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DIFFERENT EXPRESSION SIGNATURES OF GENES RELATED TO PRRSV INFECTION AND RESISTANCE IN HEALTHY LUNG TISSUES FROM FOUR CHINESE PIG BREEDS

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is a chronic disease, which is related to reproductive and respiratory and caused by PRRS virus (PRRSV) binding to specific porcine receptors in pigs. Different pig breeds have different infection and resistance abilities to PRRSV. To study the expression levels of genes related to PRRSV infection and resistance in healthy lung tissues form different pig breeds, we collected lung tissue from healthy Tibetan pigs (TB), Wuzhishan pigs (WZS), and Meishan pigs (MS), as well as Bama mini-pigs (BM). For each breed, we used three independent biological replicates. Real-time fluorescence quantitative PCR (RT-qPCR) was performed to detect the expression levels of *CD163*, *CD169*, *CD151*, *MYD88*, *TRAF6*, *TLR3*, *IFN-α*, *IFN-γ*, *IL-1β*, *IL-6*, *IL-8*, *CCl4*, *NF-kB*, and *CD86*. The results showed that *CD163*, *CD169*, *CD151*, *IFN-α*, *IFN-γ*, *IL-1β*, *CCl4* and *NF-kB* had significant differences among different breeds. Our results of different related gene expression among pig breeds might provide new insights into the genetic strategies for improvement of resistance to PRRSV infection in pigs.

Key words: PRRSV receptor genes, IFN-α, IFN-γ, IL-1β, CCl4, NF-kB

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV), which only infects pigs (Butler *et al*. 2014). Pigs of all ages are susceptible to PRRSV, and infections of piglets within one month and of pregnant sows are the most serious, with the highest infection rate and mortality. Infected pigs mainly suffer from respiratory tract symptoms such as high fever, panting, and acute dyspnea, and reproductive failure such as low-quality semen for boars and abortion and stillbirth for pregnant sows (Collins *et al*. 1992). In 1996, PRRS was first identified in China, and there is a massive outbreak in June 2006 (Zhou and Yang 2010). PRRS is the most important infectious disease currently affecting the global pig production industry.

PRRSV, a single-stranded positive-sense RNA virus with envelope, belongs to the family Arteriviridae (Xiao *et al*. 2010). The main target cells of PRRSV infection are macrophages, and endothelial cells, especially in lungs, lymphoid tissues and placenta (Kim *et al*. 1993; Duan *et al*. 1997; Lawson *et al*. 1997). The process of PRRSV infection includes adsorption, internalization, entry, uncoating, replication, assembly of viral components, and releasing newly synthesized virions (Xiao *et al*. 2010). PRRSV envelope proteins interact with cellular receptors of PRRSV including cluster of differentiation 163 (CD163), Heparin sulfate (Hs), Vimentin, Sialic acid adhesin (Sn/CD169), and Cluster of differentiation 151 (CD151) (Butler *et al.* 2014). *In vitro*, the efficiency of PRRSV infection can be enhanced by nearly 20-fold when low levels of IFNα are used to stimulate the expression of Sn/CD169 in macrophages during the first two days post infection (Delputte *et al*. 2007). Additionally, CD163 knockout porcine macrophages and pigs resist PRRSV infection (Barranco *et al.* 2012). These findings indicate that the expression level of cellular receptors of PRRSV can affect the efficiency of PRRSV infection.

After any viral infection, adequate activation of the host's innate immune system is critical to prevent viral replication and invasion of mucosal tissues, and more importantly, to initiate a strong adaptive immune response against intracellular pathogens (Koyama *et al.* 2008). However, the innate and adaptive immune responses induced by PRRSV infection are poor. The occurrence of this poor response is not completely related to immune regulation and virus clearance, and depends on the age and immune status of most pigs (Loving *et al.*

2015). Nursery pigs are more susceptible to PRRSV than adult animals because their innate immune systems are poorly developed and have limited viral immune avoidance strategies (Butler *et al.* 2014). PRRSV engineered to express IFN has been shown to limit the replication of co-infecting PRRSV, but the effects on PRRSV-specific adaptive immunity in vivo have not been elucidated (Sang *et al.* 2012; Sang *et al*. 2014). In contrast, myeloid differentiation factor 88 (MyD88), tumor necrosis factor receptor associated factor 6 (TRAF6), toll like receptor 3 (TLR3), IFN-α, IFN-γ, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), chemokine 4 (CCL4), NF-kB and cluster of differentiation 86 (CD86) have been demonstrated as important in regulating innate and adaptive immunity (Bautista and Molitor 1999; Tak and Firestein 2001; Bose and Banerjee 2003; Lee and Kleiboeker 2005; Borden *et al*. 2007; Li *et al*. 2010; Barranco *et al.* 2012; Negash *et al*. 2013; Chen *et al.* 2014; Yang *et al*. 2018). High expression of these genes is thus likely involved in the regulation of innate and adaptive immunity and may contribute to resistance to PRRSV infection.

Many studies revealed that susceptibility of pigs to PRRSV is affected by genetic background (Thanawongnuwech *et al*. 1998; Petry *et al*. 2005; Vincent *et al*. 2005). There are many pig breeds in China (Zhang *et al*. 1983), and different breeds of pigs have different resistance to PRRSV: Tibetan (TB), and Meishan (MS) pigs are resistant to PRRSV (Kang *et al.* 2017), while Wuzhishan (white, WZS), Bama minipigs (BM) are sensitive to PRRSV (Pan *et al*. 2015). However, the expression differences of PRRSV membrane receptor genes and key immune-related genes among different pig breeds in China have not been detected yet. Therefore, the lung tissues of healthy TB, WZS, MS and BM pigs were used to detect the expression of PRRSV membrane receptor genes including *CD163*, *CD169*, *CD151*, and key immune-related genes including *MyD88*, *TRAF6*, *TLR3*, *IFN-α*, *IFN-γ*, *IL-1β*, *IL-6*, *IL-8*, *CCL4*, *NF-kB* and *CD86* by real-time fluorescence quantitative PCR.

MATERIALS AND METHODS

Sample collection: Left lung tissue samples of 30-day-old TB (Tibet), WZS (Beijjing), MS (Tianjin) and BM (Beijing) were collected from four different breeding farms in China. For each breed, we used three purebred pigs. These pigs were all raised under standard indoor conditions. All lung tissues were quick-frozen in liquid nitrogen, then transported to the laboratory on dry ice, and stored at -80 °C for subsequent experiments.

Selection of candidate genes and primer design: According to the literature, 14 candidate genes were selected, and beta-2-microglobulin (B2m) was used as an internal reference gene. Primer sequence information is shown in Table 1. Primers were synthesized by Beijing New Times Zhonghe Technology Co., Ltd.

Table 1 Primers for the candidate reference genes and their parameters derived from RT-qPCR data analysis.

Gene symbols	Full name of gene symbols	Primer sequences	Reference
CD151	Cluster of differentiation 151	F: CCTACCTGGCCACAGCCTAC R: ACAGGCGCAGCAGGTTCCGA	(Zong *et al*. 2017)
CD163	Cluster of differentiation 163	F: ATTCATCATCCTCGGACCCAT R: CCCAGCACAACGACCACCT	(Li *et al*. 2017)
IL-1β	Interleukin-1β	F: CCCAAAAGTTACCCGAAGAGG R: TCTGCTTGAGAGGTGCTGATG	(Wang *et al*. 2015)
IL-8	Interleukin-8	F: AGTTTTCCTGCTTTCTGCAGCT R: TGGCATCGAAGTTCTGCACT	(Shabir *et al*. 2018)
IFN-α	Type Ⅰ interferon-α	F: TCCAGCTCTTCAGCACAGAG R: AGCTGCTGATCCAGTCCAGT	(Barranco *et al*. 2012)
SN/CD169	Sialoidsin	F: AGCAGCCGAACGCAGGAT R: TTCTGGTCTTTGAGCTTCGTCC	(Novakovic *et al.* 2016)
CD86	Cluster of Differentiation 86	F: GTTCCTATCCACCAGATGAGT R: GAAGAGACACCCTGATTGATAC	(Rodriguez-Gomez *et al*. 2015)
TRAF6	TNF-Receptor Associated Factor 6	F: GGGAACGATACGCCTTACAA R: CTCTGTCTTAGGGCGTCCAG	(Islam *et al*. 2016)
CCL4	Chemokine CCL4	F: CTCTCCTCCAGCAAGACCAT R: CAGAGGCTGCTGGTCTCATA	(Yang *et al.* 2018)
IFN-γ	Type Ⅰ interferon-γ	F: GGAGCATGGATGTGATCAAG R: GAGTTCACTGATGGCTTTGC	(Barranco *et al.* 2012)
IL-6	Interleukin-6	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA	(Zhang *et al*. 2018)
MyD88	Myeloid differentiation factor	F: GGAACAGACCAACTATCGGC	(Sun *et al*. 2019)

	88	R: GAGACAACCACTACCATCCG	
TLR3	Toll like receptor 3	F: GCATTGCCTGGTTTGTTA	(Meng *et al*. 2018)
		R: CTGGGAGACCATGATATTGA	
NF-kB	Nuclear factor kappa B	F: GGGACTACGACCTGAATGCT	
		R: GGGCACGGTTGTCAAAGAT	
B2m	Beta-2-microglobulin	F: CTCACTGTCTGGCCTGGATG	
		R: GGCGGATGGAACCCAGATAC	

RNA extraction and cDNA synthesis: Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and quality of RNA were determined using a Quawell UV-Vis Spectrophotometer Q5000. The RNA samples with OD260/OD280 ratios of 1.8~2.1 and OD260/OD230 ratios of more than 2.0 were selected for the experiment. Inverse transcription to cDNA was performed using the cDNA Synthesis Kit (TaKaRa, RR047A, Japan). The RNA and cDNA were stored at -80 °C.

Real-time quantitative PCR: RT-qPCR was performed according to TaKaRa SYBR Premix EX Taq kit instructions (TaKaRa, RR420A, Japan). The reaction system was 15 μL including 0.8 μL cDNA, 6.1 μL ddH_2O, 7.2 μL TB Green nucleic acid dye, 0.3 μL Dye II reference dye, 0.3 μL forward primer, 0.3 μL reverse primer. Three biological replicates were used, each with three technical repeats. The reaction was performed in QuantStudio5 real-time PCR system (Applied Biosystems, USA), with following conditions: 95 °C for 5 min. 95 °C 15 s, 60 °C 45 s, 40 cycles. 95 °C 15 s, 60 °C 1 min, 95 °C 15 s.

Data statistics: The expression levels of all genes were converted into relative expression levels of $2^{-\Delta Ct}$ (ΔCt = Ct (target gene) −Ct (internal reference gene)), and the data were imported into GraphPad 8.0 for calculation (Motulsky 2015). A *t*-test was used to analyze data. $^{*}(p < 0.05)$ represents significant difference, ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$) represents extremely significant differences.

RESULTS

Expression of PRRSV specific receptor in lung tissues from different breed pigs: The expression levels of PRRSV specific receptors *CD163*, *CD169* and *CD151* in TB, WZS, MS, and BM pigs are shown in Figure 1. The expression levels of *CD163* in different breeds were in the order of MS > TB > WZS > BM. The expression of *CD163* in BM was extremely significantly lower than that in TB pigs ($p<0.0001$), MS pigs ($p<0.001$) and WZS pigs ($p<0.01$). The *CD163* expression level in MS pigs was significantly higher than that in TB ($p<0.05$) and WZS pigs ($p<0.05$) (Figure 1A). The expression levels of *CD169* in different pig breeds ordered from high to low were TB > MS > WZS > BM. The expression of *CD169* in TB was extremely significantly higher than that in WZS ($p<0.001$) and BM pigs ($p<0.001$). In MS pigs, *CD169* expression was extremely significantly higher than that in BM pigs ($p<0.01$), and significantly higher than that in WZS pigs ($p<0.05$) (Figure 1B). The expression level of *CD151* in different pig breeds ordered from high to low were TB > MS > WZS > BM pig. The expression of *CD151* in BM pigs was extremely significantly lower than that in TB ($p<0.001$), WZS ($p<0.001$) and MS pigs ($p<0.0001$) (Figure 1C).

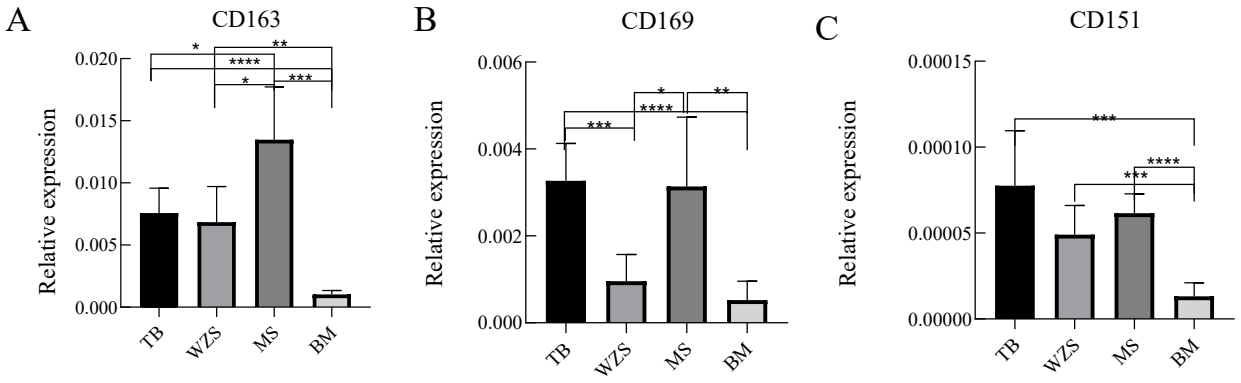

Figure 1 Expression of PRRSV specific receptor gene in lung tissue of different breeds of pigs
A. Expression of *CD163*. B. Expression of *CD169*. C. Expression of *CD151* in different pig breeds. * ($p < 0.05$) represents significant difference, ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$).

Expression of immune response-related genes in lung tissue from different pig breeds: The expression of immune response-related genes *MyD88*, *TRAF6*, *TLR3*, *IFN-α* and *IFN-γ* in lung tissues of different pig breeds a shown in Figure 2. The expression levels of *MyD88* in different varieties were in the order of TB > WZS ≈ MS > BM pigs. The *MyD88* expression in BM pigs was extremely significantly lower than that in MS pigs (p<0.01) (Figure 2A). The expression levels of *TLR3* in different breeds from high to low were WZS > BM > MS ≈ TB. The expression level of *TLR3* in TB pigs was extremely significantly lower than that in BM pigs (p<0.01, Figure 2B). The expression levels of *TRAF6* in different breeds from high to low were ordered as TB > MS > BM > WZS. *TRAF6* was significantly higher in MS pigs than in WZS pigs (p<0.05) (Figure 2B). The expression levels of *IFN-α* in different breeds were in the order of MS > TB > WZS > BM. The expression levels of *IFN-α* in TB (p<0.01) and MS pigs (p<0.01) were extremely significantly higher than that in BM pigs (Figure 2D). The expression levels of *IFN-γ* in different breeds from high to low were TB > MS > WZS > BM. The expression level of *IFN-γ* in TB pigs was extremely significantly higher than that in WZS pigs (p<0.001) and higher than BM pigs (p<0.05). The expression level in WZS pigs was significantly lower than that in MS pigs (p<0.05) (Figure 2E).

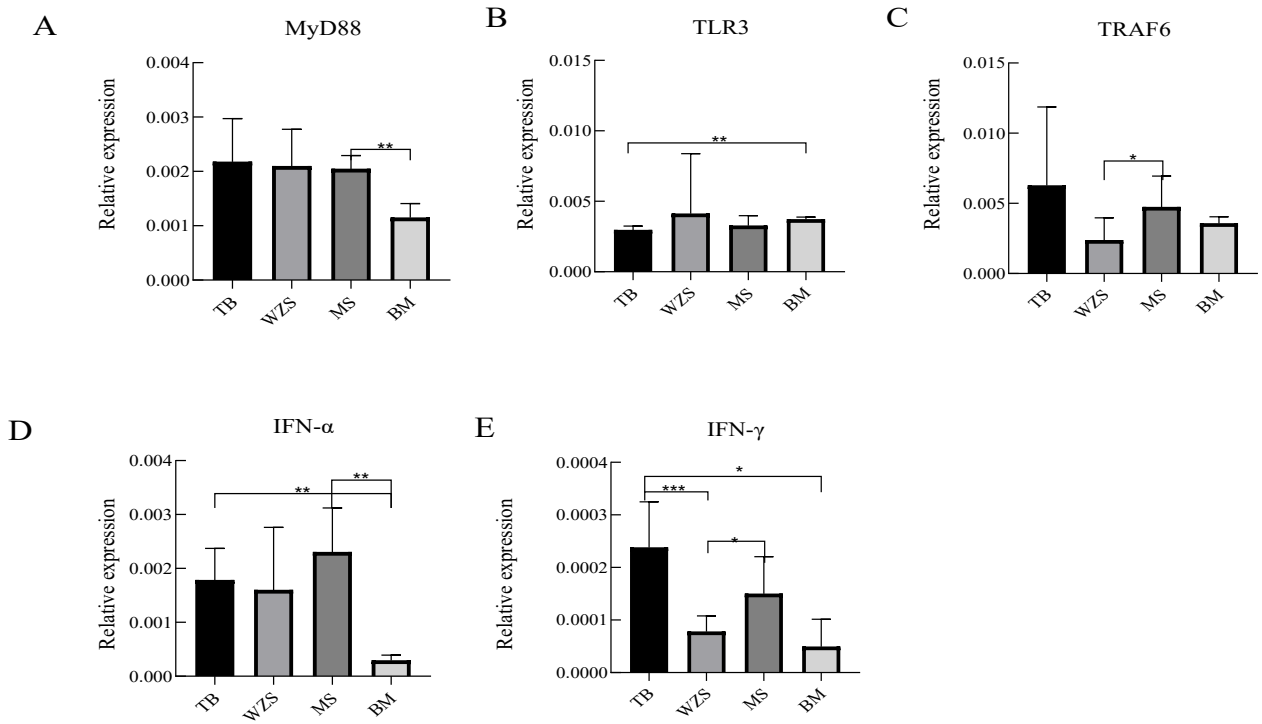

Figure 2 Expression of immune response related genes in lung tissues of different breeds of pigs
A. Expression of *MyD88*. B. Expression of *TRAF6*. C. Expression of *TLR3* in different pig breeds. D. Expression of *IFN-α*. E. Expression of *IFN-γ*.

Expression of inflammatory response related genes in lung tissues from different pig breeds: The expression of inflammatory response-related genes *IL-1β*, *IL-6*, *IL-8*, *CCL4*, *NF-kB* and *CD86* in lung tissues of four breeds of pigs is shown in Figure 3. The expression levels of *IL-1β* in different breeds from high to low were BM > MS > WZS ≈ TB. The expression level of *IL-1β* in BM was extremely significantly higher than that in TB (p<0.01), and was significantly higher than that in MS (p<0.05) and WZS (p<0.05). The *IL-1β* expression level in MS pigs was significantly higher than that in TB (p<0.05) and WZS pigs (p<0.05) (Figure 3A). The expression levels of *IL-6* in different breeds from high to low were ordered as BM > TB > MS > WZS. The expression level of *IL-6* in BM was significantly lower than that in WZS (p<0.05) and MS pigs (p<0.05) (Figure 3B).

The expression levels of *IL-8* in different breeds were in the order of WZS > TB > BM > MS, but there was no significant difference among the four pig breeds (Figure 3C). The expression levels of *NF-kB* in different pigs were in the order of BM > TB > WZS ≈ MS. The expression level of *NF-kB* in TBZ, WZS, and MS pigs was significantly lower than that in BM pigs (p<0.01, Figure 3D). The expression levels of *CCL4* in different breeds from high to low were ordered as TB > MS > WZS > BM. The expression of *CCL4* in TB pigs was

extremely significantly higher than that in WZS pigs (p<0.01), and significantly higher than that in MS (p<0.05) and BM pigs (p<0.05). The expression in MS pigs was significantly higher than that in BM (p<0.01) and in WZS pigs (p<0.05) (Figure 3E). The expression level of *CD86* in different breeds from high to low were TB > MS > WZS > BM. The expression level of *CD86* in TB and MS pigs was extremely significantly higher than that in BM pigs (p<0.01, Figure 3F).

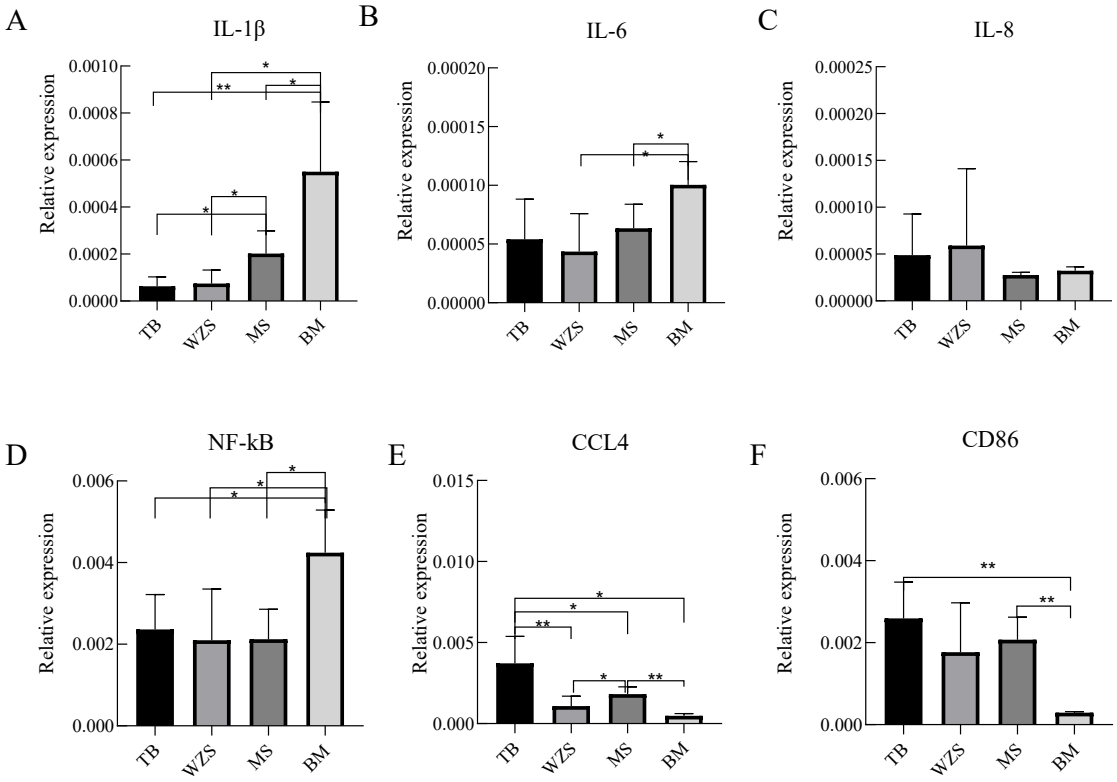

Figure 3 Expression of inflammatory response related genes in lung tissues of different breeds of pigs
A. Expression of *IL-1β*. B. Expression of *IL-6*. C. The expression of *IL-8*. D. Expression of *CCL4*. E. Expression of *NF-kB*. F. Expression of *CD86*.

DISCUSSION

The ability of pigs to resist PRRS disease is controlled by multiple genes. On the one hand, different expression of pathogen receptor genes in pigs may lead to different pathogen adsorption capacities. On the other hand, the host response ability differs in infection with the same virus, so the symptoms are also different. HP-PRRSV was used to infect Tibetan, Zang Mei black (Tibetan × Meishan), and Yorkshire pigs, and results showed that viral RNA was identified in all 15 organs of Zang Mei black and Yorkshire pigs, but not in the spleen, heart, liver, thyroid, kidney, submandibular gland, or small intestine of the Tibetan pigs during infection (Kang *et al*. 2018). In addition to different distribution of the virus after infection, the expression levels of PRRSV receptor genes were also different (Kang *et al*. 2017). Wuzhishan (white), Bama, Lantang pigs, and Juema minipigs were inoculated with virulent strain NVDC-JXA1 of PRRSV, and local binary hybrid pigs were used as control, and mortality rates showed that BM and WZS were the most sensitive to NVDC-JXA1 (Pan *et al.*2015).

CD163 functions directly in PRRSV invasion of susceptible cells (Darwich *et al*. 2010). In 2016, Whitworth *et al* reported, *CD163* knockout pigs were not infected with PRRSV-2 (Whitworth *et al*. 2016). A study by Barranco *et al*. showed that *CD163* knockout porcine macrophages could resist PRRSV-1 and PRRSV-2 infection (Barranco *et al*. 2012). In 2020, Xu *et al* generated *CD163* and *pAPN* double gene knockout (DKO) pigs. After 12 hours of infection, the PRRSV viral load of DKO porcine alveolar macrophages (PAMs) was significantly lower than that of wild type PAMs. During the whole experiment, the body temperature of wild-type pigs was higher than 40 °C from the first day of infection with PRRSV, and there were symptoms such as loss of appetite, tachypnea, cough, fatigue, drowsiness and difficulty walking. However, the body temperature of DKO pigs was normal, with only a short period of cough

and diarrhea (Xu *et al*. 2020). This indicated that *CD163* knockout pigs could resist PRRSV completely.

During PRRSV infection, *CD169* binds to the glycoprotein V platelet (GP5 - M) protein dimer of viral particles and adsorbs on the surface of macrophages, which can participate in the interaction between cells and viruses (Singleton *et al*. 2016). Using PK-15 cells to construct *CD169* over-expressing cells, it was found that PRRSV can enter cells but cannot initiate viral uncoating (Xia *et al.* 2018). Studies showed that *CD151* can directly bind to the 3'-UTR of PRRSV during Marc-145 infection. The infectivity of PRRSV to Marc-145 cells transfected with monkey-derived *CD151* protein was significantly increased (Shanmukhappa *et al.* 2007). In addition, the infectivity of PRRSV had significantly decreased in Marc-145 cells where the expression of *CD151* was knocked down (Shanmukhappa *et al.* 2007). These results suggested that *CD163*, *CD169*, *CD151* are crucial in PRRSV infection in susceptible cells. Here, we found that under physiological conditions, gene expression of PRRSV receptor *CD163*, *CD169*, *CD151* differed in different kinds of pig lung tissue. The differential expression of these genes may be one reason for the different sensitivities of different pig breeds to PRRSV.

Pigs resist the invasion of PRRSV by producing immune factors. At the same time, the virus will use different mechanisms to escape this reaction to achieve sustained infection. The cytokine interferon family is key component of innate and acquired immunity and the first line of defense against viral infection (Borden *et al*. 2007). *IFN-α* and *IFN-γ* are mainly distributed in the cytoplasm of macrophages and participate in innate immune response through antiviral activity. *IFN-α* is produced by white blood cells induced by viruses. Its main functions are to inhibit viral reproductive activity, anti-tumor activity, strengthening the ability of NK cells to kill viral infected cells, thereby inhibiting the proliferation and diffusion of viruses, and enhancing the level of cellular immune response in the body. *IFN-γ* has an immune regulation function and antiviral activity, and is essential in controlling acute infection caused by virus. Bautista and Molitor (Bautista and Molitor 1999) reported that the *IFN-γ* factor produced by lymphocytes can inhibit the replication of PRRSV in macrophages by blocking the normal synthesis of viral proteins and enhancing the ability of macrophages to produce superoxide anion. After infected with PRRSV, the expression of IFN-α increased to third day and then decreased in pigs (Barranco *et al.* 2012). Tongcheng pigs have stronger PRRSV resistance than Yorkshire pigs and higher *IFN-γ* activity after infection (Li *et al.* 2010). The expression of *IFN-α* and *IFN-γ* may be related to the resistance of pigs to PRRS. Here, the expression levels of *IFN-α* and *IFN-γ* were significantly different between different pig breeds, suggesting that the high expression of these two genes may be one of the indicators for selecting PRRSV-resistant pigs.

IL-1β is an effective proinflammatory cytokine, mainly produced by monocytes, macrophages, and lymphocytes, that is central in regulating immune and inflammatory responses (Bose and Banerjee 2003). PRRSV can induce *IL-1β* mRNA expression and protein secretion, and the TLR4/MyD88 signaling cascade and its downstream signaling pathways *NF-kB*, ERK1/2 and p38 MAPK can induce *IL-1β* protein secretion (Negash *et al.* 2013). After recombination of swine IL-1β gene sequence on PRRSV (vP129/swIL1β), high expression of swine IL-1β can effectively prevent the virus from infecting pigs (Lawson *et al*. 2012). *CCL4* is a virus-induced inflammatory factor, which may be related to different antibody responses during PRRSV immunization (Yang *et al.* 2018). The expression of *CCL4* was induced by PRRSV infection in microglia, and was significantly increased in the cerebral cortex and cerebellum of HP-PRRSV infected pigs (Chen *et al.* 2014) .*NF-kB* is central in inflammatory response and participates in multiple stimuli to control the expression of many inflammatory genes (Tak and Firestein 2001). PRRSV infection activates *NF-kB* in various ways, one of which may be through the degradation of I-kB protein (*NF-kB* inhibitor) to activate *NF-kB* (Lee and Kleiboeker 2005). Our results showed that the expression levels of *IL-1β*, *CCL4* and *NF-kB* were significantly different among different pig breeds, suggesting that the high expression levels of *IL-1β*, *CCL4* and *NF-kB* might be another indicator for selecting PRRSV-resistant pigs.

Conclusions: Here, the expression of 14 genes related to virus recognition, immunity and pro-inflammatory were detected in healthy lung tissues from four pig breeds. Results showed that three PRRSV-specific receptor genes (*CD169*, *CD163* and *CD151*), two immune factors (*IFN-α* and *IFN-γ*), and three pro-inflammatory factors (*IL-1β*, *CCL4* and *NF-kB*) had significant difference expression signatures among different pig breeds. The lower expression of *CD169*, *CD163*, and *CD151*, and higher expression of *IFN-α*, *IFN-γ*, *IL-1β*, *CCL4*, and *NF-kB* may be used for the genetic selection of pigs with more resistance to PRRSV infection.

Author Contributions: YP designed and managed the project. YS and JL performed the experiment and analyzed the data, XZ collected biological samples. YS wrote the manuscript. ZF, YY, and YP revised the paper. All authors approved the final version of the manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

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