Pulmonary Surfactant Protein A Is Expressed in Mouse Retina by Müller Cells and Impacts Neovascularization in Oxygen-Induced Retinopathy

Faizah Bhatti,^{1,2} Genevieve Ball,¹ Ronald Hobbs,² Annette Linens,¹ Saad Munzar,¹ Rizwan Akram,¹ Alistair J. Barber,³ Michael Anderson,^{1,4} Michael Elliott,² and Madeline Edwards¹

¹Neonatal Perinatal Medicine, Department of Pediatrics, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States

²Department of Ophthalmology and Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States

³Department of Ophthalmology, Pennsylvania State University Hershey College of Medicine, Hershey, Pennsylvania, United States

⁴Department of Biostatistics and Epidemiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States

Correspondence: Faizah Bhatti, The Children's Hospital, University of Oklahoma Health Sciences Center, 1200 Everett Drive, 7th Floor North Pavilion, Oklahoma City, OK 73104, USA; faizah-bhatti@ouhsc.edu.

Submitted: November 21, 2013

Accepted: October 25, 2014

Citation: Bhatti F, Ball G, Hobbs R, et al. Pulmonary surfactant protein A is expressed in mouse retina by Müller cells and impacts neovascularization in oxygen-induced retinopathy. *Invest Ophthalmol Vis Sci.* 2015;56:232–242. DOI:10.1167/iovs.13-13652

PURPOSE. Surfactant protein A (SP-A) up-regulates cytokine expression in lung disease of prematurity. Here we present data that for the first time characterizes SP-A expression and localization in the mouse retina and its impact on neovascularization (NV) in the mouse.

METHODS. Retinal SP-A was localized in wild-type (WT) mice with the cell markers glutamine synthetase (Müller cells), neurofilament-M (ganglion cells), glial acid fibrillary acid protein (astrocytes), and cluster of differentiation 31 (endothelial cells). Toll-like receptor 2 and 4 (TLR-2 and TLR-4) ligands were used to up-regulate SP-A expression in WT and myeloid differentiation primary response 88 (MyD88) protein (necessary for NFκB signaling) null mouse retinas and Müller cells, which were quantified using ELISA. Retinal SP-A was then measured in the oxygen-induced retinopathy (OIR) mouse model. The effect of SP-A on retinal NV was then studied in SP-A null (SP-A^{-/-}) mice.

RESULTS. SP-A is present at birth in the WT mouse retina and colocalizes with glutamine synthetase. TLR-2 and TLR-4 ligands increase SP-A both in the retina and in Müller cells. SP-A is increased at postnatal day 17 (P17) in WT mouse pups with OIR compared to that in controls ($P = 0.02$), and SP-A^{-/-} mice have reduced NV compared to WT mice ($P = 0.001$) in the OIR model.

CONCLUSIONS. Retinal and Müller cell SP-A is up-regulated via the NFκB pathway and up-regulated during the hypoxia phase of OIR. Absence of SP-A attenuates NV in the OIR model. Thus SP-A may be a marker of retinal inflammation during NV.

Keywords: Müller cells, neovascularization, oxygen-induced retinopathy

Collectins are important collagen-containing, calcium-dependent innate immunity molecules that recognize pathogen-associated molecular patterns by binding with their sugar moieties.¹ These moieties include mannose binding lectin (MBL), pulmonary surfactant protein A (SP-A) and SP-D, and the conglutinins CL-L1 and CL-P1.² They are important in recognizing and mediating neutralization of viruses, bacteria, and fungi, clearance of apoptotic and necrotic cells, and resolution of inflammation.^{2–4} Although several receptor targets for these molecules have been identified, the significance and role of downstream inflammatory mediators and their systemic functions are still largely under investigation.⁵ The pulmonary surfactant proteins SP-A and SP-D play key roles in pulmonary immunity.⁶ Deficiency or dysregulation of SP-A is implicated in developmental pulmonary pathologies including respiratory disease syndrome of prematurity and its sequelae of chronic lung disease or bronchopulmonary dysplasia.^{7–9} Interestingly, these molecules have been found in organs other than those in the pulmonary system.^{10–12} SP-A has been

identified in vaginal and amniotic fluid, synovial fluid, the gastrointestinal tract, and the ocular surface,¹³ thus broadening the possible function of these immunity-regulating proteins. SP-A on the corneal surface has been found to be an important mediator of bacterial clearance in the lacrimal/ocular system.^{14–16} Investigation to this point has focused on the presence and role of SP-A and SP-D in the corneal and lacrimal system, including tear fluid, but little is known about the presence of SP-A or its potential role in the retina.^{13,17,18}

Deficiency of surfactant protein is a major contributor to lung disease of prematurity, as the premature lung has not yet acquired transcriptional potential sufficient to express surfactant and surfactant proteins.¹⁹ After birth, various stressors, including hyperoxia, sepsis, and cytokines, can increase surfactant production.^{7,20} Toll-like receptor 2 (TLR-2) and TLR-4 are both known to increase SP-A in response to ligands such as cytokines and lipopolysaccharides (LPS) through the nuclear factor kappa light chain enhancer of activated B cells (NF-κB). The myeloid differentiation primary response

(MyD88) protein is necessary for activation of NF- κ B, leading to increased transcription of cytokines and cell survival factors.

Babies born prematurely are at risk for retinopathy of prematurity (ROP), specifically, retinal and intravitreal neovascularization (NV). Beyond nutritional status and hyperoxia, the risk of ROP is also increased by inflammation and sepsis.^{21,22} Therapeutic strategies that target neovascularization at the level of oxygen-dependent angiogenic mediators are under investigation.^{23–25} Immunity-related signaling pathways that impact retinal and systemic angiogenesis in premature infants have not been well described.¹³ Two important observations thus emerge: first, SP-A interacts with a host of cytokine-mediating receptors throughout the body and has been found in ocular structures; and second, SP-A dysregulation plays an important role in preterm lung disease. Therefore SP-A up-regulated in the retina in response to inflammatory signals may lead to aberrant blood vessel growth. In this study we addressed the following hypotheses: (1) SP-A is present in retinal tissue; (2) retinal SP-A expression is up-regulated by TLR-2 and -4 ligands; and (3) SP-A expression is increased in OIR and the absence of SP-A attenuates retinal NV in the OIR model.

MATERIALS AND METHODS

We addressed our first hypothesis by visualizing the expression of SP-A *in vivo* in ocular structures and retinas in mice from P0 to P21 and in 6-week-old WT adult mice. In order to address our second hypothesis, we quantified up-regulation of SP-A in whole-retina homogenate after intravitreal injection of TLR-2 and TLR-4 ligands. Protein expression was also measured in human Müller cells (MIO-M1) treated with the same ligands. Finally, we measured SP-A expression in WT mouse retinas during OIR and evaluated the effect of the absence of SP-A on NV in the mouse OIR model.

Animals

C57BL/6J WT mice, MyD88 null (MyD88^{-/-}) mice and SP-A null (SP-A^{-/-}) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The gene targeting strategy of the SP-A^{-/-} mice has been described previously in detail.²⁶ The SP-A^{-/-} mice were previously bred on a background of black Swiss mice, which are known to be homozygous for the recessive retinal degeneration 1 mutation (rd1^{-/-}) of the Pde6b gene.²⁷ The SP-A^{-/-} mice were therefore mated with C57BL/6J mice to outbreed the rd1^{-/-} mutation in six consecutive generations. Genotyping was confirmed by PCR. All mice were maintained on a 12-hour light/dark cycle and fed standard mouse chow. All procedures with animals were performed in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee.

Localization and Expression of SP-A in Mouse Retina

Retina and Lung Tissue Harvest from WT Mouse Pups. Wild-type mice were euthanized, and their retinas were collected at postnatal day 0 (P0), P2, P5, P7, and P14 and in adulthood (ages varied from P48 to 6 months of age). Mice at P0 and P2 were deeply anesthetized via cryoanesthesia and decapitated, and mice at P5 and older were euthanized by CO₂ asphyxiation. One eye was enucleated whole and placed in

fixative (PreFer; Anatech, Ltd., Battlecreek, MI, USA) for 15 minutes at room temperature. The other eye was dissected, and the retina was harvested for whole-retina homogenate. Adult lung tissue was analyzed simultaneously to serve as a positive control for immunohistochemistry (IHC). Lung tissue from all developmental time points was used to compare SP-A expression. Whole-retina and lung tissue homogenates were prepared by addition of 100 to 150 μ L of lysis buffer (Invitrogen, Grand Island, NY, USA) with protease inhibitor cocktail (Millipore, Billerica, MA, USA) to each retina. The protease inhibitor cocktail contained 4-benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, E-64 protease inhibitor, and leupeptin hemisulfate. The tissue was sonicated and centrifuged, and the supernatant containing the total protein was placed in clean microcentrifuge tubes. Whole-tissue lysate protein concentration was then measured using a commercial kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's recommendations. A total of six mice were assessed per time point, which had been determined by power analysis to be able to detect a 30% difference in protein concentration with a β error of 0.2 and an α of 0.05. ELISA was performed to quantify SP-A concentration as detailed below.

IHC of Tissue Cross-Sections. Tissues (retina and lung) were embedded in paraffin and sectioned at 5 μ m onto glass slides. After deparaffinization, each tissue section was blocked in 10% horse serum in Tris-buffered saline-0.3% Triton for 60 minutes. Sections were then incubated in the following primary antibodies overnight at 4°C: rabbit anti-SP-A (1:100 dilution; Life Sciences, St. Petersburg, FL, USA); rat anti-CD31 for endothelial cells (1:40 dilution; Dianova GmbH, Hamburg, Germany); mouse monoclonal anti-glutamine synthetase (GS) for Müller cells (1:200 dilution; clone GS-6; Millipore); chicken anti-gial fibrillary acidic protein (GFAP) for astrocytes (1:500 dilution; Novus Biologicals, Littleton, CO, USA), and chicken antineurofilament M (NF-M) for ganglion cells (1:100 dilution; Millipore). Sections were then incubated with Alexa Fluor 488- and 594-conjugated secondary antibodies (Invitrogen) and examined by confocal microscopy (SP2 model confocal microscope; Leica Microsystems GmbH, Buffalo Grove, IL, USA). All images shown are maximum projections from z-stacks through the entire tissue section. Primary antibody omission controls were also performed for all antibodies (data not shown).

Evaluation of Retinal and Müller Cell SP-A Expression in Response to TLR-2 and -4 Stimulation

Intravitreal Injection of TLR-2 and TLR-4 Ligands.

Adult mice were used for this experiment because intravitreal injection of mouse pups was technically difficult and did not provide reproducible results. Six-week-old WT mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100:10 mg/kg). Animals received either 1 μ g TLR-2 ligand Pam3Cys-Ser-(Lys)4 trihydrochloride (Pam3Cys) (Sigma-Aldrich Corp., St. Louis, MO, USA) or 1 μ g TLR-4 ligand LPS or phosphate-buffered saline (PBS) in a total volume of 1 μ L PBS vehicle. Injections were performed intravitreally using a 36-gauge needle mounted on a 10- μ L syringe (Hamilton Co., Reno, NV, USA). The tip of the needle was inserted under the guidance of a dissecting microscope (Wild M650 model; Leica, Bannockburn, IL, USA) through the dorsal limbus of the right eye. The animals were euthanized at various time points after the injections, the retinas were harvested, and whole-retina homogenates were prepared by addition of 100 to 150 μ L lysis buffer (Invitrogen) with protease inhibitor cocktail as de-

scribed above (Millipore) to each retina. The tissue was sonicated and centrifuged, and the supernatant containing the protein was placed in clean tubes. Whole-tissue lysate protein concentration was then measured using a commercial kit (Pierce Biotechnology) following the manufacturer's recommendations. The same experiment was then repeated in MyD88^{-/-} mice in order to evaluate the contribution of the NF- κ B pathway to SP-A expression. Ten mice were included in each experimental treatment group, and only 1 retina (left) was included for analysis. This was determined by power analysis to detect a 30% difference in protein concentration within groups with a β error of 0.2 and an α of 0.05.

Müller Cell Culture and Treatment With TLR-2 and TLR-4 Ligands. MIO-M1 cells are an immortalized human Müller cell line, which were a kind gift from G. Astrid Limb, University College of London.^{28,29} Cells were grown and maintained on 6-well tissue culture treated glass plates (Corning, Tewksbury, MA, USA) in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% PBS in a humidified incubator in 5% CO₂ at 37°C. Growth medium was changed every 4 to 5 days. When MIO-M1 cells were 80% confluent, they were treated at various time points with 1 μ g/ μ L LPS, or Pam3Cys in 3 mL growth medium, or in control medium alone. Three wells of cells were used per experimental group per time point. Medium and cells were harvested at 1, 6, 12, and 24 hours after treatment and were saved for protein analysis. Once the treatment medium was removed, cells were washed in PBS, and 200 μ L lysis buffer was added to each plate (NP 40 lysis buffer Invitrogen) containing 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 1% Nonidet P40, and 0.02% NaN₃. Protease inhibitor cocktail (Millipore) at 1 $\times$ concentration was added to the lysis buffer just prior to use (Calbiochem, Carlsbad, CA, USA). The cells were sonicated briefly (5–10 seconds) and centrifuged (12,000g at 4°C for 10 minutes). Supernatant was carefully removed and divided into aliquots in clean tubes.

Quantification of SP-A Protein Expression by ELISA. SP-A expression was quantified in whole-retina homogenates as well as MIO-M1 whole-cell lysates by a commercially available ELISA kit (USCNK Life Science, Inc., Wuhan, China), according to the manufacturer's instructions. Briefly, the microwells of a 96-well plate were coated with diluted, purified anti-mouse SP-A monoclonal antibody. The wells were washed, and nonspecific sites were blocked. Diluted, purified mouse SP-A standards (0.625–40 ng/mL) and retinal lysates were added to the antibody-coated wells, and the plate was incubated for 2 hours at 37°C. The plate was then washed and incubated with biotin-conjugated anti-mouse polyclonal SP-A antibody, followed by avidin-horseradish peroxidase, and 3,3', 5,5'-tetramethylbenzidine substrate solution. The reaction was stopped by adding 2 N H₂SO₄, and the color change was measured by reading it at 450 nm, using an iMark microplate reader (Bio-Rad, Hercules, CA, USA).

Effect of SP-A on Retinal Neovascularization

SP-A Expression in Oxygen-Induced Retinopathy. Oxygen-induced retinopathy was induced in WT and SP-A^{-/-} mice by using a previously published technique.³⁰ Briefly, all mouse pups were maintained in room air until P7. At P7, pups were placed with their dams in a poly(methyl methacrylate) (Plexiglas) chamber and exposed to 75% oxygen, using the OxyCycler C42 system (Biospherix, Lacuna, NY, USA), while a second set of pups ($n = 6$) were kept in room air to serve as controls. The dams were replaced every 48 hours with healthy dams because adult mice become sick after exposure to hyperoxia. After 5 days, the pups and dams in the oxygen chamber were returned to room air at P12 and maintained

there until P17. SP-A protein concentrations from whole-retina homogenates were measured by ELISA as described above. Retinas from WT animals were examined at P7 prior to oxygen exposure, at P12 after hyperoxia (vaso-obliterative phase), and at P17 (neovascular phase) at the time of completion of OIR. Six animals were included in each group, which had been determined by power analysis to be able to detect a 30% difference in protein concentration with a β error of 0.2 and an α of 0.05.

Quantification of Neovascularization in SP-A^{-/-} Mice After OIR. To examine the effect of the absence of SP-A on neovascularization, WT and SP-A^{-/-} mouse pups were exposed to OIR and compared to room air (RA) controls. At P17, mouse pups were euthanized, and their retinas were flat mounted for CD31 immunostaining. Total retinal area, area of vaso-oblation (VO), and area of NV were analyzed using a well-established and standardized method.³¹ Briefly, ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) with NV SWIFT macro plug-in (kindly shared by Lois Smith, PhD, Boston, MA, USA) was used, and the total retinal area, the area of vaso-oblation, and the area of NV were quantified. NV was expressed as a percentage of total retinal area. Six animals were included in each group, which had been determined by power analysis to be able to detect a 30% difference in NV with a β error of 0.2 and an α of 0.05.

Statistical Analysis

The mean concentrations of SP-A protein measured by ELISA across the developmental stages were compared by one-way ANOVA with post hoc analysis by Newman-Keuls multiple comparisons test. Unpaired Student's *t*-tests were used to analyze each group treated with TLR ligands compared to controls. A *P* value of <0.05 was considered significant. Areas of neovascularization in OIR were also compared by Student's *t*-tests, with a *P* value of <0.05 considered significant. All protein concentrations were expressed as mean nanograms per milligram (ng/mg) total protein $\pm$ standard error of the mean (SEM).

RESULTS

Pattern of SP-A Expression in the Developing Postnatal Mouse Retina

Lung expression according to IHC results is characterized by a speckled appearance in the cells lining the alveolar spaces (Figs. 1a–c). As the mouse matures, the pattern of staining is enhanced. In order to confirm and validate antibody staining in visual structures in comparison to those in previously published reports, the ciliary body was visualized at P0 and P5 (Figs. 1d, 1e) as were the cornea (Fig. 1m) of neonatal and adult C57BL/6J mice. SP-A expression was positive in the epithelial lining of the cornea at the level of Bowman's layer. Marked staining was also seen in the ciliary body. SP-A expression at the optic nerve head (ONH) at P0 (Fig. 1n) and blood vessel staining in the superficial retinal vascular layer in close proximity to the vitreal surface were visualized (Fig. 1l).

The developmental expression of SP-A in mouse pup retina cross-section was assessed from P0 to P17 by IHC. SP-A was present at P0 in the mouse retina (Fig. 1f). SP-A first appeared in the developing inner neuroblastic layers on the innermost (vitreal) surface of the retina. In addition, the ONH showed marked expression of SP-A at P0 (Fig. 1n). Over the next 2 weeks (Figs. 1g–j), SP-A localized in proximity to developing

FIGURE 1. SP-A localization in C57BL/6j mouse pups from P0 to adulthood. (a–c) SP-A in pulmonary tissue at P0 and P5 and in an adult mouse. SP-A is packaged with lipids and then secreted to the alveolar surface from type II cells. (d, e) SP-A in the ciliary body at P0 and P5. At P0, SP-A staining is prominent at the ONH and is also seen in close association with developing and mature blood vessels. SP-A then appears along the vitreal surface of the developing retina (f–j). By P14, SP-A is penetrating into the deeper layers of the retina and is prominent in the inner plexiform layer. The ONH has prominent staining which is well marked through adulthood (k). Blood vessels show positive SP-A staining, which begins at P0 and is clearly delineated by 6 weeks of age (m). (k) Representative section from an adult cornea with marked staining in the basal layers of the corneal epithelium (green, anti-SPA and nuclei were counterstained with 4',6-diamidino-2-phenylindole [DAPI]). Magnification: $\times 40$.

retinal blood vessels as well as the developing inner and outer plexiform layers. Further enhancement occurred over the superficial surface of the retina (vitreal surface), close to developing blood vessels (Fig. 1j). Prominent staining was noted in the inner plexiform and inner nuclear layers.

Quantitative analysis of SP-A protein concentrations in the same mouse pups (P0–P17) was performed by ELISA and compared to the expression of SP-A in the lung at the same time points (Fig. 2). The time of development retinal SP-A protein expression over the first week of life was similar to the pattern seen in the lung, although 3-fold lower in concentration. In the lung, the SP-A level at P0 was 94.5 ± 10.1 ng/mg, declined on P7 to 49.9 ± 6.2 ng/mg, and then increased by P17 to 61.3 ± 3.1 ng/mg (Fig. 2a). There was significantly less SP-A expressed at all developmental stages than at P0, as measured by one-way ANOVA ($P=0.02$). In the retina (Fig. 2b), the SP-A level was 16.6 ± 0.6 ng/mg at P0, decreased to 7.2 ± 0.4 ng/mg at P5, and then increased to 16.9 ± 1.2 ng/mg at P17. Again, there were significant differences in concentrations ($P=0.01$) when developmental stages were compared to day of birth, P0.

SP-A Colocalizes With the Müller Cell Marker in Mouse Retina

Using IHC, we observed that SP-A immunoreactivity colocalized with GS-positive cells in mouse retina cross-sections and was prominent in the mature adult mouse (Fig. 3). GS is a marker for Müller glial cells in the retina. This was present at the ONH as well as peripherally in the retina. SP-A did not colocalize with ganglion cells as demonstrated by NF-M expression (Fig. 4) or with GFAP (data not shown). Of note, the staining pattern with CD31 showed that SP-A staining was distinct from that of CD31 but in close association with the vasculature (Fig. 5). Therefore, Müller cells are likely the primary SP-A-expressing cells in the mature mouse retina, but SP-A is also associated with blood vessels in the retina.

TLR-2 and TLR-4 Ligands Up-Regulate SP-A Expression In Vivo in Mouse Retina

Because pulmonary SP-A expression is known to be up-regulated by TLR-2 and TLR-4 activation, we hypothesized that

FIGURE 2. Quantification of SP-A protein by ELISA in whole-tissue homogenate in C57BL/6J mice, from P0 to adulthood. (a) Pulmonary tissue; (b) expression in retinal tissue. Levels are expressed as nanograms of total protein per milligram. Pulmonary SP-A levels were significantly decreased at P7 compared to those at P0 ($*P < 0.05$), and retinal SP-A was significantly decreased at both P2 and P5 compared to that at P0 ($*P < 0.05$). Error bars are standard errors.

TLR ligands up-regulate retinal SP-A expression. In order to test this hypothesis, TLR ligands were used to increase expression of SP-A protein in whole-retina samples *in vivo*. Adult mice were used, as consistent results were not obtained by injection in the small mouse pups. Intravitreal injections of the TLR-2 ligand Pam3Cys and the TLR-4 ligand LPS were administered to adult mice, and then SP-A expression was measured in whole-retina homogenate by ELISA. Injection of LPS significantly increased retinal SP-A levels compared to those of controls ($P = 0.0098$), with a peak observed 12 hours after injection (Fig. 6). Injection of Pam3Cys also up-regulated SP-A compared to injection with PBS ($P = 0.05$) with a peak observed at 6 hours after injection. In order to determine whether the increase in SP-A was mediated by NF- κ B, the same injections were also given to MyD88 $^{-/-}$ mice (in which the NF- κ B pathway is inactive). In these mice, there was no up-regulation of SP-A, suggesting that it is up-regulated in retina through MyD88 and NF- κ B-mediated TLR-2 and TLR-4 signaling.

SP-A Expression Is Up-Regulated *In Vitro* in Müller Cells by TLR-2 and TLR-4

In order to confirm that Müller cells express SP-A, we treated MIO-M1 cells *in vitro* with Pam3Cys and LPS. SP-A expression peaked at 6 hours after treatment with LPS ($P = 0.006$) and at 12 hours after stimulation with Pam3Cys ($P = 0.007$), as measured by ELISA (Fig. 7), in comparison to cells stimulated with medium alone. SP-A expression was also quantified in the

cell medium from the stimulated cells and was significantly increased compared to cells stimulated with control medium for Pam3Cys ($P = 0.024$) and LPS ($P = 0.05$). Therefore, Müller cells express and secrete SP-A in response to TLR-2 and TLR-4 ligand activation.

Retinal SP-A Protein Was Increased at the Neovascular Stage of OIR

Although the absence of SP-A attenuated NV, it was not clear whether up-regulation occurred more in the hyperoxic or hypoxic phase of OIR. Therefore, retinal homogenates were analyzed for SP-A protein levels via ELISA in WT mice at P7 prior to hyperoxia; at P12 immediately after the mice were removed from 75% hyperoxia; and then at P17, at the end of the hypoxic phase, and compared to RA controls at all time points. Although SP-A expression was slightly higher, there were no significant differences between the SP-A concentrations in OIR mice at P12 and those in RA controls ($P = 0.27$). At P17, in the OIR mice, the SP-A concentration was significantly increased (1.33 ± 0.09 ng/mg) compared to that in RA controls (0.97 ± 0.12 ng/mg; $P = 0.02$) (Fig. 8).

Absence of SP-A Attenuates the Neovascular Stage in OIR

Development of the retinal vasculature was similar under basal conditions (control RA experiment) in both the WT and SP-A $^{-/-}$ mouse retinas, as seen in retinal flat mounts at P17 (Figs. 9e, 9h). By P17, both groups of mice had complete and normal appearing vascular patterns extending to the edge of the retina. Next, OIR was induced in SP-A $^{-/-}$ and WT mice. The total retinal areas were measured at P17 and found to be similar in both groups of animals. The area of vaso-oblivation at P12 was not statistically different from that in WT animals compared to SP-A $^{-/-}$ mice. SP-A $^{-/-}$ mice had a 50% decrease in neovascular area compared to WT ($P = 0.001$), as shown in Figure 9k. In addition, the density of vascular tufts was decreased (Fig. 9f, 9i, large arrows). The absence of SP-A in the SP-A $^{-/-}$ mouse was confirmed by performing IHC with SP-A antibody staining of the WT and SP-A $^{-/-}$ mouse; results showed SP-A staining in the WT mouse, absence of staining in flat mounts from the SP-A $^{-/-}$ mouse, and SP-A staining in retinal cross-sections and SP-A $^{-/-}$ mouse (Figs. 9a–d). CD31 staining of the endothelial cells was present in both flat mounts and cross-sections.

DISCUSSION

This study is the first to characterize developmental and adult SP-A expression in the mouse retina, and shows that retinal NV is associated with increased levels of SP-A in the OIR mouse model. We elucidated the expression of this protein in the developing mouse retina under basal conditions and showed that it follows the same time pattern of expression previously established in the pulmonary system.²⁰ In addition, we showed that the absence of SP-A is associated with a decrease in NV in the mouse retina during OIR.

SP-A and Its Expression in Retina

SP-A belongs to a superfamily of “c-type lectins.” Multiple receptor targets of SP-A have been identified, including TLR-2 and TLR-4^{32–36}; C1qR^{37,38}; SPR210³⁹; SIRPalpha, calreticulin and CD91⁴⁰; gp340⁴¹; and CD14.⁴² TLR-activated TRAF6 pathways are known to induce angiogenesis through activation of MyD88 and NF- κ B.⁴³ Although it is important for lung

FIGURE 3. SP-A and GS IHC. Retinal cross-section costained with anti-SP-A (green) and anti-GS (red). Merged images show colocalization, which suggests that Müller cells express SP-A from the time they are differentiated at P5. Images are representative of the ONH and peripheral retina for P5 to P10. Magnification: $\times 40$.

pathology, extrapulmonary SP-A has been described in the intestine,^{10,44} corneal surface and lacrimal system,^{45,46} amniotic and vaginal fluid,^{12,47} and recently, in brain tissue.⁴⁸ Multiple epitope sites on the protein make detection a challenge, but newer ELISA methods have had greater reproducibility in protein detection.

The pattern of SP-A expression was visualized in not only the retina but also in other ocular structures such as the cornea and ciliary body. This finding was similar to that reported previously in human ocular structures.¹³ This observation confirmed the specificity of the antibody used for SP-A detection. Glutamine synthetase has been shown to be a relatively specific marker of Müller cells in both the developing

and mature retina.⁴⁹ Several studies have shown that differentiation of Müller cells and glial cell elements in the mouse retina begins quite early. Colocalization of SP-A was highly prominent with GS at P5. Previous studies have shown that GS and the L-glutamate-L-aspartate transporter (GLAST) are highly sensitive markers for Müller cells. There are reports showing that GS and GLAST express early in human retinal development at 14 weeks gestation.⁵⁰ Other markers of Müller cell differentiation in the mouse retina are also present at this point in development.⁵¹ Detection of SP-A in cells also expressing GS suggests that SP-A may be involved in the differentiation of various glial cell types in the retina. SP-A expression in the Müller cells may be a protective response to

FIGURE 4. SP-A and NF-M IHC. Retinal cross-section costained with anti-SP-A (green) and anti-NF-M (red). Merged images show that SP-A did not colocalize with ganglion cells. This was similar for the developmental images and the ONH (data not shown). Magnification: $\times 40$.

FIGURE 5. SP-A and CD31 IHC. Retinal cross-section costained with anti-SP-A (green) and anti-CD31 (red) at P0 and in the adult. SP-A appears to associate closely with the developing vasculature at P0 but does not colocalize with CD31. Magnification at $\times 120$ of the developing blood vessel from the retinal artery shows the vessel cross-section with SP-A toward the periphery of the endothelial layer. This may represent staining in the periphery of the endothelial cell distinct from SP-A or expression in junctional or other mural cell elements. Magnifications: $\times 63$, $\times 100$, and $\times 120$.

systemic inflammation. Although our data show that Müller cells express SP-A, other glial cells (astrocytes), or mural cells such as pericytes, are other candidates for expression, as they are intimately involved in maintaining the blood-retina barrier. Further studies are being undertaken in our laboratory to evaluate these other cell types for SP-A expression. The staining pattern of SP-A in association with the vascular bed suggests that it may play a part in maintenance of the blood retina homeostatic barrier. This is currently under further investigation in our lab.

SP-A Up-Regulation Is Mediated by TLRs and NF κ B Pathway

SP-A has been known to increase in response to TLR receptor activation as well as to act as a TLR ligand. Regardless of cause, an increase in SP-A expression is associated with up-regulation of proinflammatory cytokines. In the context of the developing blood vessels, this may alter the expression of pro-angiogenic signaling molecules such as VEGF as well as molecules that maintain blood vessel integrity (e.g., angiopoietin-1/2).⁵² The in vivo expression of SP-A in the mouse retina after intravitreal injection of TLR-2 and TLR-4 ligands demonstrates that inflammatory pathways induced by common bacterial patho-

gens (e.g., LPS-mediated TLR-4 activation) led to an increase in retinal SP-A levels. Although our data are from adult mice, several studies have shown an increased risk of ROP in the face of maternal chorioamnionitis with postnatal infections and sepsis.^{21,53} Our in vivo studies demonstrated that SP-A expression was more pronounced for LPS-mediated TLR-4 activation, although Pam3Cys-mediated TLR-2 activation was found to occur earlier (12 hours vs. 6 hours). The pattern of SP-A expression in Müller cells was similar to that observed in vivo when treated with the same ligands. These data show that Müller cells express SP-A under inflammatory signal activation and that SP-A is also secreted by the cells, as shown by a significant increase in protein in cell culture medium. Further study will require measuring SP-A protein in the neonatal mouse retina after local or systemic inflammatory insults. Elucidating SP-A expression under the effects of TLR ligand activation is only the first step in understanding its role in vascular development.

Increased SP-A Correlates With NV in OIR and Its Absence Attenuates NV

Our in vivo data are intriguing because deficiency of SP-A leads to only a modest increase in vaso-obliteration but a significant

FIGURE 6. TLR ligand up-regulated SP-A expression in retinal whole-tissue homogenate as measured by ELISA. *Black bars* represent C57BL/6J (WT) mice, whereas *gray bars* show MyD88^{-/-} mice. Expression was measured after in vivo intravitreal injection of either PBS control, Pam3Cys (TLR-2 ligand), or LPS (TLR-4 ligand). Levels are expressed as nanograms of total protein per milligram. Statistically significant differences in protein concentrations in WT mice were compared with those in MyD88^{-/-} mice (* $P < 0.01$) and between PBS and Pam3Cys and between PBS and LPS (## $P < 0.01$). *Error bars* depict SEM.

FIGURE 7. Toll-like receptor ligand up-regulated SP-A expression in MIO-M1 cell homogenate (a) and cell medium (b) as measured by ELISA. MIO-M1 cells were grown to confluency and then treated with either PBS control, TLR-2 ligand Pam3Cys, or TLR-4 ligand LPS. Cells were harvested after 6, 12, and 24 hours. SP-A concentrations were measured in cell homogenate as well as in cell growth medium from treated cells and was expressed as ng/mg of total retinal protein. SP-A was significantly increased in Pam3Cys-treated cells and medium after 12 hours and in LPS-treated cells and medium after 6 hours of treatment ($P < 0.0001$). *Error bars* represent standard errors of the mean.

FIGURE 8. Retinal SP-A expression as measured by ELISA in WT mice in OIR and RA mouse pups. C57BL/6J mice were divided into two groups and exposed to either OIR or kept in room air. SP-A protein concentrations were measured in the retinal homogenates at P7 (baseline), P12 (after hyperoxia, representative of vaso-obliterative stage), and P17 (after normoxia, which is the neovascular stage). Protein concentrations are expressed as nanograms of total retina protein per milligram. SP-A was significantly increased in the OIR mouse pups at P17 after neovascularization (* $P = 0.002$). *Vertical bars* at each time point represent 95% CI.

decrease in NV. What is unclear is whether the decrease in NV is a direct effect of the obliteration of normal vascular growth occurring in the hyperoxic environment or secondary to the subsequent hypoxia, although the increased expression of SP-A in the neovascular stage suggests that it correlates with an increase in angiogenic factors that is known to occur in the second phase of OIR in both rodents and humans.⁵⁴ The decrease of NV in the mouse retina during OIR is a crucial observation in the context of prematurity as deficiency of several important surfactant proteins has been implicated in lung disease of prematurity, and this may possibly also contribute to ROP. Inflammation in premature infants is a major cause of pulmonary and systemic disease. The developing retina is particularly vulnerable to excessive inflammation and reactive oxygen species⁵⁵ as multiple neuronal and vascular structures are not yet established at 23 weeks gestation in humans,⁵⁶ which is the current lowest gestational age at which infants are deemed viable. Thus, the entire vascular system of the retina may develop ex-utero in prematurely delivered infants and therefore must develop under highly stressed conditions.

The question arises, will the role of SP-A protein be anti- or pro-inflammatory in the developing retina? SP-A has been shown to inhibit⁵⁷⁻⁵⁹ release of cytokines, and mice deficient in SP-A have been found to be more prone to morbidity and mortality.⁶⁰ An elevation of SP-A levels was found in the lungs of rats exposed to hyperoxia from days 3 to 10.⁶¹ It is possible that a similar increase in SP-A occurs in the retina that may be an attempt to decrease local inflammation and protect the developing retina. In the second stage of ROP (neoproliferative stage), which occurs several weeks after birth, SP-A levels are well established in infants, and systemic inflammation in babies who have bacterial sepsis may cause further increases in SP-A expression, compromising the neurovascular unit and blood-retina barrier. Targeting hyperoxia-related angiogenic factors has decreased the incidence of permanent vision loss, but this is not yet a validated, safe therapy in the developing preterm neonate. Debilitating ROP continues to be diagnosed in many infants. Inhibiting inflammatory pathways may be a viable adjunctive therapy in the treatment of pathological retinal neovascularization in preterm sick infants, so SP-A expression

FIGURE 9. Quantification of neovascularization in P17 OIR C57BL/6J and SP-A^{-/-} mouse CD31 stained retinal flat mounts. (a) Wild-type and (b) SP-A^{-/-} retinal flat mounts stained with SP-A (green) and CD31 (red). SP-A^{-/-} retina shows absence of SP-A expression. (c) Wild-type retina cross-section and (d) SP-A^{-/-} section, again showing absence of staining for SP-A. Wild-type mice in OIR (e, f) show decrease in neovascularization compared to SP-A^{-/-} (h, i) mice (large arrows show NV tufts). This is quantified on flat mounts as shown (g, j), and when quantified, NV was reduced to almost 50% (k), which was statistically significant (**P* = 0.002). Small arrows (a, c) show SP-A associating with the vascular structures. Large arrows (f, i) are directed at NV tufts. Error bars show standard errors. OIR flat mount magnification: $\times 4$.

and signaling may represent a new molecular target in this work, and we are now working to uncover its role in neonatal mice.

In conclusion, we have shown that SP-A is present in the mouse retina at birth and expresses in a developmental pattern over the first few weeks of life, colocalizing with Müller cell markers and in proximity to developing blood vessels. SP-A expression is inducible in both the mouse retina and in cultured Müller cells, and absence of MyD88 attenuates this increase. SP-A up-regulation correlates with the NV phase of OIR, and absence of SP-A attenuates NV in the mouse OIR model. Further work is currently underway in order to determine the angiogenic factors regulated by SP-A in the neonatal mouse retina.

Acknowledgements

The authors thank G. Astrid Limb, University College of London, for providing the MIO-M1 cells; the Histology Core Facility and Imaging Core Facility; Mark Dittmar, Manager, Animal Facilities at Dean McGee Eye Institute; and Jim Henthorn, Cell Cytology Facility, University of Oklahoma Health Sciences Center.

Supported by a Knights Templar Eye Foundation Pediatric Ophthalmology Career Starter Grant and US National Institutes of Health P20 RR017703-10 Pilot Project Award.

Disclosure: F. Bhatti, None; G. Ball, None; R. Hobbs, None; A. Linens, None; S. Munzar, None; R. Akram, None; A.J. Barber, None; M. Anderson, None; M. Elliott, None; M. Edwards, None

References

- Sastry K, Ezekowitz RA. Collectins: pattern recognition molecules involved in first line host defense. *Curr Opin Immunol*. 1993;5:59-66.
- Holmskov U, Thiel S, Jensenius JC. Collections and ficolins: humoral lectins of the innate immune defense. *Annu Rev Immunol*. 2003;21:547-578.
- Awasthi S. Surfactant protein (SP)-A and SP-D as antimicrobial and immunotherapeutic agents. *Recent Pat Antiinfect Drug Discov*. 5:115-123.
- Wright JR. Immunomodulatory functions of surfactant. *Physiol Rev*. 1997;77:931-962.
- Kishore U, Greenhough TJ, Waters P, et al. Surfactant proteins SP-A and SP-D: structure, function and receptors. *Mol Immunol*. 2006;43:1293-1315.
- Crouch E, Hartshorn K, Ofek I. Collectins and pulmonary innate immunity. *Immunol Rev*. 2000;173:52-65.
- Nagai H, Ogawa Y, Eguchi H, et al. Pulmonary surfactant apoprotein-A in neonates with different respiratory disorders. *Acta Paediatr Jpn*. 1992;34:636-641.

8. Hallman M, Haataja R, Marttila R. Surfactant proteins and genetic predisposition to respiratory distress syndrome. *Semin Perinatol.* 2002;26:450-460.
9. Nkadi PO, Merritt TA, Pillers DA. An overview of pulmonary surfactant in the neonate: genetics, metabolism, and the role of surfactant in health and disease. *Mol Genet Metab.* 2009;97:95-101.
10. Lin Z, deMello D, Phelps DS, Koltun WA, Page M, Floros J. Both human SP-A1 and Sp-A2 genes are expressed in small and large intestine. *Pediatr Pathol Mol Med.* 2001;20:367-386.
11. Luo JM, Wan YS, Liu ZQ, Wang GR, Floros J, Zhou HH. Regularity of distribution of immunoreactive pulmonary surfactant protein A in rat tissues. *Int J Mol Med.* 2004;14:343-351.
12. MacNeill C, Umstead TM, Phelps DS, et al. Surfactant protein A, an innate immune factor, is expressed in the vaginal mucosa and is present in vaginal lavage fluid. *Immunology.* 2004;111:91-99.
13. Brauer L, Kindler C, Jager K, et al. Detection of surfactant proteins A and D in human tear fluid and the human lacrimal system. *Invest Ophthalmol Vis Sci.* 2007;48:3945-3953.
14. Mun JJ, Tam C, Kowbel D, et al. Clearance of *Pseudomonas aeruginosa* from a healthy ocular surface involves surfactant protein D and is compromised by bacterial elastase in a murine null-infection model. *Infect Immun.* 2009;77:2392-2398.
15. Ni M, Tam C, Verma A, et al. Expression of surfactant protein D in human corneal epithelial cells is upregulated by *Pseudomonas aeruginosa*. *FEMS Immunol Med Microbiol.* 2008;54:177-184.
16. McCormick CC, Hobden JA, Balzli CL, et al. Surfactant protein D in *Pseudomonas aeruginosa* keratitis. *Ocul Immunol Inflamm.* 2007;15:371-379.
17. Rantamaki AH, Telenius J, Koivuniemi A, Vattulainen I, Holopainen JM. Lessons from the biophysics of interfaces: lung surfactant and tear fluid. *Prog Retin Eye Res.* 2011;30:204-215.
18. Schicht M, Posa A, Paulsen F, Brauer L. The ocular surfactant system and its relevance in the dry eye [in German]. *Klin Monbl Augenbeilkd.* 227:864-870.
19. Boggaram V, Mendelson CR. Transcriptional regulation of the gene encoding the major surfactant protein (SP-A) in rabbit fetal lung. *J Biol Chem.* 1988;263:19060-19065.
20. ter Horst SA, Fijlstra M, Sengupta S, Walther FJ, Wagenaar GT. Spatial and temporal expression of surfactant proteins in hyperoxia-induced neonatal rat lung injury. *BMC Pulm Med.* 2006;6:8.
21. Dammann O, Brinkhaus MJ, Bartels DB, et al. Immaturity, perinatal inflammation, and retinopathy of prematurity: a multi-hit hypothesis. *Early Hum Dev.* 2009;85:325-329.
22. Sood BG, Madan A, Saha S, et al. Perinatal systemic inflammatory response syndrome and retinopathy of prematurity. *Pediatr Res.* 67:394-400.
23. Chen J, Stahl A, Hellstrom A, Smith LE. Current update on retinopathy of prematurity: screening and treatment. *Curr Opin Pediatr.* 2011;23:173-78.
24. Clark D, Mandal K. Treatment of retinopathy of prematurity. *Early Hum Dev.* 2008;84:95-99.
25. Kong L, Mintz-Hittner HA, Penland RL, Kretzer FL, Chevez-Barrios P. Intravitreal bevacizumab as anti-vascular endothelial growth factor therapy for retinopathy of prematurity: a morphologic study. *Arch Ophthalmol.* 2008;126:1161-1163.
26. Korfhagen TR, LeVine AM, Whitsett JA. Surfactant protein A (SP-A) gene targeted mice. *Biochim Biophys Acta.* 1998;1408:296-302.
27. Clapcote SJ, Lazar NL, Bechard AR, Wood GA, Roder JCNH. Swiss and Black Swiss mice have retinal degeneration and performance deficits in cognitive tests. *Comp Med.* 2005;55:310-316.
28. Lawrence JM, Singhal S, Bhatia B, et al. MIO-M1 cells and similar Müller glial cell lines derived from adult human retina exhibit neural stem cell characteristics. *Stem Cells.* 2007;25:2033-2043.
29. Limb GA, Salt TE, Munro PM, Moss SE, Khaw PT. In vitro characterization of a spontaneously immortalized human Müller cell line (MIO-M1). *Invest Ophthalmol Vis Sci.* 2002;43:864-869.
30. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101-111.
31. Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc.* 2009;4:1565-1573.
32. Awasthi S, Brown K, King C, Awasthi V, Bondugula RA. TLR4-interacting surfactant protein-A-derived peptide suppresses TNF-alpha release from mouse JAWS II dendritic cells. *J Pharmacol Exp Ther.* 2011;336:672-681.
33. Guillot L, Balloy V, McCormack FX, Golenbock DT, Chignard M, Si-Tahar M. Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. *J Immunol.* 2002;168:5989-5992.
34. Ohya M, Nishitani C, Sano H, et al. Human pulmonary surfactant protein D binds the extracellular domains of Toll-like receptors 2 and 4 through the carbohydrate recognition domain by a mechanism different from its binding to phosphatidylinositol and lipopolysaccharide. *Biochemistry.* 2006;45:8657-8664.
35. Yamada C, Sano H, Shimizu T, et al. Surfactant protein A directly interacts with TLR4 and MD-2 and regulates inflammatory cellular response. Importance of supratrimeric oligomerization. *J Biol Chem.* 2006;281:21771-21780.
36. Henning LN, Azad AK, Parsa KV, Crowther JE, Tridandapani S, Schlesinger LS. Pulmonary surfactant protein A regulates TLR expression and activity in human macrophages. *J Immunol.* 2008;180:7847-7858.
37. Malhotra R, Haurum J, Thiel S, Sim RB. Interaction of C1q receptor with lung surfactant protein A. *Eur J Immunol.* 1992;22:1437-1445.
38. Nepomuceno RR, Henschen-Edman AH, Burgess WH, Tenner AJ. cDNA cloning and primary structure analysis of C1qR(P), the human C1q/MBL/SPA receptor that mediates enhanced phagocytosis in vitro. *Immunity.* 1997;6:119-129.
39. Chroneos ZC, Abdolrasulnia R, Whitsett JA, Rice WR, Shepherd VL. Purification of a cell-surface receptor for surfactant protein A. *J Biol Chem.* 1996;271:16375-16383.
40. Gardai SJ, Xiao YQ, Dickinson M, et al. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell.* 2003;115:13-23.
41. Holmskov U, Lawson P, Teisner B, et al. Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule. *J Biol Chem.* 1997;272:13743-13749.
42. Sano H, Chiba H, Iwaki D, Sohma H, Voelker DR, Kuroki Y. Surfactant proteins A and D bind CD14 by different mechanisms. *J Biol Chem.* 2000;275:22442-22451.
43. Pollet I, Opina CJ, Zimmerman C, Leong KG, Wong F, Karsan A. Bacterial lipopolysaccharide directly induces angiogenesis through TRAF6-mediated activation of NF-kappaB and c-Jun N-terminal kinase. *Blood.* 2003;102:1740-1742.

44. Bourbon JR, Chailley-Heu B. Surfactant proteins in the digestive tract, mesentery, and other organs: evolutionary significance. *Comp Biochem Physiol A Mol Integr Physiol.* 2001;129:151-161.
45. Brauer L, Johl M, Borgermann J, Pleyer U, Tsokos M, Paulsen FP. Detection and localization of the hydrophobic surfactant proteins B and C in human tear fluid and the human lacrimal system. *Curr Eye Res.* 2007;32:931-938.
46. Ni M, Evans DJ, Hawgood S, Anders EM, Sack RA, Fleiszig SM. Surfactant protein D is present in human tear fluid and the cornea and inhibits epithelial cell invasion by *Pseudomonas aeruginosa*. *Infect Immun.* 2005;73:2147-2156.
47. Sati L, Seval-Celik Y, Demir R. Lung surfactant proteins in the early human placenta. *Histochem Cell Biol.* 2010;133:85-93.
48. Meng F, Shi L, Cheng X, et al. Surfactant protein A promoter directs the expression of Cre recombinase in brain microvascular endothelial cells of transgenic mice. *Matrix Biol.* 2007;26:54-57.
49. Bringmann A, Wiedemann P. Müller glial cells in retinal disease. *Int J Ophthalmol.* 2012;227:1-19.
50. Georges P, Cornish EE, Provis JM, Madigan MC. Müller cell expression of glutamate cycle related proteins and anti-apoptotic proteins in early human retinal development. *Br J Ophthalmol.* 2006;90:223-228.
51. Walcott JC, Provis JM. Müller cells express the neuronal progenitor cell marker nestin in both differentiated and undifferentiated human foetal retina. *Clin Exp Ophthalmol.* 2003;31:246-249.
52. David S, Kumpers P, van Slyke P, Parikh SM. Mending leaky blood vessels: the angiopoietin-Tie2 pathway in sepsis. *J Pharmacol Exp Ther.* 2013;345:2-6.
53. Manzoni P, Maestri A, Leonessa M, Mostert M, Farina D, Gomitato G. Fungal and bacterial sepsis and threshold ROP in preterm very low birth weight neonates. *J Perinatol.* 2006;26:23-30.
54. Hartnett ME, Penn JS. Mechanisms and management of retinopathy of prematurity. *N Engl J Med.* 2012;367:2515-2526.
55. Wilkinson-Berka JL, Rana I, Armani R, Agrotis A. Reactive oxygen species, Nox and angiotensin II in angiogenesis: implications for retinopathy. *Clin Sci (Lond).* 2013;124:597-615.
56. Roth AM. Retinal vascular development in premature infants. *Am J Ophthalmol.* 1977;84:636-640.
57. Alcorn JF, Wright JR. Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway. *Am J Physiol Lung Cell Mol Physiol.* 2004;286:L129-136.
58. Borron P, McIntosh JC, Korfhagen TR, Whitsett JA, Taylor J, Wright JR. Surfactant-associated protein A inhibits LPS-induced cytokine and nitric oxide production in vivo. *Am J Physiol Lung Cell Mol Physiol.* 2000;278:L840-L847.
59. Agrawal V, Smart K, Jilling T, Hirsch E. Surfactant protein (SP)-A suppresses preterm delivery and inflammation via TLR2. *PLoS One.* 2013;8:e63990.
60. Phelps DS, Umstead TM, Quintero OA, Yengo CM, Floros J. In vivo rescue of alveolar macrophages from SP-A knockout mice with exogenous SP-A nearly restores a wild type intracellular proteome; actin involvement. *Prot Sci.* 2011;9:67.
61. White CW, Greene KE, Allen CB, Shannon JM. Elevated expression of surfactant proteins in newborn rats during adaptation to hyperoxia. *Am J Respir Cell Mol Biol.* 2001;25:51-59.