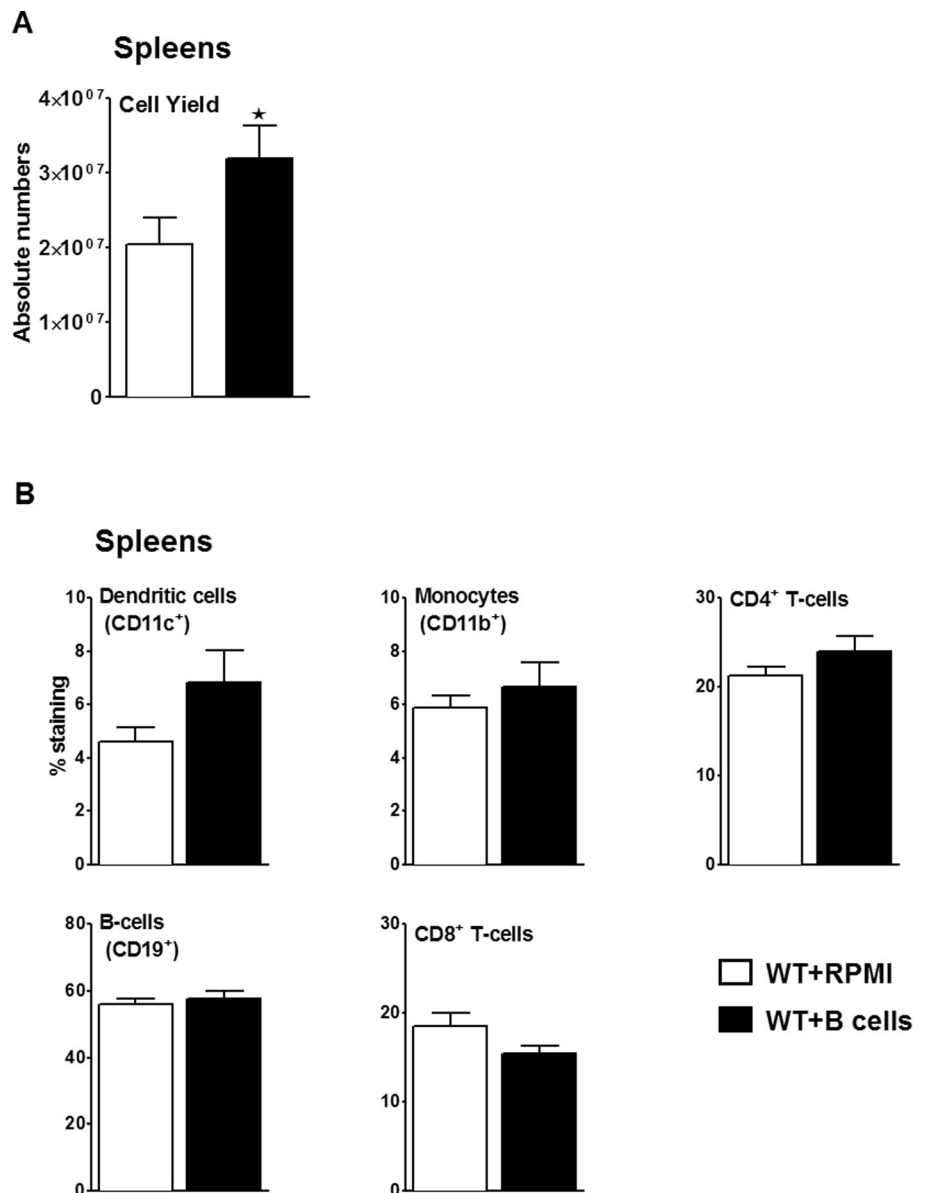
of RPMI Vehicle (no cells). * $p < 0.05$. **b** Representative 2,3,5 triphenyl-tetrazolium chloride stained cerebral sections 96 h following 1 h of MCAO. Localization of the ischemic lesion differed between WT mice receiving intravenous IL10⁺ B-cells (right column) vs. RPMI Vehicle (no cells) (left column) 24 h after MCAO. * $p < 0.05$

Table 1 Neurological deficit score distribution and median scores at various reperfusion time points following 1 h of middle cerebral artery occlusion (MCAO) in male C57BL/6J (wild-type, WT) treated intravenously with RPMI (Vehicle) or 5 million IL10⁺ B-cells 24 h after MCAO

Experimental Groups	Distribution Of Neurological Deficit Scores During Reperfusion																								
	1 h					24 h					48 h					72 h					96 h				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
WT+RPMI (n=28)	0	0	19	9	0	0	13	15	0	0	0	19	8	1	0	0	19	6	3	0	5	14	5	4	0
WT+IL10 ⁺ B Cells (n=30)	0	0	17	13	0	0	13	14	3	0	0	20	7	3	0	4	20	5	1	0	13	13	2	2	0
Experimental Groups	Median Neurological Deficit Scores During Reperfusion																								
	1 h					24 h					48 h					72 h					96 h				
	<i>(P=0.4268)</i>					<i>(P=0.5711)</i>					<i>(P=0.8513)</i>					<i>(P=0.0830)</i>					<i>(P=0.0235)</i>				
WT+RPMI (n=28)	2					2					1					1					1				
WT+IL10 ⁺ B Cells (n=30)	2					2					1					1					1#				

$p < 0.01$ compared to WT+RPMI

Fig. 2 Treatment with IL-10⁺ B-cells, 24 h after the induction of MCAO, ameliorates splenic atrophy 96 h after MCAO, mononuclear cells were isolated from spleens of RPMI or IL-10-GFP⁺ B-cell recipient WT mice and analyzed for: **a.** Total cell count via hemocytometer. Values represent mean numbers (\pm SEM) of indicated cell subsets from 28 to 30 mice in each group, from at least 8 separate experiments; **b.** Comparison of CD11c⁺ dendritic cells, CD11b⁺ monocytes, CD19⁺ B-cells, CD4⁺ and CD8⁺ T-cell subpopulations. Values represent mean numbers (\pm SEM) of indicated cell subsets, gated on live leukocytes (by PI exclusion), from 8 to 12 mice of each group, from at least 3 separate experiments. Statistical analysis was performed with Student's *t*-test to compare between RPMI and IL-10-GFP⁺ B-cell recipient mice. Significant differences between sample means are indicated ($*p \leq 0.05$)



were significantly higher (~30–40 million cells/mouse, $p=0.049$) 96 h following MCAO (Fig. 2a).

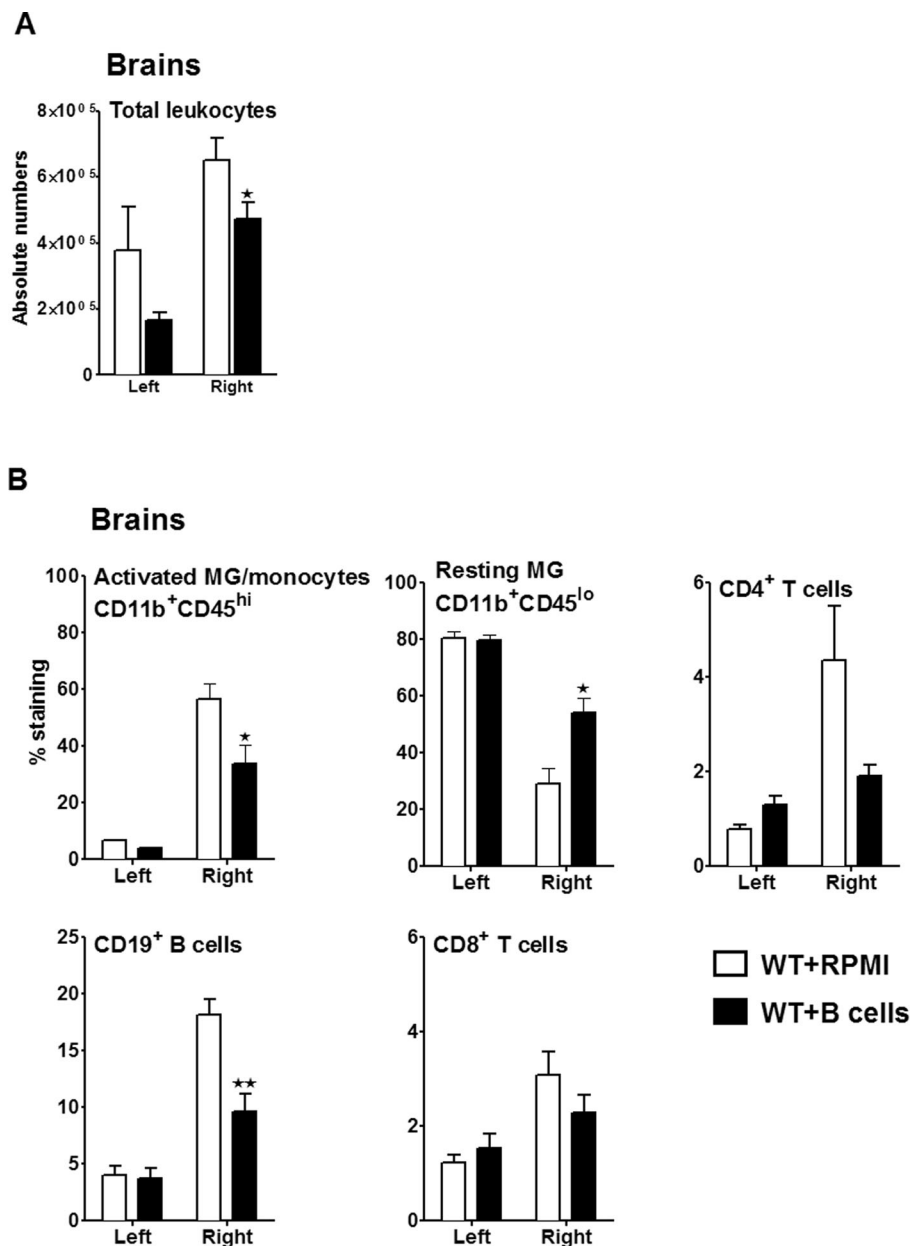
Given the partial restoration of splenic cell numbers in the IL-10⁺ B-cell-treated group, we further evaluated the frequencies of specific surviving splenic cell types. As shown in Fig. 2a, none of the spleen cell subpopulations (percentages or absolute cell numbers) were significantly different between the B-cell vs. Vehicle treated MCAO mice, although CD4⁺ T-cells, CD11b⁺ monocytes and CD11c⁺ dendritic cells trended higher, CD19⁺ B-cells did not change and CD8⁺ T-cells trended lower. Since the transferred IL-10-GFP⁺ B-cells comprised only ~1–2 % of the total splenocytes from WT recipient mice (data not shown), it was evident that the increase in spleen cell numbers was not contributed by the transferred

cells, but rather due to the influence of the transferred cells on recipient splenocyte distribution. Moreover, recipients of IL-10⁺ B-cells had more viable splenocytes 96 h after reperfusion compared to Vehicle control mice (~70 % vs. 45 % propidium iodide (PI)⁻ cells, data not shown). Thus, splenic atrophy was partially prevented upon transfer of IL-10⁺ B-cells 24 h after MCAO induction.

Transfer of IL-10-GFP⁺ B-cells to WT mice, 24 h post-MCAO, leads to reduced cerebral inflammatory cell infiltration but increased resting microglial (MG) cells

Our earlier studies demonstrated that with a reduction in the infarct volumes there is also a reduction in infiltrating

Fig. 3 Transfer of IL-10-GFP⁺ B-cells to WT mice, 24 h post-MCAO, leads to reduced cerebral inflammatory cell infiltration 96 h after MCAO. Mononuclear cells were isolated from brains of RPMI and IL-10-GFP⁺ B-cell recipient WT mice and were analyzed for: **a** Total cell count via hemocytometer. Values represent mean numbers (\pm SEM) of indicated cell subsets from 14 to 15 mice per group, from at least 5 separate experiments; and **b** Percent frequencies of CD11b⁺CD45^{high} activated microglia (MG)/monocytes, CD11b⁺CD45^{lo} resting MG, CD4⁺ T-cells, CD8⁺ T-cells and CD19⁺ B-cells obtained from the non-ischemic (left) and ischemic (right) hemispheres of WT recipient mice by Flow cytometry. Values represent mean numbers (\pm SEM) of indicated cell subsets from 6 mice from WT mice transferred with medium or IL-10-GFP⁺ B-cells after 24 h of MCAO, from at least 3 separate experiments. Statistical analysis was performed with ANOVA followed by Tukey’s multiple comparison post-hoc test. Significant differences between sample means are indicated (* $p\leq 0.05$ and ** $p\leq 0.01$) as compared to the ischemic right hemisphere of medium-treated WT recipient mice



Spleens

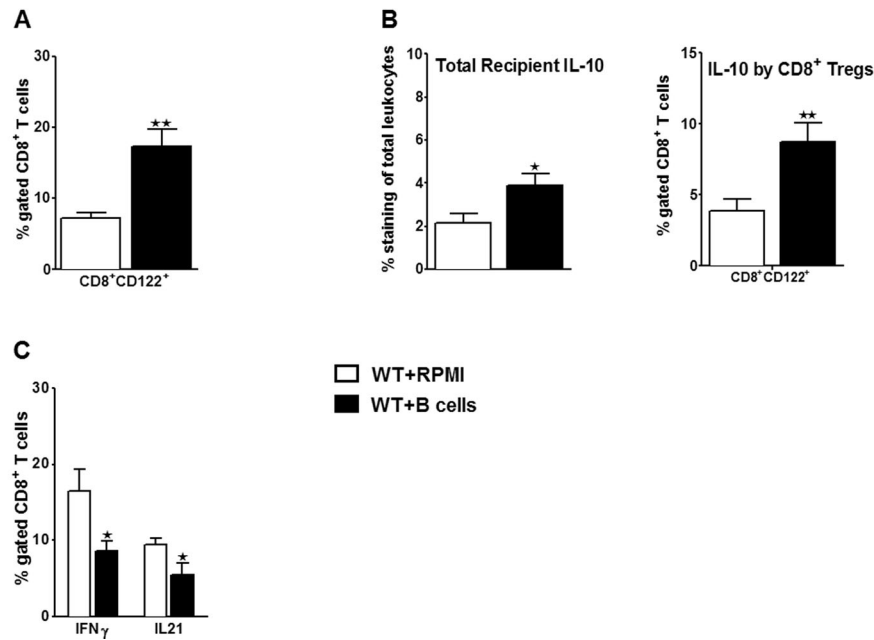


Fig. 4 Treatment with IL-10⁺ B-cells, 24 h post-MCAO, leads to a significant increase in the CD8⁺ T regulatory cell population and its anti-inflammatory status in the spleens of recipient mice Splenocytes were isolated from RPMI or IL-10-GFP⁺ B-cell recipient WT mice 96 h after MCAO and assessed for: **a.** Frequency of CD8⁺CD122⁺ T-cells. Data are representative of 5 independent experiments (mean±SEM); **b.** analyzed for IL-10-production by GFP⁻ cells using IL-10 APC mAb for detection by Flow cytometry and for the expression of IL-10 (GFP⁻) on

gated CD8⁺CD122⁺ T-cells; Data are representative of 5 independent experiments with spleens processed from 9 to 10 individual mice (mean±SEM); and **c** expression of IFN-γ and IL-21 production by the gated splenic CD8⁺ T cells. Data are representative of 2 independent experiments with spleens processed from 5 to 6 individual mice (mean±SEM). Significant differences between the groups were determined using Student's *t*-test and are indicated (**p*≤0.05 and ***p*≤0.01)

inflammatory cells into the ischemic brain hemispheres of the B cell-sufficient mice (Bodhankar et al. 2013, 2014). To determine whether the delayed transfer of IL-10-GFP⁺ B-cells to WT mice similarly reduces cellular infiltration into brain after MCAO, we enumerated the total number of live leukocytes obtained from the left (contralateral, MCAO-

unaffected) and right (ipsilateral; MCAO-affected) hemispheres. The Vehicle-treated MCAO mice demonstrated a massive infiltration of leukocytes in the ischemic hemisphere after 96 h of reperfusion, but this cellular infiltration was significantly reduced (*p*=0.042) in the MCAO-affected hemispheres of the IL-10-GFP⁺ B-cell-recipient WT mice

Brains

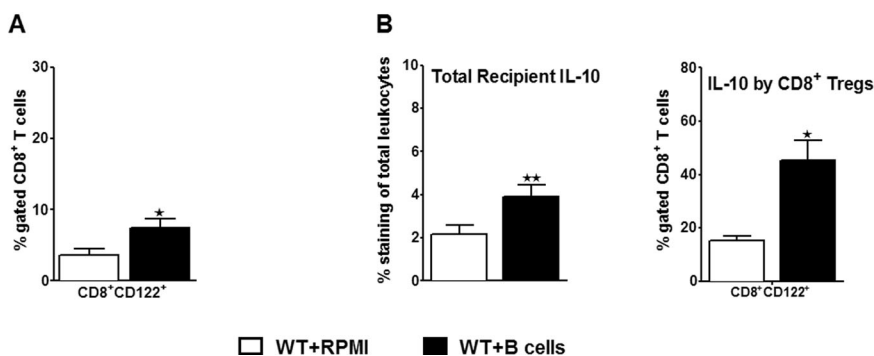


Fig. 5 Treatment with IL-10⁺ B-cells, 24 h post-MCAO, leads to a significant increase in the CD8⁺ T regulatory cell population and its anti-inflammatory status in the ischemic hemisphere of the brain of recipient mice Brain leukocytes from ischemic hemispheres of RPMI and IL-10-GFP⁺ B-cell transferred WT recipient mice were harvested 96 h after MCAO and assessed for: **a.** Frequency of CD8⁺CD122⁺ T-cells. Data are representative of 5 independent experiments (mean±

SEM); and **b.** analyzed for IL-10-production on GFP⁻ cells using IL-10 APC mAb for detection by Flow cytometry and for the expression of IL-10 (GFP⁻) on gated CD8⁺CD122⁺ T-cells; Data are representative of 5 independent experiments with spleens processed from 9 to 10 individual mice (mean±SEM). Significant differences between the groups were determined using Student's *t*-test and are indicated (**p*≤0.05 and ***p*≤0.01)

(Fig. 3a). Further flow cytometric evaluation of the leukocyte composition in the brains of the IL-10-GFP⁺ B-cell-treated vs. Vehicle-treated mice after 96 h of reperfusion revealed reduced frequencies of activated CD11b⁺CD45^{high} MG/macrophages ($p=0.047$) and infiltrating CD19⁺ B-cells ($p=0.003$) and an increased frequency of resting MG cells ($p=0.020$) in the ipsilateral hemispheres of the MCAO-induced B-cell-transferred group (Fig. 3b).

Treatment with IL-10⁺ B-cells, 24 h post-MCAO, results in a significant increase in CD8⁺ T regulatory cells and reduced inflammation in both the spleen and the ischemic brain hemisphere of recipient mice after 96 h reperfusion

Our previous studies demonstrated that prophylactic treatment with IL-10⁺ B-cells given 24 h prior to MCAO induced immunoregulation in the periphery but not locally in the affected brain hemisphere, potentially mediated in part by a recently identified CD8⁺ regulatory T-cell sub-population called CD8⁺CD122⁺ Tregs (Bodhankar et al. 2013, 2014). We thus addressed whether *therapeutic* treatment of MCAO mice with IL-10⁺ B-cells 24 h post MCAO could also induce CD8⁺CD122⁺ Tregs. As shown in Figs. 4 and 5, treatment with IL-10⁺ B-cells resulted in a significant increase in the frequency of CD8⁺CD122⁺ Tregs in both the spleen (Fig. 4a, $p=0.002$) and the ipsilateral brain hemisphere (Fig. 5a, $p=0.039$) after 96 h reperfusion. Moreover, there were significant increases in the spleen and the ischemic brain hemisphere in percentages of total recipient GFP⁻ IL-10⁺ leukocytes (Fig. 4b, $p=0.025$ in spleen; and Fig. 5b, $p=0.049$ in brain) and IL-10⁺CD8⁺CD122⁺ Tregs (Fig. 4b, $p=0.007$ in spleen; and Fig. 5b, $p=0.005$ in brain). It is noteworthy that the IL-10⁺CD8⁺CD122⁺ Tregs comprised ~10 % of CD8⁺ cells in the spleen and >40 % of CD8⁺ cells in the ischemic brain hemisphere after 96 h reperfusion in IL-10⁺ B-cell treated mice with MCAO. Moreover, in spleen, the increase in IL-10⁺CD8⁺CD122⁺ Tregs appeared to have anti-inflammatory activity, potentially accounting for a significant reduction in percentages of IFN- γ ⁺ and IL-21⁺ CD8⁺ T-cells (Fig. 4c, $p=0.037$).

Comparatively, other regulatory cell types were less apparent in MCAO mice treated therapeutically vs. prophylactically with IL-10⁺ B-cells. We previously demonstrated significant increases in the frequencies of Foxp3⁺CD4⁺ Tregs as well as CD1d^{hi}CD19⁺ Bregs in IL-10⁺ B-cell recipient vs. control mice when the IL-10⁺ Bregs were transferred 24 h before the induction of MCAO. As shown in Fig. 6a, the frequencies of CD4⁺Foxp3⁺ Tregs were significantly higher in the spleens of IL-10⁺ B cell-recipient mice, but there was no difference in the frequency of CD1d^{hi}CD19⁺ Bregs. Having previously demonstrated that B-cells are the major producers of IL-10 post-MCAO (Ren et al. 2011), we here similarly evaluated IL-10 production by splenic Breg cells. As shown in Fig. 6b, the

total recipient GFP⁻ IL-10⁺ percentage expressed by recipient Breg (CD1d^{hi}CD19⁺) cells in spleens was not significantly different ($p=0.106$) in the B-cell-recipient vs. control mice with MCAO. However, unlike the IL-10⁺CD8⁺CD122⁺ Tregs, there was no significant increase in the CD4⁺Foxp3⁺ Tregs or IL-10⁺CD1d^{hi}CD19⁺ Bregs in the ischemic hemisphere of the brain of MCAO mice after 96 h reperfusion (Fig. 6c, d). Notably, a low percentage of transferred GFP⁺IL-10⁺CD19⁺ B-cells was also found in both the spleen (~1 %) and ischemic brain hemisphere (~3 %) of the recipient mice (Fig. 6b, d), indicating their persistence in the periphery and infiltration into the brain after MCAO.

IL-10⁺GFP⁺ B-cells, transferred 24 h after MCAO, inhibit the activation and suppress the pro-inflammatory states of T-cells and monocytes in the periphery

To further evaluate the possible regulatory effects of the dominant CD8⁺CD122⁺ suppressor Tregs generated after the transfer of IL-10⁺ B-cells, 24 h post-MCAO, we evaluated changes in expression of activation markers and cytokines by splenic monocytes and T-cells that might be influencing the splenic milieu by Flow Cytometry. CD11b⁺ splenic monocytes from IL-10⁺ B-cell treated mice had significantly lower expression of MHCII ($p=0.003$), TNF- α ($p=0.026$) and IL-1 β ($p=0.0002$) and similarly, splenic T-cells had lower expression of CD69 ($p=0.049$ for CD4⁺ and $p=0.046$ for CD8⁺ T-cells) and IL-21 ($p=0.01$ for CD4⁺ T-cells) (Fig. 7a, b) compared to splenocytes from Vehicle-treated mice.

Transfer of IL-10⁺ B-cells, 24 h after MCAO induction, reduces the overall pro-inflammatory milieu in the ischemic brain hemisphere

In order to assess the treatment effect of IL-10⁺ B-cells on the expression of immune-related genes in the ischemic hemisphere of the brain, a 96-gene real time PCR assay was performed. mRNA was isolated from the MCAO-affected brains of three Vehicle-treated and four IL-10⁺ B-cell-treated mice. Equivalent amounts of cDNA were pooled and the expression levels of various immune-related genes in the B-cell-treated samples were analyzed relative to the Vehicle-treated (control) samples. The immune array data indicate >2-fold upregulation of the *CD8*, *IL4* and *IL10* genes as well as several genes in the cell death (*Agtr2*, a blood flow regulator) and signal transduction pathways (*Stat1* and transcription factors *Tbx21*, *Nfatc3* and *Nfatc4*) in the MCAO-affected brain hemispheres of B-cell treated vs. Vehicle-treated mice (Fig. 8a). Two notable downregulated genes were *Icam1*, an adhesion molecule, and a chemokine receptor, *Cxcr3*. Expression of five of these genes were validated using RT-PCR on individual samples, and as demonstrated in Fig. 8b, the expression trends confirmed the changes observed in the gene

array. Moreover, the expression level for *IL33*, which can induce IL-10-producing B-cells that counteract mucosal inflammatory responses in the gut (Sattler et al. 2014), was shown to be significantly increased ($p=0.0012$), whereas the expression level of *MMP9*, known to be closely implicated in cerebral ischemia (Clark et al. 1997; Rosenberg et al. 1996), was significantly decreased ($p=0.048$) (Fig. 8c) in the CNS of B-cell treated vs. Vehicle-treated MCAO mice. Finally, CD3⁺ T-cells but not CD11b⁺ monocytes from in the ipsilateral brain hemispheres of the B-cell transferred group demonstrated a significantly reduced capacity to produce TNF- α after 96 h of reperfusion ($p=0.008$; data not shown).

Discussion

The results presented above demonstrate for the first time a major neuroprotective role for IL-10-producing B-cells in

treating MCAO in WT male mice 24 h after occlusion, a time point well beyond the ~4 h tPA treatment window. This therapeutic approach resulted in the generation of a dominant IL-10⁺CD8⁺CD122⁺ Treg population associated with reduced CNS inflammation.

IL-10, an anti-inflammatory cytokine, is produced mainly by Th2-lymphocytes, Treg and Breg cell subsets and also by other cells such as monocytes, macrophages and microglial cells. It can inhibit IL-1 and TNF- α and decrease both cytokine receptor expression and receptor-induced activation. IL-10 is increased in brain tissue after stroke (Pelidou et al. 1999; Strle et al. 2001) and acts locally to limit stroke pathogenesis. Pre-clinical data indicated that IL-10 deficient mice have a larger lesion size after MCAO (Grilli et al. 2000), and systemic administration (Spera et al. 1998) and gene transfer of IL-10 (Ooboshi et al. 2005) in animal models could decrease brain injury after stroke (Kim et al. 1996). Clinical data showed that low IL-10 levels predict an increased risk of stroke (van Exel et al. 2002). Taken together, IL-10 delivered in a biologically

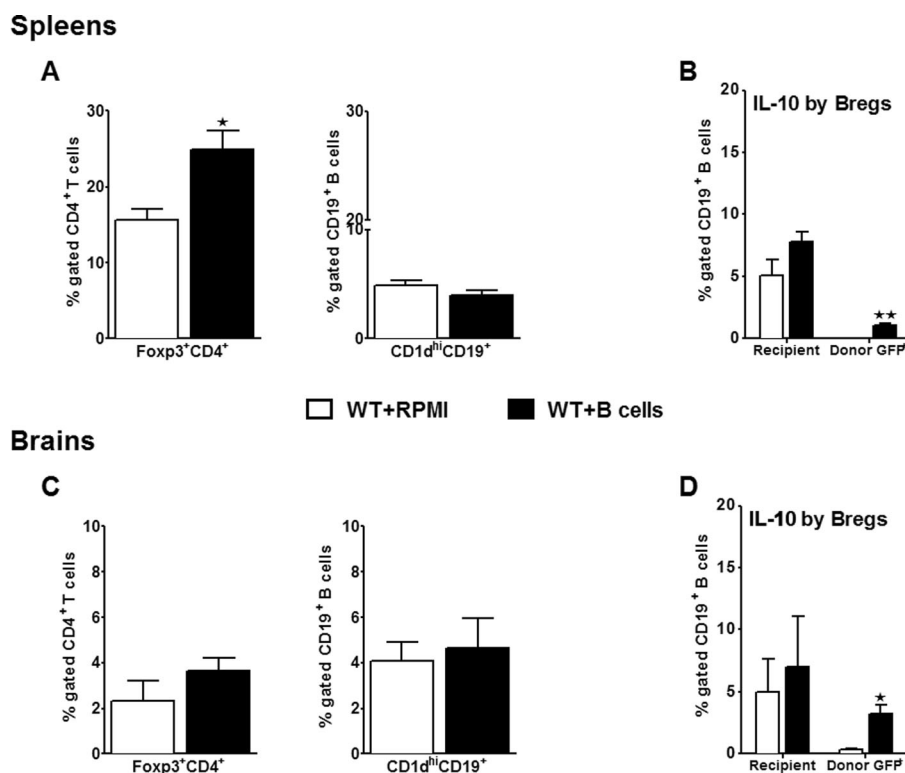


Fig. 6 Treatment with IL-10⁺ B-cells, 24 h post-MCAO, leads to a significant increase of the FoxP3⁺CD4⁺ T-cells in spleen but not the ischemic brain hemisphere, with no changes in recipient Breg cells in spleen or brain. Splenocytes and brain leukocytes from RPMI and IL-10-GFP⁺ B-cell transferred WT recipient mice were harvested 96 h after MCAO and assessed for: **a.** expression of FoxP3⁺CD4⁺ T-cells and CD1d^{high}CD19⁺ regulatory B-cells in the spleens. Data are representative of 2 independent experiments with spleens processed from 6 to 7 individual mice (mean±SEM); **b.** analyzed for IL-10-production on GFP⁻ cells using IL-10 APC mAb for detection and/or detecting the

GFP⁺CD19⁺ cells (i.e. IL-10-GFP⁺ transferred B-cells) by Flow cytometry; **c.** expression of FoxP3⁺CD4⁺ T-cells and CD1d^{high}CD19⁺ regulatory B-cells in the ischemic hemispheres of the brains. Data are representative of 2 independent experiments with spleens processed from 6 to 7 individual mice (mean±SEM); and **d.** analyzed for IL-10-production on GFP⁻ cells using IL-10 APC mAb for detection and/or detecting the GFP⁺CD19⁺ cells (i.e. IL-10-GFP⁺ transferred B-cells) by Flow cytometry. Significant differences between the groups were determined using Student's *t*-test. (* $p\leq 0.05$ and ** $p\leq 0.01$)

Spleens

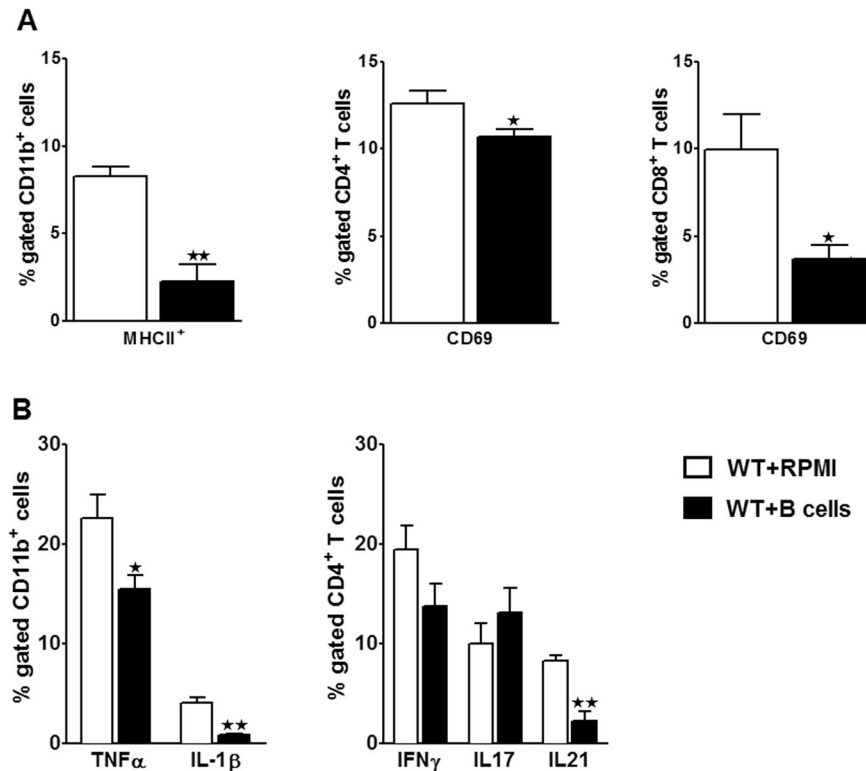


Fig. 7 IL-10⁺GFP⁺ B-cells, transferred 24 h after MCAO, inhibit the activation and suppress the pro-inflammatory states of T-cells and monocytes in the periphery. Splenocytes were isolated from RPMI or IL-10-GFP⁺ B-cell recipient WT mice, 96 h after MCAO and assessed for: **a**. expression of MHC class II on gated CD11b⁺ monocytes and expression of activation marker, CD69, on gated CD4⁺ and CD8⁺ T-cells; and **b** CD11b⁺ monocytes were analyzed for TNF- α and IL-1 β production and

CD4⁺ T-cells were analyzed for IFN- γ , IL-17⁺ and IL-21⁺ production by Flow cytometry. Values represent mean percentages (\pm SEM) of indicated cell subsets from 5–6 mice of each group, from at least 2 separate experiments. Statistical analysis was performed with Student's *t*-test to compare between RPMI and IL-10-GFP⁺ B-cell recipient vs. Vehicle control MCAO mice. Significant differences between sample means are indicated (* p ≤0.05 and ** p ≤0.01)

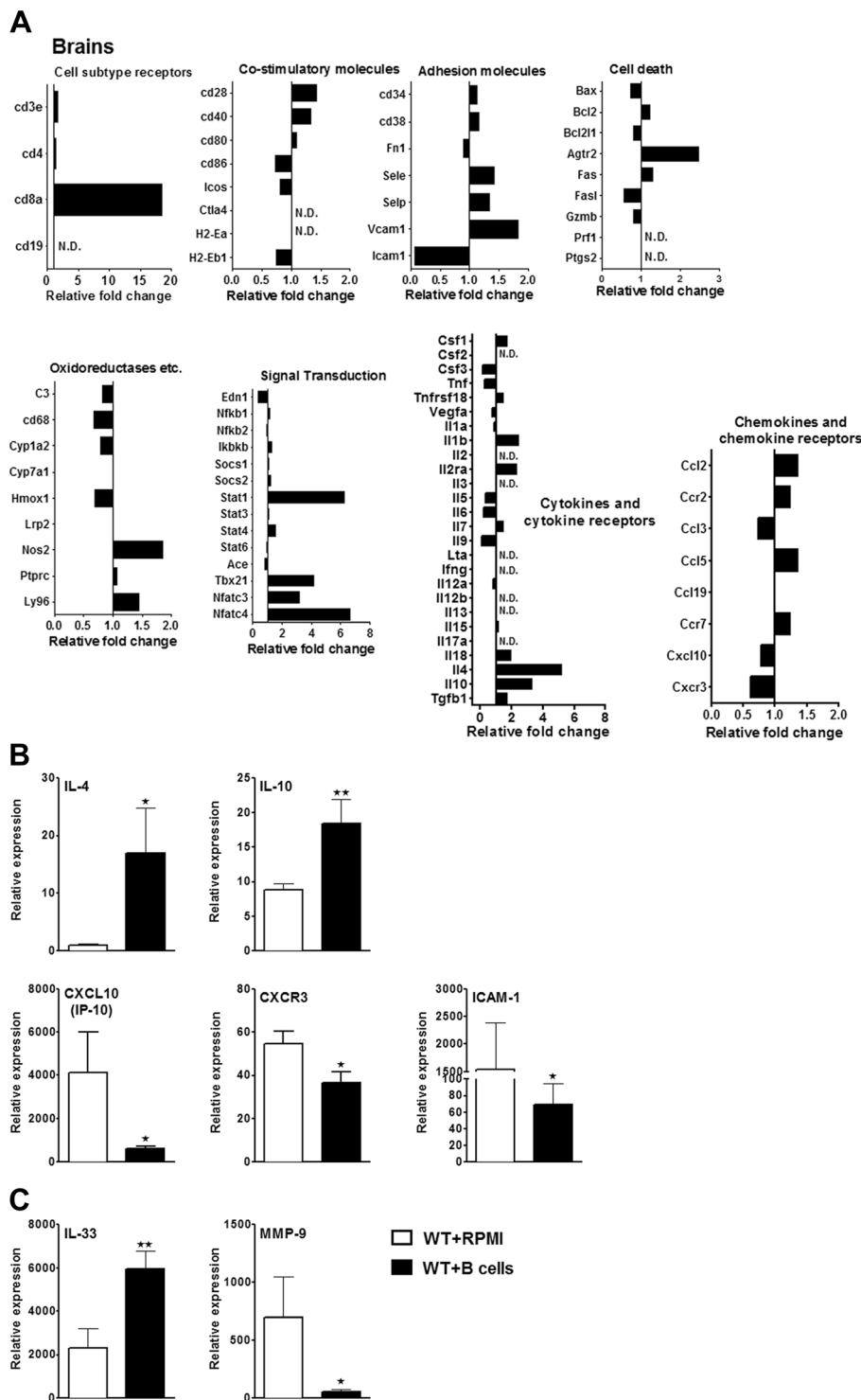
compatible manner by regulatory B- and T-cells could be a highly effective therapy for the treatment of ischemic stroke (Jin et al. 2013).

Transfer of IL-10⁺ B-cells 24 h after MCAO-induction led to a significant reduction in infarct volumes. Protection from MCAO-induced damage was observed mainly in the cortex and ischemic hemisphere but not in the striatum, perhaps reflecting the difficulty of IL-10-secreting B-cells to reverse early MCAO-induced damage in moderately vascularized brain regions. Also, from the neurological deficit scores, it was apparent that the protection rendered to the MCAO mice that received IL-10⁺ B-cells only became significant at the 96 h time-point. This delay in the treatment effect suggests that the nature of the immune changes generated in the recipient mice may involve adaptive as well as innate responses.

To further discern the regulatory role of IL-10⁺ B-cells on the developing infarct, we focused on the fate of the transferred IL-10-secreting B-cells and their impact on peripheral responses and the infiltrating leukocytes and resident cells within the ischemic hemisphere. Due to their derivation from

GFP⁺ donor mice, it was possible to distinguish the GFP⁺ IL-10-secreting donor B-cells from GFP⁻ recipient pathogenic and regulatory cells in mixed populations by FACS after 96 h reperfusion in MCAO mice. Indeed, the donor GFP⁺ B-cell population (5 million were injected 24 h after MCAO) constituted only about 0.6 % of the splenocyte population and about 0.3 % of the cells in the ischemic brain hemisphere of MCAO mice. The result of this delayed transfer had an enormous impact on the outcome of the MCAO insult, however, reducing stroke lesion size by >20 %, ameliorating survival of splenocytes from 20 to >30 million cells (>50 % improvement) and inhibiting infiltration of B-cells and activated macrophages and enhancing numbers of resident MG cells in the ipsilateral brain hemisphere (Fig. 3).

The relatively small numbers of surviving GFP⁺ IL-10-secreting donor B-cells suggested that other recruited regulatory cell types might be selectively induced after treatment with the donor B-cells, including CD4⁺Foxp3⁺ Tregs, CD8⁺CD122⁺ Tregs and CD19⁺CD1d^{hi} Bregs observed in previous studies (Bodhankar et al. 2013, 2014; Offner et al.



2006b). Of these three regulatory cell types, the best candidate was the IL-10⁺CD8⁺CD122⁺ subpopulation. These cells were significantly increased in B-cell vs. Vehicle treated MCAO mice in the spleen and the ischemic brain hemisphere (Figs. 4 and 5), constituting ~1.5 % of both total splenocytes and ipsilateral brain cells. In contrast, CD4⁺Foxp3⁺ Treg-cells were significantly increased in the spleen but not the brain of B-cell treated vs. Vehicle-treated MCAO mice and CD19⁺CD1d^{hi} Breg-cells although present, were not different in B-cell treated vs. Vehicle-treated MCAO mice.

By calculating the percentages of total recovered cells for each regulatory subset, it is possible to compare the actual numbers of each subtype present in spleen and ipsilateral brain hemisphere from MCAO mice. Thus, in spleen there were ~165,000 donor IL-10-secreting B-cells, ~360,000 CD8⁺CD122⁺ Tregs, ~330,000 CD19⁺CD1d^{hi} Bregs and 1.9 million CD4⁺Foxp3⁺ Tregs. In ipsilateral brain tissue there were ~1,500 donor IL-10-secreting B-cells, ~5,600 CD8⁺CD122⁺ Tregs, ~3,500 CD19⁺CD1d^{hi} Bregs and ~400 CD4⁺Foxp3⁺ Tregs. From this information, it is clear that although all the different regulatory subsets were present and could contribute to immunoregulation in spleen and brain of MCAO mice, the CD4⁺Foxp3⁺ Tregs were predominant in spleen, whereas the CD8⁺CD122⁺ Tregs were predominant in ischemic brain. Unlike the appearance of CD8⁺CD122⁺ Treg cells in spleen *but not CNS* after prophylactic treatment with IL-10⁺ B-cells 24 h *prior to* MCAO (Bodhankar et al. 2013, 2014), the appearance of the CD8⁺CD122⁺ Treg cells in the ischemic brain hemisphere in mice treated 24 h *after* MCAO indicated an important new activity of the transferred IL-10⁺ B-cells; that is, their ability to induce migration of the CD8⁺CD122⁺ Treg cells from the periphery into the CNS where local immunoregulation could take place.

CD8⁺CD122⁺ T-cells are naturally occurring regulatory T-cells that secrete copious levels of IL-10 that has been shown to be the key effector molecule for transducing regulatory activity (Endharti et al. 2005; Rifa'i et al. 2004). The *in vivo* relevance of CD8⁺CD122⁺ regulatory T-cells has been proven by experiments involving cell transfer into lymphocyte-deficient mice (Endharti et al. 2011; Lee et al. 2008; Mangalam et al. 2012). CD8⁺CD122⁺ regulatory T-cells are known to recognize activated T-cells through direct cell-to-cell contact involving conventional class I MHC molecules (Okuno et al. 2013) and to inhibit the target T-cells without additional antigen-presenting cells (Suzuki et al. 2008). Consistent with this description, our studies demonstrated reduced expression of the CD69 activation molecule on both CD4⁺ and CD8⁺ splenic T-cells as well as significantly inhibited levels of IL-21 by CD4⁺ T-cells and IFN- γ and IL-21 by CD8⁺ T-cells from spleen (Figs. 4 and 7). Such inhibition of IL-21 could indeed be beneficial to stroke outcome as indicated in a recent report showing that T-cell production of this cytokine can induce brain injury after MCAO (Clarkson et al.

2014). Additional regulatory effects observed included reduced expression of MHCII, TNF- α and IL-1 β by splenic CD11b⁺ monocytes and enhanced expression of IL-4, IL-10 and IL-33, but reduced expression of ICAM-1, CXCL10, CXCR3 and MMP-9 in the ischemic brain hemisphere (Figs. 7-9).

Taken together, our present study demonstrates that transfer of IL-10⁺ B-cells, 24 h after MCAO, markedly reduced infarct volume in recipient mice. The MCAO-protected B-cell recipient mice had reduced numbers of activated, inflammatory T-cells, decreased infiltration of T-cells, increased regulatory subpopulations, predominantly the CD8⁺CD122⁺ Treg subset, and a less inflammatory milieu in the ischemic hemispheres as compared to the control mice. Our studies for the first time demonstrate a major immunoregulatory role for IL-10⁺ B-cells in treating MCAO in WT mice even at a time point well beyond the tPA treatment window, leading to the generation of a dominant CD8⁺CD122⁺ Treg population in the ischemic brain hemisphere, thus implicating their potential role in post-stroke immunoprotection. The effective treatment of MCAO recipient mice with IL-10⁺ B-cells that fostered not only a favorable stroke outcome but also decreased associated degenerative factors known to worsen stroke adds promise to this treatment for the future.

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Authors' contribution SB designed and performed the immunology experiments, carried out statistical analyses, prepared graphics and wrote the manuscript; YC performed the MCAO procedures, carried out statistical analyses, prepared the graphics and wrote the methods and results for infarct volume data; AL assisted in tissue preparations and acquisition of immunological data; AAV critiqued and edited the manuscript; SJM and JAS directed study design and data analysis of the MCAO experiments and edited the manuscript; HO directed the overall study, supervised the immunological studies and data analysis and edited the manuscript. All authors read and approved the final version of the manuscript.

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