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## Neutralizing Anti-Interleukin-1 $\beta$ Antibodies Reduce Ischemia-Related Interleukin-1 $\beta$ Transport across the Blood-Brain Barrier in Fetal Sheep

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### Abstract

Hypoxic ischemic insults predispose to perinatal brain injury. Pro-inflammatory cytokines are important in the evolution of this injury. Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a key mediator of inflammatory responses and elevated IL-1 $\beta$  levels in brain correlate with adverse neurodevelopmental outcomes after brain injury. Impaired blood-brain barrier (BBB) function represents an important component of hypoxic-ischemic brain injury in the fetus. In addition, ischemia-reperfusion increases cytokine transport across the BBB of the ovine fetus. Reducing pro-inflammatory cytokine entry into brain could represent a novel approach to attenuate ischemia-related brain injury. We hypothesized that infusions of neutralizing IL-1 $\beta$  monoclonal antibody (mAb) reduce IL-1 $\beta$  transport across the BBB after ischemia in the fetus. Fetal sheep were studied 24-h after 30-min of carotid artery occlusion. Fetuses were treated with placebo- or anti-IL-1 $\beta$  mAb intravenously 15-min and 4-h after ischemia. Ovine IL-1 $\beta$  protein expressed from IL-1 $\beta$  pGEX-2T vectors in *E. Coli* BL-21 cells was produced, purified, and radiolabeled with <sup>125</sup>I. BBB permeability was quantified using the blood-to-brain transfer constant ( $K_i$ ) with <sup>125</sup>I-radiolabeled-IL-1 $\beta$ . Increases in anti-IL-1 $\beta$  mAb were observed in the brain of the mAb-treated group ( $P < 0.001$ ). Blood-to-brain transport of <sup>125</sup>I-IL-1 $\beta$  was lower ( $P < 0.04$ ) across brain regions in the anti-IL-1 $\beta$  mAb treated than placebo-treated ischemic fetuses. Plasma <sup>125</sup>I-IL-1 $\beta$  counts were higher ( $P < 0.001$ ) in the anti-IL-1 $\beta$  mAb

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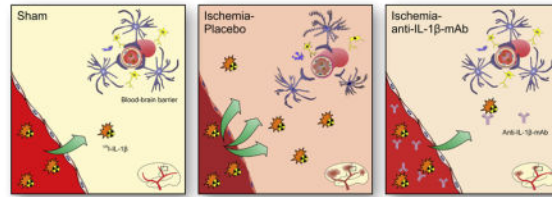
### DISCLOSURE/CONFLICT OF INTERESTS

Yow-Pin Lim is employed by ProThera Biologics. All other authors have no duality or conflicts of interests to declare.

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than placebo-treated ischemic fetuses. Systemic infusions of anti-IL-1 $\beta$  mAb reduce IL-1 $\beta$  transport across the BBB after ischemia in the ovine fetus. Our findings suggest that conditions associated with increases in systemic pro-inflammatory cytokines and neurodevelopmental impairment could benefit from an anti-cytokine therapeutic strategy.

## Graphical Abstract



## Keywords

antibodies; blood-brain barrier; cytokines; Interleukin-1 $\beta$ ; fetus; hypoxia-ischemia; sheep

## INTRODUCTION

Hypoxic-ischemic brain injury represents a major component of neurologic abnormalities originating in the perinatal period (Volpe, 2012). Insults to the brain before and after birth can impair normal developmental processes in the brain and result in long-term neurodevelopmental impairment including developmental delays, cerebral palsy, and seizure disorders (Vannucci and Perlman, 1997).

The BBB is a selective diffusion barrier composed of highly specialized endothelial cells interconnected by an intricate network of basal lamina containing pericytes and perivascular antigen presenting cells (Abbott et al., 2010). This barrier separates the brain parenchyma from the systemic circulation, thereby preserving and maintaining a normal physiologic environment critical for brain function. We have previously demonstrated that impaired BBB function is an important component of hypoxic ischemic brain injury in the ovine fetus (Chen et al., 2012).

Pro-inflammatory cytokines are important mediators in pathways associated with perinatal brain injury caused by a variety of insults (Leviton et al., 2011, McAdams and Juul, 2012). Interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- $\alpha$ ) are cytokines with both anti-inflammatory and pro-inflammatory properties that have been implicated as essential for brain development but also as critical factors contributing to the initiation and propagation of inflammatory signals in the brain secondary to hypoxic-ischemic injury (Pantoni et al., 1998, Ferriero, 2004). Systemic inflammatory states such as sepsis, necrotizing enterocolitis and prolonged mechanical ventilation are known to be associated with adverse neurodevelopmental outcomes in preterm and full term neonates (Stoll et al., 2004, Walsh et al., 2005, Bose et al., 2013). It has long been postulated that pro-inflammatory cytokines originating in the systemic circulation could cross from the circulation into the brain through the BBB to injure the brain (Dammann and Leviton, 1997).

However, definitive experimental evidence was lacking, particularly in immature subjects, until our recent work (Sadowska et al., 2015).

IL-1 $\beta$  is an important pro-inflammatory cytokine that contributes to hypoxic-ischemic brain injury (Rothwell, 1999). It is a key mediator of the inflammatory cascade, results in apoptosis, and is activated by the caspase biological pathway (Thornton et al., 2006, Brough and Rothwell, 2007). There is growing evidence to suggest that IL-1 $\beta$  is important in various inflammatory states and neurodegenerative disorders in the CNS (Ginsberg, 2003). There could be several potential sources of IL-1 $\beta$  in the brain such as blood borne or endogenously produced IL-1 $\beta$  originating within the brain from neurons, microvascular endothelial cells, astrocytes, microglia and oligodendrocyte progenitor cells (Vela et al., 2002). Increased IL-1 $\beta$  expression in astrocytes has been reported in periventricular leukomalacia in neonatal human and rat brain (Kadhim et al., 2001). IL-1 $\beta$  protein expression is also increased in the cerebral cortices after ischemic injury in the ovine fetus (Sadowska et al., 2012). Elevated levels of serum IL-1 $\beta$  have been observed in encephalopathic infants with abnormal neurodevelopmental outcomes (Bartha et al., 2004). Furthermore, direct intracerebral injections of IL-1 $\beta$  protein result in brain damage (Cai et al., 2004) and intracerebroventricular injections of IL-1 receptor antagonists reduce caspase 3 activity, decrease apoptosis and reduce brain damage after exposure to hypoxic-ischemic insults in young rats (Hu et al., 2005).

We have recently generated and purified anti-ovine IL-1 $\beta$  monoclonal antibody (mAb) using reported methodologies (Rothel et al., 1997), and confirmed its sensitivity, specificity, and neutralizing capacity against ovine IL-1 $\beta$  *in vitro* (Chen et al., 2013). In addition, ovine IL-1 $\beta$  protein is transported across the BBB of the adult mouse brain (Threlkeld et al., 2010). Systemic infusions of this neutralizing IL-1 $\beta$  mAb results in penetration of the mAb into brain and attenuation of the ischemia-related endogenous increases in IL-1 $\beta$  protein concentrations in the brain suggesting that the anti-IL-1 $\beta$  mAb infusions have important specific biological effects upon the IL-1 $\beta$  levels after ischemia in fetal brain (Chen et al., 2015). Furthermore, we have recently shown that systemically produced IL-1 $\beta$  is able to cross the fetal BBB (Sadowska et al., 2015). Therefore, a novel approach to perinatal brain injury could be the use of an agent that could reduce the transfer of the systemic cytokines across the fetal BBB.

We have used a preclinical translational fetal sheep model with ischemia reperfusion related brain injury (Gunn et al., 1997). The neurodevelopmental maturity of fetal sheep at 127 days of gestation is approximately similar to that of the near term human fetus (Back et al., 2012). This makes the fetal sheep a very useful model to study inflammatory processes related to perinatal hypoxic-ischemia brain injury (Hutton et al., 2007, Jellema et al., 2013). The objective of the current study was to test the hypothesis that systemic intravenous infusions of neutralizing anti-IL-1 $\beta$  mAb decrease IL-1 $\beta$  cytokine transport across the BBB after ischemia in the fetus.

## EXPERIMENTAL PROCEDURES

All procedures were approved by the Institutional Animal Care and Use Committees of The Alpert Medical School of Brown University and Women & Infants Hospital of Rhode Island, and in accordance with the National Institutes of Health Guidelines for the use of experimental animals.

### Surgical preparation of animals, experimental groups, and study design

Surgery was performed on 10 mixed breed ewes at 119–121 days of gestation (full term = 148–150 days). The surgical techniques have been previously described in detail (Stonestreet et al., 1993, Gunn et al., 1997). The ewes were anesthetized by an intravenous injection of ketamine (10 mg/kg, Putney, Inc. Portland, ME, USA) before intubation and general anesthesia maintained with 2–3% isoflurane in oxygen. In brief, a midline incision was made to expose the uterus, and the fetus was partially exposed for instrumentation. Polyvinyl catheters were placed into brachial vein for placebo or mAb and isotope administration. Catheters were also placed in fetal brachial artery for blood sampling, heart rate, and blood pressure monitoring. An amniotic fluid catheter was placed as a referent for fetal arterial blood pressures.

The fetal carotid arteries were exposed the lingual arteries and vertebral-occipital anastomoses ligated to restrict non-cerebral and vertebral blood flow to the brain (Gunn et al., 1997). Two inflatable 4-mm vascular occluders (In Vivo Metric, Healdsburg, CA, USA) were placed around each carotid artery in addition to perivascular ultrasonic flow probes (Transonic Systems Inc., Ithaca, NY, USA) caudal to the occluders.

After surgery, the ewes were individually housed in cages in a 12 h light dark cycled room with four cages per room. The ewes had ad libitum access to food and water and were given Ampicillin 1 g (Mylan Laboratories, Rockford, IL, USA) and Gentamicin 130 mg (MWI Veterinary Supply, Boise, ID, USA) intramuscularly for 3 days after surgery. The fetal catheter patency was maintained by flushing with heparinized saline (10 U/ml) and filling the catheters with heparin (1000 U/ml) every other day. The fetal sheep were studied after 6–7 days of recovery from surgery at 125–128 days of gestation. The fetuses in this study were approximately 85 percent of full term sheep gestation at the time of study as the duration of gestation in this breed of sheep is typically 148–150 days. The sheep were randomly assigned to placebo-ischemic (n=5) and anti-IL-1 $\beta$  mAb ischemic groups (n=5). In a commitment to limit unnecessary experimentation on this large animal resource, we also included results from five instrumented non-ischemic control sham fetal sheep from our previous work (Sadowska et al., 2015). The inclusion of these fetal sheep was justified because the studies were performed in the same laboratory, by the same team, over a contemporary period using the same experimental methodology and procedures as in the current study.

The design of our study is shown schematically in Fig. 1. After obtaining baseline determinations of physiologic variables, brain ischemia was induced by inflation of the carotid artery occluders with 0.154M NaCl for 30-minutes. Thereafter, the occluders were deflated and reperfusion continued for 24 hours and twenty minutes. Anti-IL1 $\beta$  mAb

(4.7±0.2 mg/kg, mean±SEM) or equivalent volumes of placebo (0.154 M NaCl, 15 ml) were infused intravenously 15 minutes and 4 hours after initiation of brain ischemia. The initial infusions of the anti-IL-1 $\beta$  mAbs or placebo were given over 2-hours beginning 15 minutes after ischemia. Additional infusions of anti-IL-1 $\beta$  mAbs or placebo were also given over 2 hours beginning 4 hours after ischemia. The infusion paradigm was designed to achieve early-sustained increases in systemic mAb levels in order to expose the cerebral microvasculature to the mAb for a prolonged time interval after ischemia and before the onset of BBB permeability studies (Chen et al., 2015). The BBB permeability studies were performed over 20 minutes 24 hours after the onset of brain ischemia with <sup>125</sup>I-IL-1 $\beta$  as the blood-to-brain tracer. The radiolabeled protein was rapidly injected intravenously and the arterial plasma isotope concentrations measured sequentially before and after the injection of <sup>125</sup>I-IL-1 $\beta$  during the 20-minute BBB study. Brain vascular volumes were determined with technetium-99m (<sup>99m</sup>Tc, Cardinal Health, RI, USA) radiolabeled red blood cells (RBCs) given to the fetus two minutes before the end of the studies (Chen et al., 2012). Briefly, 4 ml of whole blood obtained from the ewe was incubated with Tin-pyrophosphate for 15 min and <sup>99m</sup>Tc for an additional 15 minutes. The <sup>99m</sup>Tc-labeled RBCs were washed, resuspended in 0.154 M NaCl to a volume of 4 ml, and administered intravenously to the fetus. The efficiency of radiolabeling of the RBCs exceeded 99%.

At the end of the studies, both the ewe and fetus were sacrificed with intravenous pentobarbital (100–200 mg/kg). The fetal brain was quickly removed and the fetal body and brain were weighed. One half of the fetal brain was dissected as previously described for regional BBB permeability analysis (Stonestreet et al., 1996, Chen et al., 2015, Sadowska et al., 2015). The remainder of the brain was frozen for determination of anti-IL-1 $\beta$  mAb concentrations in selected areas of the cerebral cortex.

### Physiological determinations

Fetal pH, blood gases, heart rate, mean arterial blood pressure, hematocrit and carotid arterial blood flow values were measured at baseline, at the end of brain ischemia, and sequentially after brain ischemia as previously reported (Chen et al., 2012). Blood gases and pH were measured on a blood gas analyzer (model 248, Siemens, Washington, DC, USA) corrected to 39.5°C. Carotid arterial blood flow was measured by flow probes (Small Animal Blood Flow Meter: T206, Transonic Systems Inc. Ithaca, NY, USA) connected to a PowerLab Data Acquisition System (Colorado Springs, CO, USA) in order to ascertain that carotid blood flow was reduced during ischemia. Carotid arterial blood flow and cerebral blood flow are closely related even though carotid flow does not directly reflect cerebral blood flow (van Bel et al., 1994). Carotid arterial blood flow was calculated as a sum of left and right carotid arterial blood flow averaged over 5 min at each measurement time and reported as ml/min.

### IL-1 $\beta$ protein production and radiolabeling

Recombinant ovine IL-1 $\beta$  protein encoded by pGEX-2T vectors [Commonwealth Scientific and Industrial Research Organization (CSIRO) Industries, Victoria, Australia] was generated and purified using previously described methods (Frangioni and Neel, 1993, Rothwell, 1999, Chen et al., 2013) in *E. Coli* BL-21 cells with few additional modifications. The protein was

further purified with Diethylaminoethyl-Convective Interaction Media (DEAE-CIM) anion-exchange monolithic resins (BIASeparations, Villach, Austria) and TSKgel G3000SW Size Exclusion chromatographic column (Tosoh Bioscience, King of Prussia, PA, USA). We have previously confirmed the biological activity of the ovine IL-1 $\beta$  protein by showing that this protein stimulates the expression of the downstream transcription factor NF- $\kappa$ B in cell culture and pre-incubation of the IL-1 $\beta$  protein with anti-IL-1 $\beta$  mAbs reduced the effects of IL-1 $\beta$  on NF- $\kappa$ B expression in mononuclear cells (Frangioni and Neel, 1993, Rothwell, 1999, Chen et al., 2013).

Purified IL-1 $\beta$  protein was labeled radioactively with iodine-125 (<sup>125</sup>I-IL-1 $\beta$ ) using the chloramine-T method (Banks et al., 1991, Sadowska et al., 2015). Two mCi of <sup>125</sup>I-, 5  $\mu$ g of IL-1 $\beta$  protein, and 10  $\mu$ L of 1 mg/ml chloramine-T (Sigma-Aldrich, ST. Louis, MO) were combined and mixed. The reaction was terminated with 10  $\mu$ L of 10 mg/ml sodium metabisulfite (Sigma, ST. Louis, MO, USA) after 60 seconds and the iodinated protein was separated from the free iodine on a PD-10 desalting column (GE Healthcare, Newark, NJ, USA). The percent of the <sup>125</sup>I-radiolabel tightly bound to the purified ovine IL-1 $\beta$  protein was calculated by an acid precipitation assay using trichloroacetic acid (Banks et al., 1991, Sadowska et al., 2015). The efficiency of iodine-125 radioactive labeling of IL-1 $\beta$  protein was calculated as the percent of the labeled iodinated IL-1 $\beta$  protein contained in the acid precipitated protein pellet and was determined according to the following equation (Banks et al., 1991, Sadowska et al., 2015):

$$\text{Efficiency of radioactive labeling (percent acid precipitation)} = \frac{\text{Pellet CPM}}{(\text{Pellet CPM} + \text{Supernatant CPM})} \times 100.$$

The radioactive labeling efficiency, *e.g.* percent of acid precipitation, exceeded 98 percent in the preparations that were used for the ischemic placebo and ischemic mAb treated groups. The integrity of the iodine-125 radioactive labeled IL-1 $\beta$  protein in the fetal plasma remained greater than 85% fifteen minutes after intravenous administration. The calculated specific activity of the <sup>125</sup>I-IL-1 $\beta$  was 200 Ci/g.

### Anti-IL-1 $\beta$ mAb production and purification

Ovine anti-IL-1 $\beta$  mAb was generated using mouse hybridoma cells as previously described (Rothel et al., 1997) and purified using techniques that we reported (Chen et al., 2013) with some additional modifications. The immunoglobulin G (IgG) from the anti-IL-1 $\beta$  mAb was purified from cell culture supernatants by affinity chromatography on Protein G Sepharose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.3) and neutralized to pH 7.4 by adding 1 M Tris buffer. The eluted antibodies were passed through an anion-exchange column (CIMmultus QA, BIASeparations, Wilmington, DE, USA) to remove potential endotoxin contamination. Bound antibodies were eluted from the column by a buffer containing 200 mM NaCl, whereas the endotoxin was retained on the column. Finally, the antibody solution was concentrated using a Millipore ultrafiltration device with 30-kDa exclusion membrane and passed to 0.2-micron syringe sterile filter (Millipore Corp., Chicago, IL, USA) (Chen et al., 2015). The pGEX-2T vectors and mouse hybridoma cells were kindly provided by the



Commonwealth Scientific and Industrial Research Organization (CSIRO, Livestock Industries, Victoria, Australia).

### **Indirect ELISA for measurement of anti-IL-1 $\beta$ mAb in fetal plasma and cerebral cortex**

An indirect ELISA was used to detect anti-IL-1 $\beta$  mAb concentrations in fetal plasma and in the cerebral cortex using previously reported procedures (Chen et al., 2015). A portion of the cerebral cortex was obtained in order to measure the anti-IL-1 $\beta$  mAb neutralizing antibody concentration. The concentrations of anti-IL-1 $\beta$  mAb could not be measured in all of the brain regions, in which BBB permeability was measured, because the remaining quantity of residual tissue was not sufficient for analysis in the smaller brain regions.

The tissue was prepared to measure parenchymal anti-IL-1 $\beta$  mAb uptake as follows. Frozen brain tissue (0.53–0.64 g) was placed in 1 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 mM benzethonium chloride (Sigma-Aldrich, Inc., St. Louis, MD, USA), 0.1% sodium-dodecyl-sulfate (SDS, Bio-Rad Laboratories, Hercules, CA, USA), 1% Igepal (Sigma-Aldrich), and 0.5% sodium deoxycholate (Sigma-Aldrich). The tissues were homogenized on ice for 30 seconds, and sonicated for 10 seconds at 10 mV. After 30 minutes incubation on ice, the homogenates were centrifuged at 16,000 *g* for 30 min. The supernatant was aliquoted and frozen at –80°C until analysis. The lower limit of detection in this assay was 50 pg/ml.

In brief, purified ovine IL-1 $\beta$  protein diluted to 0.5  $\mu$ g/ml in coating buffer was added to a 96-well ELISA plate (100  $\mu$ l/well) and incubated overnight at 4 °C. The nonspecific binding sites in the coated wells were blocked with 200  $\mu$ l of blocking solution (1% sodium casein in PBS) for 2 h at room temperature following multiple washes in PBS. A standard curve of anti-IL-1 $\beta$  mAb with a range from 50 pg/ml to 100 ng/ml was created by duplicate serial dilutions across the plate. Similar volumes of diluted plasma samples (1:100 or 1:400) or brain extracted samples (1:5 or 1:10) in dilution buffer (0.05% Tween 20 plus blocking solution) were added to the remaining wells in duplicate. The plate was incubated at room temperature for 2 h followed by multiple washes in PBS. HRP-conjugated goat anti-mouse secondary antibody (Zymed, San Francisco, CA, USA) diluted 1:1000 was added (100  $\mu$ l) to each well. The plate was incubated for 1 h at room temperature followed by washes in PBS as described above.

The plates were read on a micro-plate reader (Model 680, Bio-Rad Laboratories) at 450 nm. A standard curve was plotted using a three-parameter logistic regression analysis model (SigmaPlot, Systat Software, Inc., San Jose, CA, USA). Non-specific mouse anti-sheep IgG1 mAb (AbD Serotec, Raleigh, NC, USA) was used as a negative control in the indirect ELISA. Experiments were repeated two or more times until the correlation coefficient of the standard curve was > 0.99.

### **Calculation of anti-IL-1 $\beta$ mAb concentration in fetal brain parenchyma**

Accurate concentration of anti-IL-1 $\beta$  mAb in the brain parenchyma was determined by correcting for the quantity of anti-IL-1 $\beta$  mAb that was contained within the brain vascular space using published methods (Banks et al., 2002, Banks et al., 2005). This could be accurately determined because the brain vascular space was measured with <sup>99</sup>mTc-labeled

RBCs and the fetal hematocrit (Chen et al., 2012, Chen et al., 2015). The following equations were used in the calculations:

- a. Total brain to plasma ratio ( $\mu\text{l/g}$ ) = Amount of mAb in brain tissue (ng)  $\div$  Concentration mAb in plasma (ng/ $\mu\text{l}$ ) X weight of brain tissue (g)
- b. Corrected brain to plasma ratio ( $\mu\text{l/g}$ ) = Total brain to plasma ratio ( $\mu\text{l/g}$ ) – plasma volume ( $\mu\text{l/g}$ )
- c. Plasma volume ( $\mu\text{l/g}$ ) = Blood volume ( $\mu\text{l/g}$ )  $\times$  (1 – Hct / 100)
- d. Amount of anti-IL-1 $\beta$  mAb in brain parenchyma (ng/g brain) = Corrected brain to plasma ratio ( $\mu\text{l/g}$ )  $\times$  mAb concentration in plasma (ng/ $\mu\text{l}$ ) and brain parenchymal concentrations of anti-IL-1  $\beta$  mAb

### BBB permeability measurements

The blood-to-brain transfer constant was measured 24 hours after ischemia with the  $^{125}\text{I}$ -labeled IL-1 $\beta$  ovine protein as we have recently reported (Sadowska et al., 2015).  $^{125}\text{I}$ -IL-1 $\beta$  ( $25.5 \pm 3.0 \mu\text{Ci/kg}$ , mean  $\pm$  SEM) was rapidly injected intravenously and the arterial plasma isotope concentrations were obtained at fixed intervals of times before and after the injection until 20 minutes of study. Brain vascular volumes values determined by administering technetium-99m ( $^{99\text{m}}\text{Tc}$ ) radiolabeled red blood cells (RBCs) to the fetus were used from like study groups in our earlier report based on the same experimental design and methodology (Chen et al., 2012). The fetal brain was dissected to measure regional BBB permeability. Tissue and plasma samples were processed as previously described for quantification of  $^{125}\text{I}$ -IL-1 $\beta$  radioactivity (Sadowska et al., 2015). Determinations of the plasma isotope concentration profile and radioisotope in the brain parenchyma facilitated accurate calculation of the blood-to-brain transfer constant (Sadowska et al., 2015).

$$K_i (\mu\text{l} \bullet \text{g Brain}^{-1} \bullet \text{min}^{-1}) \text{ given by: } K_i = A_{\text{br}} / \int_0^t c_p(T) dT$$

Where  $A_{\text{br}}$  is the amount of isotope that crossed the BBB from blood to brain during the study ( $\text{dpm} \bullet \text{g}^{-1}$ ), and  $c_p$  is the isotope concentration in plasma ( $\text{dpm} \bullet \mu\text{L}^{-1}$ ) at the time  $t$  (min).  $A_{\text{br}}$  is obtained by correcting the total amount of isotope measured in the tissue  $A_m$  ( $\text{dpm} \bullet \text{g}^{-1}$ ) for the residual part remaining in the brain vasculature space, which is measured by  $^{99\text{m}}\text{Tc}$ -labeled RBCs. Thus,  $A_{\text{br}} = A_m - V_p c_p$ , where  $V_p$  is the blood volume of brain tissue ( $\mu\text{l} \bullet \text{g}^{-1}$ ) and  $c_p$  is the concentration of tracer in the terminal plasma sample ( $\text{dpm} \bullet \text{g}^{-1}$ ).  $V_p = A_m^\dagger / c_p^\dagger$ , where  $A_m^\dagger$  and  $c_p^\dagger$  have the same definitions as  $A_m$  and  $c_p$  above except that they apply to the  $^{99\text{m}}\text{Tc}$ -labeled RBCs (Sadowska et al., 2015).

### Statistical analysis

All results are expressed as mean  $\pm$  SEM. Physiological, biochemical, carotid arterial blood flow values, and plasma concentrations of anti-IL-1 $\beta$  mAb were compared by two-factor repeated measures analysis of variance (ANOVA) between the placebo infused ischemic and anti-IL-1 $\beta$  mAb infused ischemic groups. Regional BBB permeability within and between the three groups were also compared by two-factor ANOVA for repeated measures. One-way

ANOVA was used to detect differences among the groups with respect to changes in BBB permeability within each brain region. If significant differences ( $P < 0.05$ ) were found by ANOVA, the groups were further compared by the Newman-Keuls *post hoc* test. A two group T-test was used to compare values, in which two groups were compared, such as the brain parenchymal anti-IL-1 $\beta$  mAb levels between the placebo infused ischemic and anti-IL-1 $\beta$  mAb infused ischemic groups. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### Physiological variables

The gestational ages ( $126.5 \pm 0.3$ ,  $126.4 \pm 0.5$ ,  $126.4 \pm 0.5$ , Mean  $\pm$  SEM) and body weights ( $2952.2 \pm 296.4$ ,  $2845.9 \pm 388.4$ ,  $3246.3 \pm 326.1$ ) in the sham non-ischemic, placebo ischemic and anti-IL-1 $\beta$  mAb ischemic groups, respectively did not differ. The brain weight was lower ( $P = 0.01$ ) in the placebo ischemic treated ( $32.5 \pm 2.5$ ) compared with the anti-IL-1 $\beta$  mAb ischemic ( $43.2 \pm 2.7$ ) and sham non-ischemic ( $41.7 \pm 1.6$ ) groups. However, the brain to body weight ratios did not differ among the three groups.

Fetal arterial pH, PO<sub>2</sub> and PCO<sub>2</sub>, arterial base excess, heart rate, mean arterial blood pressure (MABP), hematocrit, and carotid arterial blood flow values during the study are summarized in Table 1. All baseline values were within the physiological range for our laboratory and did not differ between the placebo ischemic and anti-IL-1 $\beta$  mAb ischemic treated groups. The physiological variables also did not differ between the placebo ischemic and anti-IL-1 $\beta$  mAb ischemic treated groups after brain ischemia during reperfusion. Carotid artery occlusion resulted in immediate significant reductions in carotid arterial blood flow with values approaching zero within seconds of the onset of ischemia and did not differ between the placebo ischemic and anti-IL-1 $\beta$  mAb ischemic treated groups. Carotid arterial blood flow values remained in this range for the 30-minute duration of occlusion after which they returned to values similar to the baseline measurements and did not differ between the placebo ischemic and anti-IL-1 $\beta$  mAb ischemic treated groups.

### Anti-IL-1 $\beta$ mAb concentrations in fetal plasma and brain parenchyma

Plasma levels of anti-IL-1 $\beta$  mAb were increased at 4 hours after intravenous administration of the anti-IL-1 $\beta$  mAb in mAb treated ischemic group (Fig. 2A, ANOVA: main effects for mAb concentration over study time,  $F = 15.9$ ,  $n = 5$ ,  $P = 0.005$ ) and remained elevated up to the end of the studies. The peak level of anti-IL-1 $\beta$  mAb in the plasma was observed 4 h after ischemia ( $139 \pm 35.7$  ng/ml). As expected, the mAb was not detectable in the placebo treated ischemic group. The intravenous infusions of the anti-IL-1 $\beta$  mAb in the ischemic animals resulted in a considerable (Fig. 2B,  $F = 42.8$ ,  $n = 5$ ,  $P = 0.0001$ ) accumulation of the anti-IL-1 $\beta$  mAb within the brain parenchyma at the end of the studies.

### BBB permeability measurement ( $K_i$ )

The BBB permeability studies performed 24 h after brain ischemia showed that the  $K_i$  values measured with the radiolabeled IL-1 $\beta$  protein differed significantly between the sham operated non-ischemic, placebo treated ischemic, and anti-IL-1 $\beta$  mAb treated ischemic

groups (ANOVA: main effects for groups across brain regions,  $F=4.04$ ,  $n=5$  in each group,  $P=0.04$ , Fig. 3). The  $K_i$  values were significantly lower across the brain regions of the ischemic animals administered anti-IL-1 $\beta$  mAb compared with the placebo treated ischemic fetal sheep (ANOVA: main effects for groups across brain regions,  $F=6.02$ ,  $n=5$  each group,  $P=0.03$ , Fig. 3). Although inspection of Fig. 3 suggests that the  $K_i$  values in the anti-IL-1 $\beta$  mAb treated ischemic group appeared lower compared with the sham non-ischemic group, the  $K_i$  values were not significantly lower across the brain regions of the anti-IL-1 $\beta$  mAb treated ischemic than the sham group (ANOVA: main effects for groups across brain regions,  $F=5.40$ ,  $n=5$  each group,  $P=0.05$ , Fig. 3). Post hoc analysis revealed that there were no differences between the sham placebo and the ischemic mAb within each brain region. In addition, analysis of the mean percent reduction in the  $K_i$  values in the anti-IL-1 $\beta$  mAb ischemic group (Fig.3, closed bars) compared with the placebo ischemic group (hatched bars) suggested variations in the extent of the reductions between the brain regions as a result of mAb treatment, which ranged from 78% to 93% in various brain regions.

The  $K_i$  values measured with radioactive IL-1 $\beta$  protein also differed across the cerebral cortical regions (frontal, parietal and occipital cortices) among the sham operated non-ischemic, placebo treated ischemic, and anti-IL-1 $\beta$  mAb treated ischemic groups (ANOVA: main effects for groups across brain regions,  $F=5.45$ ,  $n=5$  each group,  $P=0.02$ , Fig. 4). The  $K_i$  values were significantly lower across cerebral cortical regions (frontal, parietal and occipital cortices) in the anti-IL-1 $\beta$  mAb treated ischemic than in the placebo treated ischemic animals (ANOVA: main effects for group across brain regions,  $F=7.44$ ,  $n=5$  each group,  $P=0.02$ , Fig. 4). The  $K_i$  values were not significantly lower across the cerebral cortical regions of the anti-IL-1 $\beta$  mAb treated ischemic compared with the sham operated non-ischemic fetal sheep (ANOVA: main effects for groups across brain regions,  $F=5.26$ ,  $n=5$  each group,  $P=0.05$ , Fig. 4).

### IL-1 $\beta$ radioisotope levels in the fetal plasma

Fetal plasma IL-1 $\beta$  radioisotope levels were determined during the BBB permeability studies and were normalized as the log of the radioisotope values over the duration of the BBB permeability studies. IL-1 $\beta$  radioisotope levels differed among the sham operated non-ischemic, placebo treated ischemic, and anti-IL-1 $\beta$  mAb treated ischemic groups over time (ANOVA: interaction for groups over time,  $F=10.3$ ,  $n=5$  each group,  $P<0.001$ , Fig. 5), but ranged during the study from about 30–250 dpm/ $\mu$ l (0.05–0.4 ng/ml). IL-1 $\beta$  radioisotope levels were significantly higher over the duration of the study (ANOVA: main effects for groups across over time,  $F=22.7$ ,  $n=5$  each group,  $P<0.001$ , Fig. 5) in the anti-IL-1 $\beta$  mAb treated ischemic group compared with the placebo treated ischemic group. The IL-1 $\beta$  radioisotope levels were also significantly higher over the duration of the study (ANOVA: interactions for groups over time,  $F=14.3$ ,  $n=5$  each group,  $P<0.001$ , Fig. 5) in anti-IL-1 $\beta$  mAb treated ischemic group compared with the sham operated non-ischemic group. The IL-1 $\beta$  radioisotope levels did not differ between the sham operated non-ischemic and placebo treated ischemic groups over time (ANOVA: interaction for groups over time,  $F=0.14$ ,  $n=5$  each group,  $P=0.72$ , Fig 5).

## DISCUSSION

The BBB represents the largest interface between the systemic circulation and brain microenvironment and provides both anatomical and physiological protection for the CNS by regulating the passage of blood borne substances into the brain (Abbott et al., 2006). Hypoxic ischemic and inflammatory insults may modify or impair the function of this interface (Abbott et al., 2006, Chen et al., 2012). Although there is a plethora of information suggesting that increased permeability and dysfunction of the BBB represents an important component of CNS pathologies in adult disorders, ranging from ischemic stroke to neurodegenerative disorders and infection (Abbott and Friedman, 2012, Rochfort and Cummins, 2015), the significance of abnormalities in the BBB contributing to brain injury in perinatal medicine has been less well appreciated. Neuroinflammatory cascades that accompany BBB dysfunction in adults have been strongly linked to elevated levels of pro-inflammatory cytokines (Rochfort and Cummins, 2015). Inflammation is now a well-recognized component of hypoxic ischemic brain damage in the neonate as well (Ferriero, 2004). The focus of the current study was to improve our understanding of the potential for cytokine neutralizing strategies to reduce pro-inflammatory cytokine transport across the BBB after hypoxic ischemic brain insults in the fetus.

Bilateral carotid occlusion resulted in similar decreases in perfusion in the placebo treated ischemic and anti-IL-1 $\beta$  mAb treated ischemic groups (Chen et al., 2012, Chen et al., 2015, Sadowska et al., 2015). Carotid occlusion in fetal sheep is associated with a watershed pattern of damage that includes both neuronal and white matter injury (Williams et al., 1992, Petersson et al., 2002, Back et al., 2012). Thirty minutes of ischemia was selected because this duration of ischemia is associated with consistently severe injury from which rescue is still feasible (Gunn et al., 1997).

The dose of anti-IL-1 $\beta$  mAb administered to the fetal sheep was based upon previous work examining the role of treatment with antibodies on brain ischemia in adult rats (Lavine et al., 1998). We adjusted the dose to account for the larger blood volume of the fetus and the potential for mAbs to be sequestered within the placenta (Stonestreet et al., 1989, Chen et al., 2015, Zhang et al., 2015). Two doses of mAbs were given after ischemia over an extended time to allow for sustained exposure of the endothelial microvasculature to mAbs before the BBB permeability studies were performed (Chen et al., 2015, Zhang et al., 2015). The highest plasma level of anti-IL-1 $\beta$  mAb,  $139 \pm 35.7$  ng/ml, was observed at 4 h of reperfusion and the final concentration at 24 h of reperfusion remained elevated ( $108 \pm 25.4$  ng/ml) suggesting that the half-life of anti-IL-1 $\beta$  mAb could be more than 24 hours in the fetal circulation. In the present study, we administered the mAbs early after the ischemic insult. However, there often is a delay in the initiation of therapy in the clinical setting after exposure of infants to hypoxic ischemic brain insults (Shankaran et al., 2010). Therefore, additional pharmacokinetic studies are required to determine the half-life and time to steady-state plasma concentrations of anti-IL-1 $\beta$  mAb to examine further the potential of this mAb as a therapeutic agent.

The novel findings of our study are that systemic intravenous infusions of anti-IL-1 $\beta$  mAb administered after ischemia resulted in substantial accumulation of mAb within the fetal

brain parenchyma and significantly decreased transport of the IL-1 $\beta$  cytokine across the fetal BBB. These findings suggest that systemically infused cytokine-neutralizing mAb has substantial biological effects either on circulating pro-inflammatory cytokines and/or on the cerebral microvascular endothelial cells of the BBB to diminish ischemia-augmented transfer of IL-1 $\beta$  across the BBB in the fetus. Although it still remains to be determined how cytokines directly interact with the microvascular endothelium of the BBB, pro-inflammatory cytokines could originate from local sources within the neurovascular unit including the endothelium, from peripheral circulating cells or from systemic sources to injure the BBB (Rochfort and Cummins, 2015). Presently, we cannot determine the exact mechanism(s) by which systemic infusions of the neutralizing anti-cytokine mAb reduced the transport of IL-1 $\beta$  protein across the fetal BBB. However, two possibly interrelated mechanism(s) are that mAb-cytokine binding in the peripheral circulation reduces the availability of the free IL-1 $\beta$  protein to cross the BBB and that the mAb-cytokine complex is less able to interact with the IL-1 transporter located at the barrier (Banks et al., 1991). Supporting these potential mechanisms are our findings that radioactive levels of IL-1 $\beta$  were higher in the blood of antibody-treated animals (see Fig. 5). In addition, the molecular weight of the antibody (180 kDa) and IL-1 $\beta$  (16 kDa) combined (196 kDa) is not much greater than that of the antibody alone. Moreover, we have shown that the antibody levels are higher in the mAb ischemic than the placebo treated group (see figure 2B). Therefore, the potential mechanisms described above are only logical if the cytokine is crossing the BBB by using its transporter rather than to leakage across the fetal BBB.

In the current study, we demonstrated that ischemia accentuates the transfer of the IL-1 $\beta$  protein across the BBB 24 h after ischemic injury, extending our previous findings that such accentuation occurs 4 h after ischemia (Sadowska et al., 2015). BBB permeability was measured 24 h after ischemia in order to enable exposure of the cerebral microvasculature endothelium to cytokine neutralizing antibodies for a sustained period after the onset of ischemia and before performing the BBB permeability studies. We recently have shown that systemic infusions of anti-IL-1 $\beta$  mAb reduce ischemia-related increases in non-specific BBB permeability when measured with a biologically inert molecule (Chen et al., 2015). In this study, we used  $^{125}\text{I}$ -IL-1 $\beta$  as the tracer for BBB permeability determinations in contrast to our former studies, in which we used  $\alpha$ -[ $^{14}\text{C}$ ]-aminoisobutyric acid (AIB, 103 Da) as the tracer (Chen et al., 2012, Chen et al., 2015). The results from our sham control fetal sheep suggest that permeability measured with  $^{125}\text{I}$ -IL-1 $\beta$  is only approximately half that of AIB, even though IL-1 $\beta$  is 165 fold larger than AIB. The differences are most likely explained by the presence of a specific IL-1 transporter across the BBB because previous work has shown that there is a proportionately greater propensity for cytokines to cross the BBB despite their large size as a result of the saturable transport systems (Banks et al., 1991, Banks et al., 1994, Banks et al., 2001). Nonetheless, the increase in IL-1 $\beta$  transport across the barrier appears to be greater after ischemia relative to the sham control treated animals in the current study, when compared with the fold increase AIB permeability after ischemia relative to the sham controls in our former work (Figure 4 in our former publication) (Chen et al., 2015).

The exact mechanisms by which IL-1 $\beta$  protein is transported across the cerebral microvasculature endothelial cells of the BBB cannot be discerned by our *in vivo* methods in

fetal sheep (Sadowska et al., 2015). We also cannot ascertain whether the ischemia related transport of IL-1 $\beta$  into the brain represents increased transport via paracellular or transcellular routes (Engelhardt and Sorokin, 2009, Abbott et al., 2010, Sadowska et al., 2015). However, extensive work in adult rodents has firmly established that cytokines are able to cross the BBB via direct penetration (Banks et al., 1994, Banks et al., 1995, Banks et al., 2001). We have previously demonstrated that the ovine IL-1 $\beta$  protein crossed the BBB of the normal mouse by a saturable transport mechanism (Threlkeld et al., 2010). In addition, previous work has shown that IL-1 $\beta$  is transported across porcine cerebral microvascular endothelial cells *in vitro* via a temperature sensitive, microtubule dependent, saturable receptor, potentially via a type II IL-1 receptor (Skinner et al., 2009). Therefore, it is likely that the IL-1 $\beta$  protein crossed the BBB in the fetal sheep via direct penetration and/or by a transport mechanism. In support of this contention, previous studies of CNS injury have shown that increased transporter activity rather than BBB disruption accounted for increased cytokine uptake in the CNS (Pan et al., 1996).

In our previous report (Chen et al., 2015), we showed that systemic administration of anti-IL-1 $\beta$  mAb decreased BBB permeability after ischemia when measured with the biologically inert molecule AIB. This finding further suggests that the anti-cytokine neutralizing antibody does not reduce the IL-1 $\beta$  transport merely by neutralizing and inhibiting systemic cytokines, but could also have direct effects upon the cerebral vascular endothelium of the barrier. In studies using *in vitro* models of the BBB, it has been shown that IL-1 $\beta$  regulates the function and immune profile of human cerebral microvascular endothelial cells of the BBB (O'Carroll et al., 2015). IL-1 $\beta$  also increases the expression of adhesion molecules ICAM-1 and other pro-inflammatory cytokines, increases paracellular permeability and decreases transendothelial electrical resistance even under basal conditions in the microvascular endothelial cells of the BBB (Labus et al., 2014). Therefore, it remains likely that the anti-IL-1 $\beta$  mAb could affect BBB permeability in the microvascular endothelium by mitigating these effects of IL-1 $\beta$  on the neurovascular unit (Abbott et al., 2010), thereby reducing inflammatory activation in the barrier endothelium. Neutralization of IL-1 $\beta$  by the anti-IL-1 $\beta$  mAb could not only reduce the transport of the protein into the brain, but could also modulate the function of the cells of the neurovascular unit (Abbott et al., 2010) to reduce the relay of other inflammatory signals. This could then attenuate the transfer of inflammatory signals into the brain independent of IL-1 $\beta$  transport across the BBB. The consequences of the lower BBB permeability on developing brain in anti-IL-1 $\beta$  mAb treated ovine sham control fetuses cannot be determined by our study. In our previous work, we have established that there is an ontogenic decrease in BBB permeability in ovine fetus from 60% gestation to the mature brain in adult sheep (Stonestreet et al., 1996). Whether the anti-IL-1 $\beta$  mAb also modulates and penetrates an intact BBB under non-ischemic conditions would be an important focus for future study, which would further elucidate the action of this mAb in the normal developing fetal BBB.

Several lines of evidence appear to suggest that this therapy could have neuroprotective properties. Interleukin-1 is a key mediator of neuronal injury, and the naturally occurring interleukin-1 receptor antagonist has been reported to have beneficial effects in experimental studies of stroke (Denes et al., 2011, Maysami et al., 2016). In adult rats, the IL-1 receptor antagonist, IL-1RA reduced brain injury induced by focal cerebral ischemia (Clark et al.,

2008). Nonetheless, inflammation can have both beneficial and detrimental effects in the brain after injury (Le Thuc et al., 2015), therefore, the effects of the anti-IL-1 $\beta$  mAb on the neuropathological changes after brain ischemia in fetal sheep brain remain to be determined. However, based upon the recent findings in adult rodents (Clark et al., 2008, Denes et al., 2011, Maysami et al., 2016), it appears that inhibition of IL-1 $\beta$  transport across the BBB could potentially ameliorate perinatal brain injury. We have also not measured other cytokine levels or inflammatory markers in the systemic circulation or brain parenchyma after treatment with the anti-IL-1 $\beta$  mAbs. Commercially available assays such as the multiplex cytokine assay are not available for sheep. In our experience, cytokine assays designed for mouse, rat or human do not work in sheep. However, it would be feasible to measure levels of IL-6 cytokine in ovine fetal circulation in future using our specifically designed ovine IL-6 cytokine assay (Zhang et al., 2015).

The findings of our study are represented schematically in Fig. 6. Systemic IL-1 $\beta$  is able to cross the fetal BBB in non-ischemic sham ovine fetuses (A) (Sadowska et al., 2015). Increased transfer of the systemic IL-1 $\beta$  cytokine across the BBB is observed 24 h after ischemic brain injury, which is not altered by the systemic normal saline infusions (B). Systemic infusions of anti-IL-1 $\beta$  mAb neutralizing antibodies after ischemia attenuates the transfer of systemic IL-1 $\beta$  cytokine across the BBB and also results in anti-IL-1 $\beta$  mAb accumulation in brain parenchyma ovine fetuses (C).

## CONCLUSIONS

In summary, the novel findings of our study are that systemically infused anti-IL-1 $\beta$  mAb substantially reduces the transport of IL-1 $\beta$  cytokine across the BBB after ischemia in the ovine fetus at least in part by complexing with the free IL-1 $\beta$  in the systemic circulation. There is ample evidence to suggest that clinical conditions such as necrotizing enterocolitis, sepsis and chronic ventilation, which are associated with elevations in systemic cytokines, also place neonates at high risk for adverse neurodevelopmental outcomes (Dammann et al., 2001, O'Shea, 2002, Stoll et al., 2004, Walsh et al., 2005, O'Shea et al., 2011, Bose et al., 2013, Leviton et al., 2013, O'Shea et al., 2013). In order for systemically produced cytokines to contribute to/or cause brain injury in the neonate, they would first need to cross the BBB and/or stimulate an inflammatory response at the endothelium of the BBB as has previously postulated (Dammann and Leviton, 1997, Dammann et al., 2001, Dammann and Leviton, 2004). In the current study, we not only confirm the transfer of a brain injury inciting cytokine across the BBB in response to ischemia, but also show that the increased transfer can be decreased by systemically administered anti-cytokine specific mAb. Therefore, we propose that conditions such as sepsis, necrotizing enterocolitis, and prolonged ventilation in neonates could potentially benefit from an anti-cytokine therapeutic strategy. Thus, our finding that a modest dose of systemically administered anti-IL-1 $\beta$  mAb can penetrate ovine fetal brain and diminish the ischemia related transfer of IL-1 $\beta$  cytokine across BBB could have translational potential in perinatal brain injury.



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## LIST OF ABBREVIATIONS

<b>ANOVA</b>	Analysis of variance
<b>AIB</b>	$\alpha$ -aminoisobutyric acid
<b>PO<sub>2</sub></b>	arterial oxygen tension
<b>PCO<sub>2</sub></b>	arterial carbon dioxide tension
<b>K<sub>i</sub></b>	blood-to-brain transport constant
<b>CSIRO</b>	Commonwealth Scientific and Industrial Research Organization
<b>CPM</b>	counts per minute
<b>DEAE-CIM</b>	Diethylaminoethyl-Convective Interaction Media
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>E. Coli</b>	Escherichia coli
<b>HRP</b>	horse radish peroxidase
<b>IgG</b>	immunoglobulin G
<b>IL-1<math>\beta</math></b>	interleukin-1 beta
<b>IL-1RA</b>	interleukin-1 receptor antagonist
<b>IL-6</b>	interleukin-6
<b>125I</b>	Iodine-125
<b>MABP</b>	mean arterial blood pressure
<b>mean <math>\pm</math> SEM</b>	mean $\pm$ standard error of the mean
<b>mAb</b>	monoclonal antibody
<b>NaCl</b>	sodium chloride
<b>PBS</b>	phosphate buffered saline

<b>RBS</b>	red blood cells
<b><sup>99m</sup>Tc</b>	technetium-99m
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor alpha

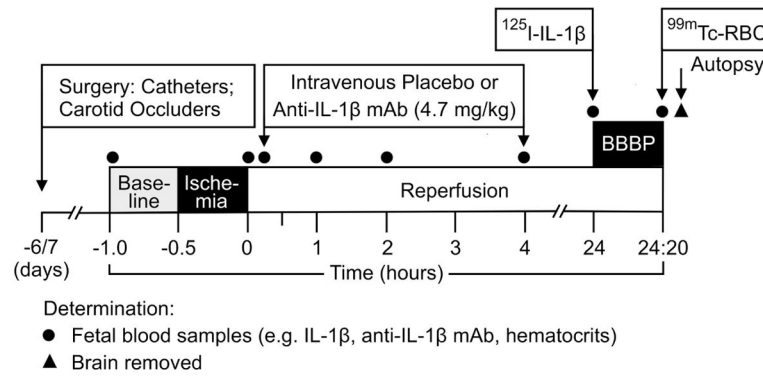
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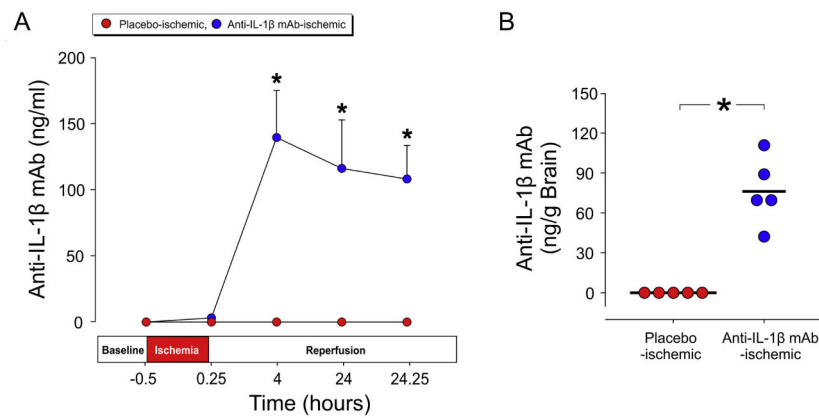
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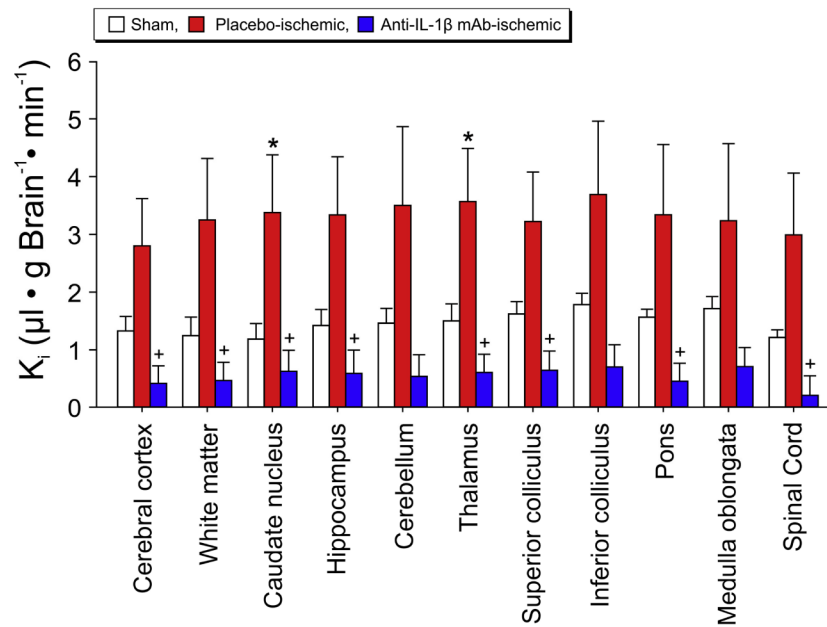


**Fig. 1.**

Study design. Surgical preparation was 6–7 days before the onset of the studies. After the baseline determinations, ischemia was induced for thirty minutes by inflation of the bilateral carotid artery occluders. At the end of ischemia, the occluders were deflated and reperfusion continued for 24 hours and twenty minutes. In the sham operated control sheep, the occluders were not inflated. Fifteen minutes and 4 hours after the end of ischemia placebo (0.154M NaCl) or anti-IL-1 $\beta$ mAb (5 mg/kg) was given intravenously to the fetus. The BBB permeability studies were performed over twenty minutes at the end of the 24 hours of reperfusion.  $^{125}\text{I}$ -IL-1 $\beta$  was administered intravenously at the onset of the BBB permeability studies. The brain vascular space was measured by administering  $^{99\text{m}}\text{Tc}$ -labeled red blood cells two minutes before the end of the study. At the end of the studies, both the ewe and fetus were sacrificed with intravenous pentobarbital (100–200 mg/kg). The fetus and brain were weighed, and the brain was removed for analysis.

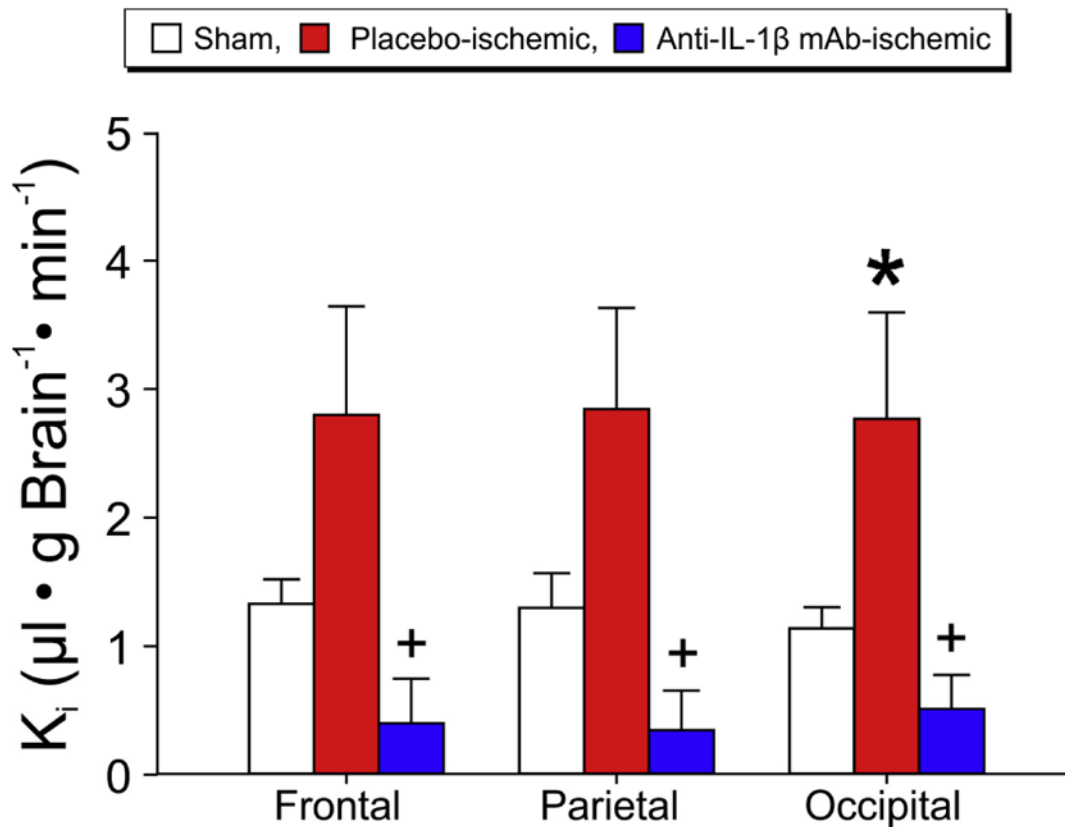
**Fig. 2.**

**A.** Concentration (ng/ml) of anti-IL-1 $\beta$  mAb in fetal plasma in placebo-ischemic (hatched circles, n=5), and anti-IL-1 $\beta$  mAb ischemic (closed circles, n=5) groups, plotted on the Y-axis against study time in hours on X-axis. Increase in anti-IL-1 $\beta$  mAb concentrations in fetal plasma were detected 4 h after the onset of infusions. \*P=0.005 vs. baseline values. **B.** Scatter grams of anti-IL-1 $\beta$  mAbs within the brain parenchyma plotted on the Y-axis in ng/g brain tissue for placebo-ischemic (hatched circles, n=5), and anti-IL-1 $\beta$  mAb - ischemic (closed circles, n=5) groups on X axis, \*P=0.0001 vs. placebo-ischemic. Values were corrected for the residual amount of anti-IL-1 $\beta$  mAb remaining within the brain vascular space as detailed in the methods (Banks et al., 2002, Banks et al., 2005).

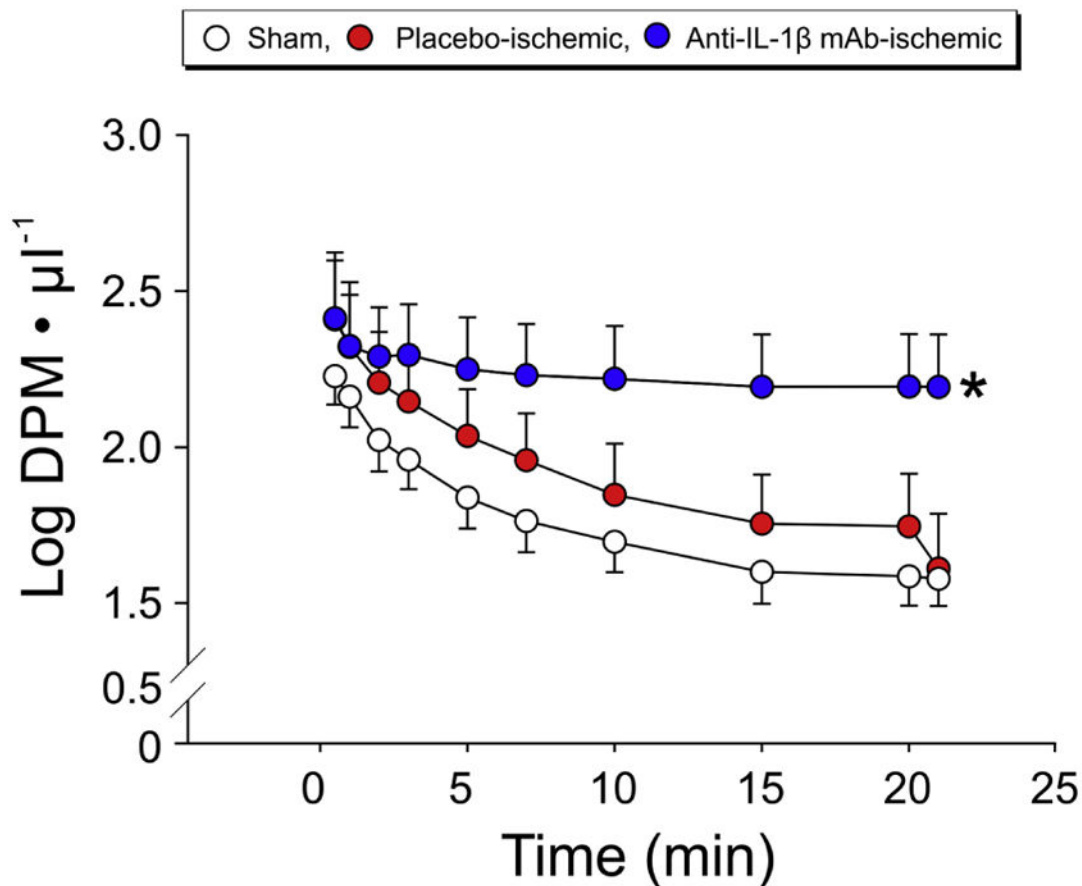


**Fig. 3.** Blood-to-brain transfer constants ( $K_i$ ) in brain regions by study group measured with  $^{125}\text{I}$ -labeled IL-1 $\beta$  ovine protein. Blood-to brain transfer constants ( $K_i$ ) plotted on the Y-axis in the sham (open bars, n=5), placebo-ischemic (hatched bars, n=5), and anti-IL-1 $\beta$  mAb ischemic (closed bars, n=5), groups plotted for the brain regions on the X-axis. Values are Mean  $\pm$  SEM. \*P=0.04 versus sham, + P=0.03 vs. placebo-ischemic group.

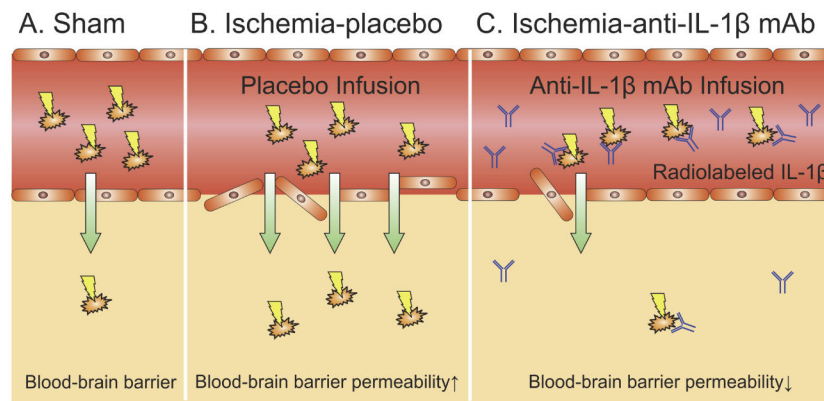




**Fig. 4.** Blood-to-brain transfer constants ( $K_i$ ) in the sham (open bars, n=5), placebo-ischemic (hatched bars, n=5) and anti-IL-1 $\beta$  mAb ischemic (closed bars, n=5), groups plotted for the frontal, parietal and occipital cerebral cortices. Values are Mean  $\pm$  SEM, \*P=0.02 versus sham, +P=0.02 vs. placebo-ischemic group.



**Fig. 5.** Fetal plasma IL-1 $\beta$  radioisotope levels normalized as log of radioisotope values in sham (open circle, n=5), placebo-ischemic (hatched circle, n=5) and anti-IL-1 $\beta$  mAb-ischemic (closed circle, n=5) groups plotted against duration of time during the BBB permeability studies. Values are Mean  $\pm$  SEM, \*P < 0.001 vs. placebo-ischemic and sham groups.



**Fig. 6.**

Systemic infusions of anti-IL-1 $\beta$  mAb neutralizing antibodies reduce the transport of the IL-1 $\beta$  cytokine across the BBB after ischemia. A) Systemic IL-1 $\beta$  is able to cross the fetal BBB in non-ischemic sham ovine fetuses. B) Increased transfer of systemic IL-1 $\beta$  cytokine across the BBB is noted after ischemia reperfusion brain injury, which is not altered by systemic placebo infusion of normal saline in ovine fetuses. C) Systemic infusions of anti-IL-1 $\beta$  mAb neutralizing antibodies in ischemic ovine fetuses significantly attenuates the transfer of systemic IL-1 $\beta$  cytokine across the BBB and also results in anti-IL-1 $\beta$  mAb accumulation in brain parenchyma.

**Table 1**

Fetal arterial pH, blood gases, base excess, heart rate, mean arterial blood pressure, and hematocrit values by study group.

Group	Baseline	Hours after Ischemia									
		0	0.25	1	2	4	24				
pH											
	Placebo-I/R	7.40 ± 0.03	7.38 ± 0.03	7.37 ± 0.02	7.36 ± 0.02	7.37 ± 0.02	7.37 ± 0.02	7.36 ± 0.02	7.37 ± 0.02	7.36 ± 0.02	7.36 ± 0.02
	mAb-I/R	7.39 ± 0.03	7.37 ± 0.03	7.37 ± 0.02	7.37 ± 0.03	7.37 ± 0.03	7.36 ± 0.03	7.36 ± 0.03	7.37 ± 0.03	7.37 ± 0.03	7.37 ± 0.03
Arterial PO <sub>2</sub> (mm Hg)											
	Placebo-I/R	21 ± 1	22 ± 2	23 ± 2	24 ± 2	24 ± 2	22 ± 1	22 ± 1	21 ± 2	21 ± 2	21 ± 2
	mAb-I/R	22 ± 1	23 ± 1	23 ± 1	24 ± 1	23 ± 1	22 ± 1	22 ± 1	23 ± 1	23 ± 1	23 ± 1
Arterial PCO <sub>2</sub> (mm Hg)											
	Placebo-I/R	50 ± 2	49 ± 1	46 ± 2	44 ± 2	46 ± 2	49 ± 2	49 ± 2	45 ± 3	45 ± 3	45 ± 3
	mAb-I/R	49 ± 1	51 ± 3	49 ± 2	51 ± 2	49 ± 2	51 ± 1	53 ± 2	47 ± 3	47 ± 3	47 ± 3
Arterial Base Excess (mEq/L)											
	Placebo-I/R	5 ± 2	2 ± 3	1 ± 3	1 ± 3	1 ± 2	2 ± 2	2 ± 2	1 ± 2	1 ± 2	1 ± 2
	mAb-I/R	3 ± 2	4 ± 3	3 ± 1	3 ± 1	2 ± 2	2 ± 2	2 ± 1	2 ± 3	2 ± 3	2 ± 3
Heart Rate (beats/min)											
	Placebo-I/R	184 ± 11	155 ± 11	141 ± 11	153 ± 6	141 ± 11	151 ± 17	146 ± 11	167 ± 14	167 ± 14	167 ± 14
	mAb-I/R	147 ± 7	150 ± 8	145 ± 4	156 ± 4	145 ± 4	135 ± 9	153 ± 14	168 ± 16	168 ± 16	168 ± 16
MABP (mm Hg)											
	Placebo-I/R	46 ± 2	46 ± 6	46 ± 8	47 ± 9	46 ± 8	47 ± 7	43 ± 7	50 ± 4	50 ± 4	50 ± 4
	mAb-I/R	48 ± 6	44 ± 6	46 ± 7	47 ± 7	46 ± 7	42 ± 9	45 ± 6	46 ± 2	46 ± 2	46 ± 2
Carotid Blood Flow (ml/min)											
	Placebo-I/R	53 ± 10	47 ± 6	48 ± 6	48 ± 6	48 ± 6	43 ± 5	47 ± 7	63 ± 14	63 ± 14	63 ± 14
	mAb-I/R	54 ± 4	48 ± 2	53 ± 8	50 ± 4	53 ± 8	58 ± 6	58 ± 7	53 ± 3	53 ± 3	53 ± 3
Hematocrit (%)											
	Placebo-I/R	33 ± 1	ND	ND	ND	ND	ND	33 ± 3	30 ± 2	30 ± 2	30 ± 2
	mAb-I/R	32 ± 1	ND	ND	ND	ND	ND	31 ± 2	31 ± 2	31 ± 2	31 ± 2

Values are mean ± standard error of the mean; Placebo-I/R = Placebo treated ischemic group, n = 4-5; mAb-I/R = anti-IL-1β mAb treated ischemic-reperfusion groups, n = 4-5; Arterial PO<sub>2</sub> and PCO<sub>2</sub> are oxygen and carbon dioxide pressures, respectively; MABP, mean arterial blood pressure; ND = indicates measurements that were not determined. These measurements were not determined in order to limit blood sample withdrawal from the fetuses and some measurements were not considered critical to the study outcomes.

\* P<0.05 versus baseline values.

Time point zero signifies the time point immediately after ischemia was terminated. pH, blood gases, and mean blood pressures were not determined during ischemia. Results are not reported for the five instrumented non-ischemic control fetal sheep because these values were previously reported (Sadowska et al., 2015).