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Neonatal alveolar macrophages upregulate cytokine gene transcription via p38 MAPK signaling but fail to secrete cytokines in response to Respiratory Syncytial Virus

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
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CHAPTER ONE. GENERAL INTRODUCTION

LITERATURE REVIEW

Respiratory Syncytial Virus: An introduction

Respiratory Syncytial Virus (RSV) is the leading cause of lower respiratory tract illness in infants worldwide, causing severe bronchiolitis and pneumonia. In the United States alone, there are approximately 75,000 to 125,000 RSV-related hospitalizations and 20,000 deaths per year, with an associated cost of $300 million (1-3). Premature and newborn infants, as well as elderly and immunocompromised individuals, are the groups most susceptible to severe RSV infections. Nearly 70% of children are infected within the first year of life and reinfection is common throughout adulthood (4, 5). Neonatal RSV infection is of special significance not only because of the acute disease it causes but also because of the suggested link between early viral infection and the development of allergic asthma later in life (6).

Bovine Respiratory Syncytial Virus (BRSV) is the etiological agent responsible for similar respiratory tract disease in cattle. BRSV infection can cause severe disease alone as well as in concert with other viral or bacterial pathogens as part of the bovine respiratory disease complex (7). BRSV infection causes severe pneumonia in young calves, and is responsible for considerable economic loss in the cattle industry worldwide. Similar to infection rates in humans, 70% of calves born each year have been infected with BRSV by the age of 9 months to a year (8).

While BRSV vaccines are commercially available, they are not 100% protective, and fail to provide long-term immunity. Current challenges facing BRSV vaccination are the limited efficacy and duration of protection, the need to vaccinate immunologically immature

neonates, and the interference of maternal antibodies to RSV. The two main goals in developing an efficacious RSV vaccine are: to activate cell-mediated immunity to clear the virus, and to provide long-lasting protection by inducing both T cell memory and antibody production to prevent reinfection (9).

There is no safe and effective RSV vaccine currently available for use in humans. In the 1960s, a formalin-inactivated RSV (FI-RSV) vaccine was tested in infants and children with unexpectedly disastrous results. Vaccinated individuals developed severe RSV infections following natural exposure to the virus, of which two cases proved fatal (10). It has been shown that priming with different RSV vaccines elicits a Th2 immune response upon secondary live RSV infection rather than a protective Th1 response. Studies using recombinant vaccinia viruses expressing the individual RSV G or F proteins primed a Th2 or Th1 response, respectively (11, 12). In addition, the adjuvant used in RSV vaccine preparations can also affect the immune response (9). The failed FI-RSV vaccine was alum-adjuvanted, and led to vaccine-enhanced Th2 disease in test subjects. *Quillaja* saponins have been used as a successful adjuvant in commercially available BRSV vaccines, and prime Th1-type protection (9). Additional research with RSV subunit vaccines and synthetic CpG-oligodeoxynucleotide adjuvants has also been shown to elicit a Th1 response in mice and rats, although one study reported enhanced lung pathology following challenge (13). Further research based on these findings could contribute to the development of promising hRSV and BRSV vaccine candidates. Challenges in human RSV vaccine research are the same as for BRSV, and include more stringent safety testing and regulations due to the history of and potential for vaccine-associated disease enhancement (9).

Virus structure and genomic organization

RSV is an enveloped, non-segmented, negative sense RNA virus belonging to the *Pneumovirus* genus of the family *Paramyxoviridae.* It acquires its lipid envelope upon budding from the host cell, which contains the virally-encoded glycosylated (G), fusion (F) and small hydrophobic (SH) proteins (Figure 1). The matrix protein (M) forms a layer on the inner surface of the lipid envelope, surrounding the viral genome and associated ribonucleoprotein complex, which is made up of the large (L) RNA-dependent-RNA-polymerase, the nucleocapsid (N) protein and the phosphoprotein (P).

The virion also contains two non-structural proteins (NS1 and NS1) which are believed to be regulatory in function, controlling viral RNA transcription and replication as well as host anti-viral IFNα/β signaling (14). The RSV virion also contains two additional matrix proteins, the M2 factors M2-1 and M2-2, which are encoded by the same mRNA. M2-1 is a transcriptional elongator and anti-termination factor, and M2-2 is a regulatory protein that mediates the switch between viral gene transcription and replication(8).

The viral genome is around 15,000 nucleotides long and helical in shape, encoding for 10 viral mRNAs which are translated into 11 viral proteins. Starting at the 3' end, the genes are transcribed in the following order, with those genes closer to the 3' end having a higher frequency of transcription due to a polar transcription gradient: NS1, NS2, N, P, M, SH, G, F, M2, and L. Each viral gene is flanked by conserved sequences signaling the polyadenylation/termination of one transcript and a start signal for the transcription of the next downstream gene (15). The full length viral genome also serves as the template for replication. The switch from transcription to the initiation of replication of the genome is

mediated by the presence of the M2-1 protein, as well as 5' regulatory sequences within the genome itself (16).

Viral attachment and entry

Currently, the specific mechanism by which RSV virions attach and enter host cells is not completely understood. Viral envelope proteins, F, G, and SH are thought to act in concert to bind to their specific host cell surface receptors, which remain unidentified. Studies have implicated Toll-like Receptor-4 and CD14 surface molecules in binding the F protein, and the fractalkine receptor (CX3CR-1) in binding the G protein (17, 18). Receptor binding induces the reorganization of cellular membrane proteins and the recruitment of lipid rafts and additional receptors to the binding site. Upon fusion, the viral lipid envelope becomes integrated into the host cell membrane, and virions enter the cytoplasm (15, 19). This viral uptake induces signaling pathways and ultimately activates NF-kB transcription factor.

Animal models of RSV

Animal models of RSV infection can be extremely valuable research tools for studying factors related to disease pathogenesis. Animal models have many advantages and can be used to gain a better understanding of biological processes in order to predict how the human immune response will react to different stimuli including pathogens and vaccines. Manipulation and evaluation of animal models can help focus human clinical trials in terms of optimal dosages and routes of administration, and most importantly, decreasing the potential for adverse side effects (3).

Formalin-inactivated BRSV (FI-BRSV) vaccines have been shown to have similar detrimental effects upon secondary virus exposure as the FI-RSV vaccine used in children in

the 1960s, suggesting that the BRSV model may offer important insight into human RSV vaccine research. One study reported that while vaccination with FI-BRSV did induce viral G and F protein-specific IgG, no neutralizing antibodies were produced (20). Further studies have shown similar results with partial protection, but also indicate disease enhancement in vaccinated individuals following secondary exposure to BRSV. In one study, vaccinated calves had lower lung lesion scores, but exhibited an earlier onset of severe clinical signs with a resulting 30% mortality rate (21). It is suggested that the disease enhancement is due to a priming of a Th-2 inflammatory response, resulting in eosinophilic infiltration and high levels of circulating BRSV-specific IgE (6, 22, 23).

While cattle are the natural host for BRSV, other livestock species have been shown to become naturally infected with BRSV, including sheep, goats, llamas and bison (23-26). This fact, along with the structural and antigenic similarities between the human and bovine viruses, makes the use of animal models a valuable tool for studying RSV infection (27, 28). The lamb model of RSV infection is similar in terms of age-dependent susceptibility, with infected animals exhibiting similar clinical signs, disease progression, and pathological lesions as human infants infected with RSV (28-32).

Host immune response to RSV

There is a great deal of variation in disease severity associated with RSV infection in children, which is likely a result of differences in regulation of the host response to the virus. It is unknown whether more severe RSV-associated pathology results from an overzealous immune response, a Th2-biased immune response, or an inadequate neonatal immune response (33).

Early innate immune responses to RSV infection shape the adaptive immune response through the production of immune mediators. Early cytokine and chemokine expression induce trafficking of macrophages, eosinophils, neutrophils and NK cells to the lungs, leading to the activation of B and T cells. Differential regulation of these events may be responsible for virus clearance as well as disease exacerbation (9).

Neonatal immune response

There is evidence that a strong Th2 bias exists in the fetal immune system, as a mechanism for limiting dangerous inflammatory responses. Increased levels of TNF-α and IL-1β have been shown to not only induce premature labor but may also be a factor for spontaneous abortion (34). Increased IFN-γ at the fetal-maternal interface has been described to damage placental integrity and cause fetal loss as well (35). In this regard, it appears that a Th2-biased immune response is beneficial through fetal development and at the time of birth, although it may contribute to the susceptibility of neonates to viral and bacterial infections during this time.

The neonatal immune system is faced with the challenges of protecting against infection while limiting inflammation, as well as transitioning to an antigen-rich environment after birth (36). The respiratory tract is constantly exposed to antigen, and early infections likely play an important role in shaping the developing immune responses. Studies in support of the hygiene hypothesis suggest that LPS-stimulation might inhibit the development of allergic disease by skewing the immune response away from the Th2 bias.

Because of their roles in innate immunity and antigen presentation, neonatal macrophage function is likely to be a major determinant of immune system maturation and development. However, defects have been reported in neonatal macrophage function.

Macrophages isolated from neonatal mouse spleens were shown to have an increased IL-6 and IL-10 secretion, but failed to secrete IL-1β, IL-12 or TNF-α, possibly due to reduced TLR-4 and CD14 surface expression, or the suppressive effects of IL-10 (37). Another study using AMΦs from a rat model reported similar findings, where LPS-stimulated AMΦs exhibited decreased capacity to produce nitric oxide and IL-1β, as well as impaired phagocytic ability(38).

Neonatal AMΦs isolated from premature infants with chronic lung disease had increased IL-1β and IL-6 secretion compared to healthy controls, indicating these cells had the capacity to produce these cytokines under the right conditions (39). Macrophages derived from cord blood monocytes secreted IL-6 and TNF-α quickly in response to in vitro RSV infection (40). In a lamb model, neonatal BRSV infection was shown to induce a mixed cytokine response in AMΦs, as evidenced by increased IL-4 and IL-10 gene transcripts in addition to significantly increased IL-1β and IL-8 gene expression (41). The specific role of the AMΦ in initiation and modulation of the immune response to RSV in terms of active cytokine secretion remains unknown.

Alveolar macrophages

Alveolar macrophages (AMΦs) are a primary innate immune cell in the lung, and are responsible for pathogen clearance, antigen presentation and cytokine production. They are the most abundantly recovered cell type from bronchio-alveolar lavage (BAL) fluid, and are one of the first cell types to come into contact with inhaled microbes, toxins and antigens (42). AMΦ functions in host defense include phagocytosis and intracellular killing of pathogens, secretion of oxygen metabolites, and cytokine production. Initial pro-

inflammatory cytokine production recruits additional immune cells including neutrophils to the site of infection. This response is necessary for mounting an effective immune response, although it must be tightly regulated so as not to obstruct alveolar air exchange (43). AMΦs appear to be somewhat self-regulating in that they also have anti-inflammatory activities. Following resolution of infections, AMΦs phagocytose apoptotic neutrophils to prevent the unintentional release of their contents. This event reduces the release of pro-inflammatory cytokines and stimulates anti-inflammatory IL-10 and TGF-β production instead (44).

RSV and alveolar macrophages

It has been shown that AMΦs can support RSV replication by in vitro and in vivo studies (45, 46). Early in vitro studies reported that human AMΦs are susceptible to RSV, but that infection is abortive. Infected AMΦs exhibited increased TNF-α, IL-6 and IL-8 cytokine secretion, of which only TNF-α appeared to be replication-dependent. Treatment of bronchial epithelial cells with cell culture supernatants from RSV-infected AMΦs did not appear to confer any protection, indicating that the cytokine production from these cells has a role in modulating host inflammatory responses rather than direct anti-viral activities (47). Using a mouse model, another study reported that activated AMΦs had decreased viral loads compared with resting AMΦs, while increasing doses of virus resulted in an increased TNF-α release, but a decrease in cytotoxic killing and production of reactive oxygen species (48).

Large animal models of BRSV have shown bovine and ovine AMΦs to be permissive to infection with variable effects on AMΦ functions (49-53). In vitro infection of bovine AMΦs with BRSV proved to be 80% abortive, indicating that some infected cells can, in fact, produce infective virus (49). These infected AMΦs also exhibited decreased phagocytic

and killing functions, as well as an increase in neutrophil chemotactic factor release (49). Variable effects on macrophage function were observed in an ex vivo study, where BRSV inoculation was reported to inconsistently alter the AMΦ functions of phagocytosis, lysosomal killing and respiratory burst (53).

BRSV located within ovine AMΦs has been positively identified by immunofluorescence (41, 52). BRSV infection of ovine AMΦs induces the transcription of both proinflammatory (IL-1, IL-6, IL-8, IL-12p40, TNFα) and immunomodulatory (IL-4, IL-10) cytokine genes (41). Current gene expression data supports the suggestion of a mixed cytokine response of RSV-infected AMΦs, although whether or not this leads to translation and secretion of active cytokines is unknown.

Th2 cytokines and RSV

To date, only one study has shown a correlation between the development of wheezing and childhood asthma later in life to severe RSV bronchiolitis as infants (54). This association could be a result of two possibilities: either severe RSV is a causal agent for childhood asthma, or individuals who are already predisposed to asthma and allergy may be more susceptible to severe RSV infection.

Although RSV has been implicated as a risk factor for the development Th2-type conditions, conflicting data from research to date prevents the RSV host immune response from fitting one definitive paradigm. Increased IL-4/IFN-γ ratios have been observed in RSV-infected children compared to healthy controls, as well as in children with more severe RSV-induced bronchiolitis compared with those with more mild symptoms, suggesting an association between RSV and Th2 cytokine production (54, 55). In contrast, other groups

have found no such association. No correlation between clinical severity and Th2 cytokine production was found in infants under 6 months of age with mild or severe RSV infection, as shown by low plasma levels of IL-4 and IL-10 compared with increased plasma levels of IL-6 and IL-8 (56). In another study, there was no correlation found between wheezing in RSV-infected infants and children and detection of Th2 cytokines in naso-pharyngeal secretions. A correlation was found, however, between MIP-1α production and disease severity (57).

Moreover, the role of IL-10 expression in RSV infection is controversial. Early studies reported that RSV infection effectively suppressed early immunoregulatory cytokines by inducing the expression of IL-10 (58). However, other studies have shown that host IL-10 production actually inhibits viral replication, and could have a potentially protective function (59). RSV-bronchiolitis was found to be associated with an increased inflammatory response, with measured increases in IL-6, IL-8, IL-10, IFN-γ and MIP-1β, although those children with higher levels of inflammatory cytokines needed less extensive oxygen therapy (60).

Taken together, these results indicate that RSV can induce both Th1 and Th2 cytokine expression, making it difficult to isolate those host factors which may contribute to more severe disease and which may actually have a protective role in children. Further research is also needed to elucidate the various factors contributing to the vast differences in host responses.

Immunomodulatory cytokines

Interleukin-4 (IL-4)

IL-4 is the main Th2-polarizing cytokine, and is responsible for the production of cytokines which trigger the differentiation of naïve T helper cells into Th2 cells, and

B cell Ig class switching to IgE (61, 62). IL-4 is produced by immune cells including T cells, mast cells, basophils, eosinophils, as well as AMΦs (62, 63). As cytokine production specifically by AMΦs affects the development of an adaptive immune response, IL-4 production in these cells could effectively skew the Th1/Th2 cytokine balance in favor of a Th2 response. The contribution of IL-4 protein secreted by AMΦs and its effect on the immune response to RSV is currently unknown.

Splice variants of the IL-4 gene have been shown to exist in humans and cattle, and are suggested to have IL-4-antagonistic activity (64-66). The splice variants IL-4δ2 and IL-4δ3 result from the removal of exon 2 and exon 3, respectively, and are normally coexpressed with IL-4 (64, 66). In humans, IL-4δ2 is preferentially expressed in cells from BAL fluid compared with PBMCs, and inhibits IL-4-induced T cell proliferation (64). Recent work in a bovine model of tuberculosis has suggested a role in protective immunity for IL-4δ3 expression (67). As IL-4 antagonists, the IL-4 splice variants could have a protective role in modulating the effects of IL-4 production during RSV infection. It will also be important for future studies reporting IL-4 production to specify the detection of full-length versus splice variant mRNA or protein.

Interleukin-10 (IL-10)

IL-10 is another anti-inflammatory cytokine induced during RSV infection. It was initially described in as “cytokine synthesis inhibitory factor” because of its immunosuppressive functions (68). It has been shown to inhibit Th1 cytokine production as well as antigen-presenting cell functions (69, 70). In the lung, IL-10 is produced by alveolar macrophages in response to LPS stimulation and viral infections (71, 72). RSV has been

shown to induce IL-10 secretion by healthy adult AMΦs(58). IL-10 production by AMΦs during RSV infection could be a key factor in promoting the Th2 bias by inhibiting Th1 cytokine production.

Signaling pathways

NFAT pathway

NFAT (nuclear factor of activated T cells) is the transcription factor responsible for the expression of IL-4 and other cytokine genes in T cells and mast cells (73, 74). The mechanism of IL-4 production by RSV-infected AMΦs is currently unknown. Activation of the NFAT pathway in AMΦs by RSV could be a strategy employed by the virus to induce IL-4 gene expression and disrupt the Th1/Th2 balance in favor of a Th2 response.

NFAT is activated upon calcium signaling in the cell, which activates calcium ion-calmodulin binding, and the subsequent activation of calcineurin. Calcineurin functions to dephosphorylate NFAT, which in turn exposes multiple nuclear localization signals, and allows NFAT to translocate to the nucleus (Figure 2). In the nucleus, active NFAT binds DNA and its cofactor, AP-1 to induce cytokine gene transcription (75, 76).
The immunosuppressive drugs, cyclosporin A (CsA) and tacrolimus (FK506), block calcineurin/NFAT interaction and prevent the activation and nuclear translocation of NFAT (77, 78). Use of these drugs in research can be used to determine if IL-4 production in AMΦs occurs in a NFAT-dependent or NFAT-independent manner.

NF-κB pathway

NF-κB is a well characterized inducible transcription factor made up of 3 subunits: p65 (Rel A) transactivator, p50 (RelB, c-Rel, NF-κB1), and p52 (NF-κB2)(79). p50-p65

heterodimers make up the NF-κB complex, which contains a nuclear localization sequence (NLS). IκB family members regulate NF-κB activity by binding and masking the NLS (80).

RSV attachment and entry activates intracellular signaling cascades that activate the IκB kinase family members IKKα and IKKβ, which phosphorylate IκB bound to NF-κB (81). IκB phosphorylation induces its degradation and exposure of the NF-κB NLS. This event allows NF-κB to translocate to the nucleus where it binds and activates cytokine gene transcription (79).

RSV has been shown to activate the NF-κB pathway in epithelial cells and alveolar macrophages both in vitro and in vivo, resulting in the production of IL-1, TNF-α, IL-6, IL-10, and IFN-β cytokines and the chemokines IL-8, MCP-1, MIP-1α (82, 83). Initial RSV binding to TLR4 or another host cell receptor may induce the signaling cascade necessary for NF-κB activation. In an in vivo mouse model, NF-κB activation was shown to be TLR-4-dependent but viral replication-independent with the use of UV-inactivated virus, as determined by electrophoretic mobility shift assay of nuclear extracts from whole lung tissue (84). Other studies have used UV-inactivated RSV both in vitro and in vivo, and have shown that it induces IL-8, IL-6, TNF-a, and IL-10 cytokine gene expression, suggesting early recognition of the virus by innate pattern recognition receptors and induction of NF-κB gene transcription (33, 41). Multiple signaling pathways including TLR-4 signaling, PI3-kinase signaling and the p38 MAPK pathways have been suggested to play a role in the immune response to RSV (19). It is currently unknown which specific pathways or signaling molecules may be most critical in shaping the host immune response to RSV infection.

Mitogen-activated protein kinase (MAPK) signaling

MAPK pathways are the signal transduction cascades responsible for the production of IL-10 and other cytokines in alveolar macrophages. MAPK signaling is a highly conserved phosphorylation cascade activated in response to multiple extracellular stimuli such as environmental stresses and pro-inflammatory stimuli (85) (Figure 3). MAP kinases were first identified as insulin-induced protein kinases, and were shown to be regulated by the dual phosphorylation of specific tyrosine and threonine residues by MAPK kinases (86). The MAPK kinases (MEK) themselves are activated by MEK kinases by another specific dual phosphorylation event (87, 88). MAPK activation is tightly regulated by MAPK phosphatases (MKPs), also known as dual-specificiy phosphatases (DUSPs), which dephosphorylate the active-site threonine and/or tyrosine residues as part of negative feedback regulation (88). To date, three different MAPK pathways have been identified: extracellular signal-regulated kinases (ERK), c-jun-N-terminal kinase (JNK) and p38 MAPK (89).

The p38 MAPK pathway is well-characterized, and has been shown to be necessary for cytokine gene transcription in LPS-stimulated AMΦs (72, 90). It has also been shown to activate cytokine gene expression in response to viral infections, including RSV (91, 92). Activation of p38 MAPK modulates cytokine expression by phosphorylation of its two main targets, nuclear transcription factors and MAPK-activated protein kinase-2 (MAPKAP2). Activation of nuclear transcription factors directly induces cytokine gene expression, while MAPKAP2 activation affects mRNA stabilization. Cytokine genes with AU-rich elements (ARE) in the 3’untranslated regions are targets for ARE-binding proteins activated by

MAPKAP2. Activated ARE-binding proteins then bind and stabilize mRNA transcripts containing AREs, preventing their degradation (93).

MAPK inhibitors

Small molecule inhibitors of MAPK signaling have been a valuable tool for studying these pathways, and are also being investigated as potential therapeutics for inflammatory diseases (94). SB203580 is a pyridinyl imidazole compound that specifically binds the ATP-binding site on p38 MAPK, causing a conformational change in the key interacting residues (89, 95). This competitive inhibition effectively blocks the downstream actions of p38, and has been widely used in MAPK research to date. Many studies using SB203580 have shown expression of IL-6, IL-8, IL-1β, TNF-α and IL-10 cytokines to be p38-MAPK dependent in rat and human models (72, 91, 96, 97). SB203580 treatment has been shown, however, to have variable effects in different cell types. In one study, p38 MAPK inhibition by SB203580 treatment decreased IL-6 and TNF-α gene expression in L929 fibrosarcoma cells and whole blood, but increased expression from 4-4 murine macrophages and peritoneal macrophages (98). This observation suggests that p38 MAPK activation of cytokines may be cell-type specific, and could possibly have species-specific results as well.

Treatment with SB203580 decreased IL-10 production in LPS-induced human AMΦs, as well as IL-1β and TNF-α production in RSV-infected epithelial cells (72, 92). The effect of p38 MAPK inhibition on IL-10 production in RSV-infected AMΦs has yet to be shown, and would provide insight into how the anti-inflammatory response of these cells is controlled.

OBJECTIVES AND SPECIFIC AIMS

Statement of the Problem

As described, RSV infection disrupts the Th1/Th2 cytokine balance, in favor of a predominantly Th2 immune response, which is a likely factor in the association between neonatal RSV infection and the development of allergies and asthma later in life. The overall aim of the experiments described in the following chapter was to address the role of AMΦs in terms of cytokine expression and secretion during RSV infection. These experiments examined cytokine responses in freshly isolated AMΦs from both neonatal and adult sheep infected in vitro with BRSV.

Objective 1

Given that AMΦs upregulate the gene expression of IL-4 upon BRSV infection, determine if full-length IL-4 or splice variant mRNA transcripts are produced. The production of IL-4 splice variant mRNA could potentially protect against IL-4 responses induced by RSV infection. As described, IL-4 splice variants are normally coexpressed with full length IL-4 and result from removal of either exon 2 or exon 3 from full length IL-4 mRNA. Because the IL-4 splice variants are suggested to have IL-4-antagonistic activities, it is important to determine whether the changes in IL-4 mRNA levels are full length transcripts or the inactive splice variants.

<u>Hypothesis 1</u>

IL-4/IL-4δ2 and IL-4/IL-4δ3 ratios will be increased in RSV-infected AMΦs compared to uninfected controls.

Specific aim 1

IL-4 and IL-4 splice variant mRNA was measured by real-time PCR. Taqman assay with oligonucleotide primers and fluorescent probes were used for increased sensitivity in detecting full length IL-4 versus splice variant mRNA.

Objective 2

Determine if the increases seen in IL-4 mRNA in RSV-infected AMΦs results in an increased level of secreted IL-4 protein, which could contribute to the overall Th2 environment seen during RSV infection in both neonates and adults. Increased IL-4 secretion from neonatal AMΦs could help in explaining more severe disease seen in infants compared with adults.

Hypothesis 2

RSV-infected AMΦs will secrete increased amounts of IL-4 protein due to the increase in IL-4 gene expression. Secreted IL-4 protein from neonatal AMΦs will be higher than adults.

Specific aim 2

Optimized IL-4 ELISA for detection of ovine IL-4 protein, and measured IL-4 protein levels secreted in cell culture supernatants from neonatal and adult AMΦs infected with RSV.

Objective 3

Determine if IL-10 is secreted by AMΦs in response to RSV infection, which could be a contributing factor to the modulation of the immune response.

Hypothesis 3

AMΦs produce the anti-inflammatory cytokine IL-10 in response to RSV infection, and IL-10 levels produced in neonatal AMΦs will be higher than that from adult cells.

Specific Aim 3

Measured IL-10 protein levels in cell culture supernatants from neonatal and adult AMΦs infected with RSV or stimulated with mitogen.

Objective 4

Determine the proinflammatory IL6, IL-8 and IL-1β cytokine production by RSV-infected AMΦs from neonates and adults.

Because RSV pathogenesis is associated with a mixed immune response and the production of both immunomodulatory and proinflammatory mediators, AMΦ production of proinflammatory cytokines may contribute to the differences observed in the severity of disease observed in neonates compared to adults.

Hypothesis 4

In addition to immunomodulatory cytokine gene transcription, AMΦs upregulate the proinflammatory cytokines IL-6, IL-8 and IL-1β in response to RSV infection. Increases in IL-1β gene transcription will lead to increases in IL-1β protein secretion by AMΦs due to RSV infection, with higher levels from neonatal AMΦs.

Specific aim 4

Quantified IL-6, IL-8 and IL-1β mRNA expression by real time PCR using SYBR green chemistry. Measured IL-1β protein secretion by ELISA.

Objective 5

Elucidate the role of MAPK signaling pathways involved in cytokine expression by RSV-infected AMΦs through the use of SB203580, the specific chemical inhibitor for p38 MAPK.

Hypothesis 5

Selective inhibition of p38 MAPK signaling will decrease IL-10 and pro-inflammatory cytokine production by RSV-infected AMΦs.

Specific aim 5

In vitro RSV infection was initiated after pre-incubation with the p38 MAPK inhibitor, SB203580. Cytokine expression was quantified by real time PCR using SYBR green chemistry. Secreted IL-4, IL-10 and IL-1β protein was detected by ELISA.

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Figure 1. RSV virion structure. RSV is an enveloped, non-segmented, negative sense RNA virus. It acquires its lipid envelope upon budding from the host cell, which contains the virally-encoded glycosylated (G), fusion (F) and small hydrophobic (SH) proteins. The matrix protein (M) forms a layer on the inner surface of the lipid envelope, surrounding the viral genome and its associated ribonucleoprotein complex, made up of the large (L) RNA-dependent-RNA-polymerase, the nucleocapsid (N) protein and the phosphoprotein (P). M2 factors M2-1 and M2-2 are associated with the M protein layer.

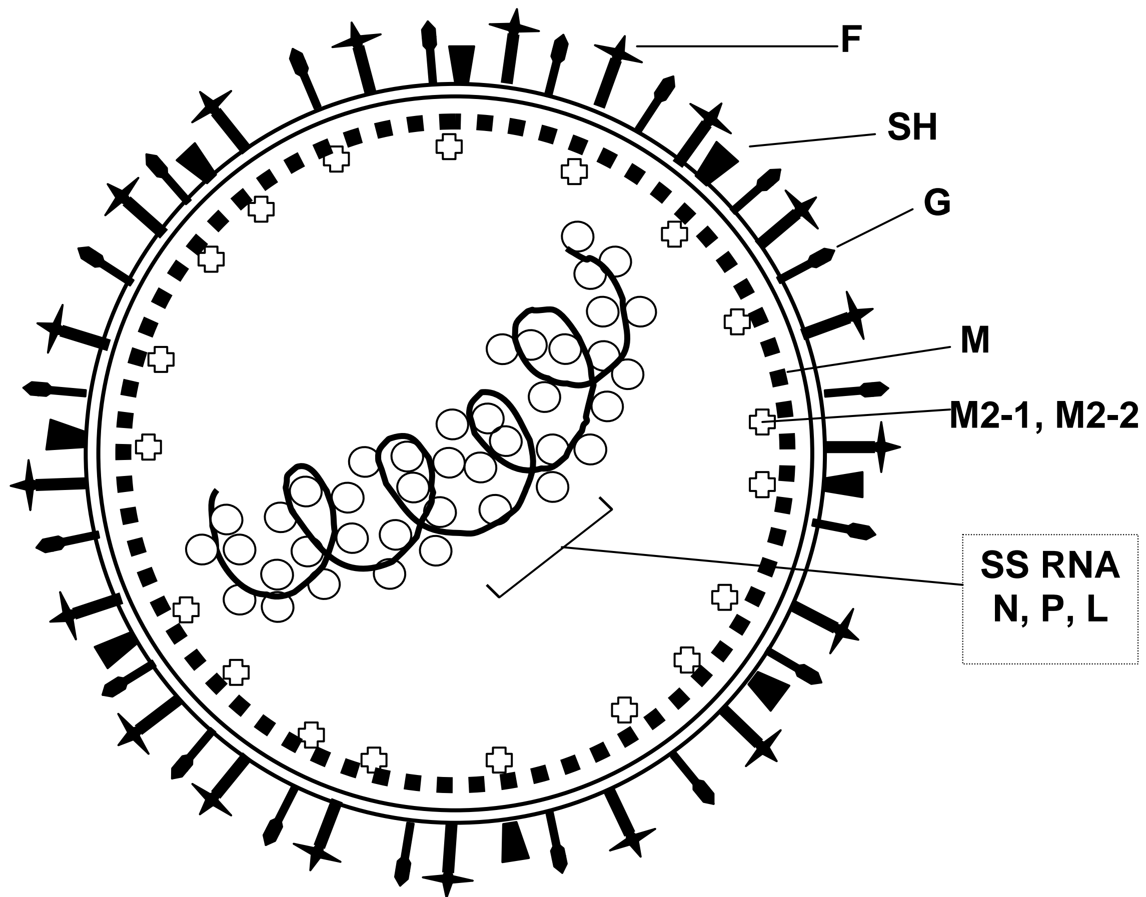

Figure 1. Adapted from Valarcher and Taylor. Veterinary Research 38 (2007) 153-180.

Figure 2. Proposed pathway leading to IL-4 production in macrophages. NFAT is activated upon calcium signaling in the cell, which activates calcium ion-calmodulin binding, and the subsequent activation of calcineurin. Calcineurin functions to dephosphorylate NFAT, which in turn exposes multiple nuclear localization signals, and allows NFAT to translocate to the nucleus. In the nucleus, active NFAT binds DNA and its cofactor, AP-1 to induce cytokine gene transcription. Cyclosporin A (CsA) and tacrolimus (FK506), block calcineurin/NFAT interaction and prevent the activation and nuclear translocation of NFAT.

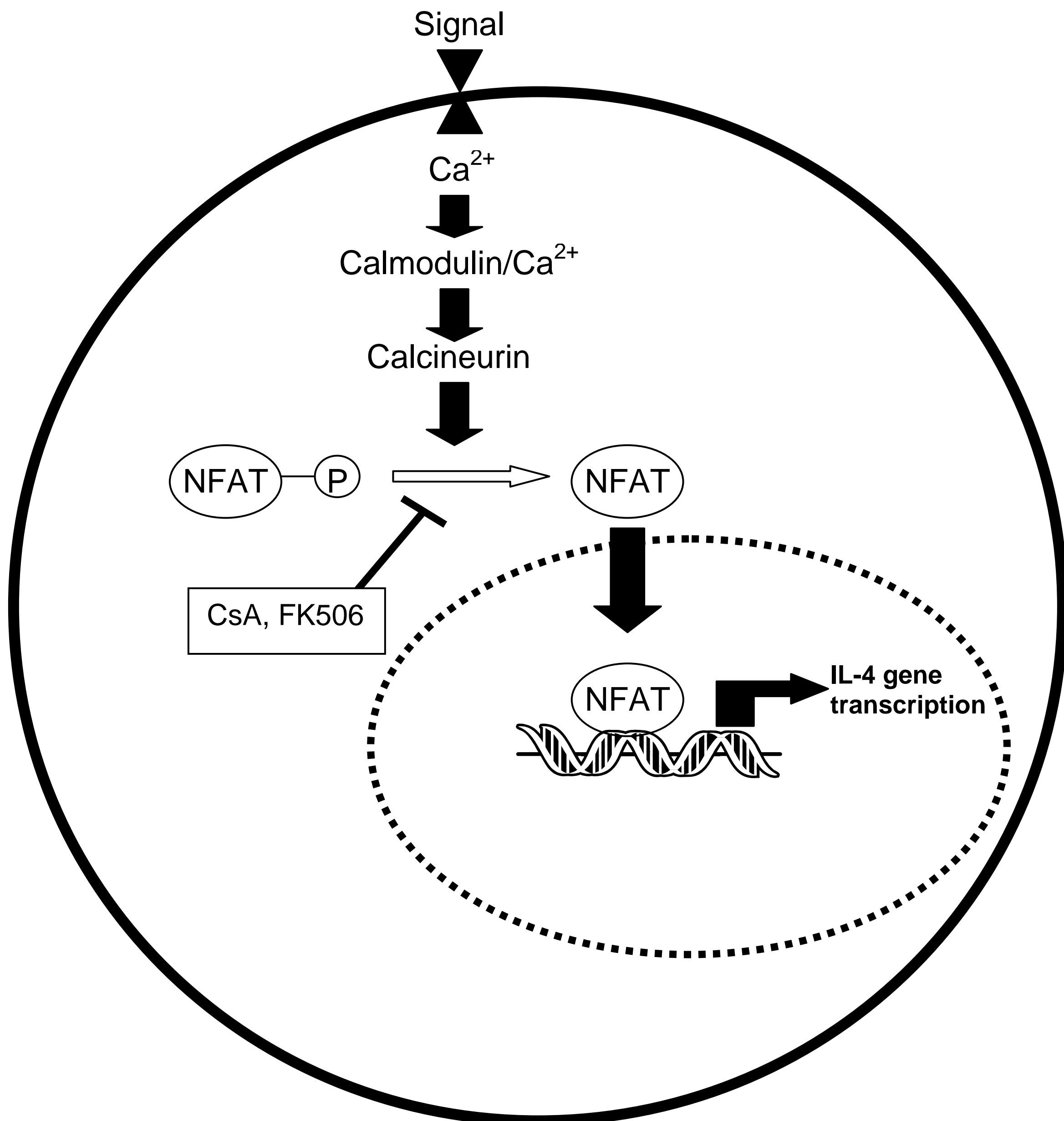

Figure 2. Adapted from Serfling et al. The International Journal of Biochemistry & Cell Biology 36 (2004) 1166-1170.

Figure 3. Overview of p38 MAPK signaling in alveolar macrophages. MAPK signaling is activated in response to multiple extracellular stimuli such as environmental stresses and pro-inflammatory stimuli. Upon activation, MEK kinases (MEKK) phosphorylate MAPK kinases (MEK) by a specific dual phosphorylation event. Active MEK phosphorylates MAP kinases including p38, ERK and JNK by another dual phosphorylation event. Multiple targets of p38 include MAPK-activated protein kinase-2 (MAPKAP2) and nuclear transcription factors.

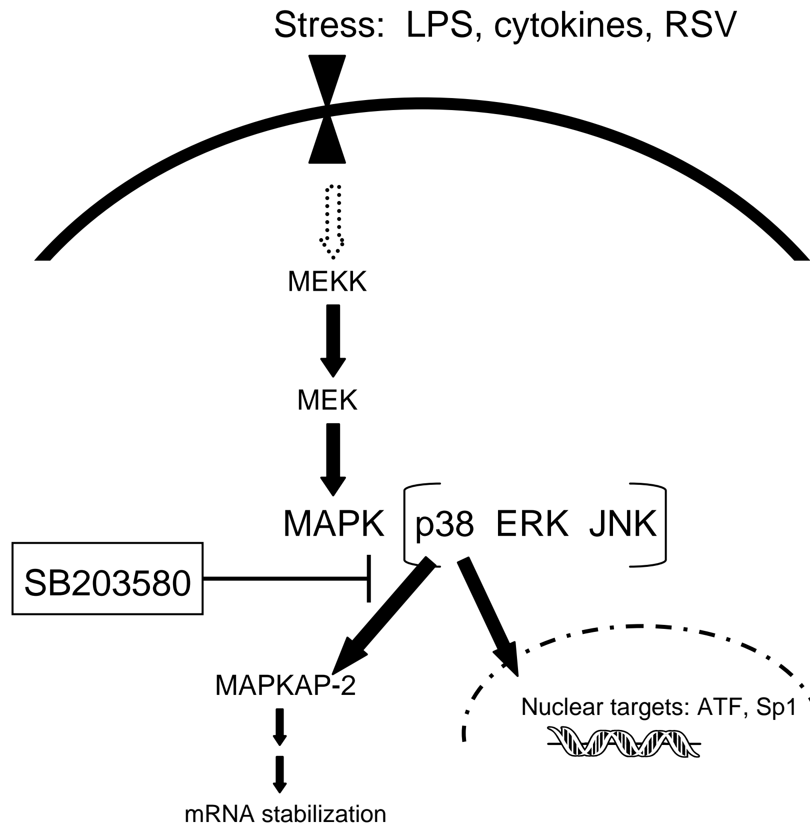

Figure 3. Adapted from Haddad et al. Cellular Signalling 15 (2003) 255-267.

CHAPTER TWO. NEONATAL ALVEOLAR MACROPHAGES UPREGULATE CYTOKINE GENE TRANSCRIPTION BUT FAIL TO SECRETE CYTOKINES IN RESPONSE TO RESPIRATORY SYNCYTIAL VIRUS

A paper to be submitted to *Cellular Immunology*

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Abstract

Respiratory syncytial virus (RSV) is a leading cause of bronchiolitis and pneumonia in premature and newborn infants. We examined the role of alveolar macrophages (AMΦs) in a model of RSV infection. Additionally, the contribution of MAPK signaling to cytokine expression was delineated via p38 inhibitor SB203580. AMΦs isolated from neonatal and adult sheep were infected with BRSV in vitro with or without SB203580 pre-treatment. Expression of cytokine mRNA and protein secretion was determined. Results showed that RSV infection induced IL-1β, IL-4, IL-6, IL-8, and IL-10 gene expression. Furthermore, RSV induction of IL-6, IL-8 and IL-1β mRNA in neonatal and adult AMΦs, as well as IL-10 protein from adult AMΦs, was p38 MAPK-dependent. Significantly higher IL-1β mRNA was detected in RSV-infected neonatal AMΦs than adult cells. Paradoxically, neonatal AMΦs did not secrete IL-1β, IL-4, or IL-10. Inhibition of cytokine secretion by RSV may contribute to the increased susceptibility and severity of disease in neonates.

1. Introduction

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract illness in infants worldwide, causing severe bronchiolitis and pneumonia. Premature and newborn infants are most susceptible to RSV infection, in addition to elderly and immunocompromised individuals. Neonatal RSV infection is of particular interest due to the suggested link between early RSV infection and the development of allergic asthma later in life (1-3).

Bovine Respiratory Syncytial Virus (BRSV) is the etiological agent responsible for similar respiratory tract disease in cattle. BRSV causes severe pneumonia in young calves, and is responsible for considerable economic loss in the cattle industry worldwide. Additionally, BRSV induces a Th2-biased immune response in calves, as indicated by the production of virus-specific IgE and IL-4 in lymph cells from infected calves (4).

Modulation of the immune response to RSV and Th2 cytokine production are possible factors in the association between neonatal RSV infection and the development of allergic asthma. AMΦs are critical for early innate immune responses in the lung through phagocytic activity and antigen presentation to immune effectors. Production of cytokines by AMΦs affects the development of an adaptive immune response, and could effectively skew the Th1/Th2 cytokine balance.

Previous gene expression studies have shown RSV-infected AMΦs upregulate immunomodulatory IL-4 and IL-10 mRNA transcripts, which could contribute to an overall Th2-type environment in the lung (5). Splice variants of the IL-4 gene have been shown to exist in humans and cattle, and are suggested to have IL-4-antagonistic activity (6-9). The splice variants IL-4δ2 and IL-4δ3 result from the removal of exon 2 and exon 3, respectively,

and are normally coexpressed with full-length IL-4. As IL-4 antagonists, the IL-4 splice variants could have a protective role in modulating the effects of IL-4 production during RSV infection. Therefore, it is imperative to differentiate between full-length IL-4 and the biologically-inactive splice variants. Here, we report that IL-4 mRNA transcripts induced by BRSV in AMΦs contain the full-length sequence and are not splice variant mRNA.

Cytokine production by AMΦs is critical for the switch from innate to adaptive immunity. To further characterize the effects of cytokine production by AMΦs on the immune response to BRSV infection, we investigated IL-4 and IL-10 secretion in addition to gene expression. Adult human AMΦs secrete IL-4 protein upon stimulation with PMA and ionomycin and IL-10 protein following LPS stimulation, but this has yet to be shown in neonatal AMΦs (10, 11). IL-4 protein secretion was not detected from neonatal or adult AMΦs treated with either BRSV or the combination of PMA and ionomycin in vitro. Neonatal AMΦs secreted negligible amounts of IL-10 protein in response to either BRSV or mitogen stimulation, while adult AMΦs secreted increased levels of IL-10 protein following BRSV or mitogen stimulation.

The data presented herein suggest that neonatal AMΦs are not a source of anti-inflammatory cytokines during RSV infection. Rather, pro-inflammatory cytokine production by AMΦs could contribute to the lung pathology and inappropriate immune response observed during neonatal RSV infection. The induction of IL-6, IL-1β and IL-8 gene transcripts by RSV has been previously reported in peripheral blood monocytes from human infants, as well as AMΦs from mice and sheep (5, 12, 13). Here, we investigated whether the increases in cytokine message are translated into increases in secreted protein

from AMΦs. Increased expression of pro-inflammatory cytokine genes IL-1β and IL-8 were detected in BRSV-infected AMΦs obtained from both neonatal and adult sheep. Significantly more IL-1β mRNA transcripts were detected in BRSV-infected AMΦs from neonates compared with those from adult sheep. However, negligible IL-1β protein was detected from BRSV-infected AMΦs.

To date, the effect of BRSV infection on signaling pathways and mechanisms of cytokine production has not been well-studied in neonatal AMΦs. Here, we examined the role of p38 MAPK signaling in BRSV-infected AMΦs from both neonates and adult sheep through the use of a specific chemical inhibitor of p38 MAPK, SB203580. IL-6, IL-8 and IL-1β gene expression levels were lower in neonatal and adult AMΦs pre-treated with SB203580 prior to BRSV- or LPS-stimulation compared to BRSV- or LPS-stimulation alone.

2. Materials and Methods

2.1. Sample collection

Lungs were harvested from healthy adult sheep (n=8) or neonatal lambs (n=8) and lavaged with sterile PBS. Bronchioalveolar lavage fluid was centrifuged at 805 x g for 15 minutes, resuspended in sheep-specific alveolar macrophage culture medium (RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 2% sheep serum, 25mM HEPES, 2mM L-glutamine, 1% antibiotic/antimycotic and 0.02mg/mL Gentamicin) and plated in 150x15mm plastic petri dishes. Cells were incubated for 2h at 37°C/5%CO_2 to allow for macrophage adherence. Adherent cells were washed 2 times with sterile PBS and gently scraped into 15mL sheep alveolar macrophage medium. Harvested cells were pelleted by centrifugation at 453 x g for 10 minutes at 4°C and then resuspended in sheep alveolar macrophage medium

for counting by hemacytometer. $5x10^5$ cells were plated in 200µl medium per well in 96-well round-bottom tissue culture plates and incubated at 37°C/5%CO_2.

2.2. p38 MAPK inhibition

Designated AMΦs were pre-incubated with p38 MAPK inhibitor SB203580 (Biomol, Plymouth Meeting, PA) diluted in sheep alveolar macrophage medium at 25µM in-well concentration for 1 hour at 37°C/5%CO_2. This working concentration was determined in preliminary experiments.

2.3. In vitro BRSV infection and mitogen stimulation

BRSV-375 was added at a multiplicity of infection (moi) of 1 pfu/cell and incubated at 37°C/5%CO_2 for 90 minutes. Cells were washed 2 times and fresh culture medium added. LPS-stimulated cells were treated with LPS from E. coli 055:B5 (Sigma, St. Louis, MO) at 1µg/mL in-well concentration. Phorbol-12-myristate-13-acetate (PMA) and ionomycin (Sigma) were added at 1µg/mL and 0.5µg/mL, respectively in PMA/ionomycin-stimulated AMΦs. Cells were collected in RLT lysis buffer from RNeasy Mini kit (Qiagen, Valencia, CA) at 2, 6, 12, 24h for RNA isolation and stored at -80°C until use. Supernatants were collected and frozen at 24h, 48h and 72h for protein detection by ELISA.

2.4. RNA extraction and First-Strand cDNA synthesis

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions, including an on-column RNase-free DNase digestion step to remove any contaminating genomic DNA. RNA yield and purity were determined on GeneQuant-pro spectrophotometer (GE Healthcare, Piscataway, NJ). 400 ng total RNA was

reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and oligo$(dT)_{12\text{-}18}$ primers (Invitrogen).

2.5. Real-time PCR Taqman assay for IL-4 (full length), IL-4δ2 and IL-4δ3 mRNAs

Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed using the SuperScriptIII Platinum One-Step qRT-PCR kit with ROX (Invitrogen) in a 30 µl reaction volume with 250-500 ng total RNA. No-RT control (NRC) samples were performed with Platinum qPCR Supermix-UDG with ROX (Invitrogen).

Oligonucleotide primer and probe sets used are listed in Table 1 and were modified from published bovine sequences (Waldvogel 2004). qRT-PCR reactions were run on an Applied Biosystems 7300 Real-time PCR System with the following conditions: 49°C for 30 min, 95°C for 2 min, followed by 50 cycles of 95°C for 15s, 50°C for 30s and 72°C for 30s.

2.6. Real-time PCR SYBR green assay for IL-4, IL-10, IL-6, IL-8, IL-1β mRNAs

Oligo(dT) cDNA was diluted 1:10, and 2µl used per reaction. Real-time PCR reactions were performed using SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA) in a 20µl reaction volume. Primers used are listed in Table 2 and reaction conditions were as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 15s and 50°C for 1 min, and a dissociation step of 95°C for 15s, 50°C for 20s, 95°C for 15s and 60°C for 15s.

Expression of ovine polyubiquitin gene was used as the endogenous control for calculation of dCt values. Relative quantification of RT-PCR data was determined by the $2^{-(\mathrm{dd}Ct)}$ method (Livak and Schmittgen, 2001).

2.7. IL-4 Enzyme-linked immunosorbent assay (ELISA)

Secreted IL-4 protein was detected in cell culture supernatants by sandwich ELISA. Briefly, Immunolon 2HB microtiter plates (Thermo Scientific, Franklin, MA) were coated with 100μl per well of mouse anti-bovine IL-4 antibody clone CC313 (Serotec, Raleigh, NC) diluted 5μg/mL in 0.5M carbonate-bicarbonate buffer (pH 9.6), and incubated at 4°C overnight. Plates were washed 5 times between each step with 300μl per well of washing buffer (PBS + 0.05% Tween20), and blocked with 100μl blocking buffer (PBS + 1% BSA + 0.05% Tween20) per well for 1h at 37°C. Serial dilutions of recombinant bovine IL-4 protein standard and sample supernatants were added in duplicate wells and incubated for 1h at 37°C. Biotinylated mouse-anti-bovine IL-4 antibody clone CC314 (Serotec, Raleigh, NC) was diluted to 2.5μg/mL in blocking buffer and 100μl added per well for detection. Streptavidin-HRP conjugate (GE Healthcare, Piscataway, NJ) was diluted 1:1000 in blocking buffer and 100μl added per well. Wells were developed with 100μl per well of ABTS substrate (US Biological, Swampscott, MA). Color development was measured after 10 min by measuring absorbance at 405 nm with a FlexStation3 microplate reader (Molecular Devices, Sunnyvale, CA). Recombinant bovine IL-4 protein (Serotec, Raleigh, NC) was to generate a standard curve, and used to calculate concentration of IL-4 in sample supernatants. The limit of detection of this assay was found to be 32pg/mL.

2.8. IL-10 ELISA

Secreted IL-10 was detected in cell culture supernatants by sandwich ELISA. Briefly, MaxiSorp microtiter plates (Nunc, Rochester, N.Y.) were coated with 100μl per well mouse anti-bovine IL-10 antibody (Serotec, Raleigh, NC) diluted 4μg/mL in 0.5M

carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 37°C. Plates were washed 5 times between each step with 300µl per well of washing buffer (PBS + 1% Tween 80) per well and then blocked with 100µl blocking buffer (PBS + 1% gelatin) per well for 1h at 37°C. Serial dilutions of recombinant bovine IL-10 protein standard and sample supernatants were added in duplicate wells and incubated for 1h at 37°C. Biotinylated mouse anti-bovine IL-10 (Serotec, Raleigh, NC) detection antibody was added per well at 1µg/mL in blocking buffer. Streptavidin-HRP conjugate (GE Healthcare, Piscataway, NJ) was diluted 1:800 in blocking buffer and 100µl added per well. Wells were developed with 100µl per well of ABTS substrate (US Biological, Swampscott, MA). Color development was measured after 30 min by measuring absorbance at 405 nm with a FlexStation3 microplate reader (Molecular Devices, Sunnyvale, CA). Standard curves were generated from recombinant bovine IL-10 protein (J.C. Hope, Institute of Animal Health, Compton, UK) and were used to calculate concentration of IL-10 ELISA units/mL in sample supernatants.

2.9. IL-1β ELISA

Secreted IL-1β was detected in cell culture supernatants by ELISA. Briefly, MaxiSorp microtiter plates (Nunc, Rochester, N.Y.) were coated with 100µl per well rabbit anti-bovine IL-1β antibody (Thermo Scientific, Rockford, IL) diluted 5µg/mL in 0.5M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 37°C. Plates were washed 5 times between each step with 300µl per well of washing buffer (PBS + 0.05% Tween 20) per well and then blocked with 100µl blocking buffer (Milk blocking buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD)) per well for 1h at 37°C. Serial dilutions of recombinant bovine IL-1β protein standard and sample supernatants were added in duplicate

wells and incubated for 1h at 37°C. Biotinylated rabbit anti-bovine IL-1β (Thermo Scientific, Rockford, IL) detection antibody was added per well at 10μg/mL in blocking buffer. Streptavidin-HRP conjugate (GE Healthcare, Piscataway, NJ) was diluted 1:250 in blocking buffer and 100μl added per well. Wells were developed with 100μl per well of TMB microwell substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Color development was measured after 20 min by measuring absorbance at 650 nm with a FlexStation3 microplate reader (Molecular Devices, Sunnyvale, CA). Standard curves were generated from recombinant bovine IL-1β protein (Serotec, Raleigh, NC) and were used to calculate concentration of IL-1β in sample supernatants.

3. Results

3.1. Alveolar macrophages produce full-length IL-4 mRNA transcripts in response to BRSV infection.

AMΦs harvested from neonatal and adult sheep lungs were stimulated in vitro with BRSV or PMA and ionomycin to assess IL-4 gene expression and protein secretion. Specific primers and probe sets were used to detect full-length IL-4 or splice variant mRNA transcripts by RT-PCR. BRSV induced a 7-fold increase in full-length IL-4 expression and a 2-fold increase in adult and neonatal AMΦs, respectively, at 24h post-infection compared to mock-treated controls. PMA and ionomycin-treated AMΦs had significant increases in full-length IL-4 transcription as well, with an average of 10 and 20-fold increase in full-length IL-4 expression at 6h post-infection in neonates and adults (Figure 1). No splice variant mRNA was detected in any of the samples in any of the treatment groups.

To assess whether the observed increases in IL-4 mRNA transcripts leads to increases in IL-4 protein levels, cell-culture supernatants from the in vitro BRSV- or PMA and ionomycin-treated AMΦs were harvested at 24 and 48h, and tested for secreted IL-4 protein by ELISA. Dilutions of recombinant bovine IL-4 protein were used to create a standard curve, and the limit of detection of the assay was found to be 32pg/ml. Cell-culture supernatants from magnetically-sorted ovine $CD4^+$ T cells isolated from peripheral blood, stimulated with PMA and ionomycin for 24h were included as a positive control. IL-4 protein was not detected in supernatants from BRSV-infected or PMA and ionomycin-stimulated AMΦs (data not shown).

3.2. IL-10 production by adult alveolar macrophages is induced by in vitro BRSV infection or LPS stimulation and is dependent on p38 MAPK signaling.

AMΦs harvested from neonatal and adult sheep were treated in vitro with SB203580, to block p38 MAPK signaling upon BRSV-infection or LPS-stimulation. IL-10 mRNA transcripts detected by real-time PCR were expressed relative to control AMΦs. No significant differences in IL-10 expression were observed in either neonatal or adult AMΦs treated with BRSV or LPS compared to mock-treated cells. Adult AMΦs pre-incubated with SB203580 prior to LPS stimulation exhibited a trend of lower IL-10 mRNA transcripts compared to LPS treatment alone (Figure 2).

Secreted IL-10 protein was measured by sandwich ELISA. Treatment with either BRSV, LPS, or PMA and ionomycin increased IL-10 secretion from adult AMΦs. Pre-incubation with SB203580 effectively decreased IL-10 secretion in mock-treated, BRSV-infected and LPS-treated AMΦs from adult sheep to levels lower than controls, on average

(Figure 3B). Minimal IL-10 protein was detected in cell culture supernatants from neonatal AMΦs infected with BRSV or treated with PMA/ionomycin (Figure 3A). The outliers observed in neonatal AMΦ IL-10 secretion represent samples from the same animal. The high level of IL-10 detected in the mock-infected sample suggests a pre-existing condition, and therefore changes in IL-10 secretion in samples from this animal can not be specifically attributed to the in vitro BRSV infection or SB203580 treatment.

3.3. BRSV infection induces increased pro-inflammatory cytokine gene expression in AMΦs from neonates compared to adults

Transcription of proinflammatory cytokine genes IL-6, IL-8 and IL-1β were measured in response to in vitro BRSV infection in neonatal and adult AMΦs in the presence or absence of SB203580 pre-treatment. No significant differences were observed in IL-6 mRNA transcripts between mock- and BRSV-infected AMΦs in neonates or adults. However, LPS stimulation of adult AMΦs induced a two-fold increase in expression at 24h. Pre-treatment with SB203580 prior to LPS stimulation induced less IL-6 transcription in adult AMΦs compared to cells treated with LPS only (Figure 4A).

IL-8 gene expression was increased in both neonatal and adult AMΦs infected with BRSV compared to mock-infected cells, with a two-fold increase at 12h in both age groups (Figure 4B). BRSV-treated AMΦs that were pre-incubated with SB203580 exhibited IL-8 gene expression either equal to or less than that of control cells. LPS stimulation of adult AMΦs induced a significant ($p<0.05$) 5.5-fold increase in IL-8 transcription compared to mock-treated control cells. Pre-treatment with SB203580 prior to LPS stimulation induced only a 4.5-fold increase in IL-8 expression compared to controls (Figure 4B).

BRSV-treatment of neonatal AMΦs induced a significant ($p<0.01$) increase in IL-1β transcripts compared with mock-treated cells, which was reduced by pre-incubation with the p38 MAPK inhibitor SB203580. Neonatal AMΦs pre-incubated with SB203580 had IL-1β transcription levels less than or equal to levels in mock-treated cells. No significant differences in IL-1β gene expression were observed in adult AMΦs treated with BRSV or LPS (Figure 4C).

3.4. Neonatal AMΦs are not a significant source of IL-1β protein during RSV infection

Freshly isolated AMΦs from adult (n=8) or neonatal (n=8) sheep were stimulated in vitro with BRSV, following pre-incubation with p38 MAPK inhibitor, SB203580, or culture medium alone. LPS- and PMA and ionomycin- stimulated AMΦs were included as positive controls. Cell culture supernatants were harvested at 24h or 48h, and frozen at -80C until use. Supernatants were screened for IL-1β protein by sandwich ELISA. Low levels of secreted IL-1β were detected at 24h in neonatal AMΦs infected with BRSV with or without SB203580 pretreatment (Figure 5A). An increase in IL-1β protein secretion by neonatal AMΦs was observed only in the subset treated with PMA and ionomycin (Figure 5A). Adult AMΦs secreted increased levels of IL-1β in response to LPS-stimulation, which was quantitatively larger than that in mitogen-stimulated neonatal AMΦs. No increase in IL-1β secretion was observed in adult AMΦs following BRSV infection at 24h (Figure 5B). Similar results were observed at 48h, with less IL-1β detected in supernatants from both neonatal and adult AMΦs (Figure 6A and 6B). No significant effects of p38 MAPK inhibition on IL-1β protein secretion were observed in either age group at 24 or 48h.

4. Discussion

The aim of the present study was to further elucidate the role of the AMΦ during the host response to RSV infection in neonates and adults. In this study, we compared cytokine production in AMΦs from neonatal (age ≤ 7d) and adult (age 1-3y) sheep. Here, we report that RSV infection induces the expression of both anti-inflammatory (IL-4, IL-10) and pro-inflammatory (IL-8, IL-1β) cytokine genes in AMΦs. Moreover, p38 MAPK signaling was shown to be necessary for IL-6, IL-8 and IL-1β gene transcription in neonatal and adult AMΦs, as well as IL-10 protein secretion from adult AMΦs. Significantly higher IL-1β mRNA was detected in RSV-infected AMΦs from neonates compared to adults. However, IL-1β, IL-4, or IL-10 protein was not detected in cell culture supernatants from RSV-infected neonatal AMΦs.

It has been previously shown that AMΦs elicit a mixed cytokine response following RSV infection, as indicated by increases in both pro- and anti-inflammatory cytokine gene expression (5, 14). To further investigate this response, the first aim of these studies was to explore how the changes in cytokine gene expression are reflected in corresponding changes in cytokine protein secretion.

Because of the suggested link between neonatal RSV infection and the development of Th2-associated conditions later in life (1, 2), we first examined IL-4 production. Recent work has reported the detection of IL-4 splice variants, IL-4δ2 and IL-4δ3, in humans, mice and cattle, which are formed from the removal of exon 2 or exon 3, respectively (6-9). IL-4 splice variant mRNA is translated into biologically inactive protein that acts as a competitive inhibitor for the IL-4 receptor, thus blocking IL-4 activation of other cells. In a bovine model

of tuberculosis, a disease known to elicit a Th1 immune response, cattle that had increased levels of IL-4δ3 transcripts also had no visible lung pathology following *Mycobacterium bovis* infection (15). This suggests a role for IL-4 splice variants in protection against Th1-type infections. In addition, Th2 responses are associated with greater full-length IL-4 to IL-4 splice variant ratios (8, 16). Based on this information, we hypothesized that full-length IL-4 mRNA would be dominantly expressed relative to IL-4 splice variant mRNA during RSV infection. To test this, we used specific primer and probe sets for full-length IL-4, IL-4δ2 and IL-4δ3 to determine which are induced in BRSV-infected AMΦs. Our results show that the increases in IL-4 gene expression are in fact full-length transcripts, and not either of the splice variants. Full-length IL-4 expression in BRSV-infected AMΦs was increased 7-fold over mock-infected cells at 24hpi.

Recently, human AMΦs were shown to secrete IL-4 protein in response to phorbol-12-myristate-13-acetate and calcium ionophore stimulation (10). In our system, however, IL-4 protein secretion was undetectable from either adult or neonatal AMΦs by ELISA. The amount of IL-4 protein necessary to elicit an effective response is unknown. It is possible that minimal but similar amounts of IL-4 protein elicit a stronger T cell response in neonates compared with adults, which could contribute to the disregulated Th2 response observed in asthmatic children with a previous history of RSV infection (1). However, our data suggest that AMΦs are not a significant source of IL-4 protein during RSV infection in our model. IL-4 produced in the lung during RSV infection is likely to be secreted from another cell type, such as T cells or infiltrating monocytes (17-19).

IL-10 is another anti-inflammatory cytokine produced by AMΦs that is a critical regulator of inflammation and homeostasis in the lung (20). Previous work has shown increases in IL-10 gene expression in neonatal AMΦs infected with RSV, which could contribute to an overall anti-inflammatory environment in the lungs (5). Here, we measured IL-10 protein by RSV-infected AMΦs. Our data demonstrate increases in secreted IL-10 protein in BRSV-infected and LPS-stimulated AMΦs from adults. This data is supported by other studies documenting increases in IL-10 protein in AMΦs from adult humans (21). Interestingly, neonatal AMΦs did not secrete appreciable amounts of IL-10 protein in response to BRSV infection or mitogen stimulation. Overall, our data suggest a restricted capacity of neonatal AMΦs to produce anti-inflammatory cytokines.

This apparent deficiency in the ability of the neonatal AMΦs to produce key anti-inflammatory cytokines could result in a disregulated and/or destructive pro-inflammatory response to RSV. Our results demonstrate increases in IL-8 and IL-1β gene expression in RSV-infected AMΦs from both adults and neonates, with significantly more IL-1β transcripts produced in neonatal cells compared to adult cells. No change in IL-6 gene expression was observed in RSV-infected AMΦs from either age group. This observation differs from previous RSV studies which have reported increases in IL-6 from neonatal AMΦs or monocytes infected with RSV (5, 14, 22, 23).

A major difference was observed between neonatal and adult AMΦ transcription of IL-1β. Due to the significant increase in neonatal IL-1β mRNA transcription in response to RSV infection, we hypothesized that a coordinated increase in secreted IL-1β protein would be detected. However, minimal IL-1β protein was detected from neonatal AMΦs infected

with BRSV or stimulated with PMA and ionomycin. This disconnect between transcription of message and translation of protein could be explained by RSV interference with the post-transcriptional or post-translational processing of IL-1β.

Previous studies have reported equivocal results of IL-1β secretion in response to RSV infection. Increased IL-1β mRNA and protein was shown in RSV-infected human cord blood monocytes, as well as in AMΦs from BALB/c mice at 6-8 weeks of age (24, 25). Significantly higher IL-1β protein levels, however, were detected in monocyte cell lysates compared to cell culture supernatants (25). This suggests that intracellular IL-1β is not fully-processed to form active and secreted IL-1β protein from RSV-infected cells. IL-1β is normally synthesized as a precursor protein, pro-IL-1β, that is specifically cleaved by caspase-1 to expose a secretory signal sequence (26, 27). Interferon regulatory factor-1 (IRF-1) is a transcriptional activator of caspase-1, which is translated into pro-caspase-1, and requires cleavage by caspase-8 for activation (26). Interference of any of these regulatory processes by RSV could effectively prevent the secretion of IL-1β protein from AMΦs, regardless of increased transcription or translation levels. RSV has been shown to enhance IRF-1 and caspase-1 gene expression in a human alveolar epithelial cell line, however this response has not yet been investigated in AMΦs (24).

Interestingly, we report that adult AMΦs secrete higher levels of IL-1β in response to mitogen stimulation than neonatal AMΦs, which could be a result of increased levels of caspase-1, caspase-8 or IRF-1 in stimulated adult AMΦs. Interference with signaling pathways by RSV has previously been shown to modulate host cellular immune responses by blocking cytokine production (28, 29). RSV NS2 protein inhibits IFNα/β signaling and host

anti-viral activity by mediating the degradation of Stat2, as a mechanism of evading the host immune response (28). Both the NS1 and NS2 proteins of RSV have been shown to block the induction of type I interferons in epithelial cells and macrophages (28-30). This mechanism of evasion utilized by RSV resulted in less pro-inflammatory cytokine expression, with decreased levels of RANTES, IL-8 and TNF-α (31). Interaction of RSV and host signaling components may also play a role in the control of IL-1β regulation at the level of transcription, translation, or secretion.

The second major aim of these studies was to elucidate the signaling pathways activated by RSV infection and provide insight into the mechanism of cytokine gene transcription and protein secretion in AMΦs. To date, p38 MAPK signaling has been implicated in the expression of IL-10, IL-6, IL-β and IL-8 cytokine genes in macrophages (11, 32-35), although this is the first study to examine its role specifically in RSV-infected AMΦs from neonates and adults. Here, we report that specifically blocking p38 MAPK activation with the chemical inhibitor SB203580 resulted in decreased levels of IL-8 and IL-1β mRNA in response to RSV-infection or LPS-stimulation. Our data suggest a role for p38 MAPK signaling in cytokine production in RSV-infected AMΦs. Interestingly, IL-10 transcription was not affected by p38 MAPK inhibition, but IL-10 protein secretion from adult AMΦs was reduced, suggesting a role for p38 MAPK in the post-transcriptional processing of IL-10 mRNA in the RSV-infected AMΦ. MAP kinase-activated protein kinase 2 (MAPKAP-2) is one cellular target of active p38 which could become activated during RSV infection. MAPKAP-2 activates AU-rich element-binding proteins which bind to AU-rich regions in the 3'untranslated regions of mRNA transcripts to prevent self-folding and

degradation (36). Preferential activation of MAPKAP-2 by RSV could function to stabilize IL-10 mRNA transcripts, which could explain the increases in IL-10 protein detected in cell culture supernatants without a preceding increase in transcription.

In summary, our data indicate that AMΦ function is modulated by RSV infection. Based on this study, neonatal AMΦs are not a significant source of IL-4, IL-10 or IL-1β cytokines during RSV infection. IL-10 protein secretion, as well as IL-6, IL-8 and IL-1β gene expression, was found to be dependent on p38 MAPK signaling. Although increased IL-1β transcripts were detected in neonatal AMΦs, a corresponding increase in secreted protein was not observed. Inadequate cytokine production by neonatal AMΦs may provide some explanation for the increase in host susceptibility to RSV and resultant secondary infections in infants.

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Table 1. Taqman primers and probe sets for detecting ovine IL-4.

Primer name	Sequence (5'-3')	Accession no.
Full-length IL-4 Forward	CAT GCA TGG AGC TGC CTG TA	NM_001009313
Full-length IL-4 Reverse	AAT TCC AAC CCT GCA GAA GGT	
Full-length IL-4 Probe	FAM-TGC TGC CCC AAA GAA CGC AAC TG-TAMRA	
Il-4δ2 Forward	AGA GAT CAT AA AAC GCC GAA CAT	NM_001009313
IL-4δ2 Reverse	CTC AAT TCC AGT CCT ACA GAA	
IL-4δ2 Probe	FAM-CCT CAC ATC GCA GAA GAA CGC AAC TGA GAA GGA-TAMRA	
IL-4δ3 Forward	CAT GCA TGG AGC TGC CTG TA	NM_001009313
IL-4δ3 Reverse	CGT ACT TGT ACT CGT CTT GGC	
IL-4δ3 Probe	FAM-TGC CCC AAA GAC CTG TTC TGT GAA TGA A-TAMRA	
Polyubiquitin 3'UTR Forward	GGT GGC TGT TAA TTC TTC AG	NM_001009202
Polyubiquitin 3'UTR Reverse	AA TGG CTA GAG TGC AGA ACG AT	
Polyubiquitin 3'UTR Probe	FAM-TTC ATA ATG CTC AGT GAT GG-MGBNFQ	

Table 2. SYBR green primer sets for detecting ovine cytokine genes.

Primer name	Sequence (5'-3')	Accession number
IL-4 Forward	GGA CTT GAC AGG AAT CTC	Z11897
IL-4 Reverse	CTC AGC GTA CTT GTA CTC	
IL-10 Forward	GAT GCC ACA GGC TGA GAA CC	U11421
IL-10 Reverse	GCG AGT TCA CGT GCT CCT TG	
IL-6 Forward	GAG TTG CAG AGC AGT ATC	X62501
IL-6 Reverse	GGC TGG AGT GGT TAT TAG	
IL-8 Forward	AAG CTG GCT GTT GCT CTC	X78306
IL-8 Reverse	GGC ATCAGAA GTT CTG TAC TC	
IL-1β Forward	ATG GGT GTT CTG CAT GAG	X54796
IL-1β Reverse	AAG GCC ACA GGA ATC TTG	
Polyubiquitin 3'UTR Forward	GGT GGC TGT TAA TTC TTC AG	NM_001009202
Polyubiquitin 3'UTR Reverse	AA TGG CTA GAG TGC AGA ACG AT	

Figure 1: Relative quantification of full-length IL-4 mRNA transcripts expressed in response to stimulation with PMA and ionomycin or BRSV, compared with mock-treated cells. AMΦs isolated from adult (n=6) or neonatal (n=8) sheep lungs were stimulated in vitro with PMA (1µg/ml) and ionomycin (0.5µg/ml) or BRSV (moi = 1). Cells were lysed at various timepoints for total RNA isolation. Total RNA was used as template for RT-PCR reactions using SuperScriptIII Platinum One-Step qRT-PCR kit (Invitrogen). BRSV and PMA/ionomycin treatments both induced full-length IL-4 mRNA transcription. Data are representative of 3 separate experiments.

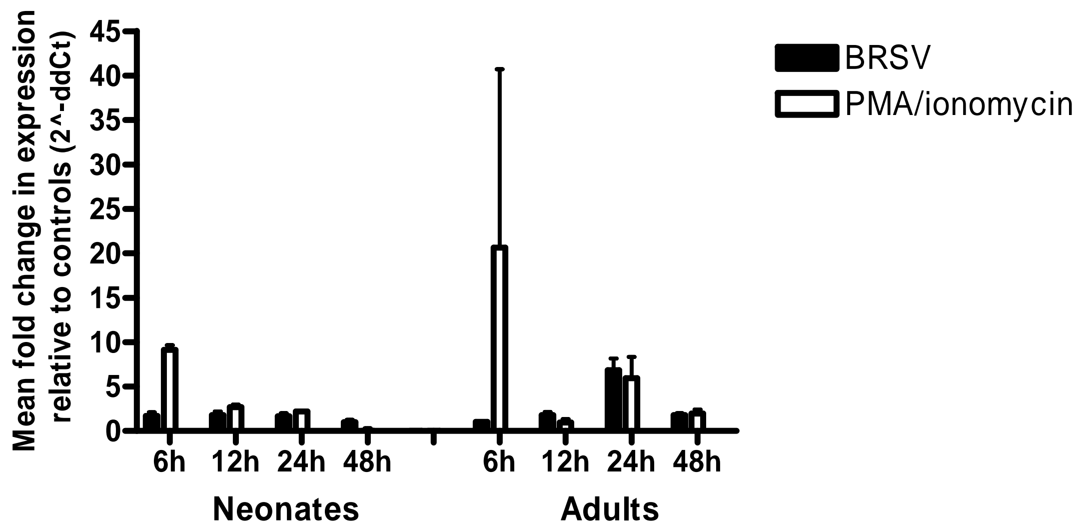

Figure 1.

Figure 2: Relative quantification of IL-10 mRNA expression in ovine AMΦs in response to in vitro stimulation. AMΦs isolated from adult sheep (n=8) and neonatal lambs (n=8) were preincubated with p38 MAPK inhibitor SB203580 for 1h. BRSV (moi =1) was added for 90 min and then cells were washed. LPS (1μg/ml) was added to a subset of adult AMΦs. Cells were lysed at designated timepoints and total RNA isolated. Oligo(dT) cDNA was transcribed and used as template for SYBR green real-time PCR. Results are reported as fold-changes in expression compared to mock-treated cells normalized to endogenous control, ovine polyubiquitin. No significant differences were observed in neonatal AMΦs or adult AMΦs pre-treated with SB203580, treated with BRSV or LPS. Data are representative of 4 separate experiments.

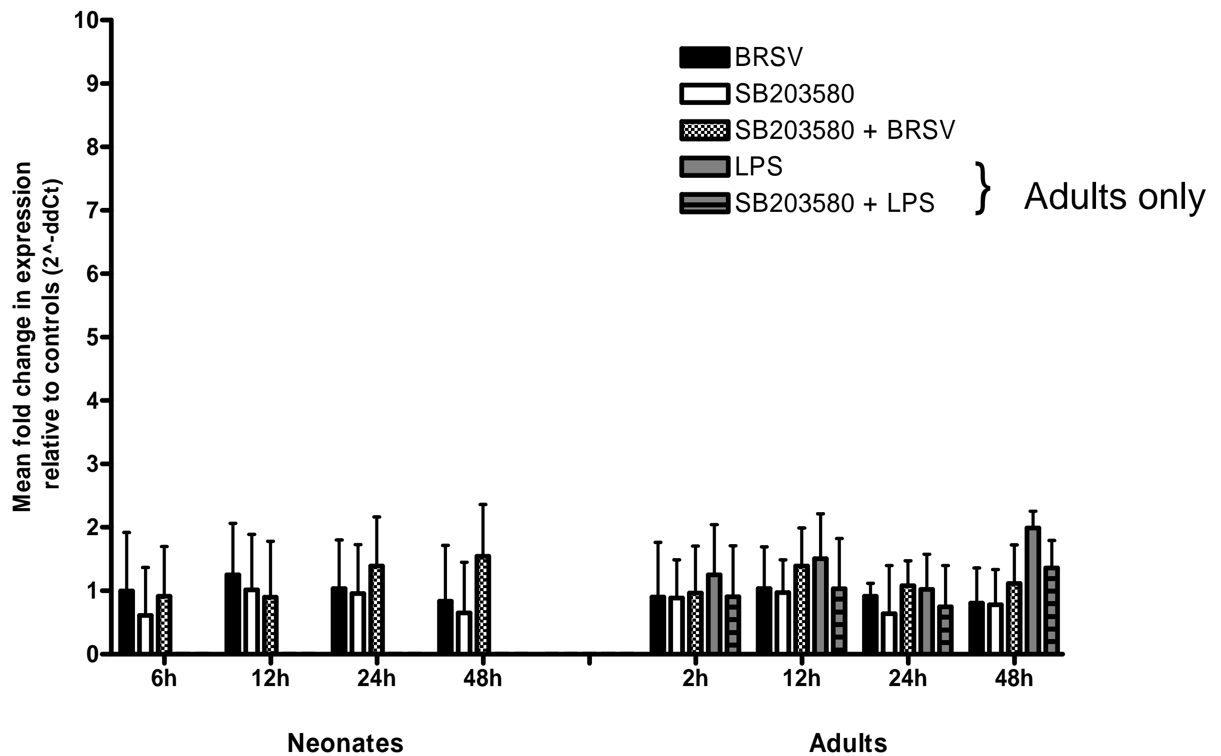

Figure 2. Relative quantification of IL-10 mRNA expression in ovine AMΦs in response to in vitro stimulation

Figure 3: IL-10 protein secretion (units/mL) by ovine AMΦs following p38 MAPK inhibition and in vitro BRSV or mitogen stimulation. AMΦs isolated from neonates (n=8) and adults (n=8) were pre-incubated with p38 MAPK inhibitor, SB203580, for 1h. BRSV (moi=1), PMA (1μg/ml) and ionomycin (0.5μg/ml), and LPS (1μg/ml) were added to designated wells. Cell culture supernatants were harvested at 48h and frozen at -80C until use. Secreted IL-10 was measured by ELISA in IL-10 ELISA units/mL as determined by recombinant bovine IL-10 standard curve. In general, neonatal AMΦs (A) did not secrete appreciable amounts of IL-10 protein following BRSV infection or stimulation with PMA/ionomycin. In adult AMΦs (B), treatment with either BRSV, LPS or PMA/ionomycin induced an increase in IL-10 protein secretion compared to mock-treated cells. Pre-treatment with SB203580 decreased average levels of IL-10 protein in cell-culture supernatants in mock-, BRSV- and LPS-treated AMΦs. Data are representative of 4 separate experiments.

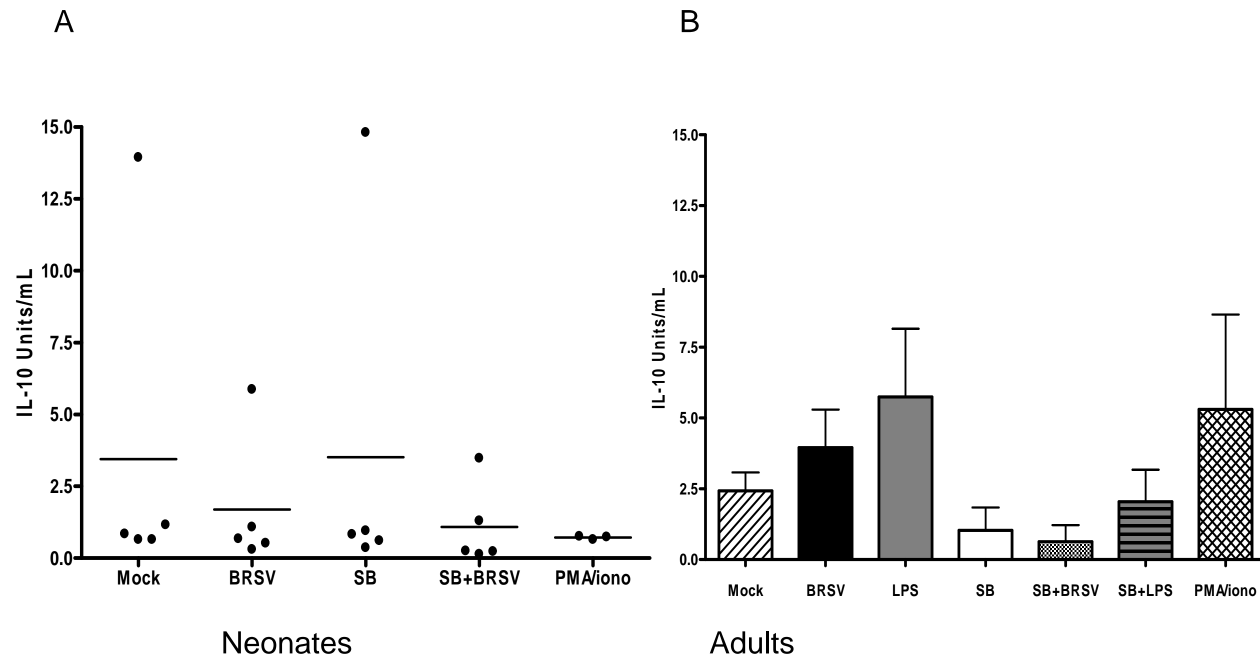

Figure 3. IL-10 protein secretion (units/mL) by ovine AMΦs following p38 MAPK inhibition and in vitro BRSV or mitogen stimulation.

Figure 4: Relative quantification of pro-inflammatory cytokine gene transcripts produced by AMΦs stimulated in vitro. Neonatal (n=8) and adult (n=8) AMΦs were pre-incubated with p38 MAPK inhibitor, SB203580, for 1h prior to treatment with BRSV (moi=1) or LPS (1μg/ml). Cells were lysed in RLT buffer and total RNA isolated for cDNA synthesis. Oligo(dT) cDNA template was used for real-time PCR assay with SYBR green chemistry. Results are reported as fold-change in expression compared to mock-treated cells normalized to ovine polyubiquitin as an endogenous control. No significant differences were observed in IL-6 mRNA transcripts between mock- and BRSV-treated AMΦs in neonates or adults, while LPS-treatment of adult AMΦs induced a two-fold increase in expression (A). IL-8 and IL-1β gene expression was significantly increased in neonatal AMΦs treated with BRSV compared with control cells ($p<0.01$), while significant increases in adult AMΦ IL-8 and IL-1β gene transcripts were observed only in LPS-treated cells ($p<0.05$) (B) and (C). Data are representative of 4 separate experiments.

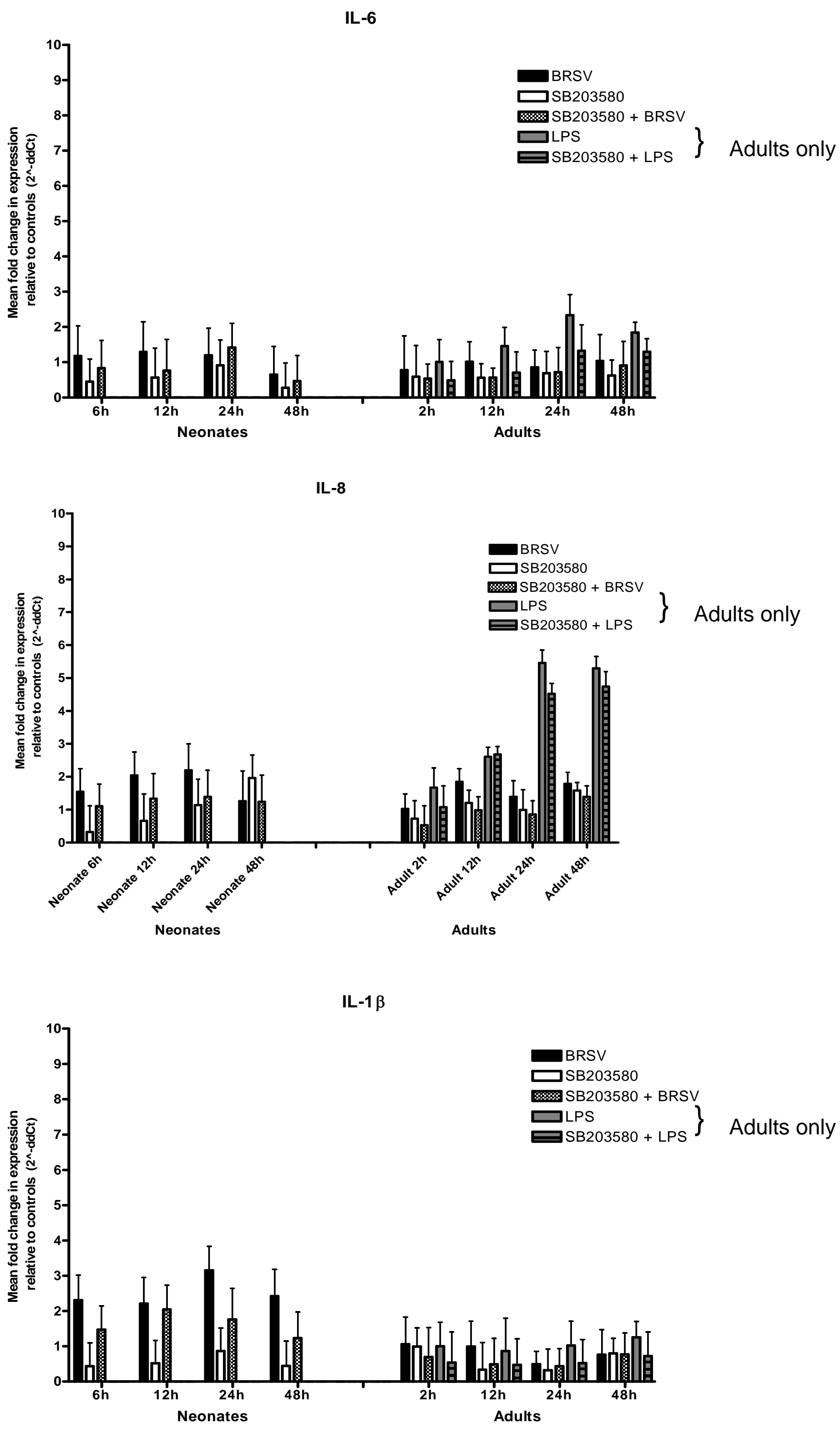

Figure 4. Relative quantification of pro-inflammatory cytokine gene transcripts produced by AMΦs stimulated in vitro.

Figure 5: IL-1β protein secretion by ovine AMΦs following p38 MAPK inhibition and in vitro BRSV or mitogen stimulation. AMΦs isolated from neonates (n=8) and adults (n=8) were pre-incubated with p38 MAPK inhibitor, SB203580, for 1h. BRSV (moi=1), PMA (1μg/ml) and ionomycin (0.5μg/ml), and LPS (1μg/ml) were added to designated wells. Cell culture supernatants were harvested at 24h and frozen at -80C until use. In general, neonatal or adult AMΦs did not secrete appreciable amounts of IL-1β protein following BRSV infection. Data are representative of 4 separate experiments.

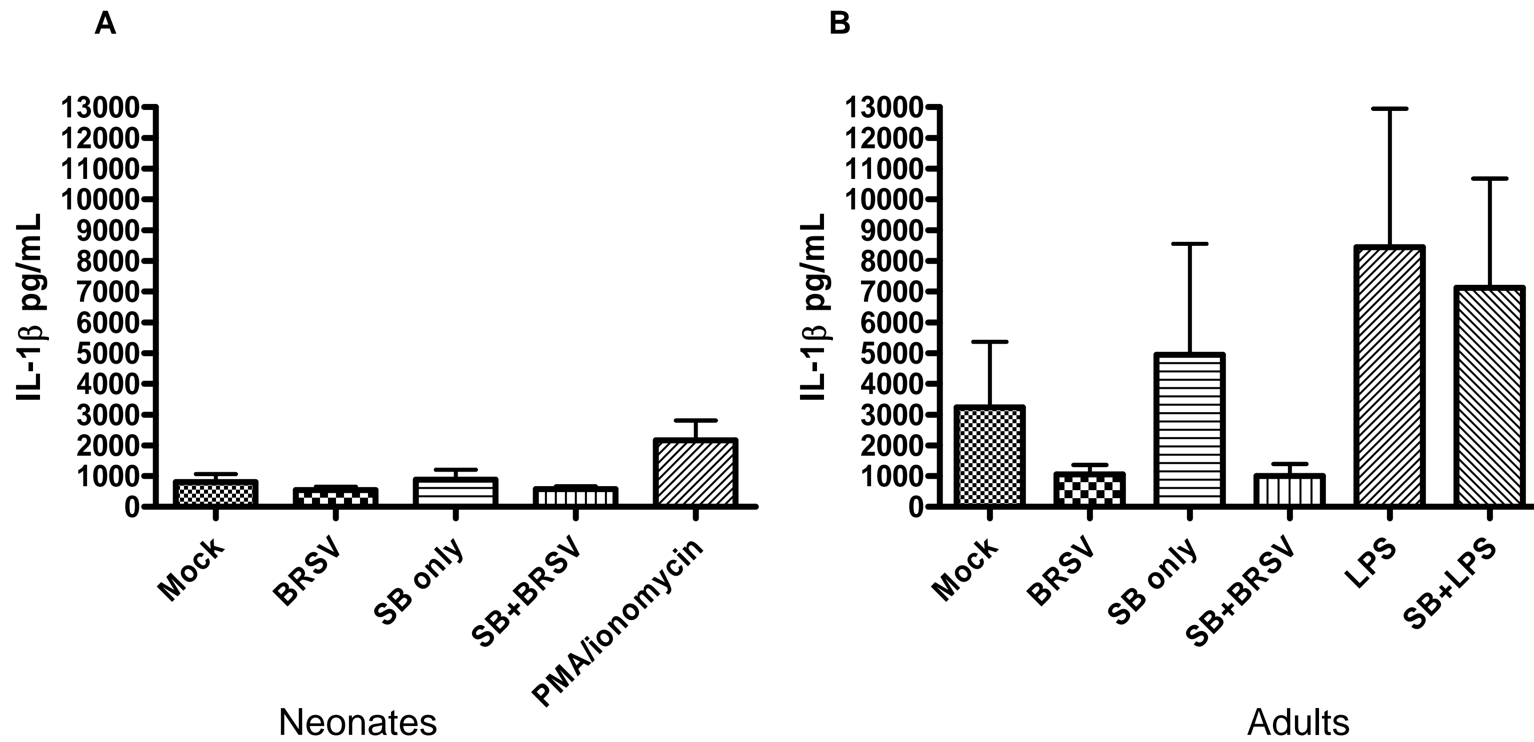

Figure 5. IL-1β protein secretion by ovine AMΦs following p38 MAPK inhibition and in vitro BRSV or mitogen stimulation (24h)

Figure 6: IL-1β protein secretion by ovine AMΦs following p38 MAPK inhibition and in vitro BRSV or mitogen stimulation. AMΦs isolated from neonates (n=8) and adults (n=8) were pre-incubated with p38 MAPK inhibitor, SB203580, for 1h. BRSV (moi=1), PMA (1μg/ml) and ionomycin (0.5μg/ml), and LPS (1μg/ml) were added to designated wells. Cell culture supernatants were harvested at 48h and frozen at -80C until use. In general, neonatal or adult AMΦs did not secrete appreciable amounts of IL-1β protein following BRSV infection. Data are representative of 4 separate experiments.

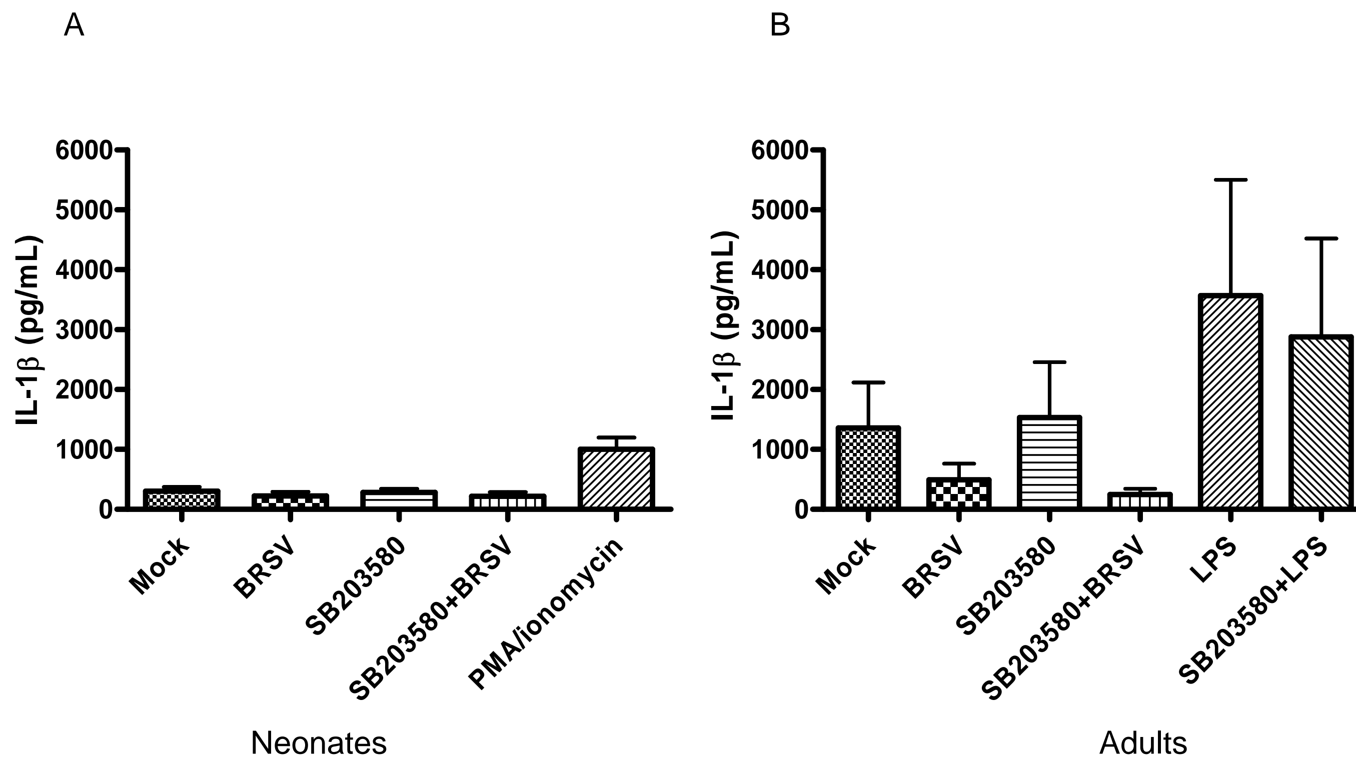

Figure 6. IL-1β protein secretion by ovine AMΦs following p38 MAPK inhibition and in vitro BRSV or mitogen stimulation (48h)

CHAPTER 3. GENERAL CONCLUSIONS

GENERAL DISCUSSION

RSV remains the leading cause of lower respiratory tract illness in infants worldwide. Nearly 70% of children are infected by the age of 1, and nearly 100% are infected by the age of 2, with high rates of reinfection (1, 2). This is likely a result of an ineffective immune response to RSV mounted in the neonate, leaving them susceptible to serious secondary infections in addition to RSV infection alone. Alveolar macrophages (AMΦs) are critical innate immune cells in the lung, as first-defenders against inhaled antigens (3). In addition to pathogen recognition and antigen presentation, cytokine production by AMΦs helps to shape adaptive immune responses in the lung. The studies described here explored the role of AMΦ during RSV infection in neonates compared to adults in terms of cytokine expression and secretion. Based on previous work, RSV infection induces a mixed cytokine response in AMΦs as determined by the upregulation of both pro- and anti-inflammatory cytokine genes in neonates (4). However, based on these current experiments, there appear to be functional differences in the ability of the neonatal AMΦ to secrete cytokine protein compared with adult AMΦs.

Objective 1. The production of IL-4 splice variant mRNA could potentially protect against IL-4 responses induced by RSV infection. As described previously, IL-4δ2 and IL-4δ3 are normally coexpressed with full length IL-4 and result from removal of either exon 2 or exon 3 from full length IL-4 mRNA (5, 6). IL-4 splice variants have been shown to be preferentially expressed in cattle that were protected following natural exposure to *Mycobacterium bovis* in a bovine tuberculosis model (7). This suggests a role for IL-4 splice

variants in the Th1-type immune response elicited by *M. bovis* (6, 7). In another study, human patients with chronic asthma had increased full-length IL-4 to IL-4δ2 ratios compared to healthy control subjects (8). Based on this information, we hypothesized that the ratio of full-length IL-4 to IL-4 splice variants expressed in AMΦs following BRSV infection would be similarly increased compared to mock-infected controls.

This set of experiments compared full-length IL-4 and IL-4 splice variant expression in response to BRSV infection. In vitro BRSV infection of sheep AMΦs induced the transcription of full-length IL-4. No IL-4 splice variant mRNA, however, was detected in any of the test samples. This could be due to the fact that IL-4δ2 and IL-4δ3 are expressed infrequently or intermittently in sheep AMΦs, regardless of infection state.

Objective 2. These experiments determined if the increases seen in IL-4 mRNA in RSV-infected AMΦs results in an increased level of secreted IL-4 protein, which could contribute to the overall Th2 environment seen during RSV infection in both neonates and adults (9-12). Increased IL-4 secretion from neonatal AMΦs could help in explaining more severe disease seen in infants compared with adults. Here, we measured IL-4 protein by sandwich ELISA. In our system, secreted IL-4 protein was not detected from adult or neonatal AMΦs following BRSV infection or mitogen stimulation. This suggests that AMΦs are not a significant source of IL-4 protein, and IL-4 production during RSV infection is likely from another cell type in the lung such as activated T cells or infiltrating monocytes.

Objective 3. After determining that AMΦs are not a likely source of IL-4 protein during RSV infection, we examined whether they produce another critical anti-inflammatory mediator, IL-10. IL-10 has been shown to inhibit Th1 cytokine production as well as

antigen-presenting cell functions (13, 14). In the lung, IL-10 is produced by alveolar macrophages in response to LPS stimulation and viral infections, including RSV (15-17). Increases in IL-10 secretion by BRSV-infected AMΦs could suppress Th1 responses and contribute to the overall Th2 cytokine bias in the lung. In this study, neonatal AMΦs did not secrete appreciable amounts of IL-10 in response to BRSV infection or mitogen stimulation. Adult AMΦs, however, produced IL-10 protein following BRSV infection, and stimulation with LPS or the combination of PMA and ionomycin. This suggests that IL-10 production may contribute to the decreased susceptibility of adults to RSV infection.

Objective 4. Because RSV pathogenesis is associated with a mixed immune response and the production of both immunomodulatory and proinflammatory mediators, AMΦ production of proinflammatory cytokines may contribute to the differences observed in the severity of disease observed in neonates compared to adults. Here, we assessed proinflammatory IL6, IL-8 and IL-1β cytokine expression in RSV-infected AMΦs from neonates and adults by real-time PCR with SYBR green. While no change was observed in IL-6 transcription in neonatal or adult AMΦs in response to BRSV infection, IL-8 and IL-1β expression were both increased. An interesting difference between neonatal and adult AMΦs was noted in the transcription of IL-1β following BRSV infection, with significantly higher expression from neonatal cells. Based on this observation, we hypothesized that the increases in IL-1β gene transcription will lead to increases in IL-1β protein secretion by AMΦs in response to RSV infection, with higher levels from neonatal AMΦs. Surprisingly, RSV infection did not induce IL-1β protein secretion in our system. This observation suggests a post-

transcriptional control mechanism employed by the virus which would limit the pro-inflammatory response.

The secretion of active IL-1β is a highly regulated event. IL-1β is first synthesized as an inactive precursor, pro-IL-1β, which must be specifically cleaved by caspase-1 for activation (18, 19). Caspase-1 itself is synthesized as inactive pro-caspase-1 in response to interferon regulatory factor-1 (IRF-1), and requires cleavage by caspase-8 for its own activation (18). Blocking any of these critical regulatory steps would prevent the effective secretion of IL-1β from BRSV-infected AMΦs, which could contribute to the disease susceptibility of neonates.

Objective 5. These experiments explored the role of p38 MAPK signaling in BRSV-infected AMΦs from neonates and adults. p38 has been shown to be required for cytokine gene expression in other models and cell types (17, 20-23), and here we describe its role in BRSV-infected AMΦs. Neonatal and adult AMΦs were pre-incubated with a specific chemical inhibitor of p38 MAPK, SB203580, for 1h prior to in vitro BRSV infection. Inhibition of p38 MAPK effectively reduced IL-8 and IL-1β gene transcription in response to BRSV-infection or LPS-stimulation. IL-10 protein secretion from adult AMΦs was also reduced in those cells pre-incubated with SB203580 prior to stimulation with BRSV or LPS. This observation, along with the fact that IL-10 message was not significantly different in SB203580-treated AMΦs compared to those pre-incubated with culture medium alone, suggests a role for p38 MAPK in addition to its known role of initiation of transcription. A cytoplasmic target of p38 MAPK, MAPK-activated protein kinase-2 (MAPKAP-2), functions to stabilize mRNA transcripts by activating AU-rich element-binding proteins.

These proteins bind to AU-rich regions in the 3'untranslated regions of mRNAs and enhance stability by preventing self-folding and degradation of the transcripts (24). By inhibiting p38 MAPK activation of MAPKAP-2, the decreased stability of mRNA transcripts could lead to the reduced amount of secreted protein. Based on our findings, manipulation of this pathway by RSV could lead to increased cytokine gene transcripts and protein in infected AMΦs.

RECOMMENDATIONS FOR FUTURE RESEARCH

The findings reported in this thesis only scratch the surface of intracellular signaling pathways utilized during RSV infection of AMΦs. We report an essential role for p38 MAPK signaling on both cytokine gene transcription as well as IL-10 protein secretion. p38 MAPK is one of three major MAPK enzymes, along with ERK and JNK. Different specific chemical inhibitors are available for blocking ERK and JNK activation, and could be used to explore their role in cytokine production by RSV-infected AMΦs. One study, using human AMΦs stimulated with LPS, implicated a role for ERK and JNK for IL-10 production (17) although this has yet to be described during RSV infection. It would also be interesting to focus on the upstream activators and downstream targets of each of the MAPK proteins. The effect of RSV on total protein levels and phosphorylation states of MAPK proteins and their regulators in AMΦs could help describe the mechanism responsible for cytokine expression and production in these cells. Further description of the mechanisms leading to differential regulation of cytokine production in neonatal and adult AMΦs during RSV infection could help explain the differences in disease susceptibility and severity seen in neonates.

Another interesting direction to take would be to further explore the regulation of IL-1β in the neonatal AMΦ. As described, IL-1β protein is formed as an inactive precursor,

pro-IL-1β, that is cleaved by caspase-1 prior to secretion. Caspase-1 itself is transcriptionally regulated by IRF-1, and requires cleavage by caspase-8 to become fully active (18). In a human epithelial cell line, RSV induced increased caspase-1 transcription and secretion following increased IRF-1 induction, although this has not been described for AMΦs (19). Examining the levels of IRF-1, caspase-1, and caspase-8 could provide important insight into the observed disconnect between IL-1β gene transcription and protein secretion from neonatal AMΦs infected with BRSV.

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