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Expression of select immune genes (surfactant proteins A and D, sheep beta defensin 1, and toll-like receptor 4) by respiratory epithelia is developmentally regulated in the preterm neonatal lamb

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Abstract

Preterm infants experience enhanced susceptibility and severity to respiratory syncytial virus (RSV) infection. Terminal airway epithelium is an important site of RSV infection and the extent of local innate immune gene expression is poorly understood. In this study, expression of surfactant proteins A and D (SP-AD), sheep beta defensin 1 (SBD1), and toll-like receptor 4 (TLR4) mRNA were determined in whole lung homogenates from lambs. SP-AD and TLR4 mRNA expression increased ($p<0.05$) from late gestation to term birth. In addition, gene expression of LCM-retrieved type II pneumocytes (CD208+), adjacent epithelium (CD208−) and bronchial epithelium demonstrated that bronchiole-alveolar junction epithelium (combined CD208+/−) had significant ($p<0.05$) developmental increases in SP-AD, SBD1 and TLR4 mRNA, whereas CD208+ cells had statistically significant increases only with SP-A mRNA. Using immunofluorescence, SP-AD antigen distribution and intensity were also greater with developmental age. These studies show reduced SBD1, SP-AD, and TLR4 expression in the preterm lung and this may underlie enhanced RSV susceptibility.

Keywords

Antimicrobial peptide; Beta-defensin; Lung; Preterm infant; Sheep; Surfactant protein; Toll-like receptor

1. Introduction

Respiratory syncytial virus (RSV) is a pneumo-virus that causes significant seasonal respiratory disease especially in young children [1]. Infants born at <32 weeks gestation (37+ weeks = term) are hospitalized for respiratory infections fourteen times more often than full-term infants, and preterm infants have an elevated risk for severe RSV infection [2,3]. Factors that may contribute to increased RSV severity at preterm include smaller airways and a lack of maternal neutralizing antibodies as well as reduced innate immune activity such as: reduced

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innate cytokine responses and phagocytosis by monocytes secondary to RSV [4], reduced monocyte function [5], impaired neutrophil activity [6], impaired lung epithelial cell repair [7], and altered responses to lipopolysaccharide [8].

Study of the basic mechanisms in pediatric RSV infection is limited by technical difficulty, practical and ethical concerns. Investigation of the mechanisms in RSV infection requires a documented animal model with comparable disease manifestations. Late-term fetal and preterm lambs have proven valuable as pulmonary models for the study of surfactant expression and regulation, ventilation-induced injury, congenital diaphragmatic hernia, chorioamnionitis, sleep apnea and persistent pulmonary hypertension in the newborn [9–13]. They have also been useful for study of RSV, parainfluenza virus-3 (PIV-3) infection and other infectious respiratory diseases [10,14]. Sheep infected with ovine RSV strains have consistency with human RSV disease including increased susceptibility in newborns, lesions, and similar innate immune genes [15–17]. Recently, preterm lambs were shown to have reduced RSV clearance compared to full-term lambs, which is similar to age-dependent disease seen in preterm RSV infected infants [2,3,15].

A major site for clinical RSV infection is airway epithelium and little is known about developing epithelia's capacity for innate immune response. Cell specificity of CD208 (also known as dendritic cell-lysosomal associated membrane protein) expression in human, murine and ovine lungs is histologically, morphologically, biochemically and ultrastructurally consistent with type II pneumocytes [18]. The type II pneumocyte is an important cell in this region as it functions not only as a stem cell for alveolar type I pneumocytes, but also as regulator of surfactant and innate immune molecule expression in the terminal airway. Cellular expression products of the terminal airway that have putative (direct or indirect) anti-viral activity include surfactant proteins A and D (SP-A and SP-D, respectively) [19], toll-like receptor 4 (TLR4, [20]) and beta defensins [18]. A role for these immune factors in paramyxoviral clearance was recently characterized by increased expression levels of ovine surfactant proteins A and D (SP-A, SP-D) and sheep beta defensin 1 (SBD1) in PIV3-infected newborn lambs and the timing of this increased expression was associated with viral clearance [10]. TLR4, a pattern recognition receptor, has been shown to be critical in activating immune responses to RSV and decreased TLR4 expression is associated with severe RSV disease in infants and mice [20,21]. In vivo, epithelia's innate immune response to RSV infection is poorly defined and developmental expression may contribute to the age-dependent clearance and RSV disease-severity seen in infants and lambs.

The hypothesis of this work is that target anti-RSV innate immune genes are developmentally and anatomically regulated in respiratory epithelium. Of particular interest is cellular expression by terminal airway epithelium, since these cells are the major site of clinical infection, innate immune defense and regeneration.

2. Materials and methods

2.1. Lambs

Five lambs at 115 days of gestation (80% gestation), four lambs from 130 days of gestation (90% gestation), four lambs at full-term, natural birth and four lambs 15 days old were acquired (C-section or natural birth) from date mated commercial ewes as previously described [15]. For C-section lambs (days 115 and 130), lambs were extracted from the uterus and euthanized with sodium pentobarbital. Natural birth lambs (term and 15-day old) were euthanized within 6 h of birth (term) or at 15 days of age.

2.2. Laser capture microdissection (LCM) tissue preparation

Lung tissues were embedded in OCT media and placed over blocks of dry ice until the tissues were snap frozen. All samples were then transferred immediately to −80 °C for storage. Tissues were cut with a cryostat (6 μm sections) onto glass slides at −25 °C. The sections were stored first at −80 °C for durations of anywhere from 0 to 8 days without noticeable loss of immunofluorescence or real-time qPCR signals from all such sections.

2.3. Immunofluorescent detection of CD208+ cells

Upon retrieval from −80 °C for use in IHC-LCM, slides were defrosted (30 s) and quickly transferred into cold nuclease-free PBS (made with HPLC-grade water, Fisher Scientific, Hanover, IL, PBS tablets and 0.1% Tween 20, Sigma, St. Louis, MO, USA) for a series of cold PBS washes. Next, blocking solution comprised of 1% normal goat serum “NGS” (Sigma) and 1% normal swine serum “NSS” (Invitrogen) in Common Antibody Diluent (BioGenex) was added to the sections for 5 min. Then sections were incubated for 10 min with a primary: biotinylated-secondary-antibody complex (prepared by mixing equal volumes of primary monoclonal mouse anti-human CD208 IgG [Cat. No. IM3448, Beckman Coulter] and a 1 mg/ml solution of biotinylated goat anti-mouse secondary IgG [Kirkegaard & Perry]) with an additional amount of BioGenex Common Antibody Diluent (comprising 28% of the final primary-biotinylated-secondary-mixture volume). Slides were subsequently washed three times (cold nuclease-free PBS+0.1% Tween 20), then Cy3streptavidin reagent (Rockland, Inc.), diluted 1:300 with Common Antibody Diluent (BioGenex, containing 1% NGS and 1% NSS) was added to the sections for 10 min, followed again by three washes. For dehydration prior to LCM, sections were put through a series of graded ethanol baths and xylene.

2.4. LCM conditions

Just before LCM, slides were dried for 15 min under a laminar flow hood and immediately placed inside a desiccator (with fresh desiccant) until subjected to LCM. LCM was routinely performed on each section within 40±20 min of their removal from ultra-fresh xylene. Typical LCM laser power settings (on the PixCell II LCM system, Arcturus) used were 80 milliwatts (mW) for a duration of 800 μs for a calculated spot size of 10 μm. Briefly, approximately 100–300 cells of a particular cell type, as identified during LCM by IF–HC and morphology, were laser captured onto HS LCM caps (Arcturus). In this study, type II pneumocytes were identified with CD208+ immunofluorescence and laser captured. Using a new HS LCM cap, the CD208 − cells (adjacent bronchiolar epithelium and type I pneumocytes) were collected. These were immediately adjacent to the previously detached CD208+ cells. Lastly, on a different HS LCM cap, the cells of bronchial epithelium were laser captured. After all cells of interest were harvested onto HS LCM caps, total RNA was isolated.

2.5. RNA isolation from LCM samples on HS LCM caps

Immediately post-laser capture, 10 μl of Lysis Buffer (Invitrogen) was pipetted directly onto captured cells in the center of each HS LCM cap, fitted onto 0.5 ml nuclease-free microfuge tubes (ABI) and placed on ice. After each LCM session, all HS LCM cap-sample tube assemblies were removed from the ice container and incubated at 75 °C for 12 min in a heating block (Arcturus). After the extraction incubation, the tubes were vortexed and microfuged. Next, 1.6 μl 10× DNase I buffer (Invitrogen) and 5 μl of DNase I (Amplification Grade DNase I, Invitrogen) were added and vortexed, spun down, and incubated. Next, to minimize non-specific adhesion of RNA to positively charged plastic surfaces (in tubes and plates), we added glycogen to each sample [22]. The glycogen stock solution was made by diluting Ultrapure Glycogen (Invitrogen) with nuclease-free water (MidWest Scientific) and 18.4 μl of a 1.902 μg/μl glycogen solution was added to each sample. The final concentration of glycogen was 0.2 μg/μl in each reaction well. After glycogen addition, samples were vortexed and spun down

for collection. Isolates were heated for 5 min at 65 °C (in a GeneAmp 2400 thermocycler, Perkin Elmer/ABI) followed by snap cooling on ice and storage at −80 °C to ensure template linearity.

2.6. One-step real-time qPCR

RNA samples (from above) were spun down, and 6 μl of each was used as "RNA template" in 30 μl fluorogenic one-step real-time qPCR reactions as carried out in 96-well PCR reaction plates (ABI) using a GeneAmp 5700 Sequence Detection System for detection and relative quantification of four mRNA targets: ovine surfactant protein A (SP-A), ovine surfactant protein D (SP-D), sheep beta-defensin-1 (SBD1) and ovine toll-like receptor 4 (TLR4). The qPCR Master Mix and RT enzyme were used as suggested and provided in a commercially available kit (TaqMans® One-Step qPCR Master Mix Reagents Kit, ABI). Each of the 30 μl one-step real-time qPCR reactions contained: 15 μl One-Step Master Mix, 0.25 U/μl Multiscribe™ RT enzyme, 0.4 U/μl RNAse inhibitor, appropriate forward and reverse primer and fluorogenic probe concentrations (Table 1), nuclease-free water, and 6 μl of each LCM-(RNA template) cell extract isolate. Before use, all solutions were gently vortexed and spun down. Thermocycling conditions for fluorogenic one-step qPCR were: 35 min at 48 °C, 10 min at 95 °C, and 50 cycles of: 15 s at 95 °C and 1 min at 58 °C. We used ovine ribosomal protein S15 (ovRPS15) as the housekeeping (endogenous reference) gene, the sequence for which we received from Dr. Sean Limesand (Department of Pediatrics University of Colorado Health Sciences Center). We designed all sequence-specific oligonucleotide primers and fluorogenic probes using Primer Express v.2.0 in conjunction with the search tool BLAST (Basic Local Alignment Search Tool, National Center for Biotechnology Information). Resultant probe sequences were checked for specificity by comparing them for similarity to all other available sequences in the database. Only unique sequences and/or sequences that spanned a genomic intron were used for our SP-A, SP-D, SBD1 and TLR4 primer and probe designs. Each plate contained both target and endogenous references for all samples present on that plate, and a negative no-template control ("NTC"; nuclease-free water) for each target and endogenous reference. All samples were run in duplicate. For the LCM RNAs, each target signal mRNA level was normalized to its respective ovRPS15 signal (see Section 2.8). This was followed by calculating the average and standard error of the mean for all replicates per each particular sample group.

2.7. Whole lung homogenates

Total RNA was isolated from 0.3 g of sheep lung tissue as previously described [10]. Briefly, all total RNA isolates were assessed for quantity and purity by examining 1:50 dilutions of each by spectro-photometry at 260 and 280 nm, and DNase treatment was performed using the TURBO DNA-*free* kit from Ambion. DNase treatment mixtures were vortexed gently, and then incubated in a 2400 thermocycler (Perkin Elmer/ABI) for 30 min at 37°C. 1 μl DNase Inactivation Reagent per 10 μl solution was added to each tube. The tubes were incubated for 2 min at room temperature with intermittent vortexing every 10–15 s, then centrifuged at 10,000*g* for 1.5 min to pellet the Inactivation Reagent, and the upper transparent layer containing the RNA was transferred to a new tube. Next, complementary deoxyribonucleic acid (cDNA) synthesis was performed. Reverse transcription master mix containing 3.38% nuclease-free water, 31.17mM TRIS, 64.94mM KCl, 5.71mM $MgCl_2$, 2.08mM dNTP mix, 2.6 μM random hexamers and 0.0222 μg/μl TURBO DNase-treated RNA was heated for 5 min at 65 °C then snap-cooled on ice for at least 1 min. Samples were spun down, and RNAse inhibitor (20 U/μl, ABI) and SuperScript III RT enzyme (200 U/μl, Invitrogen) were finally added to each cooled sample reverse transcription mixture (now 200–400 μl each). The final concentrations attained of each reverse transcription component were: 3.25% nuclease-free water, 30mM TRIS, 62.5mM KCl, 5.5mM $MgCl_2$, 2mM dNTPs (0.5mM each of dATP, dCTP, dTTP and dGTP), 2.5 μM random hexamers, 3.5 U/μl SuperScript III RT enzyme, 0.4 U/μ

RNAse inhibitor and 0.021 μg/μl TURBO DNase-treated RNA. These reagents were vortexed gently, split into 100 μl amounts into nuclease-free 0.2 ml tubes (MidWest Scientific), and the tubes were placed into the GeneAmp 2400 thermocycler. Reverse transcription thermocycler conditions were: 5 min at 25 °C, 45 min at 53 °C, 15 min at 70 °C, followed by a safety hold at 4 °C. The values of target gene expression were normalized to ovRPS15 values (see Section 2.8) followed by calculation of the mean and standard error of the mean for all replicates in each particular sample group.

2.8. Quantification of fluorescent real-time PCR

The calculation for relative fluorescent PCR expression of whole lung homogenates and LCM retrieved cells was performed using a recently described mathematical model, value = $[(E_{\text{target}})^{\Delta Ct(\text{control}-\text{treated})}]/[(E_{\text{ref}})^{\Delta Ct(\text{control}-\text{treated})}]$ [23]. The calculated cellular expression of the composite bronchiole–alveolar junction (BAJ) was made by pooling the quantified expression values of the CD208+ and CD208− cells.

2.9. Statistics

To assess the cellular target gene expression trends, linear regression was used to calculate the sign (+/−) of the slope coefficient. To compare regression coefficients of each anatomic region for a given target gene, a Student's *t*-test was performed as previously described [24].

3. Results

Whole lung homogenates

In whole lung homogenates, expression of SP-A, SP-D, and TLR4 mRNA was limited in preterm lambs, but significantly increased throughout development ($p<0.05$ for each target gene, Fig. 1). Expression of SBD1 was detected most often in the day-15 group with only one lamb having detectable expression in the −30 day to term groups (data not shown).

SP-A

BAJ epithelium (combined CD208+ and CD208− cells) had significantly increased SP-A expression during development ($p = 0:01$) and greater developmental expression than the bronchial epithelium ($p = 0:07$) (Fig. 2A). LCM-retrieved CD208+ cells alone showed increased SP-A expression with development ($p = 0:03$) while CD208− and bronchial cells exhibited no statistically significant alterations.

SP-D

SP-D mRNA expression level showed significant variation between individual animals. Expression of SP-D mRNA by BAJ epithelium (combined CD208+ and CD208− cells) significantly increased during development ($p = 0:04$), while bronchial expression was sporadic late gestation to term and absent in older neonatal (15 days old) lambs (Fig. 2B).

SBD1

BAJ epithelium (combined CD208+ and CD208−) showed progressively increased SBD1 mRNA expression levels during development ($p = 0:04$), while bronchial epithelium lacked detectable expression in neonatal (term and 15 days old) lambs (Fig. 2C). SBD1 expression by BAJ epithelium was increasingly detectable after day-115 gestation.

TLR-4

While BAJ epithelium (combined CD208+ and CD208−) had progressively increased TLR4 mRNA expression levels during development ($p = 0:02$), remaining TLR4 expression levels

for specific cell types were not statistically significant, but sporadic for all time points (Fig. 2D).

Fluorescent microscopy

SP-A and SP-D antigens were present in the cytoplasm and luminal surface of multifocal respiratory epithelial cells lining bronchi, bronchioles and alveoli. Staining for SP-A was more intense and present in more cells than SP-D in both pre- and full-term lung. Staining intensity and distribution of both SP-A and SP-D protein was qualitatively less in preterm lung compared to full-term (Fig. 3 and Fig. 4).

4. Discussion

The limited expression of the innate immune genes (SP-A, SP-D, SBD1, TLR4) at preterm gestation is consistent with limited innate immunity of preterm neonates including: reduced innate cytokine responses and phagocytosis by monocytes secondary to RSV [4], reduced monocyte function [5], impaired neutrophil activity [6], impaired lung epithelial cell repair [7], and altered responses to lipopolysaccharide [8]. Such alterations in innate immunity collectively may predispose preterm infants to increased incidence and severity of respiratory infections with agents such as RSV and PIV-3. Our hypothesis was that innate immune gene expression of terminal airway epithelium (including type II pneumocytes) is developmentally regulated.

Whole lung homogenates from young lambs (ages 80% gestation to birth) had developmentally regulated gene expression of SP-D, TLR4 and SP-A. The developmental regulation of surfactant proteins is consistent with previous work on the pathogenesis of respiratory distress syndrome showing antenatal surfactant expression/synthesis in infants and lambs [25,26]. The developmental regulation of TLR4 in whole lung homogenates of lambs is a novel finding that parallels recent work on monocytes of infants showing that developmental regulation of TLR4 expression may contribute to the increased susceptibility to infection by low birth weight infants [27].

While whole lung expression of most genes at preterm was detectable with real-time qPCR, it was limited and intermittently nominal. Enrichment of specific cell populations with LCM greatly increased sensitivity of real-time qPCR detection (e.g. SBD1). This is similar to previous work in which LCM-retrieved bronchiolar epithelium showed significantly more SBD1 expression than whole lung homogenates [16]. Furthermore, the current study further characterizes the BAJ as a richer site of SBD1 developmental expression than bronchial epithelium. Although gene expression by LCM-retrieved epithelia was relatively low in preterm lambs, the levels were higher than whole lung homogenates and more easily quantified.

Of the genes assessed, SP-A expression was the most consistently expressed and its mean expression followed a progressive pattern of increase with age, especially in BAJ cells (e.g. type II pneumocytes) assessed by LCM. In humans, mice and sheep, type II pneumocytes are characterized by CD208+ expression [18]. SP-A expression has been classically linked to type II pneumocytes, however, less robust expression occurs in other cells of the lung and extrapulmonary expression has also been described [28]. Consistent with the literature, most of the developmental expression of SP-A mRNA in this study was seen in CD208+ cells with detectable expression in CD208− cells and bronchial epithelium.

SP-D mRNA expression developmentally increased in whole lung and BAJ epithelium, however, CD208+, CD208− and bronchial cells each had no significant alterations. This is likely due, in part, to the cellular sites of cellular SP-D expression. Constitutive SP-D expression occurs in type II pneumocytes with some contribution by Clara cells (non-ciliated

bronchiolar epithelium) and respiratory epithelial cells of the trachea and bronchi [29]. The lack of bronchial expression in neonatal lambs (15 days old) suggests that bronchial SP-D expression may be more relevant during late gestation to birth versus postnatally.

SBD1 expression by BAJ epithelium increased during development while bronchial epithelium did not show detectable mRNA in term and 15 days old lambs. This trend corresponded with previous work, which identified terminal airway (e.g. bronchiolar) epithelium as a major site of SBD1 expression compared to bronchial epithelium [16]. In this current study we were not able to show statistically significant alterations in specific cell lineages suggesting CD208+, CD208− and possibly other cell types collectively contribute to the innate immune expression of beta-defensins in the developing ovine lung. While this study demonstrates developmental regulation of SBD1 mRNA in BAJ epithelium, SBD1 regulation appears to be complex with increased expression seen during clearance of PIV3 infection, but there is reduced expression following *Mannheimia hemolytica* inoculation [10,16].

TLR4 mRNA expression in the BAJ region significantly increased with age, demonstrating developmental regulation. This localization was not statistically significant for cell type. The differential anatomic expression of TLR4 in term lambs (moderate reduction in BAJ expression, but increase in whole lung) was not expected. One speculative explanation is local cytokine regulation. For example, transforming growth factor-beta (TGF-beta) is a cytokine expressed during lung development and is important for epithelial differentiation and maturation needed for surfactant synthesis [30]. Recent evidence indicates TGF-beta may also inversely regulate TLR4 expression [31,32]. The net effect may be promotion of epithelial surfactant synthesis while minimizing inflammation that could complicate respiratory function in the neonate. Further work is needed to clarify this anatomic disparity.

The staining patterns of SP-A and SP-D proteins as determined by fluorescent microscopy were consistent with mRNA levels detected by real-time qPCR. That is, SP-A protein expression was more prevalent (in intensity and distribution) than SP-D in pre- and full-term lambs as was mRNA expression of these genes. Moreover, expression of both SP-A and D protein were reduced in preterm lambs compared to full-term, similar to mRNA levels. These findings suggest that preterm protein production by these genes is limited and that there is concomitance/fidelity between mRNA expression and protein production.

The regional differences (e.g. bronchi or BAJ) of innate immune gene expression were interesting. Target gene expression of bronchial epithelium did not increase with age, however, SP-D, SBD1 and TLR4 expressions were reduced/absent with advanced developmental age—which is similar to another pulmonary peptide in developing sheep, adrenomedullin [33]. That BAJ's expression of target innate immune genes increased with advancing age was suggestive that younger animals may have reduced innate immune capacity in these regions. More specifically, type II pneumocytes had significantly increased developmental expression of SP-A, while the other target genes appeared to be less regulated by age. Categorization of BAJ gene expression into cell types of type II pneumocytes (CD208+) and adjacent cells (CD208 −) confirms that type II pneumocytes are not the solitary source of innate immune gene products. Rather, BAJ cells (with heterogeneous CD208 expression) have redundancy in innate immune function.

This study demonstrates developmental gene expression and cellular localization of select innate immune genes in preterm lambs. The reduced expression of these innate immune products during development may partially explain the impaired ability to clear RSV infection and enhanced risk for respiratory infection in the preterm neonate [2,15]. Recent evidence indicates that these innate immune moieties have complex interaction patterns and synergism. For instance, SP-A's proinflammatory effect is modulated in part, through TLR4 activation

and TLR4 activation can lead to upregulation of cytokine and beta-defensin expression [34, 35]. Furthermore, some beta-defensins when co-expressed with other beta-defensins can have synergistic antimicrobial efficacy [36]. Thus, small alterations in expression of a single gene may have limited effects, but when taken collectively, significant or even subtle alterations in numerous innate immune genes may have a jointly profound influence on the host pulmonary defense status. Since the time course for alveolar development of lambs and infants is similar [37,38], the reduced expression of pulmonary innate immune factors and predisposition to enhanced disease makes the developing perinatal lamb a valuable pulmonary model for pediatric respiratory disease.

Abbreviations

BAJ	bronchiole–alveolar junction
BLAST	basic local alignment search tool
C	celsius
cDNA	complementary deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediamine tetra-acetate
HPLC	high-performance liquid chromatography
IF–IHC	immunofluorescence–immunohistochemistry
LCM	laser capture micro-dissection
mRNA	messenger ribonucleic acid
mW	milliwatts
MuLV	murine leukemia virus
PIV3	parainfluenza virus-3
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RSV	respiratory syncytial virus
RNase	ribonuclease
RT	reverse transcriptase
SBD1	sheep beta defensin 1
SP-AD	surfactant protein-AD
TLR4	toll-like receptor 4
TGF beta	transforming growth factor beta

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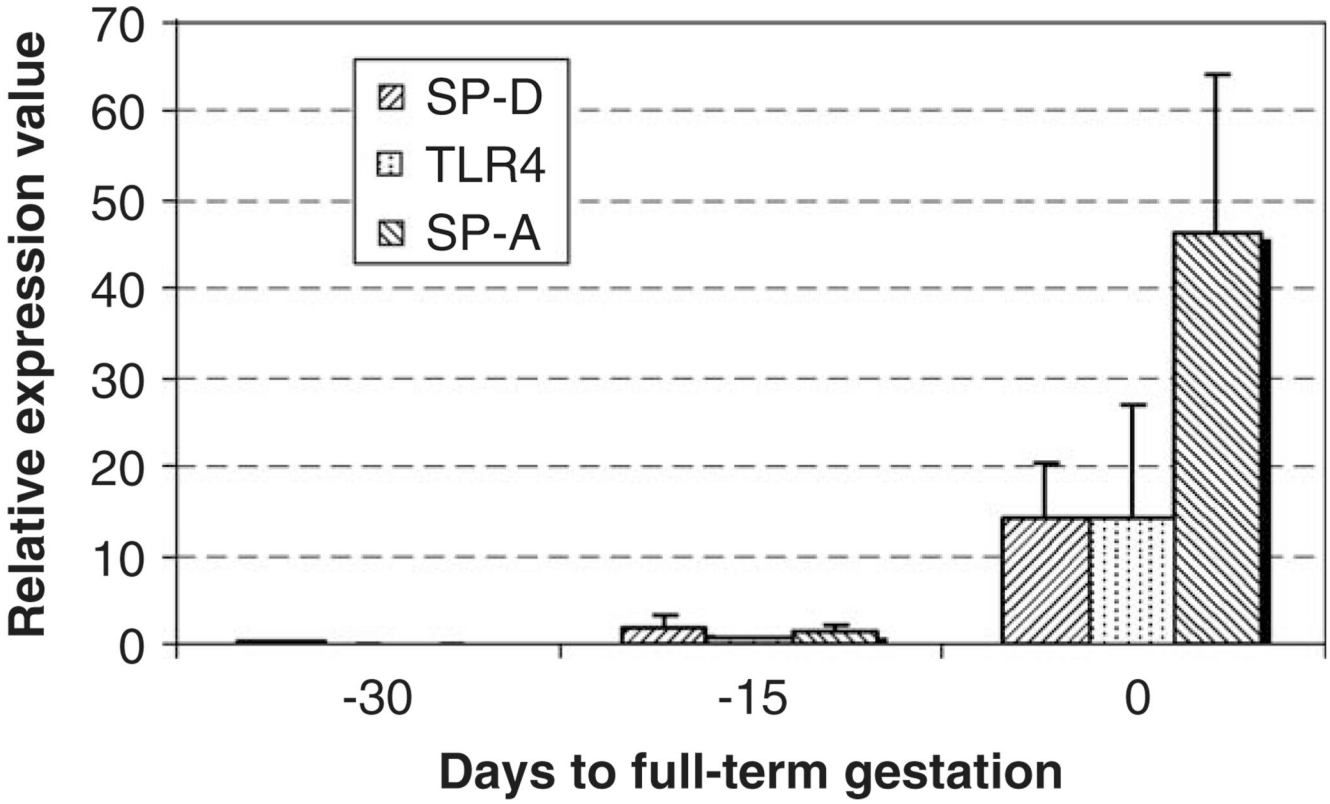

Fig. 1.

SP-D, TLR4, SP-A, and SBD1 mRNA levels in homogenized lungs of pre- and full-term lambs as determined by real-time qPCR. The relative mean expression of mRNA significantly increased with gestational age ($p<0.05$ for SP-D, TLR4 and SP-A genes). SBD1 expression was sporadic in late gestation and term lambs with low relative expression values (data not shown).

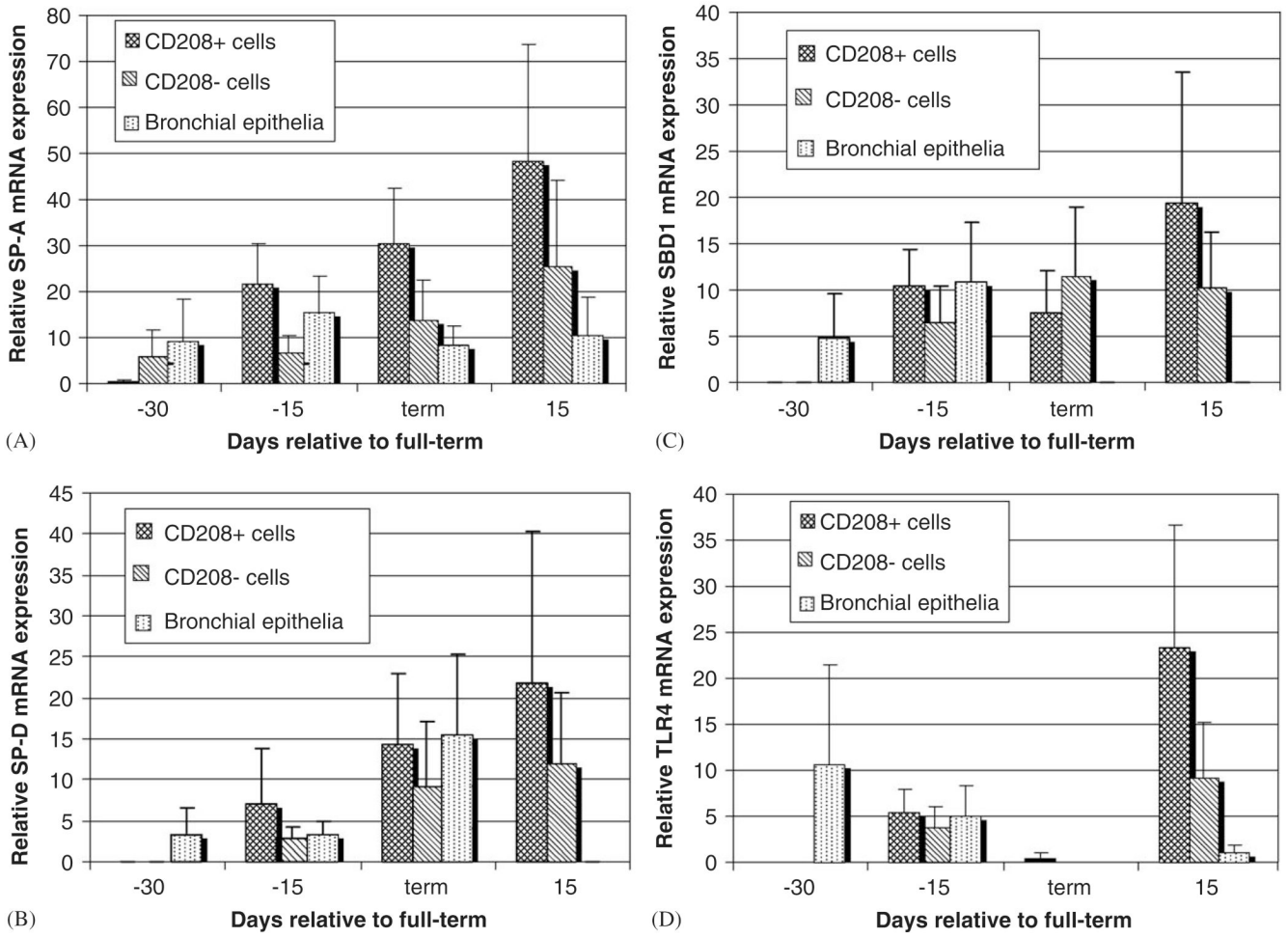

Fig. 2.

SP-A (A), SP-D (B), SBD1 (C), and TLR4 (D) mRNA levels at terminal bronchiole–alveolar junction (BAJ) epithelium (combined CD208+ and CD208− cells) and bronchial epithelium retrieved by laser capture microdissection in pre- and full-term lambs as determined by real-time qPCR. (A) BAJ epithelium (combined CD208+ and CD208−) epithelium had increased expression of SP-A ($p = 0{:}01$) during development with greater developmental expression over bronchial epithelium ($p = 0{:}07$). LCM retrieved CD208+ cells had elevated SP-A mRNA expression that increased during development ($p = 0{:}03$) while CD208− and bronchial cells each had insignificant alterations. (B) BAJ epithelium (combined CD208+ and CD208−) expressed increased SP-D mRNA levels during development ($p = 0{:}04$), while bronchial expression was sporadic through late gestation and absent in 15 days old lambs. (C) BAJ epithelium (combined CD208+ and CD208−) had progressively increased SBD1 mRNA expression levels during development ($p = 0{:}04$), while bronchial epithelium lacked detectable expression in term and 15 days old groups. (D) While BAJ epithelium (combined CD208+ and CD208−) had progressively increased TLR4 mRNA expression levels during development ($p = 0{:}02$), TLR4 expression levels were comparatively sporadic in all cell types and time points.

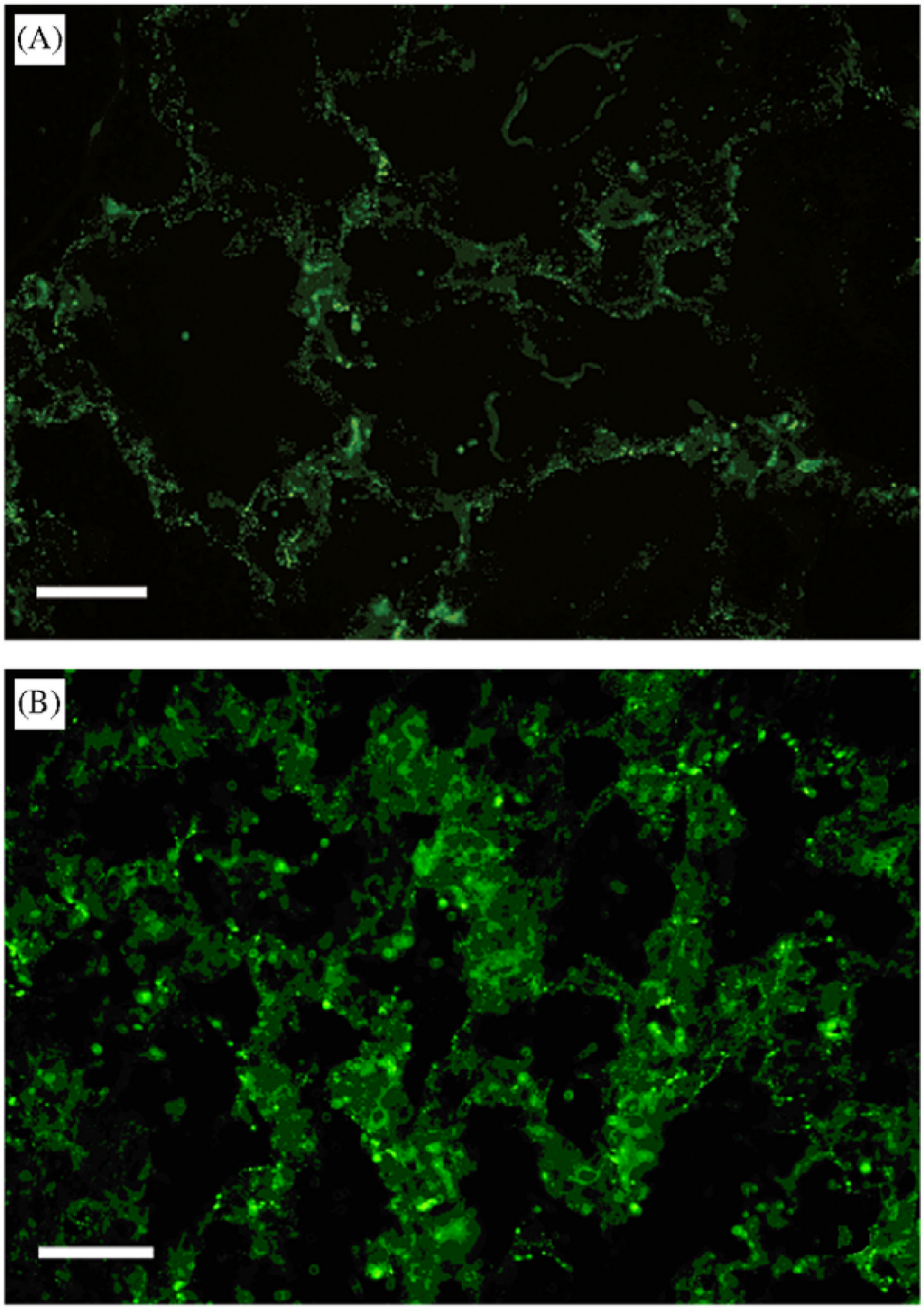

Fig. 3.
Fluorescence detection of SP-A protein expression in fetal (−17 days) (A) and term lung (B). In both sections there is antigen present in and along epithelial cells with increased intensity and distribution of SP-A antigen in the term lung. Bar = 25 μm.

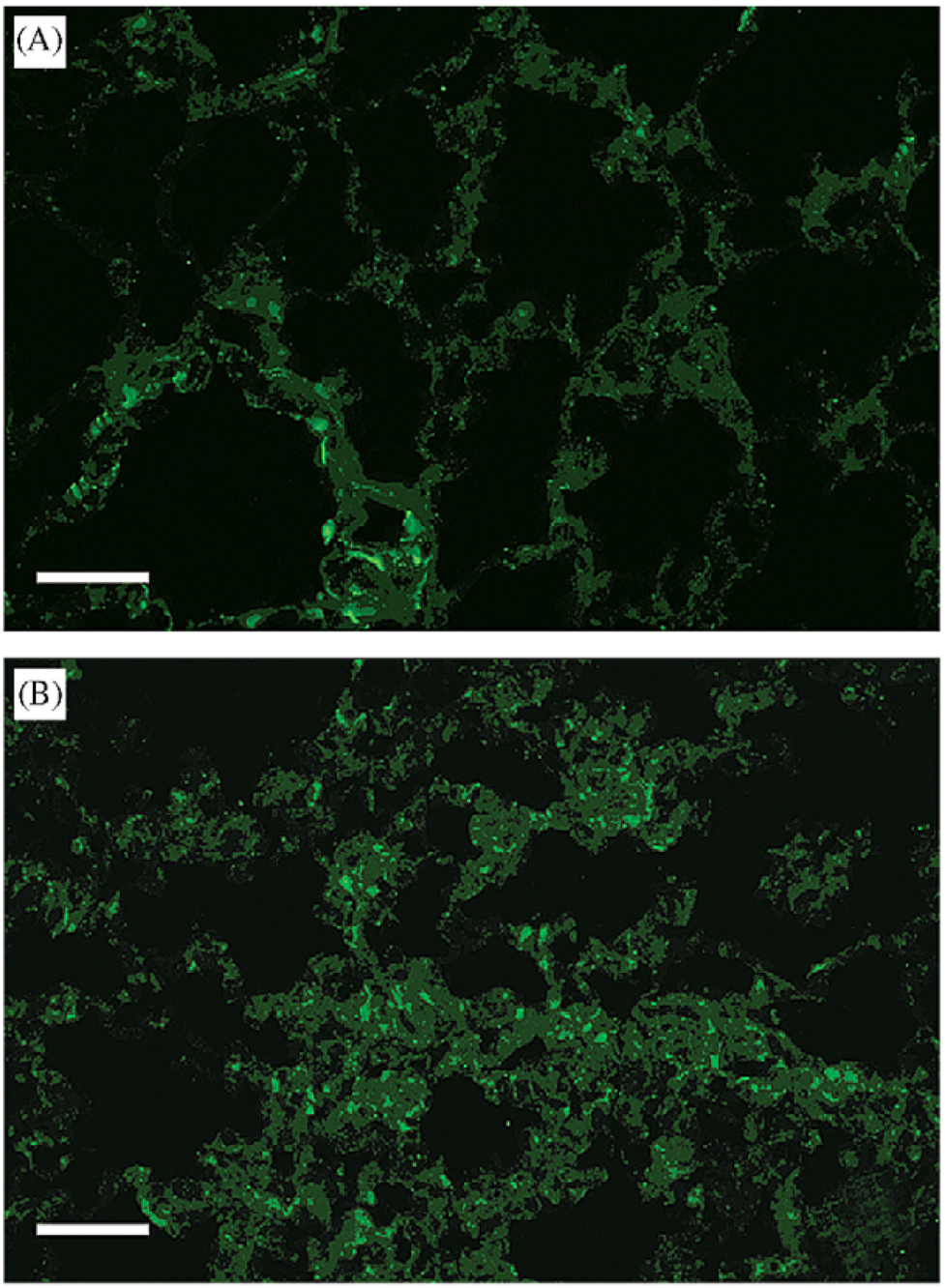

Fig. 4.
Fluorescence detection of SP-D protein expression in fetal (−17 days) (A) and term lung (B). In both sections there is antigen present in and along epithelial cells with increased intensity and distribution of SP-D antigen in the term lung. Bar = 25 μm.

Table 1

Target primer and probe sequences with optimized concentrations

SP-A	Fwd primer (300 nM):	5′-TGACCCTTATGCTCCTCTGGAT
	Rev primer (300 nM):	5′-GGGCTTCCAAGACAAACTTCCT
	Probe (50 nM):	5′-6FAM-TGGCTTCTGGCCTCGAGTGCG-TAMRA
SP-D	Fwd primer (300 nM):	5′-ACGTTCTGCAGCTGAGAAT
	Rev primer (300 nM):	5′-TCGGTCATGCTCAGGAAAGC
	Probe (100 nM):	5′-6FAM-TTGACTCAGCTGGCCACAGCCCAGAACA-TAMRA
SBD1	Fwd primer (300 nM):	5′-CCATAGGAATAAAGGCGTCTGTGT
	Rev primer (900 nM):	5′-CGCGACAGGTGCCAATCT
	Probe (150 nM):	5′-6FAM-CCGAGCAGGTGCCCTAGACACATGA-TAMRA
TLR4	Fwd primer (1000 nM):	5′-GAGAAGACTCAGAAAAGCCTTGCT
	Rev primer (1000 nM):	5′-GCGGGTTGGTTTCTGCAT
	Probe (150 nM):	5′-6FAM-TAAACCCCAGAGTCCAGAAGGAACAGCA-TAMRA
ovRPS15	Fwd primer (1000 nM):	5′-CGAGATGGTGGGCAGCAT
	Rev primer (1000 nM):	5′-GCTTGATTTCCACCTGGTTGA
	Probe (150 nM):	5′-VIC-CCGGCGTCTACAACGGCAAGACC-TAMRA

* 6FAM = fluorescent reporter dye, TAMRA = fluorescent quencher dye, VIC = fluorescent reporter dye (Applera Corporation).