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# Surfactant Protein A Attenuates Generalized and Localized Neuroinflammation In Neonatal Mice

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## Abstract

Surfactant protein A (SP-A) has important roles in innate immunity and modulation of pulmonary and extrapulmonary inflammation. Given SP-A has been detected in rat and human brain, we sought to determine if SP-A has a role in modulating inflammation in the neonatal mouse brain. Neonatal wildtype (WT) and SP-A-deficient (SP-$A^{-/-}$) mice were subjected to three models of brain inflammation: systemic sepsis, intraventricular hemorrhage (IVH) and hypoxic-ischemic encephalopathy (HIE). Following treatment, RNA was isolated from brain tissue and expression of cytokine and SP-A mRNA was determined by real-time quantitative RT-PCR analysis. In the sepsis model, expression of most cytokine mRNAs was significantly increased in brains of WT and SP-$A^{-/-}$ neonates with significantly greater expression of all cytokine mRNA levels in SP-$A^{-/-}$ mice compared to WT. In the IVH model, expression of all cytokine mRNAs was

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Ethics Approval and Consent to Participate

Mice used in this study were housed in the Center for Laboratory Animal Medicine and Care at the McGovern Medical School at the University of Texas Health Science Center at Houston, TX. All experimental protocols were approved by the Institutional Animal Care and Use Committee of McGovern Medical School (AWC-20-0066, Houston, TX).

significantly increased in WT and SP-A$^{-/-}$ mice and levels of cytokine mRNAs were significantly increased in SP-A$^{-/-}$ mice compared to WT. In the HIE model, only TNF-α mRNA levels were significantly increased in WT brain tissue while most cytokine mRNAs were significantly increased in SP-A$^{-/-}$ mice, and all cytokine mRNA levels were significantly higher in SP-A$^{-/-}$ mice compared to WT. SP-A mRNA was not detectable in brain tissue of adult WT mice nor of WT neonates subjected to the models. These results suggest that SP-A$^{-/-}$ neonatal mice subjected to models of neuroinflammation are more susceptible to generalized and localized neuroinflammation compared to WT mice, thus supporting the hypothesis that SP-A attenuates inflammation in neonatal mouse brain.

**Keywords**

surfactant; neonatal; neuroinflammation; sepsis; IVH; HIE

## 1. Introduction

The neonatal population is inherently at risk for certain neuroinflammatory processes resulting from both systemic and localized injury. Three examples of such processes are sepsis, intraventricular hemorrhage, and hypoxic-ischemic encephalopathy. All neonates are vulnerable to sepsis, a potentially life-threatening inflammatory condition that occurs as a consequence of infection, which increases peripheral production of proinflammatory cytokines and reactive oxygen species, resulting in increased permeability of the blood-brain barrier (BBB) and activation of glial cells and cytotoxic mediators (Danielski et al., 2018). Intraventricular hemorrhage (IVH), bleeding within or surrounding the ventricles of the brain that is fairly common and specific to preterm neonates, results in red blood cell lysis and hemoglobin oxidation with the release of heme which can cause sterile neuroinflammation (Erdei et al., 2020). In term neonates, hypoxic-ischemic encephalopathy (HIE) can occur as a result of oxygen deprivation to the infant brain; in such cases, neuroinflammation is a major contributor to secondary brain cell injury which accounts for a significant proportion of neuronal loss in hypoxic-ischemic encephalopathy (HIE) (Li et al., 2020). While these three inflammatory insults are similar in that they are all commonly seen clinically in neonates, they are believed to represent three completely different mechanisms of inflammation in the brain.

Production and action of pro-inflammatory cytokines, particularly IL-1β, IL-6 and TNF-α, are common factors between these three different neuroinflammatory mechanisms. Mimicking sepsis, LPS injection leads to production of these cytokines by microglia and astrocytes in the neonatal rat brain (Dammann and Leviton, 1997; Fleiss et al., 2021; McAdams and Juul, 2012). When injected into neonatal mouse brain, these cytokines lead to proliferation of astrocytes in conjunction with reduced myelination (Nesin and Cunningham-Rundles, 2000), presumably due to TNF-α-induced apoptosis of oligodendrocytes (Cai et al., 2000). These cytokines have a role in neuronal injury during hypoxic ischemia of the brain, which leads to increased secretion of IL-1β, IL-6 and TNF-α by activated microglia (Rothwell and Hopkins, 1995). In addition, the human IL-8 homologue CXCL1 has been shown to be involved in pro-inflammatory microglia activation following inflammation-

sensitized hypoxic-ischemic brain injury in neonatal rats (Serdar et al., 2020). Further research into other potential proteins involved in these neuroinflammatory mechanisms is certainly warranted.

While surfactant is well-recognized in reducing alveolar surface tension in the lungs, components of surfactant have also been demonstrated to be involved in immune system activation and regulation of inflammation. Hydrophilic surfactant proteins A (SP-A) and D (SP-D) are carbohydrate-binding lectins, or collectins, that have been found to mediate recognition and neutralization of pathogens and modulate the inflammatory response (Herbein and Wright, 2001; Kishore et al., 2001; Kishore et al., 2006; Nayak et al., 2012; Sato et al., 2003; Vieira et al., 2017; Wright, 2005). In addition to their expression in the lung, SP-A and SP-D are expressed at extra-pulmonary sites including the nervous, ocular, cardiovascular, gastrointestinal, urinary/renal, male and female genital/reproductive, integumentary, and glandular systems (Madsen et al., 2000; Nayak et al., 2012; Snyder et al., 2008; Sorensen, 2018; Stahlman et al., 2002; Vieira et al., 2017). The role of collectins, specifically SP-A, in central nervous system (CNS) inflammation is the focus of the present study.

The function of the collectins in regulation of inflammation is complex. A wide variety of ligands bind to or activate SP-A and SP-D, which then bind to downstream receptors (for a comprehensive list of these ligands and receptors, see (Vieira et al., 2017). Because of these complex and dynamic interactions, SP-A and SP-D are considered to be both pro- and anti-inflammatory molecules, which is consistent with findings in prior clinical research. SP-A and SP-D were found to have pro-inflammatory effects in the colon (Nexoe et al., 2019) and vascular system (Colmorten et al., 2019). On the other hand, SP-A and SP-D have been found to attenuate inflammation in the intestine (Liu et al., 2021; Quintanilla et al., 2015; Saka et al., 2016), kidney (Tian et al., 2017), pancreas (Liu et al., 2015), fetal tissue (Agrawal et al., 2018; Salminen et al., 2008), and vascular system (Colmorten et al., 2019). While the anti-inflammatory effects of SP-A in the CNS have not been directly investigated, several previous studies have explored SP-A expression in the context of multiple CNS disease processes (Colmorten et al., 2019; Schob et al., 2013; Schob et al., 2016; Yang et al., 2017). In addition, it is known that SP-A binds to toll-like receptor (TLR) 2 and TLR4 and modulate their activity by various mechanisms (Henning et al., 2008). The main role of TLRs during neuroinflammation is the regulation of pathways which activate astrocytes, microglia, enzymes, and cytokines during the inflammatory process (Kielian, 2006). Therefore, it is reasonable to hypothesize that SP-A may play a role in neuroinflammation.

In this study, our goal was to further examine the role of SP-A in inflammation in the neonatal mouse brain by contrasting wildtype mice and mice deficient in SP-A. We chose models of sepsis, intraventricular hemorrhage (IVH), and hypoxic-ischemic encephalopathy (HIE) as triggers of this inflammation because these pathologies have certain implications for the neonatal population. The mechanisms of these pathologies as causes of neuroinflammation have been fairly well-established and primarily involve astrocyte and microglial activation, followed by infiltration of peripheral immune cells which then release a variety of pro-inflammatory cytokines, including IL-1, IL-5, IL-6,

CXCL1, and TNF-α (Aronowski and Zhao, 2011; Gao et al., 2008; Iadecola and Anrather, 2011; Jones et al., 2018; Patel et al., 2013; Taylor and Sansing, 2013; Wang and Tsirka, 2005; Wang and Dore, 2007; Wilson and Young, 2003; Zhou et al., 2014). Other cytokines, including IL-4 and IL-10, are then triggered which exert inhibitory regulation on CNS cytokine production (Sawada et al., 1995). While previous work has examined the role of SP-A in inflammation, neuroinflammation has not been extensively investigated. Additionally, SP-A expression in the mouse brain has not been explored. In the present study, we hypothesized that SP-A as an immunomodulatory protein attenuates inflammation in the neonatal mouse brain.

## 2. Results

### 2.1 Cytokine expression in brain tissue of SP-A$^{-/-}$ mice is significantly increased compared to WT mice subjected to a sepsis model of generalized neuroinflammation.

To assess cytokine expression in WT and SP-A$^{-/-}$ mice following intraperitoneal LPS injection as a model for sepsis, qRT-PCR analysis of cytokine mRNA was performed. Expression of cytokine mRNA as a fold-change from WT control is shown in Figure 1 for both genotypes (WT or SP-A$^{-/-}$) following injection of LPS or phosphate-buffered saline (PBS). We found that control expression (mice injected with PBS) of IL-1β, IL-6, CXCL1, and TNF-α mRNA in SP-A$^{-/-}$ neonatal mouse brain was significantly higher than levels in WT brain following intraperitoneal injection of PBS, a trend reported previously in ileum of neonatal mice (Liu et al., 2021). We found statistically significant differences between WT mice injected with PBS vs LPS for IL-1β ($p < 0.001$), CXCL1 ($p = 0.047$), and TNF-α ($p = 0.001$). We also found statistically significant differences for SP-A$^{-/-}$ mice for IL-1β ($p < 0.001$), CXCL1 ($p = 0.012$), TNF-α ($p = 0.012$) and IL-10 ($p = 0.004$), indicating that the model is effective in generating inflammation. In addition, expression of all five cytokine mRNAs (IL-1β, IL-6, CXCL1, TNF-α, and IL-10) was significantly increased in the brains of SP-A$^{-/-}$ mice compared with WT mice at 24 hours following LPS injection ($p = 0.001$, $p = 0.002$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively). These results indicate that SP-A deficient mice demonstrate increased expression of inflammatory cytokines following LPS injection compared with WT controls. Interestingly, when analyzing for any sex differences in cytokine levels following the sepsis model, we found significantly greater cytokine levels in males compared to females for IL-6 (p = 0.009), CXCL1 (p = 0.019), and TNF-α (p = 0.049) (data not shown).

### 2.2 Cytokine expression in brain tissue of SP-A$^{-/-}$ mice is significantly increased compared to WT mice subjected to an IVH model of localized neuroinflammation.

To compare cytokine expression in WT and SP-A$^{-/-}$ mice following unilateral intraventricular hemoglobin injection as a model for IVH, qRT-PCR analysis of cytokine mRNA was performed. Expression of cytokine mRNA as a fold-change from WT control is shown in Figure 2 for both genotypes (WT or SP-A$^{-/-}$) following injection of hemoglobin (IVH) or phosphate-buffered saline (PBS). Similar to the results of the sepsis model, we found that control expression (mice injected with PBS) of IL-1β, IL-6, CXCL1, TNF-α, and IL-10 mRNA in SP-A$^{-/-}$ neonatal mouse brain was significantly higher than levels in WT brain following intraventricular injection of PBS. We found statistically significant

differences between WT mice injected with PBS vs hemoglobin for all five cytokine mRNAs ($p < 0.001$). We also found statistically significant differences for SP-A$^{-/-}$ mice ($p < 0.001$), indicating that the model is effective in generating localized inflammation in the brain. In addition, expression of four cytokine mRNAs (IL-1β, CXCL1, TNF-α, and IL-10) was significantly increased in the brains of SP-A$^{-/-}$ mice compared with WT mice at 4 hours following intraventricular hemoglobin injection ($p = 0.005$, $p = 0.040$, $p < 0.001$, and $p = 0.002$, respectively). These results indicate that SP-A deficient mice demonstrate increased expression of inflammatory cytokines following intraventricular hemoglobin injection compared with WT controls. However, unlike the sepsis model, we found no significant differences in cytokine levels between males and females.

### 2.3 Cytokine expression in brain tissue of SP-A$^{-/-}$ mice is significantly increased compared to WT mice subjected to an HIE model of localized neuroinflammation.

To compare cytokine expression in WT and SP-A$^{-/-}$ mice following unilateral common carotid artery ligation followed by hypoxia exposure as a model for HIE, qRT-PCR analysis of cytokine mRNA was performed. Expression of cytokine mRNA as a fold-change from WT control is shown in Figure 3 for both genotypes (WT or SP-A$^{-/-}$) as well as all three interventions (TrueSham, Sham+Hypoxia, and HIE). In regards to the control (TrueSham) group, unlike the sepsis and IVH models, we found that expression of cytokine mRNA in SP-A$^{-/-}$ neonatal mouse brain did not significantly differ from levels in WT brain. We found statistically significant differences between SP-A$^{-/-}$ mice that underwent Sham surgery with hypoxia exposure and those that underwent HIE surgery for IL-1β ($p = 0.003$), IL-6 ($p = 0.001$), CXCL1 ($p < 0.001$), and TNF-α ($p < 0.001$), indicating that the model is effective in generating inflammation. Expression of all pro-inflammatory cytokine mRNAs (IL-1β, IL-6, CXCL1, and TNF-α) was significantly increased in the brains of SP-A$^{-/-}$ mice compared with WT mice at 24 hours following HIE surgery ($p < 0.001$, $p = 0.002$, $p < 0.001$, and $p < 0.001$, respectively). IL-10, an anti-inflammatory cytokine, showed no significant difference ($p = 0.241$). These results indicate that SP-A deficient mice demonstrate increased expression of inflammatory cytokines following unilateral common carotid artery ligation followed by hypoxia exposure compared with WT controls. Again, unlike the sepsis model, we found no significant differences in cytokine levels between males and females.

### 2.4 SP-A mRNA is not detectable in mouse brain through RT-PCR analysis.

To assess expression of SP-A in the mouse brain, three lung samples and three brain sample were collected from WT and SP-A$^{-/-}$ mice at six weeks of age. Figures 4A and 4B show RT-PCR analysis at 50 cycles of mSPA-1 mRNA in WT and SP-A$^{-/-}$ mouse tissue. RT-negative mSPA-1 was used as a negative control, and beta actin as a positive control. We found that SP-A mRNA expression was detected in lung tissue of WT mice, as expected. However, contrary to previous literature reporting SP-A expression in the brains of humans and rates, SP-A expression was not detected in the brains of either WT or SP-A$^{-/-}$ mice. These results indicate that SP-A does not appear to be expressed in the mouse brain by six weeks of age at baseline.

In order to evaluate whether inflammation would increase SP-A expression in the brain to detectable levels, four to five brain samples from WT mice following each of the three

inflammatory models (sepsis, IVH, and HIE) were collected and RT-PCR analysis was again performed at 50 cycles to measure mSPA-1 mRNA (Figure 4C). One lung sample, as well as beta actin (not shown) were used as positive controls. Once again, SP-A expression was not detected in the brains of WT mice, indicating that inflammation does not appear to increase SP-A expression to a detectable level.

## 3. Discussion

The present study investigated the potential role of SP-A in neuroinflammation in the neonatal mouse. Three models of neuroinflammation were selected due to their clinical relevance in human neonates. All three models consistently demonstrated increased cytokine expression in SP-A deficient mice following neuroinflammatory insult when compared with SP-A sufficient mice. Notably, this finding remained reliable in models of focal CNS disease as well as generalized neuroinflammatory injury. Despite this outcome, we were unable to detect SP-A expression in the brains of either SP-A sufficient or SP-A deficient mice, even following an inflammatory insult.

The vast majority of research on surfactant proteins has historically focused on their location and function in the lung. However, more recent studies have focused on extra-pulmonary expression of SP-A and SP-D, and several research groups have demonstrated expression of SP-A in the CNS in humans (Schob et al., 2013; Yang et al., 2017) utilizing multiple methods, including RT-PCR, conventional PCR, Western blot, and immunohistochemistry, to evaluate SP-A expression in human brain tissue and cerebrospinal fluid (CSF). All surfactant proteins, including SP-A, were detected by each of their methods. Furthermore, they localized SP-A immunoreactivity to the tissue surrounding the microvasculature of the brain parenchyma, the choroid plexus, and the small vessels of the pineal gland. Yang and colleagues (Yang et al., 2017) performed immunohistochemical staining of SP-A in human astrocytes and microglial cells, detecting immunopositive SP-A in the cytoplasm and nuclei of human astrocytes and in the cytoplasm of microglia. However, unlike the current study, neither of these studies utilized a negative control in SP-A$^{-/-}$ mice.

Other investigators have explored expression of SP-A in the CNS in rats (Luo et al., 2004; Schob et al., 2017; Yang et al., 2017). Luo and colleagues (Luo et al., 2004) found strong immunoreactive SP-A positive signals in myelin sheaths of cerebrum, cerebellum, and walls of blood vessels. Schob and colleagues (Schob et al., 2017) also used immunohistochemistry and concluded that SP-A is abundant at the site of the BBB. Similarly, Yang and colleagues found strong immunoreactivity for SP-A in the choroid plexus, cerebellum, and glial cells. This localization of SP-A to the choroid plexus, the microvasculature, and the BBB supports a role for SP-A in protection of the CNS from pathogens, as well as a possible role in prevention of entry of peripheral immune cells into the CNS. However, once again, a negative control was not utilized in any of these investigations.

While SP-A expression in the CNS of mice had not been investigated prior to the current study, Lambertsen and colleagues (Lambertsen et al., 2014) did utilize SP-D knockout mice to investigate whether SP-D affected cerebral ischemic infarction and ischemia-induced inflammatory responses in mice. They found very low to undetectable levels of SP-D in the

normal mouse brain. Interestingly, they also found no evidence of SP-D mRNA upregulation by the parenchymal cells after the ischemic insult. These results may be consistent with our own in that mouse parenchymal cells do not demonstrate expression of collectin mRNA. Additionally, relatively few studies have previously explored the function of collectins in the CNS. SP-A expression has found to be decreased in certain central autoimmune and neuroinflammatory conditions (notably multiple sclerosis), yet expression was increased in diseases characterized by elevated intracranial pressure or ventricular enlargement (acute hydrocephalus, aqueductal stenosis, and pseudotumor cerebri) (Schob et al., 2013; Schob et al., 2016; Yang et al., 2017). Yang and colleagues (Yang et al., 2017) found that treatment of human astrocytes and microglia with lipopolysaccharide (LPS) stimulated SP-A expression, and that exogenous SP-A decreased expression of TLR4 and reduced IL-1β and TNF-α levels, concluding that SP-A likely has a role in the modulation of CNS inflammatory responses. Similar studies have been conducted investigating the effect of SP-D on CNS diseases. Kumral and colleagues (Kumral et al., 2017) found that introduction of SP-D resulted in a significant decline in apoptosis in a model of LPS-induced periventricular leukomalacia. This suggests that collectins may also suppress inflammation in the brain by promoting clearance of apoptotic cells by macrophages, similar to their role in the lung.

The current study represents the first investigation into the expression of SP-A in the CNS in mice, as well as the first exploration of the role of SP-A specifically in neonatal neuroinflammation. We utilized SP-A knockout mice as a true negative control which, while far from novel, has not been consistent throughout earlier literature. Additionally, we were able to conclusively demonstrate increased cytokine expression in SP-A deficient mice using models of both focal and generalized brain injury. Interestingly, cytokine expression in the control groups of two of the three neuroinflammatory models (the sepsis model and the IVH model) showed evidence of greater inflammation in SP-A knockout mice compared to SP-A sufficient mice. This would suggest that without any experimental inflammatory intervention, SP-A knockout mice demonstrate higher levels of baseline inflammation. This is consistent with previous work on SP-A in the gut by Liu and colleagues (Liu et al., 2021) demonstrating that intestinal levels of inflammatory cytokines are increased at baseline in SP-A knockout mice compared to wild type mice. Cytokine expression in the control group of the HIE model demonstrated no significant difference in inflammation in SP-A knockout mice compared to wild type mice. It is unclear if this difference could be because these mice were slightly older (generally closer to 9 days of age rather than 7 days), or perhaps because there was a greater degree of manipulation in this model.

Since IL-10 is anti-inflammatory, is expressed in response to inflammation, and can help resolve inflammation in the brain by down-regulating production of IL-1β and TNF-α (Opp et al., 1995), we expected IL-10 levels to increase in concert with the increase of pro-inflammatory cytokine expression, perhaps “validating” the impact of the lack of SP-A to increase inflammation in models of neuroinflammation. Indeed, our results indicate that IL-10 expression increased in SP-A$^{-/-}$ mice compared to WT mice in the models. It has been reported that SP-A inhibits LPS-induced expression of IL-10 by macrophages in the lung (Chabot et al., 2003), and in this study IL-10 expression was significantly increased in the LPS-induced model of sepsis. We feel these outcomes strengthen the argument that SP-A has a role in modulating neuroinflammation as it does pulmonary inflammation.

When analyzing for sex differences in cytokine levels following each inflammatory model, only the sepsis model of generalized inflammation showed significant differences in that males demonstrated higher cytokine levels compared to females. Several previous studies investigated sex differences in response to bacterial infection in the presence or absence of SP-A (see (Depicolzuane et al., 2021) for a brief review). Mikerov and colleagues (Mikerov et al., 2008; Mikerov et al., 2012) found that in SP-A knockout mice exposed to *Klebsiella pneumoniae* infection, infected males showed lower survival and were more predisposed to have a higher level of dissemination of infection compared to females. Additional investigation into the mechanisms for sex differences in SP-A deficient mice is a potential future direction for this project.

There are several limitations to this study. The most notable limitation is that SP-A expression in the brain was only analyzed by one method: RT-PCR. This identifies a definite future direction for our work, analysis of actual cytokine levels in the brain. Another limitation is the fairly low sample size (6-8 animals) of each intervention group, resulting in fairly large standard deviations in some analyses. Nevertheless, statistically significant differences were detected between SP-A$^{-/-}$ and WT mice. Finally, there is currently no reliable method of administering exogenous SP-A to SP-A$^{-/-}$ mice in order to demonstrate a direct impact of SP-A on reversal of inflammation. We attempted to introduce intranasal exogenous SP-A obtained via bronchoalveolar lavage of human subjects with pulmonary alveolar proteinosis to neonatal mice, but were unable to verify that SP-A was delivered to the lungs. This is another direction we hope to further investigate.

In conclusion, our study showed that neonatal mice deficient in SP-A demonstrated increased inflammation in the brain following several proinflammatory insults, both focal and generalized. This supports the hypothesis that SP-A does in fact play a role in neonatal neuroinflammation in mice, and possibly in other species. This difference occurred despite our inability to detect SP-A expression in the brains of either WT or SP-A knockout mice both at baseline and following an inflammatory insult, suggesting that either SP-A is expressed elsewhere in the body and circulated systemically to the brain, or SP-A expression needs to be analyzed by other methods designed to detect very low levels of SP-A or localize SP-A to specific sections of the brain.

## 4. Experimental Procedures

### 4.1. Animals

Wild type C57BL/6J mice (WT) were purchased from The Jackson Laboratory (#000664, Bar Harbor, ME). Mice deficient for the SP-A gene (SP-A$^{-/-}$) were obtained from Dr. Carole Mendelson (Montalbano et al., 2013) and were originally generated in the lab of Dr. Samuel Hawgood (Li et al., 2002). It has been long established in the literature that under normal conditions, SP-A null mice have no obvious phenotype other than increased susceptibility to mortality after pulmonary infections by bacteria and viruses (Depicolzuane et al., 2021; Ikegami et al., 1997). Our work and others have also shown that SP-A-deficient mice are more susceptible to mouse models of non-pulmonary injury involving inflammation (Liu et al., 2021; Vieira et al., 2017).

To reduce genetic differences in our wild type and SP-A$^{-/-}$ lines used in this study, backcrossing protocols were used as previously described (Liu et al., 2021). Briefly, SP-A$^{-/-}$ mice and WT mouse lines were crossed when first introduced in our colony and resulting heterozygotes from different sires were crossed again. From these crosses, the resulting SP-A$^{-/-}$ and WT offspring from different sires were used as breeding pairs for our studies. This backcross procedure is repeated yearly to keep genetic variation between the lines at a minimum.

All three neuroinflammatory models were performed in 7- to 10-day-old (P7-P10) mice as previous research has indicated that the brains of 7- to 10-day-old mice and rats are similar to third-trimester human fetuses (and thus premature human newborns) in regards to cellular proliferation and myelination, neuroanatomy, neurochemical indices, and neuroinformatics (Clancy et al., 2007; Dobbing and Sands, 1979; Marret et al., 1995; Romijn et al., 1991). Pups were kept with their dams at all times, with the exception of the brief period during surgery. In regards to blinding, the surgeon for the HIE model was blinded to genotype, and all tissues (SP-A$^{-/-}$ and WT) were processed and analyzed together. SP-A expression was evaluated in 6-week-old mice as previous research has indicated lower levels of SP-A in neonatal mice compared with older animals. Animals of both sexes were included in each experimental group in this study and treatment between the sexes was standardized. A total of 94 pups were used in the study.

### 4.2. Sepsis model

P7 mice were injected intraperitoneally with lipopolysaccharide (LPS-EB Ultrapure; *Escherichia coli*; strain: 0111:B4; 100 μg/kg; InvivoGen, San Diego, CA) diluted to 0.1 μL/g. The dose of LPS (100 μg/kg) used in this study has been previously shown in Sprague-Dawley rats to produce mild fever (Heida et al., 2004), transient activation of cerebral microglia, and a long-lasting increase in hippocampal excitability (Galic et al., 2012). Furthermore, peripheral LPS administration at doses of 20 μg/kg up to 100 μg/animal in mice have been shown to increase pro-inflammatory cytokine expression in the brain (Gabellec et al., 1995; Pitossi et al., 1997). Pups in the control group received the same volume of sterile phosphate-buffered saline (PBS). Pups were returned to their dams, then at 24 hours post-injection, pups were deeply sedated with isoflurane, cervically dislocated, and decapitated to harvest brain tissue. No animals died unintentionally during this procedure.

### 4.3. IVH model

P7 mice received unilateral intraventricular hemoglobin injection using a modification of a previously described rat model of neonatal IVH (Goulding et al., 2020). Prior to IVH induction, pups were sedated throughout the procedure with isoflurane (5% for induction and 2-3% for maintenance). Pups were secured in a warmed stereotaxic frame using nonrupture ear bars (Stoelting, Wood Dale, IL). The scalp was prepped with 10% povidone-iodine, and a midline skin incision was made to expose bregma. Using a stereotaxic injector (Stoelting, Wood Dale, IL) equipped with a Hamilton syringe (model 701 RN, 30G, point style 4, removable needle), the right lateral ventricle was accessed at coordinates 1 mm lateral, 3 mm posterior, and 2 mm deep from bregma. Injections of 150 mg/ml of hemoglobin (MP Biomedicals, Irvine, CA) prepared in phosphate-buffered saline (PBS) or

PBS alone (control group) were delivered at a rate of 6.67 μl per minute, until a total of 10 μl was injected. The syringe was left in place for an additional 1 minute to reduce retrograde flow upon removal. Incisions were closed with Vetbond tissue adhesive (3M Corp, St. Paul, MN), and animals were returned to their dams. All mice were then deeply sedated with isoflurane, cervically dislocated, and decapitated at 4 hours post-surgery to harvest brain tissue. No animals died unintentionally during this procedure.

### 4.4. HIE model

Unilateral HIE was induced in P8-10 mice (weight 5-6 grams) using the modified Rice-Vannucci model (Liu et al., 1999; Rice et al., 1981). After sedation with isoflurane (4% for induction and 1.5-2% for maintenance) and local anesthesia with bupivacaine infiltration to minimize pain and distress, the surgical site was cleaned with 10% povidone-iodine and a midline cervical incision was made. The right common carotid artery was isolated and occluded through 8-Watt electrocoagulation. For mice in the sham groups, the carotid artery was visualized and isolated but not cauterized. The skin incision was closed with Vetbond tissue adhesive and infiltrated with additional local anesthesia. All mice were subjected to ischemic surgery within 5 minutes. Mice then received a subcutaneous injection of 0.3 mL normal saline to prevent dehydration during recovery. After surgery, the pups were kept warm using a temperature-controlled blanket and allowed to recover for 2 hours. Two control groups (True Sham and Sham + Hypoxia) were used in order to control for the effects of hypoxia alone. To induce hypoxia, the HIE and Sham + Hypoxia groups were placed in a chamber containing 10% oxygen and 90% nitrogen at 36°C for 45 minutes. After that, the animals were replaced on a temperature-controlled blanket for 20 min and then returned to their dams. The True Sham group remained in normoxia. All mice were then deeply sedated with isoflurane, cervically dislocated, and decapitated at 24 hours post-surgery to harvest brain tissue. Overall unintended death rate for this procedure was 8.3% and was due to either surgical complications or intolerance of hypoxia.

### 4.5. Assessment of SP-A and cytokine expression

Expression of SP-A was determined via standard reverse transcription PCR analysis, while expression of cytokines IL-1β, IL-6, CXCL1, TNF-α, and IL-10 was determined via real-time quantitative reverse transcription PCR (qRT-PCR) as described previously (Liu et al., 2021). Brains were harvested, flash frozen in liquid nitrogen, then transferred to −80°C freezer until ready for RNA isolation. Brain tissue was mechanically homogenized then processed to isolate RNA using RNeasy Mini Kit (Qiagen, Germantown, MD) per manufacturer's instructions. RNA concentration was quantified using TECAN (Infinite 200 Pro, Männedorf, Switzerland), then reverse transcription was performed for complimentary DNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). For cytokine analysis, qRT-PCR was performed using a LightCycler 480II System (Roche Diagnostics, Indianapolis, IN) and the iTaq Universal SYBR Green Supermix (Bio-Rad) per manufacturer's instructions using primers specific for target mRNAs (see Table 1). Data was calculated by the comparative $C_T$ method ($C_T$, threshold cycle) and each sample was duplicated to ensure accuracy. The amplicon used as a reference in all analyses by real-time qRT-PCR was generated using primers specific for 18S rRNA (Tratwal et al., 2014). We determined that the 18S rRNA primer efficiency is 106%, which is between the 90-110%

acceptable range for PCR primer efficiency (giving an amplification of 2.07 per PCR cycle). Cytokine expression was determined via replicated $2^{-\Delta\Delta C(t)}$ values and normalized to WT control values = 1 (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

For analysis of SP-A expression in tissue, standard RT-PCR was performed using primers specific for mouse SP-A and β-actin (see Table 1). Briefly, mRNA isolated from lung and brain tissue was subjected to reverse transcription to produce cDNA which was then subjected to 50 cycles of PCR. Negative controls were included in the analysis in which the reverse transcription step was eliminated.

### 4.6. Statistical analysis

Values were graphed as average mean ± standard deviation (SD). Comparison of cytokine mRNA expression between two groups by genotype or intervention was determined via Student's t-test. Normality was examined using the Shapiro-Wilk test. In case a variable violated the normality assumption, the statistical result based on the Box-Cox transformed variable was reported. To control for false discovery rate, multiple comparison adjustment was performed using the Benjamini-Hochberg method and all the reported p-values have been adjusted for multiple comparison. We used additional Student's t-test to assess sex differences in cytokine levels with each of the models of inflammation. Statistically significant difference was defined as p-value ≤ 0.05. All statistical analyses were performed in R Statistical Software (version 4.2.0; R Core Team 2022).

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## ABBREVIATIONS:

| | |
|---|---|
| **SP-A** | surfactant protein A |
| **SP-D** | surfactant protein D |
| **CNS** | central nervous system |
| **IVH** | intraventricular hemorrhage |
| **HIE** | hypoxic-ischemic encephalopathy |
| **BBB** | blood-brain barrier |

| | |
|---|---|
| **TLR** | toll-like receptor |
| **LPS** | lipopolysaccharide |

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- Surfactant protein A (SP-A) impacts immunomodulation in extra-pulmonary tissues
- The presence of SP-A has been reported in brain tissue of adult mice
- Prematurity predisposes infants to neuroinflammation via multiple mechanisms
- Neonatal mice deficient in SP-A have increased expression of cytokines in brain tissue

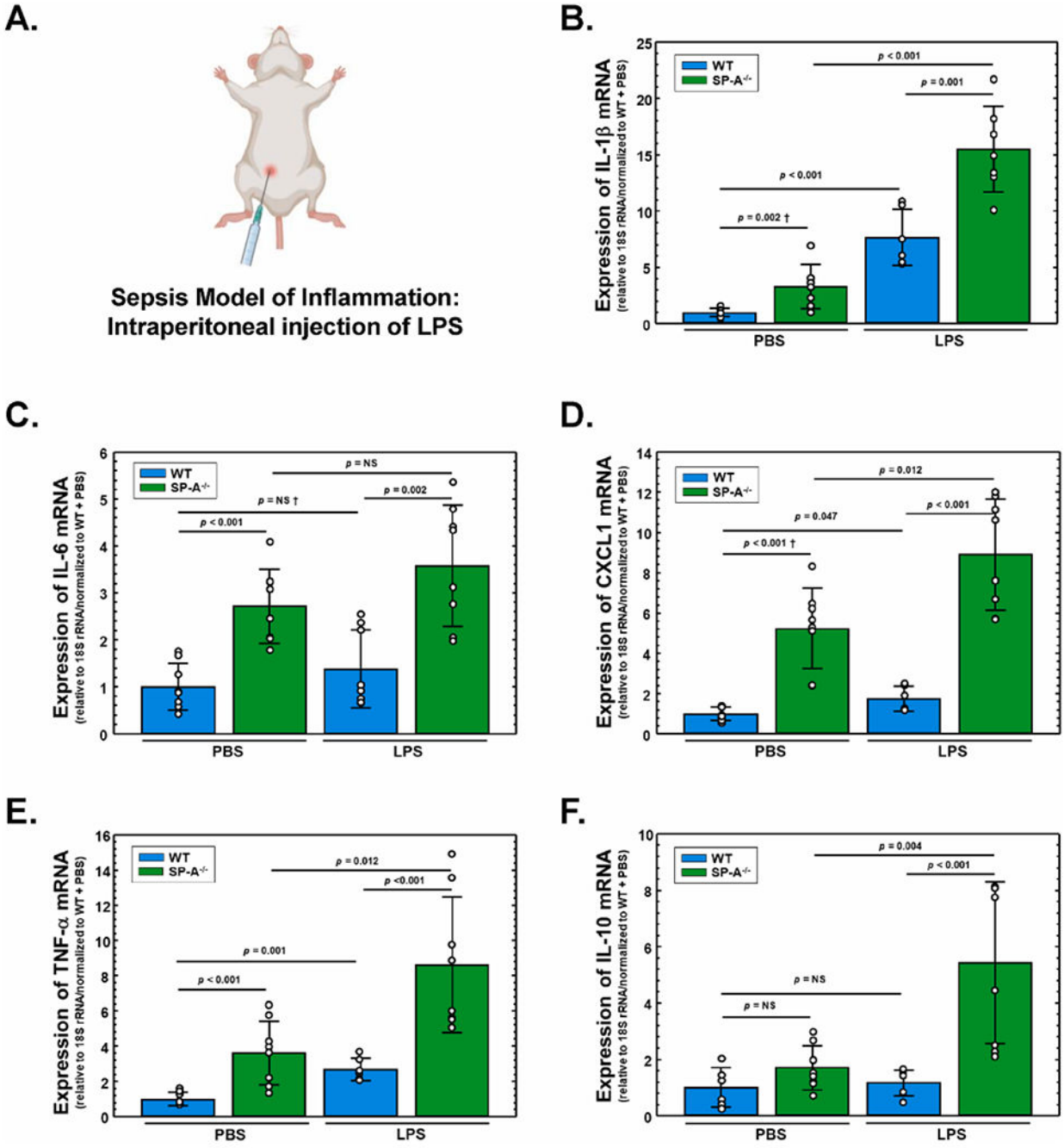


**Figure 1. Cytokine mRNA expression in brains of WT and SP-A$^{-/-}$ mice subjected to systemic inflammation.**

**A.** Neonatal mice (P7) were subjected to a model of LPS-induced systemic inflammation (image created with BioRender.com). After 24 h, mRNA was isolated from brain tissue and analyzed as described in Methods. Shown are levels of cytokine mRNA expression relative to levels in WT mice not exposed to LPS (set as 1); **B.** IL-1β mRNA relative expression, **C.** IL-6 mRNA relative expression, **D.** CXCL1 mRNA relative expression, **E.** TNF-α mRNA relative expression, **F.** IL-10 mRNA relative expression. Data shown represents the average

+/− SD; n ≥ 6 of 3 independent experiments. Significance (p) between samples are indicated by the bars. † indicates p-value after Box-Cox transformation was reported.

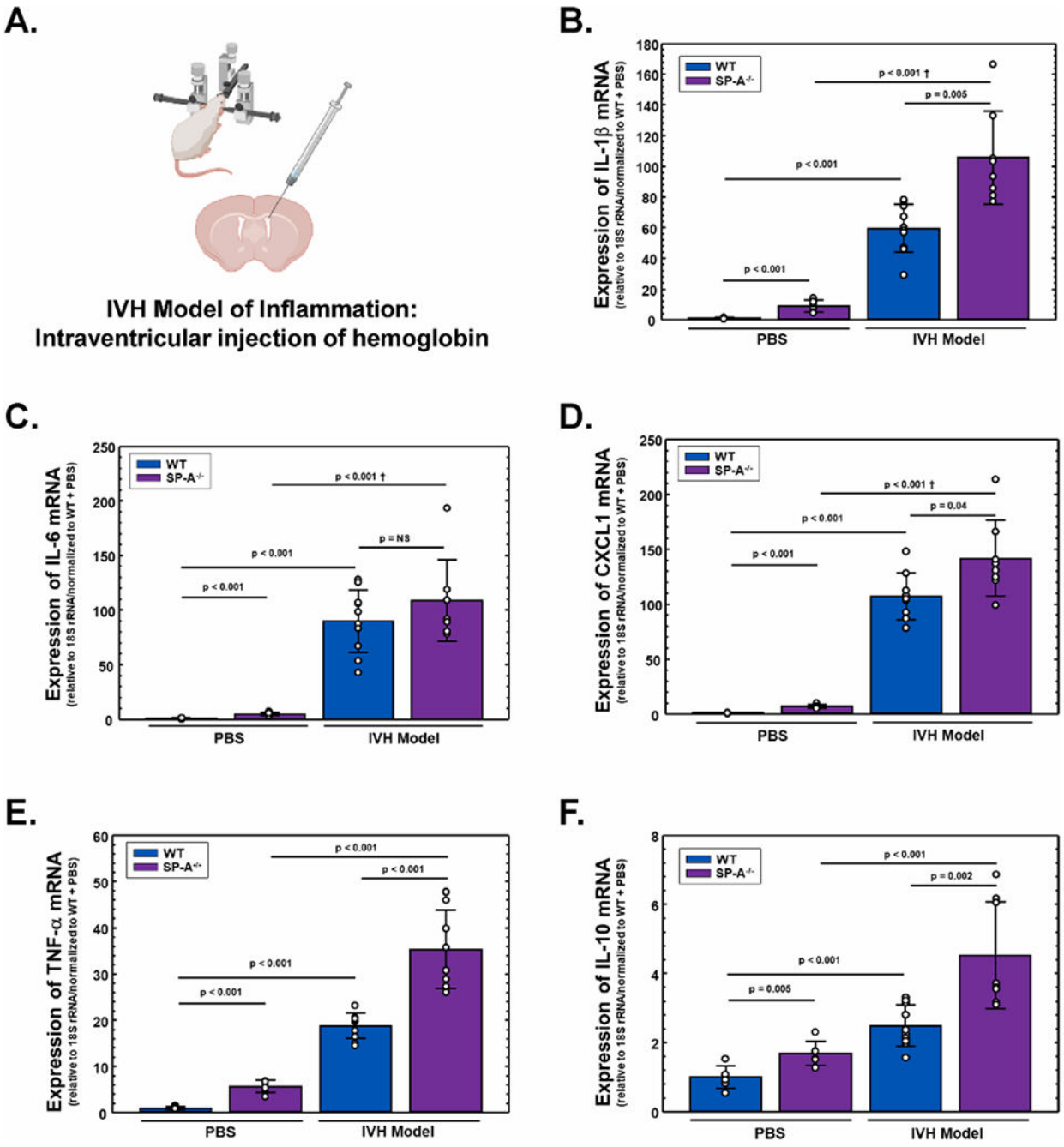


**Figure 2. Cytokine mRNA expression in brains of WT and SP-A$^{-/-}$ mice subjected to an IVH model.**

**A.** Neonatal mice (P7) were subjected to a model of IVH-induced localized inflammation (image created with BioRender.com). After 24 h, mRNA was isolated from brain tissue and analyzed as described in Methods. Shown are levels of cytokine mRNA expression relative to levels in WT mice not exposed to LPS (set as 1); **B.** IL-1β mRNA relative expression, **C.** IL-6 mRNA relative expression, **D.** CXCL1 mRNA relative expression, **E.** TNF-α mRNA relative expression, **F.** IL-10 mRNA relative expression. Data shown represents the average

+/− SD; n ≥ 6 of 3 independent experiments. Significance (p) between samples are indicated by the bars. † indicates p-value after Box-Cox transformation was reported.

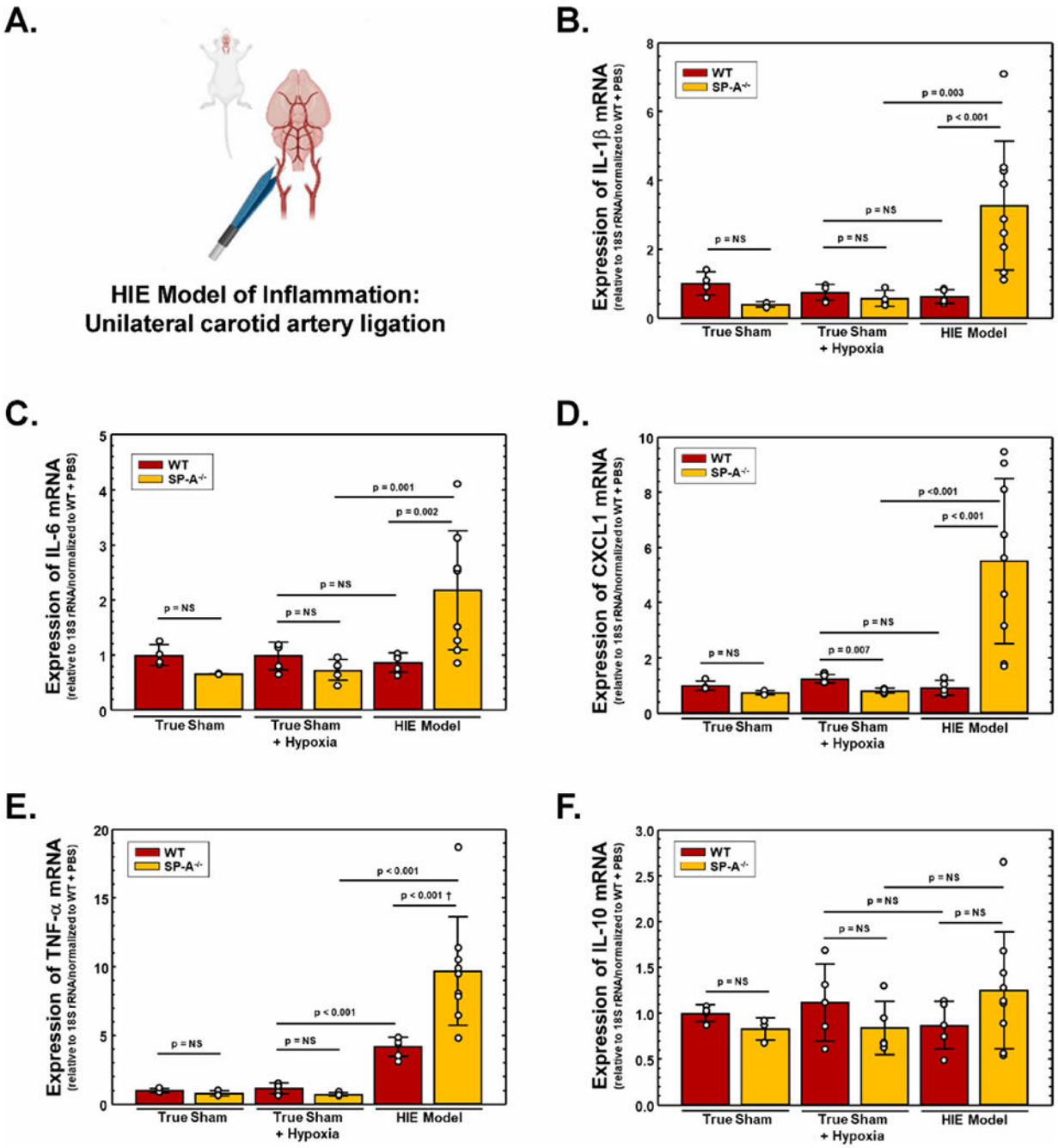


**Figure 3. Cytokine mRNA expression in brains of WT and SP-A$^{-/-}$ mice subjected to an HIE model.**

**A.** Neonatal mice (P8-10) were subjected to a model of HIE-induced localized inflammation (image created with BioRender.com). After 24 h, mRNA was isolated from brain tissue and analyzed as described in Methods. Shown are levels of cytokine mRNA expression relative to levels in WT mice not exposed to LPS (set as 1); **B.** IL-1β mRNA relative expression, **C.** IL-6 mRNA relative expression, **D.** CXCL1 mRNA relative expression, **E.** TNF-α mRNA relative expression, **F.** IL-10 mRNA relative expression. Data shown represents the average

+/− SD; n ≥ 3 of 3 independent experiments. Significance (p) between samples are indicated by the bars. † indicates p-value after Box-Cox transformation was reported.

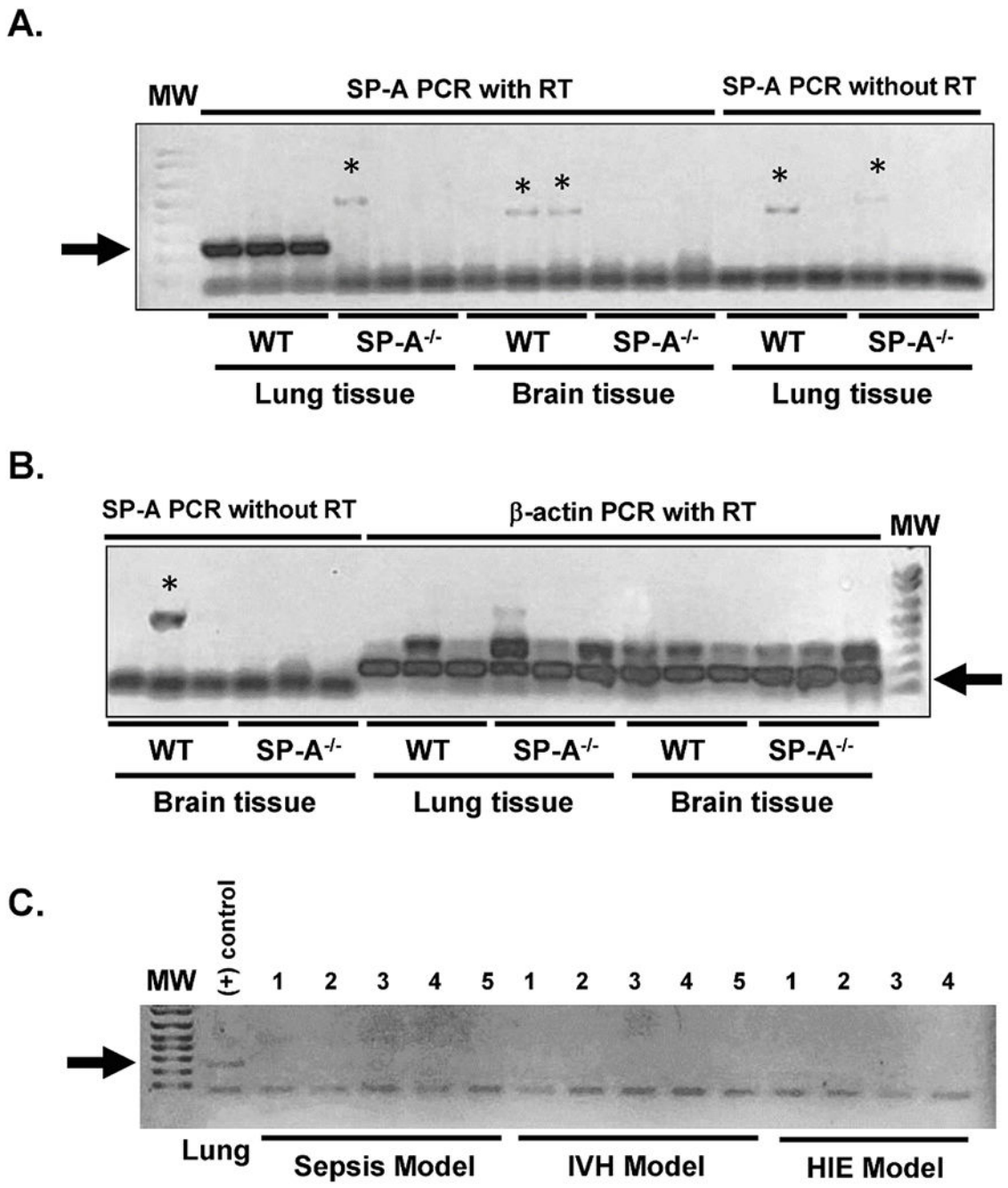


**Figure 4. Detection of mouse SP-A mRNA in WT and SP-A$^{-/-}$ lung and brain tissue.**

**A.** RNA was isolated from lung and brain tissue from 6 week-old WT and SP-A$^{-/-}$ mice. RNA was subjected to reverse transcription and the resulting cDNA subjected to 50 cycles of PCR using primers specific for mouse SP-A. Lung RNA samples were also subjected to PCR analysis without the reverse transcription step to serve as a true negative control. Shown is an image of the ethidium bromide-stained DNA following standard gel electrophoresis. The arrow indicates the expected position of the SP-A amplicon, * indicates unexpected spurious bands. **B.** Image represents a continuation of image in A in which brain

RNA samples were also subjected to PCR analysis without the reverse transcription step to serve as a true negative control. Shown is an image of the ethidium bromide-stained DNA following standard gel electrophoresis. The arrow indicates the expected position of the β-actin amplicon, * indicates unexpected spurious bands. Also included are RT-PCR reactions using β-actin primers as a positive control. **C.** Analysis of neonatal (P7-P10) WT brain tissue for expression of SP-A mRNA after being subjected to inflammatory models. Brain tissue RNA isolated from 4 or 5 WT pups subjected to the neuroinflammatory models was analyzed by RT-PCR as described above using primers specific for SPA-1 mRNA. Shown is an image of the ethidium bromide-stained DNA following standard gel electrophoresis. RT-PCR analysis of RNA isolated from a WT lung sample is included as a positive control. β-actin positive control not shown.

**Table 1.**

Sequence of primers used in real-time quantitative RT-PCR analysis.

| Target Gene | Forward Primer | Reverse Primer | Amplicon Size | Ref |
|---|---|---|---|---|
| **IL-1β** | 5’-GCCACCTTTTGACAGTGATGAG | 5’-AAGGTCCACGGGAAAGACAC | 218 bp | (Cai et al., 2017) |
| **IL-6** | 5’-TAGTCCTTCCTACCCCAATTTCC | 5’-TTGGTCCTTAGCCACTCCTTC | 75 bp | (Willis et al., 2020) |
| **CXCL1** | 5’-CTGCACCCAAACCGAAGTC | 5’-AGCTTCAGGGTCAAGGCAAG | 66 bp | (Stewart et al., 2014) |
| **TNF-α** | 5’-CAGCCTCTTCTCATTCCTGC | 5’-GGTCTGGGCCATAGAACTGA | 132 bp | (Sales et al., 2015) |
| **IL-10** | 5’-GCTCTTACTGACTGGCATGAG | 5’-CGCAGCTCTAGGAAGCATGTG | 104 bp | (Ouchi et al., 2001) |
| **SP-A** | 5’-GTGCACCTGGAGAACATGGA | 5’-TGACTGCCCATTGGTGGAAA | 177 bp | (Ferretti et al., 2016) |
| **β-actin** | 5’-CATGTACGTTGCTATCCA | 5’-CTCCTTAATGTCACGCAC | 249 bp | (Akiyama et al., 2016) |
| **18S** | 5’-GTAACCCGTTGAACCCCATT | 5’-CCATCCAATCGGTAGTAGCG | 150 bp | (Tratwal et al., 2014) |