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Increased Production of Proinflammatory Cytokines following Infection with Porcine Reproductive and Respiratory Syndrome Virus and *Mycoplasma hyopneumoniae*

Roongroje Thanawongnuwech,[1]† Brad Thacker,[2]‡ Patrick Halbur,[2] and Eileen L. Thacker[1]*

Veterinary Microbiology and Preventive Medicine[1] and Veterinary Diagnostic and Production and Medicine,[2] Iowa State University, Ames, Iowa

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Induction of the proinflammatory cytokines interleukin-1 (IL-1) (α and β), IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor alpha (TNF-α) in pulmonary alveolar macrophages (PAMs) was assessed following experimental infection with porcine reproductive and respiratory syndrome virus (PRRSV) and/or *Mycoplasma hyopneumoniae* by using in vivo and in vitro models. The in vivo model consisted of pigs infected with PRRSV and/or *M. hyopneumoniae* and necropsied at 10, 28, or 42 days postinfection. Pigs infected with both pathogens had a greater percentage of macroscopic lung lesions, increased clinical disease, and slower viral clearance than pigs infected with either pathogen alone. The pigs infected with both PRRSV and *M. hyopneumoniae* had significantly increased levels of mRNA for many proinflammatory cytokines in PAMs collected by bronchoalveolar lavage (BAL) at all necropsy dates compared to those in uninfected control pigs. Increased levels of IL-1β, IL-8, IL-10, and TNF-α proteins in BAL fluid, as measured by enzyme-linked immunosorbent assay, confirmed the increased cytokine induction induced by the pathogens. An in vitro model consisted of *M. hyopneumoniae*-inoculated tracheal ring explants cultured with PRRSV-infected PAMs. PAMs were harvested at 6 or 15 h postinfection with either or both pathogens. The in vitro study detected increased IL-10 and IL-12 mRNA levels in PAMs infected with PRRSV at all time periods. In addition, IL-10 protein levels were significantly elevated in the culture supernatants in the presence of *M. hyopneumoniae*-inoculated tracheal ring explants. The increased production of proinflammatory cytokines in vivo and in vitro associated with concurrent *M. hyopneumoniae* and PRRSV infection may play a role in the increased rates of pneumonia associated with PRRSV infection. The increased levels of IL-10 may be a possible mechanism that PRRSV and *M. hyopneumoniae* use to exacerbate the severity and duration of pneumonia induced by PRRSV and modulate the respiratory immune response.

Porcine respiratory disease complex (PRDC) remains an important economic problem for swine producers worldwide. PRDC is characterized by slow growth, decreased feed efficiency, anorexia, fever, cough, and dyspnea in finishing pigs. PRDC is multifactorial, as several viral and bacterial pathogens are typically detected. Porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* are commonly isolated from pigs with clinical signs consistent with PRDC, and despite the use of intervention strategies that include vaccines and antibiotics, control of PRDC is inconsistent at best.

Proinflammatory cytokines are believed to play an important role in porcine respiratory disease by coordinating and activating the adaptive immune response, which enables the host to eliminate offending pathogens (14, 21, 33, 34). However, if cytokine levels become excessive, tissue damage and even death of the host can occur. Therefore, determination of both the presence and the quantity of proinflammatory cytokines can lead to an increased understanding of the pathogenesis of disease and the corresponding host's immune response.

Production of proinflammatory cytokines has been shown to be associated with the development of *M. hyopneumoniae*-induced pneumonia. Increased levels of interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α) in bronchoalveolar lavage (BAL) fluid have been reported in *M. hyopneumoniae*-infected pigs (1, 2, 30). Respiratory viruses also induce proinflammatory cytokine production, although cytokine levels and the clinical manifestations of pneumonia can vary by virus (33). For example, the levels of IL-1 and TNF-α were lower in BAL fluid from pigs infected with PRRSV than in BAL fluid from pigs infected with swine influenza virus (33). This finding may provide a possible mechanism for the prolonged persistence of PRRSV following infection (38).

Previously, we demonstrated (27) that the presence of *M. hyopneumoniae* significantly increased the severity and duration of pneumonia, consistent with PRRSV infection in pigs. The mechanism used by *M. hyopneumoniae* to enhance the viral pneumonia is unknown; however, the induction of proinflammatory cytokines by *M. hyopneumoniae* may play an important role. The cytokine response to pathogens in pigs has been poorly defined and has been limited by the lack of immunological and biological assays for porcine cytokines. The availability of reagents has increased dramatically in the past several years, and a number of studies have been performed to

* Corresponding author. Mailing address: 2118 Vet. Med. Bldg., Department of VMPM, Iowa State University, Ames, IA 50011. Phone: (515) 294-5097. Fax: (515) 294-8500. E-mail: ethacker@iastate.edu.

† Present address: Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, 10330 Thailand.

‡ Present address: Intervet Animal Health, Millsboro, Del.

identify the cyokines produced by swine macrophages and monocytes (10, 35).

The goals of the study reported here were to investigate the levels of mRNA for select proinflammatory cytokines to begin to obtain a further understanding of the pathogenesis of the disease induced by these problem pathogens. Previous studies have demonstrated that each pathogen induces the production of the proinflammatory cytokines in different ways. The purpose of this study was to attempt to begin to investigate the interaction between these two pathogens at the molecular level by assessing their impacts on proinflammatory cytokines individually and together by using both in vitro and in vivo models. The cytokines measured in pigs experimentally inoculated with PRRSV and/or *M. hyopneumoniae* and necropsied at 10, 28, and 42 days postinfection (dpi) included IL-1 (α and β), IL-6, IL-8, IL-10, IL-12, and TNF-α. The levels of the IL-1β, IL-8, IL-10, and TNF-α proteins were also measured. In addition, the same proinflammatory cytokines produced by pulmonary alveolar macrophages (PAMs) infected or not infected with PRRSV and incubated with tracheal explants with or without *M. hyopneumoniae* infection were measured at 6 and 15 h postinfection (hpi) by an in vitro assay. Both the in vivo and the in vitro models described in this study have previously proven useful in investigating the proinflammatory cytokine responses by PAMs to infection with PRRSV and/or *M. hyopneumoniae* (31, 32). In an earlier study that measured in vitro cytokine production by PAMs at 24 and 48 hpi in pigs infected with PRRSV and/or *M. hyopneumoniae*, the differences in various proinflammatory cytokine levels were observed at 24 hpi. However, by 48 hpi the viabilities of PRRSV-infected PAMs were low, making accurate measurement of cytokine levels difficult.

Infection with both pathogens significantly increased the levels of the majority of the cytokines in both the in vivo and the in vitro models, suggesting that inflammation is important for inducing the lung lesions observed following coinfection with these two pathogens. Specifically, we found enhanced production of IL-10, a cytokine associated with a T-helper 2 (Th2) type of immune response, which may provide an explanation for the persistence of PRRSV in the presence of *M. hyopneumoniae*. Interestingly, the levels of IL-12, a cytokine known to induce the production of gamma interferon (IFN-γ), remained elevated up to 42 dpi in the in vivo trial and throughout the in vitro sampling time period. *M. hyopneumoniae* infection both in vitro and in vivo appeared to stimulate the production of more inflammatory cyokines than PRRSV. However, cytokine levels were often further increased in the presence of both pathogens. The results of this study provide additional information on the temporal production of proinflammatory cytokines by the lungs in response to respiratory pathogens by using both in vivo and in vitro models. This information will aid in understanding the pathogenesis of *M. hyopneumoniae* and PRRSV and their effects on the respiratory immune system.

MATERIALS AND METHODS

Animals and experimental design. For the in vivo study, 70 crossbred pigs (landrace, large white, and duroc; age, 10 to 12 days) serologically negative for PRRSV and *M. hyopneumoniae* were obtained from a commercial herd. The pigs were randomly assigned to four treatment groups with stratification by their weight on arrival. The treatment groups consisted of an uninoculated negative control group, *M. hyopneumoniae*-inoculated pigs, PRRSV-inoculated pigs, and pigs inoculated with both *M. hyopneumoniae* and PRRSV. Pigs within each group were randomly assigned to one of three necropsy dates at 10, 28, and 42 dpi. An additional six pigs (age, 7 weeks) from the same herd were used as donors for the trachea explants and the PAMs used in the in vitro study. Pigs were provided commercial diets and water ad libitum throughout the study. All animal-related procedures were conducted in accordance with the guidelines of the Iowa State University Institutional Committee on Animal Care and Use.

Challenge inocula. For the in vivo study, pigs in the appropriate groups were inoculated intranasally with $10^{4.9}$ 50% tissue culture infective doses ($TCID_{50}$s) of a highly virulent PRRSV strain (ATCC VR-2385) per 5 ml and/or *M. hyopneumoniae* strain 232, a derivative of strain 11, per 10 ml (at 10^5 color-changing units/ml) on the same day at 6 weeks of age, as described previously (27).

M. hyopneumoniae strain 91-3 (5×10^8 color changing units/ml), a highly adherent strain originally cloned from strain 232, was used to inoculate the tracheal rings in the in vitro study (39). In the in vitro study, PAMs from the appropriate groups were inoculated at a multiplicity of infection of 0.01 with the same PRRSV isolate used in the in vitro study.

Clinical evaluation and pathological examination. The pigs were evaluated daily for a minimum of 15 min for signs of clinical disease, including appetite, cough, increased respiratory rate, dyspnea, or behavioral changes. Rectal temperatures and a clinical respiratory score were obtained daily from 0 to 10 dpi and every other day from 11 to 20 dpi, as described previously (12). Pigs from each group were necropsied at 10, 28, or 42 dpi, as described previously (27). Bronchial swabs were collected aseptically for the isolation of *M. hyopneumoniae* and other bacterial pathogens. Macroscopic lesions consistent with mycoplasmal pneumonia (dark red to purple consolidated areas) were sketched on a standard lung diagram, and the proportion of the lung surface with lesions was calculated from the diagram by using a Zeiss SEM-IPS image-analyzing system (24). Pneumonia consistent with PRRSV was characterized by parenchyma that was mottled tan and rubbery in consistency and failure to collapse and was scored by a previously developed system based on the approximate volume that each lobe contributes to the entire lung: the right cranial lobe, the right middle lobe, the cranial part of the left cranial lobe, and the caudal part of the left cranial lobe each contributes 10% of the total lung volume; the accessory lobe contributes 5%; and the right and left caudal lobes each contributes 27.5% (12). These scores were then used to determine the total lung lesion score on the basis of the relative contribution of each lobe.

BAL was performed to collect epithelial lining fluid and bronchoalveolar cells, primarily consisting of PAMs (more than 85%), as described previously (20). BAL fluid and cells were collected by flushing the lungs with phosphate-buffered saline (PBS) containing 75 U of penicillin per ml and 75 μg of streptomycin (both from Sigma, St. Louis, Mo.) per ml. The BAL fluid was centrifuged, and the pelleted cells were washed twice with PBS. The pellet, which primarily consisted of PAMs, was resuspended in complete medium consisting of RPMI 1640 (Sigma) containing 5% fetal bovine serum, 75 U of penicillin per ml, and 75 μg of streptomycin per ml. The cells were counted, and the viability was determined with trypan blue dye. PAMs were kept in RNAlater (Ambion, Austin, Tex.) until they were tested.

Experimental design of in vitro study with tracheal rings and PAMs. The tracheas were removed aseptically from the six additional pigs and submerged in sterile chilled PBS. The tracheas were washed, and transverse sections (thickness, approximately 0.5 cm) were made by making an incision between the tracheal rings. Each tracheal ring was placed into a 30-mm culture plate insert (Millipore, Bedford, Mass.) containing 3 ml of complete medium. PAMs (approximately 5×10^6 cells) were reconstituted in 3 ml of complete medium and placed in a six-well plate for 2 h at 37°C in 5% CO_2 prior to inoculation with PRRSV. PAMs were collected, and assays were performed at 6 and 15 hpi with PRRSV. All tissue culture media and supplements were confirmed to be endotoxin free by standard assays. The tracheal explants and PAMs were cultured overnight at 37°C in 5% CO_2, and experiments were performed on the next day.

The experimental design is summarized in Table 1 and consisted of the same treatments described previously (32). Cells and tracheal explants from each of the donor pigs were subjected to all treatments. *M. hyopneumoniae* was inoculated onto the tracheal rings of the appropriate groups 6 h prior to their addition to the PAMs. PAMs from the PRRSV-infected groups were inoculated with PRRSV 1 h prior to the addition of the tracheal rings. The tracheal ring inserts and their supernatants were transferred to the cultured PAMs, and the mixture was incubated at 37°C in 5% CO_2 for 6 or 15 hpi prior to harvesting of the PAMs with a cell scraper. The supernatant from the tracheal cultures was dripped through the insert over the PAMs so no cell-to-cell contact between the tracheal rings and PAMs occurred. PAMs harvested at 6 and 15 hpi were placed in

TABLE 1. Experimental design of in vitro experiment

Group	PAMs	PRRSV	Tracheal explant	*M. hyopneumoniae*
Control	+	−	+	−
M. hyopneumoniae	+	−	+	+
PRRSV	+	+	+	−
M. hyopneumoniae and PRRSV	+	+	+	+

RNAlater (Ambion), according to the directions of the manufacturer, and were stored at 4°C until they were assayed.

Total RNA isolation, RT-PCR, and densitometric quantification of RT-PCR products. Total RNA isolation, the primers used, and reverse transcription-PCR (RT-PCR) procedures were performed with the PAMs collected by BAL in the in vivo study and the cultured PAMs from the in vitro study, as described previously (31, 32). RT-PCRs for IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-12 (p40), TNF-α, and cyclophilin (a housekeeping gene) were performed with a Mastercycler instrument (Eppendorf, Westbury, N.Y.) and an Access RT-PCR system (Promega, Madison, Wis.). The RT-PCR product intensity as a percentage of the cyclophilin intensity was quantitated by AlphaEase spot densitometry (Alpha Innotech Corp., San Leandro, Calif.), as described previously (36).

Cytokine ELISA. The levels of the IL-1β, IL-8, IL-10, and TNF-α proteins in the BAL fluid and culture supernatants were measured by commercial enzyme-linked immunosorbent assays (ELISAs) specific for porcine cytokines (Cytoscreen; Biosource, Camarillo, Calif.). BAL fluid and culture supernatant samples were used undiluted. The cytokine concentrations in the samples were determined by comparing the concentrations obtained to the concentrations of recombinant standards run in parallel with each assay, according to the instructions of the manufacturer.

***M. hyopneumoniae* and PRRSV isolation and serology.** During the in vivo study, *M. hyopneumoniae* was isolated from the bronchial swab specimens obtained at necropsy, as described previously (24). PRRSV isolation and titration were performed with BAL fluid collected at necropsy and with the culture supernatant at 6 and 15 hpi by an established protocol (19, 27). Briefly, monolayers of MARC-145 cells were incubated with the samples, followed by staining with anti-PRRSV monoclonal antibody SDOW-17 (South Dakota State University, Brookings) and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin. A fluorescence microscope was used to visualize specific viral antigens.

Serum was obtained from all pigs upon arrival and at 10, 28, and 42 dpi. *M. hyopneumoniae* antibody titers were determined by ELISA, as described previously (6). Known positive and negative sera were included as controls in each plate. Readings more than 2 standard deviations above the mean value for the negative control were considered positive (optical density, 0.220). All sera were tested for PRRSV antibodies by a commercially available ELISA (HerdChek: PRRS; IDEXX Laboratories, Westbrook, Maine) by the procedures provided by the manufacturer. Samples were considered positive if the calculated sample-to-positive control ratio was 0.4 or greater.

Statistical analysis. Data were subjected to analysis of variance (ANOVA). If the *P* value from the ANOVA was less than or equal to 0.05, pairwise comparisons of the different treatment groups were performed by a least-significant-difference test at a rejection level of a *P* value <0.05.

RESULTS

Clinical disease and pathology. The severity of clinical disease and the pathology associated with *M. hyopneumoniae* and PRRSV infections were similar to those found in earlier studies (13, 27, 28). Rectal temperatures and clinical respiratory scores were significantly increased in both groups of PRRSV-inoculated pigs beginning at approximately 3 dpi (13; data not shown). Coughing was observed in all groups inoculated with *M. hyopneumoniae*. Macroscopic examination revealed that the percentages of pigs with pneumonia consistent with PRRSV infection at 10 dpi were similar in both groups inoculated with PRRSV (Table 2). At 28 and 42 dpi, the increases in the severity and the duration of the viral pneumonia caused by *M. hyopneumoniae* were evident compared those of the pneumonia in the group infected only with PRRSV. In the groups challenged with *M. hyopneumoniae*, a lower than normally expected proportion (1.06% ± 0.75%) of the lung was affected by pneumonia, consistent with mycoplasmal pneumonia, as the anticipated level of pneumonia induced in our experimental model was between 8 and 12% (29, 30). As observed in an earlier study (27), the presence of PRRSV increased the severity of the mycoplasmal pneumonia at 10 dpi and at 28 and 42 dpi. The increased severity of viral pneumonia consistent with PRRSV was not dependent on the severity of the mycoplasmal pneumonia, as pigs with minimal macroscopic lesions consistent with *M. hyopneumoniae* infection had increased viral pneumonia compared to the lungs of pigs infected only with PRRSV at 28 and 42 dpi.

In vivo mRNA expression of PAMs. The levels of mRNA expression from PAMs collected from BAL fluid are summarized in Table 3. Due to the number of cytokines assayed, only data for cytokines that showed significant differences in levels at each time point are shown in Table 3. Pigs infected with both *M. hyopneumoniae* and PRRSV exhibited significantly increased levels of expression of mRNA for IL-1β, IL-8, IL-10, IL-12, and TNF-α at 10 dpi ($P < 0.05$). Pigs infected with only PRRSV had significantly ($P < 0.05$) increased levels of mRNA for IL-10 and IL-12 compared to those for the control and the *M. hyopneumoniae*-infected pigs. At 28 dpi, the levels of

TABLE 2. Summary of macroscopic lung lesions from pigs necropsied at 10, 28, and 42 dpi with either *M. hyopneumoniae* or PRRSV

Source of pneumonia and dpi[a]	% of lung with visible lesions on necropsy at indicated dpi[a,b]			
	Control	*M. hyopneumoniae*[c]	PRRSV[d]	*M. hyopneumoniae* and PRRSV
M. hyopneumoniae				
10	0.02 ± 0.05A	0.67 ± 0.37A	0.0 ± 0.0A	1.87 ± 2.00B
28	0.0 ± 0.0A	1.06 ± 0.75A	0.16 ± 0.24A	7.49 ± 4.91B
42	0.17 ± 0.24A	0.95 ± 1.13A	0.10 ± 0.15A	3.80 ± 2.44B
PRRSV				
10	0.0 ± 0.0A	0.0 ± 0.0A	36.50 ± 9.25B	43.00 ± 15.15B
28	0.0 ± 0.0A	0.0 ± 0.0A	2.33 ± 1.03A	31.60 ± 7.09B
42	0.0 ± 0.0A	0.0 ± 0.0A	1.17 ± 1.47A	18.0 ± 9.86B

[a] dpi with PRRSV and/or *M. hyopneumoniae*.
[b] The values are group means ± standard deviations. Within each row, values followed by different letters (A, B, or C) are significantly different ($P < 0.05$).
[c] As determined with lesion sketches and by image analysis.
[d] As estimated by visual observation.

TABLE 3. Average mRNA levels from PAMs from in vivo study of pigs at 10, 28, and 42 dpi with PRRSV and/or *M. hyopneumoniae*

dpi and cytokine	Avg mRNA level (%)[a]			
	Control	*M. hyo*	PRRSV	PRRSV and *M. hyo*
10 dpi				
IL-1β	27.54 ± 17.34A	55.50 ± 30.07B,C	39.66 ± 10.88A,B	67.42 ± 20.56C
IL-8	48.60 ± 38.89A	56.49 ± 29.31A	30.76 ± 35.87A	101.17 ± 40.49B
IL-10	0.0 ± 0.0A	0.0 ± 0.0A	20.12 ± 17.55B	34.99 ± 23.40B
IL-12	5.77 ± 14.13A	9.63 ± 10.58A	28.89 ± 16.99B	47.52 ± 15.58C
TNF-α	47.94 ± 22.54A,B	30.17 ± 20.87A	41.24 ± 22.79A	65.93 ± 10.43B
28 dpi				
IL-1α	27.60 ± 14.55A	100.11 ± 22.99B	79.51 ± 7.70B	90.46 ± 21.96B
IL-1β	41.16 ± 9.14A	91.92 ± 28.75B,C	76.52 ± 10.07B	102.59 ± 12.90C
IL-6	0.0 ± 0.0A	21.56 ± 11.45B	22.46 ± 8.21B	29.11 ± 16.31B
IL-8	18.80 ± 10.37A	57.79 ± 22.88B	28.95 ± 11.35A	63.05 ± 8.57B
IL-10	0.0 ± 0.0A	46.93 ± 16.81B,C	30.20 ± 14.34B	58.59 ± 20.50C
IL-12	9.56 ± 10.93A	41.80 ± 15.82B,C	29.30 ± 10.20B	55.95 ± 18.94C
TNF-α	30.16 ± 7.53A	86.95 ± 13.97B	86.70 ± 8.80B	89.69 ± 19.35B
42 dpi				
IL-8	34.70 ± 19.27A	38.75 ± 9.35A	38.88 ± 35.73A	68.62 ± 16.05B
IL-10	35.44 ± 12.39A	22.05 ± 22.27A	71.54 ± 40.33B	50.27 ± 10.20A,B
IL-12	21.64 ± 19.37A	36.37 ± 8.35A,B	47.02 ± 13.08B,C	59.65 ± 20.18C

[a] The mRNA levels are presented as the ratio of cytokine RT-PCR product band intensity cyclophilin RT-PCR product band intensity. Data are shown only for cytokines with the significant differences between inoculation groups. Values in rows with different letters (A, B, or C) are significantly different ($P < 0.001$). *M. hyo*, *M. hyopneumoniae.*

mRNA for all proinflammatory cytokines measured except IL-8 were significantly increased in pigs inoculated with either or both pathogens. IL-8 mRNA levels were no longer elevated in the pigs infected only with PRRSV. The levels of mRNA for IL-1β, IL-10, and IL-12 were significantly higher in both groups of pigs infected with *M. hyopneumoniae* than in the pigs infected only with PRRSV. No significant difference in the levels of IL-1α, IL-6, and TNF-α mRNA expression between the infected groups was observed at 28 dpi. By 42 dpi, the levels of mRNA for IL-8 and IL-12 remained elevated in the group of pigs inoculated with both pathogens. Interestingly, the levels of mRNA for IL-10 and IL-12 remained increased in the group infected only with PRRSV. Otherwise, the levels of mRNA for all other cytokines had returned to those in the control pigs. The background levels of all cytokines in all groups appeared to be increased at 42 dpi, as shown in Table 3. Since all assays were performed at the same time, it is unknown if this is a normal occurrence associated with the maturation of pigs.

Effect of in vitro infection with *M. hyoneumoniae* and PRRSV on cytokine RT-PCR products. PAMs harvested from BAL fluid yielded more than 90% macrophages with 98% viability following collection and centrifugation. At 6 hpi, PAMs from the group infected with both *M. hyopneumoniae* and PRRSV expressed significantly higher levels of mRNA for IL-1α, IL-1β, IL-12, and TNF-α than the noninfected control group (Table 4). Only data for the cytokines with levels that were significantly different between groups are shown in Table 4. The PAMs infected with only PRRSV had significantly increased levels of mRNA for IL-1α and TNF-α. No significant differences in the cytokine levels of uninfected PAMs incubated with *M. hyopneumoniae*-inoculated tracheas compared with those in the uninfected control group were observed (data

TABLE 4. Relative mRNA levels from PAMs in vitro at 6 and 15 hpi with PRRSV

hpi and cytokine	Relative mRNA level[a]			
	Control	*M. hyo*	PRRSV	PRRSV and *M. hyo*
6 hpi				
IL-1α	77.72 ± 16.34A	101.93 ± 25.08A,B	119.55 ± 45.87B	120.50 ± 45.60B
IL-1β	105.11 ± 17.77A	121.86 ± 17.27A	121.89 ± 63.2A	170.88 ± 43.65B
IL-12	22.73 ± 18.94A	31.28 ± 10.66A,B	12.20 ± 19.57A	49.87 ± 22.72B
TNF-α	96.18 ± 12.19A	130.16 ± 40.56A,B	154.56 ± 32.10C	136.94 ± 28.23B
15 hpi				
IL-1β	95.59 ± 27.00A	136.60 ± 29.28B	67.47 ± 14.54A	87.20 ± 25.79A
IL-8	77.40 ± 26.18A	131.17 ± 19.14B	106.08 ± 39.66A,B	100.90 ± 27.80A,B
IL-12	0.0 ± 0.0A	17.11 ± 19.62B	0.0 ± 0.0A	8.09 ± 19.82B
TNF-α	46.53 ± 20.59A	87.36 ± 20.04B,C	64.96 ± 9.39A,B	93.87 ± 29.48C

[a] The mRNA levels are presented as the ratio of cytokine/cyclophilin RT-PCR product band intensity. Data are shown only for cytokines with significant differences between inoculation groups. Values in rows with different letters (A, B, or C) are significantly different ($P < 0.001$). *M. hyo*, *M. hyopneumoniae.*

TABLE 5. Cytokine concentrations determined from BAL fluid from pigs experimentally infected with *M. hyopneumoniae* and/or PRRSV in vivo

dpi and cytokines	Cytokine concn (pg/ml)[a]			
	Control	*M. hyo*	PRRSV	PRRSV and *M. hyo*
10 dpi				
IL-1β	85.50 ± 84.60A	149.67 ± 44.51A,B	71.00 ± 21.41A	204.50 ± 170.80B
IL-8	106.67 ± 0.12	682.50 ± 1090.10	740.83 ± 935.24	1112.50 ± 9630.55
IL-10	2.92 ± 2.46	4.86 ± 6.02	5.95 ± 3.08	5.35 ± 3.94
TNF-α	18.50 ± 3.56A	21.67 ± 4.32A	25.50 ± 6.03A	50.00 ± 31.15B
28 dpi				
IL-1β	120.08 ± 53.93A	224.00 ± 209.52A	101.50 ± 68.57A	734.88 ± 469.90B
IL-8	19.17 ± 13.26A	935.83 ± 1153.90B	34.33 ± 25.34A	3482.80 ± 668.14C
IL-10	2.62 ± 1.17A	2.77 ± 2.15A	3.35 ± 0.96A	21.96 ± 15.42B
TNF-α	51.33 ± 9.69A	45.38 ± 10.09A	43.83 ± 8.51A	85.29 ± 51.22B
42 dpi				
IL-1β	48.00 ± 52.25A	82.38 ± 33.13A	106.20 ± 118.66A	321.08 ± 232.00B
IL-8	17.50 ± 7.71A	106.00 ± 60.86A,B	22.50 ± 24.60A	988.17 ± 1381.90B
IL-10	1.07 ± 1.28	0.48 ± 1.08	0.98 ± 1.12	2.97 ± 4.82
TNF-α	23.36 ± 10.09	27.29 ± 10.14	29.91 ± 10.75	25.50 ± 16.70

[a] Cytokine concentrations were measured by a commercial ELISA. Values in rows with different letters (A, B, or C) are significantly different ($P < 0.001$). *M. hyo*, *M. hyopneumoniae.*

not shown). At 15 hpi, noninfected PAMs incubated with *M. hyopneumoniae*-inoculated tracheal rings had significantly increased levels of mRNA for IL-1β, IL-8, IL-12, and TNF-α. At 15 hpi the levels of mRNA for IL-12 and TNF-α in the PAMs infected with both pathogens had declined compared to the levels observed at 6 hpi, but they remained significantly greater than those in the control group. No other differences in the levels of mRNA for other cytokines were observed in any treatment group at 15 hpi.

Soluble cytokine levels following *M. hyopneumoniae* and PRRSV inoculation in vivo and in vitro. The results obtained for the levels of the four cytokine proteins are summarized in Tables 5 and 6. At 10 dpi, IL-1β and TNF-α protein levels were increased in the BAL fluid from pigs infected with both *M. hyopneumoniae* and PRRSV. IL-8 protein levels were increased in both of the *M. hyopneumoniae*-infected groups at 28 dpi, with pigs coinfected with PRRSV having significantly higher levels than pigs infected with *M. hyopneumoniae* alone. The levels of the IL-8 protein in BAL fluid were very high and may have been beyond the operating range of the assay, making statistical differences difficult to interpret. Pigs infected with both pathogens also had significantly increased levels of IL-10 and TNF-α proteins in their BAL fluid at that time. By 42 dpi, only IL-1β and IL-8 protein levels were increased in the BAL fluid of the group infected with both pathogens.

The in vitro assay found significantly increased levels of IL-10 protein in the supernatants of *M. hyopneumoniae*-infected tracheal ring cultures with or without PRRSV at 6 hpi. IL-8 protein levels were significantly increased in the supernatants of the group inoculated only with *M. hyopneumoniae*. There were no changes in the cytokine protein levels in the PAMs infected with only PRRSV at either 6 or 15 hpi. At 15 hpi, the levels of proteins of all four cytokines were significantly increased in the supernatants of the group infected with *M. hyopneumoniae*.

PRRSV and *M. hyopneumoniae* isolation and serology. To evaluate the relationship of the cytokine levels to viral clearance, PRRSV was isolated from the BAL fluid of infected pigs.

TABLE 6. Cytokine concentrations determined from supernatants of cocultured tracheal rings and PAMs at 6 and 15 hpi with PRRSV in vitro

hpi and cytokine	Cytokine concn (pg/ml)[a]			
	Control	*M. hyo*	PRRSV	PRRSV and *M. hyo*
6 hpi				
IL-1β	61.00 ± 50.19	104.33 ± 50.58	6.17 ± 10.68	37.00 ± 64.09
IL-8	1.18 ± 0.12A	1.57 ± 0.52B	0.95 ± 0.21A	1.28 ± 0.33A,B
IL-10	0.125 ± 0.0A	11.69 ± 4.54B	0.33 ± 0.36A	18.36 ± 10.47B
TNF-α	24.36 ± 3.79	29.08 ± 9.61	17.42 ± 3.56	26.17 ± 17.17
15 hpi				
IL-1β	49.33 ± 18.09A	396.83 ± 337.86B	76.00 ± 34.37A	359.33 ± 197.05A,B
IL-8	3.00 ± 0.82A	13.05 ± 6.35B	2.72 ± 0.59A	10.79 ± 8.74A,B
IL-10	1.69 ± 1.56A	18.56 ± 2.86B	1.17 ± 1.80A	18.45 ± 16.00B
TNF-α	9.78 ± 5.01A,B	14.50 ± 7.95B	7.14 ± 6.13A	12.28 ± 5.09B

[a] Cytokine concentrations were measured by a commercial ELISA. Values in rows with different letters (A, B, or C) are significantly different ($P < 0.001$). *M. hyo*, *M. hyopneumoniae.*

In addition, the levels of virus in the PAM culture supernatants were determined by titration. Virus was isolated from all pigs inoculated with PRRSV at 10 dpi. At 28 dpi, 33% (two of six) of pigs infected only with PRRSV remained positive for virus, whereas 80% (four of five) of the pigs inoculated with both pathogens remained positive for virus. No virus was isolated from the BAL fluid of any pig at 42 dpi. *M. hyopneumoniae* was isolated from all *M. hyopneumoniae*-inoculated pigs at all necropsy times. No *M. hyopneumoniae* isolates were was found in either the control group or the PRRSV-inoculated group at any necropsy date.

In vitro, although no significant differences were detected between the treatment groups, at 6 and 15 hpi, PAMs in the cultures inoculated only with PRRSV tended to yield higher viral titers ($10^{2.37} \pm 10^{0.24}$ and $10^{3.12} \pm 10^{0.27}$ $TCID_{50}$s/ml, respectively) than PAMs in the group inoculated with both pathogens ($10^{2.21} \pm 10^{0.19}$ and $10^{2.75} \pm 10^{0.43}$ $TCID_{50}$s/ml, respectively).

All pigs inoculated with PRRSV developed serum antibodies to PRRSV by 10 dpi and remained seropositive for the remainder of the trial. Only two pigs from either *M. hyopneumoniae*-infected group seroconverted to *M. hyopneumoniae* antibody positivity by 42 dpi.

DISCUSSION

Induction of an innate immune response is important for the control and elimination of invading pathogens. Proinflammatory cytokines produced by macrophages have a broad spectrum of effects that contribute to the host's defense against pathogens. However, while the presence of proinflammatory cytokines can be beneficial in the control of pathogens, significant tissue damage can occur if their levels become excessive. In addition, many cytokines produced by macrophages, such as IL-10 and IL-12, are associated with the induction and implementation of either Th1 or Th2 types of immune responses. Development of a Th1 type of immune response can be crucial for host resistance and the control and elimination of intracellular pathogens such as viruses. The severity of respiratory disease associated with bacterial or viral infections in murine models has been shown to be influenced by these cytokine responses (5, 15).

The study reported here investigated the induction of a number of proinflammatory cyokines produced by macrophages in response to infection with PRRSV and/or *M. hyopneumoniae*, two common swine respiratory pathogens, by using both in vitro and in vivo models. These models enabled us to evaluate the effects of these pathogens in the respiratory tract in vivo, while the in vitro model enabled us to assess more directly the impacts of the pathogens on PAMs independently of other cells of the respiratory immune system. Other cell types, including epithelial cells, were present in the tracheal explants. Accordingly, future studies to assess the direct impact of *M. hyopneumoniae* on epithelial cells will provide further independent analysis of that specific interaction that was not assessed in this study. Together the results of this study suggest that these two pathogens have a powerful effect on cytokine production, which may play a role in the induction of disease and the persistence of the organisms in the host.

Previous studies have demonstrated that infection with *M. hyopneumoniae* induces a number of proinflammatory cytokines in vivo (1, 2, 30). The results of the study presented here confirm that *M. hyopneumoniae* appears to induce greater levels of the proinflammatory cytokines than PRRSV. In contrast, an earlier in vivo study showed that PRRSV did not induce proinflammatory cytokine production as effectively as swine influenza virus (34). PRRSV infection has been reported to induce minimal TNF-α at 10 dpi (33), which was confirmed in this study. This finding is interesting, as the maximal pathology associated with PRRSV infection occurs at this time. It has been hypothesized that PRRSV may inhibit TNF-α production by either unknown inhibitory factors or posttranscriptional control, as TNF-α has been shown to have an antiviral effect (16). In contrast, by 28 dpi, all groups of pigs infected with PRRSV and/or *M. hyopneumoniae* had significantly increased TNF-α levels, which may have been associated with the clearance of PRRSV from the respiratory tract.

IL-1β is known to induce IL-6 production (40). In previous studies of pneumonia and disease induced by *Actinobacillus pleuropneumoniae*, the appearance of IL-1, TNF-α, and IL-6 coincided with the onset of clinical respiratory disease and increased body temperature (11, 14). It is interesting that IL-6 levels were not elevated in the PRRSV-infected group at 10 dpi, when maximal pneumonia and clinical disease, including fever, occurred, but were increased at 28 dpi, when minimal pneumonia or clinical symptoms were present. It has previously been demonstrated that *M. hyopneumoniae* induces the production of IL-6, although at 42 dpi, the IL-6 levels in both *M. hyopneumoniae*-infected groups had returned to the same level as that in the negative control group, even though pneumonia remained (2). It is difficult to interpret the significance of either IL-1-β or IL-6 in this study.

IL-8 is a neutrophil chemotactic factor produced by both macrophages and epithelial cells (17). Increased levels of IL-8 mRNA were found in the pigs infected with both pathogens at all three necropsy times. PRRSV infection alone appeared to have a minimal impact on IL-8. The soluble IL-8 levels in BAL fluid detected by ELISA were extremely high and there was considerable variability in the assay results, so the data should be interpreted with caution. Despite the significant increase in IL-8 production in conjunction with *M. hyopneumonaie* infection observed in this study, large numbers of neutrophils are not associated with mycoplasmal pneumonia. *M. hyopneumoniae* has been reported to have a suppressive effect on neutrophil function (3, 8). This suppression of neutrophil function may contribute to the exacerbation of secondary bacterial infections associated with *M. hyopneumoniae* infection. However, IL-8 has also been reported to be chemotactic for T cells, which may contribute to the lymphocyte proliferation associated with mycoplasmal pneumonia (9).

IL-10, which is produced by Th2-type T cells and activated macrophages, is a potent inhibitor of macrophage function and is a potent regulatory cytokine that decreases inflammatory responses and T-cell stimulation (4). Constitutive production of IL-10 by bronchial epithelial cells in the lung may help modulate the inflammatory response to environmental irritants by suppressing macrophage function (7). In this study, the two pathogens increased the level of IL-10 production dramatically. One possible outcome that could result from the increased IL-10 levels associated with PRRSV and that could

possibly be enhanced by *M. hyopneumoniae* infection is the potential shift of the host immune response away from a Th1-type response, which would potentially be more effective in controlling PRRSV, toward a predominantly Th2-type response. A number of viruses, such as human respiratory syncytial virus and murine cytomegalovirus, weaken the host immune response by inducing IL-10 production (22, 23). A possible mechanism by which IL-10 may increase the severity of viral pneumonia is by the impact of IL-10 on macrophages. PRRSV infection has been demonstrated to induce apoptosis in bystander PAMs (26). However, IL-10 has been demonstrated to protect against apoptosis (25). The enhanced production of IL-10 by *M. hyopneumoniae* may inhibit the apoptosis of PAMs in response to PRRSV infection, thus increasing the number of macrophages susceptible to infection by the virus. In this model, PRRSV persists longer in the respiratory tract of pigs infected with both pathogens.

IL-12 induces the production of IFN and is associated with a Th1 type of cytokine profile and the production of IFN-γ. The biologically active form of IL-12 is a heterodimeric cytokine composed of two disulfide-linked protein subunits. Macrophages and dendritic cells produce the subunits that form the active subunit IL12p70, made up of subunit IL12p35 and IL12p40 molecules. Other cell types, including respiratory epithelial cells, produce only the IL12p40 subunit, which often forms a homodimer that can bind to the IL-12 receptor, blocking binding and activation by the IL12p70 molecule (37). In this study we measured the level of only the IL12p40 molecule and not that of IL12p35. Thus, while IL-12 levels may appear to be elevated, if only IL12p40 is produced, the formation of IL12p80 could occur, which would potentially delay the production of IFN-γ, as has been reported from a study that used systemic lymphocytes and an enzyme-linked immunospot assay (18). This may be an important mechanism for viral persistence; however, we did not measure IFN-γ levels in this study.

The in vitro assay system enabled us to investigate the cyokines produced by PRRSV-infected PAMs without the impact of the entire respiratory immune system. The results of this study confirm that the colonization of ciliated epithelial cells by *M. hyopneumoniae* may influence the types of cytokines produced by PAMs. Our study did not assess the effects of *M. hyopneumoniae* on tracheal epithelial cells. It should be noted that various cells in the tracheal rings could produce cytokines. In addition, chemokines may have produced some of the cytokines measured in this study and may be important for the induction of inflammation and an immune response.

Similar to the in vivo study, when *M. hyopneumoniae*-inoculated tracheas were incubated with PRRSV-infected PAMs, they induced proinflammatory cytokines at levels higher than the levels detected in tracheas infected with either agent alone. In the in vitro model, the levels of mRNA for TNF-α were significantly increased in both PRRSV-infected groups at 6 and 15 hpi, and the soluble levels of TNF-α were also increased at 15 hpi in both groups infected with *M. hyopneumoniae*. The presence of increased levels of TNF-α mRNA in PAMs infected with PRRSV in association with low levels of soluble TNF-α in the culture supernatant and BAL fluid either may have been due to the short half-life of TNF-α in culture or may suggest a posttranslational impact of PRRSV on TNF-α production. This could be an explanation for the low levels of TNF-α observed during PRRSV infection (33).

Other differences in the levels of production (compared to those measured in vivo) of cytokines produced in response to infection of the tracheal implants and/or PAMs were observed. The increase in IL-1α and IL-1β levels was less consistent in vitro, and no increase in IL-6 levels was observed any time point, including 24 hpi, in the earlier study (32). However, IL-8 levels were increased in vitro in noninfected PAMs cultured with *M. hyopneumoniae*-inoculated tracheas at 15 hpi, and increased levels of IL-8 protein were present in the supernatant at 6 and 15 hpi. These results suggest that the production of IL-8 may be an important mechanism associated with *M. hyopneumoniae*. PAMs infected with only PRRSV exhibited no increase in IL-8 levels.

Interestingly, no increase in IL-10 mRNA levels in PAMs was found in vitro either in this study or in a previous study (32). It may be that the source of IL-10 is not from PAMs and that *M. hyopneumoniae*-infected tracheal epithelial cells may have been the source of the IL-10 protein detected in the cultured medium. Normal human bronchial epithelial cells have been found to produce IL-10; however, less is known about the response of swine epithelial cells (7). IL-12 mRNA levels were upregulated in both PRRSV-infected and noninfected PAMs incubated with the *M. hyopneumoniae*-infected trachea. The previous in vitro study did not detect increased IL-12 levels in any of the treatment groups at 24 hpi (32). Since we measured only the subunit IL12p40 molecule, it is possible that *M. hyopneumoniae* induces macrophages and, possibly, epithelial cells to produce IL12p40. Further investigations should assess the specific form of IL-12 produced in response to infection with PRRSV and *M. hyopneumoniae*. Although IL-12 was produced both in vitro and in vivo in this study, the increased levels of production of IL-10 may have suppressed the formation of both IL12p40 and IL12p35 gene upregulation at the transcriptional level (4).

The specific mechanisms, cellular or subcellular, by which *M. hyopneumoniae* potentiates PRRSV-induced pneumonia remain unidentified. Using in vitro and in vivo models, we investigated the role played by proinflammatory cytokines produced in response to each of the pathogens alone or together. Both models confirmed our earlier findings, which documented that *M. hyopneumoniae* induces significant levels of proinflammatory cytokines. Further in vitro and in vivo studies that would include blocking of the effects of these cytokines as well as measurement of the impacts of the pathogens at the transcriptional levels will assist in providing a further understanding of how these two pathogens continue to induce disease and persist in pigs.

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