EFFECTS OF MANNAN OLIGOSACCHARIDE ON IMMUNE FUNCTION AND DISEASE RESISTANCE IN PIGS

BY

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DISSERTATION

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ABSTRACT

The 3 studies described below demonstrate the effects of mannan oligosaccharide (**MOS**) on immune function and disease resistance in pigs. Study 1 evaluated whether MOS in both *in vivo* and *in vitro* systems regulates cytokine production by alveolar macrophages (**AMϕ**) in response to *in vitro* microbial challenge models. The lipopolysaccharide (**LPS**)-stimulated AMϕ from MOS-fed pigs produced less tumor necrosis factor-α (**TNF-α**) ($P < 0.01$) and more IL-10 ($P = 0.051$) than AMϕ from the control-fed pigs. Dietary MOS did not affect AMϕ-produced cytokines induced by polyinosinic:polycytidylic acid (**Poly I:C**). When applied *in vitro*, MOS suppressed LPS-induced TNF-α ($P < 0.001$), but enhanced LPS-induced IL-10 ($P < 0.05$). Further, TNF-α production by AMϕ stimulated with LPS ($P < 0.05$) or Poly I:C ($P < 0.001$) was suppressed by a mannan-rich fraction (**MRF**). In order to learn if MOS interacts with LPS receptors, AMϕ were cultured with Polymyxin B, an inhibitor of LPS-activated toll-like receptor (**TLR**) 4. Although Polymyxin B completely inhibited AMϕ-produced TNF-α induced by LPS, it did not affect the ability of MOS to regulate cytokine production in the absence of LPS. When added *in vitro*, both MOS and MRF were also able to regulate constitutive production of TNF-α in the absence of LPS. Study 2 determined if various levels of dietary MOS affect growth and serum cytokine levels in nursery pigs. No effect of MOS on growth was found. There were no differences in serum levels of TNF-α and IL-10, although these levels changed over time. Study 3 showed that MOS altered nursery pigs' immune response to a porcine reproductive and respiratory syndrome virus (**PRRSV**). Infection of PRRSV reduced pig performance and leukocytes ($P < 0.01$), but increased serum inflammatory mediators and fever ($P < 0.01$). Dietary MOS

prevented leukopenia at d 3 and 7 postinfection (**PI**) and tended to improve feed efficiency. In infected pigs, MOS reduced fever at d 7 PI ($P < 0.01$) and serum TNF-α at d 14 PI ($P = 0.06$). The gene expression profile in peripheral blood mononuclear cells and bronchoalveolar lavage fluid cells at d 7 PI was characterized by using microarray and real time RT-PCR. The MOS x PRRSV interaction altered the gene expression in the above leukocytes ($P < 0.05$). In peripheral blood mononuclear cells, MOS increased the gene expression of pattern recognition receptors, cytokines, and intracellular signaling molecules in uninfected pigs, but reduced the gene expression of TLR4 and various types of key cytokines and chemokines in infected pigs ($P < 0.05$). In bronchoalveolar lavage fluid cells, MOS may promote a cytotoxic T cell immune response by enhancing MHCI mRNA expression, but reduce the expression of complement system-associated molecules and 2',5'-oligoadenylate synthetase-1. The downregulation of inflammatory responses regulated by MOS at d 7 PI was associated with several important canonical pathways such as triggering receptor expressed on myeloid cells-1 signaling, hypoxia signaling, IL-4 signaling, macropinocytosis signaling, and perhaps the alternative activation of macrophages. In summary, MOS is a potent immunomodulator in both *in vitro* and *in vivo* systems. Dietary inclusion of MOS in diets for pigs may bring benefits by boosting and maintaining the host's disease resistance while preventing over-stimulation of the immune system.

Key words: Alveolar macrophages and cytokine secretion; disease resistance; immunomodulation; mannan oligosaccharide; pigs; PRRSV

DEDICATED

TO

MY MOTHER, WIFE, AND SON FOR ALL THEIR SPIRITUAL SUPPORTS

Most of us, swimming against the tides of trouble the world knows nothing about, need only a bit of praise or encouragement -- and we will make the goal.

Jerome P. Fleishman

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LIST OF ABBREVIATIONS

ADFI	average daily feed intake
ADG	average daily gain
AMϕ	alveolar macrophages
ANOVA	analysis of variance
AP	activation protein
APP	acute phase proteins
APRIL	a proliferation-inducing ligand
ARG	arginase
BALF	bronchoalveolar lavage fluid
BW	body weight
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CON	uninfected control-fed pigs
CRP	C-reactive protein
C1QA	complement component 1
CV	coefficient of variation
d	day
DDX	dead box polypeptide
DMEM	dubelco's modified eagle medium
ELISA	enzyme-linked immunosorbent assay
FCGRT	fragment crystallizable of IgG, receptor, and transporter

FDR	false discovery rate
G:F	gain to feed ratio
GIT	gastrointestinal tract
GLM	general linear model
GluF	glucan fraction
HIF	hypoxia-inducible factor
Hp	haptoglobin
ICON	infected control-fed pigs
IFN	interferon
Ig	immunoglobulin
IL	interleukine
IMOS	infected mannan oligosaccharide-fed pigs
IPA	ingenuity pathway analysis
LPS	lipopolysaccharide
M	microfold
MCP	monocyte chemotactic protein
MHC	major histocompatibility complex
min	minutes
MIP	macrophage inflammatory protein
MOS	mannan oligosaccharide
MR	mannose receptor
MRF	mannan-rich fraction
MyD	myeloid differentiation factor

mRNA	messenger ribonucleic acid
NA	neutralizing antibodies
NRC	national research council
OAS	oligoadenylate synthetase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEC-60	peptide with N-terminal glutamic acid, C-terminal cysteine, and a total of 60 residues
PI	postinfection
PI3K	phosphoinositide 3-kinase
PMB	polymyxin B
Poly I:C	polyinosinic:polycytidylic acid
PRRS	porcine reproductive and respiratory syndrome
PRRSV	porcine reproductive and respiratory syndrome virus
PW	postweaning
p38 MAPK	p38 mitogen-activated protein kinase
p53	tumor protein 53
RT	rectal temperature
RT-PCR	reverse transcription polymerase chain reaction
SAS	statistical analysis software
S/P	sample to positive ratio
Th	T helper

TLR	toll-like receptor
TNF	tumor necrosis factor
TREM	triggering receptor expressed on myeloid cells
WBC	white blood cells
wk	week

CHAPTER 1

LITERATURE REVIEW

MANNAN OLIGOSACCHARIDE

Introduction

Glycobiology is a relatively new field of study in the world of science. In the past decades, discoveries in the field of glycobiology have revealed the critical role of carbohydrates in the mechanisms of immunity (Dwek, 1996; Munro, 2000; Axford, 2001). These discoveries will lead to the ability to use these functional carbohydrates, with a reduced use of antibiotics, in diets to improve performance and health of animals. There has of late been increasing pressure on the livestock industry to decrease the use of antibiotics due to the potential development of antibiotic resistance (Pettigrew, 2006; Stein and Kil, 2006). Among carbohydrates, mannan oligosaccharide (**MOS**), derived from the yeast cell wall of *Saccharomyces cerevisiae*, has been shown to improve animal performance and health through several mechanisms such as prevention of pathogens from binding to the gastrointestinal tract (**GIT**), alteration of GIT microbial populations, and enhancement of immune functions.

Growth Performance

Swine. Addition of MOS to a diet has resulted in a large variation in growth performance response of pigs. Some researchers have reported little response in ADG, ADFI, and G:F when MOS was fed to weaned pigs. LeMieux et al. (2003) reported that nursery pigs fed diets with 0, 0.2, or 0.3% MOS supplementation showed no improvement in growth

performance. With similar dietary inclusion levels of MOS, Davis et al. (2004a) found no effect of MOS on growth response of nursery pigs. In other studies, however, increases in growth performance of pigs have been demonstrated (Dvorak and Jacques, 1998; Kim et al., 2000; Davis et al., 2004b). Under commercial conditions, differences in growth response to dietary MOS have also been reported. Rozeboom et al. (2005) investigated effects of MOS on the performance of pigs on 2 large-scale commercial farms (A and B) and one university research farm (C). They demonstrated that growth improvements with MOS on Farms A and B were not significant, but pigs on Farm C had better ADG, ADFI, and G:F. Recently, in a study assessing performance of nursery pigs in 7 trials, the pooled data suggested that addition of MOS to a weaned pig's diet increased ADFI and ADG of piglets (Corrigan et al., 2008). Growth performance responses to MOS supplementation are variable, being especially related to growth rate of pigs (Miguel et al., 2004). The meta-analysis of Miguel et al. (2004) suggested that MOS had little or no response in pigs with high growth rate (> 180 g/d) during the first 1 to 2 wk postweaning (**PW**).

Mannan oligosaccharide has the potential to partly replace pharmacological levels of trace minerals or may be alternative to antibiotics in nursery diets. In a series of experiments, LeMieux et al. (2003) found that diets with 0.2% MOS supplementation during phase 2 increased the growth performance of pigs when excess zinc was not included in the diet. When Zn included in the diet at a level of 3000 ppm, there was no difference in growth performance between pigs fed MOS and those fed the control. Davis et al. (2004a) showed that adding MOS resulted in an improved growth response if dietary zinc levels were restricted to levels of 200 or 500 ppm. These results indicate that the amount of zinc which is commonly added to the nursery pig diet can be lowered in MOS-supplemented diets. This

may help reduce the negative impact of excess zinc on the environment because of reduced zinc excretion. Similarly, the benefit of growth in pigs fed MOS was also substantiated when the level of copper in the diet was restricted (Davis et al., 2002a). In addition, MOS may be considered alternative to antibiotics in certain cases. For instance, Rozeboom et al. (2005) evaluated the effect of MOS on the number of medical treatments, removal rate, and mortality of nursery pigs reared under commercial farm or university research farm levels. They reported that MOS was likely an alternative to tylosin and sulfamethazine as a growth promotant in nursery diets.

Most of the data obtained from the above studies, whether conducted at a university research farm or under commercial conditions, illustrate the beneficial effects of MOS on growth performance of young pigs. The favorable effect of MOS on growth performance in diets with low levels of copper and zinc seems to be promising if the proportions of these trace minerals in nursery pig diets are strictly regulated as a concern of environmental pollution. The potential of MOS as a growth-promoting alternative to antibiotics needs further investigation.

Poultry. Several studies have been conducted to investigate the effect of dietary MOS on growth performance in poultry. The inclusion of MOS in poultry diets has resulted in varying responses of growth performance. Some studies have reported no effect of MOS on the performance of poultry. In an experiment with broiler chickens, a diet with 0.5% MOS supplementation did not significantly produce better performance than the control (Geier et al., 2009). Fritts and Waldroup (2003) reported that turkey poults fed 0.05 or 0.1% MOS performed the same as those consuming the negative control. In contrast, other researchers found beneficial effects on the growth of turkeys. Zdunczyk et al. (2005) indicated that

turkeys fed diets with medium and high levels of MOS had greater BW at the age of 16 wk than those fed the control. Also, Sims et al. (2004) showed that the BW of turkeys fed 0.05 or 0.1% was heavier than that of turkeys fed the control. Furthermore, in a meta-analysis, Hooge (2004a,b) evaluated the effects of dietary MOS on the performance of broiler chickens and turkeys. The results showed that diets containing MOS significantly improved final BW compared to control diets but gave statistically equivalent BW compared to diets containing sub-therapeutic levels of antibiotics. In brief, on the basis of these available data, it is suggested that MOS has potential as a growth promoter for poultry.

Other Species. The growth-enhancing effect of MOS has also been studied in other types of animals such as cattle, rabbits, and fish. Heinrichs et al. (2003) reported that ADFI was increased in MOS-fed calves compared to antibiotic-fed calves, but no difference in growth was found during the experimental period of 5 wk. A similar response was also obtained by Terre et al. (2007) when calves were fed 4 g of MOS per day. In rabbits consuming different levels of MOS ranging from 0.05 to 0.2%, feed efficiency was improved compared to the control rabbits. In a study with rainbow trout, the results also showed that MOS improved feed efficiency as well as growth performance (Staykov et al., 2007).

Changes in Microbial Population through Agglutination of Pathogens

Mannan oligosaccharide, derived from the cell wall of *Sacchromyces cerevisiae*, is believed to promote gut health by preventing pathogens from attaching to the epithelial surface of the intestines (Kocher and Tucker, 2005). Mannan, present in MOS, offers a competitive binding site for a certain class of bacteria (Oyofo et al., 1989a, b). One of the basic modes of action of MOS is to block the attachment of pathogenic bacteria containing

Type I fimbriae to the intestinal wall of animals. Hence, the attached harmful bacteria are taken with digesta to the large intestine and then excreted in feces. In a survey, it was demonstrated that approximately 70% of 77 *Escherichia coli* strains and 53% of 30 *Salmonella* species possessing Type 1 fimbriae were sensitive to mannan (Finuance et al., 1999). In order to confirm that MOS inhibits pathogen colonization, Spring et al. (2000) screened different bacterial strains for their ability to agglutinate MOS in yeast cell preparations. Five of 7 strains of *Escherichia coli* and 7 of 10 strains of *Salmonella typhimurium* and *Salmonella enteriditis* were agglutinated by MOS and yeast cells. It has been shown that MOS changed microbial populations in the GIT of young pigs (Miguel et al., 2006). The reduction in the number of pathogenic bacteria perhaps results in a better gut microflora through which diarrhea is possibly prevented.

Fecal Score Consistency and Diarrhea

Addition of MOS to diets has been shown to result in improved fecal consistency and reduce diarrhea in several types of animals. Castillo et al. (2008) showed that pigs fed 0.2% MOS had more normally shaped feces than those fed the control. In another study with weaned pigs, the incidence of diarrhea and number of diarrhea days were lower in the MOS-fed group than in the control group (Grela et al., 2006). In calves, fecal scores were also improved when calves were fed 4 g of MOS daily for 5 wk (Heinrichs et al., 2003). The beneficial effect of MOS on fecal consistency and diarrhea may be associated with the ability of MOS to block the colonization of enteric pathogens to the GIT wall.

Immune Responses to Dietary MOS

Small oligosaccharides have been shown to exert a variety of effects on the immune system and to potentially modulate the immune responses (Bland et al., 2004). More available data about the role of oligosaccharides as immunomodulators has been increasingly generated. Among oligosaccharides, MOS has been reported to influence the innate and adaptive immunity.

Several studies have demonstrated effects of MOS on the nonspecific cellular immunity. In an *in vitro* experiment, the stimulation of phagocytic activity of rat macrophages by MOS was shown to be dose-dependent (Newman, 1995). Davis et al. (2004b) also reported that phagocytic macrophages isolated from the jejuna lamina propria of pigs fed MOS consumed a greater number of sheep red blood cells per phagocytic macrophage than did phagocytic macrophages isolated from pigs fed the control diet. These data indicate that MOS is a potent immunostimulator. On the other hand, MOS reduced the inflammatory tissue response in poultry. Cotter and Weinner (1997) showed that inclusion of MOS in the diets of replacement pullets at a level of 1 g/kg reduced the intensity of the wattle hypersensitivity reaction in both 8- and 10-wk-old pullets subjected to 3 successive exposures to antigen at 2-wk intervals commencing at 6 wk of age. The mechanism of MOS in reducing inflammation is unknown, but may be associated with the expression level of pattern recognition receptors (**PRR**) involved in antigen binding and secretion of cytokines. Singboottra et al. (2006) found that reduced expression of IL-6 by a mannan-rich fraction was mediated through a transitory decrease in the expression of toll-like receptor (**TLR**) 4. This receptor, when activated, triggers a cascade of inflammatory cytokine production.

The effects of MOS on lymphocyte proliferation and leukocyte populations have been evaluated. Mannan oligosaccharide appeared not to affect *in vitro* lymphocyte proliferation. In a series of experiments conducted by Davis et al. (2002a) and Davis et al. (2004a,b), lymphocytes from pigs fed different levels of MOS and copper or zinc were collected and lymphocyte proliferation was measured *in vitro* by unstimulating or stimulating lymphocytes with phytohemagglutinin or pokeweek mitogen. The results showed no effect of dietary MOS on lymphocyte proliferation. However, in the presence of Zn, MOS modulated lymphocyte proliferation by increasing the number of pokeweek-stimulated lymphocytes. Although not affecting *in vitro* lymphocyte proliferation, feeding MOS to animals increased the proportion of peripheral lymphocytes. Davis et al. (2004b) found that pigs fed 0.3% MOS had a greater lymphocyte percentage than those fed the control, but there was no difference in the number of lymphocytes between MOS-fed pigs and control-fed pigs. A similar response was also shown in a study of Swanson et al. (2002) in which dogs were fed 0 or 0.1% MOS. Moreover, dietary MOS intermittently modified the proportions of cluster of differentiation (**CD**) 14 and CD14 major histocompatibility complex (**MHC**) II leukocytes in the blood and jejuna lamina propria of weanling pigs (Davis et al., 2004b). The increase in CD8 T cells was also documented in MOS-fed pigs. This is very important in diseases associated with viral infections.

Mannan oligosaccharide has also been shown to influence the adaptive immunity. In poultry, Savage et al. (1996) reported an increase in plasma IgG and bile IgA in poults fed diets supplemented with 0.11% MOS. The increase in antibody response to MOS indicates that MOS may contain components which probably have the ability to elicit powerful antigenic properties. Shashidhara and Devegowda (2003), investigating influence of MOS on

infectious bursal disease virus antibody titers in broiler breeder and progeny, showed that antibody responses against infectious bursal disease virus in breeder and progeny were higher in the MOS group. In pigs, a study with sows receiving MOS 14 d prefarrowing and throughout lactation observed higher concentrations of colostral IgM compared to the untreated sows (Newman and Newman, 2001). Another study also indicated that feeding MOS to sows increased IgG and IgA levels in colostrum (O'Quinn et al., 2001). Furthermore, Franklin et al. (2005) reported that supplementation of MOS to cows during the dry period enhanced their immune response to rotavirus and tended to enhance the subsequent transfer of rotavirus antibodies to calves. In addition, Davis et al. (2004b) showed that pigs fed diets containing MOS intermittently affected selected components of the young pigs' immune function both systemically and enterically.

Several benefits from the use of MOS in animals have been discussed, but the improved immunity of animals by MOS has remained unclear. The data reviewed suggest that MOS may directly interact with immune cells and induce changes in expression of molecules involving immune regulation such as cytokines, chemokines, PRR, etc. Thus, the specific effects of MOS on immune responses need to be evaluated under various conditions. Understanding of the mechanisms of MOS in modulation of the immune responses of pigs is necessary to gain maximum benefit from MOS under practical application.

THE IMMUNE SYSTEM

Innate and Adaptive Immunity

The immune system has evolved to protect the multicelllular organism from pathogens. It can be divided into 2 systems of immunity: innate immunity and adaptive

immunity. The innate immune system is universal and the first line of host defense against infection. It non-specifically responds to an antigen at the first contact. This immune response is primarily mediated by phagocytic cells, natural killer cells, mast cells, eosinophils, and soluble molecules such as complement factors, acute phase reactants and cytokines (Janeway and Medzhitov, 2002). The phagocytic cells, especially neutrophils and monocytes/macrophages, eliminate the microorganisms by phagocytosis and subsequent degradation by intracellular enzymes (Underhill and Ozinsky, 2002). The innate immune system is characterized by immediate activation of effectors, quick response, perfect self-nonself discrimination, non-clonal distribution, and recognition of conserved molecular patterns of microbes. The antigen presenting cells, i.e. macrophages and dendritic cells, not only are capable of phagocytosis but also can process and present antigens to antigen-specific T lymphocytes (Jenkins et al., 2001; Trombetta and Mellman, 2005). In this way they form a bridge between innate and adaptive immunity.

Adaptive immunity refers to antigen-specific immune response and is more complex than the innate immunity. It provides a second, comprehensive line of defense, capable of specifically recognizing foreign antigens, developing an immunological memory of infection, and rearranging receptor gene segments. However, it takes a few days for the adaptive immunity to begin after the initial infection (Williams and Bevan, 2007). Cells involved in an adaptive immune response include T lymphocytes, B lymphocytes, and antigen presenting cells.

There are 2 main subsets of T lymphocytes, distinguished by the presence of cell surface molecules known as CD4 and CD8. T lymphocytes expressing CD4 are also known as helper T (**Th**) cells, and are regarded as being the most prolific cytokine producers

(Jenkins et al., 2001). This subset can be further sub-divided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines (Szabo et al., 2003; Lippolis, 2008). The Th1-type cytokines tend to produce the pro-inflammatory responses responsible for killing intracellular pathogens and interferon (**IFN**)-γ is the main Th1 cytokine. Excessive pro-inflammatory responses can lead to uncontrolled tissue damage, so there needs to be a mechanism to counteract this. The Th2-type cytokines include interleukins 4, 5, and 13, which are associated with the promotion of IgE and eosinophilic responses in atopic allergies, and also IL-10, which has more of an anti-inflammatory effect. In excess, Th2 responses will antagonize the Th1-mediated microbicidal action. The optimal scenario would therefore seem to be that animals should produce a well-balanced Th1 and Th2 response, suited to the immune challenge.

There are 3 main types of professional antigen-presenting cells, consisting of dendritic cells, macrophages, and B lymphocytes. These cells take up antigens in peripheral tissues, process them into proteolytic peptides, and load these peptides onto MHC class I and II molecules (Guermonprez et al., 2002; McHeyzer-Williams and McHeyzer-Williams, 2005). In addition to the professional antigen-presenting cells, any nucleated cell in the body can present a complex of antigen and MHCI bound on its surface to CD8 T cells or cytotoxic T cells. Naive CD8 precursors have no cytotoxic activity and must undergo an activation process requiring 1 to 3 d for maximal activity (Wong and Pamer, 2003; Williams and Bevan, 2007). This activation process requires T cell receptor-stimulated induction of cytokine receptors (e.g., IL-2 and IL-6), which then induce the expression of granule components, including perforin and granzymes. In contrast, macrophages and dendritic cells present antigens to Th1 cells through MHCII, whereas B cells present antigens to Th2 cells.

An antigen-presenting process occurs in secondary lymphoid organs and thereafter initiating antigen-specific immune responses, or immunological tolerance (Guermonprez et al., 2002; Trombetta and Mellman, 2005). The recognition of an antigen-MHC complex by a specific mature T lymphocyte induces clonal proliferation and differentiation into various memory T and B cells, effector T cells, and antibody-secreting plasma cells.

Pattern Recognition Receptors and MOS

The innate immune system uses several different receptors to recognize and respond to antigenic stimulators which are characteristic of microbial surfaces but are not found on the host cells (Janeway and Medzhitov, 2002). These receptors, termed PRR, are diverse, including TLR, scavenger receptors, and lectin receptors such as the mannose receptor (**MR**). In mammals TLR family is known to consist of 11 members (TLR1 to TLR11) and can recognize distinct microbial components (Takeda et al., 2003; Akira and Takeda, 2004). For instance, TLR4 is a receptor specifically involved in the recognition of lipopolysaccharide, a major cell wall component of gram-negative bacteria, whereas MR is able to recognize a wide range of gram-negative and gram-positive bacteria, yeasts, parasites, and mycobacteria (Stahl and Ezekowitz, 1998; Raetz and Whitfield, 2002). Activation of PRR triggers intracellular signaling cascades leading to the biosynthesis of cytokines, such as IL-1β, IL-6, IL-10, tumor necrosis factor (**TNF**)-α, and IFN, and induction of co-stimulatory molecules required for the adaptive immune response.

Mannan oligosaccharide has been assumed to have a direct effect on the immune cells through its mannan molecule. Recent discovery has revealed that TLR4 and MR may be involved in the recognition of mannan. It has been found that MR on macrophages and other

cells recognizes mannan (Tizard et al., 1989; Davis et al., 2002b). In other words, Singboottra et al. (2006) reported that altered cytokine expression of chicken macrophages stimulated with a mannan-rich fraction was mediated via a transitory decrease in the mRNA expression of TLR4. Additionally, it was shown that TLR4 recognized mannan and mannan-associated molecules (Sheng et al., 2006). It may be speculated that activation of macrophages by MOS is likely involved in both MR and TLR4 whose expression levels would determine the immune responses of macrophages to bacterial or viral stimulations. Variations in immunomodulatory characteristics among mannan-containing products can be ascribed to a polymerization degree of mannan (Bland et al., 2004), types of terminal linkages of mannan sequences (Young et al., 1998), or types of mannan (Djeraba and Quere, 2000; Sheng et al., 2006). Thus, evaluation of MOS effects on immune function in pigs is necessary because benefits, such as better performance and enhanced disease resistance may result from its efficient immunomodulation.

Gastrointestinal Immunity in Association with MOS

Integrity and well-being of the GIT of animals is protected by several defense mechanisms including peristaltic movements of the intestine, shedding of epithelial cells, gastric acidity, bile acids, antimicrobial peptides, mucus as well as water secretion, and balanced microflora. Mannan oligosaccharide included in a nursery pig diet may promote a healthy GIT by inhibiting intestinal colonization of enteric pathogens and enhancing the host's immunity. First, MOS contains yeast cell wall fragments. These fragments contain mannans, which competitively bind gram-negative bacteria, preventing their attachment to the intestinal mucosa (Oyofo et al., 1989a,b; Finuance et al., 1999). Because mannans are not

digestible in the intestines (Longe et al., 1982), the bound pathogens likely pass through the digestive tract. Recently, it has been demonstrated that certain bacterial strains, such as *lactobacilli, bifidobacteria,* and *L.johnsonii La1* possess immunostimulatory properties and can enhance innate immune mechanisms (Blum et al., 2002; Herich and Levkut, 2002; Desbonnet et al., 2008). Microbial populations altered by MOS therefore possibly affect mucosal and systemic immune responses of pigs. Second, increases in immunoglobulin concentrations in plasma, colostrum, and bile suggest that MOS may have a direct effect on mucosal as well as systemic immunity if it is taken up by microfold (**M**) cells located on the GIT and acts as an immunostimulatory agent. The M cells are distinctive epithelial cells and serve as a critical component in the cascade from antigen deposition at mucosal surfaces to transepithelial transport and development of mucosal immunity (Kraehenbuhl, 2000). Antigens are transported into mucosal lymphoid tissues by M cells, which are only present in the follicle-associated epithelium overlaying organized lymphoid tissue in the nasal and oral cavities as well as the intestine and bronchi (Makala et al., 2002). Generally, there has been little information about the uptake of MOS mediated by receptors of M cells, but the systemic immunity regulated by MOS may be associated with modulated mucosal immunity and function of M cells.

Lung Inflammatory Responses

Respiratory diseases caused by viruses, such as PRRSV, swine influenza, porcine respiratory coronavirus, and porcine circo virus are common in pigs (Thacker, 2001; Paton and Done, 2002). The lung is in direct and continuous exposure to the surrounding environment. In spite of continual contact with immunologically potent challenges,

inflammation in the lung is tightly controlled to keep the host animal in a healthy and non-inflamed state. Inflammatory responses can be beneficial or detrimental to the host and categorized into as acute and chronic in nature.

Inflammatory responses are typically immediate in nature and characterized by changes in vascular tissues in order to eliminate harmful stimuli, e.g. pathogens. Leukocytes, such as neutrophils, natural killer cells, macrophages and lymphocytes, various cytokines and chemokines, and other serum components play a role in both triggering and controlling inflammation (Lazarus, 1986). Intercellular communication occurs using various mediators and messengers that include cytokines, leukotrienes, prostaglandins, thromboxane, platelet-activating factor, acute phase proteins, and the various cell adhesion molecules. In the first stages of inflammation, neutrophils and natural killer cells are the first WBC population to arrive and affect the host inflammatory response (Guo and Wand, 2002; Kohlmeier and Woodland, 2009). Neutrophils and natural killer cells infiltrate the lungs in response to the various mediators of acute inflammation. During the attraction and activation of neutrophils, activated phagocytic cells, especially macrophages, are also recruited to the site of inflammation. Due to inflammation, cytokines and chemokines are secreted rapidly and early following injury or infection. Interferon-α, TNF-α, IL-1, and IL-6 are early cytokines produced during the initial stage of an infection (Murtaugh et al., 1996; Murtaugh and Foss, 2002; Van Reeth et al., 2002). Chemokines are important mediators of inflammation in the respiratory tract. Chemokines are small polypeptides that control adhesion, chemotaxis, and activation of leukocyte populations (Rot and Andrian, 2004; Allen et al., 2007). Some chemokines are constitutively expressed, whereas others are either up or downregulated in association with inflammation. A variety of serum proteins are actively involved in acute

inflammation reactions. These systems include the complement, coagulation, and kinin systems as well as the acute-phase proteins such as haptoglobin (**Hp**), C-reactive protein, and serum amyloid A (Kirschfink, 1997; Petersen et al., 2004; Parra et al., 2006). Synthesis and secretion of acute phase proteins is a dynamic process involving both systemic and metabolic changes and provides nonspecific protection early following infection.

Chronic inflammation initially follows the same pathway as an acute inflammatory response (Thacker, 2006). Chronic inflammation takes place if the acute inflammatory response is insufficient to clear the tissue of invading pathogens or substances and promote further protection and tissue repair. Pathogens that induce chronic inflammation in the respiratory tract include *Mycoplasma hyopneumoniae* and PRRSV in pigs (Wills et al., 1997; Van Reeth et al., 2002; Mateu and Diaz, 2008). Scarring which results from damaged lung parenchyma can cause respiratory problems due to reduced area for oxygen exchange. Chronic respiratory tract inflammation reduces pig performance, leading to economic loss to the producers (Neumann et al., 2005). This is especially problematic to the swine industry where chronic pneumonia due to combined respiratory pathogens is commonly seen. Therefore, prevention of excessive inflammation or successful elimination of chronic pneumonia will bring benefits to swine producers.

In short, animals are protected by the 2 major arms of defense system, the innate or nonspecific immune system and the adaptive or specific immune system. Each system has both cellular and humoral components by which it can recognize and eliminate invading organisms. The inflammatory response is beneficial in protecting animals against an infection, but can be detrimental if it is severe and inappropriately controlled. Thus, activation of the immune system is regulated through several mechanisms to maintain the

host's disease resistance as well as to prevent immune-mediated disorders. Although both of the defense systems have distinct functions, most diseases involve both arms of immune response. In addition, effects of MOS on the host's immune response may result from its interaction with the PRR of immune cells, such as MR and TLR4.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (**PRRSV**) is a positive-sense, single-stranded enveloped RNA molecule, containing 9 open reading frames encoding as many as 9 viral proteins including a membrane-spanning protein, nucleocapsid protein, glycoprotein-5 and non-structural protein-2 (Kimman et al., 2009). It belongs to the family *Arteriviridae*, which includes other members such as equine arteritis virus, simian hemorrhagic fever virus, and lactate dehydrogenase-elevating virus (Duan et al., 1997; Kawashima et al., 1999; Choi et al., 2001). The virus was first isolated in swine herds in the United States in 1987 and later in Europe in 1990 (Mengeling et al., 1996; Albina, 1997). Since then it has quickly spread throughout major swine producing countries in Asia and other parts of the world. The disease is presently a serious concern for the swine industry worldwide and causes a significant loss to swine producers (Neumann et al., 2005). The PRRSV frequently causes persistent or repeated infection in susceptible pigs and herds. Pigs vaccinated with one serotype are generally not protected against infection by heterogonous strains, indicating high genomic diversity among PRRSV isolates (Labarque et al., 2004).

The PRRSV is the most devastating pathogen impacting the swine industry and can cause porcine reproductive and respiratory syndrome, which is characterized by reproductive

failure in sows and respiratory disease in pigs of various stages of growth and development. A PRRS virus primarily infects alveolar macrophages which later spread the disease from lungs to the rest of the body via peripheral circulation (viremia). Another route of PRRSV infection is from the reproductive tract by artificially inseminating sows with infected sperm. Interaction of the virus with porcine cells, such as pulmonary alveolar macrophages and intravascular macrophages of the placenta and umbilical cord results in initial immune responses to PRRSV (Oleksiewicz and Nielsen, 1999; Thanawongnuwech et al., 2000; Riber et al., 2004). The failure in reproduction may consist of late-term abortions, increased numbers of stillborn, mummified and weak piglets, and high neonatal death loss (B∅tner, 1997; Pejsak et al., 1997; Rossow, 1998). The respiratory disease results in a high morbidity in pigs of all ages and is very severe in young pigs. The PRRS disease becomes more devastating if pigs are infected with secondary pathogens such as *Pasteurella multocida, Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Streptococcus suis, Haemophilus parasuis*, Aujeszky's virus, swine influenza virus, and circo virus. Pigs infected with PRRSV and a secondary pathogen develop more severe clinical diseases and pathological lesions than do pigs infected with PRRSV alone (Pol et al., 1997; Thanawongnuwech and Thacker, 2003; Thanawongnuwech et al., 2004). Typically, PRRSV-infected pigs showed labor-breathing and thickened alveolar septae of the interstitial pneumonia (Van Reeth et al., 1999; Choi et al., 2001; Shibata et al., 2003). Severity and duration of fever depend on PRRSV doses and strains (Halbur et al., 1996, Diaz et al. 2005; Loving et al., 2008).

Clinical Signs

Pigs infected with PRRSV may show different clinical signs depending on PRRSV strains. Pigs infected with the North American and Japanese strain of PRRSV had more intense clinical signs and pathological changes than the European strain of PRRSV. Thanawongnuwech and Thacker (2003) found that pigs infected with the American strain showed labored and accentuated abdominal breathing and increased rate of breathing beginning at d 3 PI, but no coughing was observed. Respiratory disease signs were characterized by transient dyspnoea and tachypnoea when handling pigs for rectal temperature measurement. Additionally, moderate lethargy, anorexia, and rough hair were also observed with the North American strain (Halbur et al., 1996; Suradhat and Thanawongnuwech, 2003; Escobar et al., 2004). The body temperature of pigs infected with a highly virulent Japanese PRRSV strain or North American strain was found to increase (> 40^{o}C) for up to 14 d postinfection (**PI**) or 28 d PI (Thanawongnuwech et al., 2000; Shibata et al., 2003). On the contrary, Van Reeth et al. (1999) found that pigs infected with European strain showed no respiratory signs but anorexia and lethargy for 3 to 5 d PI. The rectal temperature of the infected pigs was also moderately elevated with a peak of just above 39.5^{o}C and lasted for about 7 d PI (Lohse et al., 2004; Diaz et al., 2005).

Changes in Blood Leukocytes

PRRSV infection causes an immunosuppression in pigs by reducing the number of white blood cells (**WBC**) and lymphocytes for about 2 wk PI. Apoptosis of immune cells has been commonly observed in PRRSV-infected pigs during the early stage of infection, as monocytes/macrophages are the common targets for PRRSV infection and replication (Sur et

al., 1998; Choi and Chae, 2002; Labarque et al., 2003). According to Nielsen and B∅tner (1997), total WBC counts and lymphocyte counts were significantly decreased for a few days shortly after infection, but had returned to pre-infection levels on d 8 to 10 PI. Shibata et al. (2000) found that the number of WBC was reduced on d 3 and was recovered on d 7 PI. Shi et al. (2008) reported that the total WBC count in the PRRSV group was significantly lower than the control group on d 7 PI, but became significantly higher than the controls on d 10, 14 and 28 PI. They also showed that compared with the control group, the total number of lymphocytes in the PRRSV group was significantly lower on d 3 and 7 PI, but significantly higher on d 14 and 28 PI. Additionally, the number of B cells was reduced from d 3 to 14 PI (Shi et al., 2008). In short, PRRSV infection causes a transient leukopenia and lymphopenia in peripheral blood that resolves in about 14 d.

Innate and Humoral Responses to PRRSV Infection

The immune responses to PRRSV infection have been shown to be weak because of modulated production of antiviral molecules and inflammatory mediators. Downregulation of IFN-α and other pro-inflammatory cytokines and upregulation of IL-10 have been thought to possibly facilitate PRRSV replication and delay the host's immune response. In viral infections, the presence of double-stranded RNA induces synthesis of antiviral molecules including IFN-α which reduces viral growth (Albina et al, 1998; Le Bon et al., 2001). Levels of IFN-α in the bronchoalveolar lavage fluid (**BALF**) of PRRSV-infected pigs were much lower than in BALF of pigs infected with porcine coronavirus or swine influenza virus (Van Reeth et al., 1999). Apart from IFN-α, other inflammatory cytokine secretion is also important in the initial response to PRRSV. However, different isolates of PRRSV have

different abilities to trigger induction of cytokines including INF-α, TNF-α, IL-10, and IL-12 (Lee et al., 2004; Mateu and Diaz, 2008). For instance, involvement of TNF-α during the early response to PRRSV has been inconsistent. Tumor necrosis factor-α mRNA was increased in lung, alveolar macrophages, and peripheral blood mononuclear cells (Choi et al., 2001; Choi and Chae, 2002; Sipos et al., 2003). In contrast, other researchers found no detectable or low level of TNF-α in the BALF during European PRRSV strain infection (Van Reeth et al., 1999; Labarque et al. 2003). Although suppressing production of pro-inflammatory cytokines, PRRSV increases IL-10 and Hp. Levels of IL-10 mRNA increased in peripheral blood mononuclear cells, BALF cells, and alveolar macrophages (Suradhat and Thanawongnuwech, 2003; Thanawongnuwech and Thacker, 2003; Thanawongnuwech et al., 2004). Earlier studies showed increased levels of serum Hp in PRRSV-infected pigs from d 5 to 21 PI, indicating its important role in mediating the immune response during PRRSV infection (Asai et al., 1999; Diaz et al., 2005; Gnanandarajah et al., 2008).

Pertaining to the development of adaptive immunity, circulating antibodies can be detected as early as 5 to 14 d PI, but the antibodies are not efficient at neutralizing the virus. Anti-PRRSV IgM antibodies appeared in serum 5 to 7 d PI and all infected pigs were seroconverted by d 14 PI (Yoon et al., 1995; Joo et al., 1997). Concentrations of IgG antibodies were found by d 7 to 10 PI and peaked at d 21 to 49 PI (Vezina et al., 1996; Loemba et al., 1996). Serum IgA was detected at d 14 PI, reached a maximum at d 25 PI, and remained detectable until d 35 PI (Labarque et al., 2000). However, these Ig responses did not correspond to neutralizing antibodies (**NA**). Vezina et al. (1996) reported the isolation of PRRSV from the blood of pigs with NA. Following experimental infection, viremia may be resolved without detectable levels of NA (Diaz et al., 2006).

Collectively, the lack of an acute inflammatory response, weak antiviral activity, and inefficient antigen-specific antibodies may contribute to the establishment of persistent infection of PRRSV.

Cell-Mediated Immunity to PRRSV Infection

The changes in lymphocyte subpopulations were evaluated by several researchers. T lymphocytes were reduced during the early stage of infection. Nielsen and B∅tner (1997) found that the percentages of CD2, CD4, and CD8 T cells were significantly decreased, but quickly returned to approximately pre-infection values. The depletion of CD8 T cells was also obtained on d 2 to 5 PI (Lohse et al., 2004). The reduction in lymphocyte populations indicates a weak response to PRRSV, resulting in prolonged PRRSV infection. Lohse et al (2004) argued that CD8 T cell depletion at the early phase of PRRSV infection neither caused increased disease nor influenced the ability to clear the virus in the anti-CD8 monoclonal antibody-treated pigs. According to Xiao et al. (2004), a weak cell-mediated immune response contributes to persistent PRRSV infection and suggests that PRRSV suppresses T-cell recognition of infected macrophages. Thus, the slow but eventual resolution of PRRSV infection may be dependent on limiting permissive macrophages and on innate immune factors. Furthermore, PRRSV-specific T cell responses first appeared in peripheral blood at 4 wk PI (Bautista and Molitor, 1997). In addition, a lower number of circulating natural killer cells was found in the infected pigs for 28 d PI (Shi et al., 2008).

In summary, PRRS is an infectious disease caused by PRRSV and characterized by reproductive disorders in pregnant sows and respiratory problems in growing pigs. The PRRSV predominantly infects macrophages and monocytes in the lung and later spreads out

to other parts of the body. It causes leukopenia and lymphopenia for about 14 d PI and a weak initial innate immune response. Pro-inflammatory cytokine response is reduced and activation of natural killer cells is delayed, but the anti-inflammatory cytokine, IL-10, is increased. Furthermore, induction of acquired immunity is suppressed due to downregulated antigen presentation. There is also a delay in NA response and the NA is not efficient at eliminating the virus. In addition, PRRSV infection reduces the cell-mediated immunity. Apparently, these modulated immune responses may greatly contribute to persistent PRRSV infection.

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CHAPTER 2

MANNAN OLIGOSACCHARIDE REGULATES CYTOKINE PRODUCTION BY ALVEOLAR MACROPHAGES IN NURSERY PIGS

ABSTRACT: Mannan oligosaccharide (**MOS**) and related yeast cell wall derivatives promote growth of nursery pigs, but the underlying mechanisms are not understood. We explored the hypothesis that MOS acts to reduce systemic inflammation in pigs by evaluating cytokine production of alveolar macrophages (**AMϕ**) and serum cytokine concentrations. Pigs were fed diets containing 0.2 or 0.4% MOS for either 2 or 4 wk after weaning compared to control diets without MOS. Alveolar macrophages were collected and stimulated *in vitro* with lipopolysaccharide (**LPS**) or polyinosinic:polycytidylic acid (**Poly I:C**). Lipopolysaccharide-stimulated AMϕ from MOS-fed pigs (n = 12) produced less tumor necrosis factor-α (**TNF-α**) ($P < 0.01$) and more IL-10 ($P = 0.051$) than AMϕ from control-fed pigs (n = 6). Mannan oligosaccharide did not affect cytokine production when AMϕ were stimulated with Poly I:C. There were also no significant differences in serum levels of TNF-α and IL-10, although these levels changed over time. These results establish that feeding MOS to pigs for 2 wk reduces TNF-α and increases IL-10 following *in vitro* treatment of AMϕ with LPS. A similar pattern of cytokine production by AMϕ in response to LPS was found when MOS was directly applied *in vitro*. Mannan oligosaccharide suppressed LPS-induced TNF-α secretion ($P < 0.001$) and enhanced LPS-induced IL-10 secretion ($P < 0.05$). In a similar experiment in which a mannan-rich faction (**MRF**) replaced MOS, TNF-α production by AMϕ stimulated with LPS ($P < 0.05$) or Poly I:C ($P < 0.001$) was suppressed by MRF. These data establish that both MOS and MRF suppress LPS-induced TNF-α

production by AMϕ. To learn if MOS interacts with LPS receptors, AMϕ were cultured with Polymyxin B, an inhibitor of LPS-activated toll-like receptor 4. Although Polymyxin B completely inhibited AMϕ-produced TNF-α induced by LPS, it did not affect the ability of MOS to regulate cytokine production in the absence of LPS. When added *in vitro*, both MOS and MRF were also able to regulate constitutive production of TNF-α in the absence of LPS. Collectively, these data establish that MOS is a potent immunomodulator in both *in vitro* and *in vivo* systems as determined by reducing TNF-α and enhancing IL-10 synthesis after *ex vivo* challenge of porcine AMϕ with bacterial endotoxin.

INTRODUCTION

Mannan oligosaccharide (**MOS**), derived from the cell wall of yeast *Saccharomyces cerevisiae*, is a growth promoter in young pigs and poultry (Hooge, 2004a,b; Miguel et al., 2004). The improvement in animal performance may be due in part to the ability of MOS to inhibit attachment of pathogens with Type I fimbriae to the intestinal wall of animals (Oyofo et al., 1989a,b; Spring et al., 2000). The unattached harmful bacteria are taken with digesta to the large intestine and then excreted in feces, resulting in a healthy gut. Effect of MOS on innate and humoral immunity has been reported. The MOS enhanced the activity of phagocytes *in vitro* in a dose-dependent manner (Newman, 1995) and reduced the intensity of the wattle hypersensitivity reaction in pullets (Cotter and Weinner, 1997). In addition, feeding MOS to animals increased the immunoglobulin levels in their plasma, bile, and colostrum (Savage et al., 1996; Newman and Newman, 2001). These results show that MOS affects immune function, but the specific effects require clarification. In particular, it is important to know the impact of MOS on secretion of cytokines under various conditions.

Cytokines that are synthesized by cells of the innate immune system, such as macrophages, not only regulate immune function but also alter many metabolic processes (Johnson, 1997; Spurlock, 1997; Elsasser et al., 2008). Therefore, a balance between pro-inflammatory (e.g. IL-1, IL-6, tumor necrosis factor-α), and anti-inflammatory (e.g. IL-10) cytokines plays an important role in growth responses and in maintaining the appropriate and efficient activity of the immune system in response to surrounding immunological challenges.

The objectives of the study were (1) to determine whether MOS, when applied *in vitro* or fed to pigs, can modulate cytokine production by alveolar macrophages (**AMϕ**) stimulated with models of bacterial or viral infection and (2) to evaluate effects of dietary levels of MOS on serum cytokines and growth performance of nursery pigs.

MATERIALS AND METHODS

Pig Feeding Experiment

The experimental protocol used in this study was approved by the University of Illinois Institutional Animal Care and Use Committee. One hundred and sixty barrows and gilts from C-22 females mated to PIC line 337 boars, about 20 d old and 6.5 ± 1.1 kg BW were blocked by BW and randomly allotted to 5 treatments in a randomized complete block design. Each pen within a block had an equal distribution of males and females. Ancestry was equalized within treatment as much as possible. Immediately upon entering the nursery facility after weaning (d 0), pigs were fed the 5 experimental diets: 0% MOS supplementation as the control diet (1), 0.2% MOS through d 14 (2) or d 28 (3) postweaning (**PW**), 0.4% MOS through d 14 (4) or d 28 (5) PW. Mannan oligosaccharide was provided by Alltech, Inc. (Nicholasville, KY). There were 8 replicate pens per treatment and 4 pigs per

pen. Pigs were housed in an environmentally controlled nursery and had *ad libitum* access to feed and water at all time. Each pen measured 1.32 x 1.32 m in size with metal slatted floor and had one nipple waterer. Pigs were fed the basal diets (Table 2.1) formulated to contain the levels of all essential nutrients which met or exceeded the nutritional requirements of pigs during the nursery period (NRC, 1998).

A pig's BW was recorded at weaning (d 0) and on d 7, 14, 21, and 28 PW, and feed disappearance was measured each wk, for calculation of ADG, ADFI, and G:F for each pen.

At 14 d PW, one pig from each of 6 pens per treatment was slaughtered for collection of AMϕ, which were stimulated *in vitro* with LPS and Poly I:C as described below and concentrations of TNF-α and IL-10 in the supernatants were measured.

Blood Collection and Processing

Blood samples were collected at d 7, 14, 21, and 28 after the commencement of the experiment. One pig per replicate pen (8 pigs per treatment) was sampled, and blood samples were taken from the same pig throughout the experiment. Ten milliliters of blood from each pig were collected into glass tubes containing no anticoagulant. Blood was allowed to clot at room temperature and stored overnight at 4^{o}C before harvest of serum by centrifugation. The collected serum was frozen at -80^{o}C, and later analyzed for TNF-α and IL-10 by ELISA kits as described below.

Collection and Isolation of AMϕ

Alveolar macrophages were collected from 5 to 6-wk-old donor pigs not fed experimental diets or from pigs fed diets with different levels of MOS for 2 wk PW. Pigs

were anesthetized by intramuscular injection of a 1-mL combination of telazol, ketamine, and xylazine (2:1:1) per 23.3 kg BW. The final mixture contained 100 mg telazol, 50 mg ketamine, and 50 mg xylazine in one mL (Fort Dodge Animal Health, Fort Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway) per 1 kg of BW (Henry Schein, Inc., Indianapolis, IN).

Alveolar macrophages were collected by pulmonary lavage with 150 mL of PBS without Ca and Mg. Lavage fluid was filtered through a double layer of sterile gauze and then centrifuged at 400 x g for 15 min at room temperature. After centrifugation, lung lavage cells were washed twice with Hank's balanced salt solution and re-suspended in 5 mL of Roswell Park Memorial Institute 1640 culture medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The percentage of live cells was determined by Trypan Blue dye exclusion (Sigma-Aldrich Co., St Louis, MO), and the cells were adjusted to 1 x 10^6 cells/mL. The viability was > 97%. We used the term "alveolar macrophages" throughout this paper because the majority (93 to 97.5%) of bronchoalveolar lavage fluid cells is macrophages (Shibata et al., 1997; Dickie et al., 2009).

Culture and Stimulation of AMϕ

Alveolar macrophages were cultured in 96-well plastic tissue culture plates at a density of 1 x 10^5 cells per well and incubated overnight at 37^{o}C in a humidified 5% CO_2 incubator to allow AMϕ to adhere to the plates. The plates were washed 3 times with warm Hank's balanced salt solution to remove non-adherent cells. The adhered AMϕ were stimulated with 200 μL of yeast cell wall components or microbial stimulators as described below. The stimulated AMϕ were then incubated for 24 h more before the collection of

supernatants, which were frozen at -80^{0}C until measurement of tumor necrosis factor (**TNF)**-α and IL-10 concentrations.

The adhered AMϕ were treated in triplicates with substances to be tested, including MOS, glucan fraction (**GluF**), mannan-rich fraction (**MRF**), lipopolysaccharide (**LPS**), and polyinosinic:polycytidylic acid (**Poly I:C**). Irradiated MOS, GluF, and MRF were obtained from Alltech, Inc. (Nicholasville, KY). The GluF and MRF were also extracted from the yeast cell wall, but contained more β-glucan and mannose, respectively, than MOS. The sugar profile of the yeast components is presented in Table 2.2. The LPS and Poly I:C, purchased from Sigma-Aldrich Co. (St. Louis, MO), have been widely used as models to imitate the acute phase responses to bacterial and viral challenges, respectively.

Tests of Activity of Yeast Components In Vitro

There were 4 assays, in which replicates were untreated donor pigs. In assay 1, AMϕ were activated with increasing concentrations of MOS, GluF, or MRF, ranging from 0 to 3 mg/mL. The concentration at which the greatest TNF-α level was determined was used in subsequent assays. A pre-assay using AMϕ stimulated with varying levels of LPS or Poly I:C was also conducted. The TNF-α response of AMϕ to those stimulators peaked at 1 μg/mL of LPS, and 50 μg/mL of Poly I:C, which were used in the following assays. In assay 2, AMϕ from 6 donor pigs were stimulated with control (medium), MOS, LPS, Poly I:C, MOS plus LPS, and MOS plus Poly I:C. The AMϕ were pre-incubated with control or MOS for 30 min before the medium, LPS, or Poly I:C were added into the cultures to stimulate AMϕ. Assay 3 was identical to assay 2 but used MRF instead of MOS. In assay 4, to learn whether MOS interacts with LPS receptors, AMϕ were cultured *in vitro* in the presence (30 μg/mL) or

absence (0 µg/mL) of Polymyxin B (**PMB**, an anti-inflammatory agent) for 30 min, and then stimulated with MOS, GluF, or LPS. The concentration of PMB used was pre-determined to significantly inhibit LPS-induced TNF-α.

Measurement of Cytokines

Tumor necrosis factor-α and IL-10 levels in serum and in supernatants from AMϕ incubations were measured in duplicate by ELISA kits specific for porcine TNF-α and IL-10 (R & D Systems, Minneapolis, MN). Standards of known recombinant porcine TNF-α and IL-10 concentration were used. The minimum detectable dose of porcine TNF-α by the assay was 3.7 pg/mL. The intra- and inter-assay coefficients of variation were < 6.9% (6.2% for supernatant) and < 9.2% (10% for supernatant), respectively. The minimum detectable dose of porcine IL-10 by the assay was 3.5 pg/mL. The intra- and inter-assay coefficients of variation were < 4.2% and < 7.2%, respectively. The results were expressed in picograms per milliliter based on a standard curve.

Statistical Analyses

Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, NC). For the pig-feeding experiment, performance data were analyzed as a randomized complete block design. Pens were considered the experimental unit and the model included the effects of block and dietary treatment. The serum cytokines were analyzed as repeated measures over time on each individual pig as an experimental unit. The model included the effects of dietary treatment, day, and dietary treatment x day interaction. For cytokine concentrations in supernatants, pigs were the experimental units and the model

included the effects of dietary treatment. Data from pigs fed 0.2% MOS at d 14 PW were pooled for that treatment. Similarly, data from pigs fed 0.4% MOS at d 14 PW were pooled for that treatment. For *in vitro* assays 1, 2, 3, & 4, donor pigs were considered blocks and a pool of 3 wells was considered an experimental unit. For assay 1, the model included the effects of block and concentration of yeast components. For assay 2, the model included the effects of block, MOS, stimulant, and MOS x stimulant interaction. For assay 3, the model included the effects of block, MRF, stimulant, and MRF x stimulant interaction. For assay 4, the model included the effects of block, PMB, stimulant, and PMB x stimulant interaction. Treatment differences were compared using the least squares means procedure of SAS.

RESULTS

Supernatant and Serum Cytokines in Response to MOS Feeding

Supernatant concentrations of TNF-α associated with LPS-stimulated AMϕ from pigs fed the MOS diets were lower than ($P < 0.01$) those of AMϕ from pigs fed the control diet (Figure 2.1). No differences were observed between 0.2% and 0.4% MOS diets. The LPS-stimulated AMϕ from MOS-fed pigs produced more IL-10 than those from control-fed pigs ($P = 0.051$).

There was no effect of MOS supplementation on serum TNF-α or IL-10 throughout the experiment (Figure 2.2). The MOS x day interaction was found not to be significant. However, there was a significant effect of day on serum TNF-α and IL-10. The TNF-α levels at d 7 and 28 PW were much greater than those at d 14 and 21 PW ($P < 0.001$). The IL-10 levels at d 14 and 28 PW were greater than those at d 7 and 21 PW ($P < 0.001$).

Growth Performance

MOS supplementation had no effect on ADG, ADFI, and G:F during phases 1, 2, or 3 or over the entire experiment (Table 2.3). Feeding MOS to pigs for either 2 or 4 wk PW, regardless of dietary level, did not clearly affect ADG, ADFI, and G:F.

Modulation of LPS- or Poly I:C-Induced Cytokine Production by MOS and MRF

The TNF-α production was increased by MOS, LPS, or Poly I:C ($P < 0.01$; Figure 2.3). However, the response to MOS was much lower than the response to LPS or Poly I:C ($P < 0.01$). Additionally, in cultures stimulated with LPS, TNF-α was significantly reduced in the presence of MOS ($P < 0.001$). In contrast to the decrease in TNF-α, LPS-induced IL-10 was increased in the presence of MOS ($P < 0.05$). The MOS did not affect TNF-α or IL-10 production of Poly I:C- induced AMϕ.

The TNF-α production induced by LPS or Poly I:C was far greater than that induced by the control or MRF ($P < 0.001$; Figure 2.4), which were not different from each other. There was a significant reduction of TNF-α in cultures stimulated with either LPS ($P < 0.05$) or Poly I:C ($P < 0.001$) in the presence of MRF. Similar to MOS, MRF did not affect IL-10 production of AMϕ induced by Poly I:C.

Direct Cytokine Production with Different Stimulators

The TNF-α concentrations in supernatants of AMϕ activated by MOS (458 pg/mL) or GluF (376 pg/mL) were greatest ($P < 0.01$) at the concentration of 0.5 mg/mL and decreased as the concentrations of those stimulators were increased (Figure 2.5). When activated with MRF, AMϕ produced much less TNF-α (164 pg/mL) and a higher ($P < 0.01$) concentration

(2.5 mg/mL) of MRF was required. The level of each yeast component used range from 0 to 3 mg/mL because MRF at 2.5 mg/mL was the optimal level, not being toxic to macrophages, but stimulating cell proliferation (Singboottra, 2005).

Polymyxin B Inhibited TNF-α Induced by LPS, But Not by MOS

To learn if MOS interacts with LPS receptors, PMB (30 μg/mL) was added to cultures. Polymyxin B is a potent antibiotic that binds to LPS and neutralizes its pro-inflammatory effect. The TNF-α production of AMϕ induced by GluF (0.5 mg/mL) or LPS (1 μg/mL) was substantially inhibited (> 95.0%) by PMB treatment ($P < 0.001$; Figure 2.6). In contrast, TNF-α production induced by MOS (0.5 mg/mL) in the presence of PMB was reduced by only 4.2%. This result indicated that MOS did not interact with LPS receptors and MOS-stimulated TNF-α was not due to contamination with endotoxin because PBM reduced TNF-α induced by LPS, but not by MOS.

DISCUSSION

Previous studies have shown that MOS increase phagocytic activity and humoral immunity in animals (Newman, 1995; Savage et al., 1996; Newman and Newman, 2001). These data suggest that MOS may have direct effects on cytokine responses of phagocytes/monocytes. In our present study, it was found that MOS, whether fed to nursery pigs for 2 wk PW or applied directly *in vitro*, reduced pro-inflammatory cytokine production by AMϕ in response to *in vitro* LPS stimulation, but not clearly to Poly I:C (Figures 2.1 and 2.3). In particular, the LPS-stimulated AMϕ produced less TNF-α and more IL-10 when MOS was applied. The mechanism for this immune response was unknown, but may be

associated with the ability of MOS to modulate cell receptors-induced responses by binding to mannose receptor (**MR**). The MOS contains a high amount of mannan (as mannose, Table 2.2), which can be recognized by MR (Giaimis et al., 1993; Underhill and Ozinsky, 2002). Mannose receptor activated by MOS can interfere regular functions of other cell receptors, e.g. toll-like receptor (**TLR**) 2 or TLR4, leading to reduced production of pro-inflammatory cytokines and enhanced IL-10 (Gazi and Martinez-Pomares, 2009). In short, MOS *in vivo* and *in vitro* suppresses TNF-α and increases IL-10 production following *in vitro* treatment of AMϕ with LPS, but the mechanism for the reduced inflammatory response of AMϕ needs further investigation.

Unlike MOS, MRF appeared not to greatly induce TNF-α production by AMϕ, but suppressed the TNF-α secretion by AMϕ stimulated with either LPS or Poly I:C (Figure 2.4). Downregulation of TLR4 and MR would possibly contribute to this inhibitory effect. These receptors, if activated, trigger an intracellular signal transduction cascade, resulting in changes in cytokine synthesis. Singboottra et al. (2006) found that reduced IL-6 expression was mediated through a decreased expression of TLR4. As LPS is a ligand specific for TLR4 (Raetz and Whitfield, 2002), this could explain why there was a lowered TNF-α level in LPS-stimulated AMϕ in the presence of MRF. With respect to the suppression of AMϕ response to Poly I:C, the effect of MRF on Poly I:C-induced TNF-α is not clearly understood, but appears to be associated with the high level of mannan in MRF. The binding of Poly I:C to its specific receptor, TLR3, leads to AMϕ activation (Huang et al., 2006; Loving et al., 2006); however, TLR3 is an intracellular receptor, and thereby Poly I:C needs to be taken up by AMϕ before inducing an inflammatory response through binding to TLR3. The MR, which can recognize mannan, was suggested to be a major endocytic receptor in the

infectious entry of viruses into murine macrophages (Tizard et al, 1989; Reading et al., 2000; Davis et al., 2002). Therefore, it may be speculated that MR ligation by MRF was not significantly responsible for TNF-α release and it reduced Poly I:C-induced TNF-α by preventing MR-mediated internalization of Poly I:C because of downregulated MR expression or reduced endocytic activity of MR. Thus, the amount and structure of mannan in MRF would determine the intensity of inflammation induced by Poly I:C.

Although the immunomodulatory property of MOS has been shown as discussed above, little is known about its effect on serum cytokine concentrations in MOS-fed pigs. Cytokines not only regulate the body's immune response but also affect nutrient metabolism (Johnson, 1997; Spurlock, 1997). Therefore, this study assessed serum concentrations of TNF-α and IL-10 in association with dietary levels and feeding time of MOS. There were significant variations in serum concentrations of TNF-α and IL-10 during the course of study (Figure 2.2). This revealed that pigs exposed to the PW environment had physiologically altered levels of inflammatory mediating cytokines, so secretion of those mediators at a specific time is very critical. Activation of the immune system is tightly regulated through several mechanisms in order to maintain disease resistance and prevent immune-mediated disorders within the host. The high concentrations of IL-10 following or along with the raised levels of TNF-α may bring out the important role of this cytokine in regulation of immune and inflammatory responses. The IL-10 has been shown to suppress TNF-α production and other cytokines and chemokines to maintain the homeostasis of the immune system (Turnbull and Rivier, 1999; Moore et al., 2001). In summary, serum levels of TNF-α and IL-10 intermittently increase during the experimental period, but no clear impact of MOS on those cytokines could be substantiated.

Addition of MOS to diets in this study showed no influence on growth performance of nursery pigs (Table 2.3). Growth performance responses to MOS supplementation are variable, being especially related to growth rate of pigs (Miguel et al., 2004). The meta-analysis of Miguel et al. (2004) suggests that MOS has little or no response in pigs with high growth rate (> 180 g/d) during the first 1 to 2 wk PW. The high growth rate of weaned pigs in the present study would possibly explain why no effect of MOS on growth was statistically detected.

Further, we found that TNF-α production by AMϕ was altered differently by various yeast cell wall components. The GluF and MOS can directly induce AMϕ to secrete TNF-α. The stimulating effect of GluF is perhaps because GluF contains a high amount of β-glucan (Table 2.2). It was reported that macrophages stimulated with β-glucan produced nitric oxide, interferon-γ, TNF-α, IL-1, and IL-6 (Adachi et al., 1994; Ohno et al., 1996; Tokunaka et al., 2000). With regard to MOS, activation of AMϕ by MOS *in vitro* (Figures 2.3 and 2.5) may be associated with both β-glucan and mannan; the contribution of mannan to the immunostimulatory effect of MOS will be discussed in the next paragraph. This finding partially supports the fact that MOS enhances the phagocytic activity (Newman, 1995). Activated phagocytes secrete cytokines which in turn upregulate the expression of molecules involved in phagocytosis and digestion of ingested particles or microbes (Moore et al., 2001; Underhill and Ozinsky, 2002). In contrast to MOS and GluF, MRF seemed not to induce TNF-α production because our data were inconsistent (Figures 2.4 and 2.5). The discrepancy in immunostimulatory characteristics among mannan-containing products can be ascribed to a polymerization degree of mannan (Bland et al., 2004), types of terminal linkages of mannan sequences (Young et al., 1998), or types of mannan (Djeraba and Quere, 2000;

Sheng et al. 2006). Collectively, the results of this study suggest that MOS can directly stimulate AMϕ, but it was not sure that this would occur *in vivo*. Also, GluF, but perhaps not MRF, is a potent immunostimulant of AMϕ.

The immunostimulatory effect of MOS was not due to its interaction with LPS receptors as well as endotoxin contamination and appeared to be different from that of GluF. This was demonstrated by using PBM which indirectly inhibit the activation of LPS-mediated TLR4 by binding to LPS (Zavascki et al., 2007). First, PMB significantly inhibited TNF-α production induced by LPS, but not by MOS (Figure 2.6). Polymyxin B not only is an effective antibiotic against gram-negative bacteria (Arnold et al., 2007; Zavascki et al., 2007), but it also has the ability to suppress TNF-α secretion stimulated by an endotoxin, particularly LPS (Stokes et al., 1989; Cardoso et al., 2007). Second, if it was assumed that β-glucan in both MOS and GluF was the only immunostimulatory agent involved in the activation of AMϕ, TNF-α production induced by MOS or GluF could have been suppressed by PMB. However, lack of inhibitory effects of PMB on MOS-induced TNF-α unveiled that mannan molecules, apart from β-glucan, in MOS may play a significant role in stimulating AMϕ to secrete TNF-α. This further implied that the property of mannan in MOS was different from that in MRF because MRF did not considerably influence TNF-α production. In addition, GluF seemed to act like LPS which activates AMϕ by attaching to TLR4. It was reported that GluF upregulated the expression of TLR4 and dectin-1 in porcine AMϕ (Chaung et al., 2009).

In conclusion, the interaction between mannan and β-glucan of tested products and receptors of AMϕ may be the key factors affecting the immune responses of AMϕ. The content, type, and structure of constituent carbohydrates of yeast cell wall extracts are

important in activating AMϕ through binding to their pattern recognition receptors such as TLR4 and MR. The expression of those receptors, along with the immune activation status of AMϕ, would influence the transmission of a specific signal that results in activation of downstream signaling cascades, and thereby the production of inflammatory mediators.

Mannan oligosaccharide is a potent immunomodulator. The ability of MOS to activate AMϕ directly has important implications in the physiological mechanisms of host defense against invading bacteria or viruses. Mannan oligosaccharide *in vivo* and *in vitro* alleviated the inflammatory responses of AMϕ induced *in vitro* by bacterial endotoxin. Thus, MOS may prevent overshooting of the host animal's immune system and help pigs quickly recover after an immunological challenge. Further research, however, should be conducted to determine effects of MOS on immune function and growth performance in pigs challenged with actual bacterial or viral infections.

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FIGURES AND TABLES

Table 2.1 Composition of basal diets fed to nursery pigs during the experiment (as-fed basis)

Item	Phase[1]		
	I	II	III
Ingredients, %			
Corn	38.11	42.82	52.45
Dried whey	22.00	16.00	10.00
Soybean meal, 48%	10.00	18.00	24.00
Spray-dried animal plasma	8.00	4.00	0.00
Soy protein concentrate[2]	5.00	3.00	0.00
Select menhaden fish meal	4.01	5.49	6.25
Soybean oil	3.61	4.09	4.07
Lactose	5.60	2.80	0.00
Limestone	0.85	0.56	0.55
Dicalcium phosphate	0.67	0.92	0.48
Carbadox premix[3]	1.00	1.00	1.00
Zinc oxide	0.42	0.42	0.42
Mineral premix[4]	0.35	0.35	0.35
Vitamin premix[5]	0.20	0.20	0.20
Lysine-HCl	0.07	0.16	0.22
DL-met	0.11	0.10	0.08
L-thr	0.01	0.09	0.14

Table 2.1 (cont.)

Item	Phase[1]		
	I	II	III
Calculated composition			
ME, Mcal/kg	3.45	3.45	3.45
Standardized ileal digestible AA, %			
Lys	1.45	1.45	1.30
Met	0.41	0.43	0.42
Thr	0.94	0.94	0.84
Tryp	0.29	0.27	0.23
Val	1.10	1.03	0.89
Ile	0.87	0.87	0.79
Ca, %	0.90	0.90	0.80
Available P, %	0.55	0.55	0.40
Lactose, %	21.00	14.00	7.00

[1]Phase I, II, and III diets were fed to nursery pigs for 7, 7, and 14 d postweaning, respectively.
[2]Soycomil, Archer Daniels Midland Company, Decatur, IL.
[3]Mecadox 2.5, provided 0.055 g of carbadox per kilogram of diet, Phibro Animal Health, Fairfield, NJ.
[4]Provided as milligrams per kilogram of diet: sodium chloride, 3,000; zinc, 100 from zinc oxide; iron, 90 from iron sulfate; manganese, 20 from manganese oxide; copper, 8 from copper sulfate; iodine, 0.35 from calcium iodide; selenium, 0.30 from sodium selenite.
[5]Provided per kilogram of diet: retinyl acetate, 2,273 µg; cholecalciferol, 17 µg; DL-α-tocopheryl acetate, 88 mg; menadione sodium bisulfate complex, 4 mg; niacin, 33 mg; D-Ca-pantothenate, 24 mg; riboflavin, 9 mg; vitamin B_{12}, 35 µg; choline chloride, 324 mg.

Table 2.2 Sugar profiles of yeast components used in the study (as-is basis)

Item, %	MOS[1]	MOS[2]	GluF[3]	MRF[4]
Starch	5.75	6.35	17.06	0.98
β-glucans	34.44	29.85	66.75	15.99
Ribose	0.97	1.15	0.00	0.75
Fucose	0.00	0.00	0.00	0.00
Arabinose	2.80	0.00	0.00	0.00
Xylose	4.46	0.52	0.00	0.00
Mannose	25.59	26.58	5.68	37.90
Glucose	44.66	40.22	3.12	18.86
Galactose	3.80	3.38	4.55	2.25

[1]Bio-Mos, a commercial product that is included in pig diets; MOS: mannan oligosaccharide.
[2]A Bio-Mos preparation used in the *in vitro* assays; MOS: mannan oligosaccharide.
[3]GluF: glucan fraction.
[4]MRF: mannan-rich fraction.

Table 2.3 Growth performance of pigs fed diets with different levels of mannan oligosaccharide (MOS) for 2 or 4 weeks after weaning[1]

Item	Dietary supplementation of MOS, %					SEM
	0.0	0.2/0.0[2]	0.2/0.2[3]	0.4/0.0[2]	0.4/0.4[3]	
d 0 to 14						
ADG, g	274	250	243	252	268	16.3
ADFI, g	327	285	283	286	297	16.7
G/F, g/kg	852	883	855	873	901	19.0
d 14 to 28						
ADG, g	527	533	523	490	500	21.5
ADFI, g	746	720	710	700	685	27.8
G:F, g/kg	714	741	740	699	729	17.6
d 0 to 28						
ADG, g	400	392	383	371	384	16.2
ADFI, g	536	502	497	493	491	17.8
G:F, g/kg	783	812	798	786	815	14.5

[1]Data were means of 8 replicate pens with 4 pigs each. Pigs averaged 20 d of age and 6.5 ± 1.1 kg of initial BW.
[2]MOS was supplemented at 0.2% or 0.4% of the diet from d 0 to 14 after weaning, and pigs were fed the control diet from d 14 to 28 after weaning.
[3]MOS was supplemented at 0.2% or 0.4% of the diet from d 0 to 28 after weaning.

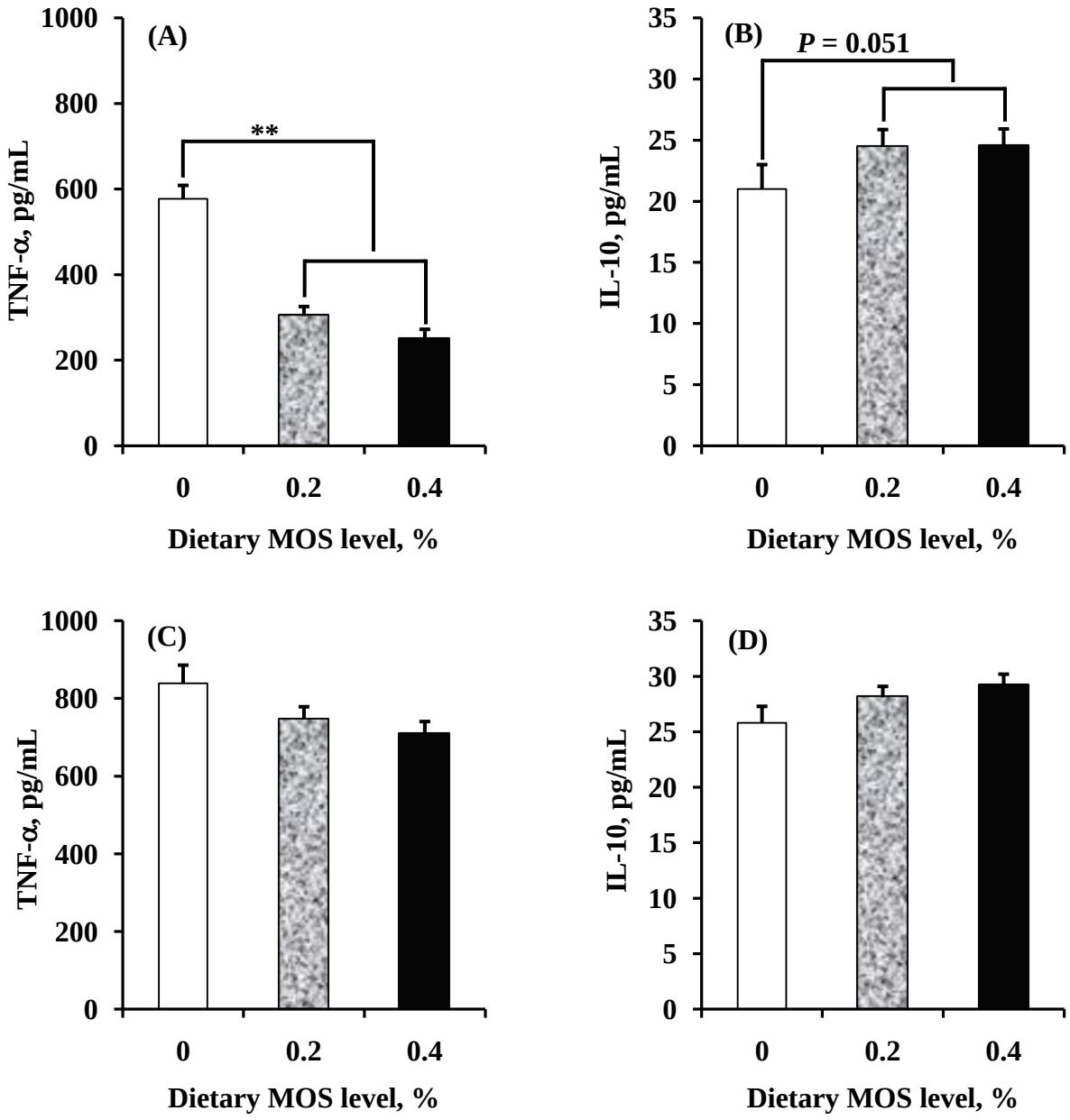

Figure 2.1 Supernatant cytokine production by alveolar macrophages (AMϕ) from pigs fed diets with 0%, 0.2%, and 0.4% mannan oligosaccharide (MOS) supplementation. (A) & (B) lipopolysaccharide-stimulated AMϕ from pigs (n = 12) fed MOS diets, regardless of inclusion levels, produced significantly less ($P < 0.01$) tumor necrosis factor (TNF-α) and more IL-10 ($P = 0.051$) than those from pigs (n = 6) fed the control diet. (C) Polyinosinic:polycytidylic acid-stimulated AMϕ from pigs fed MOS diets, regardless of inclusion levels, tended to produce less TNF-α ($P < 0.1$) than those from pigs fed the control diet. (D) There was no effect of dietary MOS on IL-10 production by polyinosinic:polycytidylic acid-stimulated AMϕ. The concentrations of lipopolysaccharide and polyinosinic:polycytidylic acid used were 1 μg/mL and 50 μg/mL, respectively. Data were means ± pooled SEM. ** $P < 0.01$.

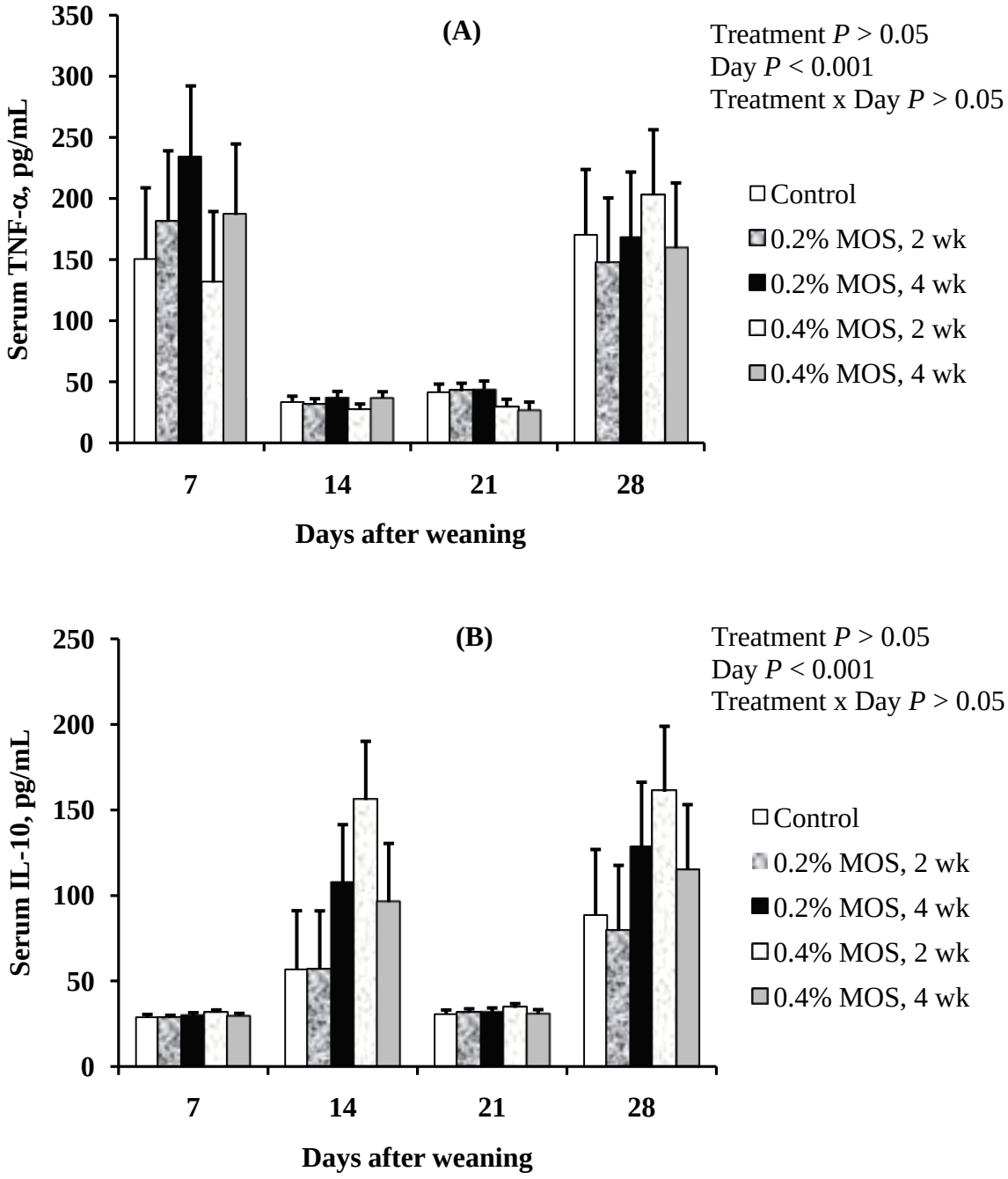

Figure 2.2 Serum tumor necrosis factor-α (TNF-α, A) and IL-10 (B) levels of pigs fed diets with 0%, 0.2%, and 0.4% mannan oligosaccharide (MOS) supplementation for 2 or 4 weeks after weaning. The TNF-α levels at d 7 and 28 postweaning were greater than those at d 14 and 21 ($P < 0.001$). The IL-10 levels at d 14 and 28 postweaning were greater than those at d 7 and 21 ($P < 0.001$). Data were means ± pooled SEM (n = 8).

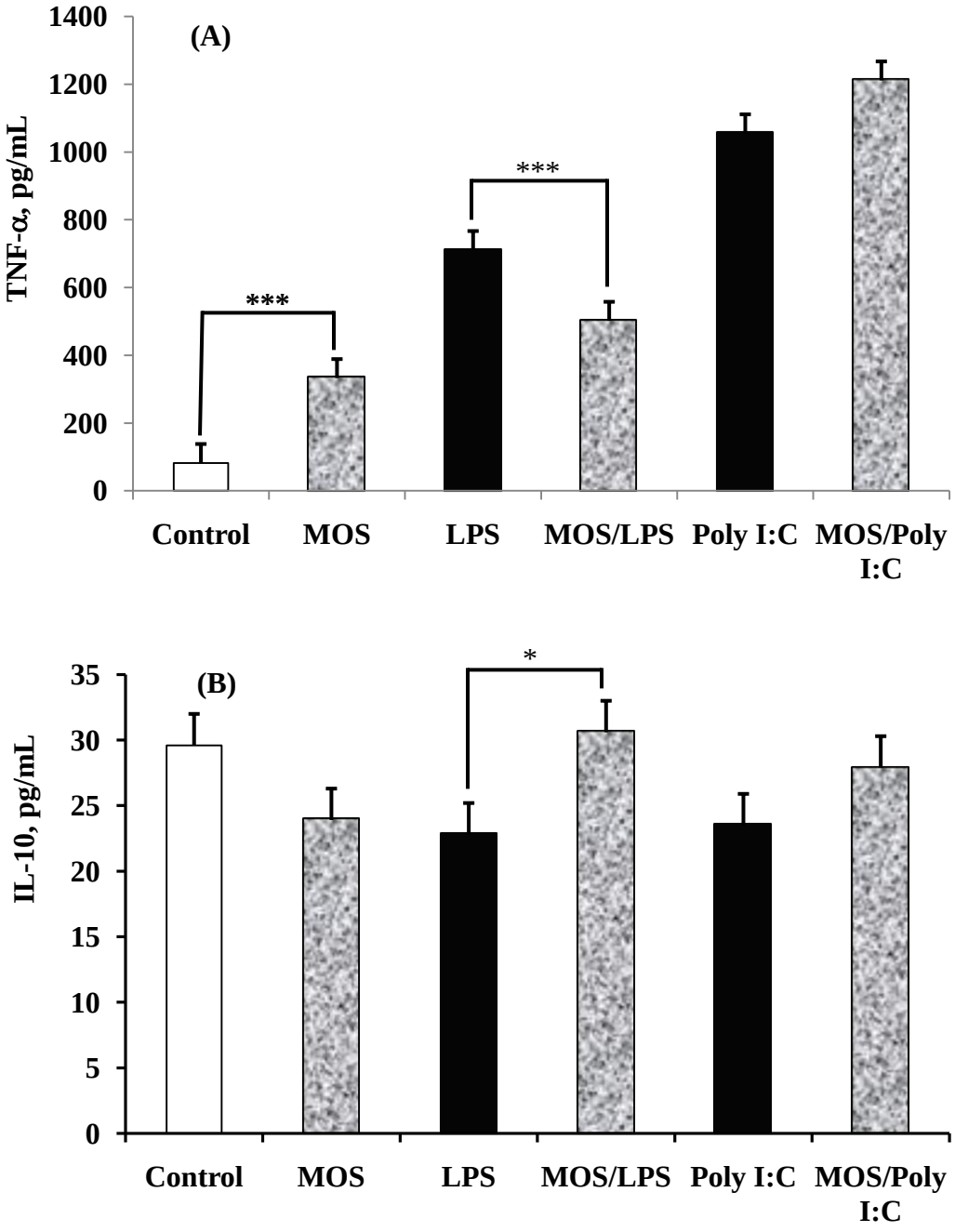

Figure 2.3 Tumor necrosis factor-α (TNF-α) or IL-10 production by alveolar macrophages stimulated *in vitro* with lipopolysaccharide (LPS, 1 µg/mL) or polyinosinic:polycytidylic acid (Poly I:C, 50 µg/mL) in the presence (0.5 mg/mL) or absence (0 mg/mL) of mannan oligosaccharide (MOS). (A) MOS activated alveolar macrophages to produce TNF-α ($P < 0.01$), but significantly suppressed LPS-induced TNF-α production ($P < 0.001$). (B) MOS significantly enhanced LPS-induced IL-10 production ($P < 0.05$). Data were means ± pooled SEM (n = 6). * $P < 0.05$, *** $P < 0.001$.

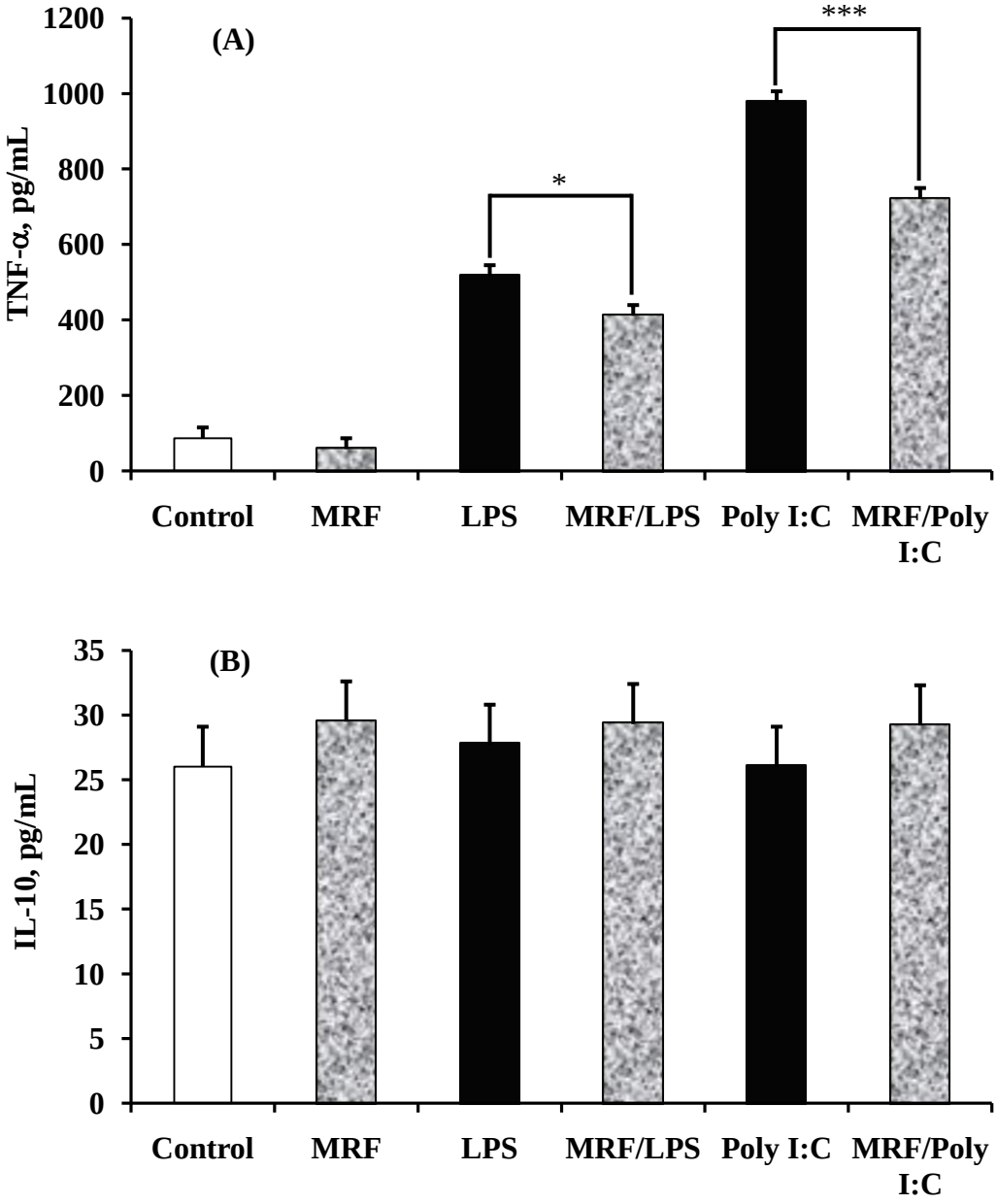

Figure 2.4 Tumor necrosis factor-α (TNF-α) or IL-10 production by alveolar macrophages stimulated *in vitro* with lipopolysaccharide (LPS, μg/mL) or polyinosinic:polycytidylic acid (Poly I:C, μg/mL) in the presence or absence of mannan-rich fraction (MRF, 2.5 mg/mL). (A) MRF significantly suppressed TNF-α production by alveolar macrophages stimulated with LPS ($P < 0.05$) or Poly I:C ($P < 0.001$). There were no differences in TNF-α production between MRF and the control ($P > 0.05$). (B) MRF did not affect LPS- or Poly I:C-induced IL-10 production ($P > 0.05$). Data were means ± pooled SEM (n = 6). * $P < 0.05$, *** $P < 0.001$.

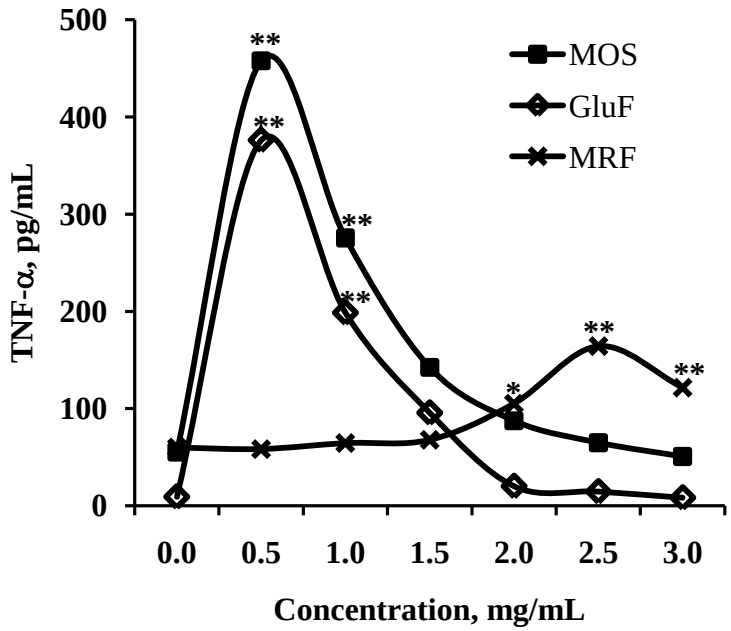

Figure 2.5 Tumor necrosis factor-α (TNF-α) production by alveolar macrophages activated with increasing concentrations of mannan oligosaccharide (MOS), glucan fraction (GluF), or mannan-rich fraction (MRF). The TNF-α response of alveolar macrophages peaked at 0.5 mg/mL MOS ($P < 0.01$), 0.5 mg/mL GluF ($P < 0.01$), and 2.5 mg/mL MRF ($P < 0.01$). Data were means of 4 replicates. * $P < 0.05$ and ** $P < 0.01$: different from the control (within a line).

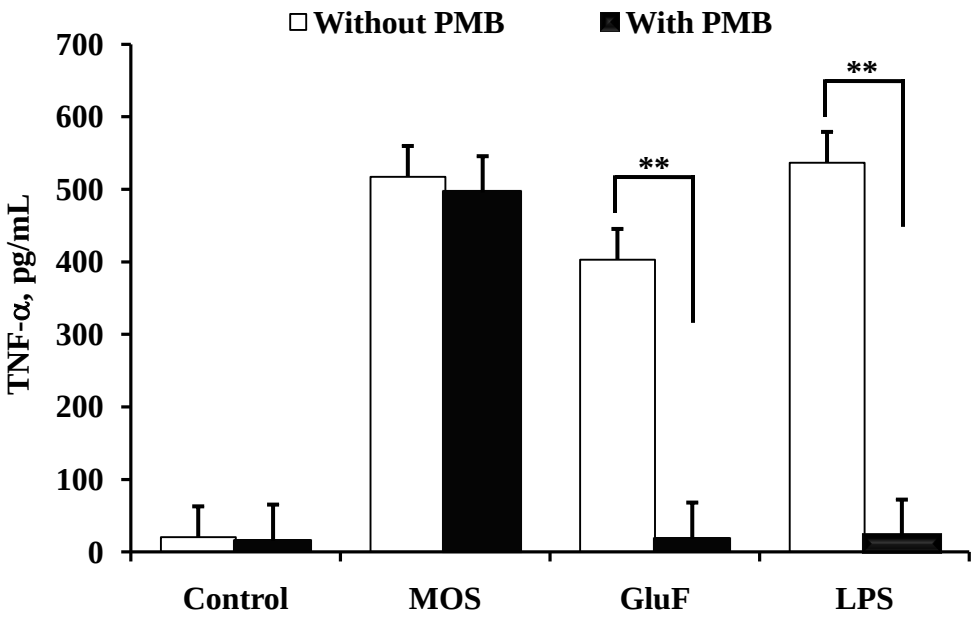

Figure 2.6 Tumor necrosis factor-α (TNF-α) production by alveolar macrophages activated with mannan oligosaccharide (MOS), glucan fraction (GluF), or lipopolysaccharide (LPS) in the presence or absence of Polymyxin B (PMB). Polymyxin B significantly inhibited TNF-α production induced by GluF or LPS ($P < 0.001$), but not by MOS ($P > 0.05$). The concentrations of MOS, GluF, LPS, and PMB used were 0.5 mg/mL, 0.5 mg/mL, 1 µg/mL, and 30 µg/mL, respectively. Data were means ± pooled SEM (n = 6). *** $P < 0.001$.

CHAPTER 3

EFFECTS OF MANNAN OLIGOSACCHARIDE ON IMMUNE RESPONSE AND GROWTH PERFORMANCE IN NURSERY PIGS EXPERIMENTALLY INFECTED WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

ABSTRACT: This study was conducted to determine whether the ingestion of mannan oligosaccharide (**MOS**) alters the immune response of nursery pigs challenged with porcine reproductive and respiratory syndrome virus (**PRRSV**). A total of 64 pigs (3 wk old), free of PRRSV, were used in 2 separate but similar trials conducted sequentially. Pigs were divided into blocks of 4 based on BW, gender, and litter origin. They were randomly assigned from within blocks to one of 4 treatments in a 2 x 2 factorial arrangement [2 types of diet: control (0%) and MOS addition (0.2%); 2 levels of PRRSV: with and without]. There were 8 replicate chambers of 2 pigs each. Pigs fed control or MOS diets for 2 wk were intranasally inoculated with PRRSV or a sterile medium at 5 wk of age. The PRRSV challenge decreased ADG, ADFI, and G:F throughout the experiment ($P < 0.01$). Feeding MOS tended to improve G:F of the pigs during d 0 to 7 ($P < 0.1$) and d 7 to 14 ($P < 0.07$) postinfection (**PI**). Serum levels of tumor necrosis factor (**TNF**)-α, C-reactive protein (**CRP**), and haptoglobin (**Hp**) were increased by PRRSV ($P < 0.01$). The MOS x PRRSV interaction tended to be significant for serum TNF-α at d 14 PI ($P = 0.06$), suggesting that the infected pigs fed MOS had lower TNF-α than those fed the control. Dietary MOS increased serum IL-10 at d 14 PI ($P < 0.05$). Further, MOS-fed pigs had greater numbers of white blood cells (**WBC**, d 3 and 7 PI) and lymphocytes (d 7 PI) than control-fed pigs ($P < 0.05$). In contrast, PRRSV decreased ($P < 0.01$) the numbers of WBC and lymphocytes until d 14 PI. Dietary MOS appeared to

increase the neutrophils in PRRSV-infected pigs, but no MOS x PRRSV interaction was found. Infection with PRRSV increased rectal temperature (**RT**) of pigs at d 3 PI ($P < 0.01$) and continued to affect the infected pigs fed the control diet until d 14 PI. The MOS x PRRSV interaction for RT was found at d 7 ($P < 0.01$) and 10 ($P < 0.06$) PI, indicating that the infected pigs fed MOS had a lower RT than those fed the control. This could explain why feed efficiency was improved by MOS. No effect of treatments on viremia or PRRSV-specific antibody was observed. These results suggest that MOS is associated with rapidly increased numbers of WBC at the early stage of infection and alleviates PRRSV-induced effects on G:F and fever. They also indicate that the reduced intensity of inflammation by MOS may be related to the decrease in serum TNF-α and Hp, and increase in serum IL-10 at the end of acute phase.

INTRODUCTION

Mannan oligosaccharide (**MOS**), extracted from the cell wall of yeast *Saccharomyces cerevisiae*, improves nursery pig performance (Kim et al., 2000; Davis et al., 2002). The improvement in animal performance is thought to be associated with reduced pathogens in the gastrointestinal tract and/or enhanced innate and humoral immunity (Newman, 1995; Savage et al., 1996; Spring et al., 2000). Although pigs' performance response to dietary MOS supplementation is varied, a 4% growth improvement on average has been documented (Miguel et al., 2004).

Pathogenic challenges trigger an inflammatory response characterized by the release of cytokines and other inflammatory mediators (Spurlock, 1997; Elsasser et al., 2008). Pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor (**TNF)-**α are known

to reduce appetite and growth; increase nutrient mobilization from the body; and alter many metabolic processes (Johnson, 1997; Spurlock, 1997). In contrast, anti-inflammatory cytokines like IL-10 are immunosuppressive (Turnbull and Rivier, 1999; Moore et al., 2001). Porcine reproductive and respiratory syndrome virus (**PRRSV**) strongly modulates pigs' immune response. Reduced leukocyte counts, delayed cell-mediated immunity, and inhibited secretion of key cytokines have been demonstrated (Lohse et al., 2004; Wang et al., 2007). Recent studies revealed that MOS is a potent immunostimulant and has the ability to alter cytokine responses of alveolar macrophages under various conditions (Che et al., 2008). Therefore, it is important to know whether dietary MOS also exerts its immunomodulatory effects on pigs' immune response to PRRSV. The objective of this study was to determine whether ingestion of MOS altered nursery pigs' immune response to a PRRSV challenge.

MATERIALS AND METHODS

Experimental Design, Animals, and Housing

Sixty four pigs (3 wk old), free of PRRSV (virology and PCR), were used in 2 separate but similar trials conducted sequentially (32 pigs per trial). Pigs were brought to the experimental site at weaning at 3 wk of age and upon arrival were placed in disease-containment chambers which have been described previously (Escobar et al., 2004). Lincomycin was administered daily via intramuscular injection for 3 d after arrival to prevent infections (11 mg/kg of BW; Pharmacia and Upjohn Co., Kalamazoo, MI).

The pigs were divided into blocks of 4 based on BW, gender, and litter origin. They were randomly assigned from within blocks to one of 4 treatments in a 2 x 2 factorial arrangement (2 types of diet: 0% MOS as the control and 0.2% MOS addition; 2 levels of

PRRSV: with and without). There were 8 replicate pens (or chambers) of 2 pigs each. One castrated male and one female were placed in each chamber. All pigs were housed in a temperature-controlled room with constant lighting and had *ad libitum* access to water and feed. Pigs were fed the experimental diets for 2 wk before being challenged with PRRSV. The basal diets (Table 3.1) were formulated to contain the levels of all essential nutrients which met or exceeded nutritional requirements of pigs during the nursery period (NRC, 1998). Treatment diets were formulated by supplementing the basal diets with 0.2% MOS throughout the experimental period.

Experimental Procedures

The experimental protocol was approved by the University of Illinois Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. The procedures for this study were adapted from the method of Escobar et al. (2004) with modifications. Having been fed the experimental diets for 2 wk, pigs in one-half of the chambers were inoculated intranasally with 2 mL of high-virulence strain of PRRSV (Purdue isolate P-129 containing 10^5 50% tissue culture infective dose). Pigs in the remaining chambers received 2 mL of sterile Dubelco's modified Eagle medium. One-half of the pigs (32 pigs) across the treatments were euthanized at d 7 postinfection (**PI**) of PRRSV and the remainder at the end of the experiment (d 14 PI).

Before being euthanized, pigs were weighed and then blood was sampled for determination of differential leukocyte counts, acute phase proteins (**APP**), cytokines, viral load, and antibody level, followed by measurement of rectal temperature (**RT**). Pigs were then euthanized to collect samples of lung tissue for histopathological assessment.

Body weight was measured weekly until the end of the experiment. Feeders were weighed daily after PRRSV inoculation between 07:00 and 08:30 h so that group ADFI could be determined. Rectal temperature was measured at d 0, 3, 7, 10, and 14 PI.

Collection of Lung Tissue

Pigs were anesthetized by intramuscular injection of a 1-mL combination of telazol, ketamine, and xylazine (2:1:1) per 23.3 kg BW. The final mixture contained 100 mg telazol, 50 mg ketamin, and 50 mg xylazine in one mL (Fort Dodge Animal Health, Fort Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway) per 1 kg of BW (Henry Schein, Inc., Indianapolis, IN). The collected lung tissue was submerged in 10% neutral buffered formalin in a 50-mL conical tube for further analysis.

Blood Collection and Processing

Blood samples were collected from the jugular vein at d 0, 3, 7, and 14 PI. Ten milliliters of blood from each pig was collected into a glass tube containing no anticoagulant. Blood was allowed to clot at room temperature and stored overnight at 4^{o}C before harvest of serum by centrifugation. Serum was analyzed for C-reactive protein (**CRP**), haptoglobin (**Hp**), TNF-α, IL-10 (R & D Systems, Minneapolis, MN), viral load, and antibody titer. A second whole blood sample collected in EDTA tubes (2 mL/pig) was used for determination of differential leukocyte counts.

Determination of Cytokines, CRP, and Hp

Serum levels of TNF-α and IL-10 were measured in duplicate by ELISA specific for porcine TNF-α and IL-10 (R & D Systems, Minneapolis, MN). Standards of known recombinant porcine TNF-α and IL-10 concentration were used. The minimum detectable dose of porcine TNF-α by the assay was 3.7 pg/mL. The intra- and inter-assay coefficients of variation were < 6.9% and < 9.2%, respectively. The minimum detectable dose of porcine IL-10 by the assay was 3.5 pg/mL. The intra- and inter-assay coefficients of variation were < 4.2% and < 7.2%, respectively. Commercially available ELISA kits specific for porcine CRP and Hp were also used (Alpco Diagnostics, Windham, NH). The serum levels were analyzed at 1:2000 and 1:10000 dilutions in duplicate for CRP and Hp, respectively. The intra-assay CV was < 10.0%. The results were expressed in picograms per milliliter based on a standard curve.

Measurement of Differential Blood Leukocytes, PRRSV Antibody, Viremia, and Lung Lesions

Differential leukocyte proportions and concentrations were analyzed on a multiparameter, automated hematology analyzer calibrated for porcine blood (Abbott, Abbot Park, IL). Antibodies against PRRSV were detected by an ELISA method according to the procedures described by the manufacturer (IDEXX, Westbrook, ME). The ELISA sample to positive (**S/P**) ratio was calculated from each serum sample of the infected pigs. An S/P ratio of 0.4 or greater was considered positive. The PRRSV was detected in the serum and the viral load was determined by PCR analysis. From each pig, 4 samples of lung lobes were collected at necropsy for histopathological examination through a microscope. Briefly, the

tissues were fixed in 10% neutral buffered formalin and processed using routine techniques, embedded in paraffin, sectioned at 5 µ and stained with hematoxylin and eosin.

Statistical Analysis

Data were analyzed as a randomized complete block design with a 2 x 2 factorial treatment arrangement by ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, NC). An individual pig was considered the experimental unit for ADG, differential leukocyte counts, serum cytokines, APP, and rectal temperature. For ADFI and G:F, a chamber was considered the experimental unit. Treatment differences were compared using the least squares means procedure of SAS. Within the infected pigs, Student's t-test was performed for assessment of viral load and antibody titer.

RESULTS

Clinical Signs

After inoculation, infected pigs showed first signs of lethargy and anorexia by d 2 PI. A reduction in ADFI was evident from d 2 to d 14 PI (Figure 3.1). Respiratory symptoms such as coughing were not detected. When handling the pigs for collection of blood samples and RT data, the infected pigs often developed patchy dermal cyanosis which lasted for a few minutes. The infection of PRRSV increased the RT of pigs at d 3 PI ($P < 0.01$) and continued to affect the infected pigs fed the control diet until d 14 PI (Figure 3.2). At d 7 PI, there was a PRRSV x diet interaction ($P < 0.01$) for RT, indicating that the infected pigs fed MOS diet (39.9^{o}C) had a lower RT ($P < 0.05$) than those that received the control (40.5^{o}C). The interaction between diet and PRRSV was also found at d 10 PI ($P < 0.06$). The diet

supplemented with MOS lowered the RT of the infected pigs. Additionally, pigs fed the control diet (40.1^{o}C) had a greater RT ($P < 0.01$) at d 14 PI as compared to those fed the MOS diet (39.7^{o}C). All pigs uninoculated with PRRSV were clinically healthy throughout the experimental period.

PRRSV Infection, Antibody Titer, and Lung Lesions

The effectiveness of the PRRSV challenge model was verified by serological and PCR tests. The serological results indicated that the pigs used in the study were free of PRRSV before inoculation. By d 3 PI, the PCR test confirmed that pigs inoculated with PRRSV were PRRSV-positive and those not inoculated with PRRSV were PRRSV-negative. All PRRSV-inoculated pigs remained viremic from d 3 to d 14 PI (Figure 3.3). Mannan oligosaccharide was not found to affect the viral load in the infected pigs at d 3, 7, or 14 PI. All pigs that were not inoculated with PRRSV remained PRRSV-free throughout the study. The PRRSV-specific antibody was detected in the infected pigs at d 7 PI (Figure 3.3), but only 7 out of 16 pigs fed the control diet and 8 of those fed the MOS diet were considered positive (an S/P ratio ≥ 0.4). The antibody titers of all infected pigs at d 14 PI were positive and greater than those at d 7 PI. The antibody titers were not different between the infected pigs fed the MOS diet and those fed the control diet at d 7 and 14 PI. Within the infected pigs with positive S/P ratios, the MOS-fed pigs had an S/P ratio of 0.65, whereas the control-fed pigs had an S/P ratio of 0.54 at d 7 PI ($P < 0.14$).

Further measurements of lung histopathology were done at d 7 and 14 PI to assure that PRRSV inoculation resulted in the expected lesions. Lung lesions typical of PRRSV infection were induced in 6 of 8 inoculated pigs by d 7 PI and 8 of 8 pigs by d 14 PI. The

PRRSV-positive pigs had lesions, whereas the PRRSV-negative pigs remained lesion-free (Figures 3.4 & 3.5). Within the infected pigs, no differences in histological appearance of the lungs were observed between pigs which received the MOS diet and those fed the control. All PRRSV-infected pigs had thickened alveolar septae typical of the interstitial pneumonia of PRRSV. Other changes that were present in all infected pigs were typical of PRRSV, such as alveolar exudation and some macrophage necrosis.

Serum Cytokines and Acute Phase Proteins

There was no effect of dietary MOS supplementation on serum TNF-α or IL-10 before PRRSV inoculation (Figure 3.6). However, serum TNF-α levels of the PRRSV-infected pigs were greater at d 7 and 14 PI than those of the uninfected pigs ($P < 0.01$). There was a MOS x PRRSV interaction ($P = 0.06$) for TNF-α at d 14 PI, indicating that the infected pigs fed the MOS diet had a lower TNF-α level than those fed the control. Serum IL-10 levels of the PRRSV-infected pigs at d 7 PI were greater than those of the uninfected pigs ($P < 0.05$). Pigs fed the MOS diet had greater IL-10 levels at d 14 PI than those fed the control diet ($P < 0.05$).

Two APP, CRP and Hp, were evaluated in this study (Figure 3.7). There were no differences in CRP and Hp before PRRSV inoculation. The CRP and Hp levels of the PRRSV-infected pigs at d 7 and 14 PI were greater than those of the uninfected pigs ($P < 0.01$).

Differential Leukocyte Counts

At d 3 PI, white blood cells (**WBC**), lymphocytes, monocytes, and eosinophils were decreased ($P < 0.05$) in the PRRSV-infected pigs (Table 3.2). Nonetheless, there was no effect of PRRSV infection on the number of neutrophils. Additionally, MOS induced an increase in WBC ($P < 0.05$). The neutrophil to lymphocyte ratio was greatly increased in the PRRSV-infected pigs ($P < 0.01$).

At d 7 PI, the PRRSV-infected pigs still had lower numbers of WBC and lymphocytes as compared to the uninfected pigs ($P < 0.01$), whereas neutrophils were increased ($P < 0.05$) in the PRRSV-infected pigs (Table 3.3). The numbers of WBC and lymphocytes remained greater in pigs fed the MOS diet than those fed the control diet ($P < 0.05$). At the end of the experiment (d 14 PI), WBC, neutrophils, and eosinophils were increased in the PRRSV-infected pigs ($P < 0.01$), but no effect of PRRSV infection on lymphocytes was observed (Table 3.4). The neutrophil to lymphocyte ratio remained high in the PRRSV-infected pigs at d 7 and 14 PI ($P < 0.01$).

Growth Performance

Before PRRSV inoculation, ADG, ADFI, and G:F were found to be insignificant between pigs which were fed the control diet and those fed the MOS diet (data not shown). During d 0 to 14 PI, ADG, ADFI, and G:F were greatly reduced in the PRRSV-infected pigs ($P < 0.01$) (Table 3.5). As noted earlier, PRRSV infection rapidly caused a significant reduction in ADFI ($P < 0.01$) of the infected pigs from d 2 to 14 PI (Figure 3.1). There was no effect of dietary MOS on pig performance, but G:F tended to improve in the MOS-fed pigs from d 0 to 7 PI ($P < 0.1$) and d 7 to 14 PI ($P < 0.07$).

DISCUSSION

Porcine reproductive and respiratory syndrome virus is considered in swine-producing countries worldwide to be a significant cause of multifactorial respiratory disease in young pigs (Albina, 1997; Van Reeth et al., 2002; Thacker, 2006). It is also known to strongly modulate the host's immune responses (Mateu and Diaz, 2008). Alveolar macrophages and monocytes are believed to be the target cells for PRRSV replication *in vivo* (Sur et al., 1998; Choi and Chae, 2002; Labarque et al., 2003). Our previous study showed that MOS, when fed to nursery pigs or applied *in vitro*, ameliorated the cytokine production of alveolar macrophages induced by *in vitro* bacterial or viral challenge models (Che et al., 2008). In this study, we demonstrated that feeding MOS to nursery pigs also altered the pigs' immune response to a PRRSV challenge and may help alleviate negative impacts of infection.

Apparent clinical signs observed in the PRRSV-infected pigs included anorexia and elevated RT. The infected pigs' loss of appetite was shown in a drop in ADFI from d 2 to 14 PI. Other studies with PRRSV have also observed a significant reduction in ADFI from d 5 to 14 PI and d 2.6 to 13 PI (Halbur et al., 1996; Escobar et al., 2004; Toepfer-Berg et al., 2004). Apart from the anorexia, fever was the most commonly witnessed clinical sign in the PRRSV-infected pigs. Severity and duration of fever depend on PRRSV doses and strains (Halbur et al., 1996, Diaz et al. 2005; Loving et al., 2008). For example, the body temperature of pigs infected with a highly virulent Japanese PRRSV strain or the North America strain continued to increase for up to 14 d PI (Shibata et al., 2003) or 28 d PI (Thanawongnuwech et al., 2000), respectively. Our study used one of the highly virulent PRRSV strains (Purdue P-129) in the North America, which often causes a high and

prolonged fever. After exposure to PRRSV, the infected pigs showed pyrexia with a peak at d 3 PI. Notably, the infected pigs fed the control diet had a high average RT of > 40.0^{o}C from d 3 to 14 PI, whereas the RT of the MOS-fed pigs declined markedly by d 7 PI. This suggests that MOS modulates the pigs' immune response to a viral infection and reduces the intensity of continuing inflammation. Hung et al. (2008) also found a reduced fever at 2 h after injection of lipopolysaccharide in pigs consuming MOS.

Furthermore, PRRSV infection brought about a significant decrease in leukocyte populations until d 14 PI. The numbers of total WBC, lymphocytes, monocytes, and eosinophils, but not neutrophils, were reduced for several days shortly after infection. The total WBC and lymphocyte counts continued to be low through d 7 PI. These results were consistent with those reported earlier (Shimizu et al., 1996; Lohse et al., 2004; Shi et al., 2008). The general decline in peripheral blood leukocyte populations indicates that apoptosis of immune cells was probably induced by PRRSV during the early stage of infection, as monocytes/macrophages are the common targets for PRRSV infection and replication (Sur et al., 1998; Choi and Chae, 2002; Labarque et al., 2003). This implies that infection with PRRSV causes the infected pigs to undergo a state of immunosuppression for at least a wk after infection. Dietary MOS prevented the leukopenia by increasing WBC and neutrophils by d 3 PI, and lymphocytes by d 7 PI. The greater numbers of WBC, neutrophils, and lymphocytes at d 3 and 7 PI in challenged pigs fed MOS suggest the immune system may have been primed by the ingestion of MOS, prompting a quick response to the viral infection. Modulation of the immune response by MOS, therefore, plays a critical role in maintaining and boosting the pigs' nonspecific immunity impaired by PRRSV before the disease-specific immunity comes into play.

The differences in clinical signs and leukocyte populations might be associated with the severity of inflammation and level of inflammatory cytokines. The early cytokines produced during the initial stage of respiratory infections are interferon-α, TNF-α, IL-1β, IL-6, and IL-8 (Van Reeth et al., 2002; Thacker, 2006). The TNF-α response to PRRSV in the present study was pronounced at d 7 and 14 PI, suggesting that this cytokine was probably involved in the early immune response. Tumor necrosis factor-α, together with IL-1, increases the expression of adhesion molecules on the vascular endothelium and leukocytes to recruit more immune cells to the site of infection. Macrophages and other monocytes may be responsible for TNF-α production. A demonstrable increase in TNF-α mRNA was evident in porcine peripheral blood mononuclear cells (**PBMC)** and in lung tissue for 10 d PI and in alveolar macrophages for 7 d PI (Choi et al., 2001; Choi and Chae, 2002; Sipos et al., 2003). So far, there has not been much information about the serum levels of TNF-α in PRRSV-infected pigs, but the increased mRNA expression of TNF-α found in PBMC appeared to support the involvement of this cytokine during the early immune response.

The serum IL-10 in the infected pigs was increased at d 7 PI. The result of our study was consistent with that reported by other researchers, who did not measure serum IL-10, but found an increase in its transcriptional level. Suradhat and Thanawongnuwech (2003) showed that levels of IL-10 mRNA were increased more rapidly in PBMC (d 5 PI) than in BALF cells (d 9 PI). The IL-10 mRNA levels were also found to be increased in AMϕ from d 10 PI (Thanawongnuwech and Thacker, 2003; Thanawongnuwech et al., 2004). The increase in IL-10 in the infected pigs may be in response to the PRRSV-induced inflammation. Interleukin-10 is secreted to inhibit activation of different types of immune cells and production of pro-inflammatory cytokines, resulting in reduced inflammatory

response (Moore et al., 2001). The production level of IL-10 is crucial in regulating a balance between pathology and protection. Furthermore, the increase in serum IL-10 of the MOS-fed pigs at the end of the acute phase indicates that there may be a shift from T helper1 to T helper2 lymphocyte response in association with dietary MOS. However, other cells such as T regulatory cells and type II macrophages are also capable of producing IL-10. Together with TNF-α data, MOS can apparently regulate the cytokine responses of pigs to a PRRSV infection and its immunomodulatory effects seem to be protective by repressing ongoing inflammation.

Pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 produced during acute phase response can induce production of several APP by hepatocytes (Eckersall et al., 1996; Petersen et al., 2004). We found that PRRSV augmented the serum levels of Hp and CRP at d 7 and 14 PI and induced a much stronger response of Hp than CRP. Earlier studies showed increased levels of serum Hp in PRRSV-infected pigs from d 5 to 21 PI indicating its important role in mediating the immune response during PRRSV infection (Asai et al., 1999; Diaz et al., 2005; Gnanandarajah et al., 2008). Acute phase proteins are primarily synthesized in IL-6-mediated hepatocytes and secreted into the blood stream; however, TNF-α has been shown to increase Hp production directly or indirectly through the induction of or in synergy with IL-6 (Nakawaga-Tosa et al., 1995; Tilg et al., 1997; Petersen et al., 2004). Thus, the reduction in the serum TNF-α level of the MOS-fed pigs at d 14 PI would in part explain the numerical decline of Hp observed. Moreover, Hp and CRP may provide a feedback mechanism by downregulating pro-inflammatory cytokine production and activity in mononuclear cells (Tilg et al., 1997; Moore et al., 2001; Petersen et al., 2002). In brief, the trends in decreased TNF-α and APP in the MOS-fed pigs at d 14 PI may indicate a sign of

inflammation slowdown and Hp may be a better indicator for assessing the health status of PRRSV-infected pigs than CRP.

In conclusion, the data suggest that MOS regulates the nursery pigs' immune responses to PRRSV infection *in vivo*. The increase in leukocytes a few days after infection, together with the decreases in RT and inflammatory mediators at the end of the acute phase, indicates that MOS has the ability to enhance the host's immune system at the early stage of infection, but thereafter suppresses ongoing immune responses and inflammation. Immunomodulation by MOS may help alleviate negative impacts of PRRSV, including inflammation and poor nutrient utilization. Further studies, however, are needed to investigate the effect of MOS on a pig's immune response to combined diseases caused by bacteria and viruses, as pigs often encounter a complex of pathogens.

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FIGURES AND TABLES

Table 3.1 Composition of basal diets fed to nursery pigs during the experiment (as-fed basis)

Item	Phase[1]		
	I	II	III
Ingredients, %			
Corn	38.11	42.82	52.45
Dried whey	22.00	16.00	10.00
Soybean meal, 48%	10.00	18.00	24.00
Spray-dried animal plasma	8.00	4.00	0.00
Soy protein concentrate[2]	5.00	3.00	0.00
Select menhaden fish meal	4.01	5.49	6.25
Soybean oil	3.61	4.09	4.07
Lactose	5.60	2.80	0.00
Limestone	0.85	0.56	0.55
Dicalcium phosphate	0.67	0.92	0.48
Carbadox premix[3]	1.00	1.00	1.00
Zinc oxide	0.42	0.42	0.42
Mineral premix[4]	0.35	0.35	0.35
Vitamin premix[5]	0.20	0.20	0.20
Lysine-HCl	0.07	0.16	0.22
DL-met	0.11	0.10	0.08
L-thr	0.01	0.09	0.14

Table 3.1 (cont.)

Item	Phase[1]		
	I	II	III
Calculated composition			
ME, Mcal/kg	3.45	3.45	3.45
Standardized ileal digestible AA, %			
Lys	1.45	1.45	1.30
Met	0.41	0.43	0.42
Thr	0.94	0.94	0.84
Tryp	0.29	0.27	0.23
Val	1.10	1.03	0.89
Ile	0.87	0.87	0.79
Ca, %	0.90	0.90	0.80
Available P, %	0.55	0.55	0.40
Lactose, %	21.00	14.00	7.00

[1]Phase I, II, and III diets were fed to nursery pigs for 7, 7, and 14 d postweaning, respectively.
[2]Soycomil, Archer Daniels Midland Company, Decatur, IL.
[3]Mecadox 2.5, provided 0.055 g of carbadox per kilogram of diet, Phibro Animal Health, Fairfield, NJ.
[4]Provided as milligrams per kilogram of diet: sodium chloride, 3,000; zinc, 100 from zinc oxide; iron, 90 from iron sulfate; manganese, 20 from manganese oxide; copper, 8 from copper sulfate; iodine, 0.35 from calcium iodide; selenium, 0.30 from sodium selenite.
[5]Provided per kilogram of diet: retinyl acetate, 2,273 µg; cholecalciferol, 17 µg; DL-α-tocopheryl acetate, 88 mg; menadione sodium bisulfate complex, 4 mg; niacin, 33 mg; D-Ca-pantothenate, 24 mg; riboflavin, 9 mg; vitamin B_{12}, 35 µg; choline chloride, 324 mg.

Table 3.2 Effect of mannan oligosaccharide (MOS)[1] and porcine reproductive and respiratory syndrome virus (PRRSV)[2] on differential leukocyte counts of pigs at d 3 postinfection[3]

Item	Control	MOS	PRRSV	MOS+PRRSV	SEM
Leukocyte count, x 10^3/μL					
White blood cell[ab]	10.76	12.71	7.66	10.16	1.08
Neutrophils	3.29	3.44	3.41	5.41	0.70
Lymphocytes[c]	6.71	8.61	3.92	4.29	0.68
Monocytes[a]	0.47	0.40	0.22	0.26	0.07
Eosinophils[c]	0.26	0.24	0.08	0.17	0.04
Neutrophils/Lymphocytes[c]	0.51	0.48	1.55	1.73	0.31

[1]Pigs were fed MOS diets starting at weaning at 2 wk before infection.
[2]Pigs were challenged with PRRSV at 5 wk of age.
[3]Values are means of 16 pigs representing each treatment.
[a]Main effect of PRRSV infection was significant ($P < 0.05$).
[b]Main effect of diet was significant ($P < 0.05$).
[c]Main effect of PRRSV infection was significant ($P < 0.01$).

Table 3.3 Effect of mannan oligosaccharide (MOS)[1] and porcine reproductive and respiratory syndrome virus (PRRSV)[2] on differential leukocyte counts of pigs at d 7 postinfection[3]

Item	Control	MOS	PRRSV	MOS+PRRSV	SEM
Leukocyte count, x 10^3/μL					
White blood cells[ab]	12.79	14.18	9.74	11.92	0.82
Neutrophils[c]	3.58	3.18	4.08	5.01	0.50
Lymphocytes[ab]	8.46	10.20	4.90	6.13	0.60
Monocytes	0.57	0.59	0.52	0.47	0.09
Eosinophils	0.14	0.16	0.19	0.26	0.05
Neutrophils/Lymphocytes[a]	0.46	0.32	0.92	0.94	0.11

[1]Pigs were fed MOS diets starting at weaning at 2 wk before infection.
[2]Pigs were challenged with PRRSV at 5 wk of age.
[3]Values are means of 16 pigs representing each treatment.
[a]Main effect of PRRSV infection was significant ($P < 0.01$).
[b]Main effect of diet was significant ($P < 0.05$).
[c]Main effect of PRRSV infection was significant ($P < 0.05$).

Table 3.4 Effect of mannan oligosaccharide (MOS)[1] and porcine reproductive and respiratory syndrome virus (PRRSV)[2] on differential leukocyte counts of pigs at d 14 postinfection[3]

Item	Control	MOS	PRRSV	MOS+PRRSV	SEM
Leukocyte count, x 10^3/μL					
White blood cells[a]	16.66	16.16	20.95	22.03	1.52
Neutrophils[a]	3.47	3.80	6.87	7.89	0.58
Lymphocytes	11.88	11.30	12.23	12.64	1.14
Monocytes	1.00	0.68	1.08	0.87	0.27
Eosinophils[a]	0.14	0.36	0.60	0.56	0.08
Neutrophils/Lymphocytes[a]	0.30	0.35	0.62	0.67	0.07

[1]Pigs were fed MOS diets starting at weaning at 2 wk before infection.
[2]Pigs were challenged with PRRSV at 5 wk of age.
[3]Values are means of 8 pigs representing each treatment.
[a]Main effect of PRRSV infection was significant ($P < 0.01$).

Table 3.5 Effect of mannan oligosaccharide (MOS)[1] and porcine reproductive and respiratory syndrome virus (PRRSV)[2] on performance of pigs after PRRSV infection

Item	n[3]	Control	MOS	PRRSV	MOS+PRRSV	SEM
d 0 to 7 postinfection						
ADG, g[a]	16	612	603	197	205	15
ADFI, g[a]	8	908	871	491	461	27
G:F, g/kg[ab]	8	676	700	405	447	19
d 7 to14 postinfection						
ADG, g[a]	8	564	654	284	295	26
ADFI, g[a]	8	950	996	571	539	45
G:F, g/kg[ac]	8	599	654	500	551	23

[1]Pigs were fed MOS diets starting at weaning at 2 wk before infection.
[2]Pigs were challenged with PRRSV at 5 wk of age.
[3]The pig was the experimental unit for ADG, whereas the pen, containing 2 pigs, was the experimental unit for ADFI and G:F.
[a]Main effect of PRRSV was significant ($P < 0.001$).
[b]Trend to main effect of diet ($P < 0.1$).
[c]Trend to main effect of diet ($P = 0.07$).

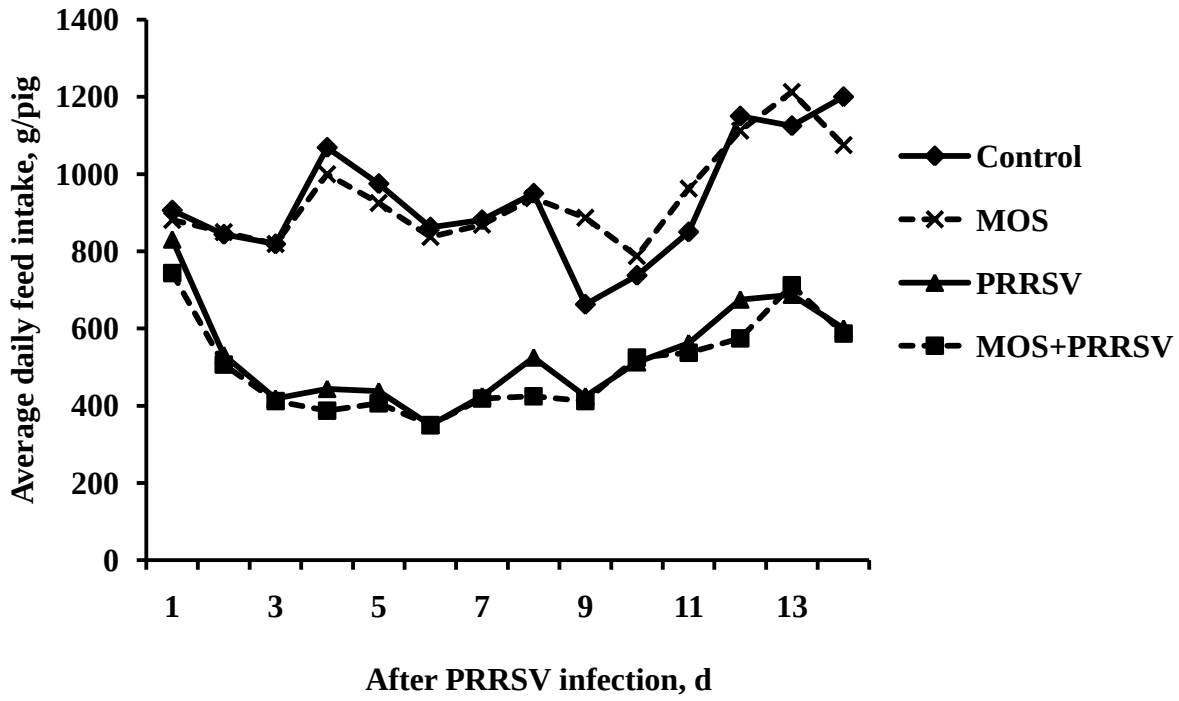

Figure 3.1 Effect of mannan oligosaccharide (MOS) and porcine reproductive and respiratory syndrome virus (PRRSV) on ADFI of nursery pigs. Pigs infected with PRRSV had a significantly lower ADFI from d 2 to 14 postinfection than the uninfected pigs ($P < 0.01$). No effect of MOS on ADFI was found.

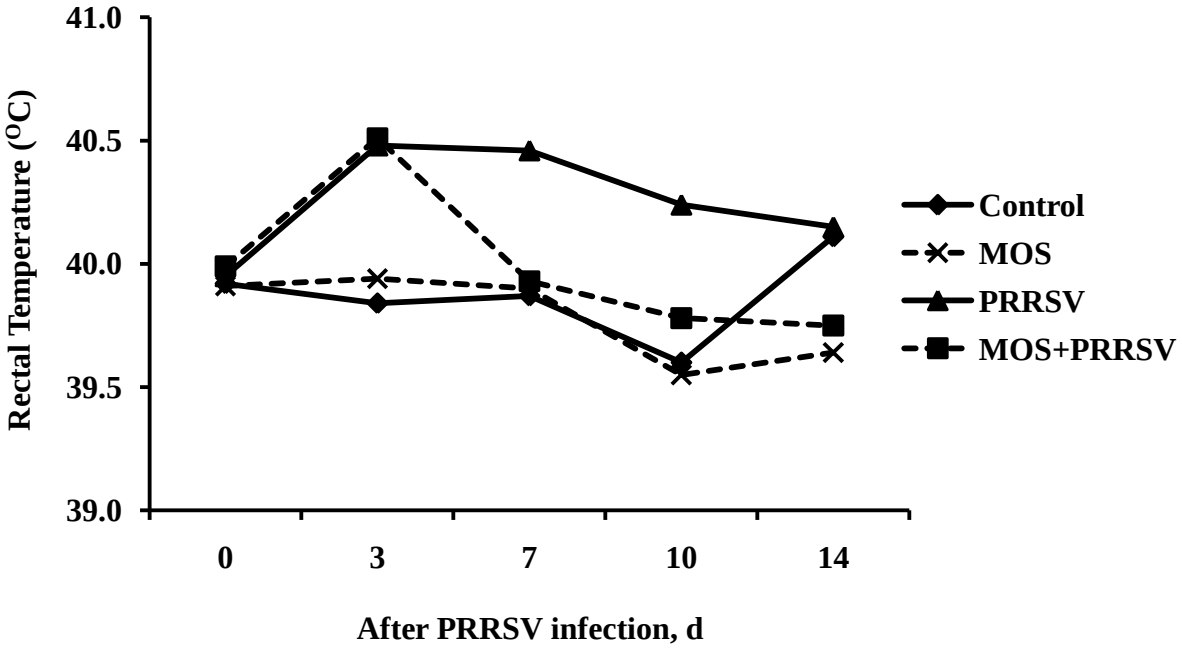

Figure 3.2 Rectal temperature (RT) in pigs fed control or mannan oligosaccharide (MOS) diets with or without infection of porcine reproductive and respiratory syndrome virus (PRRSV). The RT of the infected pigs peaked at d 3 postinfection (PI) and was significantly greater than that of the uninfected pigs ($P < 0.01$) and PRRSV continued to affect the infected pigs fed the control diet until d 14 PI. At d 7 PI, there was a PRRSV x diet interaction ($P < 0.01$) on RT, indicating that the infected pigs fed the MOS diet had a lower RT ($P < 0.05$) than those fed the control diet. Dietary MOS also lowered the RT of the infected pigs at d 10 PI ($P < 0.06$). The pigs fed the control diet had a greater RT at d 14 PI compared to those fed the MOS diet ($P < 0.01$). Values were means ± pooled SEM (d 0, 3, and 7 PI, n = 16; d 10 & 14 PI, n = 8).

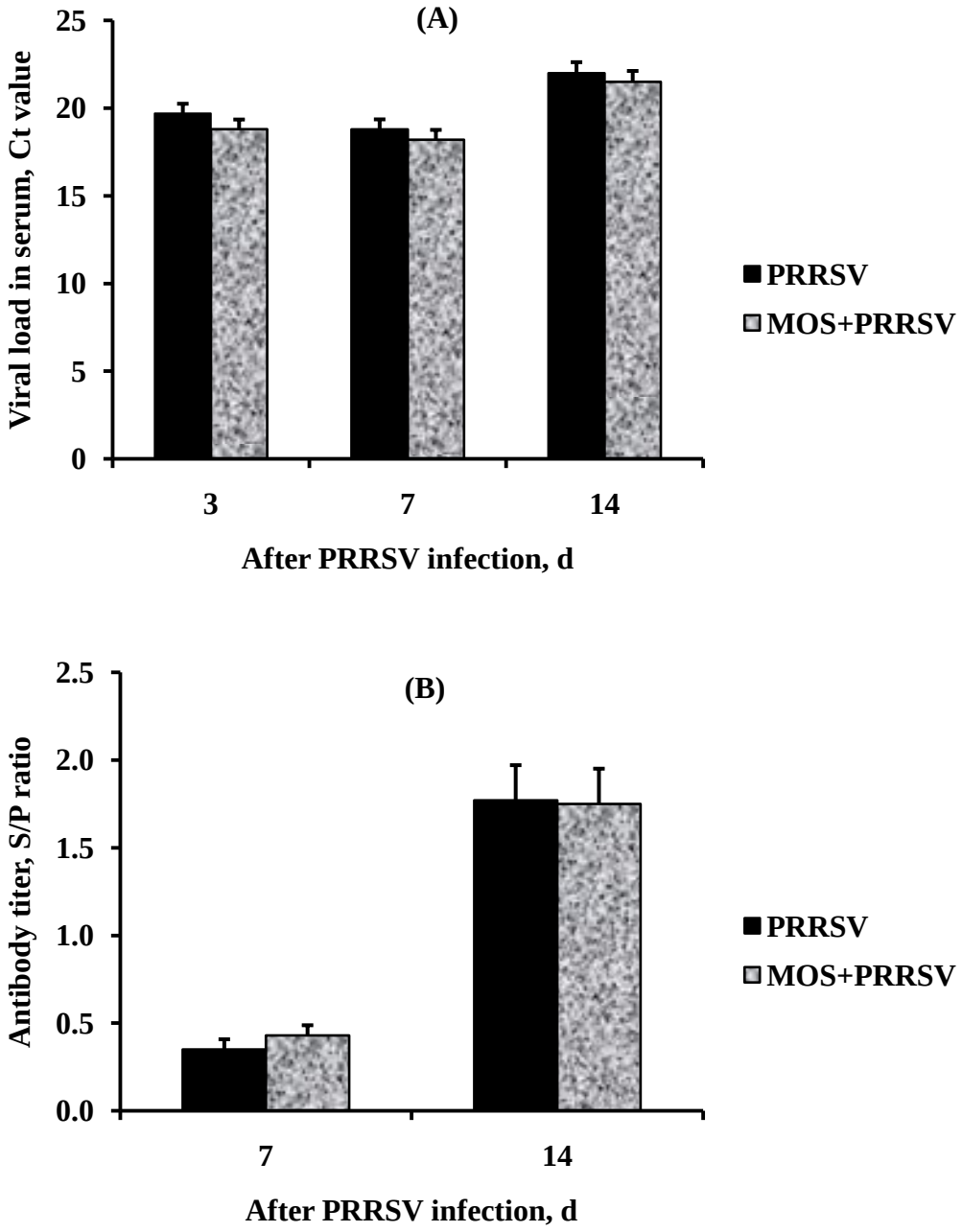

Figure 3.3 Viral load (A) and antibody titer (B) in control- or mannan oligosaccharide (MOS)-fed pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). Viral load is presented as cycle threshold (Ct) values and antibody titer as sample to positive control (S/P) ratios. Mannan oligosaccharide did not affect ($P > 0.05$) the S/P ratios and Ct values of the infected pigs postinfection (PI). Values were means ± pooled SEM (d 3 & 7 PI, n = 16; d 14 PI, n = 8).

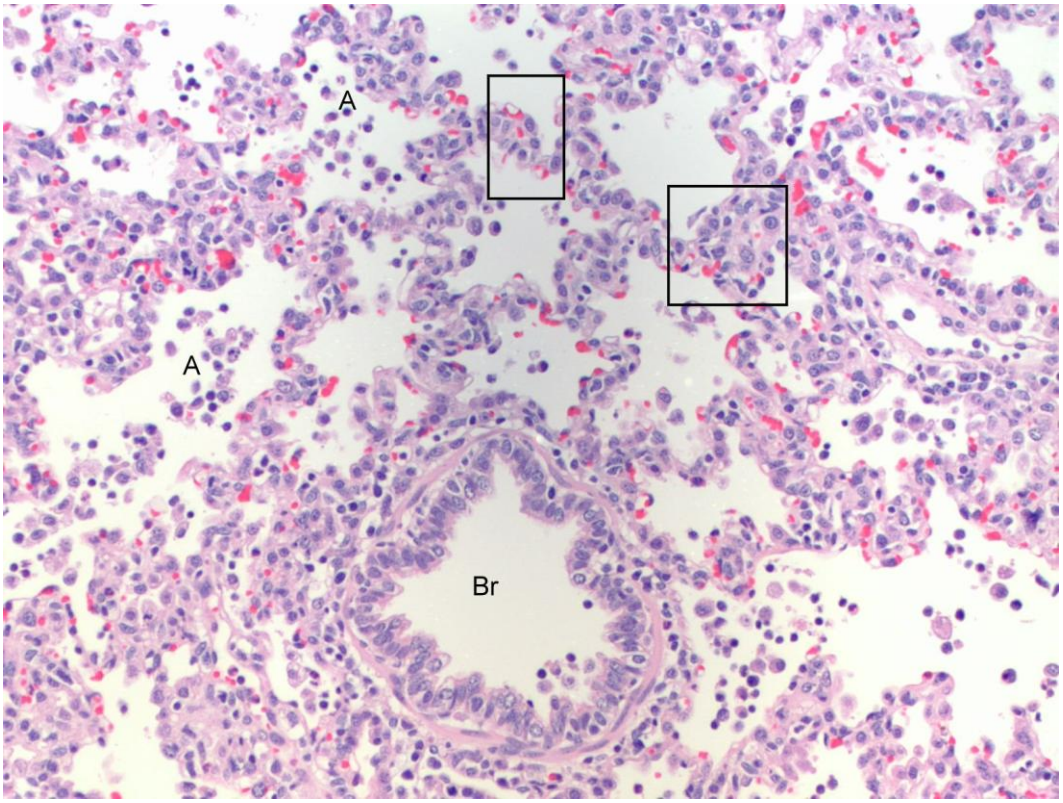

Figure 3.4 Microscopic section of lungs from pigs infected with porcine reproductive and respiratory syndrome virus. Alveoli (A) and bronchioles (Br) contained many inflammatory cells. Alveolar septa (box) were thickened typical of the interstitial pneumonia of porcine reproductive and respiratory syndrome (Hemotoxylin and Eosin stain, 200x magnification).

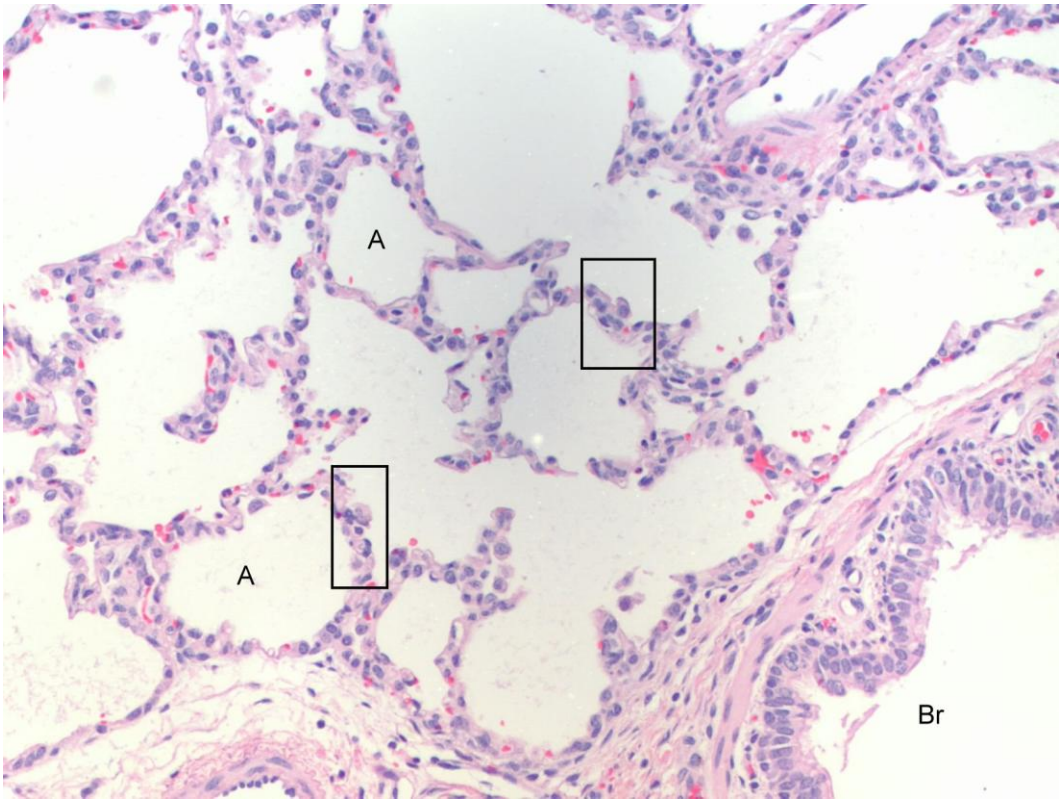

Figure 3.5 Microscopic section of normal lungs from pigs not infected with porcine reproductive and respiratory syndrome virus. There was no exudate in alveoli (A) or bronchioles (Br) and alveolar septa (box) were normal thickness (Hemotoxylin and Eosin stain, 200x magnification).

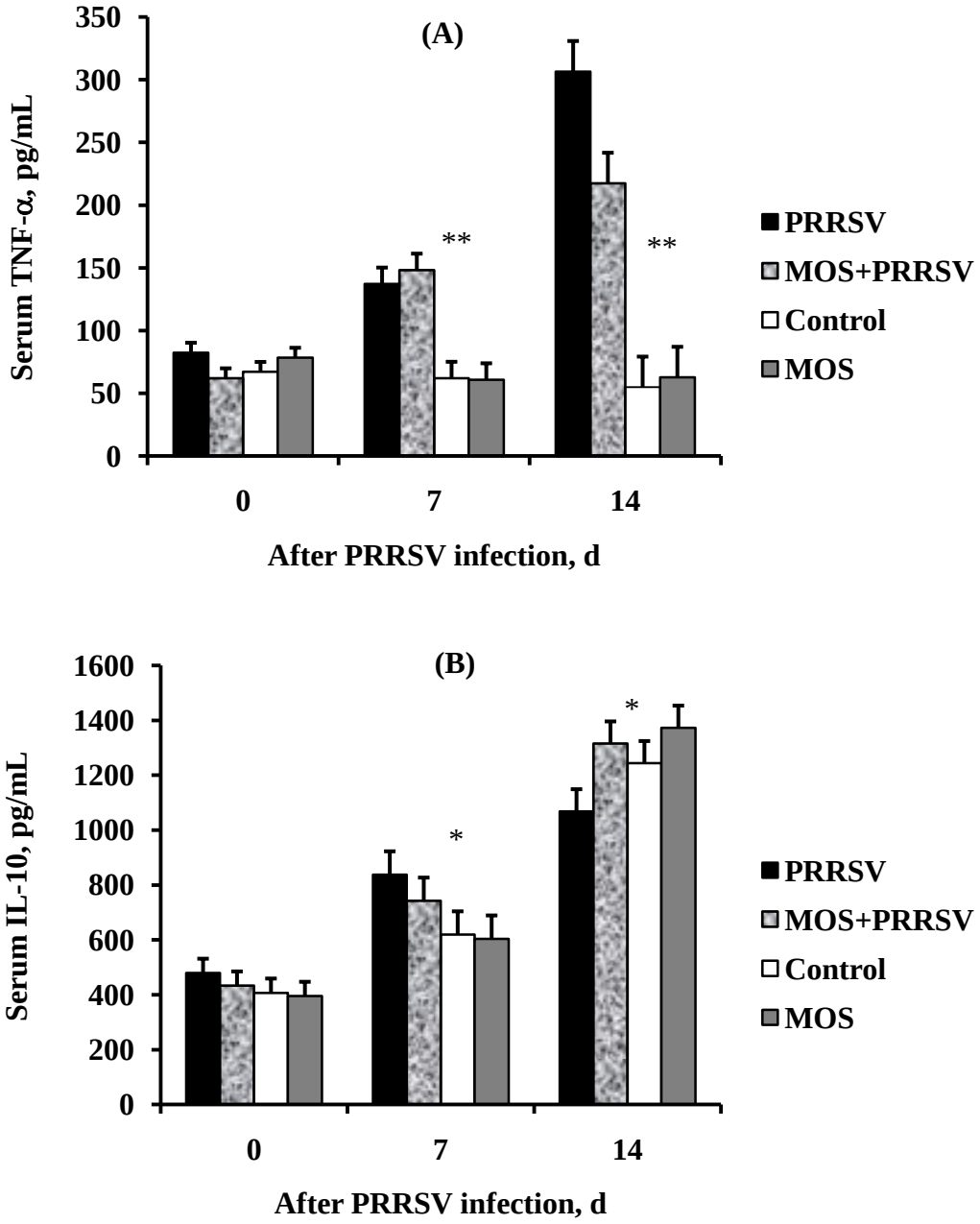

Figure 3.6 Serum tumor necrosis factor-α (TNF-α) and IL-10 levels in pigs fed control or MOS diets with or without porcine reproductive and respiratory syndrome virus (PRRSV) infection. (A) TNF-α levels of PRRSV-infected pigs were greater at d 7 (n = 16) and 14 (n = 8) postinfection (PI) than those of the uninfected pigs ($P < 0.01$). There was a PRRSV x diet interaction ($P = 0.06$) on TNF-α at d 14 PI, indicating that the infected pigs fed the MOS diet had a lower TNF-α level than those fed the control. (B) IL-10 levels of PRRSV-infected pigs were greater at d 7 PI (n = 16) than those of the uninfected pigs ($P < 0.05$). Pigs fed MOS diets had greater IL-10 levels at d 14 PI (n = 8) than those fed the control ($P < 0.05$). Values were means ± pooled SEM. * $P < 0.05$; ** $P < 0.01$.

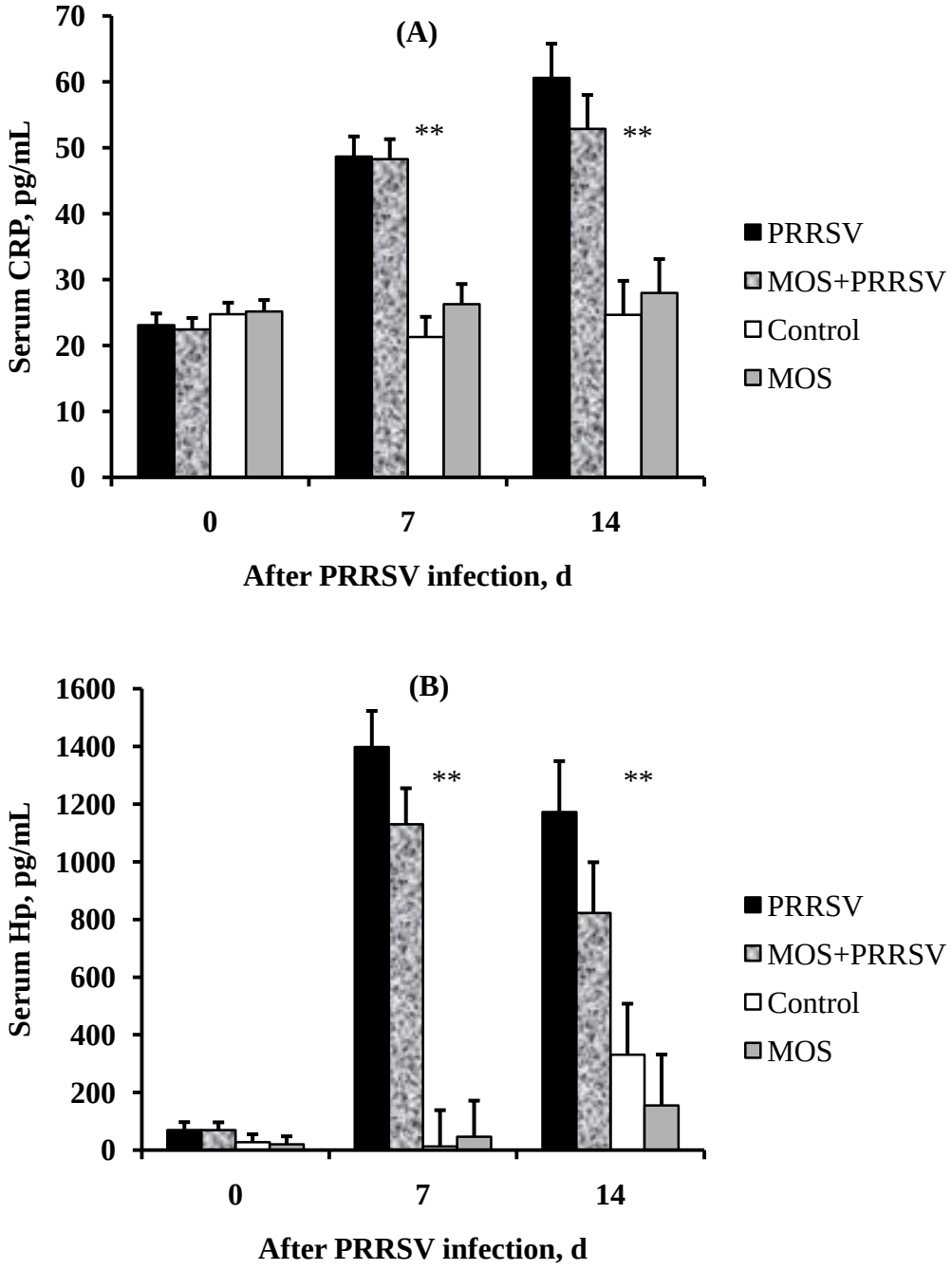

Figure 3.7 Serum C-reactive protein (CRP, A) and Haptoglobin (Hp, B) levels in pigs fed control or MOS diets with or without porcine reproductive and respiratory syndrome virus (PRRSV) infection. The CRP and Hp levels of PRRSV-infected pigs were greater at d 7 (n = 16) and 14 (n = 8) postinfection (PI) than those of the uninfected pigs ($P < 0.01$). Values were means ± pooled SEM. ** $P < 0.01$.

CHAPTER 4

MANNAN OLIGOSACCHARIDE MODULATES GENE EXPRESSION PROFILE IN PIGS EXPERIMENTALLY INFECTED WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

ABSTRACT: This study characterized gene expression in peripheral blood mononuclear cells (**PBMC**) and bronchoalveolar lavage fluid (**BALF**) cells from control-or mannan oligosaccharide **(MOS**)-fed pigs with or without PRRSV at d 7 postinfection (**PI**). Weaned pigs (3 wk old) fed 0% or 0.2% MOS diets were intranasally inoculated with porcine reproductive and respiratory syndrome virus (**PRRSV**) or a sterile medium at 5 wk old. Total RNA (3 pigs/treatment) was extracted from cells. Double-stranded cDNA was amplified, labeled, and further hybridized to the Affymetrix GeneChip Porcine Genome Array consisting of 23,937 probe sets representing 20,201 genes. Microarray data were analyzed in R using packages from the Bioconductor project. Differential gene expression was tested by fitting a mixed linear model equivalent to a 2 x 2 factorial ANOVA using the limma package. Dietary MOS and PRRSV changed the expression of thousands of probe sets in PBMC and BALF cells ($P < 0.05$). The MOS x PRRSV interaction altered the expression of more nonimmune probe sets in PBMC (977 up and 1128 down) than in BALF cells (117 up and 78 down). The MOS x PRRSV interaction ($P < 0.05$) for immune probe sets in PBMC affected genes encoding key inflammatory mediators. In uninfected pigs, gene expression of IL-1α, IL-6, myeloid differentiation factor 88, toll-like receptor (**TLR**) 4, major histocompatibility complex (**MHC**) II and dead box polypeptide 58 increased in PBMC of MOS-fed pigs ($P < 0.05$). This suggests that MOS enhances disease resistance in pigs and supports the fact that

MOS induced a rapid increase in leukocytes at d 3 and 7 PI. Within infected pigs, however, MOS reduced the expression of IL-1β, IL-6, IL-8, macrophage inflammatory protein (**MIP**)-1α, MIP-1β, monocyte chemotactic protein (**MCP**)-1, and TLR4 genes in PBMC ($P < 0.05$). This finding may explain why fever was ameliorated in infected pigs fed MOS by d 7 PI. The expression of IL-1β, IL-6, MIP-1β, MCP-1, and TLR4 genes analyzed by real time RT-PCR confirmed the microarray results. In BALF cells of infected pigs, MOS reduced the gene expression of TLR4, MHCII, and molecules associated with the complement system, but increased the gene expression of MHCI. The MOS-regulated decrease of inflammatory responses was involved in several biological pathways. In short, MOS regulated the expression of nonimmune and immune genes in leukocytes of pigs, perhaps providing benefits by enhancing immunity while preventing over-stimulation of the immune system.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (**PRRSV**) continues to be a threat for swine-producing countries worldwide (Albina, 1997; Van Reeth et al., 2002; Thacker, 2006) and is capable of impairing the host's immune responses (Mateu and Diaz, 2008). It has been shown that PRRSV reduces total leukocyte counts, delays cell-mediated immunity, inhibits key cytokines such as interferon (**IFN**)-α, and may interfere with correct antigen presentation and activation of T lymphocytes (Lee et al., 2004; Wang et al., 2007; Shi et al., 2008). Additionally, different PRRSV isolates have different abilities to induce various cytokines such as tumor necrosis factor (**TNF**)-α, IL-1β, IL-6, IL-10, and IL-12 (Sipos et al., 2003; Thanawongnuwech et al., 2004). Our recent study showed that mannan oligosaccharide (**MOS**), extracted from the yeast cell wall of *Saccharomyces cerevisiae*,

induced changes in the immune responses of pigs to PRRSV infection by d 7 postinfection (**PI**). Dietary MOS was associated with rapidly increased numbers of white blood cells (**WBC**), lymphocytes, and neutrophils in infected pigs at the early stage of infection. Feeding MOS to infected pigs reduced fever at d 7 PI and the level of serum TNF-α at d 14 PI. In addition, feeding MOS to pigs increased the serum IL-10 level in pigs at d 14 PI. Together, these data indicate that the on-going inflammation caused by PRRSV may be alleviated by MOS after d 7 PI. Therefore, this altered immune response was studied further here using the Affymetrix GeneChip Porcine Genome Array followed by a real time RT-PCR validation. The objective of this study was to characterize gene expression, measured by the Affymetrix GeneChip Porcine Genome Array, in peripheral blood mononuclear cells (**PBMC**) and bronchoalveolar lavage fluid (**BALF**) cells isolated at d 7 PI from control- or MOS-fed pigs with or without PRRSV infection.

MATERIALS AND METHODS

Experimental Design, Animals, and Housing

Sixty four pigs (3 wk old), free of PRRSV (virology and PCR), were used in 2 separate but similar trials conducted sequentially (32 pigs per trial). Pigs were brought to the experimental site at weaning at 3 wk of age and upon arrival were placed in disease-containment chambers which have been previously described (Escobar et al., 2004). Lincomycin was administered daily via intramuscular injection for 3 d after arrival to prevent infections (11 mg/kg of BW; Pharmacia and Upjohn Co., Kalamazoo, MI).

The pigs were divided into blocks of 4 pigs based on BW, gender, and litter origin. They were randomly assigned from within blocks to one of 4 treatments in a 2 x 2 factorial

arrangement (2 types of diet: 0% MOS as the control and 0.2% MOS addition; 2 levels of PRRSV: with and without). There were 8 replicate pens (or chambers) of 2 pigs each. One castrated male and one female were placed in each chamber. All pigs were housed in a temperature-controlled room with constant lighting and had *ad libitum* access to water and feed. Pigs were fed the experimental diets for 2 wk before being challenged with PRRSV. The basal diets (Table 4.1) were formulated to contain levels of all essential nutrients which met or exceeded the nutritional requirements of pigs during the nursery period (NRC, 1998). Treatment diets were formulated by supplementing the basal diets with 0.2% MOS throughout the experimental period.

Experimental Procedures

The experimental protocol was approved by the University of Illinois Institutional Animal Care and Use Committee and the Institutional Biosafety Committee. The procedures for this study were adapted from the method of Escobar et al. (2004) with modifications. Having been fed the experimental diets for 2 wk, pigs in one-half of the chambers were inoculated intranasally with 2 mL of high-virulence strain of PRRSV (Purdue isolate P-129 containing 10^5 50% tissue culture infective dose). Pigs in the remaining chambers received 2 mL of sterile Dubelco's modified Eagle medium. One-half of the pigs (32 pigs) were euthanized at d 7 PI and the remainder at the end of the experiment (d 14 PI) for collection of PBMC and BALF cells. In each of the 2 trials, one pig from each chamber of control, MOS, PRRSV, and MOS plus PRRSV was euthanized at d 7 and 14 PI. Before being euthanized, pigs were bled by jugular vein to obtain blood samples for isolation of PBMC. Then, pigs were euthanized to collect BALF cells.

Blood Collection and Isolation of PBMC

Blood samples were collected from the jugular vein at d 7 PI. Six milliliters of blood from each pig were collected into a glass tube containing anticoagulant (heparin). Peripheral blood mononuclear cells from blood were isolated by gradient centrifugation using ficoll gradient (Histopaque 1077, density = 1.077 g/mL; Sigma Chemical Company, St. Louis, MO). Three milliliters of whole blood were carefully added on the top of 3 mL of Histopaque solution in a 15-mL conical tube. The tube was centrifuged at 400 x g for 30 min at room temperature. After centrifugation, the upper layer of the opaque interface containing mononuclear cells was aspirated and transferred to a new centrifuge tube. The cells were washed twice with 10 mL of Hank's balanced salt solution and centrifuged at 250 x g for 10 min at room temperature. The cells were then washed with Roswell Park Memorial Institute 1640, pelleted by centrifugation, and resuspended in 200 µL RNAlater® (Ambion, Inc., Austin, TX). The cells were kept at -80^{o}C until used.

Collection and Isolation of BALF Cells

Bronchoalveolar lavage fluid cells were isolated from pigs at d 7 PI. Pigs were anesthetized by intramuscular injection of a 1-mL combination of telazol, ketamine, and xylazine (2:1:1) per 23.3 kg BW. The final mixture contained 100 mg telazol, 50 mg ketamine, and 50 mg xylazine in one mL (Fort Dodge Animal Health, Fort Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway) per 1 kg of BW (Henry Schein Inc., Indianapolis, IN).

Bronchoalveolar lavage fluid cells were collected by pulmonary lavage with 150 mL of PBS without Ca and Mg. Lavage fluid was filtered through a double layer of sterile gauze

and centrifuged at 400 x g for 15 min at room temperature. After centrifugation, lung lavage cells were washed twice with Hank's balanced salt solution. The cells were then washed with Roswell Park Memorial Institute 1640, pelleted by centrifugation, and resuspended in 200 µL RNAlater® (Ambion, Inc., Austin, TX). The cells were kept at -80^{o}C until used.

Total RNA Extraction and Gene Expression by Microarrays

Total RNA (3 pigs/treatment) from PBMC and BALF cells isolated at d 7 PI was extracted using TRIzol® plus PureLink^{TM} RNA Mini Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The RNA quality and quantity was assessed using the Agilent 2100 Bioanalyzer and the ND-1000 Nanodrop spectrophotometer, respectively. All samples used for further analysis had an O. D. of 1.8 to 2.1 and an RNA integrity number of ≥ 7. Double-stranded cDNA was first synthesized, purified, and employed as a template for *in vitro* amplification and labeling by using the GeneChip® Expression 3'-Amplification IVT Labelling Kit (Affymetrix Inc., Santa Clara, CA). Then, cDNA was used to synthesize cRNA which was hydrolyzed to produce fragmented cRNA in the 35-200 nucleotide size range for proper hybridization. The fragmented cRNA was labelled and further hybridized to the Affymetrix GeneChip® Porcine Genome Array. Each array consisted of 23,937 probe sets to interrogate 23,256 transcripts in pig, which represents 20,201 genes. Twenty four chips in total were used in this experiment.

Analysis of Microarray Data

Data from the PBMC samples and BALF cell samples were handled separately at all stages of the process. All quality control assessments, data processing, and statistical

analyses were done in R (R Development Core Team, 2008) using packages from the Bioconductor project (Gentleman et al., 2004) as indicated below.

Quality Control Assessment. Quality control assessment (MacDonald, 2005) showed that all arrays were of acceptable quality except that one PBMC sample was an outlier by the principle component analysis method and so was excluded from further analysis. The remaining arrays were re-processed with the Guanine Cytosine Robust Multi-Array Analysis algorithm, which performs a GC-based background-correction, does a quantile normalization between arrays and summarizes the multiple probes into one probe set value using a median polish algorithm (Wu and Irizarry, 2005).

Differential Gene Expression Analysis. Testing for differential gene expression was done by fitting a mixed linear model equivalent to a 2 x 2 factorial ANOVA using the limma package (Smyth, 2005), which uses an empirical Bayes correction that helps to improve power by borrowing information across genes (Smyth, 2004). Appropriate pairwise comparisons between tested groups and the overall interaction effect between MOS and PRRSV were pulled as contrasts from the model. There were 5 comparisons as follows: infected MOS (**IMOS**) vs. infected control (**ICON**), uninfected MOS (**MOS**) vs. uninfected control (**CON**), IMOS vs. MOS, ICON vs. CON, and MOS x PRRSV interaction. The limma model was fit and raw p-values were calculated using all 23,937 probe sets on the array, but the correction for multiple hypothesis testing using the false discovery rate (**FDR**) method (Benjamini and Hochberg, 1995) was applied as follows: First, we were particularly interested in the immune genes, so we generated one list of the immune genes based on the immune genes probe sets provided by Affymetrix's NetAffix Analysis Center on March 9, 2009. For both the PBMC and BALF cell samples, all immune probe sets that were present

on at least one array in that tissue based on Affymetrix's Call Detection Algorithm were pulled as one list and corrected using the FDR method (Affymetrix, 2009). Second, the rest of the probe sets on the array that were present on at least one array were pulled as another list and corrected using the FDR method. The numbers of present immune and other probe sets for each tissue are listed in Table 4.2.

Pattern Analysis of Gene Expression. In order to make biological sense of the analysis results, we used Ingenuity Pathway Analysis (**IPA**, version 7, Ingenuity® Systems, 2009) on the combined list of present immune probe sets and present other probe sets for each tissue. Because IPA does not support Affymetrix's porcine array, we instead used the equivalent human probe set identification as provided by Tsai et al. (2006); only 679 out of 17,167 (PBMC) and 698 out of 17,648 (BALF cells) porcine probe sets did not have a human probe set equivalent, and were discarded from the IPA analysis. We were interested in the following 3 comparisons: MOS vs. ICON, IMOS vs. MOS, and interaction. For each probe set, the fold change and FDR p-values for those 3 comparisons were input for each tissue for a total of 6 comparisons tested in IPA. We used the list of present probe sets as the background for testing over-representation in the significant lists. The criteria for calling a probe set "significant" changed slightly for each of the 6 comparisons but were determined in the following manner: First, in the case where more than one probe set mapped to the same gene, we told IPA to use the one with the largest fold-change value. Second, the cutoff for significance was originally set to FDR p-value < 0.05 and the number of genes that met this criterion and had information in Ingenuity's Knowledge Base was determined. The IPA recommends that no more than 800 significant genes be used for network construction, so we used a minimum fold change criterion to set to ~800 genes. For one comparison, the BALF

cells interaction, we increased the FDR p-value cutoff to 0.1 to have a larger number of genes to test. The specific FDR p-value and fold-change criteria for each comparison, plus the number of significant genes tested are present in Tables 4.3.

Quantitative Real Time RT-PCR

The same total RNA (3 pigs/treatment) from PBMC and BALF cells used to run the Affymetrix microarray was also employed for real time RT-PCR. First strand cDNA was produced from 3 µg of total RNA per sample using the SuperScript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) in a total volume of 20 µL. Total RNA was denatured at 65^{o}C for 5 min and annealed at 42^{o}C for 2 min. Then, the reverse transcription reaction was carried out at 42^{o}C for 50 min, followed by heat inactivation at 70^{o}C for 15 min. The reaction was collected by centrifugation, incubated with 1 µL of RNase H at 37^{o}C for 20 min before amplification of the target DNA.

In order to verify the results from the microarray, quantitative analysis of IL-1β, IL-4, IL-6, macrophage inflammatory protein (**MIP**)-1β, monocyte chemotactic protein (**MCP**)-1, toll-like receptor (**TLR**) 4, mannose receptors (**MR**), major histocompatibility complex (**MHC**) II, and Arginase (**ARG**)-1 mRNA was assayed using RT-PCR. The primers and probes were synthesized by Applied Biosystems (Foster, CA). All probes for the target genes were designed to contain 6-carboxy-fluorescein (FAM) as a fluorescent reporter dye at the 5' end and 6-carboxy-tetramethyl-rhodamine (TAMRA) as a quencher-fluorescent dye at the 3' end. The 18S ribosomal RNA was used as a housekeeping gene and dual-labeled with a 5' reporter dye (VIC) and a 3' quenching dye (TAMRA). Primer and probe sequences were generated for the target genes using the available GenBank sequence. The probe/primer pair

sequences and the amplicon lengths are shown in Table 4.4. One hundred nanograms of total RNA were assayed for each sample in triplicate. The PCR reactions contained each primer and probe, Taqman® universal PCR master mix, cDNA, and RNase-free water in a total volume of 20 µL. The real-time RT-PCR analysis was done using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster, CA). Thermal cycling conditions were 50^{o}C for 2 min and 95^{o}C for 10 min, followed by 40 cycles with 15 sec at 95^{o}C and 1 min at 60^{o}C.

RESULTS

Differential Nonimmune Gene Expression in PBMC and BALF Cells

Dietary MOS and PRRSV infection significantly changed the expression of nonimmune genes in PBMC and BALF cells at d 7 PI (Table 4.5). The interaction between MOS and PRRSV for the gene expression in PBMC was significant ($P < 0.05$) and upregulated the expression of 997 probe sets and downregulated the expression of 1,128 probe sets. For BALF cells, the MOS x PRRSV interaction increased the expression of 117 probe sets and decreased the expression of 78 probe sets ($P < 0.05$). The data indicate that the MOS x PRRSV interaction affected a greater number of probe sets in PBMC than in BALF cells. In addition, the number of probe sets affected by MOS varied dependent on the status of pigs. In uninfected pigs, dietary MOS induced the expression of many more probe sets ($P < 0.05$) in PBMC (938 upregulated and 998 downregulated) than in BALF cells (1 upregulated and 1 downregulated). However, within the infected pigs, dietary MOS altered the expression of more probe sets ($P < 0.05$) in BALF cells (1,007 upregulated and 1,318 downregulated) than in PBMC (164 upregulated and 237 downregulated).

Differential Immune Gene Expression in PBMC and BALF Cells

We found that there was a significant MOS x PRRSV interaction on the expression of immune genes in PBMC ($P < 0.05$), but not in BALF cells. Two probe sets were upregulated and 19 downregulated (Table 4.6). When comparing IMOS vs. ICON, more immune probe sets in PBMC and BALF cells were identified as downregulated (9 & 24, respectively) than as upregulated (3 & 1, respectively) ($P < 0.05$). Within the uninfected pigs, dietary MOS increased the expression of 14 probe sets and decreased the expression of one probe set in PBMC as compared to the control ($P < 0.05$).

The immune genes identified as significantly differentially expressed in PBMC are shown Table 4.7 and Figure 4.1. The MOS x PRRSV interaction affected key genes encoding inflammatory mediators. In uninfected pigs, the gene expression of cytokines, intracellular signaling molecules, and pattern recognition receptors (**PRR**) was increased in PBMC of the MOS-fed pigs as compared to the control-fed pigs ($P < 0.05$). Additionally, MOS tended to increase ($P < 0.09$) the gene expression of IL-1β, IL-1 receptor antagonist, IL-18, and alveolar macrophage-derived chemotactic factor-2 (data not shown). Within the infected pigs, however, MOS reduced the gene expression of important pro-inflammatory cytokines and chemokines in PBMC as compared to the control ($P < 0.05$).

With respect to the expression of immune genes in BALF cells, the MOS x PRRSV interaction was not significant ($P > 0.05$, Table 4.8). In uninfected pigs, there was no difference in the gene expression between MOS and CON ($P > 0.05$). Within the infected pigs, MOS downregulated the gene expression of several genes involved in inflammation and upregulated the expression of MHCI gene as compared to the control ($P < 0.05$).

Patterns of Gene Expression

To understand the effects of MOS and PRRSV on the pattern of gene expression, we used the IPA to categorize the significantly affected immune and nonimmune genes based on biological processes and molecular functions for both PBMC and BALF cells. We also examined the canonical pathways in which those genes were involved. The criteria for selection of genes to be included in the analysis were mentioned earlier. Biological functions and canonical pathways associated with the changes of gene expression in PBMC and BALF cells for 3 comparisons (interaction, IMOS vs. ICON, and IMOS vs. MOS) are presented. For each comparison, 12 biological processes and 4 canonical pathway are selectively shown.

Interaction between MOS and PRRSV. Putative functional categories of significantly affected genes in PBMC are shown in Table 4.9. For PBMC, the MOS x PRRSV interaction affected the majority of expressed genes identified by 64 biological processes in PBMC of pigs. The genes expressed were involved in many important biological processes such as cell cycle, cellular growth and proliferation, cell interaction and movement, and many other immune-related functions. These samples were taken at a stage of acute infection during which most immune cells were produced from the central immune organs and recruited to the site of infection. Particularly, genes related to hematological system development and function, inflammation, cell to cell signaling, and immune cell trafficking were activated. For BALF cells, the interaction significantly affected 74 biological processes (Table 4.10). The top biological functions in which gene expression was substantially changed comprised lipid metabolism, cell death, hematological system development and function, and many other immune-related processes.

The canonical pathways associated with PBMC genes that were differentially expressed are shown in Table 4.11. The MOS x PRRSV interaction affected 214 pathways of which 38 were significant. Most biological pathways had more genes downregulated than upregulated. Notably, the interaction downregulated the hypoxia signaling and triggering receptor expressed on myeloid cells (**TREM**)-1 signaling. Reduced mRNA expression of key transcriptional factors, tumor protein 53 (**p53**) and hypoxia-inducible factor (**HIF**)-1 indicated that the hypoxia signaling was downregulated. The significantly downregulated genes associated with TREM-1 signaling included cytokines (IL-1β, IL-6) and chemokines (IL-8, MIP-1β, MCP-1). With respect to BALF cells, of 173 pathways, 19 were significantly affected by MOS x PRRSV interaction (Table 4.12). More genes involved in these pathways were downregulated than upregulated.

Infected MOS vs. Infected CON. The results obtained showed that MOS affected a number of biological processes in PBMC and BALF cells of the infected pigs. For PBMC, 76 significant biological processes were found (Table 4.9). They included cell cycle, DNA synthesis and repair, cellular movement, hematological system development and function, and many other immune-related functions. For BALF cells, MOS significantly affected 71 biological processes, including cell to cell signaling and interaction, hematological system development and function, tissue development, lipid metabolism, and many other immune-related functions (Table 4.10).

For PBMC, it was noted that the 4 major canonical pathways involved were communication between innate and adaptive immune cells, TREM-1 signaling, p53 signaling, and protein ubiquitination (Table 4.11). The significantly affected genes participated in these pathways were more downregulated than upregulated, suggesting that

those pathways are not activated by MOS in infected pigs. Genes whose expression was repressed included IL-1β, IL-6, IL8, MIP-1α, MIP-1β, TLR9, p38 mitogen-activated protein kinase (**p38 MAPK**), cysteine-aspartic protease-6, baculoviral inhibitor of apoptosis repeat-containing 5, ubiquitin-conjugating enzymes, ubiquitin ligase, and inducible heat shock protein 90. For BALF cells, MOS impacted several functional pathways, including macropinocytosis signaling, clathrin-mediated endocytosis signaling, antigen presentation pathway, virus entry via endocytic pathway, and IL-4 signaling (Table 4.12). The genes significantly repressed were MR, integrin-β, phosphoinositide 3-kinase (**PI3K**), protein kinase C, cell division control protein 42, activating protein 2, huntingtin interacting protein-1, IL-13 receptor, corticosteroid receptor, and members of MHCII. Only CD14 mRNA which encodes a co-receptor along with TLR4 for the detection of bacterial lipopolysaccharide was upregulated.

Infected MOS vs. Uninfected MOS. For PBMC, PRRSV infection significantly influenced 66 biological processes within the MOS-fed pigs (Table 4.9). These biological processes identified consisted of antigen presentation, cell to cell signaling and interaction, hematological system development and function, and many other immune-related functions. For BALF cells, 61 biological processes were significantly affected (Table 4.10). The biological functions affected included cellular movement, cell to cell signaling and interaction, hematological system development and function, cell death, and many other immune-related functions. The results from both types of cells indicated that the immune cells from the hematological system were recruited to the site of infection.

In PBMC, the canonical pathways found were oxidative phosphorylation, dendritic cell maturation, communication between innate and adaptive immune cells, TREM-1

signaling, and other immune-related pathways (Table 4.11). The genes significantly downregulated in these pathways were TLR2, TLR4, IL-1α, IL-1β, IL-6, IL-8, MIP-1α, MIP-1β, p38 MAPK, and cluster of differentiation (**CD**) 154. The infection increased the expression of fragment crystallizable of IgG, receptor, and transporter, signal transducer and activator of transcription 1, MHCII, myeloid differentiation factor (**MyD**)-88, and cysteine-aspartic protease-1. For BALF cells, the canonical pathways affected included communication between innate and adaptive immune cells, the complement system, crosstalk between dendritic cells and natural killer cells, and many other immune-related pathways (Table 4.12). The downregulated genes included TLR4, TLR9, IL-1α, IL-1β, IL-6, IL-8, MIP-1α, MIP-1β, and MHCII. The upregulated genes included IFN-γ, IL-10, chemokine (C-C motif) ligand 5, natural killer group 2D, and CD69.

Validation of Gene Expression by Real Time RT-PCR

Based on the microarray data, 9 immune-related genes were further analyzed to validate the result of gene expression by the microarray technique. The genes analyzed included IL-1β, IL-4, IL-6, MIP-1β, MCP-1, ARG-1, MHCII, TLR4, and MR. It was found that 8 of the selected genes analyzed by RT-PCR had similar levels of relative expression as those identified by microarray (Table 4.13). Although the magnitude of the responses of those genes varied from one method to another, it did not change the trend of the responses. In addition, the IL-4 gene, which was not expressed by microarray, was also not detected by RT-PCR. There was a significant MOS x PRRSV interaction for IL-1β ($P < 0.01$), IL-6 ($P < 0.01$), MIP-1β ($P < 0.01$), MCP-1 ($P = 0.06$), TLR4 ($P < 0.01$), MR ($P < 0.05$), and ARG-1 ($P = 0.053$). In brief, the results of gene expression analysis by both methods were consistent.

DISCUSSION

Mannan oligosaccharide, when added to nursery diets, regulates the pig's immune responses (Che et al., 2009). Dietary MOS was associated with the rapidly increased WBC and lymphocytes in pigs during the first wk PI. However, the declined fever in the PRRSV-infected pigs consuming MOS by d 7 PI suggested that the intensity of on-going inflammation was gradually reduced. These altered immune responses may be associated with changes in the expression level of immune-related genes, particularly inflammation-regulating genes. Therefore, transcriptional profiling of PBMC and BALF cells by using the Affymetrix microarray and real time RT-PCR would help us better understand the host's immune response to PRRSV and the immunomodulatory role of MOS in relation to a viral infection.

Dietary MOS, PRRSV infection, and their interaction regulated the transcriptional level of a great number of nonimmune and immune genes in both PBMC and BALF cells (Tables 4.5 and 4.6). The MOS x PRRSV interaction affected a greater number of genes in PBMC than in BALF cells. Notably, in uninfected pigs, MOS appeared to alter the expression of genes in PBMC only, indicating that under an unchallenged condition, MOS has a greater impact on PBMC than BALF cells. The MOS-induced changes of gene expression in PBMC are very important in prompt triggering of an immune response because those cells circulate around the body and encounter the endogenous and exogenous stimuli (Kohlmeier and Woodland, 2009). This is supported by the fact that changes in the gene expression in BALF cells were not found between MOS and CON, but between IMOS and ICON. Also, the number of genes differentially expressed in PBMC substantially varied between the infected pigs and the uninfected ones. Interaction of the MOS-primed PBMC

with other molecules and extracellular environments during differentiation and homing at a specific tissue is critical and would have a considerable impact on their subsequent reaction against an immunogenic agent (Lefrancois and Puddington, 2006). Clearly, these results strikingly demonstrated that MOS added to the pig's diet regulates the expression of genes in both PBMC and BALF cells, and this alteration may bring about further changes in immune responses and disease resistance of pigs.

Among the differentially expressed genes in PBMC, many important immune genes involved in chemoattraction, inflammatory regulation, and pathogen detection were regulated by MOS (Table 4.7). Dietary MOS increased the mRNA expression of genes of the innate and adpative immunity, including IL-1α, IL-6, TLR4, dead box polypeptide (**DDX**) 58, CD1.1, MyD88, and MHCII in the uninfected pigs. The protein cytokines, IL-1α and IL-6, play a significant role in the host's defense mechanism. The IL-1α gene constitutively expresses in keratinocytes of the skin, epithelial cells of the cornea, granulosa cells of the ovary, and hypothalamic cells in the brain (Dinarello, 1994). The increased expression of IL-1α gene in PBMC of the uninfected pigs fed MOS is a striking finding. After being secreted, IL-1α remains mainly intracellular, but has several biologically important functions. It activates growth and differentiation factors that initiate cellular proliferation and migration events in response to immunological insults (Dinarello, 1994; Gosselin and Rivest, 2007). Recently, it has been found that IL-1α induced an antiviral state and secretion of 2 transferable antiviral factors, IFN-α and a soluble form of the low density lipoprotein receptor (Werman et al. 2008). The IL-6 cytokine has many pro-inflammatory and anti-inflammatory effects. As it can cause inflammation and fever and it also reduces inflammation by inhibiting the production of IL-1β and TNF-α and inducing anti-

inflammatory mediators such as acute phase proteins, IL-1 receptor antagonist, and IL-10 (Tilg et al., 1997; Kishimoto, 2005). Thus, the MOS-induced expression of IL-1α and IL-6 genes may enhance the pig's immunity to microbial infections and possibly provide some protection associated with IL-6 immunosuppressive effects.

Upregulation of TLR4, DDX58, CD1.1, MyD88, and MHCII genes consolidated the host's immune defense against invading pathogens and immunologic challenges (Table 4.7). Toll-like receptor 4 recognizes lipopolysaccharide on various gram-negative bacteria, whereas DDX58 codes for a PRR called retinoic-inducible gene-1 protein that can detect viruses (Takeda et al, 2003; Luo et al., 2008; Takeuchi and Akira, 2008). The CD1.1 is an MHCI-like surface glycoprotein that can be recognized by T cells. It has been suggested to play an important role at the pre-adaptive phase of immune responses to some microbial pathogens (Roark et al., 1998; Moody and Porcelli, 2003). Remarkably, the adaptor protein, MyD88 is essential for the stimulation of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, etc., and the entire range of TLR family agonists (Finberg and Kurt-Jones, 2004; Netea et al., 2004). For example, lack of MyD88 mRNA expression inhibited the IL-1 and IL-18-mediated functions (Adachi et al., 1998). Further, the MHCII which is found only on professional antigen-presenting cells, including macrophages, dendritic cells, and B cells was regulated by MOS. The increase in mRNA expression of MHCII in uninfected pigs fed MOS implies that dietary MOS is capable of promoting antigen presentation via inducing the expression of MHCII gene. Taken together with the expression of cytokine genes, it is apparent that MOS-induced upregulation of the gene expression of cytokines, intracellular signaling molecules, and PRR is immunologically very significant in recognition of

microbial pathogens, activation of the innate immune system, and initiation of a shift from an innate to adaptive immune response.

In infected pigs, MOS downregulated the mRNA expression of cytokines and chemokines such as IL-1β, IL-6, IL-8, MIP-1α, MIP-1β, and MCP-1 in PBMC (Table 4.7). The cytokines, IL-1β and IL-6, are secreted primarily by macrophages/monocytes in response to immunological challenges and can induce fever by resetting the thermoregulatory center in hypothalamus (Turnbull and Rivier, 1999; Gosselin and Rivest, 2007). In addition, cytokines and chemokines are capable of activating adhesion molecules, inducing acute phase proteins, and attracting leukocytes, particularly monocytes (Menten et al., 2002; Petersen et al., 2004; Rot and Andrian, 2004). Hence, this suggests that the reduced mRNA expression of these inflammatory mediators in the infected pigs fed MOS may have contributed to the reduced inflammatory response.

With regard to the BALF cells of infected pigs, MOS-modified molecules were associated with antigen presentation, the complement system, and 2',5'-oligoadenylate synthetase (**OAS**)-1 (Table 4.8). It was shown that PRRSV downregulated both types of MHC in dendritic cells (Loving et al., 2006; Wang et al., 2007). However, our results showed that PRRSV downregulated the expression of MHCII genes and upregulated the expression of MHCI genes in BALF cells, regardless of diet type. Mannan oligosaccharide reduced the gene expression of MHCII, but increased the gene expression of MHCI in the infected pigs as compared to the control. This implies that MOS may facilitate a cytotoxic T cell response against a viral infection. Although enhancing MHCI mRNA expression, MOS decreased the expression of genes encoding fragment crystallizable of IgG, receptor, and transporter, complement component 1, and ficolin, which are important components of the complement

system. These molecules assist in lysis and opsonization of microbes or particular antigens, leading to the clearance of invading pathogens (Holmskov et al., 2003). For instance, complement component 1 and ficolin act as an opsonin in the classical and lectin pathway, respectively. They are synthesized by hepatocytes, but also produced by alveolar macrophages and PBMC in response to infections (Matsushita and Fujita, 2002). In addition, MOS reduced the mRNA expression of OAS-1 in the infected pigs. The OAS-1 is an IFN-induced antiviral protein and expressed as an inactive enzyme, which requires double-stranded RNA for activation (Justesen et al., 2000). The active OAS-1 is involved in the formation of the activated latent ribonuclease which can suppress protein synthesis and viral growth by degrading viral and cellular RNA (Eskildsen et al., 2003). It was found that there was no difference in the IFN mRNA expression in BALF cells between the infected pigs fed MOS and those fed CON. Thus, the decrease in OAS-1 may be associated with the intracellular level of PRRSV and the double-stranded RNA generated at some stage in its life cycle (Eskildsen et al., 2003). Also, the OAS-activated latent ribonuclease has been shown to mediate viral-induced apoptosis (Durand et al., 2009). In BALF cells MOS may promote a cytotoxic T cell immune response by enhancing MHCI mRNA expression, but reduce the expression of complement system-associated molecules and OAS-1 at d 7 PI.

The prominent canonical pathways identified in PBMC are TREM-1 signaling and hypoxia signaling (Table 4.11). The TREM-1 is a cell surface receptor expressed on neutrophils and monocytes and may act as an amplifier of the immune response, promoting the secretion of inflammatory cytokines such as IL-1β, TNF-α, IL-6, and IL-8 (Bleharski et al., 2003). The activation of TREM-1 and TLR4 results in a greater increase in cytokine and chemokine production, compared to either stimulus alone (Sharif and Knapp, 2008). The

reduced mRNA expression of TLR4 accompanied by a reduction in mRNA expression of pro-inflammatory cytokines (IL-1β, IL-6, & IL-8) and chemokines (MIP-1α, MIP-1β, & MCP-1) in PBMC indicates that the PRRSV-induced inflammation is alleviated by MOS via downregulation of the TREM-1 pathway. Hypoxia is a pathological condition in which the infected pigs are deprived of an adequate supply of oxygen. The 2 important transcription factors of the pathway, p53 and HIF-1α, were downregulated, implying that the infected pigs fed the control diet experienced lack of oxygen supply and were under more severe oxidative stress as compared to those fed MOS (Acker and Plate, 2002; Sax and El-Deiry, 2003). The suppression of the hypoxia signaling pathway thus suggests a reduced intensity of PRRSV-induced inflammation in the infected pigs consuming MOS.

In BALF cells, the biological pathways of IL-4 and macropinocytosis signalings were perhaps of importance (Table 4.12). In infected pigs, MOS downregulated the IL-4 signaling pathway, suggesting that a shift from Th1 to Th2 immune response may be delayed in BALF cells in association with MOS. The downregulated genes involved in the IL-4 signaling pathway included IL-2 receptor γ, IL-4 receptor α, IL-13 receptor α, PI3K, and MHCII. The reduced expression of IL-4 receptors and PI3K causes a decreased IL-4-mediated signal leading to less transcription of IL-4 responsive genes such as IL-4 receptor α, MHCII, CD23, and IL-4 (Varin and Gordon, 2009). The reduced macropinocytosis signaling in the infected pigs was also found to be associated with MOS. Mannose receptors and β-integrin are important cell receptors, responsible for the uptake of microbial pathogens into a cell (Gazi and Martinez-Pomares, 2009). The decreased transcriptional expression of these receptors may confine the over-entry of viruses via endocytic pathways, thereby alleviating severe damage to the infected tissue.

Induction of alternative activation of macrophages in the infected pigs appeared to be associated with MOS. This functional pathway is induced by IL-4 and IL-13 (Gordon, 2003). The alternative activation of macrophages plays an important role in the protection of the host by decreasing inflammation and promoting tissue repair. Interleukin-4 inhibits expression of pro-inflammatory cytokines and chemokines and stimulates production of anti-inflammatory cytokines (Martinez et al., 2009), thus reducing inflammation. Our results showed that MOS reduced the gene expression of IL-1α, IL-6, IL-8, MIP-1α, and MIP-1β. Although no difference in the mRNA expression of IL-10 was found, the increased serum IL-10 in the MOS-fed pigs observed in the same study may reflect the involvement of this pathway (Che et al., 2009). The enhanced mRNA expression of ARG-1 in PBMC of the infected pigs fed MOS perhaps is a good indicator of the pathway and suggested initiation of tissue repair ensued (Table 4.13). Arginase 1 hydrolyzes L-arginine to urea and L-ornithine which is used to produce polyamines and proline to promote cell growth and collagen production (Varin and Gordon, 2009). One of the characteristic features of the alternative activation of macrophages is the increased expression of MR and MHCII, leading to increased antigen phagocytosis and presentation. Both MR and MHCII had greater expression in the infected pigs fed MOS than those that received the control. Mannan oligosaccharide fed to the infected pigs resulted in the expression of several marker genes associated with the alternative activation of macrophages. However, it is uncertain that MOS plays a crucial role in the induction of this pathway due to the lack of IL-4 mRNA expression.

In summary, the functional analyses of gene expression show that dietary MOS has a greater impact on the expression of genes in PBMC than in BALF cells at d 7 PI. The

increased expression of genes encoding cytokines, intracellular signaling molecules, and PRR in PBMC of uninfected pigs consuming MOS enhances the host's ability to quickly detect and mount an immune response to microbial invaders. This finding supports the fact that MOS was associated with rapidly increased leukocytes at the early stage of PRRSV infection. Within infected pigs, however, MOS reduces the gene expression of major inflammatory mediators in PBMC, possibly explaining why fever was ameliorated in the infected pigs fed MOS by d 7 PI. In BALF cells, MOS may promote the destruction of a virus through the cell-mediated immunity rather than the activation of the complement system. Our findings can provide new insights into the immunomodulatory property of MOS. This functional carbohydrate perhaps provides benefits by enhancing immunity while preventing over-stimulation of the immune system.

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FIGURES AND TABLES

Table 4.1 Composition of basal diets fed to nursery pigs during the experiment (as-fed basis)

Item	Phase[1]		
	I	II	III
Ingredients, %			
Corn	38.11	42.82	52.45
Dried whey	22.00	16.00	10.00
Soybean meal, 48%	10.00	18.00	24.00
Spray-dried animal plasma	8.00	4.00	0.00
Soy protein concentrate[2]	5.00	3.00	0.00
Select menhaden fish meal	4.01	5.49	6.25
Soybean oil	3.61	4.09	4.07
Lactose	5.60	2.80	0.00
Limestone	0.85	0.56	0.55
Dicalcium phosphate	0.67	0.92	0.48
Carbadox premix[3]	1.00	1.00	1.00
Zinc oxide	0.42	0.42	0.42
Mineral premix[4]	0.35	0.35	0.35
Vitamin premix[5]	0.20	0.20	0.20
Lysine-HCl	0.07	0.16	0.22
DL-met	0.11	0.10	0.08
L-thr	0.01	0.09	0.14

Table 4.1 (cont.)

Item	Phase[1]		
	I	II	III
Calculated composition			
ME, Mcal/kg	3.45	3.45	3.45
Standardized ileal digestible AA, %			
Lys	1.45	1.45	1.30
Met	0.41	0.43	0.42
Thr	0.94	0.94	0.84
Tryp	0.29	0.27	0.23
Val	1.10	1.03	0.89
Ile	0.87	0.87	0.79
Ca, %	0.90	0.90	0.80
Available P, %	0.55	0.55	0.40
Lactose, %	21.00	14.00	7.00

[1]Phase I, II, and III diets were fed to nursery pigs for 7, 7, and 14 d postweaning, respectively.
[2]Soycomil, Archer Daniels Midland Company, Decatur, IL.
[3]Mecadox 2.5, provided 0.055 g of carbadox per kilogram of diet, Phibro Animal Health, Fairfield, NJ.
[4]Provided as milligrams per kilogram of diet: sodium chloride, 3,000; zinc, 100 from zinc oxide; iron, 90 from iron sulfate; manganese, 20 from manganese oxide; copper, 8 from copper sulfate; iodine, 0.35 from calcium iodide; selenium, 0.30 from sodium selenite.
[5]Provided per kilogram of diet: retinyl acetate, 2,273 µg; cholecalciferol, 17 µg; DL-α-tocopheryl acetate, 88 mg; menadione sodium bisulfate complex, 4 mg; niacin, 33 mg; D-Ca-pantothenate, 24 mg; riboflavin, 9 mg; vitamin B_{12}, 35 µg; choline chloride, 324 mg.

Table 4.2 The total and detected numbers of probe sets in the Affymetrix's porcine arrays

Tissue	Immune probe sets		Other probe sets	
	Total[1]	Detected	Total	Detected
PBMC[2]	154	122	23773	17,167
BALF cells[3]	154	110	23773	17,648

[1]Provided by Affymetrix's NetAffix Analysis Center on March 9, 2009.
[2]Peripheral blood mononuclear cells.
[3]Bronchoalveolar lavage fluid.

Table 4.3 The number of genes in peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage fluid (BALF) cells used for pattern analysis of gene expression

Item		IMOS[1]-ICON[2]	IMOS[1]-MOS[3]	Interaction
PBMC	Fold-change cutoff	None	None	2
	FDR p-value cutoff[4]	0.05	0.05	0.05
	Function/pathway eligible[5]	271	301	775
	Significant genes[6]	207	230	712
BALF cells	Fold-change cutoff	2.5	8	None
	FDR p-value cutoff[4]	0.05	0.05	0.1
	Function/pathway eligible[5]	573	638	543
	Significant genes[6]	517	591	491

[1]IMOS: infected mannan oligosaccharide-fed pigs.
[2]ICON: infected control-fed pigs, a baseline for that comparison.
[3]MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison.
[4]FDR: false discovery rate.
[5]Biological and disease processes are most relevant to the genes detected; which well-characterized cell signaling and metabolic pathways are most relevant to the data obtained
[6]Differentially expressed genes.

Table 4.4 Gene specific primer sequences and PCR conditions[1]

Gene	Acc. No[2]	Forward primer (5'-3')	Reverse primer (5'-3')
IL-1β	M86725	TGCCAACGTGCAGTCTATGG	TGGGCCAGCCAGCACTAG
IL-4	NM_214123	TTCGTCCACGGACACAAGTG	GCTCCATGCACGAGTTCTTTC
IL-6	M86722	CTGGCAGAAAACAACCTGAACC	TGATTCTCATCAAGCAGGTCTCC
MIP-1β[3]	EU364894	CATACACCGTGCGGAAGCTT	CCCTTTTTGGTCTGGAATACCA
MCP-1[3]	EU682382	CGGCTGATGAGCTACAGAAGAGT	GCTTGGGTTCTGCACAGATCT
TLR4[3]	AB188301	TGTGGCCATCGCTGCTAAC	GGGACACCACGACAATAACCTT
MR[3]	AY368183	AGGCGTGTCCACTTACCACAA	TGCCTATGAGATCTTTCGTGTCA
MHCII[3]	BX088590	CTGAATGCGTTGGCCACATA	GGGTGTGTGTGGCACAGTTC
ARG-1[3]	AY039112	AGAATCCAAGGTCTGTGGGAAA	TGGTCTCCGCCCAGTACAAG

[1]Thermal cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles with 15 sec at 95°C and 1 min at 60°C.
[2]Accession number to Genbank database.
[3]MIP-1β: macrophage inflammatory protein-1β; MCP-1: monocyte chemotactic protein-1; TLR4: toll-like receptor 4; MR: mannose receptor; MHCII: major histocompatibility complex II; ARG-1: arginase-1.

Table 4.5 The number of nonimmune probe sets changed in peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage fluid (BALF) cells

Comparison[1]	PBMC[2]		BALF cells[2]	
	Upregulation	Downregulation	Upregulation	Downregulation
IMOS[3] vs. ICON[4]	164 (38%)	237 (62%)	1007 (43%)	1318 (57%)
MOS[5] vs. CON[6]	938 (48%)	998 (52%)	1 (50%)	1 (50%)
IMOS[3] vs. MOS[5]	256 (56%)	198 (44%)	3494 (45%)	4318 (55%)
ICON[4] vs. CON[6]	2243 (56%)	1791 (44%)	1083 (41%)	1585 (59%)
Interaction[7]	977 (46%)	1128 (54%)	117 (60%)	78 (40%)

[1]Comparisons set up at a false discovery rate P-value cutoff of < 0.05.
[2]The number in parentheses is the percentage of upregulated or downregulated probe sets for each tissue.
[3]IMOS: infected mannan oligosaccharide-fed pigs.
[4]ICON: infected control-fed pigs; a baseline for that comparison.
[5]MOS: uninfected mannan oligosaccharide-fed pigs; a baseline for that comparison.
[6]CON: uninfected control-fed pigs; a baseline for that comparison.
[7]Interaction = (IMOS-ICON) - (MOS-CON).

Table 4.6 The number of immune probe sets changed in peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage fluid (BALF) cells

Comparison[1]	PBMC[2]		BALF cells[2]	
	Upregulation	Downregulation	Upregulation	Downregulation
IMOS[3] vs. ICON[4]	3 (25%)	9 (75%)	1 (4%)	24 (96%)
MOS[5] vs. CON[6]	14 (93%)	1 (7%)	0 (0%)	0 (0%)
IMOS[3] vs. MOS[5]	9 (28%)	23 (72%)	15 (25%)	46 (75%)
ICON[4] vs. CON[6]	13 (93%)	1 (7%)	8 (33%)	16 (67%)
Interaction[7]	2 (10%)	19 (90%)	0 (0%)	0 (0%)

[1]Comparisons set up at a false discovery rate P-value cutoff of < 0.05.
[2]The number in parentheses is the percentage of upregulated or downregulated probe sets for each tissue.
[3]IMOS: infected mannan oligosaccharide-fed pigs.
[4]ICON: infected control-fed pigs; a baseline for that comparison.
[5]MOS: uninfected mannan oligosaccharide-fed pigs; a baseline for that comparison.
[6]CON: uninfected control-fed pigs; a baseline for that comparison.
[7]Interaction = (IMOS-ICON) - (MOS-CON).

Table 4.7 Differentially expressed genes in peripheral blood mononuclear cells of pigs

Genes	Fold-change[1]				
	MOS[2]-CON[3]	IMOS[4]-ICON[5]	IMOS[4]-MOS[2]	ICON[5]-CON[3]	Interaction[6]
IL-1α	+7.4	N.S.	-11.2	N.S.	-15.9
IL-1β	N.S.	-4.2	-15.9	N.S.	-11.6
IL-6	+6.9	-7.2	-17.0	N.S.	-49.8
IL-8	N.S.	-7.4	-40.1	N.S.	-24.6
MIP-1α[7]	N.S.	-8.2	-19.9	N.S.	-11.2
MIP-1β[7]	N.S.	-3.1	-5.1	N.S.	-4.3
MCP-1[7]	N.S.	-3.7	-9.4	N.S.	-11.1
MyD88[7]	+2.9	N.S.	N.S.	+4.9	-2.7
MHCII[7]	+2.0	N.S.	N.S.	N.S.	N.S.
TLR4[7]	+6.5	N.S.	-4.5	N.S.	-8.6
DDX58[7]	+2.5	N.S.	N.S.	+2.3	-3.1
CD1.1[7]	+2.4	N.S.	+2.3	+4.3	N.S.
PEC-60[7]	N.S.	+17.0	+11.8	N.S.	N.S.
ARG-1[7]	N.S.	+35.0	N.S.	N.S.	N.S.

[1]Genes identified as > 2 fold change up or down & a false discovery rate P-value cutoff of < 0.05; N.S.: the expression level of genes which did not meet those criteria was not shown.
[2]MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison.
[3]CON: uninfected control-fed pigs, a baseline for that comparison.
[4]IMOS: infected mannan oligosaccharide-fed pigs.
[5]ICON: infected control-fed pigs, a baseline for that comparison.
[6]Interaction = (IMOS-ICON) – (MOS-CON).
[7]MIP-1α: macrophage inflammatory protein-1α; MIP-1β: macrophage inflammatory protein-1β; MCP-1: monocyte chemotactic protein-1; MyD88: myeloid differentiation factor 88; MHCII: major histocompatibility complex II; TLR4: toll-like-receptor 4; DDX58: dead box polypeptide 58; CD1.1: cluster of differentiation 1.1; PEC-60: peptide with N-terminal glutamic acid, C-terminal cysteine, and a total of 60 residues; ARG-1: arginase-1.

Table 4.8 Differentially expressed genes in bronchoalveolar lavage fluid cells of pigs

Genes	Fold-change[1]		
	IMOS[2]-ICON[3]	IMOS[2]-MOS[4]	ICON[3]-CON[5]
APRIL[6]	-7.4	-19.4	N.S.
FCGRT[6]	-2.4	-4.1	N.S.
C1QA[6]	-10.9	-39.1	N.S.
Ficolin	-6.0	-8.3	N.S.
TLR4[6]	-2.7	-13.5	-4.5
MHCII[6]	-5.6	-25.0	-4.9
OAS-1[6]	-6.1	-16.2	N.S.
DDX58[6]	-3.5	-12.9	-3.6
MHCI[6]	+2.1	+2.8	N.S.

[1]Genes identified as > 2 fold change up or down & a false discovery rate P-value cutoff of < 0.05; N.S.: the expression level of genes which did not meet those criteria was not shown. No genes were differentially expressed for MOS x PRRSV interaction as well as between MOS and CON.
[2]IMOS: infected mannan oligosaccharide-fed pigs.
[3]ICON: infected control-fed pigs, a baseline for that comparison.
[4]MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison.
[5]CON: uninfected control-fed pigs, a baseline for that comparison.
[6]APRIL: a proliferation inducible ligand; FCGRT: fragment crystallizable of IgG, receptor, and transporter; C1QA: complement component 1, qsubunit, alpha chain; TLR4: toll-like receptor 4; MHCII: major histocompatibility complex II; OAS-1: 2',5'-oligoadenylate synthetase-1; DDX58: dead box polypeptide 58; MHCI: major histocompatibility complex I.

Table 4.9 Putative functional categories of significantly affected genes in peripheral blood mononuclear cells of pigs

Biological Function[1]	Number of Molecules[2]		
	Interaction[3]	IMOS[4]-ICON[5]	IMOS[4]-MOS[6]
Cell cycle	139 (1)	49 (1)	50 (10)
DNA replication, recombination, & repair	70 (4)	36 (2)	N.D.(12)[7]
Cellular movement	129 (10)	26 (3)	32 (8)
Hematological system development & function	132 (2)	29 (4)	57 (3)
Immune cell trafficking	56 (7)	14 (5)	41 (4)
Inflammatory response	51 (8)	13 (6)	44 (7)
Cardiovascular system development & function	58 (9)	12 (7)	25 (11)
Cell to cell signaling & interaction	93 (6)	24 (8)	47 (2)
Cellular growth & proliferation	239 (5)	31 (9)	87 (9)
Antigen presentation	49 (11)	12 (10)	43 (1)
Cell-mediated immune response	86 (3)	16 (11)	50 (5)
Humoral immune response	47 (12)	6 (12)	41 (6)

[1]Data were filtered with 2 criteria: Ingenuity pathway analysis threshold P-value ($P < 0.05$) and the corresponding microarray P-value.
[2]The number in parentheses represents the ranking of biological functions based on the P-value.
[3]Interaction = (IMOS-ICON) - (MOS-CON).
[4]IMOS: infected mannan oligosaccharide-fed pigs.
[5]ICON: infected control-fed pigs, a baseline for that comparison.
[6]MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison.
[7]N.D.: Not detected.

Table 4.10 Putative functional categories of significantly affected genes in bronchoalveolar lavage fluid cells of pigs

Biological Function[1]	Number of Molecules[2]		
	Interaction[3]	IMOS[4]-ICON[5]	IMOS[4]-MOS[6]
Cell to cell signaling & interaction	63 (5)	87 (1)	124 (2)
Hematological system development & function	66 (4)	67 (2)	133 (3)
Immune cell trafficking	39 (6)	58 (3)	88 (4)
Tissue development	32 (8)	70 (4)	73 (7)
Inflammatory response	53 (7)	56 (5)	107 (8)
Lipid metabolism	52 (1)	72 (6)	81 (12)
Cardiovascular system development & function	9 (10)	46 (7)	61 (11)
Antigen presentation	48 (2)	55 (8)	101 (6)
Cell death	151 (3)	150 (9)	205 (5)
Cellular movement	31 (11)	106 (10)	134 (1)
Cell-mediated immune response	54 (9)	50 (11)	119 (9)
Humoral immune response	53 (12)	47 (12)	99 (10)

[1]Data were filtered with 2 criteria: Ingenuity pathway analysis threshold P-value ($P < 0.05$) and the corresponding microarray P-value.
[2]The number in parentheses represents the ranking of biological functions based on the P-value.
[3]Interaction = (IMOS-ICON) - (MOS-CON).
[4]IMOS: infected mannan oligosaccharide-fed pigs.
[5]ICON: infected control-fed pigs, a baseline for that comparison.
[6]MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison.

Table 4.11 Ingenuity pathway analysis of microarray data identifies canonical pathway associated with peripheral blood mononuclear cell genes that are differentially expressed

Item[1]	Canonical pathway[2]	Score[3]	Number of genes, %		
			Significant[4]	Down[5]	Up[5]
Interaction	Hypoxia signaling	4.53	26	49	23
	TREM-1 signaling[6]	3.72	29	41	14
	Protein ubiquitination	3.03	15	46	32
	Integrin signaling	2.79	15	40	30
IMOS - ICON	Immune cell communication	3.13	17	33	12
	TREM-1 signaling[6]	2.37	14	32	22
	p53 signaling[6]	2.15	10	44	25
	Protein ubiquitination	1.97	5	45	32
IMOS - MOS	Oxidative phosphorylation	10.90	19	7	55
	Dendritic cell maturation	8.31	19	32	21
	Immune cell communication	7.05	29	36	10
	TREM-1 signaling[6]	6.62	26	36	19

[1]Interaction = (IMOS-ICON) - (MOS-CON); IMOS: infected mannan oligosaccharide-fed pigs; ICON: infected control-fed pigs, a baseline for that comparison; MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison; CON: uninfected control-fed pigs, a baseline for that comparison.
[2]Data were filtered with 2 criteria: Ingenuity pathway analysis threshold P-value ($P < 0.05$) and the corresponding microarray P-value; n = 3.
[3]The pathways were ranked by the score (score = -log(P-value)).
[4]Compared to the total number of upregulated and downregulated genes involved in that pathway.
[5]Compared to the total number of genes involved in that pathway.
[6]TREM-1: triggering receptor expressed on myeloid cells-1; p53: tumor protein 53.

Table 4.12 Ingenuity pathway analysis of microarray data identifies canonical pathway associated with bronchoalveolar lavage fluid cell genes that are differentially expressed

Item[1]	Canonical pathway[2]	Score[3]	Number of genes, %		
			Significant[4]	Down[5]	Up[5]
Interaction	IL-4 signaling	3.76	23	42	19
	LPS/IL-1 mediated inhibition of RXR function[6]	2.08	15	27	23
	Antigen presentation	2.07	24	41	13
	Pattern recognition receptors	1.90	17	33	19
IMOS - ICON	Macropinocytosis signaling	4.27	24	36	28
	Clathrin-mediated endocytosis	2.93	14	37	28
	Antigen presentation	2.69	29	38	15
	Virus entry via endocytosis	2.60	16	35	29
IMOS - MOS	Immune cell communication	4.36	41	20	16
	Complement system	3.93	32	28	42
	Crosstalk: dendritic & NK[6] cells	3.80	32	26	16
	Pattern recognition receptors	2.99	23	28	25

[1]Interaction = (IMOS-ICON) - (MOS-CON); IMOS: infected mannan oligosaccharide-fed pigs; ICON: infected control-fed pigs, a baseline for that comparison; MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison; CON: uninfected control-fed pigs, a baseline for that comparison.
[2]Data were filtered with 2 criteria: Ingenuity pathway analysis threshold P-value ($P < 0.05$) and the corresponding microarray P-value; n = 3.
[3]The pathways were ranked by the score (score = -log(P-value)).
[4]Compared to the total number of upregulated and downregulated genes in that pathway.
[5]Compared to the total number of genes involved in that pathway.
[6]LPS: lipopolysaccharide; RXR: retinoid X receptor; NK: natural killer.

Table 4.13 Verification of gene expression in peripheral blood mononuclear cells by real time RT-PCR[1]

Genes[2]	Fold change							
	MOS[3] - CON[4]		IMOS[5] - ICON[6]		IMOS[5] - MOS[3]		ICON[6] - CON[4]	
	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR
IL-1β	+2.5	+7.0	-4.6	-5.7	-15.9	-13.1	+1.1	+3.1
IL-6	+6.9	+12.0	-7.2	-12.9	-17.0	-14.0	+2.9	+11.1
MIP-1β	+1.4	+3.2	-3.1	-2.8	-5.1	-2.3	+1.2	+3.8
MCP-1	+3.0	+3.1	-3.7	-3.9	-9.4	-2.7	+1.2	+4.4
TLR4	+6.5	+5.1	-1.6	-2.1	-4.5	-3.6	+1.9	+2.9
MR	1.0	-1.9	+1.9	+1.7	+1.9	+1.9	1.0	-1.6
MHCII	+1.8	+1.9	+1.7	+1.3	+1.7	+1.4	+1.9	+2.0
ARG-1	+4.7	+3.9	+35.0	+12.2	+4.7	+5.6	+1.6	+1.8

[1]The total RNA samples (3 pigs/treatment) that were used to run Affymetrix's porcine microarray were employed for RT-PCR.
[2]The average threshold cycle values for IL-1β, IL-6, macrophage inflammatory protein (MIP)-1β, monocyte chemotactic protein (MCP)-1, arginase (ARG)-1, major histocompatibility complex (MHC) II, toll-like receptor (TLR) 4, and mannose receptor (MR) were 22.0, 28.5, 21.7, 30.4, 25.0, 24.3, 26.7, and 31.5, respectively; Eukaryotic 18S rRNA was used as an endogeneous control.
[3]MOS: uninfected mannan oligosaccharide-fed pigs, a baseline for that comparison.
[4]CON: uninfected control-fed pigs, a baseline for that comparison.
[5]IMOS: infected mannan oligosaccharide-fed pigs.
[6]ICON: infected control-fed pigs, a baseline for that comparison.

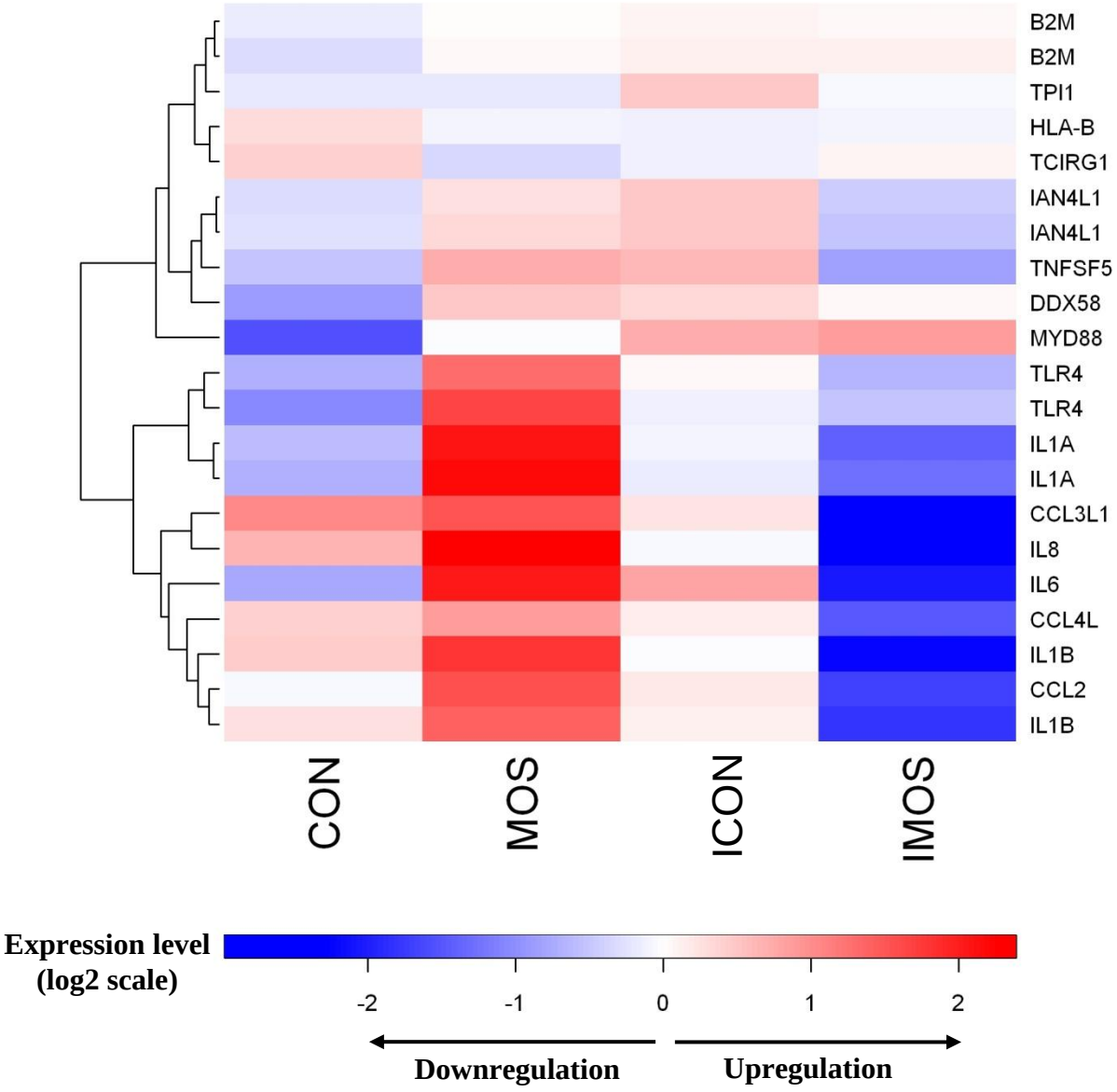

Figure 4.1 The MOS x PRRSV interaction on the expression of immune probe sets in PBMC of pigs; Levels of expression: relative to the overall mean. False discovery rate p-value cutoff: $P < 0.05$; CON: uninfected control-fed pigs; MOS: uninfected mannan oligosaccharide-fed pigs; ICON: infected control-fed pigs; IMOS: infected mannan oligosaccharide-fed pigs.

CHAPTER 5

GENERAL RESEARCH SUMMARY

Different products extracted from the yeast cell wall of *Saccharomyces cerevisiae* may have diverse immune-related properties, as each fraction differs in proportions of functional carbohydrates (mannan and β-glucan). The important aim of these studies was to determine effects of mannan oligosaccharide (**MOS**) on immune function and disease resistance in pigs. The research addressed 3 issues: (1) the *in vivo* and *in vitro* immunomodulatory properties of MOS on cytokine production of alveolar macrophages (**AMϕ**) in response to *in vitro* models of microbial challenges; (2) the effect of different levels of dietary MOS on serum cytokine concentrations and growth performance in pigs reared under regular housing conditions; and (3) the effect of dietary supplementation of MOS on immune responses and gene expression profile in pigs infected with porcine reproductive and respiratory syndrome virus (**PRRSV**).

Mannan oligosaccharide in both *in vivo* and *in vitro* systems regulated cytokine production by AMϕ in response to *in vitro* microbial challenge models. Alveolar macrophages were collected and stimulated *in vitro* with a bacterial challenge model, lipopolysaccharide (**LPS**) or a viral challenge model, polyinosinic:polycytidylic acid (**Poly I:C**). The LPS-stimulated AMϕ from pigs fed 0.2% or 0.4% MOS produced less tumor necrosis factor (**TNF)**-α and more IL-10 than those from pigs fed the diet without MOS. Similarly, when directly applied *in vitro,* MOS suppressed LPS-induced TNF-α and enhanced LPS-induced IL-10. Further, TNF-α production by AMϕ stimulated with LPS or Poly I:C was also suppressed *in vitro* by a mannan-rich fraction (**MRF**) which contains more

mannan than MOS. These results establish that both MOS and MRF reduce LPS-activated inflammatory response possibly by changing the expression of pattern recognition receptors (**PRR**) leading to modulation of activation signals and resultant immune responses. We then determined if MOS interacts with LPS receptors by culturing AMϕ with Polymyxin B, an inhibitor of LPS-activated toll-like receptor (**TLR**) 4. Although Polymyxin B completely inhibited AMϕ-produced TNF-α induced by LPS, it did not affect the ability of MOS to regulate cytokine production in the absence of LPS. It may be suggested that mannose receptor (**MR**) which can interfere with function of other cell receptors, e.g TLR4, may play a role in those immune responses. With regard to *in vitro* Poly I:C stimulation, MOS did not affect TNF-α secretion, but MRF reduced the Poly I:C-induced TNF-α. This brings up an interesting question whether more mannan in MRF contributes significantly to a much greater influence on MR expression and function, thereby affecting consequent responses of AMϕ to Poly I:C. Antigens or other molecules can be endocytosed by MR. It may be postulated that because of MRF-reduced endocytic activity of MR, less uptake of Poly I:C results in a reduction in inflammatory signaling transduction mediated by TLR3, an intracellular receptor specific to Poly I:C. Generally, these data establish that MOS is a potent immunomodulator in both *in vitro* and *in vivo* systems as determined by reducing TNF-α and enhancing IL-10 synthesis after *ex vivo* challenge of porcine AMϕ with bacterial endotoxin. However, MRF-mediated specific involvement of MR on the suppression of TLR3 activation-induced inflammation is beyond the scope of this study.

In addition, MOS and other yeast-related components were found to be able to regulate constitutive production of TNF-α in the absence of LPS or Poly I:C. Production of TNF-α by AMϕ was greatest at 0.5 mg/mL of MOS and decreased when the stimulating

concentrations of MOS increased up to 3 mg/mL. A MRF, containing more mannan than MOS, was shown to have a weaker activating effect on AMϕ. In contrast to MRF, glucan fraction which has much less mannan and more β-glucan than MOS activated AMϕ to secrete TNF-α. The direct activation of AMϕ by MOS *in vitro* indicates that it is recognized by AMϕ extracellular receptors and this recognition leads to TNF-α induction. Mannose receptors, TLR4, and dectin-1 are likely potent receptors involved in the recognition of the tested yeast components because those receptors have been shown to recognize mannan and β-glucan molecules. This aspect therefore should be further investigated to understand more details about the binding of yeast components by PRR on AMϕ activation. In brief, the ability of mannan-containing products such as MOS to constitutively regulate AMϕ-produced TNF-α in the absence of pathogen-associated stimulation is very important in maintaining and boosting the host's disease resistance.

Although MOS had a major impact on the *in vitro* cytokine production by AMϕ under various conditions, it did not seem to influence serum cytokine levels and growth performance in nursery pigs. The differences in growth and serum levels of TNF-α and IL-10 were not significant between pigs fed 0.2% or 0.4 % MOS diets and those fed the control. However, serum cytokines varied during the course of the experiment. The serum TNF-α was greater at d 7 and 28 postweaning (**PW**) than at d 14 and 21 PW, whereas serum IL-10 was increased at d 14 and 28 PW compared to d 7 and 21 PW. Cytokines not only regulate the body's immune response but also affect nutrient utilization. Thus, cytokine secretion is closely controlled in order to uphold disease resistance, but prevent any tissue damage due to over-production of pro-inflammatory cytokines. The interesting finding of this MOS feeding

experiment is that under regular housing conditions, changes in serum cytokine levels may be expected and reflect the host's reaction to any surrounding immunological stimuli.

Furthermore, feeding MOS to nursery pigs enhanced immunity while preventing over-stimulation of the immune system in response to a viral infection. Weaned pigs fed control or 0.2% MOS diets for 2 wk were intranasally inoculated with PRRSV or a sterile medium at 5 wk of age. The PRRSV infection decreased pig performance during the experimental period and the numbers of white blood cells (**WBC**) and lymphocytes through d 7 postinfection (**PI**). The infected pigs also had a febrile response and elevated levels of inflammatory mediators. In contrast, feeding MOS prevented leukopenia and lymphopenia at d 3 and 7 PI, tended to improve pig performance, and reduced fever at d 7 PI and TNF-α at d 14 PI. Rapidly increased numbers of WBC and lymphocytes at the early stage of infection demonstrate that the immune system of MOS-fed pigs is ready to react to a viral infection. This also points out that MOS enhances disease resistance, but further evaluation on increased subpopulations of lymphocytes will provide more details about specific types of lymphocytes involved in the early immune response. Additionally, decreases in fever and serum TNF-α observed in the infected pigs consuming MOS suggest that MOS is associated with reduced inflammation and may speed recovery. The increased level of serum IL-10 in MOS-fed pigs would indicate a shift from T helper (**Th**) 1 to Th2 lymphocyte response or increases in T regulatory cells and type II macrophages. Cytokines and chemokines secreted by these cells are negative regulators of Th1 responses and promote anti-inflammation.

The gene expression analysis of peripheral blood mononuclear cells and bronchoalveolar lavage fluid cells further strengthened the observations of the immune responses discussed above. In peripheral blood mononuclear cells, dietary MOS affected the

expression of immune genes encoding key inflammatory mediators. In uninfected pigs, MOS increased the mRNA expression of genes involving immune regulation, intracellular signaling molecules, and PRR. This suggests that MOS enhances the host's immune defense and supports the fact that MOS induced a rapid increase in leukocytes at the initial stage of infection. Within infected pigs, however, MOS reduced the mRNA expression of major cytokines (e.g., IL-1β, IL-6), chemokines (e.g., IL-8, MIP-1α, MIP-1β, and MCP-1), and TLR4. The decreased mRNA expression of these inflammatory regulators is likely to account for the ameliorated fever in the infected pigs fed MOS by d 7 PI. The downregulation of inflammatory responses regulated by MOS was associated with several important canonical pathways such as TREM-1 signaling, hypoxia signaling, IL-4 signaling, macropinocytosis signaling, and perhaps the alternative activation of macrophages. In bronchoalveolar lavage fluid cells MOS may promote a cytotoxic T cell immune response by enhancing MHCI mRNA expression, but downregulate the expression of molecules involved in the complement system in infected pigs at d 7 PI. It is apparent that dietary MOS changes the expression of immune genes in leukocytes of the PRRSV-infected pigs, perhaps providing benefits by enhancing immunity while preventing over-stimulation of the immune system.

In general, MOS added to nursery diets is not used to treat diseases, but should be considered a strategic feed additive that may provide some protection to pigs. Changes in PRR of leukocytes by MOS probably result in regulation of cellular activation and pathogen-induced responses. Those receptors participate in intracellular signaling, leading to target gene expression. Increased gene expression of cytokines and pathogen detection facilitates the pig's innate immune system to quickly mount an immune response against an infection and toward clearance of pathogens. However, MOS also suppresses over-reaction of the

immune system via stimulating the production of anti-inflammatory molecules and inhibiting the production of pro-inflammatory cytokines and chemokines. Thus, MOS may help prevent severe damage to infected tissues. Future research should be directed to examine gene expression of key receptors and their interaction in response to MOS and microbial challenges *in vitro*, and immune responses and performance of PRRSV-infected pigs during the recovery phase. Combined infections of bacterial and viral pathogens should be evaluated as MOS appears to reduce the intensity of inflammation due to a secondary bacterial infection.

AUTHOR'S BIOGRAPHY

Tung M. Che, the youngest in a 6-person family, was born on May 25, 1976 in Tay Ninh province in Vietnam. He grew up and completed primary, secondary, and high school educations in his hometown. Tung's internal dream, since his childhood, was to become a doctor in human medicine. Unfortunately, his father's sudden death completely ruined his long dream because of financial problems and led his life to an interesting new journey, the world of animals. One month after the sorrowing death of his father, Tung excellently passed the university entrance exam with the top 5 ranking and enrolled in an undergrad program in the field of animal science in Nong Lam University (**NLU**), Ho Chi Minh City. In 1998, he completed his B.Sc. degree with first honor graduation distinction and was a valedictorian.

In Oct. 1998, as the first honor graduate and with his excellent research work during the period of undergrad study program, Tung was offered a position as a lecturer/researcher at NLU. A few months later, Tung got a graduate research assistantship for his M.Sc. program from Putra University, Malaysia. In early 1999, Tung went to Malaysia to pursue his M.Sc. degree. In 2001, he accomplished his M.Sc degree in animal nutrition, had his first paper published, and returned to Vietnam to work as a researcher/lecturer at NLU.

In 2005, Tung started his Ph.D. program in the Department of Animal Sciences, University of Illinois at Urbana-Champaign. His Ph.D. study program was sponsored by the Vietnam Government and partly supported by Dr. James E. Pettigrew, his major advisor. In 2007, he got married to Anh T. Quach and had his first son, Van K. Che in 2009. Tung has received several prestigious awards during his Ph.D. study. Upon the completion of his Ph.D. program, Tung's plan is to work as a postdoctoral research associate for 2 more years before he returns to Vietnam. His long term goal is teaching and research.