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Sonicated Protein Fractions of *Mycoplasma hyopneumoniae* Induce Inflammatory Responses and Differential Gene Expression in a Murine Alveolar Macrophage Cell Line

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Mycoplasma hyopneumoniae is known to cause porcine enzootic pneumonia (EP), an important disease in swine production. The objective of this study was to examine the effects of sonicated protein fractions of *M. hyopneumoniae* on inflammatory response and gene expression in the murine alveolar macrophage MH-S cell line. The effects of sonicated protein fractions and intact *M. hyopneumoniae* on the gene expression of cytokines and iNOS were assessed using RT-PCR. The Annealing Control Primer (ACP)-based PCR method was used to screen differentially expressed genes. Increased transcription of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, COX-2, and iNOS mRNA was observed after exposure to the supernatant (SPT), precipitant (PPT), and intact *M. hyopneumoniae* protein. A time-dependent analysis of the mRNA expression revealed an upregulation after 4 h for IL-6 and iNOS and after 12 h for IL-1β and TNF-α, for both SPT and PPT; the fold change in COX-2 expression was less. A dose- and time-dependent correlation was observed in nitrite (NO) production for both protein fractions; however, there was no significant difference between the effects of the two protein fractions. In a differential gene analysis, PCR revealed differential expression for nine gene bands after 3 h of stimulation — only one gene was downregulated, while the remaining eight were upregulated. The results of this study provide insights that help improve our understanding of the mechanisms underlying the pathogenesis of and macrophage defenses against *M. hyopneumoniae* assault, and suggest targets for future studies on therapeutic interventions for *M. hyopneumoniae* infections.

Keywords: *M. hyopneumoniae*, sonicated protein fraction, cytokine, nitric oxide

Introduction

Porcine enzootic pneumonia (EP) is a widespread disease in pigs. It is endemic to many countries in the world and has been recognized as a major problem in swine production for almost a century, infecting a large number of pigs every year and causing huge economic losses to swine farms [11, 22]. *Mycoplasma hyopneumoniae* is the causative agent for EP, which causes a mild but chronic pneumonia and is characterized by low mortality and high morbidity [7, 12]. The disease affects the immune response system of the host, thus making the host vulnerable to infections by opportunistic pathogens like *Pasteurella multocida* [2] and porcine reproductive and respiratory syndrome virus (PRRSV) [25]. These bacteria, along with PRRSV and other agents, are also known to contribute to the porcine

respiratory disease complex.

Previous attempts at controlling *M. hyopneumoniae* infections using a commercially available vaccine succeeded in significantly reducing the clinical symptoms and lesions in the lungs of pigs; however, efforts to control the transmission of the disease have only seen limited success [13]. Vaccination alone is unlikely to be sufficient for preventing infections in pig herds.

M. hyopneumoniae has been also described to activate macrophages and monocytes to produce pro-inflammatory cytokines, including interleukin (IL)-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-18, and tumor necrosis factor-α (TNF-α) [1, 2, 4, 15, 21, 26]. Inflammatory cytokines enhance the efficiency of the immune system by increasing antigen presenting cell function to mobilize the immune response against the incoming infections [15], which is useful in understanding innate immunity against pathogens. However, the mechanism by which *M. hyopneumoniae* infection results in an induction of pro-inflammatory cytokine and nitrite (NO) production is not understood. The study of host–pathogen interactions has been stated to facilitate the understanding of host defense mechanisms against specific pathogens, which do have practical applications. Furthermore, it has been reported that an insight into the macrophage activation mechanisms and the bacterial components that initiate immune response is crucial for designing vaccines and cytokine therapies targeting the innate immune response system. In this regard, in the current study, we aimed first to examine the effects of sonicated protein fractions of *M. hyopneumoniae* on the induction of pro-inflammatory cytokines and nitric oxide production and, second, to identify differentially expressed genes in the *M. hyopneumoniae*-stimulated murine alveolar macrophage MH-S cell line.

Materials and Methods

Growth Conditions

The pathogenic strain of *M. hyopneumoniae* (ATCC 25934) was cultured in Friis medium containing 5% horse serum, 7.5% porcine serum, 3.8% freshly prepared yeast extract, 0.25% glucose, and 0.05% thallium acetate, at 37°C for 3 days, and harvested by centrifugation at 15,000 ×*g* for 30 min at 4°C. The pellets were washed three times in phosphate-buffered saline (PBS) and dispersed using a 27-gauge needle. The resulting suspension was used for determining the effect of intact *M. hyopneumoniae.* As measured using the bicinchoninic acid (BCA) method (Pierce), 0.3 mg/ml of protein corresponded to 10^{11} color changing units (CCU)/ml. Serial dilutions of solutions in tubes containing the Friis medium were used to determine the CCU as previously described [19].

Preparation of *M. hyopneumoniae* Protein Fractions

A suspension of *M. hyopneumoniae* in PBS was centrifuged at 2,000 ×*g*, and the pellet was resuspended in 2 ml of 2 M glycerol and incubated at room temperature for 10 min. The suspension was then homogenized in 2 ml of warm water using a 30-gauge needle and centrifuged at 30,000 ×*g* for 20 min. The supernatant was collected and the pelleted cytoplasmic protein was suspended in PBS. Finally, the protein quantity was measured using the BCA assay and the final protein concentration in the suspensions was adjusted to 0.2 mg/ml.

Cell Culture

Cells of the alveolar macrophage MH-S cell line (KCLB 40071, Korean Cell Line Bank, Seoul, South Korea) were cultured in RPMI supplemented with 10% fetal bovine serum, and 100 IU/ml penicillin–100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified air incubator.

Real-Time PCR

The MH-S cells (2×10^5 cells/ml) were treated with the protein for 24 h, and total RNA was extracted from the cells using Trizol reagent (Invitrogen, CA, USA). The isolated total RNA was incubated with oligo(dT) primers, and reverse transcription was performed using a thermal cycler (Mycycler, Bio-Rad). The PCR primers used in this study were (S) CAGGATGAGGACATG AGCACC, (AS) CTC-TGCAGACTCAAACTCCAC for IL-1β; (S) GTACTCCAGAAGACCAGAGG, (AS) TGCTGGTGACAACCA CGGCC for IL-6; (S) TTAACCTACGCGCTGAGTTG, (AS) CCT GTAGCCCACGRCGRAGC for TNF-α; and (S) ATGCTCCTGCTT GAGTATGT, (AS) GGAGGAAGAGGATGCGGCAGT for β-actin (as a housekeeping gene). The PCR for IL-1β, IL-6, TNF-α, and β-actin was performed with 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec, using a thermal cycler (Mycycler) and an AccuPower PCR Premix (BioNEER, Tageon, South Korea) according to the manufacturer's protocol. The RT-PCR products were separated on a 1% TAE agarose gel, stained with ethidium bromide, and photographed using Eagle-Eye (Stratagene). RT-PCR product intensity for the cytokines (as a percentage of β-actin intensity) was quantified using Eagle-Eye spot densitometry software.

Nitrite (NO) Assay

The NO level was measured by removing 100 μl of the cell-free culture medium and placing into a 96-well flat-bottom plate. One hundred microliters of Griess reagent was added into each well and incubated for 10 min at room temperature (light protected). The OD was measured at 540 nm using the VERSA max microplate reader. The amount of NO in each sample was calculated using a standard curve generated using sodium nitrite (0–100 μM in cell

culture medium). LPS was used as the positive control.

Identification of Differentially Expressed Genes Using PCR

Total RNAs from *M. hyopneumoniae*-stimulated and non–stimulated MH-S cells were extracted using Trizol (Promega) for cDNA synthesis. The extracted total RNAs were used for the synthesis of first-strand cDNAs by reverse transcription performed for 1.5 h at 42°C in a final reaction volume of 20 µl containing 3 µg of the purified total RNA, 4 µl of 5× reaction buffer (Promega, Madison, WI, USA), 5 µl of dNTPs (each 2 mM), 2 µl of 10 µM dT-ACP1 (5′-CTGTGAATGCTGCGACTACGATIIIII T(18)-3′), 0.5 µl of RNasinRNase Inhibitor (40 U/µl; Promega), and 1 µl of Moloney murine leukemia virus reverse transcriptase (200 U/µl; Promega). First-strand cDNAs were diluted by the addition of 80 µl of ultra-purified water for the GeneFishing PCR and were stored at −20°C until use.

Differentially expressed genes in the MH-S cells were screened using the Annealing Control Primer (ACP)-based PCR method using the GeneFishing DEG kit (Seegene, Seoul, South Korea) [15]. The ACP system uses primers that anneal specifically to the template and allows only genuine products to be amplified. Second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 µl containing 3–5 µl (about 50 ng) of diluted first-strand cDNA, 1 µl of dT-ACP2 (10 µM), 1 µl of 10 µM arbitrary ACP, and 10 µl of 2× Master Mix (Seegene). The PCR protocol for second-strand cDNA synthesis included one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. The second-stage PCR amplification was then performed using 40 cycles of 94°C for 40 sec, followed by 65°C for 40 sec, 72°C for 40 sec, and a 5 min final extension at 72°C. The amplified PCR products were separated on 2% agarose gel stained with ethidium bromide.

We used 120 arbitrary ACP primers, which showed specificity in PCR despite the short specific target sequences. The differentially amplified PCR fragments in the *M. hyopneumoniae*-stimulated MH-S cells were extracted from the gel using the GencleanII Kit (Q-BIOgene, USA) and directly cloned into a TOPO TA cloning vector (Invitrogen, USA) according to the manufacturer's protocol. The cloned fragments were sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

Statistical Analysis

All data were presented as means ± standard error of the mean (SEM). The data were analyzed using one-way analysis of variance and Student's t-tests (ver. 9.1, SAS, NC, USA). Differences were considered significant at $p < 0.05$.

Results

Cytokine and iNOS mRNA Expression in MH-S Cells

The sonicated protein fractions (SPT/cytoplasm and PPT/membrane proteins) and intact *M. hyopneumoniae* were found to induce the gene expression of cytokines and iNOS in the MH-S cells and increased the transcription of IL-1β, IL-6, TNF-α, COX-2, and iNOS mRNAs (Fig. 1). A time-dependent analysis of the mRNA expression revealed increased expression of IL-6, iNOS, IL-1β, and TNF-α at 4, 12, and 18 h after exposure, respectively, with both SPT and PPT, whereas COX-2 expression showed no difference after 18 h although it showed a higher expression at 2 and 4 h (Fig. 2).

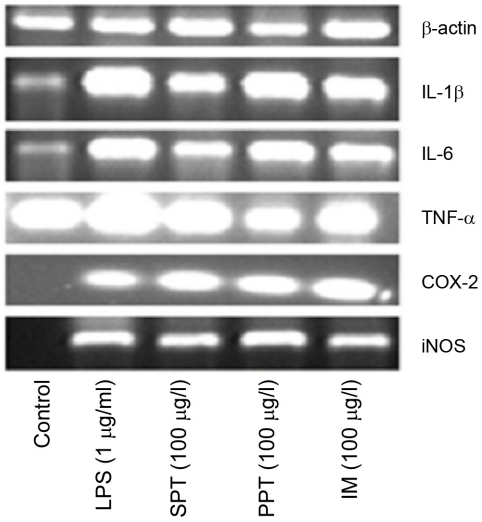

Fig. 1. Effect of *Mycoplasma hyopneumoniae* and its sonicated protein fraction on cytokine mRNA transcription in a murine alveolar macrophage MH-S cell line.
MH-S cells were treated with mycoplasma and its proteins at a concentration of 100 µg/ml for 18 h. RNA was extracted after 18 h of stimulation, and mRNA expression was evaluated using RT-PCR. Figures represent results of three independent experiments. IM, intact *M. hyopneumoniae*.

The transcription of IL-6 and COX-2 was correlated with the dose of administration of the protein fractions; however, the mRNA expression levels of TNF-α and iNOS were independent of the dose. Compared with the intact bacteria and SPT, PPT resulted in higher mRNA expression (data not shown).

Induction of NO Production in the MH-S Cells

Dose- and time-dependent induction of NO production in the MH-S cells stimulated using the supernatant and precipitate fractions of *M. hyopneumoniae* was analyzed using the cell culture medium. The result revealed a significant increase in NO production by both protein fractions as well as intact *M. hyopneumoniae* (Fig. 3A). Stimulation with SPT also resulted in significantly higher NO production than did stimulation with the intact *M. hyopneumoniae*. A dose-dependent correlation was also observed in NO production for both SPT and PPT; however,

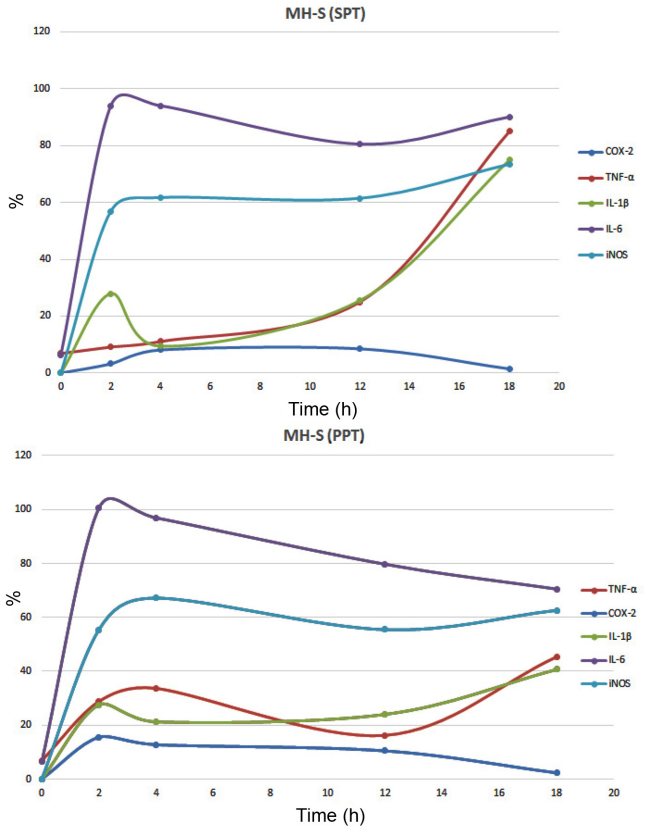

Fig. 2. Time-dependent effect of the sonicated protein fraction of *Mycoplasma hyopneumoniae* on cytokine mRNA transcription in a murine alveolar macrophage MH-S cell line.
MH-S cells were treated with SPT (supernatant protein fraction) and PPT (precipitant protein fraction) for up to 18 h. RNA was extracted after 0, 4, 6, 12, and 18 h of stimulation and mRNA expression was evaluated using RT-PCR. The amount of cytokine mRNA is shown as a percentage relative to the intensity for the RT-PCR product of β-actin. Data are represented as means ± standard error of the mean of three independent experiments.

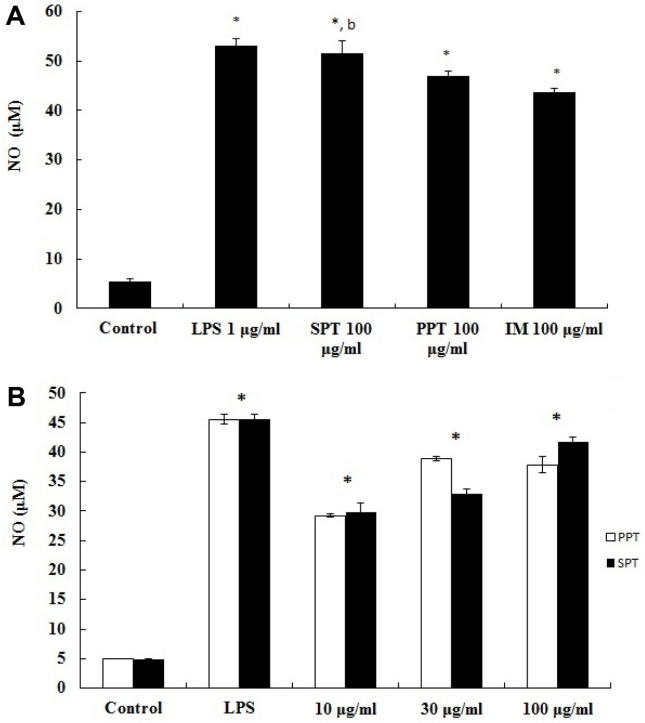

Fig. 3. Effect of *Mycoplasma hyopneumoniae* and its sonicated protein fractions on nitrite (NO) production in MH-S cells.
(**A**) Cells were treated with *M. hyopneumoniae* and its sonicated protein fractions at 100 μg/ml concentration for 18 h. (**B**) Dose effect of the sonicated protein fractions at the indicated concentrations. Culture media were collected and the NO concentration from the supernatant was measured using Griess reagent. Values correspond to the mean ± standard error of the mean of three independent experiments. *$p < 0.05$ compared with the control group.

there was no significant difference between the effects of the two protein fractions (Fig. 3B).

In addition to the dose-dependent response induced by the protein fractions, a time-dependent increase in NO production was also exhibited by the MH-S cells (data not shown).

Differentially Expressed Genes

We sought to identify other *M. hyopneumoniae*-inducible genes after we confirmed that *M. hyopneumoniae* infection induces inflammatory responses in the macrophages. *M. hyopneumoniae* (30 μg/ml) was cultured with the murine alveolar macrophage MH-S cell line for 3 h and total RNA was extracted. The RNA was then converted to cDNA, and genes were amplified using an ACP-based PCR method to identify the differentially expressed genes. The PCR products electrophoresed on 0.8% agarose gels revealed changes in nine gene bands after 3 h of stimulation — only one gene was downregulated, whereas the remaining eight were upregulated (Fig. 4A). Among the nine differentially expressed genes, the PCR fragments of two genes that were found highly upregulated were sequenced directly after cloning in a plasmid vector (Fig. 4B). The genes were confirmed to be encoding *Mus musculus* chemokine (C-C motif) ligand3 (Ccl3), also called macrophage inflammatory protein-1α (MIP-1α), and *Mus musculus* serum amyloid A 3 (Saa3). The results were confirmed using a homology search, which showed 100% identity and confirmed the genes to be associated with an inflammatory response. The gene sequences are deposited in the European Nucleotide Archive (ENA) under the accession numbers HG515535 and HG515535.

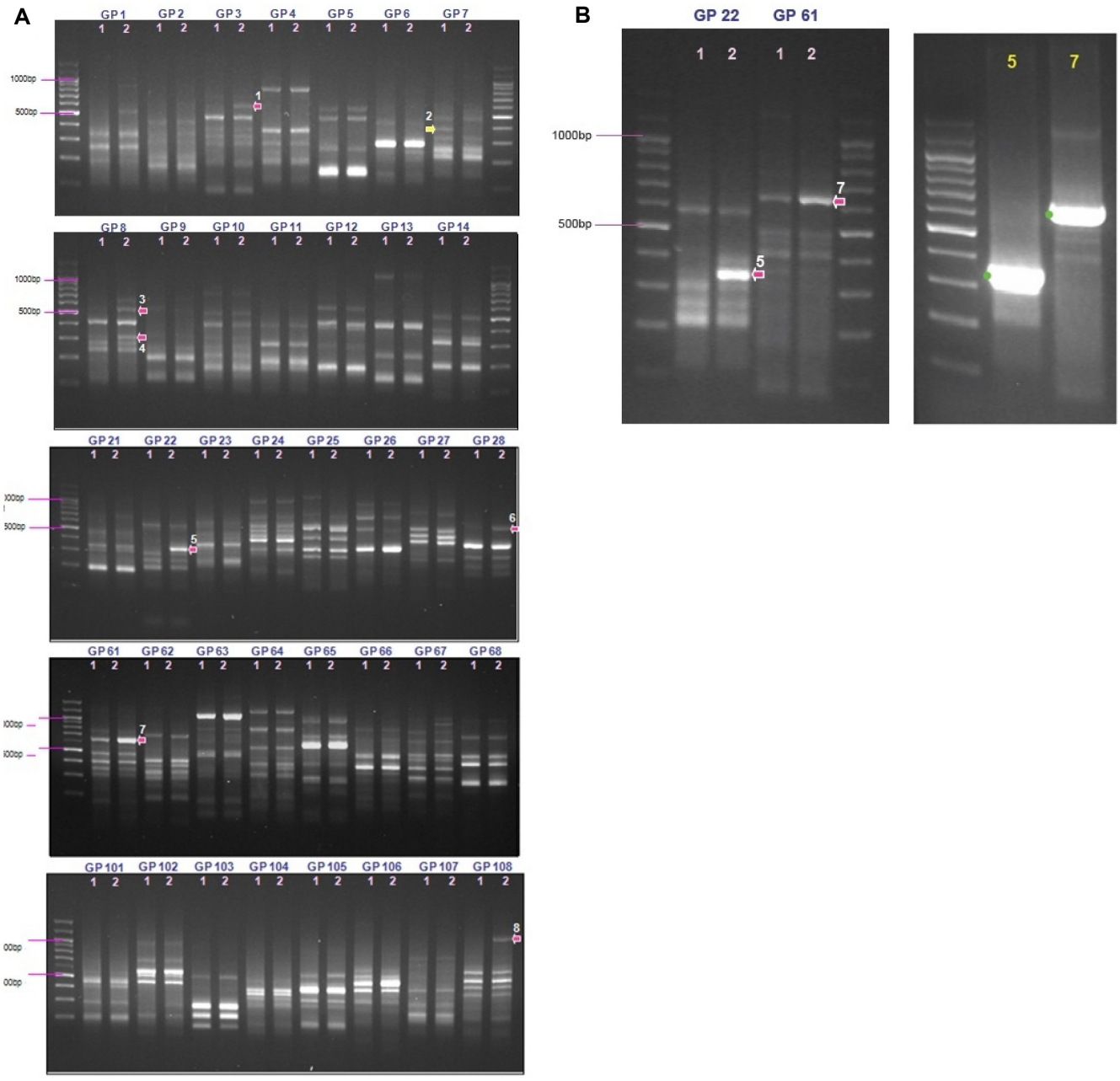

Fig. 4. The profile of differentially expressed genes of *Mycoplasma hyopneumoniae*-exposed and control MH-S cells using the Annealing Control Primer (ACP)-based PCR method and the GeneFishing DEG kit (**A**), and the re-amplified genes from the gel extract obtained using the GencleanII Kit (Q-BIOgene, Carlsbad, CA, USA) (**B**), directly sequenced using the ABI PRISM3100-AvantGenetic Analyzer.

Discussion

Despite the exhaustive studies conducted on *M. hyopneumoniae*, the pathogenesis of the infection by this organism and the causative antigens other than adhesion P97 remain elusive. Previously, the mRNA transcription of cytokines has been observed in RAW264.7 cells exposed to 100 μg/ml of intact *M. hyopneumoniae* [9]. In this study, we reconfirmed the previous results of *M. hyopneumoniae* whole protein-induced inflammation in MH-S cells and observed an increase in the transcription of COX-2, IL-1β, IL-6, TNF-α, and iNOS on exposure to the sonicated protein fractions of

M. hyopneumoniae. However, we previously performed a time-course study of the mRNA expression of cytokines using RT-PCR and found, based on the kinetics of cytokine mRNA expression, that LPS stimulation of porcine alveolar macrophages resulted in an increase in cytokine mRNA expression [3]. The time to reach the peak expression levels (14 to 24 h) by LPS were reduced to 4 to 14 h. This could be speculated from the fact that the *M. hyopneumoniae* virulence factors followed a different pathway from the inflammatory response pathway induced in macrophage cells. A difference in the time kinetics of cytokine mRNA expression after exposure to the SPT and PPT protein fractions of *M. hyopneumoniae* was also observed in this study, indicating the possibility of the same pathogenic bacterial components having a different pattern recognition receptor that could elicit an inflammatory reaction with different kinetics. In our previous study [19], we found that intact *M. hyopneumoniae* binds to a Gi/o receptor, irrespective of TLRs. Moreover, the presence of TLR2 and TLR6 on porcine alveolar macrophages upon stimulation with *M. hyopneumoniae* has been demonstrated recently by Muneta *et al*. [14]. Similar observations have been reported previously, where a subset of bacterial components was found to be acting as agonists for different TLRs — LPS for TLR4 [2, 5], lipoteichoic acid and muramyl dipeptide for TLR2 [18, 27], and heat shock proteins (HSPs) for TLR2 and TLR4 [17]. Specific to mycoplasma, its lipoprotein had been reported to act as an agonist to TLR2 in inducing the inflammatory response, which is found anchored to the membrane in some species of *Mycoplasma* and in others without the anchor domain attached to cytosolic structures [28]. This implies that our result suggests *M. hyopneumoniae* might contain a cytosolic lipoprotein that modulates the immune response in the macrophage. Furthermore, the ability of HSP components to induce a response in macrophages possibly accounts for their potency as adjuvants in preclinical and clinical vaccine trials [6, 17]. However, the increase in the expression of IL-6 upon stimulation with *M. hyopneumoniae* protein fractions was similar to that observed previously in the LPS-treated alveolar macrophages. This suggests that respiratory disease-causing gram-negative bacterial infections stimulate the expression of cytokines *in vivo*.

The results obtained for intact *M. hyopneumoniae* protein-induced NO production are also consistent with those of previous studies on *M. hyopneumoniae*-induced NO production [8, 9]. The sonicated protein fractions of *M. hyopneumoniae* induced significantly higher NO production than the whole protein in the MH-S cells. Furthermore, the induction of NO production was significantly higher for the SPT fraction than for the PPT fraction. This might be an indication of the presence of high concentrations of immunogenic proteins in the SPT protein fraction, due to which it was more potent in inducing the production of cytokines and nitric oxide in the macrophages.

Although the ACP-based PCR method used for identifying differentially expressed genes had limitations, in that it uses only 120 independent arbitrary primers, allowing the analysis of a maximum of 120 genes compared with microarray gene analysis that allows the simultaneous analysis of thousands of genes, the ACP-based PCR method provides additional information about the pathogenesis of *M. hyopneumoniae* and the inflammatory responses in the MH-S cells in response to exposure to *M. hyopneumoniae* proteins. We found two differentially expressed genes, MIP-1α and Saa3, associated with the inflammatory response in MH-S cells stimulated with *M. hyopneumoniae*. MIP-1α is involved in macrophage activation, which results in the recruitment of T cells, neutrophils, and additional macrophages to the sites of action [24]. Although the Saa3 gene is a pseudogene that does not show mRNA or protein expression in humans, in pigs, which are common hosts for pathogenic *M. hyopneumoniae,* unlike in other species, the major acute phase protein has the characteristics of Saa3 rather than Saa1 or Saa2 [23]. This suggests a possible role of Saa3 in the inflammatory responses; investigations into the macrophages in pigs are required to gain further insights on this topic.

In summary, the results of the current study provided insights that improve our understanding of bacterial pathogenesis and macrophage defense mechanisms, and suggest targets for therapeutic interventions. Further studies directed towards identifying the specific soluble protein/peptide responsible for the pathogenesis, as well as the underlying mechanisms and the potential of these proteins for use in vaccine development, are under way.

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