Regulatory B Cells Limit CNS Inflammation and Neurologic Deficits in Murine Experimental Stroke

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Evaluation of infarct volumes and infiltrating immune cell populations in mice after middle cerebral artery occlusion (MCAO) strongly implicates a mixture of both pathogenic and regulatory immune cell subsets in stroke pathogenesis and recovery. Our goal was to evaluate the contribution of B cells to the development of MCAO by comparing infarct volumes and functional outcomes in wild-type (WT) versus B-cell-deficient $\mu\text{MT}^{-/-}$ mice. The results clearly demonstrate larger infarct volumes, higher mortality, more severe functional deficits, and increased numbers of activated T cells, macrophages, microglial cells, and neutrophils in the affected brain hemisphere of MCAO-treated $\mu\text{MT}^{-/-}$ versus WT mice. These MCAO-induced changes were completely prevented in B-cell-restored $\mu\text{MT}^{-/-}$ mice after transfer of highly purified WT GFP⁺ B cells that were detected in the periphery, but not the CNS. In contrast, transfer of B cells from IL-10^{-/-} mice had no effect on infarct volume when transferred into $\mu\text{MT}^{-/-}$ mice. These findings strongly support a previously unrecognized activity of IL-10-secreting WT B cells to limit infarct volume, mortality rate, recruitment of inflammatory cells, and functional neurological deficits 48 h after MCAO. Our novel observations are the first to implicate IL-10-secreting B cells as a major regulatory cell type in stroke and suggest that enhancement of regulatory B cells might have application as a novel therapy for this devastating neurologic condition.

Introduction

Animal data clearly support a biphasic effect of stroke on the peripheral immune system. The initial phase is characterized by early signaling from the ischemic brain to spleen, resulting in a massive production of inflammatory factors, transmigration of splenocytes to the circulation and infiltration of stroke-damaged areas of the brain by inflammatory polymorphonuclear cells, macrophages, T cells and B cells (Offner et al., 2006a). This early activation phase is followed by compensatory systemic immunosuppression that is manifested within days of focal stroke by a profound (90%) loss of immune T and B cells in the spleen and

thymus and reduced T-cell activation (Offner et al., 2006b, 2009). These changes were accompanied by an increase in splenocytes that were overtly apoptotic or committed to the apoptotic pathway (i.e., that were TUNEL positive, Annexin V⁺, or PI⁺).

To evaluate the contribution of lymphocytes to stroke severity, we performed middle cerebral artery occlusion (MCAO) in SCID mice that are genetically deficient in T and B lymphocytes (Hurn et al., 2007). Cortical and total infarction volumes were strikingly smaller in the SCID mice with MCAO compared with wild-type (WT) C57BL/6 control mice ($p < 0.01$). The smaller infarct size in SCID versus WT mice indicated that ~40% of the stroke damage observed within the first 22 h of MCAO involves the inflammatory T and/or B cells that have migrated from the periphery into the evolving infarct. However, the initial inflammatory ischemic insult may also induce regulatory responses that limit CNS damage.

A promising regulatory candidate was the CD4⁺CD25⁺FoxP3⁺ T-cell population (Tregs), which increased significantly 96 h after MCAO (Offner et al., 2006b). However, depletion of Tregs using Foxp3^{DTR} mice (Kim et al., 2007) did not affect infarct volume or behavioral evaluations in mice (Ren et al., 2011). Our failure to implicate Tregs in limiting brain lesion volume after MCAO is of general importance to the field because of the increased interest in using CD4⁺Foxp3⁺ Tregs as a possible therapeutic approach in stroke. Our results differed from a recent report (Liesz et al., 2009) in which depletion of the CD25⁺ population with anti-CD25 mAbs significantly increased brain infarct volume and worsened functional outcome. Although these

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Figure 1. Deficiency of B cells exacerbates ischemic infarct volume and behavioral outcome after MCAO. **A**, Infarct volume, corrected for the presence of edema, at 48 h of reperfusion after 60 min of MCAO given as bar graphs are visualized (mean $\pm$ SEM). Statistical analysis was performed with the Student's *t* test. There was a significant difference in infarct volumes between WT and μ MT^{-/-} mice; $p < 0.05$. Groups: WT ($n = 15$); μ MT^{-/-} ($n = 12$). **B**, Representative TTC-stained cerebral sections of the MCAO modeled to analyze infarct volume. Localization of the ischemic lesion differed between WT and μ MT^{-/-} mice. **C**, B-cell-deficient μ MT^{-/-} mice had no significant functional worsening before reperfusion, but significantly worsened functional outcome at 24 h ($p = 0.02$) and 48 h ($p = 0.002$) after reperfusion. The statistical analysis was performed using the Mann–Whitney *U* test. Groups: WT ($n = 35$); μ MT^{-/-} ($n = 35$).

Table 1. Physiological parameters at baseline, mid-MCAO, and end-MCAO in WT and μ MT^{-/-} mice

Variables	WT ($n = 4$)			μ MT ^{-/-} ($n = 3$)		
	Baseline	Mid-MCAO	End-MCAO	Baseline	Mid-MCAO	End-MCAO
Arterial blood pH	7.38 $\pm$ 0.06	7.40 $\pm$ 0.08	7.4 $\pm$ 0.06	7.41 $\pm$ 0.06	7.43 $\pm$ 0.05	7.43 $\pm$ 0.01
PaCO ₂ (mmHg)	32 $\pm$ 4	32 $\pm$ 7	34 $\pm$ 8	30 $\pm$ 5	29 $\pm$ 5	27 $\pm$ 2
PaO ₂ (mmHg)	141 $\pm$ 19	137 $\pm$ 19	141 $\pm$ 11	145 $\pm$ 7	149 $\pm$ 8	144 $\pm$ 8
MABP (mmHg)	83 $\pm$ 5	83 $\pm$ 5	75 $\pm$ 7	87 $\pm$ 3	85 $\pm$ 7	70 $\pm$ 11
Temperature (°C)	36.6 $\pm$ 0.6	36.6 $\pm$ 0.4	36.3 $\pm$ 0.3	36.4 $\pm$ 0.3	36.4 $\pm$ 0.4	36.3 $\pm$ 0.6
rCBF (%)	100 $\pm$ 0	13 $\pm$ 7	18 $\pm$ 9	100 $\pm$ 0	12 $\pm$ 0	7 $\pm$ 2

There were no differences in physiological parameters determined between the genotypes. PaCO₂, arterial CO₂ tension; PaO₂, arterial O₂ tension; MABP, mean arterial blood pressure; rCBF, regional cerebral blood flow.

effects cannot be attributed solely to CD4⁺CD25⁺Foxp3⁺ Tregs due to a wider expression of CD25, this study did implicate activated T- and/or B-cell populations in a regulatory role.

Recent studies have described powerful regulatory effects of B lymphocytes on inflammatory responses (LeBien and Tedder, 2008; Lund, 2008). Depletion of B cells worsened disease severity in models of multiple sclerosis, and transfer of WT B cells provided protection against disease induction (Matsushita et al., 2010). The ability of the CD1d^{high}CD5⁺CD19⁺ regulatory B-cell subset to limit CNS injury is likely associated with the well recognized anti-inflammatory effects of IL-10 in the CNS (Fillaire et al., 2002; Mann et al., 2007). We thus evaluated the effects of regulatory B cells in stroke. Our data demonstrate the profound impact of endogenous regulatory B cells on limiting the infarct volume and neurological deficits after ischemic stroke, and further identify the cellular targets of this highly protective B-cell population.

Materials and Methods

Animals. B-cell-deficient μ MT^{-/-} mice on the C57BL/6 background were bred at the VA Animal Resource Facility. Age-matched 8–12-week-old C57BL/6 mice (WT, Jackson Laboratory) were used as control mice for MCAO induction. Green fluorescent protein (GFP⁺) mice (bred at the VA Animal Resource Facility), WT and IL-10^{-/-} mice (the Jackson Laboratory) on the C57BL/6 background were used as B-cell donors to restore B-cell function in B-cell-deficient mice. Animals were bred and

cared for according to institutional guidelines in the Animal Resource Facility at the Veterans Affairs Medical Center, Portland, OR. All experiments were performed under approved institutional protocols from the VA and Oregon Health & Science University.

MCAO model. The mice were subjected to MCAO as previously published (Offner et al., 2006b) by reversible right MCA occlusion (60 min) under isoflurane anesthesia, followed by 48 h of reperfusion. Body and head temperatures were controlled at 37 $\pm$ 0.5°C. Occlusion and reperfusion were verified in each animal by laser Doppler flowmetry (Moor Instruments).

Quantification of infarct. As previously published, brains were collected at 48 h for standard 2,3,5-triphenyltetrazolium chloride (TTC) histology and digital image analysis of infarct volume. To control for edema, corrected infarct volume is expressed as a percentage of the contralateral structure (i.e., cortex, striatum, or total hemisphere).

Neurological deficit score. Neurological function was evaluated using a 0–5-point scale neurological score: 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; 4 = no spontaneous walk or in a comatose state; 5 = death. The scores were assessed in a blinded fashion.

Cell isolation. Peripheral blood mononuclear cells were prepared by using red cell lysis buffer (eBioscience) following manufacturer's instructions. Single-cell suspensions from lymph nodes (LNs) (superficial cervical, mandibular, axillary, lateral axillary, superficial inguinal, and mesenteric) and spleens were prepared by mechanical disruption. For preparation of inflammatory cells in brain infarction, each mouse was transcardially perfused with 30 ml of saline to exclude blood cells. The forebrain was dissected from the cerebellum and suspended in RPMI-1640 medium. The suspension was digested with type IV collagenase (1 mg/ml, Sigma-Aldrich) and DNase I (50 μ g/ml, Roche Diagnostics) at 37°C for 45 min in a shaker at 180 times per minute. Inflammatory cells were isolated by 37–70% Percoll (GE Healthcare) density gradient centrifugation according to a method described previously (Campanella et al., 2002). Inflammatory cells were removed from the interface for further analysis. The cells were then washed twice with RPMI-1640 medium, counted, and resuspended in stimulation medium containing 10% FBS for phenotyping.

Analysis of cell populations by FACS. Anti-mouse antibodies used for this study included: CD19 (1D3, BD PharMingen), CD45 (30-F11, Invitrogen), CD11b (M1/70, eBioscience), MHCII (2G9, BD PharMingen), Gr1 (IA8, BD Horizon), CD3 (17A2, eBioscience), IFN- γ (XMG1.2, eBioscience), TNF- α (MP6-XT22, BD PharMingen), and IL-10 (JES5-16E3, eBioscience). Single-cell suspensions were washed with staining medium (PBS containing 0.1% Na₃N and 2% FCS). After incubation with appropriate mAbs and washing, cells were acquired with LSRII Fluorescence Activated Cell Sorter (BD Biosciences). For each experiment, cells were stained with appropriate isotype control antibodies to establish background staining and to set quadrants before calculating the percentage of positive cells. Data were analyzed using FlowJo software (TreeStar).

CD19⁺ B-cell sorting and transfer. B cells were isolated for transfer experiments by negative magnetic cell sorting (Miltenyi Biotec), with GFP⁺, WT, and IL-10^{-/-} mice as donors. μ MT^{-/-} mice were injected intraperitoneally with a total of 5×10^7 CD19⁺ B cells for all cell transfer experiments. The purity of B cells was >99%. The distribution of GFP⁺CD19⁺ B cells was evaluated 48 h after transfer in recipient μ MT^{-/-} mice.

IL-10 staining. Intracellular IL-10 expression was visualized by modification of a previously published immunofluorescence staining protocol (Yanaba et al., 2008). Briefly, isolated leukocytes or purified cells were resuspended (2×10^6 cells/ml) in complete medium [RPMI-1640 media containing 10% FCS, 1 mM pyruvate, 200 μ g/ml penicillin, 200 U/ml

streptomycin, 4 mM L-glutamine, and 5×10^{-5} M 2- β -ME with LPS (10 μ g/ml), PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μ g/ml)] (all reagents are from Sigma-Aldrich) for 5 h. For IL-10 detection, Fc receptors were blocked with mouse Fc receptor-specific mAb (2.3G2; BD PharMingen) before cell surface staining, and then 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 stained with APC-conjugated anti-IL-10 mAb (JES5-16E3, eBioscience). Isotype-matched mAbs served as negative controls to demonstrate specificity and to establish background IL-10 staining levels.

Immunohistochemistry. Brains, lymph nodes, and spleens were collected from perfused μ MT $^{-/-}$ mice that received adoptively transferred GFP $^{+}$ B cells after 60 min of MCAO and 48 h reperfusion. Tissues were fixed with 4% buffered formalin, paraffin embedded, and sectioned. Sections were incubated with anti-GFP (Cell Signaling Technology), followed by incubation with secondary biotinylated antibody (goat anti-rabbit, Cell Signaling Technology) and staining with Vectastain ABC Peroxidase Kit (Vector Laboratories) and 3', 3'-diaminobenzidine (Sigma-Aldrich). Nuclear staining was performed with hematoxylin (Sigma-Aldrich).

Statistical analysis. Data were reported as means $\pm$ SEM. Statistical analyses were performed using the appropriate test indicated in the figure legends as follows: Student's *t* test for infarct volume; Mann-Whitney *U* test for neuroscores; and Fisher's exact test for comparison of mortality rates incurred from surgery and induction of MCAO. *p* values ≤ 0.05 were considered statistically significant.

Results

B-cell deficiency exacerbates stroke outcomes and alters cerebral inflammatory cell invasion

B-cell-deficient μ MT $^{-/-}$ mice sustained significantly larger total hemisphere infarcts ($p < 0.05$) relative to WT mice (Fig. 1A). Representative histologic staining of injured brain is shown in Figure 1B. These data clearly implicate the role of B cells in limiting histological damage after MCAO. Mortality was also higher in B-cell-deficient versus WT mice (11/35 vs 3/35, respectively; $p = 0.017$). Neurological scores were similar among all animals as assessed during MCAO immediately before reperfusion (Fig. 1C) ($p = 0.36$). However, B-cell-deficient mice exhibited worse functional outcomes at both 24 h (Fig. 1C) ($p = 0.02$) and 48 h of reperfusion (Fig. 1C) ($p = 0.002$) compared with WT mice. To confirm that the ischemic insult was equivalent among all animals, relevant physiological parameters were assessed before and during MCAO. As is shown in Table 1, rectal temperature, mean arterial blood pressure, arterial blood gases, and pH were comparable between groups. Similarly, intras ischemic cortical blood flow, as estimated by laser Doppler flowmetry, was not different between B-cell-deficient and WT mice.

Leukocytes are major effectors of inflammatory damage after experimental brain ischemia (Gee et al., 2007; Wang et al., 2007). To determine whether the lack of B cells altered leukocyte composition in brain after MCAO, numbers of infiltrating Gr1 $^{+}$ neutrophils, CD3 $^{+}$ T cells, CD11b $^{+}$ CD45 low microglia, and CD11b $^{+}$ CD45 high macrophages were evaluated by flow cytometry. After 48 h reperfusion, the accumula-

Figure 2. A–D, B cells reduce the infiltration of inflammatory cells into the ischemic brain after MCAO. Analysis of Gr1 $^{+}$ neutrophils (A), CD3 $^{+}$ T cells (B), CD11b $^{+}$ CD45 low microglia (MG) (C), and CD11b $^{+}$ CD45 high macrophages (Mac) (D) counted in the nonischemic (left) and ischemic (right) hemispheres. Values represent mean numbers ($\pm$ SEM) of indicated cell subsets from five mice in each group. Statistical analysis was performed with ANOVA followed by *post hoc* Tukey's test, respectively. Significant differences between sample means are indicated (***) $p < 0.0001$.

Figure 3. A–F, B cells reduce the activation of infiltrating inflammatory cells in the ischemic brain after MCAO. Determinations of IFN- γ $^{+}$ CD3 $^{+}$ T cells (A), TNF- α $^{+}$ CD3 $^{+}$ T cells (B), MHCII $^{+}$ microglia (MG) (C), TNF- α $^{+}$ MG (D), MHCII $^{+}$ macrophages (Mac) (E), and TNF- α $^{+}$ Mac (F) quantified in the nonischemic (left) and ischemic (right) hemispheres from WT and μ MT $^{-/-}$ mice after MCAO. Values represent mean numbers ($\pm$ SEM) of indicated cell subsets from five mice of each group. Statistical analysis was performed with ANOVA followed by *post hoc* Tukey's test, respectively. Significant differences between sample means are indicated (***) $p < 0.0001$.

tion of all of these leukocyte subtypes was significantly greater in the affected hemisphere of MCAO-treated μ MT $^{-/-}$ mice compared with MCAO-treated WT mice (Fig. 2A–D). Lack of B cells in μ MT $^{-/-}$ mice further permitted significant increases in the absolute number of IFN- γ - and TNF- α -secreting CD3 $^{+}$ T cells and MHC class II $^{+}$ and TNF- α -secreting microglia and macrophages in the ipsilateral hemisphere of MCAO mice at 48 h of reperfusion (Fig. 3A–F). In addition to the cell types mentioned above, we detected 6576 ± 829 CD19 $^{+}$ B cells per hemisphere in sham-treated brains ($n = 5$), not different from 7092 ± 637 “resident” B cells in naive brains ($n = 5$). In MCAO mice, there were modest but significant increases to 9766 ± 1832 B cells in the nonischemic hemisphere and $12,282 \pm 824$ B cells in the ischemic hemisphere ($n = 5$).

Figure 4. Distribution of GFP⁺CD19⁺ B cells in $\mu\text{MT}^{-/-}$ mice at 48 h reperfusion after 60 min MCAO. **A**, 99% enrichment of GFP⁺CD19⁺ B cells from spleens of GFP⁺ C57BL/6 donors and adoptive intraperitoneal transfer of 50 million B cells into $\mu\text{MT}^{-/-}$ mice. **B**, Transferred GFP⁺CD19⁺ B cells were not detected in either the nonischemic (left brain) or ischemic (right brain) hemispheres, but were observed in blood, LNs, spleen (SP), and peritoneal cavity of recipient mice by FACS at 48 h reperfusion after 60 min MCAO. **C**, Transferred GFP⁺ B cells were visualized in LNs and spleens but not in the brains by immunohistochemical staining at 48 h reperfusion after 60 min MCAO.

Adoptive transfer of B cells to $\mu\text{MT}^{-/-}$ mice reduces ischemic infarct size and improves neurological deficits

To specifically implicate B cells as the protective cell type, highly enriched populations of B cells were transferred from WT donors to B-cell-deficient recipient mice before MCAO. As illustrated in Figure 4A, CD19⁺ B cells were obtained and enriched to 99% purity by negative selection from splenocytes of transgenic GFP⁺ mice, and 50 million GFP⁺CD19⁺ B cells were injected intraperitoneally into $\mu\text{MT}^{-/-}$ mice 1 d before MCAO. The B-cell-deficient animals that received adoptively transferred GFP⁺CD19⁺ B cells had reduced infarct volumes ($p > 0.05$) after MCAO compared with no-cell-transfer (PBS) controls (Fig. 5A,B), as well as a lower mortality rate ($p = 0.05$). Consistent with smaller infarction size, neurological outcome scores were also improved in B-cell-restored $\mu\text{MT}^{-/-}$ mice with stroke after 48 h reperfusion compared with no-cell-transfer (PBS) control mice (Fig. 5C). We also confirmed that the adoptive transfer of CD19⁺ B cells from C57BL/6 WT donors (GFP⁺) limited stroke infarct size and functional outcome (data not shown). These findings clearly demonstrate that WT CD19⁺ B cells can restore improved ischemic outcomes in B-cell-deficient $\mu\text{MT}^{-/-}$ mice. However, after 48 h of reperfusion transferred GFP⁺ B cells could be detected in blood, LNs, spleen, and peritoneal cavity, but not in ischemic (Fig. 4B, left brain) or nonischemic (Fig. 4B, right brain) of recipient $\mu\text{MT}^{-/-}$ mice by FACS. Immunohistochemical staining showed GFP⁺ cells distributed in lymph nodes and spleens, but not in nonischemic (Fig. 4C, L) or ischemic (Fig. 4C, R) hemispheres.

B cells, but not T cells, are the major producer of IL-10 in MCAO mice

Because of the significant B-cell-dependent activity in limiting stroke infarct size and functional outcome demonstrated above, we hypothesized that the protective actions of CD19⁺ B cells might be linked to IL-10 production, a major regulatory cytokine known to be produced by both B cells and T cells. Thus, intracellular staining of IL-10 was performed in CD19⁺ B cells and CD3⁺ T cells harvested from immune organs after MCAO and stimulated *ex vivo* with LPS, PMA, and ionomycin. As is shown in Figure 6, an increased percentage of IL-10-secreting CD3⁺ negative cells was observed in MCAO WT mice but not in $\mu\text{MT}^{-/-}$ mice after 48 h reperfusion in blood, but not in spleen or lymph nodes. These IL-10-secreting cells in blood were identified as CD19⁺ B lymphocytes (Fig. 7). These data demonstrate enhanced availability of B cells with potential to limit ischemic and neurological outcomes after MCAO through secretion of IL-10.

Adoptive transfer of IL-10^{-/-} B cells to $\mu\text{MT}^{-/-}$ mice does not reduce ischemic infarct size or improve neurological deficits

To specifically address the mechanism of B cells as the protective cell type producing IL-10, highly enriched populations of B cells were transferred from IL-10^{-/-} donors to B-cell-deficient recipient mice before MCAO. As shown in Figure 8, A and B, the B-cell-deficient animals that received adoptively transferred IL-10^{-/-} B cells did not exhibit significantly reduced infarct vol-

Figure 5. Transfer of GFP⁺CD19⁺ B cells reduces infarct volume and improves behavioral outcome of μ MT^{-/-} B-cell-deficient mice. **A**, Transfer of 50 million GFP⁺CD19⁺ B cells resulted in reduced infarct volume (mean $\pm$ SEM) at 48 h of reperfusion after 60 min MCAO. Statistical analysis was performed using the Student's *t* test. There was a significant difference of infarct volumes between no cell (PBS) ($n = 12$) and GFP⁺ B cell ($n = 10$) transferred μ MT^{-/-} mice (* $p > 0.05$). **B**, Representative TTC-stained cerebral sections of the MCAO modeled to analyze infarct volume. Localization of the ischemic lesion differed between no cell and B-cell-transferred μ MT^{-/-} mice. **C**, Transfer of GFP⁺ B cells did not change behavioral outcome in B-cell-deficient mice before reperfusion or after 24 h of reperfusion, but significantly improved functional outcome after 48 h of reperfusion compared with controls (* $p = 0.04$). The statistical analysis was performed using the Mann–Whitney *U* test.

Figure 6. IL-10-secreting non-T cells, but not T cells are increased after activation *ex vivo* in WT but not B-cell-deficient mice with MCAO. CD3⁺ T cells and CD3⁻ non-T cells from blood, lymph nodes, and spleen were evaluated for intracellular expression of IL-10 after activation with LPS/PMA/ionomycin after 60 min of sham or MCAO treatment and 48 h of reperfusion in WT and μ MT^{-/-} mice. IL-10-secreting cells increased in blood are from CD3⁻ non-T cells after MCAO, and no increase in IL-10⁺CD3⁺ T cells was found at this time point in blood, LNs, or spleen tissue from MCAO mice. The leukocyte population was defined via forward scatter and sideward scatter parameters. Representative plots for one mouse show frequencies of IL-10-producing cells among total lymphocytes within the indicated gates. Results represent one of five independent experiments producing similar results.

umes after 60 min MCAO followed by 48 h reperfusion compared with no-cell-transfer (PBS) controls. The mortality rate of the PBS and IL-10^{-/-} B-cell transfer groups was 7 of 16 and 6 of 14, respectively. Moreover, there were no differences of

Figure 7. IL-10-secreting B cells are increased after activation *ex vivo* in the blood of WT mice with MCAO. CD19⁺ B cells from blood, lymph nodes, and spleen were evaluated for intracellular expression of IL-10 after activation with LPS/PMA/ionomycin after 60 min sham or MCAO treatment and 48 h reperfusion in WT mice. CD19 staining was used as the initial gate for identifying B cells. Representative plots for one mouse show increased frequencies of IL-10-producing cells among total B cells in blood within the indicated gates. Results represent one of three independent experiments producing similar results.

Figure 8. Transfer of IL-10^{-/-} CD19⁺ B cells does not alter infarct volume or improve behavioral outcome of μ MT^{-/-} mice. **A**, Transfer of 50 million CD19⁺ B cells from IL-10^{-/-} mice had no effect on infarct volume of total hemisphere (mean $\pm$ SEM) at 48 h of reperfusion after 60 min of MCAO. Statistical analysis was performed using the Student's *t* test. There was no significant difference in infarct volumes between no-cell-transfer (PBS) ($n = 9$) and IL-10^{-/-} B-cell-transferred ($n = 8$) μ MT^{-/-} mice. **B**, Representative TTC-stained cerebral sections of the MCAO modeled to analyze infarct volume. Localization of the ischemic lesion did not differ between no-cell-transfer and IL-10^{-/-} B-cell-transferred μ MT^{-/-} mice. **C**, Transfer of IL-10^{-/-} B cells did not significantly improve functional outcome after 24 or 48 h of reperfusion. The statistical analysis was performed using the Mann–Whitney *U* test.

neurological outcome scores after 24 and 48 h of reperfusion between PBS and IL-10^{-/-} B-cell transfer groups. Together, these data clearly demonstrate that WT CD19⁺ B cells can restore improved ischemic outcomes that were shown to be lacking in B-cell-deficient μ MT^{-/-} mice through the secretion of IL-10.

Regulatory B cells inhibit inflammatory responses in the periphery of MCAO mice

To further evaluate possible regulatory effects of B cells on T-cell cytokine production during MCAO, inflammatory factors were quantified in blood and spleens after 60 min of MCAO and 48 h of reperfusion in WT mice, WT B-cell-restored μ MT^{-/-} mice, and IL-10^{-/-} B-cell-restored μ MT^{-/-} mice. As is shown in Figure 9, the percentages of both IFN- γ - and TNF- α -secreting CD3⁺ T cells were significantly increased in blood and spleen from B-cell-deficient versus WT mice, with a reduction to WT levels of these peripheral T cells in μ MT^{-/-} mice after resto-

Figure 9. Increased cytokine production by T cells after MCAO in peripheral blood and spleen of B-cell-deficient $\mu\text{MT}^{-/-}$ mice is normalized after B-cell restoration. **A, B.** CD3⁺ T cells from blood and spleen were evaluated for intracellular expression of IFN- γ (**A**) and TNF- α (**B**) after 60 min of MCAO and 48 h of reperfusion. CD3 staining was used as the initial gate for identifying T cells. Representative plots for one mouse show frequencies of IFN- γ - or TNF- α -producing T cells among total T cells within the indicated gates. Bar graphs indicate mean $\pm$ SEM percentages of T cells that produced IFN- γ or TNF- α in one representative experiment with five mice per group. Statistical analysis was performed using ANOVA followed by *post hoc* Tukey's test, respectively. Significant differences between sample means are indicated (* p < 0.05; ** p < 0.01).

ration with WT B cells, but not with IL-10^{-/-} B cells. Thus, WT B cells with the potential for IL-10 secretion limited both inflammatory cytokine production of peripheral T cells (Fig. 9) and infiltration of inflammatory T cells (Fig. 3A, B) into the MCAO-affected hemisphere during MCAO.

Discussion

Much has been learned about factors that worsen or modulate stroke severity in animal models such as MCAO. Of importance are the effects on and contribution of the immune system in MCAO. The occlusion triggers early signaling from the ischemic brain to spleen, resulting in a massive production of inflammatory factors and transmigration of splenocytes to the circulation and brain. Whereas inflammatory cells from the periphery have now been shown to contribute to CNS damage and cell death, other regulatory immune cells can reduce inflammation and limit damage within the brain. A major conundrum in the immunology of stroke is how to enhance the early immunoregulation that limits CNS inflammation while preventing excessive systemic suppression. To do this in a strategic manner requires a full understanding of the involved inflammatory and regulatory immune pathways. To this end, we evaluated regulatory B cells from the peripheral immune system that can diminish stroke lesion size and protect from neurological damage.

The results presented above demonstrate the previously unrecognized activity of WT B cells to limit infarct volume and functional neurological deficits as well as to inhibit activation and recruitment of inflammatory T cells, macrophages, and microglia into the growing CNS infarct after experimental stroke in mice. These regulatory activities were not only significantly decreased in MCAO-treated B-cell-deficient $\mu\text{MT}^{-/-}$ mice, but also were fully restored after passive transfer of WT B cells, thus implicating unequivocally the protective activity of regulatory B cells. These regulatory functions were associated with increased percentages

of IL-10-secreting CD19⁺ B cells in blood, but not IL-10-secreting T cells, including Tregs that have received much previous attention as possible immune regulators in stroke (Liesz et al., 2009). Our novel observations are the first to implicate B cells as a major regulatory cell type in stroke.

We demonstrated previously that the loss of B and T cells in SCID mice resulted in smaller infarct volumes (Hurn et al., 2007). Consistent with this concept, lymphocyte-deficient RAG-1^{-/-} mice sustained smaller infarct volumes and improved neurological deficit after MCAO (Yilmaz et al., 2006), and splenectomized rats given permanent MCAO exhibited reduced neurodegeneration and numbers of activated microglia, macrophages, and neutrophils in brain tissue (Ajmo et al., 2008). In the study by Yilmaz et al. (2006), CD4⁺ and CD8⁺ T cells contributed largely to postischemic intravascular inflammatory and prothrombotic responses in cerebral venules, and thus apparently could not account for the observed reduction in infarct volumes at 24 h postinjury. However, unlike our current results, their study did not detect improvement in MCAO outcomes in B-cell-deficient

mice, perhaps due to differences in the animal model or experimental paradigm (Yilmaz et al., 2006). Another recent study (Kleinschnitz et al., 2010) failed to observe a protective effect of adoptively transferred B cells in RAG-1^{-/-} mice treated with MCAO, a result possibly explained by the use of only 10 million transferred B cells (compared with 50 million cells used in our study) as well as the genetically induced lack of potentially neurotoxic T cells that would need to be present to observe protective B-cell effects. It is noteworthy that we observed significant changes in neurological scores after MCAO, further strengthening our observations of worsened tissue injury size in B-cell-deficient mice (Fig. 1). Both the previous studies and our present data were obtained in animals at fairly early recovery time points, when full maturation of the infarct has not yet occurred. This early window of observation is a limitation for all of these studies. We chose a 48 h recovery time to accommodate a potentially high mortality rate in the B-cell-deficient $\mu\text{MT}^{-/-}$ mice treated with a standard MCAO and to minimize survivorship effects that are present in an animal that lacks all B-cell functions.

As for regulatory T cells, there remains substantial discord. The recent report by Liesz et al. (2009) found that depletion of the CD25⁺ population with anti-CD25 mAbs significantly increased brain infarct volume and worsened functional outcome. These effects were attributed to CD4⁺CD25⁺Foxp3⁺ Tregs, even though the anti-CD25 mAbs only depleted ~50% of this Treg phenotype. On the other hand, our recent study using conditional Treg-deficient mice failed to implicate CD4⁺CD25⁺Foxp3⁺ Tregs as having protective activity against MCAO (Ren et al., 2011). It should be noted that CD25, the IL-2 receptor chain- α , has a broad expression on early progenitors of the T- and B-cell lineages, as well as on activated mature T and B cells. Thus, although our study did not demonstrate larger infarct volumes in Treg-deficient mice, it did not exclude the contribution of other regu-

latory cells. From this perspective, the data from these previous reports do not preclude our current identification of regulatory B cells in MCAO.

A key function of B cells is their secretion of IL-10 (Carter et al., 2011), an anti-inflammatory cytokine that has been studied extensively in stroke (Planas et al., 2006). IL-10-deficient mice develop larger infarcts after permanent focal ischemia (Grilli et al., 2000), whereas administration of IL-10 to the lateral ventricle (Spera et al., 1998) or intraperitoneally in combination with hypothermia (Dietrich et al., 1999), by adenoviral vectors (Ooboshi et al., 2005), after induction of mucosal tolerance by IL-10-producing MOG (myelin oligodendrocyte glycoprotein)-reactive T cells (Frenkel et al., 2005), or by transgenic overexpression of IL-10 (de Bilbao et al., 2009), all reduced infarct volume. Moreover, IL-10 was found to prevent neuronal damage induced by excitotoxicity *in vitro* (Grilli et al., 2000). In the clinic, early worsening of stroke was associated with lower IL-10 plasma levels in patients with subcortical infarcts or lacunar stroke, but not in patients with cortical lesions (Vila et al., 2003). Conversely, excessive levels of IL-10 may predispose to increased infections (Chamorro et al., 2006). Together, these findings suggest that local secretion of IL-10 may be preferable to systemic delivery. Our study demonstrated that B cells are the major producer of IL-10 in WT mice and are enriched in the blood after MCAO (Figs. 6, 7), thus providing locally secreted IL-10 that would be missing in B-cell-deficient mice.

Expression of CD19 is almost completely restricted to B cells but may also be expressed in follicular dendritic cells, with some phenotypes shared with plasmacytoid dendritic cells (Bao et al., 2011). A minor population of splenic dendritic cells expresses CD19 and has been shown to mediate aspects of T-cell suppression through IFN signaling (Mellor et al., 2005). In our study, we used a negative B-cell-sorting strategy, which depleted mature T cells, NK cells, and myeloid lineage cells by anti-CD43 antibody. Thus, the significant protective effect in stroke provided by highly purified B cells would not likely be affected by the few remaining dendritic cells or other cell types. Although we cannot rule out that other protective mediators from regulatory B cells (Bregs), such as transforming growth factor (TGF- β) (Tian et al., 2001) or direct cellular interactions, could contribute to B-cell-mediated protection, we concentrated on IL-10 as a universal protective mechanism used by Bregs. IL-10 potentially reduced infarct size in normal mice in a previous study, and our present report strongly supports the contention that B cells do not protect against ischemic injury (Fig. 8) or peripheral inflammation (Fig. 9) in the absence of IL-10.

In the adoptive transfer assay, we detected a small percentage of engrafted B cells in peripheral blood and immune organs after stroke (Fig. 4*B,C*), even though some B cells were retained in the peritoneal cavity 3 d after adoptive transfer (Fig. 4*B*). Higher engraftment has been reported previously in μ MT^{-/-} using 1.5×10^7 transferred B cells (Roth and Mamula, 1997), compared with the 5×10^7 CD19⁺ B cells used in the current study. Some of the transferred B cells in our study likely did not survive due to ischemia-induced B-cell depletion (Offner et al., 2006), although it has been reported that resting B cells may survive for at least 2 months after transfer into recipient mice (Gray, 1988). In the current study, transferred GFP⁺ B cells could not be detected in the brain (Fig. 4), which suggested that the regulatory effects of Bregs in this model could occur entirely in the periphery (Fig. 9). However, the stronger inflammatory response in CNS (Figs. 2, 3) of μ MT^{-/-} mice could be from infiltrating inflammatory cells from the periphery. Moreover, CNS microglia might

be directly or indirectly activated (Fig. 3) by inflammatory components.

Recent studies have identified a subpopulation of CD1d^{high} CD5⁺CD19⁺ “regulatory B cells,” and we also investigated changes in these CD1d^{high}CD5⁺ Bregs in stroke. To our surprise, the IL-10-secreting population was not restricted to either the CD1d^{high} or the CD5⁺ population poststroke (data not shown). We thus conclude that IL-10 is more specific than the CD1d^{high}CD5⁺CD19⁺ B-cell subset markers for identification of Bregs in stroke. In conclusion, our study provides new insights into the endogenous inflammatory response after acute brain ischemia. Specifically, we have described a previously unknown role for B cells as cerebroprotective immunomodulators after stroke, a function that affects diverse cytokine-dependent and cellular inflammatory targets through the anti-inflammatory effects of IL-10.

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