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Ontogeny of the Immune Response in the Ovine Lung

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Abstract

Perinatal lambs are increasingly appreciated as a model to study respiratory infections of premature and newborn human infants. To explore the relationship between developmental age and immunological competence in the respiratory tract, the basal levels of expression of genes involved in innate and adaptive immune functions in the lung were examined in pre-term lambs (115 days and 130 days), at birth (145 days) and post-partum (15 days and 3 years old). Our results show that innate immune genes (TLRs-3, -4, -7, -8; SP-A, SP-D, and SBD1) were differentially expressed through development; cytokines (IFN- γ , IL-6, TNF- α) and chemokines (IL-8, MCP-1) were low during gestation and post-partum but maximal at birth; genes involved in adaptive immunity (PD-1, PD-L1, TGF- β) were present in pre-term and newborn lung, but were lower in adult lung. The results suggest that pre-term and neonatal lambs may be able to mount an immune response following infection, but that the response may not be optimal. Our studies provide an important set of comparative data on the ontogeny of lung immunity in sheep and set a framework for studies on age-dependent susceptibility to respiratory pathogens.

Keywords

Lung; Preterm infant; Neonate; Sheep; Immune response

INTRODUCTION

Mortality and morbidity rates associated with respiratory diseases are highest in neonates and young children (Lanari et al., 2007; Lawn et al., 2005). The susceptibility to respiratory infections has traditionally been attributed to the immaturity of the neonatal immune system with well-described deficits in innate and adaptive immune functions, including a reduced response to antigenic challenge, a decreased plasma concentration of complement components, decreased production of T-helper 1 (Th1) cytokines, type I interferons, MHC class II expression on antigen presenting cells (APCs), an impaired neutrophil function and recruitment, and a reduced antibody responses from B cells (reviewed in (Adkins et al., 2004; Levy 2007)). More recently, the high-susceptibility of neonates to infection has been ascribed to a shift towards a Th2-immune response (Adkins et al., 2004).

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The study of human fetal-neonatal development requires the use of animal models with similar gestation, immune response, disease pathogenesis, cardiovascular and pulmonary functions to humans. Several reports have emphasized the similarities of the ovine perinatal lung to the human lung, including the stages of lung development in the fetus, the distribution and cellular composition of respiratory epithelia of airways, distal bronchioles and alveoli, and the presence of submucosal glands involved in oxidative responses (Scheerlinck et al., 2008; Sow et al., 2009; Thurlbeck 1975). In addition, the ability to induce premature birth in lambs provides an excellent opportunity to study the mechanisms underlying the age-dependent susceptibility to respiratory pathogens.

In fact, pre-term and newborn lambs have proven to be invaluable pulmonary models in the studies of respiratory syncytial virus infection (Belknap et al., 1995; Lehmkuhl and Cutlip 1979; Meyerholz et al., 2004; Sow et al., 2011), alcohol exposure on lung development (Lazic et al., 2007; Sozo et al., 2009), and on innate immune responses by pulmonary epithelia (Grubor et al., 2004; Grubor et al., 2006; Kawashima et al., 2006). To determine the effects of developmental age on lung immunity, we evaluated the expression levels of genes involved in innate and adaptive immune functions in fetal sheep.

MATERIALS AND METHODS

Animal Experiments

All animal use and experimental procedures were approved by Iowa State University's Animal Care and Use Committee. Healthy date-mated commercial pregnant ewes were acquired from Iowa State University's Laboratory Animal Services. A Caesarian section was performed to remove fetuses at an estimated gestational age of 115 days ($n = 3$) and 130 days ($n = 3$) regardless of their sex. A normal ovine gestation is 145–150 days. In addition, newborn lambs at 145 days gestation ($n = 4$) and 15 days post-partum lambs ($n = 4$) of either sex, as well as ewes of approximately 3 years of age (adult) ($n = 4$) were acquired from Iowa State University's Laboratory Animal Services. These animals lacked any evidence of respiratory disease. All animals used in the study were euthanized with sodium pentobarbital intravenously. Samples from the right lung were taken post-mortem, placed into cryovials, and immediately snap-frozen in liquid nitrogen, then stored at -80°C .

RNA Isolation

Total RNA was isolated from the right lung as follows: lungs were weighed and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) using an Omni TH homogenizer (Omni International, Marietta, GA). The homogenate was vortexed, and nuclease-free chloroform was added to each sample and spun at $12,000\times g$ for 10 minutes. The aqueous layers were transferred into microfuge tubes containing nuclease-free 2-propanol (Fisher Chemical, Fairlawn, NJ). The pellets obtained after centrifugation ($12,000\times g$ for 10 minutes) were washed twice in ice-cold 75% nuclease-free ethanol, after which the supernatant was removed, and the pellets were allowed to air-dry. The pellets were resuspended in nuclease-free 0.1 mM EDTA, pH 7.0. RNA concentrations and purity were measured by absorbance readings at 260 nm and 280 nm. The RNA samples were further subjected to DNase treatment using TURBO DNase (TURBO DNA-free kit, Ambion, Austin, TX). RNA samples were recovered after DNase treatment and diluted 1:10 with nuclease-free water (Ambion) and RNase OUT (Invitrogen) for one-step qPCR use.

One-step Real-time qPCR

Prior to final qPCR analysis, a test plate was run for each target of interest to identify the best RNA dilution ranges in which PCR inhibition was not observed, and where amplification efficiencies were $>80\%$. Test plate setup parameters and analysis were

performed using PREXCEL-Q (P-Q) (Gallup and Ackermann 2006). After a careful study of the test plate analysis, 1:10 RNA samples were further diluted to their ideal ranges, on a per-sample and per-target basis. Next, the diluted RNA samples were used as “RNA templates” in 20 µl fluorogenic one-step real-time qPCR reactions.

Each reaction contained RNA sample, 775 nM primers, 150 nM TaqMan probe (5'-6FAM, 3'-TAMRA-quenched or 5'-6FAM, 3'-MGBNFQ-quenched), nuclease-free water, 5.5 mM MgSO₄, SuperScript III RT/Platinum Taq mix (Invitrogen), and One-step reaction mix with ROX (Invitrogen). The samples were placed in duplicate wells of a 96-well qPCR reaction plate (Applied Biosystems, Inc., Forest City, CA). Two negative, no-template control wells were also added to the qPCR plate. The plates were run in the GeneAmp 5700 Sequence Detection System (Applied Biosystems) for detection and relative quantification of targets at 15 min, 55°C; 2 min, 95°C; and 50 cycles of 15 s at 95°C; 30 s at 58°C. The results were analyzed using the GeneAmp 5700 software and Excel files. No RT control (NRC) reactions were run for all standards and samples using Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen) to ascertain lack of genomic DNA (gDNA) contamination of the RNA samples. In all cases, contaminating gDNA signals were found to be >10 cycles away from corresponding one-step qPCR signal Ct values.

Processing of Lung Samples for Protein Quantification

Lung samples were placed in a Tissue Protein Extraction Reagent (T-PER; Fisher Science, Hanover Park, IL) at a ratio of 1g tissue/20 ml T-PER. After the addition of protease inhibitors cocktail tablets (Roche), the samples were homogenized using an Omni TH homogenizer (Omni International). Protein concentration was determined using the Coomassie Plus Protein Assay Reagent (Fisher).

Western Blot Analysis

Lung homogenates (35 µg) were separated by electrophoresis on 4–20% polyacrylamide gels (Fisher) and transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were blocked in blocking buffer (TBS + 0.05% tween 20 + 4% BSA) for 1h and incubated with mouse anti-bovine TNF- α (1:1000) for 3 hrs at room temperature (AbD Serotec). After several washes with TBS + 0.05% tween 20, membranes were incubated with Alexa Fluor 488 F(ab')₂ fragment of goat anti-mouse IgG (1:2000) for 1h at room temperature. Blots were scanned using the Typhoon 9410 Variable Mode Imager (GE Healthcare, Piscataway, NJ).

Statistical Analysis

The results were analyzed by one-way ANOVA using SAS version 9.1 (SAS Institute, Cary, NC). Mean values of relative mRNA expression levels of genes from animals born 115 days before gestation, animals born 130 days before gestation, 15-day-old animals, and adult animals were compared to newborn animals at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ and expressed as means $\pm$ SE.

RESULTS

Genes Involved in the Innate Immune Response Are Differentially Expressed in the Ovine Lung During Development

Invasion of microorganisms can be sensed in mammals by Toll-like receptor (TLR) family members, culminating in the activation of a range of host defense responses. We first sought to investigate the expression levels of TLRs transcripts throughout ontogeny in the ovine lung. TLR3, TLR4, TLR7 and TLR8 were all expressed in the lung, even early during gestation (Fig. 1). Compared to levels in the newborn lung, TLR3 was significantly

increased in 115-day-old animals (** $p < 0.01$) and 130-day-old animals (* $p < 0.05$). Although 15-day-old post-partum (PP) animals had significantly low levels of TLR3 (*** $p < 0.001$), no differences in expression were seen between newborn and adult animals (Fig. 1A).

The expression levels of TLR4 followed a progressive linear increase (*** $p < 0.001$) with age, except in the newborn, where the levels were low (46.3-fold less than levels seen in 115-day-old animals, Fig. 1B). The patterns of expression of TLR7 were variable throughout ontogeny (Fig. 1C): while the levels were similar between newborn animals and 115-day-old animals, 130-day-old animals and adult animals had significantly higher levels of TLR7 expression than newborns (** $p < 0.01$), but 15-day-old PP animals had significantly lower levels of TLR7 than newborn animals (** $p < 0.01$). TLR8 levels followed an age-dependent increase, with maximal expression in adult animals (*** $p < 0.001$, Fig. 1D). TLRs have also been reported to be differentially expressed during development in the ovine spleen and skin (Nalubamba et al., 2008).

Mammalian neonates are known to have an increased susceptibility to respiratory pathogens. SP-A and SP-D are members of the collectin family that can enhance the clearance of such pathogens. We found that SP-A and SP-D were present in the ovine lung at similar levels from 115 days of gestation to adulthood (Fig. 1), but that levels were significantly decreased at 15 days PP over newborn levels (* $p < 0.05$ for SP-A, Fig. 1E; *** $p < 0.001$ for SP-D, Fig. 1F). A similar pattern of SP-A expression during ontogeny has been reported in the rat lung, with SP-A content increasing from gestation to birth, then decreasing until day 5 after birth (Ogasawara et al., 1991).

The expression of antimicrobial peptides is important to host defense. We determined the expression of SBD-1 during development and found that mRNA levels were low from gestation to birth, then significantly increased at day 15PP and in adults (Fig. 1G).

Genes Involved In Inflammation and Chemotaxis are Expressed at Maximal Levels at Birth in the Ovine Lung

We measured the expression levels of the pro-inflammatory cytokines IFN- γ , IL-6 and TNF- α in the lung (Fig. 2) and found that IFN- γ , TNF- α and IL-6 had maximal expression levels at birth, but a significant decrease at either spectrum of age (115 days of gestation (* $p < 0.05$ for all targets) and adult animals (* $p < 0.05$ for IFN- γ , Fig. A2; *** $p < 0.001$ for IL-6, Fig. 2B; ** $p < 0.01$ for TNF- α , Fig. 2C). We determined that the relative expression level of TNF- α in the newborn and 15 days PP was approximately two-fold higher than the levels of IFN- γ and IL-6 in the same animals (Fig. 2). Protein detection in this model organism is difficult; however, an attempt was made to detect protein levels of TNF- α by western blot. TNF- α protein levels correlated with mRNA levels throughout ontogeny (Fig. 2D).

Because of their important role in lymphocyte trafficking, we assessed the mRNA expression of IL-8 and MCP-1. Similar to the cytokines, we found that the chemokines IL-8 and MCP-1 followed a similar pattern of expression throughout development: the levels increased from 115 days of gestation to birth, with a maximal expression at birth, then decreased to adulthood (Fig. 2E and Fig. 2F). The presence of cytokines and chemokines very early during fetal development, and their progressive increase towards birth suggests a contribution of these cytokines in the early development of the lung immune system.

Genes Involved in the Regulation of T Cell Functions Are Significantly Decreased in Lungs of Adult Animals

CD4⁺ and CD8⁺ T cells have a central role in cell-mediated immunity. PD-1 and its ligands PD-L1 and PD-L2 are recently described molecules that negatively regulate T cells function

by causing cell anergy and a reduced capacity to generate cytotoxicity (Keir et al., 2006; Keir et al., 2007; Okazaki and Honjo 2006; Sharpe et al., 2007). Studies of T cell regulation have also demonstrated an essential role for the cytokine TGF- β in T cell development, tolerance and the immune response (reviewed in Li and Flavell, 2008).

We examined the developmental progression of PD-1, PD-L1 and TGF- β in the lung. We found that the mRNA levels of these regulatory molecules did not significantly change from 115 days of gestation to 15 days PP, but decreased significantly in adult animals (Fig. 3). Thus, the expression of genes that can modulate or interfere with the effector function of T cells and the regulation of immune responses is developmentally regulated in the ovine lung.

DISCUSSION

The primary immune responses of fetuses/neonates are characterized by weak responses to vaccines and a high susceptibility to infectious diseases compared to those of healthy adults. In this study, we performed a gene expression profile analysis in the ovine lung to quantitatively measure the contribution of the innate immune response during development *in vivo*. Genes were chosen in different groups of immunological function to reflect role of the different facets of the immune system. The key findings of our study are:

1. Despite the immature immune system ascribed to mammalian fetuses-and neonates, lungs of pre-term and newborn lambs express numerous-host defense molecules, including TLRs, surfactant proteins, antimicrobial-proteins, cytokines, chemokines, and genes involved in adaptive immunity;
2. TLRs exhibit distinct expression patterns through development;
3. Cytokines/chemokines are low during gestation and post-partum, but are-maximal at birth;
4. Genes involved in negative regulation of T cell function are expressed-throughout development but are decreased in adult animals.

The results suggest that pre-term and newborn lambs have the capacity to mount an immune response to invading pathogens; however their response may not necessarily be optimal to fight an infection due to the relatively low basal level of expression.

We determined that TLRs 3, 4, 7 and 8 were differentially expressed in the lung across the age groups. Our results are partly in agreement with those of Hillman et al. (2008), who recently showed that newborn and adult animals had similar levels of TLR3 mRNA, and that adult animals had significantly higher levels of TLR4 mRNA than newborn animals. However, they did not find statistical differences in the expression levels of TLR3 and TLR4 between 130 days gestation and newborn animals. The levels of TLRs in sheep skin and spleen have been reported previously (Nalubamba et al., 2008). Although newborn animals were not included in the study, it was reported that the levels of TLR7 and TLR8 in the spleen were significantly higher than that of pre-term lambs, and that the levels of TLR4 in the skin were higher than that of pre-term lambs (Nalubamba et al., 2008). There were also no reported significant differences in TLR3 expression across age groups in the neonatal ovine skin or spleen.

Surfactant proteins (SP) are members of the collectin family, characterized by a collagen domain linked to a calcium-dependent lectin domain (Kishore et al., 2006). SP-A and SP-D have critical roles in the lung's primary defense against inhaled pathogens and are important components of innate immunity by binding and opsonizing microorganisms, and activating macrophages. We found that SP-A and SP-D had similar patterns and levels of expression across age groups, with a significant decrease in expression at day 15 PP. Similar results

were previously reported for ovine SP-A (Tan et al., 1999). That study also demonstrated that glucocorticoid treatment could enhance the expression of surfactant proteins in preterm lambs. In a recent article, we reported that vascular endothelial growth factor (VEGF) can also be used to enhance the expression levels of immune genes, including surfactant proteins, in neonatal lambs (Sow et al., 2009). The presence of surfactant proteins early during gestation suggests that the fetus is already endowed with primary lung innate immune defense mechanisms.

The increased susceptibility of human newborns to infection has been reported to be, in part, a consequence of their impaired Th1 immune response (Adkins et al., 2004; Klein and Remington 2001). To determine whether preterm and newborn lambs had a similar impairment of the immune response, we analyzed the expression of pro-inflammatory cytokines IFN- γ , TNF- α and IL-6, which contribute to Th1 immunity.

We found an age-dependent progression of these cytokines, whose expression culminated at birth and decreased at either spectrum of age. The chemokines IL-8 and MCP-1 also exhibited a similar pattern of expression through development. The increased expression of cytokines and chemokines during development may contribute to the maturation of the fetal immune system by mediating and regulating leukocytic infiltration and activation in the fetal lung. It is also possible that the up-regulated gene expression observed in the pre-term and newborn animals could be due to the effects of birth, maternal hormones, or even environmental factors during the first hours of birth, however influence of such would be seen across all groups.

Given that the development of the immune system, in particular adaptive immunity, in neonates is not completely understood and that the interaction of co-inhibitory/stimulatory molecules belonging to the CD28-B7 family, such as the PD genes, with their ligands (Greenwald et al., 2005), plays important roles in regulating T cell responses, we sought to evaluate the differences in PD-1 and PD-L1 expression throughout ontogeny. PD-1, which is expressed on activated T, B, and myeloid cells (Ansari et al., 2003; Liang et al., 2003; Rodig et al., 2003; Salama et al., 2003), was expressed during gestation in the ovine lung, and decreased at adulthood. PD-L1, which is expressed on resting cells and induced upon activation of T, B, myeloid, dendritic, and endothelial cells (Ansari et al., 2003; Liang et al., 2003; Rodig et al., 2003; Salama et al., 2003), was expressed at low levels during gestation but increased with developmental age, except in adult animals, where its expression was significantly decreased. Neonates have previously been reported to have the ability to develop a mature T cell response (Petty and Hunt 1998). The presence of PD-1 and PD-L1 in pre-term and newborn lambs suggests that myeloid cells are expressed early during gestation and that some of these cells are activated. In the ovine spleen, T cell responses are reported to be active by day 77 (Maddox et al., 1987; Press et al., 1993).

In summary, our data demonstrates that fetal sheep express the same spectrum of immune genes as do adults, but that the level of expression of some genes is dependent on developmental age. Although genes involved in host defense are present at appreciable levels in pre-term and newborn lambs when compared to adult sheep, the presence of comparable levels of those host defense molecules may not necessarily translate to the appropriate immune response to invading pathogens in the younger animals.

Further analysis of protein expression and function during ontogeny is needed in order to determine the fidelity of gene expression and functional protein product. It will also be important to test concomitant pulmonary infection, pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) in order to fully test responsiveness of the immune defense system in fetuses and neonates. This work may

provide new therapeutic strategies to address respiratory pathogens not previously considered.

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Figure 1. Relative mRNA expression levels of TLR3 (A), TLR4 (B), TLR7 (C), TLR8 (D), SP-A (E), SP-D (F) and SBD1 (G) were measured by qPCR in lungs of lambs. The data represent the mean $\pm$ SE ($n = 3$ for lambs at 115 days of gestation, $n = 3$ for lambs at 130 days of gestation, $n = 4$ for newborn lambs, $n = 4$ for lambs 15 days PP, $n = 4$ for 3-year old adult lambs). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by ANOVA when compared to newborn lambs.

Figure 2.

Relative mRNA expression levels of IFN- γ (A), IL-6 (B), TNF- α (C), IL-8 (E) and MCP-1 (F) were measured by qPCR in lungs of lambs. The data represent the mean $\pm$ SE ($n = 3$ for lambs at 115 days of gestation, $n = 3$ for lambs at 130 days of gestation, $n = 4$ for newborn lambs, $n = 4$ for lambs 15 days PP, $n = 4$ for 3-year old adult lambs). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by ANOVA when compared to newborn lambs. Protein levels of TNF- α (D) were detected in lungs of all animals by Western blot. β -actin loading controls were also included.

Figure 3.

Relative mRNA expression levels of PD-1 (A), PD-L1 (B) and TGF-β (C) were measured by qPCR in lungs of lambs. The data represent the mean ± SE (n = 3 for lambs at 115 days of gestation, n = 3 for lambs at 130 days of gestation, n = 4 for newborn lambs, n = 4 for lambs 15 days PP, n = 4 for 3-year old adult lambs). *p < 0.05; ***p < 0.001 by ANOVA when compared to newborn lambs.