

Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition

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Parturition is timed to begin only after the developing embryo is sufficiently mature to survive outside the womb. It has been postulated that the signal for the initiation of parturition arises from the fetus although the nature and source of this signal remain obscure. Herein, we provide evidence that this signal originates from the maturing fetal lung. In the mouse, secretion of the major lung surfactant protein, surfactant protein A (SP-A), was first detected in amniotic fluid (AF) at 17 days postcoitum, rising progressively to term (19 days postcoitum). Expression of IL-1 β in AF macrophages and activation of NF- κ B in the maternal uterus increased with the gestational increase in SP-A. SP-A stimulated IL-1 β and NF- κ B expression in cultured AF macrophages. Studies using *Rosa 26 Lac-Z* (B6;1295-Gt(rosa)26Sor) (*Lac-Z*) mice revealed that fetal AF macrophages migrate to the uterus with the gestational increase in AF SP-A. Intraamniotic (i.a.) injection of SP-A caused preterm delivery of fetuses within 6–24 h. By contrast, injection of an SP-A antibody or NF- κ B inhibitor into AF delayed labor by >24 h. We propose that augmented production of SP-A by the fetal lung near term causes activation and migration of fetal AF macrophages to the maternal uterus, where increased production of IL-1 β activates NF- κ B, leading to labor. We have revealed a response pathway that ties augmented surfactant production by the maturing fetal lung to the initiation of labor. We suggest that SP-A secreted by the fetal lung serves as a hormone of parturition.

surfactant protein-A | macrophage | uterus | NF- κ B | labor

The signaling mechanisms that promote increased uterine contractility and the initiation of labor at term remain unclear. In mammalian pregnancy, uterine quiescence is maintained by elevated circulating levels of progesterone (P₄) (1) whereas parturition is associated with a decline in maternal levels of circulating P₄ (2). However, in humans, levels of circulating P₄ and of uterine progesterone receptor (PR) remain elevated throughout pregnancy and into labor (1). Nonetheless, a critical role for functional inactivation of PR in the initiation of labor in humans is supported by the finding that treatment of pregnant women with RU486, a PR antagonist, increases uterine contractility and initiates labor (3). Mice carrying a targeted deletion in the gene for 5 α -reductase type 1 fail to deliver at term (4), suggesting that local metabolism of P₄ within the cervix and uterus also is required for spontaneous labor. Recently, we observed that parturition in humans and mice is associated with a pronounced decline in uterine levels of coactivators known to enhance PR transcriptional activity (5). We suggest that, during pregnancy, uterine quiescence is maintained by increased PR transcriptional activity whereas spontaneous labor is initiated by a series of molecular events that negatively impact PR function.

It seems that labor in women, both preterm and term, is associated with an inflammatory response (6). Increased levels of interleukins in amniotic fluid (AF) and infiltration of the myometrium by neutrophils and macrophages is evident (7). In preterm labor, intraamniotic (i.a.) infection may provide the stimulus for increased AF interleukins and leukocyte migration (8). However, the source of uterine leukocytes and stimulus for

this inflammatory response in normal term labor remain unknown. Previous studies have suggested that the signal for parturition may arise from the fetus (9–11). However, the activating factor(s) and mechanism of action remain undefined.

In this study, we used the mouse as a model to test the hypothesis that augmented surfactant secretion by the maturing fetal lung into AF provides the signal for macrophage migration and the ensuing inflammatory response leading to labor. Pulmonary surfactant, a developmentally regulated lipoprotein produced by lung type II cells, reduces alveolar surface tension and is essential for normal breathing (12). Surfactant production is initiated in fetal lung during the latter third of gestation; inadequate surfactant production by lungs of prematurely born infants can result in respiratory distress syndrome (13). Lung surfactant contains several associated proteins [surfactant protein A (SP-A), SP-B, SP-C and SP-D]. Although SP-B and SP-C are lipophilic and play important roles in reduction of alveolar surface tension (14), SP-A and SP-D are C-type lectins (collectins), which act within the lung alveolus to mediate the innate immune response to microbial pathogens (15). SP-A activates alveolar macrophages, increasing cytokine production and NF- κ B activation (15–17). However, it should be noted that the concept that SP-A acts as an inflammatory agent is controversial (15, 18).

SP-A gene expression is initiated in fetal lung after \approx 80% of gestation is completed, reaching maximal levels before birth (19). Human AF SP-A levels increase from <3 μ g/ml at 30 weeks gestation to >24 μ g/ml at term (20). SP-A expression in human fetal lung is stimulated by IL-1 acting through NF- κ B (21) and by cAMP (21, 22). Administration i.a. of IL-1 to fetal rabbits increased fetal lung expression of SP-A (23) and induced preterm birth (24). The incidence of respiratory distress syndrome is decreased in infants born prematurely to women with chorioamnionitis, supporting a role for increased AF cytokines in fetal lung maturation and surfactant synthesis. In this study, we observed that SP-A, secreted by mouse fetal lung into AF after gestation day 17, activates fetal AF macrophages, causing them to migrate to the uterine wall where they activate NF- κ B, resulting in increased uterine contractility and parturition.

Materials and Methods

Animal Surgery for Injection of Substances into AF or AF Removal. All animal studies were approved by the Institutional Animal Care and Research Advisory Committee of University of Texas Southwestern Medical Center. Eight-week-old female ICR (an outbred Institute for Cancer Research strain) mice were housed with ICR male mice overnight. Mice found to have vaginal plugs

Abbreviations: SP-A, surfactant protein A; AF, amniotic fluid; *Lac-Z* mice, B6;1295-Gt(rosa)26Sor mice; i.a., intraamniotic; β -gal, β -galactosidase; dpc, days postcoitum; LPS, lipopolysaccharide; P₄, progesterone; PR, progesterone receptor; TLR, Toll-like receptor.

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at 8:00 a.m. were considered to be 0.5 days postcoitum (dpc). Timed-pregnant ICR mice at 15 dpc were anesthetized with Avertin (2,2,2 tribromoethanol, tert isoamyl alcohol, Sigma-Aldrich). The right uterine horn was gently pulled through a 1-cm incision made above the visible ovarian fat pad, and substances (50 μ l) were injected through the exposed uterine wall into all amniotic sacs with a sterile 31-gauge half-inch needle (Becton Dickinson). The left uterine horn was untouched. The right uterine horn was returned to the abdominal cavity, the abdominal muscle wall was closed with ETHICON 5-0 Chromic Gut sutures (Becton Dickinson), and skin was closed by using 9-mm wound clips (AUTOCLIP, Becton Dickinson). The mice were kept on a warm pad and returned to cages after recovery.

Isolation of Macrophages from AF. AF was aspirated from exposed amniotic sacs of 15–19 dpc mice by using a sterile 22-gauge half-inch needle. We estimate that there are \approx 200 μ l of AF in each amniotic sac at 15 dpc. Numbers of AF macrophages increased from 2 to 30 per μ l of AF from 15–19 dpc. Macrophages isolated from the AF by adherence to plastic culture dishes were maintained in RPMI medium 1640 for 60 min, incubated in the absence or presence of SP-A (10 μ g/ml) for 30 min, and processed for RNA isolation or immunohistochemical analyses.

Semiquantitative RT-PCR. Total RNA was extracted from AF macrophages by the one-step method of Chomczynski and Sacchi (25) (TRIzol, Invitrogen). First-strand cDNA synthesis reaction was catalyzed by SuperScript II RNase H-reverse transcriptase (RT) (Invitrogen). Amplification of target cDNAs was performed by PCR, as described (5). 18S rRNA transcript was used as a reference to evaluate data from the exponential phase of PCR amplification. Primers were as follows: IL-1 β , forward, 5'-CTGGAGAGTGTGGATCCCAAG-3'; reverse, 5'-GCCCAAGGCCACAGGTATTT-3'; and NF- κ B (p65), forward, 5'-ACCAAGACCCACCCACCAT-3'; reverse, 5'-GCAGAGCCGCACAGCATTCA-3'.

Immunohistochemistry. Primary antibodies used were anti-F4/80 (1:100; Serotec) and anti- β -galactosidase (anti- β -gal) (1:100; Novus Biologicals, Littleton, CO). Secondary antibodies used were biotinylated anti-rabbit IgG (Amersham Pharmacia), FITC-conjugated anti-rabbit IgG, and Texas red-conjugated anti-rat IgG (Jackson ImmunoResearch). Immunoreactivity using biotinylated secondary antibodies was detected with a Vectastain Elite ABC kit (Vector Laboratories) and a Vector Nova Red detection kit (Vector Laboratories), by using standard fixation and staining protocols (5).

Lac-Z (B6;129S-Gt(rosa)26Sor) Mouse Model. To determine whether AF and uterine macrophages were of maternal or fetal origin, Lac-Z transgenic mice, which express β -gal in all cell types, were used (26). Heterozygous Lac-Z male mice were crossed with wild-type ICR females to generate pregnant ICR females carrying 50% heterozygous Lac-Z embryos. Mouse uteri and AF macrophages were analyzed for β -gal activity by using reagents and protocol provided by Specialty Media (Lavellette, NJ).

Immunoblot Analysis. Mouse AF, tissue lysates and nuclear extracts, prepared as described (27), were fractionated in gradient polyacrylamide gels (Invitrogen) and transferred onto Hybond-P (Amersham Pharmacia). Blots were probed by using rabbit antibodies for IL-1 β , p50, p65, CD-68 (1:500) (Santa Cruz Biotechnology), and SP-A (22); and with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) (Amersham Pharmacia) as the secondary antibody. Immunoreactive bands were visualized by using ECL-detection (Amersham Pharmacia).

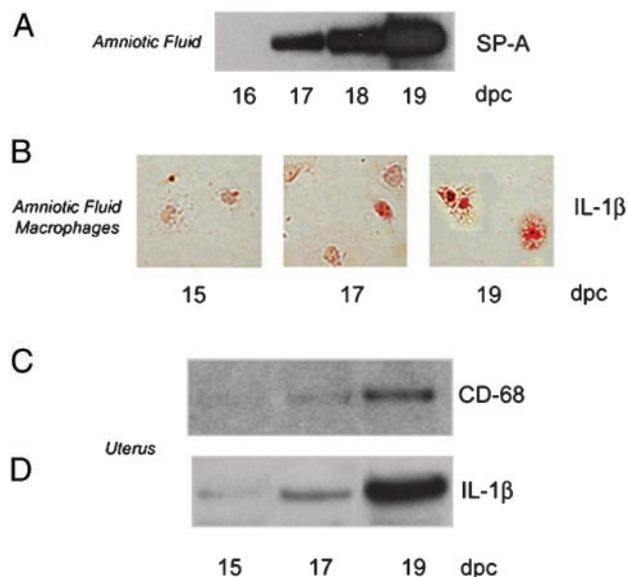


Fig. 1. IL-1 β expression in AF and uterine macrophages increases in association with increased SP-A secretion into AF. (A) SP-A is first detected in mouse AF at 17 dpc and increases markedly toward term. AF isolated from ICR mice at 16–19 dpc was analyzed for SP-A by immunoblotting. Shown is a representative immunoblot of an experiment repeated in three gestational series of mice. (B) IL-1 β protein increases to detectable levels in mouse AF macrophages toward term. AF macrophages were subjected to immunohistochemical analysis for IL-1 β . Immunohistochemical analysis shown is representative of findings obtained using macrophages from three different gestational series of mice. (C) Macrophage infiltration of the pregnant uterus increases toward term. Uteri of 15, 17, and 19 dpc mice were analyzed for CD-68 levels by immunoblotting. The immunoblot shown is representative of findings obtained using uteri from three different gestational series of mice. (D) IL-1 β levels increase in the pregnant uterus toward term. IL-1 β protein levels in uteri of 15, 17, and 19 dpc mice were analyzed by immunoblotting. The immunoblot shown is representative of findings obtained using uteri from three different gestational series of mice.

SP-A Purification. Human alveolar proteinosis lavage was delipidated by using isopropyl ether/1-butanol (28). Serum albumin was removed by extraction on a DEAE-Affigel blue gel column (Bio-Rad). Endotoxin contamination was removed with polymyxin B agarose (Sigma). Remaining endotoxin content was assessed by using a QCL-1000 LAL assay kit (BioWhittaker). Purity of the delipidated, DEAE-Affigel blue- and polymyxin B-treated SP-A preparation was assessed by Coomassie blue staining and immunoblot analysis by using an antibody raised against SP-A (22) (Fig. 6 A and B, which is published as supporting information on the PNAS web site). A control solution, largely depleted of SP-A, was prepared by passing the SP-A-enriched preparation over a D-Mannose column (Sigma) (29). The SP-A-depleted preparation was examined for remaining SP-A content by immunoblot analysis (Fig. 2).

Results

Augmented Secretion of SP-A into AF During Late Gestation Is Associated with Increased Expression of IL-1 β mRNA by AF Macrophages and Increased Numbers of Macrophages Within the Uterine Wall. SP-A gene expression serves as an excellent marker of fetal lung maturity (30). SP-A mRNA is first detectable in fetal mouse lung at 17 dpc and increases to term (31). SP-A in mouse AF, was evident at 17 dpc and increased markedly through 19 dpc (Fig. 1A). AF levels of SP-A, compared with known concentrations of purified SP-A by immunoblotting, were determined to be \approx 30 μ g/ml at 19 dpc (data not shown). In human pregnancy, AF

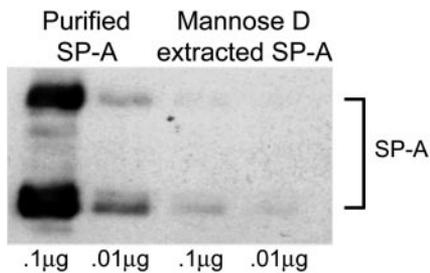


Fig. 2. Purified SP-A and SP-A-depleted preparation. SP-A isolated from the alveolar proteinosis material and the mannose-D-depleted SP-A preparation were analyzed for levels of SP-A by immunoblotting. The latter contained markedly reduced levels of SP-A.

levels of SP-A increase from $<3 \mu\text{g/ml}$ at 30 weeks gestation to $>24 \mu\text{g/ml}$ at term (20).

We postulated that SP-A may interact with AF macrophages, inducing their activation and migration to the maternal uterus. Immunocytochemistry of AF macrophages revealed that IL-1 β , a marker for macrophage activation, was detectable at 17 dpc and increased markedly at term (Fig. 1B). This increase in AF macrophage IL-1 β was temporally associated with concomitant increases in levels of the macrophage marker CD68 (Fig. 1C) and of IL-1 β (Fig. 1D) in the maternal uterus.

Injection i.a. of 15 dpc Pregnant Mice with SP-A Causes Preterm Labor.

To evaluate the capacity of SP-A to initiate the cascade of events that give rise to the initiation of labor *in vivo*, SP-A ($3 \mu\text{g}$ per sac) containing $<3 \text{ fg}$ of lipopolysaccharide (LPS) per microgram of SP-A (after polymyxin B treatment) (see *Materials and Methods* and Fig. S6) was i.a. injected into mice at 15 dpc, a time when SP-A is undetectable in AF. The amount of SP-A injected resulted in an AF concentration comparable to that normally found at term ($\approx 15 \mu\text{g/ml}$). Parallel groups of mice were i.a. injected with equivalent amounts of protein from a preparation largely depleted of SP-A on a mannose D column (Fig. 2). Fourteen of 17 mice injected with SP-A delivered premature fetuses surrounded by intact amniotic membranes from the injected uterine horn at 16–17 dpc (Table 1 and Fig. 7, which is published as supporting information on the PNAS web site) but not from the uninjected horn, suggesting a local effect of SP-A. The fetuses in the uninjected horn were reabsorbed. Spontaneous preterm labor occurred despite elevated maternal serum P₄ levels at 16 dpc (SP-A-injected mice, in labor, $96.5 \pm 4.4 \text{ ng/ml}$ P₄, $n = 3$; untreated mice, $91.9 \pm 2.1 \text{ ng/ml}$ P₄, $n = 3$). Mice i.a. injected with the SP-A-depleted preparation manifested normal term labor ($n = 4$) (Table 1). To ensure that any remaining LPS

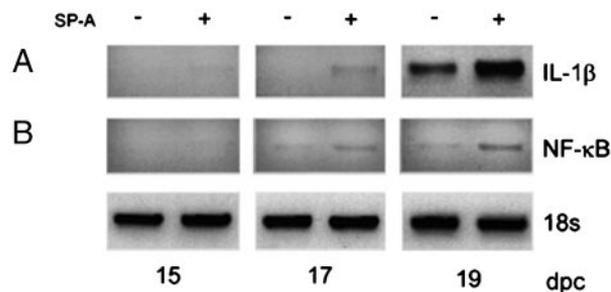


Fig. 3. IL-1 β and NF- κ B mRNA levels in mouse AF macrophages increase as term approaches and are stimulated by SP-A treatment. AF macrophages obtained from mice at 15, 17, and 19 dpc were incubated with or without $10 \mu\text{g/ml}$ SP-A for 30 min. IL-1 β and p65 mRNA levels were assessed by RT-PCR. This experiment was repeated three times with similar results.

in the SP-A preparation was insufficient to induce labor, 15 dpc mice were i.a. injected with $\approx 9 \text{ fg}$ of LPS per sac; these mice delivered at term ($n = 4$) (Table 1). To deplete endogenous AF SP-A, 15 dpc mice ($n = 6$) were i.a. injected with rabbit anti-human SP-A IgG (22) (150 ng of IgG protein per amniotic sac); these mice all delivered late ($27.5 \text{ h} \pm 9.7 \text{ h}$) (Table 1). A control group of 15 dpc mice ($n = 3$) were i.a. injected with nonimmune rabbit IgG (150 ng of IgG protein per amniotic sac); these mice delivered at term (Table 1). Based on these results and the correlation of AF levels of SP-A with IL-1 β expression in AF macrophages and with macrophage infiltration of the pregnant uterus (Fig. 1), we postulate that secretion of SP-A by the maturing fetal lung near term may provide the stimulus for activation and migration of AF macrophages to the maternal uterus, resulting in the uterine inflammatory response observed in term labor.

Expression of IL-1 β and NF- κ B in Mouse Amniotic Fluid Macrophages Is Stimulated by SP-A.

To assess the role of SP-A in AF macrophage activation, semiquantitative RT-PCR was used to analyze effects of a 30-min incubation with SP-A ($10 \mu\text{g/ml}$) on IL-1 β and NF- κ B p65 mRNA levels in mouse AF macrophages isolated at 15, 17, and 19 dpc. IL-1 β (Fig. 3A) and p65 (Fig. 3B) mRNA levels were undetectable in AF macrophages at 15 dpc and increased toward term. SP-A treatment increased IL-1 β and p65 mRNA at each gestational time point (Fig. 3A and B). These findings suggest that SP-A has the capacity to activate AF macrophages and enhance their production of proinflammatory cytokines, possibly through stimulation of NF- κ B expression.

Macrophages of Fetal Origin Infiltrate the Maternal Uterus in Late Gestation.

We postulated that the AF macrophages that invade the uterus at term arise from the fetus. To test this hypothesis,

Table 1. Injection of SP-A into all amniotic sacs of one uterine horn of 15 dpc pregnant mice causes preterm labor whereas injection of antibody to SP-A delays labor

Injection	No. of mice		
	Preterm parturition	Term parturition	Delayed parturition
SP-A ($3 \mu\text{g}$ per sac)	14/17 (3.0 ± 0.5 days preterm)	3/17	—
SP-A-depleted eluate	—	4/4	—
LPS (9 fg per sac)	—	4/4	—
SP-A antibody	—	—	6/6 (27.5 ± 9.7 h postterm)
Nonimmune rabbit IgG	—	3/3	—

SP-A ($3 \mu\text{g}$ per sac) i.a. injected into 15 dpc mice caused preterm parturition in 14 of 17 mice. To control for unknown contaminants in the SP-A preparation, 15 dpc mice were i.a. injected with an SP-A-depleted preparation ($n = 4$). These mice delivered at term. To control for levels of LPS remaining in the SP-A preparation, 15 dpc mice were i.a. injected with LPS (9 fg of LPS per sac) ($n = 4$); these mice delivered at term. Endogenous AF levels of SP-A were immunodepleted by i.a. injection of 15 dpc mice with SP-A antibody (150 ng per sac) ($n = 6$). These mice delivered at least 24 h late. To control for nonspecific effects of rabbit IgG, 15 dpc mice were i.a. injected with 150 ng of rabbit nonimmune IgG (Sigma) per sac ($n = 3$); these mice delivered at term.

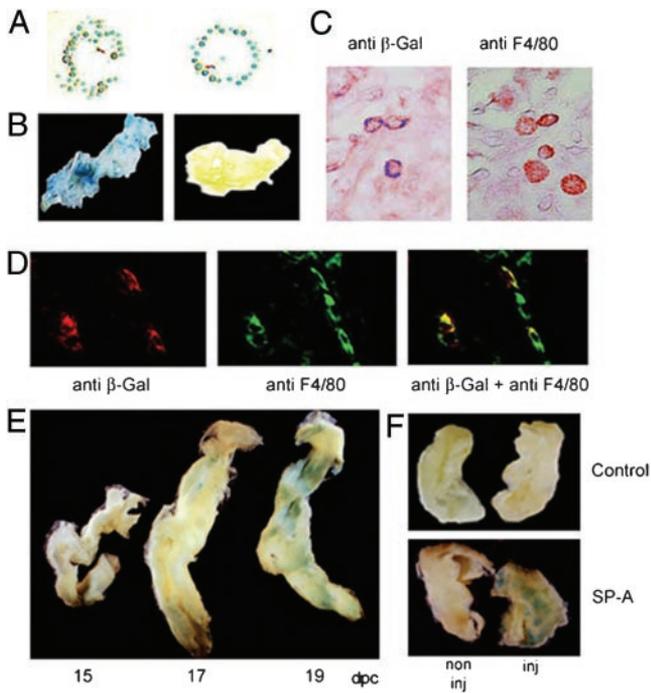


Fig. 4. AF macrophages of fetal origin invade the maternal uterus near term. (A) AF macrophages are fetal in origin. β -gal staining was detected in cytoplasmic vacuoles of macrophages isolated from AF surrounding *Lac-Z* embryos at 17 dpc. (B) Cells of fetal origin infiltrate the maternal uterus near term. β -gal staining was detected in uteri of wild-type mice carrying *Lac-Z* embryos at 17 dpc (Left). Uteri from wild-type mice carrying wild-type pups were negative (Right). (C) Macrophages of fetal origin infiltrate the maternal uterus. Immunohistochemical analysis of serial sections of uteri from 17 dpc mice carrying *Lac-Z* embryos revealed that β -gal-expressing cells are immunopositive for F4/80. (D) β -gal and F4/80 colocalize in uterine cells. Dual immunofluorescence of uteri from 17 dpc mice carrying *Lac-Z* embryos revealed colocalization of β -gal and F4/80. (E) Cells of fetal origin infiltrate the maternal uterus in increasing numbers toward term. β -gal-expressing cells were first detectable in uteri of the wild-type ICR females carrying *Lac-Z* embryos at 17 dpc and increased to term. (F) SP-A injection into all amniotic sacs of one uterine horn on 15 dpc mice increases infiltration of fetal cells into the maternal uterus. Fifteen dpc mice carrying *Lac-Z* embryos were i.a. injected with SP-A or vehicle (Control). Postsurgery (4.5 h), the injected (inj) and noninjected (non inj) uterine horns were assayed for β -gal-positive cells.

uteri, AF macrophages, and embryos from wild-type ICR females ($n = 3$) carrying $\approx 50\%$ *Lac-Z* heterozygous embryos were assayed for β -gal activity at 17 dpc. Macrophages isolated from amniotic sacs containing *Lac-Z*-positive embryos contained β -gal activity (Fig. 4A), demonstrating that AF macrophages are of fetal origin. The uterus of the wild-type mother carrying the *Lac-Z* embryos also was positive for β -gal activity whereas the uterus of a wild-type mother carrying wild-type embryos was negative (Fig. 4B). Immunohistochemical analysis of serial sections of the wild-type uterus carrying *Lac-Z* embryos indicated that cells staining for β -gal also were positive for the macrophage cell marker F4/80 (Fig. 4C). Using dual immunofluorescence, β -gal colocalized with F4/80 (Fig. 4D). Not all of the cells that were immunopositive for F4/80 stained for β -gal (Fig. 4C and D) because only $\approx 50\%$ of the fetuses carried the *Lac-Z* gene, and a proportion of the uterine macrophages may be of maternal origin. These findings indicate that macrophages that invade the pregnant uterus at term are at least, in part, of fetal origin.

To analyze temporal changes in fetal macrophage migration to the maternal uterus, uterine tissues of wild-type ICR females carrying *Lac-Z* embryos were stained for β -gal activity at 15–19

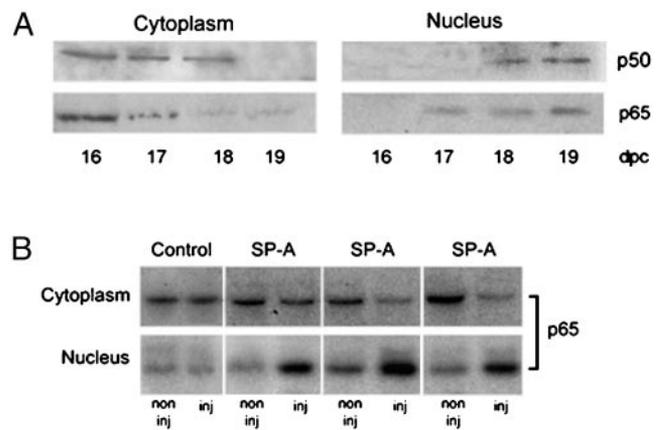


Fig. 5. Nuclear localization of NF- κ B proteins increase in mouse uterus as term approaches and is further increased by SP-A injection into amniotic fluid. (A) NF- κ B activation increases in the pregnant mouse uterus as term approaches. Nuclear and cytoplasmic fractions obtained from uteri of mice at 16, 17, 18, and 19 dpc were subjected to immunoblotting by using antibodies against p50 and p65. This immunoblot is representative of findings obtained using pregnant mice from three independent gestational series. (B) Injection of SP-A into all amniotic sacs of one uterine horn on 15 dpc increases activation of NF- κ B in the pregnant uterus. Fifteen dpc mice were i.a. injected either with SP-A or vehicle (Control). The injected mice were killed 4.5 h after surgery. Uterine cytoplasmic and nuclear fractions prepared from the injected (inj) and contralateral noninjected (non inj) horns were analyzed for p65 by immunoblotting.

dpc. β -gal staining in the uterus was first detectable at 17 dpc and increased markedly toward term (Fig. 4E).

Injection i.a. of SP-A Causes Migration of Fetal Macrophages to the Injected Horn of the Maternal Uterus. Wild-type ICR females were crossed with heterozygous *Lac-Z* males; SP-A ($3 \mu\text{g}$ per sac) was i.a. injected at 15 dpc. β -gal activity was assayed in the injected and contralateral uterine horns 4.5 h postsurgery. β -gal activity was detectable in the uterine horn containing injected amniotic sacs as early as 4.5 h after SP-A injection (Fig. 4F). By contrast, β -gal was undetectable in the contralateral uterine horn containing amniotic sacs injected with $3 \mu\text{g}$ of protein from the SP-A-depleted preparation (Fig. 4F). These findings suggest that increased SP-A levels in AF stimulate migration of fetal macrophages to the pregnant uterus, resulting in the activation of the inflammatory cascade shown in Fig. 1.

NF- κ B Activation Occurs in the Pregnant Uterus in Late Gestation. To determine whether the increased AF macrophage migration to the uterus during late gestation was associated with NF- κ B activation, NF- κ B p50 and p65 levels were analyzed in uterine cytoplasmic and nuclear fractions by immunoblotting. Remarkably, cytoplasmic levels of p50 and p65 decreased in the uterus between 16–19 dpc, in association with a coordinate increase in nuclear levels of these proteins (Fig. 5A). This result suggests that an inflammatory stimulus in the pregnant uterus near term promotes nuclear translocation and activation of p65 and p50.

Injection i.a. of SP-A Causes Local NF- κ B Activation in the Injected Horn of the Maternal Uterus. To determine the effect of i.a. injection of SP-A on uterine NF- κ B activation, SP-A ($3 \mu\text{g}$ per sac) or the SP-A-depleted preparation ($3 \mu\text{g}$ per sac) (control) was i.a. injected at 15 dpc ($n = 3$); subcellular localization of p65 was analyzed in the injected and contralateral uterine horns 4.5 h later. Nuclear levels of p65 were increased in the uterine horn containing SP-A-injected amniotic sacs, as compared with the

Table 2. Injection of the NF- κ B inhibitor SN50 into all amniotic sacs of one uterine horn of 15 dpc mice delays parturition

Injection	No. of mice		
	Preterm parturition	Term parturition	Delayed parturition
SN50 (10 pg per sac)	—	—	6/6 (28.0 \pm 8.9 h postterm)
SN50mut (10 pg per sac)	—	4/4	—
SN50 + SP-A	—	6/6	—

The NF- κ B inhibitor SN50 ($n = 6$) or the inactive mutant peptide SN50mut ($n = 4$) were i.a. injected into 15 dpc mice. A delay in parturition (28 h \pm 8.9 h) was observed in the SN50-injected mice. All SN50mut-injected mice delivered at term. To determine whether activation of uterine NF- κ B is critical for the effect of SP-A to induce preterm labor, 15 dpc mice were coinjected with SN50 and SP-A ($n = 6$); these animals delivered at term.

uninjected contralateral horn and the uterine horn injected with the SP-A-depleted preparation (Fig. 5B).

Inhibition of NF- κ B Activation in the Pregnant Uterus by Intra-amniotic Injection of SN-50 Delays the Onset of Labor. To determine whether activation of endogenous NF- κ B plays a role in the initiation of labor, the NF- κ B inhibitor peptide SN50 (32) was i.a. injected into mice at 15 dpc ($n = 3$) (10 pg per sac). The inactive peptide SN50mut (32) or vehicle were i.a. injected into 15 dpc mice ($n = 3$) as a control. At 17 dpc, uterine nuclear levels of p50 and p65 were reduced in SN50-treated animals as compared with SN50mut- and vehicle-injected controls (Fig. 8, which is published as supporting information on the PNAS web site). Mice i.a. injected with SN50 ($n = 6$) and allowed to proceed to term displayed a delay in the onset of labor (28 h \pm 8.9-h delay) whereas mice injected with vehicle (data not shown) or SN50mut delivered at term (Table 2). These findings suggest that activation of NF- κ B in the pregnant uterus plays a critical role in the initiation of labor. To determine whether the induction of preterm labor by SP-A is mediated by means of NF- κ B activation, 15 dpc mice ($n = 6$) were i.a. injected with both SP-A and the NF- κ B inhibitor SN50. In contrast to mice injected with SP-A alone, coinjected mice delivered at term, suggesting that the induction of preterm labor by SP-A requires activation of NF- κ B (Table 2).

Discussion

Herein, we provide evidence that the maturing fetal lung transmits a hormonal signal for the initiation of parturition through augmented secretion of SP-A into AF near term. As mentioned above, it has been suggested that the signal for parturition may arise from the fetus. Mitchell *et al.* (11) reported that a substance(s) in human AF secreted by fetal kidney caused a marked increase in prostaglandin E₂ (PGE₂) production by cultured human amnion cells. Others suggested that a stimulus may emanate from the maturing fetal lung. Lopez Bernal *et al.* (9) found that pulmonary surfactant isolated from human AF stimulated PGE synthesis by discs of human amnion. They suggested that surfactant phospholipids secreted into AF by fetal lung provide a source of arachidonic acid precursor for prostaglandin synthesis by the avascular amnion. Johnston and colleagues (10) proposed that platelet-activating factor, a component of the fetal lung surfactant lipids secreted into AF at term, may play an important role in activation of myometrial contractility at term.

We observed that SP-A levels were first detectable in mouse AF at 17 dpc and increased dramatically to term. The gestational increase in AF SP-A was associated with elevated expression of IL-1 β and NF- κ B in AF macrophages, increased macrophage migration to the maternal uterus, and enhanced levels of IL-1 β and nuclear NF- κ B in uterine tissue. Treatment of AF macrophages with SP-A *in vitro* enhanced IL-1 β and NF- κ B expression, suggesting that increased cytokine production during late ges-

tation is stimulated by endogenous SP-A. The critical role played by SP-A in the initiation of labor was further supported by the finding that injection of SP-A into mouse AF at 15 dpc caused preterm parturition at 16–17 dpc whereas injection of SP-A antibody delayed parturition by >24 h. Our findings using *Lac-Z* mice indicate that AF macrophages of fetal origin migrate to the maternal uterus in increasing numbers toward term and that increased AF levels of SP-A stimulate fetal macrophage migration. We propose that the SP-A-induced macrophage migration initiates an NF- κ B-signaling cascade within the uterus leading to labor. The importance of NF- κ B signaling in the initiation of labor was further supported by the finding that injection of the NF- κ B inhibitor SN50 into mouse AF delayed the onset of term labor and inhibited induction of preterm labor by SP-A.

It should be noted, however, that *SP-A*^{-/-} mice (33), as well as transgenic mice that overexpress SP-A under control of the *SP-C* gene promoter (34), have no reported parturition defects. On the other hand, in the latter study (34), there was no evidence presented for up-regulated secretion of SP-A into AF by lungs of the transgenic fetuses. Furthermore, we suggest that a critical number of pups either lacking or overexpressing SP-A in one uterine horn is required for an alteration in timing of parturition to occur. Thus, if only 50% of the fetuses were SP-A deficient or overexpressed SP-A, a parturition defect may not be evident. Additionally, based on the present findings in mice injected i.a. with SP-A antibody, we suspect that *SP-A* null mice may manifest a delay in parturition of only \approx 24 h, which may not have been noted. This finding suggests the presence of other “fail-safe” mechanisms for the initiation of parturition. Like SP-A, the related collectin SP-D is developmentally regulated in fetal lung late in gestation, reaching maximal levels at term (35, 36). SP-D protein also is evident in human amniotic fluid although its levels increase only modestly after 32 weeks of gestation (37), in contrast to the pronounced rise in SP-A (20, 37). Although SP-D levels remained unchanged in lung tissues of adult transgenic mice that overexpressed recombinant SP-A (34) and in adult *SP-A* null mice (33), no information was provided regarding SP-D levels in the fetal lungs.

SP-A plays an important role in innate immunity within the lung alveolus (15, 16). SP-A treatment of human peripheral blood mononuclear cells, lung alveolar macrophages, and monocyte cell lines caused increased expression of proinflammatory cytokines (reviewed in ref. 16), as well as rapid activation of NF- κ B (38). Additionally, SP-A stimulates macrophage migration (39) and chemotaxis (40), which seems to occur by directional actin polymerization (41).

SP-A seems to interact with two members of the Toll-like receptor (TLR) family on macrophages, TLR2 (42) and TLR4 (43). TLR2 serves as receptor for peptidoglycan in cell walls of Gram-positive bacteria (44) whereas TLR4 is a component of the LPS receptor complex in cell walls of Gram-negative bacteria (45). Both TLRs mediate signal transduction pathways leading to NF- κ B activation. Recent evidence suggests that TLR4 mediates

induction of preterm labor in mice treated with LPS (46). Thus, preterm labor associated with bacterial infection and labor at term may be mediated by similar receptors and signaling pathways although the ligands that bind these receptors differ.

We postulate that SP-A-induced activation of NF- κ B increases uterine contractility by two mechanisms. On the one hand, activated NF- κ B increases expression of target genes that cause increased myometrial contractility, such as *cyclooxygenase-2* (*COX-2*) (47), which catalyzes the rate-determining reaction in the biosynthesis of prostanoids. On the other hand, NF- κ B p65 has been reported to interact with PR to reduce its DNA binding and transcriptional activity (48). In this manner, p65 may antagonize PR activation of target genes that modulate uterine quiescence. We previously observed a decline in histone acetylation within the pregnant uterus at term, caused by a marked decrease in expression of uterine coactivators with intrinsic HAT activity. This decline in coactivators and in histone acetylation may also cause a loss of PR function, leading to the initiation of

labor (5). The cellular mechanism(s) for this decline in coactivator expression has not as yet been determined.

In conclusion, our studies provide important evidence that augmented production of SP-A by the maturing fetal lung at term provides a key hormonal stimulus for the cascade of inflammatory signaling pathways within the maternal uterus that culminate in the enhanced myometrial contractility leading to parturition. This hormonal signal, transmitted to the uterus by fetal AF macrophages reveals that the fetal lungs are sufficiently developed to withstand the critical transition from an aqueous to an aerobic environment.

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