KATIENE RÉGIA SILVA SOUSA

IMMUNOMODULATION OF COMMERCIAL LINE AND PIAU BRAZILIAN NATURALIZED BREED PIGS IN RESPONSE TO VACCINATION AGAINST MYCOPLASMA HYOPNEUMONIAE AND PASTEURELLA MULTOCIDA TYPE D

Thesis presented to the Animal Science Graduate Program of the Universidade Federal de Viçosa, in partial fulfillment of the requirements for degree of *Doctor Scientiae.*

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To my mother Maria do Socorro.

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BIOGRAPHY

Katiene Régia Silva Sousa, daughter of José Maria de Sousa and Maria do Socorro Silva Souza, born on January 29, 1980 in São Luís, Maranhão, Brazil. She began her studies in Veterinary at the Universidade Estadual do Maranhão in 2000. In March of 2006, she began a Master of Genetic and Breeding at the Universidade Federal de Viçosa and she completed her final examination on February 26, 2008 to obtain the title of *Magister Scientiae*. In March of 2008 she began the doctoral graduation program in Animal Breeding at the Animal Science Department of the Universidade Federal de Viçosa. On july 28, 2011, she completed her thesis defense to obtain the title of *Doctor Scientiae* in Animal Science.

SUMMARY

RESUMO vii

ABSTRACT ix

GENERAL INTRODUCTION 1

REFERENCES 6

CHAPTER 1 10

Differential expression of Toll-like receptors and cytokines in response to vaccination against *Mycoplasma hyopneumoniae* in Piau breed and a commercial line 10

ABSTRACT 10

RESUMO 12

1 INTRODUCTION 14

2 MATERIAL AND METHODS 16

- *2.1 Animals and tissue collection* 16
- *2.2 Nitrite assay estimation of NO production* 16
- *2.3 RNA isolation and cDNA synthesis* 17
- *2.4 Real Time PCR* 17
- *2.5 Statistical analysis:* 20

3 RESULTS 21

4 DISCUSSION 26

5 CONCLUSION 31

REFERENCES 32

CHAPTER 2 39

Toll-like receptors and cytokine mRNA expression profiles in porcine peripheral mononuclear cells and bronchoalveolar lavage cells in response to vaccination against *Pasteurella multocida* type D in two different genetic groups 39

ABSTRACT 39

RESUMO 41

1 INTRODUCTION 43

2 MATERIAL AND METHODS 45

- *2.1 Animals and tissue collection* 45
- *2.2 Nitrite assay estimation of NO production* 45
- *2.3 Lymphoid cell preparation* 46

    - *2.3.1 Cell cultures* ... 46
  - *2.4 RNA isolation and cDNA synthesis* ... 46
  - *2.5 Real Time PCR* ... 47
  - *2.6 Statistical analysis* ... 49
- **3 RESULTS** ... 50
- **4 DISCUSSION** ... 57
- **5 CONCLUSION** ... 63
- **REFERENCES** ... 64
- **GENERAL CONCLUSIONS** ... 71

RESUMO

SOUSA, Katiene Régia Silva, D.Sc., Universidade Federal de Viçosa, Julho de 2011. **Imunomodulação de suínos de linhagem comercial e da raça naturalizada brasileira Piau em resposta à vacinação contra *Mycoplasma hyopneumoniae* e *Pasteurella multocida* tipo D**. Orientador: Simone Eliza Facioni Guimarães. Co-orientadores: Paulo Sávio Lopes e Sérgio Oliveira de Paula.

Doenças respiratórias continuam a ser uma grande causa de perdas econômicas na produção de suínos. Tem havido relativamente pouco progresso no controle de doenças respiratórias, apesar do desenvolvimento contínuo de novas vacinas e antibióticos. Objetivou-se no presente estudo, comparar a resposta imune inata e adaptativa de suínos da raça naturalizada brasileira Piau e de uma linhagem comercial frente a vacinação contra *Micoplasma hyopneumoniae* e *Pasteurella multocida* tipo D. Para cada grupo genético, o sangue periférico das células mononucleares (PBMC) foi coletado antes e 10 dias depois de cada dose de vacinação de 12 leitões fêmeas soro-negativas para os patógenos em estudo para análise de RT-qPCR. Às dez semanas de idade, os baços dos animais foram coletados para ensaio de proliferação celular e, amostras de lavado broncoalveolar (BALF) foram coletadas para mensurar a produção de óxido nítrico e RT-qPCR. RNA foi extraído de células de PBMC e BALF, foi feita transcrição reversa e o RT-qPCR foi feito usando o sistema de detecção de fluorescência SYBR Green, usando como controle endógeno os genes GAPDH e HPRT1 para PBMC e BALF, respectivamente. Para o experimento com *Micoplasma hyopneumoniae* como patógeno, a linhagem comercial teve maior expressão de mRNA para TLR2, TLR4, TLR6 e TLR10 e as citocinas IL6 e TNFα depois da vacinação que antes da vacinação, enquanto os animais da raça Piau mostraram maior expressão para TLR6, TLR10, TNFα e TGFβ. Em relação às amostras de BALF, os animais vacinados da raça Piau tiveram maior diferença de expressão para os genes TLR4, TLR10, TNFα e TGFβ que os animais não vacinados da mesma raça. Por outro lado, os animais comerciais vacinados mostraram diferença significativa para TNFα. Quando se estudou a resposta específica para *Pasteurella multocida*, os animais comerciais tiveram expressão significativa para os genes TLR2, TLR4, TLR6, TLR10, IL2, IL6, IL8, IL10, IL12, IL13 e TNFα entre os tempos estudados, após duas doses da vacina; enquanto os animais Piau tiveram somente diferença de expressão para a citocina antiinflamatória TGFβ. Para expressão gênica em células de BALF dos animais comerciais vacinados, houve maiores níveis de mRNA de IL6 e TNFα que os não vacinados da mesma raça. Por outro lado, os animais vacinados Piau tiveram um aumento significativo para TNFα e TGFβ quando comparados aos não vacinados Piau. Portanto, foram observadas diferenças entre os animais Piau e os comerciais frente à vacinação contra *Micoplasma hyopneumoniae* e *Pasteurella multocida* tipo D, tais como, expressão de mRNA de receptores TLR2, TLR4, TLR6 e TLR10 e citocinas IL2, IL6, IL8, IL10, IL12, IL13, TNFα e TGFβ. Esses resultados são sugestivos de diferenças genéticas entre as raças que podem influenciar na susceptibilidade e na resistência às doenças.

ABSTRACT

SOUSA, Katiene Régia Silva, D.Sc., Universidade Federal de Viçosa; July, 2011. **Immunomodulation of commercial line and Piau Brazilian Naturalized breed pigs in response to vaccination against *Mycoplasma hyopneumoniae* and *Pasteurella multocida* type D.** Adviser: Simone Eliza Facioni Guimarães. Co-advisers: Paulo Sávio Lopes and Sérgio Oliveira de Paula.

Respiratory diseases continue to be the greatest cause of economic losses to swine production. There has been relatively little progress in the control of respiratory diseases, despite the continuous development of new vaccines and antibiotics. The objective of this study was to compare innate and adaptive immune response in Piau Brazilian Naturalized breed and commercial line in response to vaccination against *Mycoplasma hyopneumoniae* and *Pasteurella multocida* type D. For each genetic group, peripheral blood mononuclear cells (PBMC) were collected before and 10 days after vaccination against each pathogen from 12 serum-negative female piglets for RT-qPCR analysis. At 10 weeks of age, bronchoalveolar lavage fluid BALF samples were collected to measure nitric oxide production and RT-qPCR. Spleens samples were collected for lymphocytes proliferation studies. RNA was extracted from PBMCs and BALF cells, reverse transcripted and the RT-qPCR was performed using SYBR green fluorescence system, using GAPDH and HPRT1 genes as endogenous control for PBMC and BALF cells, respectively. For the *Mycoplasma hyopneumoniae* experiment, commercial line had higher expression of TLR2, TLR4, TLR6, TLR10, IL6 and TNFα mRNA after vaccination in comparison to before vaccination, while Piau animals showed higher expression only for TLR6, TLR10, TNFα e TGFβ. In relation to BALF samples, vaccinated Piau pigs showed higher differential expression for TLR4, TLR10, TNFα e TGFβ than unvaccinated Piau. In contrast, vaccinated commercial piglets showed significant difference for TNFα. When specific response in PBMC was studied in *Pasteurella multocida* analysis, commercial animals had stronger mRNA expression for TLR2, TLR4, TLR6, TLR10, IL2, IL6, IL8, IL10, IL12, IL13 and TNFα, comparing the time points studied after two doses of vaccine; on the other hand, Piau breed had only differential expression for TGFβ anti-inflammatory cytokine. For mRNA expression in BALF, vaccinated commercial group had higher levels of IL6 and TNFα than unvaccinated animals. In contrast, vaccinated Piau animals had increased TNFα and TGFβ mRNA in comparison to the unvaccinated Piau piglets. In conclusion, it was observed breed differences between Piau Brazilian Naturalized and commercial pigs in response to vaccination against *Mycoplasma hyopneumoniae* and *Pasteurella multocida* type D, such as expression of mRNA TLR2, TLR4, TLR6 and TLR10 and IL2, IL6, IL8, IL10, IL12, IL13, TNFα and TGFβ. These results are suggestive of genetic differences that may influence in disease susceptibility/ resistance.

GENERAL INTRODUCTION

Respiratory diseases continue to be the greatest cause of economic losses to swine production. There has been relatively little progress in the control of respiratory diseases, despite the continuous development of new vaccines and antibiotics (Van Reeth and Nauwynck, 2000; Glass, 2004). *Mycoplasma hyopneumoniae* is the causative agent of porcine enzootic pneumonia commonly complicated by opportunistic infections by other bacteria. The main clinical sign associated with *M. hyopneumoniae* infection is a sporadic, dry, non-productive cough (Choi et al., 2006). This disease makes the animals get in to progressive atrophic rhinitis, which is caused by *Pasteurella multocida* type D, gram-negative bacteria, normally found in the respiratory tract (Conceição and Dellagostin, 2006).

M. hyopneumoniae and *P. multocida* attaches to the cilia of tracheal epithelial cells, causing a reduction in ciliary action. After colonization of the cilia, lymphocytes infiltrate the site of infection, recruiting macrophages, neutrophils and other cellular immune responses. Once recruited to the site of infection, macrophages and neutrophils use superoxides, such as hydrogen peroxide, nitric oxide and superoxide anions, to kill bacteria during respiratory burst (Schafer et al., 2007). The response of the host immune system causes the lesions seen in the lung tissue of infected swine by increasing phagocytic and cytotoxic activities of macrophages and initiating a chronic inflammatory response (Sarradell et al., 2003).

The primary function of the immune system is to identify and eliminate pathogens. The immune system is subdivided into the innate and adaptive immunity. In a broad sense, the innate immune system is composed of anatomic, physiologic, phagocytic and inflammatory barriers. The aforementioned barriers enable the innate immune system to provide the first line of defense against infectious disease (Warner et al., 1987). The innate responses are induced through Toll-Like Receptors (TLRs), which are type 1 trans-membrane proteins situated in the cell membrane (TLR1, 2, 4, 5, 6, 10 and 11) or in the membrane of endosomes (TLR3, 7, 8 and 9). They are one class of pattern-recognition receptors (PRR) and recognize a wide variety of pathogen associated molecular patterns (PAMP) (Table 1) and can stimulate specific responses to different pathogens (Takeda et al., 2003; Harris et al., 2006; Uenish and Shinkai, 2009; Kumar et al., 2009; Jungi et al., 2011).

Table 1 Toll-like receptors and known microbial ligands

TLR family	Microbial ligands
TLR1	Tri-acyl lipopetides (bacteria, mycobacteria)
TLR2	Lipoprotein/lipopetides (a variety of pathogens) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) Glycolipids (*Treponema maltophilum*) Zymosan (fungi)
TLR3	Double-stranded RNA (virus)
TLR4	LPS (Gram-negative bacteria) HSP60 (*Chlamydia pneumonia*)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopetides (mycoplasma)
TLR7 or 8	U-rich ssRNA
TLR9	CpG DNA (bacteria)
TLR10	Ligand unknown
TLR11	Uropathogenic bacteria

Based on: Harris et al. (2006); Takeda et al. (2003)

The end product of microbial recognition by cells of the innate response is the activation of intracellular signalling pathways that initiate cellular processes, such as activation of microbicidal killing mechanisms, the production of pro-inflammatory and anti-inflammatory cytokines, and the production of costimulatory molecules required for antigen presentation to the acquired immune system (Imler et al., 2001; Kumar et al., 2009). Cytokines are mediators of innate and acquired immunity that initiate and coordinate cellular and humoral responses aiming to eradicate pathogens (Van Reeth and Nauwynck, 2000). The adaptive immunity improves on repeated exposure to a given infection and includes a specific immunological memory. This ability to develop an immunological memory is utilized at vaccination, when the host is immunized by a killed or attenuated living microorganism or its components, in order to evoke specific immunity to natural infection with the same microorganism (Johansson et al., 2002).

The development of a disease by an animal is the result of the interaction between the genotype of the individual and the environment. Disease occurs when environmental insult meets genetic predisposition and the genetic control of the resistance to disease is highly complex and involves immune system (Warner et al., 1987).

In agreement with Jovanovic et al. (2009), it is very important to distinguish between the following two terms: resistance and tolerance. Resistance refers to the ability of the individual host to resist infection or to control the pathogen life cycle, for example, to limit proliferation or transmission of infection to other hosts. Tolerance may be defined as the ability of a host to tolerate infection and show little or no measurable detriment, that is, minimal effects of disease. If the goal is to stop the spread of infection to another population, as like zoonoses, resistance to disease is far more advantageous than tolerance.

Genetic investigations involving animal resistance to infections caused by pathogens of varying etiologies can be determined as follows: selection of locally adapted breeds, the implementation of cross-breeding methods geared at introducing genes significant in the expression of genetic resistance/tolerance towards pathogens or identification of genetic markers linked to resistance, and the selection of individuals highly resistant to specific pathogen (Clapperton et al., 2005; Lewis et al., 2007).

In accordance to Bergman et al. (2010), the use of domestic animals enable analysis of populations exposed to different selection criteria and environmental challenges and comparisons to the wild ancestor can clarify the evolutionary process. Recently, studies based on genetic selection of pigs resistant to different pathological agents have been performed (Reiner et al., 2002b; Thanawongnuwech et al., 2004; Rehm et al., 2009) and quantitative trait loci (QTL) have been identified in pigs (Edfors-Lilja et al., 1998; Reiner et al., 2002a; Wimmers et al., 2009).

In addition, the availability of the pig whole genome sequence will contribute toward revealing the molecular mechanisms controlling phenotypes by identifying genetic factors and by improvement in vaccine responsiveness. It also may play an increasingly significant role in pork production, by integrating “omics” techniques and bioinformatics tools to reduce the incidence of disease and respond more rapidly to the changing demands of consumers (Glass, 2004; Lewis et al., 2007; Chen et al., 2007).

The Piau Brazilian Naturalized pig breed is considered fat-type, used to be reared in small farms, supplying farmers with meat and a large amount of fat (Sollero et al., 2008). These animals, originated from other breeds introduced by Portuguese settlers in the 16th century, have also been influenced by Dutch and African pig breeds (Vianna, 1985). Guimarães and Lopes (2001) reported these animals have singular characteristics such as rusticity, adaptability to poor conditions of management and feeding, and a great resistance to diseases.

The purpose of this experiment was to determine differences in expression of Toll-like receptors and pro-inflammatory and anti-inflammatory cytokines in porcine blood mononuclear cells and bronchoalveolar lavage cells between two different genetic groups, Piau Brazilian naturalized breed and a commercial line, before and after vaccination against *Mycoplasma hyopneumoniae* and *Pasteurella multocida* type D. It was also observed the production of nitric oxide in response to those pathogens and the ability lymphocyte proliferation in response to *Pasteurella multocida* type D.

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CHAPTER 1
Differential expression of Toll-like receptors and cytokines in response to vaccination against *Mycoplasma hyopneumoniae* in Piau breed and a commercial line

ABSTRACT

Mycoplasma hyopneumoniae, the primary pathogen of enzootic pneumonia, occurs worldwide and causes major economic losses to the pig industry; the organism adheres to and damages the ciliated epithelium of respiratory tract. The aim of the present study was to investigate expression of toll-like receptors and cytokines responses as well as nitric oxide production in response to vaccination against *Mycoplasma hyopneumoniae* in peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage fluid (BALF) cells from two different genetic groups, Piau Brazilian Naturalized breed and a commercial line. For each genetic group, peripheral blood was collected just before and 10 days after vaccination from 6 serum-negative female piglets. At 10 weeks of age, BALF samples were collected to measure nitric oxide (NO) production and RT-qPCR analysis. RNA was extracted from PBMCs and BALF cells, reverse transcripted and the RT-qPCR was performed using SYBR green fluorescence system, using GAPDH and HPRT1 genes as endogenous controls, respectively. There was significant difference expression for TLR2, TLR4, TLR6 and TLR10, IL6, TNFα and TGFβ in PBMC for commercial line, before and after vaccination; while in PBMC from Piau breed differential expression for mRNA TLR6, TLR10, TNFα and TGFβ was observed. For BALF cells, the vaccinated Piau pigs showed increased levels for TLR4, TLR10, TNFα and TGFβ in comparison to unvaccinated Piau; in contrast, commercial had no differential expression for the studied genes, except mRNA TNFα expression. These results suggested that vaccine against *Mycoplasma hyoneumoniae* modulates the differential immune response of Piau breed and commercial line pigs by inducing mRNA TLR2, TLR4, TLR6, TLR10, IL6, TNFα and TGFβ expression, which are suggestive of genetic differences. These breed differences seem to play a role in determining resistant/susceptibility of pigs to mycoplasmosis.

Key words: immune response, mycoplasmosis, pigs, RT-qPCR

RESUMO

Micoplasma hyopneumoniae é o patógeno primário da pneumonia enzoótica que ocorre em todo o mundo e causa maiores perdas econômicas na indústria suína. O organismo adere e causa danos ao epitélio ciliado do trato respiratório. Objetivou-se comparar a expressão de receptores *toll-like* e citocinas assim como mensurar a produção de óxido nítrico em resposta a vacinação contra *Micoplasma hyopneumoniae* em células mononucleares do sangue periférico (PBMCs) e do lavado broncoalveolar (BALF) de dois grupos genéticos, raça naturalizada brasileira Piau e a linhagem comercial. Para cada grupo genético foi coletado sangue periférico antes e 10 dias depois da vacinação de 6 leitões fêmeas soro-negativas. Às dez semanas de idade, as amostras de BALF foram coletadas para mensurar a produção de NO e realizar a análise de RT-qPCR. O RNA foi extraído de células de PBMC e BALF, a transcrição reversa e RT-qPCR foram feitas e utilizou-se o sistema de fluorescência SYBR Green. Os genes GAPDH e HPRT1 foram utilizados como controle endógeno, respectivamente. Houve diferença de expressão para TLR2, TLR4, TLR6, TLR10, IL6, TNFα e TGFβ em PBMC para a linhagem comercial, antes e depois da vacinação; enquanto nas células de PBMC para a raça Piau houve diferença de expressão para TLR6, TLR10, TNFα e TGFβ. Para as células do BALF, os animais Piau vacinados mostraram níveis aumentados para TLR4, TLR10, TNFα e TGFβ em comparação aos não vacinados; enquanto os animais comerciais não tiveram diferencial de expressão para os genes estudados, exceto para o gene TNFα. Os resultados obtidos sugerem que a vacina contra *Micoplasma hyopneumoniae* modula as diferentes respostas imunes em suínos da raça Piau e da linhagem comercial através da indução da expressão de TLR2, TLR4, TLR6, TLR10, IL6, TNFα e TGFβ que é sugestiva de diferenças genéticas. Essas diferenças de raças parecem desempenhar um papel importante na determinação de suínos resistente/susceptíveis a micoplasmose.

Palavras-chave: micoplasmose, resposta imune, RT-qPCR, suíno

1 INTRODUCTION

Bacterial lipoproteins (LP) are one of the integral structural components of the cell wall-less microorganisms mycoplasmas and are common immunostimulatory compounds and strong activators of monocytes /macrophages; besides being potent initiator of inflammatory responses in micoplasmosis (Esche et al., 2000; Into et al., 2004). *Mycoplasma hyopneumoniae* (Mhp) is the causative agent of enzootic pneumonia, but more importantly, it is a primary component of the porcine respiratory disease complex. The impact of disease caused by Mhp leads to significant economic losses in the swine industry. Mhp is an extracellular pathogen that resides primarily on the ciliated respiratory epithelium of the lower respiratory tract, where it induces a delayed humoral immune response in the pig (Asai et al., 1996; Lorenzo et al., 2006; Schafer et al., 2007; Zhang et al., 2011).

Innate immunity acts as a sentinel for the immune system and is quickly activated after recognition of the microbial pathogens. Innate immune cells express various pattern recognition receptors (PPRs), for example Toll-like receptors (TLR), which recognize pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002; Akira et al., 2006; Kumar et al., 2009). When a TLR ligand binds to the receptor, an intracellular signal transduction cascade is triggered, altering the pattern of gene expression in the cell (Janeway and Medzhitov, 2002; Akira et al., 2006). Many adjuvants of vaccine are believed to be mimics of TLR ligands, so TLRs turn out to be important for immune responses to vaccines as well as natural disease (Miller et al., 2009).

From the recognition of invading pathogens, the innate immune system triggers the production of various cytokines, which appear to play an important role in the pathological process and development of lesion during porcine enzootic pneumonia, which enables the host to eliminate offending pathogens (Into et al., 2004; Rodríguez et al., 2004). Production of pro-inflammatory cytokines has been shown to be associated with the development of *Mycoplasma hyopneumoniae*-induced pneumonia (Thacker et al., 2000; Thanawongnuwech et al., 2004; Lorenzo et al., 2006).

Differences in production of immune molecules have a deep influence on response to pathogens and they are associated with disease resistance and susceptibility (Lazarus et al., 2002). Studies related to transcriptomic analysis front of antigen in specific population, as well as involving local and domestic breeds have been accomplished to discover differential expressed genes (Clapperton et al., 2005; Benga et al., 2009) in the innate and adaptive immune response. The Piau Brazilian Naturalized breed has singular characteristics as rusticity, adaptability to poor conditions of management and feeding, and a great resistance to diseases, besides being considered fat-type (Silva et al., 2009), which make them proper for studies aiming to improve the knowledge concerning host-pathogen interaction and susceptibility/resistance diseases. Moreover, they were used in QTL mapping using divergent crosses (Sousa et al., 2011a; Pinto et al., 2010, Silva et al., 2009; Paixão et al., 2008), association studies (Peixoto et al., 2009) and transcriptional analysis (Serão et al., 2010; Sollero et al., 2011; Sousa et al., 2011b).

Thus, the aim of this study was to examine the oxide nitric production and expression of Toll-like receptors and cytokines in two genetically distinct groups of pigs, Piau Brazilian Naturalized breed and a commercial line, vaccinated against *Mycoplasma hyopneumoniae* in order to better understand some of the molecular mechanisms involved in protective immunity against this pathogen.

2 MATERIAL AND METHODS

2.1 Animals and tissue collection

All procedures regarding the use of animals were approved by Universidade Federal de Viçosa Animal Care and Use Committee. Female piglets negative for *Mycoplasma hyopneumoniae* (6 Piau Brazilian Naturalized breed and 6 Commercial White line) from the pig farm at Universidade Federal de Viçosa (Viçosa, MG, Brazil) were utilized. Blood samples from *sinus orbitalis* were collected just before and 10 days after the first (7º day of life) vaccination (intramuscular, inactivated vaccine, aqueous adjuvanted, SUVAXYN RESPIFEND MH, Fort Dodge) into tubes containing EDTA (0,5M, pH 8).

At 10 weeks of age, the vaccinated (6 animals each breed) and unvaccinated animals (3 animals each breed) were sacrificed by sodium pentobarbital injection 2.5% (25 mg/mL) for bronchoalveolar lavage fluid (BALF) collection. The trachea was canulated and the lungs were flushed with 40 mL ice cold sterile phosphate buffered saline (PBS, pH-7.4) and the recovered volume was placed in sterile tubes and were kept in ice and, subsequently, centrifuged for 504 g for 10 minutes. The supernatants and pellets were stored at -80ºC until nitric oxide dosage and RT-qPCR analysis, respectively.

2.2 Nitrite assay estimation of NO production

Pigs at 10 weeks of age, vaccinated (6 animals each breed) and unvaccinated (3 animals each breed) groups used for NO evaluation. Nitrite concentration in the medium was quantified by a colorimetric assay based on the Green et al. (1982). Briefly, 100 µL aliquots of supernatants were added to an equal volume of freshly prepared Griess reagent. In the system, nitrite ions react with 1% sulfanilamide in 5% ortho-phosphoric acid/0.1% N-1 naphthylethylenodiamine dihydrochloride to yield an azo-chromophore, the absorbance for measurement was 540 nm. The nitrite levels in supernatants were calculated by comparing the optical density (OD) readings against the nitrite standard curve. Data was expressed as µg/mL.

2.3 RNA isolation and cDNA synthesis

PBMCs were isolated by a 1.077 g/mL density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). Cells were washed three times in PBS 1 X. The total RNA was extracted from PBMCs (from 6 animals each breed, before and after vaccination) and BALF cells (6 vaccinated and 3 unvaccinated animals each breed) using RNeasy® Mini Kit protocol (Qiagen), and DNAse digested by DNAse RNAse free kit (Qiagen) according to manufacter's instructions. RNA yield and quality were assessed using the NanoVue Plus spectrophotometer (GE Healthcare) and Agilent 2100 BioAnalyzer (Agilent Technologies), respectively. Equivalent amounts of RNA were reverse transcribed with SuperScript III/RnaseOut Enzyme Mix (Invitrogen Life Technologies) to evaluated gene expression before and after vaccination against *Mycoplasma hyopneumoniae* and at 10 weeks of age.

2.4 Real Time PCR

Quantitative real-time PCR was performed using SYBR Green fluorescent detection system ABI Prism 7300 Sequence Detection Systems (Applied Biosystems). Primers were designed using PrimerQuest according to the software guidelines (http://www.idtdna.com/Scitools/Applications/Primerquest/). The gene-specific primers are listed in Table 1. The reactions were performed in duplicate. The thermal cycling conditions were denaturation at 95 ºC for 10 min, 40 cycles of 30 s melting at 95 °C followed by 30 s of annealing and extension at 60 °C. The efficiency of each gene was accessed in order to choose the best combination of cDNA and primer concentration in the following reactions. Efficiency of reactions was calculated according to the equation $E = 10^{(-1/slope)}$ (Pfaffl, 2004).

The genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HPRT1 (hypoxanthine phosphoribosyltransferase 1) were used as endogenous control for PBMC and BALF, respectively, once stability of genes was assessed using the statistical algorithms GeNorm (Vandesompele et al., 2002). The data was presented using ΔCt values ($\Delta Ct = Ct_{target} - Ct_{endogenous}$) and relative gene expression in $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001). Also, understanding that higher ΔCt values means lower transcriptional expression of a specific gene for a specific sample and lower ΔCt values means higher transcriptional expression.

Table 1 Primer sequences used for real-time qPCR

Gene	Primer sequence	Product length (bp)	NCBI ID
TLR 2	F-AGCACTTCCAGCCTCCCTTTAAGT R-TACTTGCACCACTGCTCTTTCACA	154	NM_213761
TLR 4	F-TGTCAGATAAGCGAGGCCGTCATT R-TCATAGGTGCTTTCACCTCTGCCA	146	NM_001113039
TLR 6	F-ACCTGCCTGGATGTGGTTCCTTTA R-TTCTGGCAGCTCTGGAAGAAGTCA	100	NM_213760
TLR 10	F-TGAACTCTACTTTGGCCACCACCA R-TCCATGAGAGCTTTCAGTGCAGGA	126	NM_001030534
IL 2	F-TGCATTGCACTAACCCTTGCACTC R-GCAGCAATGGCTCCAGTTGTTTCT	91	NM_213861
IL 6	F-TGCTGGATAAGCTGCAGTCACAGA R-TATGTGCCCAGTGGACAGGTTTCT	189	NM_214399
IL 8	F-TCCGTGGCTCCCAAGAATTTCTCA R-TGACCAGCACAGGAATGAGGCATA	85	NM_213867
IL 10	F-AAGTCCGACTCAACGAAGAAGGCA R-AGTGGATGCAGCTGTTCTCAGACT	119	NM_214041
IL12	F-TGACAACCCTGTGCCTTAGCAGTA R-AGAAGCTTTGCATTCATGGCCTGG	82	NM_213993
IL13	F-TCTGGTCATTGCTCTCACCTGCTT R-AATGAGCTCCTTGAGGGCTGTAGA	85	NM_213803
TNFα	F-AAGACACCATGAGCACTGAGAGCA R-TTTGACATTGGCTACAACGTGGGC	173	NM_214022
TGFβ	F-CACCAAGAACCTGCTTTCGGCTTT R-GGCTGCTTTCTTGGCTTCCTTCAA	157	NM_214015
GAPDH*	F-GCAAAGTGGACATTGTCGCCATCA R-TCCTGGAAGATGGTGATGGCCTTT	160	NM_001206359
HPRT1*	F- TTGTGGTAGGCTATGCCCTTGACT R- ACTAAGCAGCTGGCCACAGAACTA	194	NM_001032376

*Endogenous control: GAPDH - glyceraldehyde-3-phosphate dehydrogenase and HPRT1- hypoxanthine phosphoribosyltransferase 1

2.5 Statistical analysis:

A completely random design with two factors in split splot model was utilized for the gene expression experiment for PBMCs and a completely random design with two factors for BALFs. For PBMC expression, the fixed effects included breed (Piau or commercial), time (before and after vaccination) and interaction breed x time. For BALF, fixed effects included breeds (Piau and commercial), treatment (vaccinated and unvaccinated animals) and interaction breed x treatment. The ΔCt results were analyzed using MIXED procedures/ SAS software (version 9.0) in order to perform the variance analysis and the contrast comparisons. The NO production data are presented as the mean ± standard deviation of the mean and the difference between means of experimental groups were performed using one-way analysis of variance following by Bonferroni test. Differences that provided $p<0.05$ were considered to be statistically significant.

3 RESULTS

The present work demonstrates that NO production from BALF cells were not statistically different among vaccinated genetic groups (Piau and commercial) and unvaccinated (p>0.05) (Figure 1).

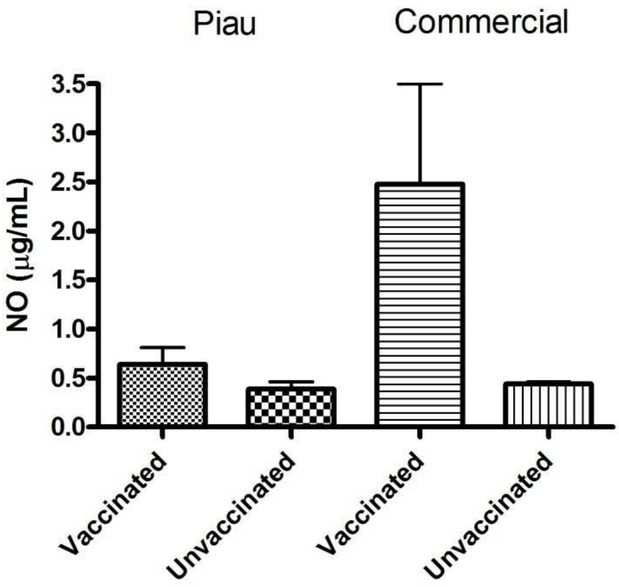

Figure 1. Effect of vaccine against *Mycoplasma hyopneumoniae* on nitrite production. Nitric oxide (NO) production by bronchoalveolar lavage fluid (BALF) cells from Piau and Commercial vaccinated compared to unvaccinated pigs at 10 weeks of age. Data are the mean ± standard deviation.

In this experiment the results about mRNA expression of immune function genes differed between pig breeds in the PBMCs in response to vaccination against Mhp are in Table 2.

Table 2 Toll like receptors (TLRs), pro-inflammatory and anti-inflammatory cytokines gene expression in PBMC from Piau breed (P) and commercial line (C) pigs.

Genes	Genetic group	Before vaccination (7 □ day)	After vaccination (17 □ day)
TLRs			
TLR2	P	4.86 ± 0.39^{Aa}	4.60 ± 0.39^{Aa}
	C	5.63 ± 0.39^{Aa}	3.81 ± 0.39^{Ba}
TLR4	P	5.65 ± 0.80^{Aa}	4.69 ± 0.80^{Aa}
	C	6.88 ± 0.80^{Aa}	5.17 ± 0.80^{Ba}
TLR6	P	5.73 ± 0.80^{Aa}	4.08 ± 0.80^{Ba}
	C	5.93 ± 0.80^{Aa}	3.48 ± 0.80^{Ba}
TLR10	P	4.95 ± 0.49^{Aa}	3.52 ± 0.49^{Ba}
	C	4.78 ± 0.49^{Aa}	2.80 ± 0.49^{Ba}
“Cytokines”			
IL2	P	9.75 ± 0.92^{Aa}	7.54 ± 0.92^{Aa}
	C	9.19 ± 0.92^{Aa}	6.96 ± 0.92^{Aa}
IL6	P	10.57 ± 0.97^{Aa}	10.01 ± 0.97^{Aa}
	C	10.49 ± 0.97^{Aa}	7.08 ± 0.97^{Bb}
IL8	P	7.10 ± 1.10^{Aa}	6.43 ± 1.10^{Aa}
	C	6.79 ± 1.10^{Aa}	5.33 ± 1.10^{Aa}
IL10	P	6.56 ± 1.12^{Aa}	6.29 ± 1.12^{Aa}
	C	6.70 ± 1.12^{Aa}	5.46 ± 1.12^{Aa}
IL12	P	$8.53 \pm 0{,}80^{Aa}$	7.16 ± 0.80^{Aa}
	C	8.55 ± 0.80^{Aa}	6.95 ± 0.80^{Aa}
IL13	P	9.30 ± 0.96^{Aa}	8.43 ± 0.96^{Aa}
	C	10.31 ± 0.96^{Aa}	8.50 ± 0.96^{Aa}
TNFα	P	4.81 ± 0.96^{Aa}	2.91 ± 0.96^{Aa}
	C	5.23 ± 0.96^{Aa}	2.02 ± 0.96^{Ba}
TGFβ	P	5.03 ± 0.42^{Aa}	3.35 ± 0.42^{Ba}
	C	6.06 ± 0.42^{Aa}	3.43 ± 0.42^{Ba}

Gene expression levels are presented as the average ΔCt ± standard error. Values followed by different letters within the same genetic group (A or B) or within each column between different genetic group for each gene (a or b) denote significant differences between treatments ($P \leq 0.05$).

The TLR2 ($p<0.002$), TLR4 ($p<0.03$), TLR6 ($p<0.0002$) and TLR10 ($p<0.0008$) levels were found to be elevated in the PBMC after vaccination in the commercial animals (Table 2). For Piau pigs, the difference in expression was higher only for TLR6 ($p<0.003$) and TLR10 ($p<0.006$) (Table 2), as lowest ΔCt values correspond to larger expressions. The approach enabled the identification of two TLRs differentially expressed in BALF cells between vaccinated and unvaccinated Piau groups, but not in commercial line (Figure 2).

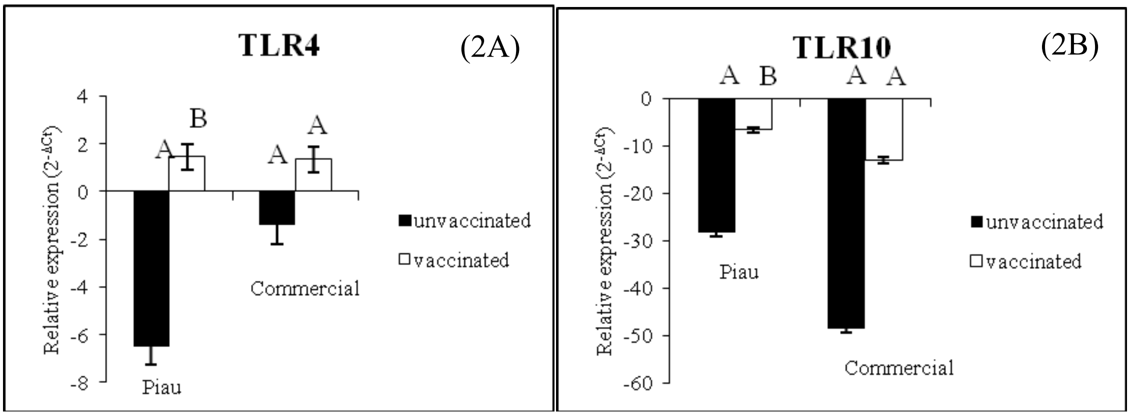

Figure 2. Significant relative expression ($2^{-\Delta Ct}$) in bronchoalveolar lavage fluid cells between vaccinated and unvaccinated within genetic groups (Piau and commercial) for Toll-like receptor 4 (TLR4, panel 2A, p=0.0362) and Toll-like receptor 10 (TLR10, panel 2B, p=0.0778). Bars without common superscripts letters differ.

The pathogen recognition by TLRs leads to synthesis and secretion of inflammatory cytokines and other mediators and for this reason we studied the gene expression of cytokines before and after vaccination against Mhp to observe differences within and between the studied genetic groups.

We found that expression of IL6 in PBMC from commercial animals was higher after vaccination than before vaccination (10.49 vs 7.08, p=0.0541), while in Piau breed no difference was seen for that gene (Table 2). In addition, we analyzed after vaccination time between breeds and the commercial animals showed the highest mRNA IL6 expression (Table 2, p=0.048). Still on the table 2, TNFα gene after vaccination, presented to be significantly higher in commercial pigs (5.23 vs 2.02, p=0.01); while in Piau animals it was not observed. The TGFβ gene expression in PBMC increased significantly in both genetic groups, 5.03 vs 3.35 in Piau breed (p=0.021) and 6.06 vs 3.43 in commercial line (p=0.002), respectively before and after vaccination.

In relation to the cytokines expression from BALF samples, we observed differential expression of TNFα in vaccinated in comparison with the unvaccinated groups, for both breeds (Figure 3A). Furthermore, ten days post vaccination, the level of transcripts of TGFβ was different in Piau breed; however, commercial line did not differ between before and after vaccination (Figure 3B). On the other hand, the others studied cytokines did not show difference after vaccination in genetic groups.

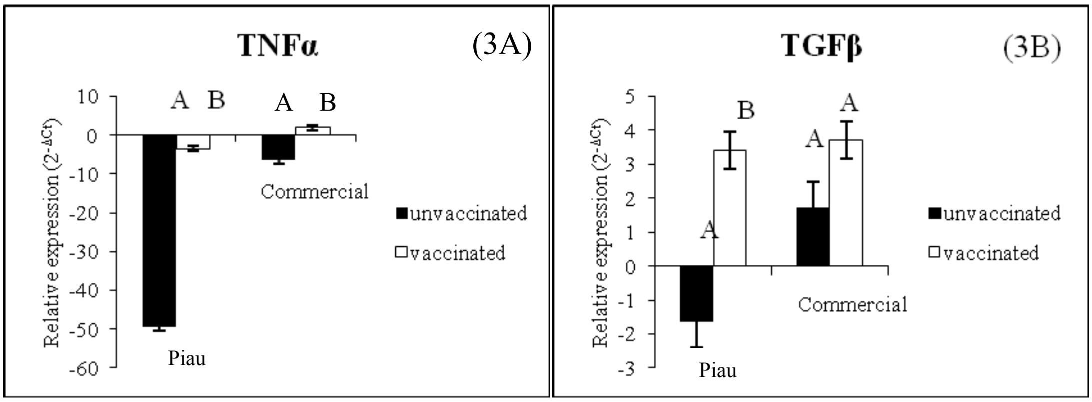

Figure 3. Significant relative expression values ($2^{-\Delta Ct}$) between vaccinated and unvaccinated within genetic groups (Piau and commercial) for TNFα (3A, p=0.0063 for Piau and p=0.0094 for commercial pigs) and TGFβ (3B, p=0.0207 for Piau and

p=0.2643 for commercial pigs) for bronchoalveolar lavage fluid cells. Bars without common superscripts letters differ.

The unvaccinated groups in both breeds showed differential expression for TNFα gene in BALF cells, being lower in Piau in comparison with unvaccinated commercial pigs, but when we analyzed the expression after vaccination, a higher expression in commercial in comparison to Piau breed was detected, once commercial group had a lowest ΔCt (data not shown, Figure 4A). Also, a different expression for IL10 gene in vaccinated animals was observed, being higher in Commercial pigs (Figure 4B). The other genes studied had no differential expression in BALF cells comparing both genetic groups, before and after vaccination.

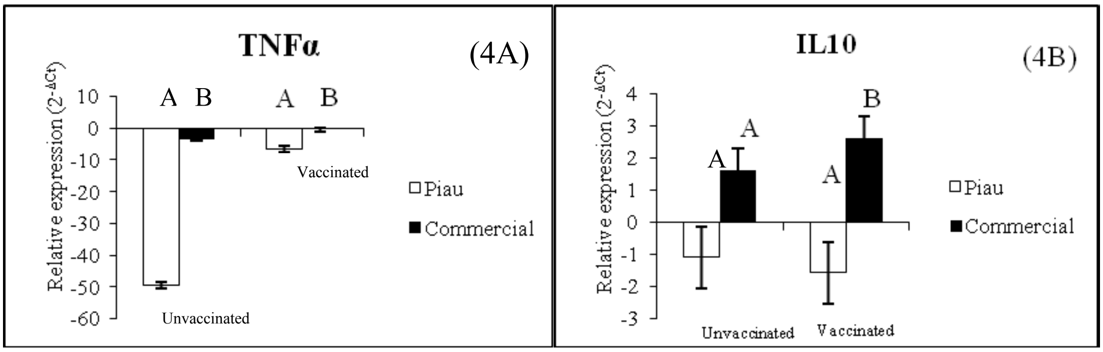

Figure 4. Significant relative expression values ($2^{-\Delta Ct}$) for TNFα between breeds (panel 4A, unvaccinated p=0.0519 and vaccinated p=0.0156) and IL10 (panel 4B, unvaccinated p=0.558; vaccinated p=0.0519) in bronchoalveolar lavage fluid cells. Bars without common superscripts letters differ.

4 DISCUSSION

The swine industry can be improved by the development of more resistant pigs. Thus, it is necessary to better understand pig immunology. Studies on genetic resistance in pigs have begun years ago (Warner et al., 1987; Edfors-Lilja et al., 1994; Mallard et al., 1998; Nguyen et al., 1998) and more recently, the immune performance of pigs divergently selected for humoral response is been evaluated (Clapperton et al., 2005; Wimmers et al., 2009).

The macrophage is the most important cell of the immune system and it is involved in beginning the innate immune responses, through phagocytosis and nitric oxide (NO) production, to eliminate pathogens. Therefore, macrophages are essential for the control of infectious agents (Guimarães et al., 2011). The outcome of macrophage-pathogen interaction depends upon several factors as the genetic background of the host (Qureshi et al., 2003). Excess release of mediators, such as TNFα and NO, are believed to be correlated with pathogenesis of several bacterial infections (Sharma and Kabilan, 2000). The induction of NO synthesis during infection is thought to be mediated by the pathogens themselves or pathogen secreted products in synergy with cytokines (Weid et al., 1994), while macrophages represent a major source of NO production. Also, *in vivo* studies have shown that TNFα and IFN-γ have a major role in the upregulation of NO production during infection (Gazzinelii et al., 1993).

TLRs have evolved to specifically recognize the respective PAMPs. TLR2 is vital to the recognition of peptidoglycan (a Gram-positive pathogen component) and lipoproteins. TLR2 forms an heterodimer with other TLRs, TLR1 or TLR6. An heterodimer of TLR2 and TLR6 cooperatively recognize mycoplasmal diacylated lipopeptide (Takeuchi et al., 2001). Levels of TLR2 mRNA expression did not differ markedly before and after vaccination in PBMCs from Piau pigs. This may be because the extreme bias of expression toward TLR2 prevents the formation of sufficient functional heterodimers of TLR6, once TLR6 exert their function after association with TLR2 and determine specific recognition of pathogen-derived molecules (Takeuchi et al., 2001).

TLR4 also showed different expression in commercial pigs PBMC samples, on the other hand in BALF cells, the commercial line did not present differential expression. TLR4 specifically recognizes lipopolysaccharide and the lipoteichoic acid components of Gram-negative and Gram-positive bacterial cell walls, respectively (Kopp and Medzhitov, 2003; Thomas et al., 2006). Hasan et al. (2005) showed that human TLR10 is a functional receptor, and can function as an homodimer (by itself) or as an heterodimer when associated with TLR1 or TLR2; but the ligands for TLR10, which are still unidentified, will clarify the role of this receptor in the innate immune system.

Induction of an innate immune response is essential for the control and elimination of offending pathogens as well as the quantification of cytokine gene expression is important for knowledge of the initiation and regulation of inflammatory and immune response in pigs, in other words, pathogen-host interaction. Pro-inflammatory cytokines have a wide spectrum of effects that contribute to the host's defense against pathogens and their presence can be beneficial in the control of pathogens (Thanawongnuwech et al., 2004). Production of pro-inflammatory cytokines has been reported to be associated with the development of *M. hyopneumoniae*-induced pneumonia. Increased levels of interleukin-1, IL6, and TNFα in bronchoalveolar lavage fluid have been showed in *M. hyopneumoniae*-infected pigs (Asai et al., 1994; Thacker et al., 2000).

In our study there was no difference in the expression of pro-inflammatory cytokines, except TNFα which had grater expression in PBMC and BALF cells from both breeds, and IL6 in PBMC from commercial pigs after vaccination. TNFα is among the first cytokines that are produced in the lungs during infection (Van Gucht et al., 2004). TNFα is a major cytokine detected in Mhp infection and contributes to lymphocyte accumulation and exacerbation of pneumonia (Asai et al., 1993). Muneta et al. (2003) suggested that blocking of TLR2 and TLR6 may regulate the overproduction of pro-inflammatory cytokines, such as TNFα, and contribute to the prevention of mycoplasmal pneumonia in pigs.

Asai et al. (1994) demonstrated that Mhp induces the production of IL6 at 2 weeks post-inoculation, but at 42 days post inoculation, the IL6 levels in both Mhp-infected groups had returned to the same levels as in the negative control, even though pneumonia remained. In our study, the IL6 mRNA levels from PBMC in the vaccinated commercial pigs (17 days of age, 10 days after vaccination) were higher than before vaccination. In contrast, it is interesting that IL6 levels were not elevated in the BALF samples, at 10 weeks of age in the vaccinated commercial pigs what is according to Asai (1994), because IL6 levels were found reduced with increasing days post vaccination. IL6 can be induced by TNFα and IL1 induces the release of acute phase reactants by the liver, and in turn switches off pro-inflammatory cytokine production. Due to their diverse actions, cytokines such as IFN-α and IL6 have been classified as “pro-inflammatory” by some researches and as “anti-inflammatory” by others (Van Reeth et al., 2002), because it is can exacerbate inflammatory process as well as reducing inflammatory response, suppressing the production of TNFα.

Although differential IL8 expression was not observed in this experiment, this chemokine has been reported to contribute to lymphocyte proliferation associated with mycoplasmal pneumonia (Thanawongnuwech et al., 2004). No significant difference in the levels of IL12 in PBMC and BALF cells in response to vaccination against Mhp between breeds was observed. IL12 plays a critical role in modulating the balance between the protective immunity provided by Th1 and Th2 lymphocytes in the host defense system.

Besides pro-inflammatory cytokines, we also examined the production of IL10, which is mainly produced by Th2 and regulatory T lymphocytes, but also by activated macrophages. IL10 is a potent anti-inflammatory cytokine capable of reducing the inflammatory response, suppressing the production of IL1 and TNFα (Van Gucht et al., 2004), but in our case there was not differential expression in PBMC and BALF between studied breeds. Also, no differential expression for mRNA IL13 was observed in response to vaccination among studied groups. IL13 which is produced by several cell types and it acts inhibiting the production of pro-inflammatory cytokines. It seems likely that pig IL13 substitutes IL4 in regulating immune response (Raymond and Wilkie, 2003).

Other studied anti-inflammatory cytokine was TGFβ, which had increased expression after vaccination in PBMC from commercial pigs (p=0.0021) and Piau animals (p=0.0212) in comparison to before vaccination in the same animals; moreover, there was differential expression in BALF from vaccinated Piau in comparison to unvaccinated Piau (p=0.0207, Figure 3B). The TGFβ is a cytokine with diverse function and its immuneregulatory properties are primary suppressive, including down-regulating MHC expression, cytokine production, T and B cell proliferation, macrophage activation and macrophage respiratory burst activity. In addition, TGFβ inhibits the development of immunopathology to self or nonharmful antigens without compromising immune responses to pathogen (McCartney and Wahl, 1994; Li et al., 2006).

Since TNFα mRNA was predominant in the present study, it strongly suggests that TNFα may play a crucial role in antibody response in pigs in response to vaccination against Mhp. According with Reddy et al. (2000), the presence of specific antibodies might interfere with the ability of the bacteria to induce cytokine production. Moreover, vaccines based on killed, such those used in the present study, or attenuated organisms as immunogen may have limitations, including poor cytotoxic T lymphocytes mediated immunity (Murtaugh and Foss, 2002). Aqueous vaccines generally promote immunity within 8-10 days after vaccination; a secondary injection is required to maintain immunity at protective levels (Cox et al., 2003) after 6 months.

The activation of immune mechanisms either by vaccination or by challenge with pathogens may generally define the defense phenotype of an individual, which is probably dictated by a certain genetic disposition. These aspects might possibly influence the different susceptibility/resistance of different individuals/breeds (Petry et al., 2007; Benga et al., 2009). This is of interest in animal production, as selection and breeding of resistant phenotypes can improve the control of infectious diseases.

Nicholas (2005) reported that the resistance involves a large number of genes and that which adquired naturally during the course of the evolution of a breed will probably present greater challenges to a pathogen than resistance that has been created by artificial selection within a population during few generations. Identifying genetic variants that control responsiveness to vaccines and disease resistance and investigating gene expression differences can together make possible to select animals for improved responsiveness to vaccination (Glass, 2004). This latter argument raises the issue of introgression of resistance genes from naturally resistant breeds into commercial populations. Moreover; the findings of the present study reinforces the need of the genetic analysis of local adapted breeds as sources of uncovered genetic variability.

5 CONCLUSION

These results suggested that vaccine against *Mycoplasma hyoneumoniae* differently modulates the immune response of Piau breed and commercial line pigs by inducing toll-like receptors and pro-inflammatory and anti-inflammatory cytokines which could serve as molecular indicators for an effective immune response and these can be suggestive of genetic differences which can play a role in determining resistance/susceptibility of pigs to *Mycoplasma hyopneumoniae.*

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CHAPTER 2

Toll-like receptors and cytokine mRNA expression profiles in porcine peripheral mononuclear cells and bronchoalveolar lavage cells in response to vaccination against *Pasteurella multocida* type D in two different genetic groups

ABSTRACT

Progressive atrophic rhinitis is a highly prevalent, contagious pig respiratory disease that occurs worldwide. The disease causes significant economic losses in the swine industry. The aim of this study was to compare toll-like receptors and cytokines expression as well as nitric oxide production and lymphocytes production in response to vaccination against *Pasteurella multocida* type D in peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage (BALF) cells from two different genetic groups, Piau Brazilian Naturalized breed and a commercial line. For each genetic group, peripheral blood was collected just before and 10 days after of each vaccination from 6 serum-negative female piglets. At 10 weeks of age, BALF samples were collected to measure nitric oxide (NO) production and RT-qPCR analysis. Spleens were collected for lymphocyte proliferation. RNA was extracted from PBMCs and BALF cells, reverse transcripted and the RT-qPCR was performed using SYBR green fluorescence system, using GAPDH and HPRT1 genes as endogenous control, respectively. There were no significant difference in NO production and lymphocytes proliferation across genetic groups. In PBMC, commercial line showed difference in mRNA coding for TLR2, TLR4, TLR6 and TLR10 genes and for cytokines IL2, IL6, IL8, IL10, IL12, IL13 and TNFα across times point studied; while Piau breed had only differential expression for TGFβ anti-inflammatory cytokine. For gene expression from BALF, vaccinated commercial group had higher levels of TLR4, TLR6, TLR10, IL6 and TNFα than unvaccinated pigs, while vaccinated Piau animals had increased mRNA coding for

TNFα and TGFβ. In conclusion, commercial line had higher expression for Toll-like receptors and cytokines in comparison to Piau breed in response to vaccination against *Pasteurella multocida* type D.

Key words: gene expression, immune response, *Pasteurella multocida* resistance, pig

RESUMO

A Rinite Atrófica Progressiva é uma doença respiratória contagiosa de suínos, altamente prevalente, que ocorre no mundo inteiro. A doença provoca significativas perdas econômicas na suinocultura. Objetivou-se comparar a expressão dos receptores *Toll-like* e citocinas em células do sangue periférico (PBMC) e do lavado broncoalveolar (BALF) assim como mensurar a produção de óxido nítrico (NO) em BALF e a produção de linfócitos de dois grupos genéticos diferentes, raça naturalizada brasileira Piau e uma linhagem Comercial, em resposta a vacinação contra *Pasteurella multocida* tipo D. Para cada grupo genético, foi coletado sangue periférico de 6 leitões fêmeas soro-negativas, antes e depois de cada dose da vacina. Às 10 semanas de idade, as amostras de BALF foram coletadas para mensurar a produção de NO e realizar a análise de RT-qPCR. Os baços foram coletados para proliferação de linfócitos. RNA foi extraído de células de PBMC e BALF, foi feita transcrição reversa e o RT-qPCR foi feito usando o sistema de fluorescência SYBR green, usando como controle endógenos GAPDH e HPRT1, respectivamente. Não houve diferença significativa na produção de NO e na proliferação de linfócitos. Nas células de PBMC, a linhagem Comercial mostrou diferença no mRNA que codifica para os genes TLR2, TLR4, TLR6 e TLR10 e para as citocinas IL2, IL6, IL8, IL10, IL12, IL13 e TNFα entre os tempos estudados; enquanto na raça Piau teve diferencial de expressão apenas para a citocina antiinflamatória TGFβ. Para a expressão gênica em células de BALF, o grupo comercial vacinado teve maiores níveis de TLR4, TLR6, TLR10, IL6 e TNFα que os animais não vacinados do mesmo grupo, enquanto os animais vacinados Piau tiveram aumento de expressão para os genes TNFα e TGFβ. Em conclusão, a linhagem comercial teve maior expressão para os receptores *Toll-like* e as citocinas em comparação a raça Piau em resposta a vacinação contra *Pasteurella multocida* tipo D.

Palavras-chave: expressão gênica, resposta imune, resistência a *Pasteurella multocida*, suíno

1 INTRODUCTION

Pasteurella multocida is a widespread and costly pathogen responsible for both progressive atrophic rhinitis (the capsular type D) and pneumonia in swine (the capsular type A) (de Jong, 2006) and it is considered a complicating agent of pneumonic process, especially those that result in pleuritis. The disease is characterized by conchal atrophy and various degrees of facial distortion. These signs are accompanied by poor growth of fattening pigs (Haesebrouck et al., 2004).

Following respiratory bacterial infection the host reacts with defense mechanisms of the innate immune system, such as pathogen recognition, phagocytosis, respiratory burst, and production of nitric oxide as well as of cytokines (Janeway and Medzhitov, 2002; Gobert et al., 2005). An important aspect of the innate immune system is the recognition and discrimination of potential pathogens from non-pathogenic. Receptors of the innate immune system that have a large role in recognition events include Natural Killer activating receptors, scavenger receptors, mannose receptors and Toll like receptors (TLR). Arguably, the most important of these receptors are the TLR, which represent a class of pattern recognition receptors (Janeway and Medzhitov, 2002).

Toll like receptors on innate immune cells recognize pathogen-associated molecular patterns (PAMPs) which are conserved microbial molecules and they instruct the immune system on how to respond to invading microorganisms (Janeway and Medzhitov, 2002; Pulendran and Ahmed, 2006). Well-described PAMPs include bacterial surface components such as peptidoglycan and lipopolysaccharide (Akira et al., 2006). To date, 13 members of the TLR family have been discovered in mammals (Jungi et al., 2010) and each TLR family detects distinct microbial PAMPs and triggers the activation of specific signaling pathways, leading to the transcription of inflammatory cytokines (Kawai and Akira, 2010) and costimulatory molecules that control immune response (Janeway and Medzhitov, 2002).

Cytokines are mediators that serve to effect and regulate the immune response, and its role in immunoregulation and immunomodulation has been extensively studied both in mice and in pigs immunized or infected with a diversity of infectious agents (Mansfield et al., 1998). This plays an important role in determining the inflammatory phenotype produced during infection (Parker et al., 2007). The defense pehenotype of an individual may be defined by the immune mechanisms, which is probably dictated by a certain genetic disposition; once there are breed differences and additive genetic variation in pigs in response to pathogens (Rehm et al., 2008; Petry et al., 2007, Reiner et al., 2002, Van Diemen et al., 2002). These aspects may influence a different individual's /breeds' susceptibility/resistance (Reiner et al., 2002, Rehm et al., 2008).

The Piau Brazilian Naturalized breed is originated from breeds introduced by Portuguese settlers in the XVI century (Vianna, 1985) and main characteristics of these animals are great resistance to diseases, adaptability to poor conditions of management and feeding, and rusticity. This pig breed is further characterized by low performance, small litter size, and especially large accumulation of subcutaneous fat (Silva et al., 2009).

The aim of the study was to compare immune response characterized as Toll-like receptors and cytokines mRNA expression, oxide nitric production and lymphocytes proliferation in Piau Naturalized Brazilian breed and in a commercial line in response to vaccination against *Pasteurella multocida* type D.

2 MATERIAL AND METHODS

2.1 Animals and tissue collection

All procedures regarding the use of animals were approved by Universidade Federal de Viçosa Animal Care and Use Committee. Female piglets negative for *Pasteurella multocida* type D and *Mycoplasma hyopneumoniae* (6 Piau Brazilian Naturalized breed and 6 commercial line) from the pig farm at Universidade Federal de Viçosa (Viçosa, MG, Brazil) were utilized. Blood samples from *sinus orbitalis* were collected before first dose of vaccination (t0 - 21º day of life) and 10 days after (t1 - 31º day of life) and before second dose of vaccination (t2 - 42º day of life) and 10 days after vaccination (t3 - 52º day of life) (intramuscular, autogenous vaccine, oil adjuvanted, MICROVET) into tubes containing EDTA (0,5M, pH 8).

At 10 weeks of age, the vaccinated (6 animals each breed) and unvaccinated animals (3 animals each breed) were sacrificed by sodium pentobarbital injection 2.5% (25 mg/mL) for spleens and bronchoalveolar lavage fluid (BALFs) collection. The trachea was canulated and the lungs were flushed with 40 mL ice cold sterile phosphate buffered saline (PBS, pH-7.4) and the recovered volume was placed in sterile tubes and were kept in ice and, subsequently, centrifuged for 504 g for 10 minutes. The supernatants and pellets were stored at -80ºC until nitric oxide dosage and RT-qPCR analysis, respectively.

2.2 Nitrite assay estimation of NO production

Pigs at 10 weeks of age, vaccinated (6 animals each breed) and unvaccinated (3 animals each breed) groups used for NO evaluation. Nitrite concentration in the medium was quantified by a colorimetric assay based on the Griess reaction et al. (1982). Briefly 100 µL aliquots of supernatants were added to an equal volume of freshly prepared Griess reagent. In the system, nitrite ions react with 1% sulfanilamide in 5% ortho-phosphoric acid/0.1% N-1 naphthylethylenodiamine dihydrochloride to yield an azo-chromophore, the absorbance for measurement was 540 nm. The nitrite levels in supernatants were calculated by comparing the optical density (OD) readings against the nitrite standard curve. Data was expressed as µg/mL.

2.3 Lymphoid cell preparation

The spleens were collected from pigs at 10 weeks of age (6 vaccinated animals each breed) in ice cold sterile phosphate buffered saline (PBS 1X) and kept in ice and taken to the laboratory. The pieces of spleens were finely minced with scissors, and dissociated into single-cell suspension in chilled PBS 1X by repeated passage by micropipette in a sterile chamber. Lysis buffer (5 mL) was added to resulting suspensions and they were placed in 15 mL polypropylene tubes on ice for 10 minutes and then centrifuged 504 g for 10 minutes and discarded supernatant. The pellets were suspended in 1 mL chilled sterile PBS 1X, and viable cell counts performed employing trypan blue exclusion test (Shapiro, 1988).

2.3.1 Cell cultures

Suspension of 6.4 x 10^6 of live cells in 100 µL was dispensed in duplicate into wells of microtest plates in 100 µL RPMI medium. The cells were stimulated by Concanavalin A (2.5 µg/mL) or *Pasteurella multocida* type D (Past; 5 µg/mL). The cells were cultivated in 5% CO_2 incubator at 37ºC for 24 hours. Neutral red (0.6 mg/ 200 µL) was added to each well. After a further 4 h incubation, supernatant was discarded by a vacuum pump and added 100 µL DMSO. The optical density of the released neutral red was measured at 550 nm.

2.4 RNA isolation and cDNA synthesis

PBMCs were isolated by a 1.077 g/mL density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). Cells were washed three times in PBS 1 X. The total RNA was extracted from PBMCs (6 animals each genetic group, before and after vaccination) and BALF cells (6 vaccinated and 3 unvaccinated animals each breed) using RNeasy® Mini Kit protocol (Qiagen), and DNAse digested by DNAse RNAse free kit (Qiagen) according to manufacter's instructions. RNA yield and quality were assessed using the NanoVue Plus spectrophotometer (GE Healthcare) and Agilent 2100 BioAnalyzer (Agilent Technologies), respectively. Equivalent amounts of RNA were reverse transcribed with SuperScript III/RnaseOut Enzyme Mix (Invitrogen Life Technologies) to evaluated gene expression before and after vaccination´s dose against *Pasteurella multocida* type D and at 10 weeks of age.

2.5 Real Time PCR

Quantitative real-time PCR was performed using SYBR Green fluorescent detection system ABI Prism 7300 Sequence Detection Systems (Applied Biosystems). Primers were designed using PrimerQuest according to the software guidelines. (http://www.idtdna.com/Scitools/Applications/Primerquest/) The gene-specific primers used were listed in Table 1. The reactions were performed in duplicate. The thermal cycling conditions were denaturation at 95 ºC for 10 min, 40 cycles of 30 s melting at 95 °C followed by 30 s of annealing and extension at 60 °C. The efficiency of each gene was accessed in order to choose the best combination of cDNA and primer concentration in the following reactions. Efficiency of reactions was calculated according to the equation $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2004).

The genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and HPRT1 (hypoxanthine phosphoribosyltransferase 1) were used as endogenous control for PBMC and BALF, respectively, once stability of genes was assessed using the statistical algorithms GeNorm (Vandesompele et al., 2002). The data was presented using ΔCt values ($\Delta Ct = Ct_{target} - Ct_{endogenous}$) and relative expression in $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001). Also, understanding that higher ΔCt values means lower transcriptional expression of a specific gene for a specific sample and lower ΔCt values means higher transcriptional expression.

Table 1 Primer sequences used for real-time qPCR

Gene name	Primer sequence	Product length (bp)	NCBI ID
TLR 2	F-AGCACTTCCAGCCTCCCTTTAAGT R-TACTTGCACCACTGCTCTTTCACA	154	NM_213761
TLR 4	F-TGTCAGATAAGCGAGGCCGTCATT R-TCATAGGTGCTTTCACCTCTGCCA	146	NM_001113039
TLR 6	F-ACCTGCCTGGATGTGGTTCCTTTA R-TTCTGGCAGCTCTGGAAGAAGTCA	100	NM_213760
TLR 10	F-TGAACTCTACTTTGGCCACCACCA R-TCCATGAGAGCTTTCAGTGCAGGA	126	NM_001030534
IL 2	F-TGCATTGCACTAACCCTTGCACTC R-GCAGCAATGGCTCCAGTTGTTTCT	91	NM_213861
IL 6	F-TGCTGGATAAGCTGCAGTCACAGA R-TATGTGCCCAGTGGACAGGTTTCT	189	NM_214399
IL 8	F-TCCGTGGCTCCCAAGAATTTCTCA R-TGACCAGCACAGGAATGAGGCATA	85	NM_213867
IL 10	F-AAGTCCGACTCAACGAAGAAGGCA R-AGTGGATGCAGCTGTTCTCAGACT	119	NM_214041
IL12	F-TGACAACCCTGTGCCTTAGCAGTA R-AGAAGCTTTGCATTCATGGCCTGG	82	NM_213993
IL13	F-TCTGGTCATTGCTCTCACCTGCTT R-AATGAGCTCCTTGAGGGCTGTAGA	85	NM_213803
TNFα	F-AAGACACCATGAGCACTGAGAGCA R-TTTGACATTGGCTACAACGTGGGC	173	NM_214022
TGFβ	F-CACCAAGAACCTGCTTTCGGCTTT R-GGCTGCTTTCTTGGCTTCCTTCAA	157	NM_214015
GAPDH*	F-GCAAAGTGGACATTGTCGCCATCA R-TCCTGGAAGATGGTGATGGCCTTT	160	NM_001206359
HPRT1*	F- TTGTGGTAGGCTATGCCCTTGACT R- ACTAAGCAGCTGGCCACAGAACTA	194	NM_001032376

*Endogenous control: GAPDH - glyceraldehyde-3-phosphate dehydrogenase and HPRT1- hypoxanthine phosphoribosyltransferase 1

2.6 Statistical analysis

A completely random design with two factors in split splot model was utilized for the gene expression experiment for PBMCs and a completely random design with two factors for BALFs. For PBMC expression, the fixed effects included breed (Piau or commercial), time (before and after each dose of vaccination) and interaction breed x time. For BALF, fixed effects included breeds (Piau and commercial) and treatment (vaccinated and unvaccinated animals) and interaction breed x treatment. The ΔCt results were analyzed using MIXED procedures/ SAS software (version 9.0) in order to perform the variance analysis and the contrast comparisons, respectively. The NO production and lymphocytes proliferation data are presented as the mean ± standard deviation of the mean and the difference between means of experimental groups were performed using one-way analysis of variance following by Bonferroni test. Differences that provided $p<0.05$ were considered to be statistically significant.

3 RESULTS

The present work demonstrates that nitric oxide production by BALF cells was stimulated and varied among the individuals tested, but did not show significant differences among genetic groups (Figure 1).

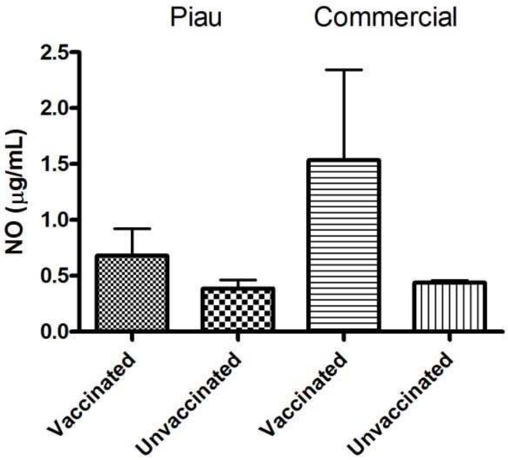

Figure 1. Effect of vaccine against *Pasteurella multocida* type D on nitrite production. Nitric oxide (NO) production by bronchoalveolar lavage fluid (BALF) cells from vaccinated Piau and vaccinated commercial compared to unvaccinated pigs at 10 weeks of age. Data are the mean ± standard deviation.

To assess in vitro cellular immune responses, *Pasteurella multocida*-specific proliferation was measured after vaccination in both groups, at 10 weeks of age. The variations in the proliferative response of lymphocytes between both genetic groups had no significant difference (Figure 2).

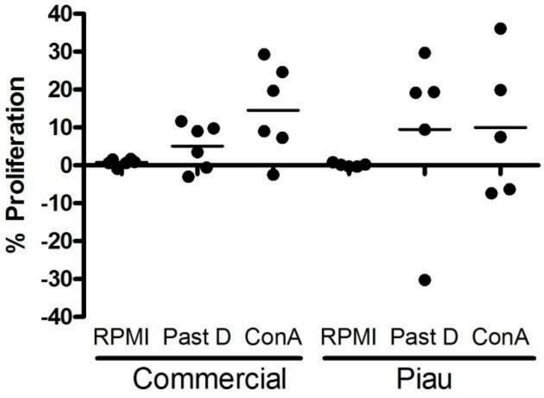

Figure 2. Cellular proliferation stimulated with ConA and *Pasteurella multocida* (Past D) after second dose of vaccine, at 10 weeks of age.

The results of Toll like receptors and cytokine gene expression from PBMC are shown in Table 2, where the statistically significant differences at the comparison across time-points within the same group are reported. In terms of ΔCt, the Piau breed showed no differences in TLRs expression in response to two doses of vaccination and, consequently, there were no differences in cytokines expression, except by TGFβ (Table 2). Instead, commercial pigs had higher TLR2, TLR4, TLR6, TLR10, IL8 and IL10 mRNA expressions after first dose of vaccination (Table 2). TNFα gene expression showed a significant increase at 42 days post-vaccination period (PE) (t1), and reaching basal levels at 52 days PE (t3) (Table 2 and Figure 4) in commercial line. IL2, 6, 12 and 13 rose significantly after second dose of vaccine and TGFβ expression showed no differences in commercial pigs (Table 2 and Figure 4).

Table 2. Toll like receptors (TLRs), pro-inflammatory and anti-inflammatory cytokine gene expressions in PBMC of Piau breed (P) and commercial line (C) pigs in the pre and 10 days post-vaccination period.

	Group	First vaccination (21° day)		Second vaccination (42° day)	
		Before (t0-21 days)	After (t1-31 days)	Before (t2-42 days)	After (t3-52 days)
TLRs					
TLR2	P	3.46 ± 0.53^{A}	4.84 ± 0.53^{A}	4.49 ± 0.53^{A}	5.10 ± 0.53^{A}
	C	2.92 ± 0.53^{A}	1.80 ± 0.53^{B}	2.61 ± 0.53^{B}	1.61 ± 0.53^{B}
TLR4	P	6.80 ± 0.75^{A}	8.16 ± 0.75^{A}	6.30 ± 0.75^{A}	6.07 ± 0.75^{A}
	C	$7,44 \pm 0,75^{A}$	$5,70 \pm 0,75^{B}$	$3,19 \pm 0,75^{B}$	$1,81 \pm 0,75^{B}$
TLR6	P	3.56 ± 0.52^{A}	4.49 ± 0.52^{A}	3.58 ± 0.52^{A}	3.37 ± 0.52^{A}
	C	3.14 ± 0.52^{A}	1.96 ± 0.52^{B}	1.80 ± 0.52^{B}	0.35 ± 0.52^{B}
TLR10	P	2.14 ± 0.60^{A}	2.78 ± 0.60^{A}	1.83 ± 0.60^{A}	2.38 ± 0.60^{A}
	C	1.53 ± 0.60^{A}	0.91 ± 0.60^{B}	0.98 ± 0.60^{A}	-0.42 ± 0.60^{B}
“Cytokines”					
IL2	P	6.63 ± 1.09^{A}	6.30 ± 1.09^{A}	6.66 ± 1.09^{A}	6.80 ± 1.09^{A}
	C	7.39 ± 1.09^{A}	5.57 ± 1.09^{A}	6.29 ± 1.09^{A}	2.16 ± 1.09^{B}
IL6	P	8.77 ± 1.27^{A}	7.43 ± 1.27^{A}	8.23 ± 1.27^{A}	8.99 ± 1.27^{A}
	C	10.78 ± 1.27^{A}	7.78 ± 1.27^{A}	8.29 ± 1.27^{A}	3.15 ± 1.27^{B}
IL8	P	6.91 ± 0.83^{A}	6.59 ± 0.83^{A}	5.99 ± 0.83^{A}	6.15 ± 0.83^{A}
	C	5.36 ± 0.83^{A}	3.24 ± 0.83^{B}	3.75 ± 0.83^{A}	2.00 ± 0.83^{B}
IL10	P	7.15 ± 0.87^{A}	6.71 ± 0.87^{A}	5.82 ± 0.87^{A}	6.12 ± 0.87^{A}
	C	5.56 ± 0.87^{A}	3.42 ± 0.87^{A}	4.13 ± 0.87^{A}	2.08 ± 0.87^{B}
IL12	P	5.46 ± 1.11^{A}	5.24 ± 1.11^{A}	6.53 ± 1.11^{A}	7.10 ± 1.11^{A}
	C	6.85 ± 1.11^{A}	4.84 ± 1.11^{A}	5.08 ± 1.11^{A}	0.62 ± 1.11^{B}
IL13	P	6.52 ± 1.40^{A}	5.61 ± 1.40^{A}	7.57 ± 1.40^{A}	7.80 ± 1.40^{A}
	C	8.29 ± 1.40^{A}	5.48 ± 1.40^{A}	6.07 ± 1.40^{A}	0.55 ± 1.40^{B}
TNFα	P	6.08 ± 0.79^{A}	6.99 ± 0.79^{A}	4.06 ± 0.79^{A}	3.49 ± 0.79^{A}
	C	4.01 ± 0.79^{A}	1.24 ± 0.79^{B}	-0.57 ± 0.79^{B}	1.97 ± 0.79^{A}
TGFβ	P	0.76 ± 0.51^{A}	1.35 ± 0.51^{AB}	2.32 ± 0.51^{B}	2.03 ± 0.51^{B}
	C	0.26 ± 0.51^{A}	0.80 ± 0.51^{A}	0.82 ± 0.51^{A}	0.63 ± 0.51^{A}

Gene expression level (mean ΔCt ± standard error) before and 10 days after of dose of vaccination. Different superscript letters indicate a statistical difference ($p<0.05$) across time points within the same genetic group (A or B).

The comparison between breeds in different times had statistically significant differences in TLRs expressions, mainly after first dose of vaccination (Figure 3). While differential IL2, 6, 12 and 13 mRNA expression was higher in t3 (52° day of age), after second dose of vaccination (Figure 4). For IL8 mRNA expression as well as for IL10 had differences between breeds in t1 (31° day) and t3 (52° day) (Figure 4). TNFα gene expression in PBMC showed a high difference from 31 (t1) and 42 (t2) days PE between both breeds, while TGFβ only in 42 (t2) days PE.

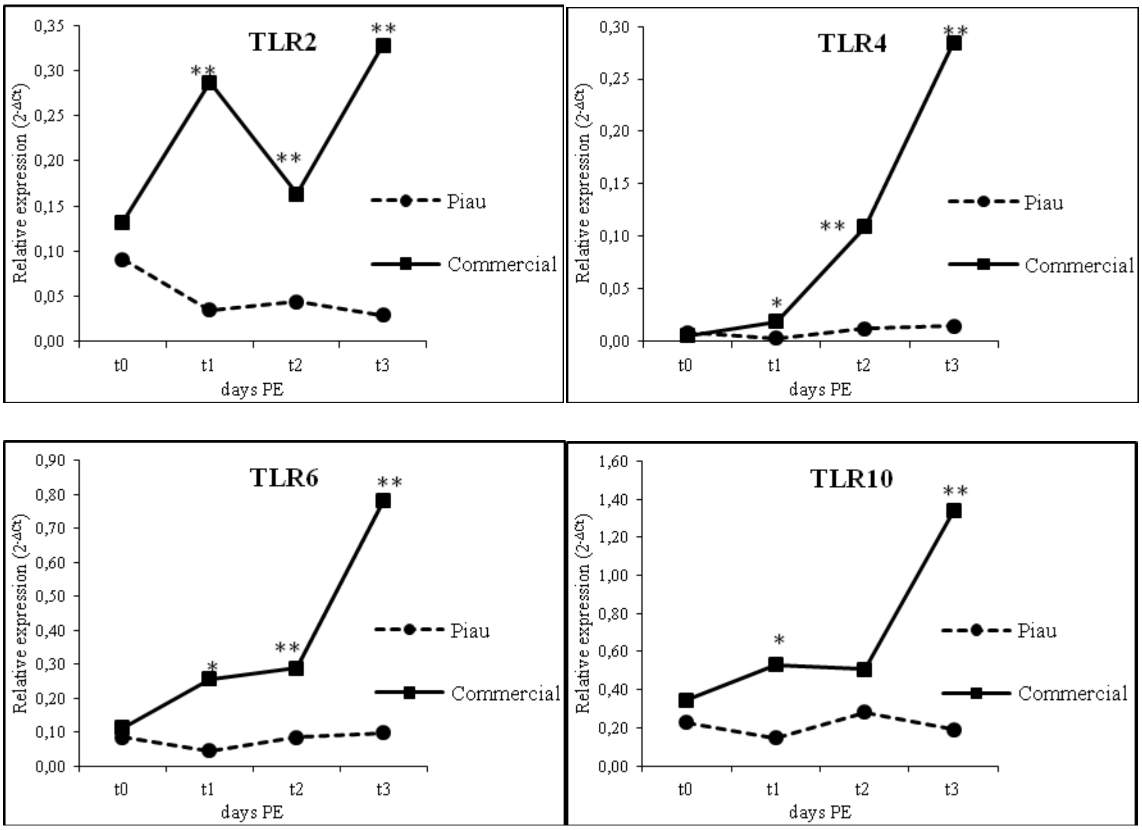

Figure 3. Course of Toll like receptors 2, 4, 6 and 10 in PBMC of Piau and commercial pigs in the pre and post-vaccine (PE). Data are shown as $2^{-\Delta Ct}$. Asterisk (* or **) indicates a statistically significant difference ($p<0.05$ or $p<0.01$, respectively) between Piau and commercial breed animals across time-points (t0-21, t1-31, t2-41 and t3-51 days PE).

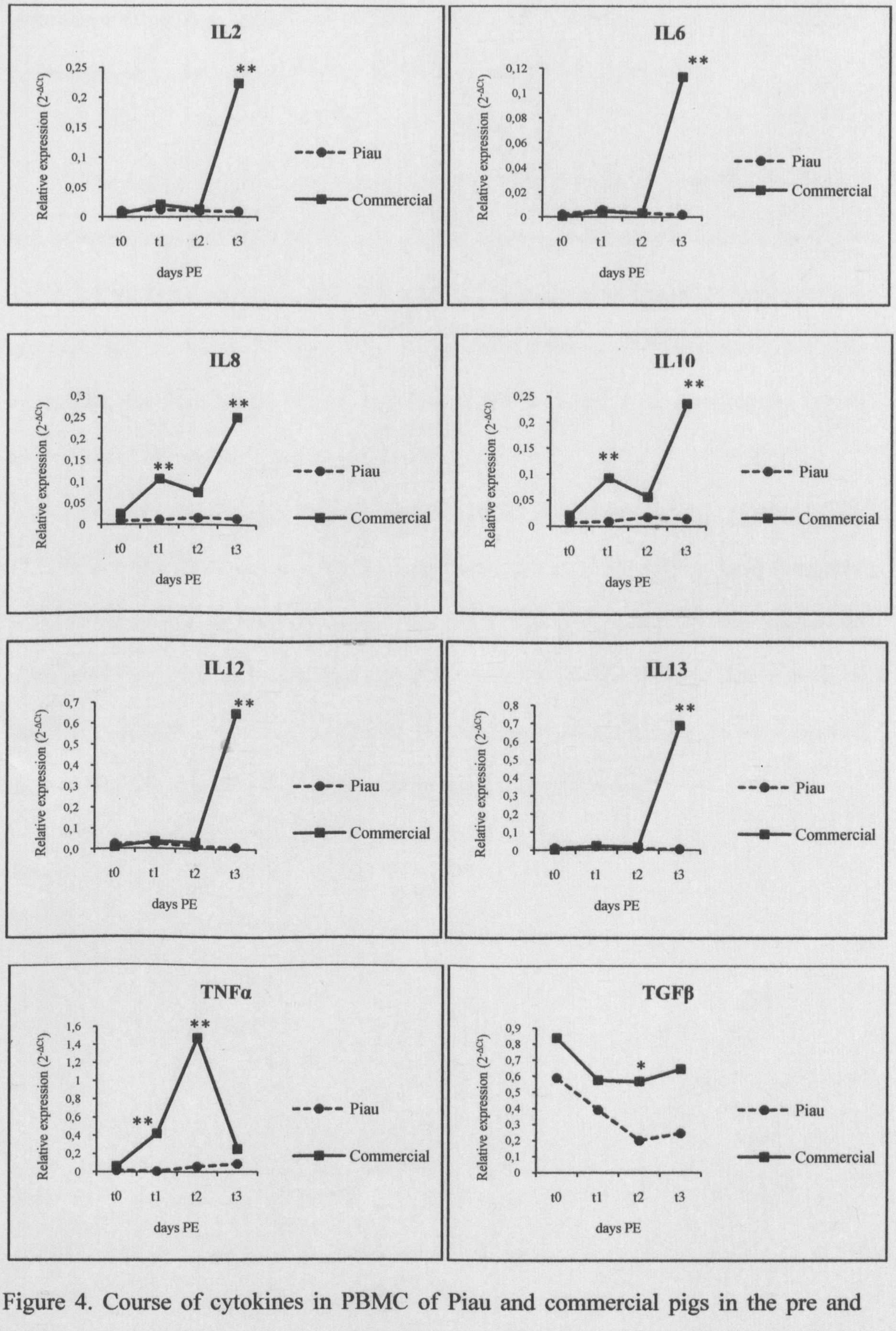

Figure 4. Course of cytokines in PBMC of Piau and commercial pigs in the pre and post-vaccine (PE). Data are shown as $2^{-\Delta Ct}$. Asterisk (* or **) indicates a statistically

Figure 4. Course of cytokines in PBMC of Piau and commercial pigs in the pre and post-vaccine (PE). Data are shown as $2^{-\Delta Ct}$. Asterisk (* or **) indicates a statistically significant difference ($p<0.05$ or $p<0.01$, respectively) between Piau and commercial breed animals across time-points (t0-21, t1-31, t2-41 and t3-51 days PE).

The comparison of gene expression from BALF cells between the vaccinated and unvaccinated pigs within the same genetic group and between breeds are shown in Table 3. Piau breed showed no difference for TLRs expression between vaccinated and unvaccinated, in terms of ΔCt. In addition, there were no differences in cytokines expression for Piau breed, except TNFα and TGFβ, which had significantly higher expression in the vaccinated group (Table 3).

On the contrary, significant differences in mRNA encoding TLR4, TLR6, TLR10, IL6 and TNFα were recorded when vaccinated commercial pigs were compared with unvaccinated commercial pigs. Also in the present study, in the vaccinated commercial animals showed a significant increase for TLR6, TLR10, IL6, IL8, IL10 and TNFα gene expression compared to the vaccinated Piau animals. In unvaccinated pigs, only TNFα had different expression between breeds (Table 3).

Table 3. Toll like receptors (TLRs), pro-inflammatory and anti-inflammatory cytokines gene expressions in BALF from Piau breed (P) and commercial line (C) pigs.

Genes	Genetic group	Unvaccinated	Vaccinated
TLRs			
TLR2	P	0.47 ± 0.44^{Aa}	1.13 ± 0.31^{Aa}
	C	0.43 ± 0.44^{Aa}	0.32 ± 0.31^{Aa}
TLR4	P	2.77 ± 0.95^{Aa}	0.58 ± 0.67^{Aa}
	C	3.07 ± 0.95^{Aa}	-0.17 ± 0.67^{Ba}
TLR6	P	3.68 ± 0.47^{Aa}	3.31 ± 0.33^{Aa}
	C	3.42 ± 0.47^{Aa}	1.82 ± 0.33^{Bb}
TLR10	P	4.57 ± 0.55^{Aa}	4.36 ± 0.39^{Aa}
	C	5.25 ± 0.60^{Aa}	3.02 ± 0.39^{Bb}
"Cytokines"			
IL2	P	8.54 ± 0.76^{Aa}	9.01 ± 0.53^{Aa}
	C	8.11 ± 0.76^{Aa}	8.60 ± 0.53^{Aa}
IL6	P	8.06 ± 1.06^{Aa}	8.60 ± 0.75^{Aa}
	C	8.20 ± 1.06^{Aa}	5.12 ± 0.75^{Bb}
IL8	P	-0.07 ± 1.02^{Aa}	0.18 ± 0.72^{Aa}
	C	-0.93 ± 1.02^{Aa}	-2.20 ± 0.72^{Ab}
IL10	P	0.70 ± 1.02^{Aa}	0.57 ± 0.72^{Aa}
	C	-0.23 ± 1.02^{Aa}	-1.85 ± 0.72^{Ab}
IL12	P	7.60 ± 0.66^{Aa}	8.76 ± 0.47^{Aa}
	C	7.24 ± 0.66^{Aa}	7.49 ± 0.47^{Aa}
IL13	P	7.88 ± 0.81^{Aa}	8.68 ± 0.58^{Aa}
	C	7.99 ± 0.81^{Aa}	8.18 ± 0.58^{Aa}

TNFα	P	4.98 ± 1.02^{Aa}	1.08 ± 0.73^{Ba}
	C	1.85 ± 1.02^{Ab}	-1.03 ± 0.73^{Bb}
TGFβ	P	0.61 ± 0.75^{Aa}	-2.04 ± 0.53^{Ba}
	C	-1.12 ± 0.75^{Aa}	-2.09 ± 0.53^{Aa}

Expression level (mean ΔCt ± standard error) of gene from BALF cells at 10 weeks of age. Different superscript letters indicate a statistical difference ($p<0.05$) between vaccinated and unvaccinated within the same breed (within row, A or B) and same group (vaccinated or unvaccinated) in different breeds for each gene (within column, a or b).

4 DISCUSSION

The experimental animals were serum-negatives for *Mycoplasma hyopneumoniae* and microbiological investigations performed for *Pasteurella multocida* type D gave consistently negative results from these animals on the 21st day of age (t0). No monitoring was done to determine if there was seroconversion to *M. hyopneumoniae* at any considered time. There are no data of illness caused by virus or bacterial infections in the farm being studied. Therefore, if significant differences were obtained, these were mainly attributed to vaccination.

The present work demonstrated that bronchoalveolar lavage cells of pigs are able to produce NO; even without a significant different production between breeds. Several studies have analyzed Toll-like receptors and cytokine gene expression *in vivo* and *in vitro* and/or cytokine secretion in the blood or bronchoalveolar lavage cells during experimental infection by bacteria and virus (Van Diemen et al., 2002; Thomas et al., 2006; Petry et al., 2007; Alvaréz et al., 2008; Rehm et al., 2008; Liu et al., 2009; Lone et al., 2009). The present study investigated the induction of some Toll-like receptors and cytokines in blood mononuclear cells and bronchoalveolar lavage cells from Piau breed and commercial line in response to vaccination against *P. multocida* type D.

The TLRs expression were identified in commercial line, soon after the first dose of vaccine, but Piau pigs had no different expression for TLR in PBMC. In BALF cells from commercial pigs there were differences in all TLR, except TLR2. TLR2 is vital to the recognition of peptidoglycans (a Gram-positive pathogen component) and lipoproteins. TLR2 forms a heterodimer with TLR6 which recognizes mycoplasmal diacylated lipopeptide (Shinkai et al., 2006). TLR2 has been associated with Th1 responses (Patel et al., 2005), regulatory T cell responses (van der Kleij et al., 2002), and most recently, Th2 responses (Redecke et al., 2004). Although, the TLR4 is the best recognition receptor of LPS, the responsiveness of TLR2 to LPS has been described (Armstrong et al., 2004). TLR4 specifically recognizes lipopolysaccharide and the structurally similar lipoteichoic acid components of Gram-negative and Gram-positive bacterial cell walls, respectively (Kopp and Medzhitov, 2003; Thomas et al., 2006). TLR10 had different expression in both samples, PBMC and BALF, from commercial animals, but further investigation of the ligands for TLR10, which are still unidentified, will elucidate the role of this receptor in the innate immune system (Hasan et al., 2005).

Liu et al. (2001) reported increased TLR2 mRNA in peripheral blood monocytes obtained from pigs infused with LPS. However, detection of porcine TLR mRNA was conducted using a human TLR2 probe. Alvarez et al. (2006) established that cloning of porcine TLR4 gene from alveolar macrophages and also determined that this gene could also be detected by RT-PCR in porcine cells and tissues. Medzhitov et al. (1997) observed high levels of TLR expression in peripheral blood leukocytes as well as Akashi et al. (2000) in monocytes, dendritic cells, B cells and T cells.

Differences in production of immune molecules such as TLRs that are involved in the recognition of pathogen-associated molecules have a profound influence on response to pathogens and are closely associated with disease resistance and susceptibility (Lazarus et al., 2002). The primary consequences of TLR activation are activation of MAPK, NF-κB, IRF-3, etc, which all lead to the production of cytokines and regulate the innate and adaptive immunity (Chen et al., 2008).

We evaluated gene expression of systemic cytokine in PBMC with objective of assessing cytokine temporal patterns. The monocytes cells, innate and acquired cytotoxic cells, regulatory cells are represented by PBMC and their proportion and functional status are expression of immune cells recruitment, migration and activations against pathogens (Van Reeth et al., 2000). Furthermore, we also analyzed gene expression in BALF cells, once cytokines regulate immune response and the spectrum of cytokines induced is related to the stimulus.

Genetic variation for disease resistance is due to effects of genes that regulate immune response, along with environmental effects. In general, commercial pigs had greater expression in PBMC of the studied genes (IL2, IL6, IL8, IL10, IL12, IL13 and TNFα) in response to vaccination than PBMC from Piau pigs. Vaccinated commercial group had higher levels of expression of IL6 and TNFα in BALF cells than unvaccinated commercial pigs, at 10 weeks of age; while vaccinated Piau animals had increased TNFα and TGFβ mRNA in BALF cells. This variation is likely due to the variability within pig populations in their innate and acquired immune responses.

Reduced expression of inflammatory and regulating cytokines may also protect the host against excessive inflammation and tissue damage, decreasing the risk of immunopathology (Chen et al., 2008) as seen in Piau pigs, once the overproduction of inflammatory cytokines within the lung may exacerbate the direct effects of *Pasteurella multocida*.

Indeed, a number of research models have been established that indicate the importance of genetic diversity within a population as crucial to response during the spread of infectious disease or to modulation of the immune system (Mallard et al., 1998; Wilkie and Mallard, 1999). Mallard et al. (1998) have seen that after 8 generations of selection for antibody and cell-mediated immune response, high, low, and control lines had diverged for growth rate, antibody response to various antigens, and response to *Mycoplasma hyorhinis*. Wilkie and Mallard (1999) concluded that genetic variation in response to certain antigens and to *M. hyorhinis* exists. Though pathways and mechanisms involved in resistance were not characterized, it was concluded that the genetic variation was polygenic, regulating both innate resistance and acquired immunity.

IL2 had higher expression, with a peak at t3 (52 days of age) in PBMC from commercial animals. It is related with cells mediated immune response. IL6 expression was detected in BALF of the vaccinated commercial pigs and in PBMC after second dose in commercial animals, too. IL6 is a multifunctional cytokine that is produced and released by diverse populations of cells and has important roles in regulation of the immune response, differentiation and proliferation of various cells, and signal transduction pathways (Baarsch et al., 1995). Antibody response may be mediated by Th-2 cytokines such as IL6. This argument is based on observations that IL6 induces differentiation of IL4-producing cells (Reddy et al., 2000).

TNFα was detected in PBMC as well as BALF from commercial pigs. TNF responses have been recorded in circulation of pigs after i.v. endotoxin administration and during fatal Gram-negative sepsis (Maroushek Boury et al., 1997). Differences in the duration of the TNFα responses could be due to the presence of soluble TNF receptors or other inhibitory factors present in porcine serum which interferes with the detection of TNF (Fossum et al., 1998). TNFα induces monocytes to secrete other cytokines, which are essential in the control of infection but are also involved in tissue lesions. Besides that, TNFα stimulates production of IL10 to regulate its own production (Diament et al., 2002).

Possibly, commercial pigs had a quick, strong response to vaccine, once had produced different level of IL8 in PBMC. More IL8 was circulating in the blood, providing innate protection to shed the bacteria quickly (Baarsch et al.,1995), once the chemokine IL8 recruits degranulating neutrophil, which are phagocytic cells that play a major role in defense of a host against infection (Petry et al., 2007). Exposure of porcine alveolar macrophages to LPS induces rapid and prolonged production of this chemokine, however, low levels of the proinflammatory cytokine, TNFα, are also required (Thacker, 2006).

The slow induction of IL10 transcripts at t2 in PBMC from commercial group was in contrast to the induction of TNFα proinflammatory cytokine, which showed a peak induction between t1 and t2 after vaccination (Figure 4). IL10 is a major anti-inflammatory cytokine by acting primarily on antigen-presenting cells such as macrophages and dendritic cells. In addition, it inhibits activation of macrophages and reduces the production of IL-1, TNFα and IFNγ (Samarasinghe et al., 2006).

There was increase in IL12 levels after second dose of vaccination in PBMC from commercial pigs (Table 2 and Figure 4), but no increase in IL12 mRNA from BALF cells from commercial pigs (Table 3) neither PBMC and BALF cells from Piau animals, too (Table 2 and 3). IL12 induces the production of IFN, activates Natural killer cells and stimulates the differentiation of CD4 T cells into Th1 subset in acquired immunity, which plays a critical role in killing infected target cells and, suppresses induction of the Th2-linked response (Thanawongnuwech et al., 2004; Chen et al., 2008). IL13 expression was higher in PBMC from commercial animals, at t3. This cytokine shares its activity with IL4 such as anti-inflammatory and pro-inflammatory immune response (Zarlenga et al., 2004).

Based on the elevated pro-inflammatory response of PBMCs from commercial pigs, no or lower anti-inflammatory TGFβ expression was expected, because anti-inflammatory cytokines such as IL2 and IL12 showed increased levels at t3. (Table 2 and Figure 4). Amadori et al. (2009), in a global view, indicated that inflammatory cytokines contribute to important strategies of environmental adaptation of farm animals. According to the Thacker (2006), the cytokines produced by phagocytic cells are influenced by their phenotype, the initiating stimulant, location, and possibly animal species.

As Piau is a local adapted breed, it showed different expression pattern for both Toll-like receptors and cytokines when compared to commercial animals, showing that there is genetic variation between both studied breeds and suggesting that these pigs may be resistant because there were not differences in its expression for toll-like receptors and pro-inflammatory cytokines in response to vaccination. Jonsson (1991) described that the process of domestication, including selective breeding for specific characteristics, may be considered as a first step in genetic improvement. Genetic groups which were not submitted to the same selection process or still maintain the original genetic bases from the beginning are genetic reservoirs to investigate economically important characteristics as well as realize comparisons of native breeds with domestic pigs that can elucidate on the evolutionary process (Jonsson, 1991; Bergman et al., 2010).

In addition, a number of research models have been established that indicate the importance of genetic diversity within a population as crucial to response during the spread of infectious disease (Jovanovic et al., 2009), besides that it should help to reveal novel control mechanisms, target genes and pathways functions that encode resistance and protective responses (Lewis et al., 2007). Indeed, the findings in the experiment reinforce the need of genetic analysis of local adapted breeds, such as Piau, as sources of uncovered genetic variability.

5 CONCLUSION

The Piau Brazilian Naturalized breed and commercial line pigs responded differently to vaccination against *Pasteurella multocida* type D indicating that genetic variation exists, once the animals produced different amount of toll-like receptors and cytokines from PBMC and BALF cells. The commercial line had higher immune responsiveness, therefore, suggesting high sensibility to vaccination. In addition, this study is the first report to evaluate gene expression in Piau Brazilian Naturalized pigs related to immune response, therefore, more studies should be conducted to confirm the resistance of these animals, once genetic selection for disease resistance must focus on improving the ability of the pig to produce an effective response to disease challenge and, simultaneously, on maintaining production performance.

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GENERAL CONCLUSIONS

We observed breed differences between Piau Brazilian Naturalized and Commercial pigs in response to vaccination against *Mycoplasma hyopneumoniae* and *Pasteurella multocida* type D, such as Toll-like and cytokines mRNA expression. Breed differences in the immune traits measured in this experiment could be explained by breed differences in adaptability to environment, once Piau is a local adapted breed and it is source of uncovered genetic variability. These results are suggestive of genetic differences in these traits in response to studied vaccines.

Furthermore, they also indicate that these populations may be appropriate founder breeds for QTL studies attempting to detect genome regions containing genes contributing to differences in immune response. Additional studies would be required to determine what active protein molecules are present, as this cannot simply be concluded from the transcript data. Hence, there is a need for analysis at the proteomic level and if these immune traits can act as markers of resistance to infectious disease.