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Intrastriatal B-cell administration limits infarct size after stroke in B-cell deficient mice

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Abstract

Recent evidence emphasizes B-cells as a major regulatory cell type that plays an important role in limiting the pathogenic effects of ischemic stroke. The aim of the current study was to extend this initial observation to specifically examine the infiltration of regulatory B-cells and to determine if the effect of B-cells to limit the inflammatory response to cerebral ischemia is mediated by their action centrally or peripherally. Our data demonstrate the increased presence of a regulatory B-cell subset in the affected hemisphere of wild-type mice after middle cerebral artery occlusion (MCAO). We further explored the use of a novel method of stereotaxic cell delivery to bypass the blood brain barrier (BBB) and introduce CD19⁺ B cells directly into the striatum as compared to peripheral administration of B-cells. Infarct volumes after 60 minutes of MCAO and 48 hours of reperfusion were determined in B-cell deficient μ MT^{-/-} mice with and without replacement of either B-cells or medium. Infarct size was significantly decreased in cerebral cortex after intrastriatal transfer of 100,000 B-cells to μ MT^{-/-} mice vs. controls, with a comparable effect on infarct size as obtained by 50 million B-cells transferred intraperitoneally. These findings support the hypothesis that B-cells play a protective role against ischemic brain injury, and suggest that that B-cells may serve as a novel therapeutic agent for modulating the immune response in central nervous system inflammation after stroke.

Keywords

regulatory B-cells; experimental stroke; intrastriatal transfer; uMT mice

INTRODUCTION

The devastating neuropathological impact of ischemic stroke in experimental and clinical stroke is augmented by systemic activation of inflammatory immune cells that infiltrate the developing lesion (Ajmo et al. 2008; Campanella et al. 2002; Gee et al. 2007; Offner et al.

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2006a; Yilmaz et al. 2006). However, there is renewed interest in the protective role of regulatory T- and B-cells that can *suppress* inflammation and limit central nervous system damage induced by infiltrating pro-inflammatory cells (Carter et al. 2011; Akiyoshi et al. 2011; Frenkel et al. 2005; Hurn et al. 2007; Liesz et al. 2009). We recently turned our focus towards regulatory B-cells known to mediate protection against other inflammatory CNS conditions (Mann et al. 2007; Matsushita et al. 2010; Yanaba et al. 2008) and found that B-cell deficient $\mu\text{MT}^{-/-}$ mice developed larger infarct volumes, higher mortality and more severe functional deficits compared to wild-type (WT) mice (Akiyoshi et al. 2011). Moreover the B-cell deficient mice had increased numbers of activated T-cells, macrophages, microglial cells and neutrophils in the affected brain hemisphere. MCAO-induced changes were completely prevented in B-cell-replaced mice after transfer of highly purified WT, but not IL-10-deficient B-cells, thus implicating IL-10-secreting B-cells as a major regulatory cell type in stroke (Akiyoshi et al. 2011; Offner and Hurn 2012).

In our previous study, we found that intraperitoneal (i.p.) transfer of 50 million B-cells one day prior to MCAO reduced infarct size to a level similar to that of wild-type (WT) mice when lesions were assessed 48 h after 60 min MCAO (Akiyoshi et al. 2011). Surprisingly, however, when GFP⁺ B-cells were transferred i.p., we were unable to detect them in either the ipsilateral or contralateral hemispheres of the brain, suggesting that the regulatory effect occurred mainly in the periphery. Interestingly, about 30% of the transferred B-cells remained in the peritoneal cavity and only a small percentage of the B-cells (~0.3–1.5%) migrated into blood, spleen and lymph nodes. These results raised questions as to whether protection by B cells is mediated by their action in CNS or the periphery. They also raised concerns about the ability of the i.p. transferred B-cells to migrate freely to all tissues and whether regulatory B-cells might be more efficient in suppressing infarct development if injected directly into the brain. We thus evaluated the presence of the B10 CD19⁺CD1d^{hi}CD5⁺ regulatory B-cell subset in the brain during MCAO in WT mice and the ability of enriched populations of IL-10-competent B-cells to reduce infarct size when injected directly into the striatum just prior to induction of MCAO. The results of our study clearly establish that intrastriatal injection of relatively small numbers of B-cells can dramatically reduce infarct size in the cortex, thus implicating local as well as systemic regulatory effects of the transferred B-cells.

MATERIALS AND METHODS

Animals

B-cell deficient $\mu\text{MT}^{-/-}$ mice on a C57BL/6J background were bred at the VA Animal Resource Facility. Age-matched 8 – 12 week old male C57BL/6J mice (WT, the Jackson Laboratory, Bar Harbor, ME, USA) were used as WT control mice for MCAO induction. Green fluorescent protein male mice (GFP⁺) mice on a C57BL/6J background (bred at the VA animal facility) and C57BL/6J mice (the Jackson Laboratory, USA) were used as B-cell donors to restore B-cell function in B-cell deficient $\mu\text{MT}^{-/-}$ 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.

Middle Cerebral Artery Occlusion Model

Transient focal cerebral ischemia was induced using reversible middle cerebral artery occlusion (MCAO) via the intraluminal filament technique as previously described (Akiyoshi et al. 2011; Zhu et al. 2010) with modifications under isoflurane (induction 5.0% and maintenance 1.5–2.0%) followed by 48 h of reperfusion. Head (temporalis muscle) and body (rectal) temperatures were controlled at $36.3 \pm 0.9^\circ\text{C}$ (mean $\pm$ SD) throughout MCAO

surgery and during manipulations to induce reperfusion with warm water pads and heating lamp. A laser Doppler probe (Model DRT4, Moor Instruments Ltd., Oxford, England) was affixed to the right side of the skull at the mid-point of ear to eye distance to monitor cortical perfusion and verify vascular occlusion and reperfusion. A 2 cm incision was opened in the middle of the anterior neck. After the right common carotid artery was ligated with 6-0 silk (ETHICON, Inc., Somerville, NJ, USA), a 6-0 ETHILON nylon monofilament (ETHICON, Inc., Somerville, NJ, USA) with a heat-blunted silicone-coated tip (230–250 μ m diameter) was inserted into the right internal carotid artery via the external carotid artery until the laser Doppler flowmetry (LDF) value dropped to <30% of baseline. In Sham MCAO groups, the filament was placed within the internal carotid artery but not advanced to achieve MCAO.

Neurologic deficit scores were determined during ischemia and at 24 h and 48 h of reperfusion to confirm ischemia and to assess equivalence of ischemic injury, respectively, rather than to determine specific functional outcomes according to the following graded scoring system: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to the affected side; 3, unable to bear weight on the affected side; and 4, no spontaneous locomotor activity or barrel rolling. After occlusion was achieved, the incision was sutured with 6-0 Vicryl sutures (ETHICON, Inc., Somerville, NJ, USA), anesthesia was discontinued, and each mouse was then placed in a separate cage with a warm water pad and heating lamp. At the end of the ischemia period (60 min), the mice were briefly re-anesthetized, the laser Doppler probe was repositioned over the same site on the skull, and the occluding filament was withdrawn for reperfusion. Mice were then allowed to recover and were observed according to the experimental protocol.

Infarct Volume Analysis

Brains were collected at 48 h reperfusion for infarct volume analysis. Each mouse brain was cut into five 2 mm-thick coronal sections. Slices were then incubated in 1.2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St Louis, MO, USA) for 10 min for each side, fixed in 10% formalin overnight, and then photographed with a digital camera. Images were analyzed in a masked fashion with SigmaScan Pro 5.0 Software (Systat, Inc., Point Richmond, CA). Cortical and striatal infarction volumes were determined through subtraction of the ipsilateral noninfarcted volume of the structure of interest from the volume of the contralateral structure. This value was then divided by the volume of the contralateral structure and multiplied by 100 to yield the infarction volume as a percent of the contralateral structure.

Isolation of B-cells from Male Mice

Splenic CD19⁺ B-cells were purified using paramagnetic bead-conjugated antibodies (Abs) and AutoMACS (Miltenyi Biotec). CD19⁺ B-cells, isolated from WT or GFP⁺ donor mice (positive fraction) by a CD19 cell isolation kit, were suspended in RPMI 1640 medium at a concentration of 100,000 cells/2 μ L and injected into the right striatum of μ MT^{-/-} mice. The purity of the isolated B cells was 95%.

Stereotaxic Injections

A novel method of stereotaxic cell delivery was used to directly inject CD19⁺ B-cells into brain tissue and bypass the blood brain barrier (BBB). Mice were anesthetized under isoflurane anesthesia and placed in a stereotaxic apparatus (Model 1900 Stereotaxic Alignment System, David Kopf Instruments, Tujunga, CA). After midline incision of the scalp, the vertex area was exposed, and a small hole was drilled according to the following coordinates: 0.5 mm anterior to the bregma, 2.0 mm right lateral to the midline. A stainless steel cannula with a 30-gauge needle on a 2 μ L Hamilton syringe was inserted stereotactically through the hole to reach right striatum (3.4 mm below the skull). A solution

containing 100,000 CD19⁺ B-cells or RPMI 1640 medium in 2 μ L was injected at a rate of 0.25 μ L/min. The skin above the injection site was closed using tissue adhesive (3MTM VetbondTM Tissue Adhesive 1469SB, Fischer Scientific). Mice were monitored for 2 to 4 h after recovery from anesthesia.

Immunofluorescent Staining of B-cells

Mononuclear cells were isolated from ipsilateral and contralateral brain hemispheres after 60 min MCAO and 48 h reperfusion, labeled with anti-CD19, anti-CD1d and anti-CD5 monoclonal antibodies (mAb) and evaluated by FACS for CD19⁺ B-cells and for the CD1d^{hi}CD5⁺ B10 B-cell subset as described (Yanaba et al. 2008). 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-beta-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 5h. 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 mAb 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 intrastriatal injections of GFP⁺ B-cells after 60min MCAO and 48 hours reperfusion. Tissues were fixed with 4% buffered formalin, paraffin embedded and sectioned. Sections were incubated with anti-GFP (Cell Signaling), followed by incubation with secondary biotinylated antibody (goat anti-rabbit, Cell Signaling) and staining with VECTASTAIN ABC Peroxidase Kit (Vector) and 3', 3'-diaminobenzidine (Sigma-Aldrich). Nuclear staining was carried out with hematoxylin (Sigma-Aldrich).

Statistical Analysis

Data were reported as means $\pm$ SEM. Statistical analyses were performed using one-way ANOVA with post-hoc Newman-Keuls test or Student's *t* test for comparison of infarct volumes. P-values <0.05 were considered statistically significant.

RESULTS

Presence of Regulatory B-cells in MCAO Lesions May Limit Infarct Size

Our previous report demonstrated that infarct volume after MCAO was significantly increased in B-cell deficient μ MT^{-/-} mice, thus implicating B-cells in limiting CNS damage from stroke (Akiyoshi et al. 2011). A small but powerful subset of B-cells, called B10, has been described recently in other inflammatory diseases and might also play a key role in limiting inflammation and infarct volume in stroke (Yanaba et al. 2008). B10 cells express the CD1d^{hi}CD5⁺ phenotype and secrete high levels of the anti-inflammatory cytokine, IL-10, that can inhibit glial cell neurotoxicity and improve stroke outcomes (de Bilbao et al. 2009; Grilli et al. 2000; Planas et al. 2006). To determine if B10 cells might be present in the developing brain infarct after MCAO, we evaluated total CD19⁺ B-cells and the B10 cell subtype in the CNS from MCAO- and Sham MCAO-treated C57BL/6 mice. Although total

B-cells in the CNS were only modestly affected by MCAO (Fig. 1A), CD1d^{hi}CD5⁺ B10 cells were highly enriched in the ipsilateral (right) vs. contralateral (left) hemispheres of MCAO mice 48 h after a 60 min occlusion (36.7% vs. 6.3% of CD19⁺ B-cells, Fig. 1B). In contrast, no difference was observed in the corresponding hemispheres of Sham MCAO-treated mice (13.4% vs. 14.8%, Fig. 1B), thus establishing the local presence of the B10 B-cell subset as a potentially important regulatory element during MCAO lesion formation.

Intrastriatal Injection of B-cells

To validate intrastriatal injection and determine longevity of the injected cells, 100,000 GFP⁺CD19⁺ B-cells were injected in 2 μ L directly into the striatum of WT C57BL/6J mice and analyzed in brain histological sections by fluorescent microscopy. As shown in Fig. 2, the injected B-cells were indeed localized to the striatum and could be observed in the tissue for a period of at least 7 days.

Direct Injection of IL-10 Competent B-cells into the Striatum Limits Cortical Infarct Size More Efficiently than Intraperitoneal B-cell Transfer

Having established a suitable method, CD19⁺ B-cells were isolated and enriched to >95% purity from non-GFP⁺ WT donors and evaluated for their potential to secrete IL-10 prior to their injection into the striatum of recipient WT mice. As is shown in Fig. 3, the B-cell population indeed had enhanced production of IL-10. This cell population of B-cells or medium alone was then loaded into a Hamilton syringe and 100,000 B-cells or 2 μ L medium were injected into the striatum of B-cell deficient μ MT^{-/-} mice 1 day prior to induction of 60 min MCAO followed by 48 h reperfusion. As is shown in Fig. 4, B-cell transferred mice had significantly smaller cortical infarct size (measured as % of contralateral cortex after correction for edema) compared to medium-transferred or control (no transfer) mice. In contrast to intrastriatal injection, the i.p. transfer route (Figure 5) required 50 million GFP⁺ B-cells from WT (unimmunized) donors to B-cell deficient μ MT^{-/-} mice to achieve a statistically significant reduction in cortical infarct using the same protocol (transfer 1 day prior to 60 min MCAO followed by 48 h reperfusion), thus demonstrating increased efficacy of B-cell dependent protection using the intrastriatal route of injection.

DISCUSSION

The data presented above demonstrate clearly that regulatory B10-cells are enriched in the ipsilateral hemisphere during MCAO and that intrastriatal injection of only 100,000 CD19⁺ B-cells produced an equivalent or greater protective effect, limiting stroke lesion size, than 50 million B-cells given by i.p. injection. The fact that the injected B-cells did not exacerbate stroke severity even though they were not specifically enriched for B10-cells or other regulatory B-cell subtypes indicates that the default native mixture of B-cells from unimmunized WT mice favors immunoregulation over inflammation. Moreover, because the source of both intrastriatal and i.p. injections involved sorted CD19⁺ B-cells that were not further enriched, the data strongly support a >500-fold increase in potency of the regulatory B-cells given by the intrastriatal route. This result is reflected by the lack of detectable GFP⁺ B-cells observed in the brains of MCAO mice after i.p. transfer of B-cells (Akiyoshi et al. 2011), which could be the result of ischemia-induced apoptosis in the periphery (Offner et al. 2006b) or impaired trafficking of B-cells from the peritoneal cavity to the brain after MCAO. Moreover, the low frequency of regulatory B-cells in the i.p. injected CD19⁺ fraction (B10 cells ~1–2%, (Yanaba et al. 2008)) could further limit detection of these cells in the brain.

The increased potency of regulatory effects enabled by intrastriatal injection of B-cells represents a major technical step toward the identification of cellular subtypes that can influence lesion size in stroke due to the requirement for relatively small cell numbers. Our preliminary studies have established that the B10 subset constitutes only 0.5–0.7% of CD19⁺ B-cells isolated from unimmunized WT mice, with similar percentages expected for other IL-10-secreting B-cell populations including the T2-MZ and B1a subsets (DiLillo et al. 2010). Given the administration of 100,000 WT CD19⁺ B-cells, the actual number of intrastriatally injected B10 cells would be only 500–700 cells. However, it is well accepted that IL-10 introduced into the CNS can reduce infarct volume (Carter et al. 2011; Planas et al. 2006), so perhaps a more accurate estimation of regulatory cell numbers might be those that can secrete IL-10 ($7.35\% = 7,350$ IL-10⁺ CD19⁺ B-cells, Fig. 3). Recovery of such small numbers of regulatory B10 cells, as well as comparable numbers of other regulatory B-cell subtypes from individual mouse donors is feasible and should allow definitive assessment of their respective contributions to limiting stroke lesion development in B-cell deficient mice. Moreover, intrastriatal transfer of even larger numbers of highly enriched regulatory B-cell subsets should enable a determination of the potential of B-cell therapy to *reduce* infarct size in WT B-cell sufficient mice with MCAO.

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Figure 1. Characterization of B-cells and B10 cells in brains of Sham MCAO and MCAO mice (*A*) A relatively low percentage of CD19⁺B cells are observed in the brains of Sham MCAO and MCAO C57BL/6J mice. (*B*) Regulatory B10-cells (CD19⁺ CD1d^{hi}CD5⁺) are highly enriched in the ipsilateral (right) brain hemisphere after 60 min MCAO and 48 h reperfusion.

Figure 2. Transfer of GFP⁺ B cells from male GFP mice into the striatum of WT C57BL/6J recipient mice

Splenic CD19⁺ B cells were isolated from GFP⁺ mice, enriched using paramagnetic bead-conjugated antibodies and AutoMACS (Miltenyi Biotec) to >95% purity and injected into striatum of C57BL/6J mice (100,000 cells/2 μ L). Brains of recipient mice were perfused and tissue was isolated at the indicated time points after injections for histological evaluation. Tissues were fixed with 4% buffered formalin, paraffin embedded and sectioned. Slides were analyzed by fluorescent microscopy (Olympus BX40, Japan).

Figure 3. B cells from spleens of naïve mice secrete IL-10 after activation ex vivo
CD19⁺ B cells from spleens of naïve C57BL/6J donor mice were AUTOMACS sorted, cultured with LPS/PMA/Ionomycin for 5 hours and evaluated for IL-10 secretion by flow cytometry.

Figure 4. Stereotaxic transfer of CD19⁺ B cells reduces infarct volume of $\mu\text{MT}^{-/-}$ B-cell-deficient mice

(A) Stereotaxic transfer of 100,000 CD19⁺ B cells in 2 μL resulted in reduced infarct volume (mean $\pm$ SEM) in cortex but not striatum at 48 h of reperfusion after 60 min MCAO. Statistical analysis was performed using one-way ANOVA with post-hoc Newman-Keuls test. Infarct volume was decreased in cerebral cortex in B-cell-transferred $\mu\text{MT}^{-/-}$ mice (n=11) compared to medium-transferred $\mu\text{MT}^{-/-}$ mice (n=11, * p <0.05) or to control $\mu\text{MT}^{-/-}$ mice (n=10, ** p <0.01) while cortical infarct volume was comparable between control and medium-transferred $\mu\text{MT}^{-/-}$ mice (p =0.374). No differences were seen in striatal infarct volume between the treatment groups (p =0.342). (B) Representative TTC-stained brains from B-cell-transferred, medium transferred and control $\mu\text{MT}^{-/-}$ mice.

Figure 5. Intraperitoneal transfer of GFP⁺ CD19⁺ B-cells reduces infarct volume of $\mu\text{MT}^{-/-}$ B-cell-deficient mice

Transfer of 50 million GFP⁺CD19⁺ B-cells resulted in reduced infarct volume (mean $\pm$ SEM) in cortex but not striatum at 48 h of reperfusion after 60 min MCAO. Statistical analysis was performed using Student's *t* test. Note significant difference of cortical (**p*<0.05) but not striatal (*p*=0.354) infarct volumes between groups receiving B-cells (*n* = 7) vs. untreated control mice (*n* = 10).