A survey of an eight-breed cross swine heterogeneous population demonstrates the associations among immune cell traits, lung lesions and *Mycoplasma* abundances

qing Zhang

State Key Laboratory for Swine Genetic Improvement and Production Technology, Jiangxi Agricultural University

jing Li

State Key Laboratory for Swine Genetic Improvement and Production Technology, Jiangxi Agricultural University

tao Huang

State Key Laboratory for Swine Genetic Improvement and Production Technology, Jiangxi Agricultural University

yifeng Zhang

State Key Laboratory for Swine Genetic Improvement and Production Technology, Jiangxi Agricultural University

wenwu Xu

State Key Laboratory for Swine Genetic Improvement and Production Technology, Jiangxi Agricultural University

lusheng Huang

State Key Laboratory for Swine Genetic Improvement and Production Technology, Jiangxi Agricultural University

huashui Ai

State Key Laboratory for Swine Genetic Improvement and Production Technology, Jiangxi Agricultural University

bin Yang (✉ binyang@live.cn)

Jiangxi Agricultural University <https://orcid.org/0000-0002-0689-0628>

Research

Keywords: pig, pulmonary disease, Mycoplasma, immune cells, routine blood test

Posted Date: May 22nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-28981/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Developments of pulmonary diseases, often accompanied by infections of bacteria, severely affect meat production and welfare of pigs. This study investigated the association of lung lesions and *Mycoplasma* levels inferred from 16S rRNA sequencing of bronchoalveolar lavage fluid with 57 immune cells and 25 hematological traits in 307 pigs at age of 240 days from an eight-breed heterogeneous cross.

Result: At a false discovery rate threshold of 0.05, we found that the greater severity of lung lesions were significantly associated with higher CD8+ to CD3+ cell ratio (CD8+/CD3+), neutrophil to lymphocyte ratio (NLR), and standard deviation of red blood cell volume distribution width (RDW-SD), and lower CD4-CD8-/CD3+, CD3+CD4-CD8-/PBMCs, CD14-CD16-/PBMCs, mean corpuscular hemoglobin concentration (MCHC), lymphocyte count (LYM) and lymphocyte count percentage (LYMR), reflected an status of inflammation, immune suppression and hypoxia of the pigs accompanying the development of the lung lesion. The *Mycoplasma* abundance showed positive correlations with neutrophil count (NEU), neutrophil count percentage (NEUR), neutrophil-to-lymphocyte ratio (NLR), monocyte count (MON), RDW-CV, and RDW-SD, and negative correlations with MCHC, LYM, and LYMR, these correlations are largely consistent with those of lung lesions, supporting the comorbidity of lung lesions and *Mycoplasma* infection. We also observed a nonlinear association that the sharp increases in NEU and NEUR occurred only when *Mycoplasma* abundance raised to a level above the population-average.

Conclusion: This study showed that the pigs from an eight-breed cross heterogeneous population reared under standardized housing conditions suffered lesion averagely covered 40% of lung, and the lung lesions were significantly linked to load of *Mycoplasma* in bronchoalveolar lavage fluid. We further demonstrated that the lung lesion and load of *Mycoplasma* perturb a large variety of immune and hematological traits. These associations provide helpful insights into the changes of host immune status in response to mycoplasma relevant lung diseases in pigs.

1. Background

Respiratory disease is common in pig production industry, often associated with multiple microbial infection, and causes great treatment burden and economic loss (1). Enzootic pneumonia in pigs is a type of respiratory disease links to infection of pathogenic bacteria such as *Mycoplasma pneumoniae* and accompanies with development of lung lesions (2). The lung tissue of pigs is accessible at slaughter, which allows for direct evaluation of lung lesions and quantification of lung microbiota or viruses from bronchoalveolar lavage through serological tests, PCR, or sequencing technology (3, 4). For example, a study of 125 farrow pigs found that *Mycoplasma hyopneumoniae*, porcine reproductive and respiratory syndrome virus, and swine influenza viruses are the major pathogens involved in the development of pneumonia-like lung lesions (4). We measured microbial communities from 20 lung samples with extreme lung lesion phenotypes from a pig heterogeneous population derived from eight founder breeds,

and found that *Mycoplasma* is the major pathogen associated with the severity of lung lesions and significantly affect the growth performance of pigs (3).

The host immune and hematological systems have essential functions in resisting pathogen and maintaining animal health (5, 6). Clarifying the associations between immune and hematological cells with the lung lesion and pathogenic bacteria would provide knowledge that is useful to diagnose, prevent, and treat respiratory diseases. It has been reported that the ratio of CD4 + T cells, NK cells, and B cells to white blood cells was lower in Landrace pigs that were selected to be resistant to *Mycoplasma* than the control group (7). Despite progresses have been made, studies aimed to reveal the links between immune and hematological responses of pigs to respiratory disease and bacterial infection remain limited.

In this study, we investigated the correlation of lung lesions and *Mycoplasma* levels with 57 immune and 25 hematological traits measured in 307 generation 7 (G7) pigs at age of 240 ± 10 days from the pig heterogeneous population. We expected that the sample size of more than 300 individuals together with broad genetic and phenotypic variations of the population would offer reasonable power to found biologically meaningful associations.

2 Materials And Methods

2.1 Experiment design

This work is a cross section association study largely takes advantage from the design of the pig heterogeneous population. The pig heterogeneous population was created by crossing eight founder pig breeds consisting of 4 western commercial breeds (Large White, Duroc, Landrace and Pietrain) and 4 Chinese indigenous breeds (Bamaxiang, Tibetan, Erhualian and Laiwu), with major objectives to investigate genetic basis of various complex traits of pig. The eight founder breeds conferred greater genetic and phenotypic diversity in the heterogeneous population than a normal F_2 or three-cross populations. Moreover, as all the pigs were reared in uniform and standardized conditions and slaughtered at approximately the same age, we expected that the environmental confounding effect on the phenotype-phenotype association results are well controlled. Another unique feature of this work is that each individual were simultaneously measured with multiple phenotypes including the lung lesion, microbiome composition from bronchoalveolar lavage fluid through 16S rRNA sequencing, immune cell using flow cytometry and hematological traits from routine blood test, which is rarely reported in pigs, therefore, an integrative analysis on these traits could provide novel information on the host response to pulmonary bacterial infection and lesion in pigs (Fig. 1A).

2.2 Animals

All animals investigated in this study are from generation 7 individuals of the heterogeneous population. Each individual is expected to contain ancestry from the eight founder breeds. The population was kept under uniform indoor conditions with *ad libitum* water supply at the experimental farm in Jiangxi

Agricultural University (Nanchang, China), and fed with corn-soybean feed (Jiangxi Huada Group) containing 3100 kJ digestive energy, 15.5% crude protein, 0.78% lysine, 0.6% calcium, 0.35% phosphorus and 0.55% salt. Each pig was vaccinated with seven vaccines including Classical Swine Fever Vaccine, Live (Cell line origin, PULIKE Biological Engineering, INC., China) and Porcine Circovirus Type 2 vaccine, Inactivated (Strain SH, PULIKE Biological Engineering, INC., China) at 15 days; Swine Streptococosis Vaccine, Inactivated (*Streptococcus equi subsp. Zooepidemicus* + *Streptococcus suis* type 2, Shanghai HILE bio-technology Co.,ltd, China) and Swine Pseudorabies Vaccine, Live (Strain Bartha K-61, Laboratories HIPRA,S.A., Spain) at 24 days; Swine Foot and Mouth Disease Bivalent Vaccine, Inactivated (Strain Re-O/MYA98/JSZ/2013 + Strain Re-A/WH/09, China Agricultural Vet.Bio.Science and Technology Co.,ltd., China) at 32 days; Porcine Reproductive and Respiratory Syndrome Vaccine, Live (Strain CH-1R, PULIKE Biological Engineering, INC., China) and Glasser's Disease Vaccine, Inactivated (*Haemophilus parasuis*, Laboratories HIPRA,S.A., Spain) at 40 days. The piglets were weaned at 46 days and male pigs were castrated at 90 days. All of the pigs were slaughtered at 240 ± 10 days of age after fasting 24 hours.

2.3 Measurement of the lung lesions and microorganism levels

Detailed measurement of lung lesion score and microorganism were described in our previous studies with a few adjustments (3). Briefly, the whole lung tissue was harvested during the slaughter and photographed from both anterior and posterior sides. The lung lesions were scored from the photographs; lungs were divided into seven sections, including apical lobe, cardiac lobe, and diaphragmatic lobe at both the left and right side, and the intermediate lobe. Then, each section was assigned a score of 0–5 corresponding to a lesion proportion of 0%, 0–20%, 20–50%, 50–75%, 75–90% and > 90%. We assigned the weights to the seven sections of lung according to their volume, which is 5%, 6%, 7%, 8%, 32%, 36% and 5% for left and right apical lobe, left and right cardiac lobe, left and right diaphragmatic lobe and intermediate lobe, respectively. Finally, the lesion score of the lungs was calculated as a weighted sum of lesion scores for the different sections of lung from both the anterior and posterior sides.

The bronchoalveolar lavage samples were collected within 20 min poster slaughter. After photographing, we infused about 100 ml sterile PBS buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate, and 2 mM potassium phosphate) into each lung with the trachea cut off, and gently pressed the lung sample for 2 min. About 50 ml lung lavage fluid was collected. The lavage fluid sample was centrifuged at 4000 g for 30 min at 4 °C, the pellet was transferred to a 2 ml sterile centrifugation tube, and stored at -80 °C for subsequent DNA extraction. DNA was extracted from the lavage samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The V3-V4 region of 16S rRNA gene was amplified using barcode-indexed universal bacterial primers. The amplicons were then purified and sequenced on an Illumina MiSeq sequencing platform (Illumina, San Diego, CA, USA). The raw sequence reads were filtered as previously described (3). The Operational taxonomic units (OTUs) were clustered using UCLUST cluster algorithm in USEARCH v7.01090 (8). The taxonomy of each 16S rRNA gene was analyzed using RDP v2.2 (9) based on Green-Genes v13.5 reference database (10). The genera level of *Mycoplasma* used in this study was determined in previous study (3).

2.4 Flow cytometry analysis

Peripheral blood samples from the neck arteries were collected during slaughter and stored in lithium-heparin tubes at room temperature for further analysis. We used flow cytometry (BD Accuri C6) to measure the absolute and relative number of immune cells using six antibodies by three combinations/panels: 1) CD3 FITC (BD Biosciences), CD4 Alexa Fluor 647 (BD Biosciences), and CD8 PE (BD Biosciences); 2) CD14 (Bio-Rad) combined with Alexa Fluor 647 - conjugated AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch) and CD16 RPE (Bio-Rad); and 3) CD163 FITC (Bio-Rad). For each panel of antibodies, 100 μ l peripheral blood was incubated for 30 min at 4°C with specific antibodies. Red cells were lysed for 15 min at room temperature (20–25°C) by erythrocyte lysis buffer (BD Biosciences). The cells were washed by 1 ml PBS and centrifuged at $300 \times g$ for 10 min. The supernatants were removed and the pellets were re-suspended in 500 μ l PBS (pH = 7.4), and analyzed on an Accuri C6 flow cytometer. Finally, we obtained immune cell traits for 307 G7 pigs including 176 males and 131 females (**Additional file 1: Table. S1**).

2.5 Measurement of the hematological traits

The routine blood test was implemented using BC-5000 Vet blood analyzer (Mindray) approximately three hours post slaughter (**Additional file 1: Table. S1**). The parameters measured included the white blood cell counts (WBC), neutrophil count (NEU), neutrophil ratio (NEUR), monocyte count (MON), monocyte ratio (MONR), lymphocyte count (LYM), lymphocyte ratio (LYMR), eosinophil count (EOS), eosinophil ratio (EOSR), basophil count (11), basophil ratio (BASR), red blood cell counts (RBC), hematocrit (HCT), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), coefficient of variation in red blood cell volume distribution width (RDW-CV), standard deviation of red blood cell volume distribution width (RDW-SD), platelet count (PLT), mean platelet volume (MPV), plateletcrit (PCT), and platelet volume distribution width (PDW) (**Additional file 1: Table. S1**). In addition, we calculated neutrophil-to-lymphocyte ratio (NLR) and eosinophil-to-basophil ratio (EBR), which are regarded as the basic markers of inflammation in human blood tests(6). Finally, we collected hematological traits from 177 G7 animals.

2.6 Statistical analysis

The summary statistics of phenotypic traits were calculated by R program (12). The repeatability of a trait was calculated by $\sigma_A^2 / (\sigma_A^2 + \sigma^2)$, σ_A^2 and σ^2 are between and within sample variance components, respectively, that were computed using ANOVA in the R program (12). The phenotypic records that deviated from mean ± 4 standard deviations of population were set to be missing. The phenotype values of the immune and hematological traits were first log2 transformed, and then corrected for potential covariates including age, sex, batch of experiment, and PBMCs concentration (the latter only for immune cell traits). Two statistical approaches, Pearson correlation and quadratic regression, were employed to investigate the association of lung lesions and *Mycoplasma* levels with immune cell and hematological traits. The correlation between a pair of traits was computed using *cor* function in R. The quadratic regression was performed by *lm* function in R based on following formula:

$$y=ax+bx^2$$

Where **y** is a vector of corrected phenotypic values of lung lesion or microorganism, and **x** represents a vector of corrected phenotypic values of immune or hematological trait. The significance thresholds of the Pearson correlations were determined by the false discovery rate (FDR) calculated from 1000 permutation tests, in which we randomly shuffled the labels of phenotype data to calculate the null distribution of association statistics.

3. Results

3.1 Descriptions of phenotypes

The summary statistics of traits investigated in this study are detailed in **Additional file 1: Table. S1**. The average lung lesion score was 1.96, indicating on average, 40% (1.96/5) of an individual pig's lung developed lesions. We determined the composition of bacteria at genera level, and investigated their correlation with lung lesion score across 625 G7 individuals. The results showed that *Mycoplasma* displayed much more significant correlation (P value = 3.3×10^{-15}) with lung lesion than the other bacteria genera (Fig. 1B). In light of this result, we focused on the *Mycoplasma* in the subsequent analyses. The coefficient of variation for the *Mycoplasma* levels is 1.16, which is greater than the coefficient of variations for the immune cell and hematological traits measured in the same population (**Additional file 1: Table. S1**), reflecting a large variability of *Mycoplasma* levels in the lung of heterogeneous pigs that were reared in uniform housing and feeding environments. We profiled 57 immune and 25 hematological traits in arterial blood samples of 138–307 G7 individuals (**Additional file 1: Table. S1**). The average repeatability of the 57 immune cell traits estimated from 8–27 pairs of replicated samples was 0.895, ranging from 0.629–0.996, suggesting measurement of immune cell phenotypes was reliable.

Both of the immune cell and routine blood test parameters were measured from the arterial blood, which directly come from lung, therefore, it could reflect the situation of lung more accurately than venous blood (13). Out of 307 individuals investigated in this study, we also measured immune cells from venous blood of 58 pigs at age of 120 days. Interestingly, the immune cell counts measured from the venous blood samples of 120 days pigs were highly correlated with those measured in the arterial blood of the same individuals at 240 days of age (Fig. 2), indicating that the arterial blood can also reflect inter-individual difference in blood cell counts in venous blood, thus the results generated in this study are potentially comparable with those studies using venous blood.

3.2 Association of immune traits with lung lesion and *Mycoplasma* abundances

First, we assessed the Pearson correlations between the immune cell traits and lung lesion score (**Table. 1**). At a FDR threshold of 0.05, we observed 4 significant correlations, including the positive correlation of

CD8+/CD3+ ($r = 0.27$, P value = 4.1×10^{-6}), and negative correlation of CD4-CD8-/CD3+ ($r = -0.21$, P value = 2.1×10^{-4}), CD3 + CD4-CD8-/PBMCs ($r = -0.20$, P value = 6.6×10^{-4}), and CD14-CD16-/PBMCs ($r = -0.25$, P value = 1.5×10^{-4}) with the lung lesion score (**Table. 1** and Fig. 3A, C). The average CD8+/CD3 + cell ratio in the top 10% of individuals with the highest lung lesion score was 71.8%, which was significantly higher than the 64.4% observed in the top 10% of the individuals with the lowest lung lesion scores (Fig. 3B). By comparison, the CD3 + CD4-CD8-/PBMCs decreased from 0.58% in the top 10% of healthy individuals to 0.39% in the top 10% of individuals with severe lung lesions (Fig. 3D). None of the immune cell traits were associated with *Mycoplasma* levels.

Table 1

Significant correlations of immune cell and hematological traits with lung lesion and mycoplasma levels

Trait Category	Traits	N	Lung lesion score		Mycoplasma	
			r	p-value	r	p-value
Immune traits	CD8+/CD3+	294	0.26	1.1E-5	0.13	0.03
	CD4-CD8-/CD3+	294	-0.21	3.5E-4	-0.10	0.09
	CD3 + CD4-CD8-/PBMCs	294	-0.20	6.6E-4	-0.14	0.02
	CD14-CD16-/PBMCs	201	-0.26	1.6E-4	-0.17	0.01
Hematological parameters	NEU	166	0.10	0.19	0.33	1.5E-5
	LYM	166	-0.34	1.1E-5	-0.22	5.0E-3
	MON	166	0.02	0.78	0.29	1.5E-4
	NEUR	166	0.21	7.3E-3	0.31	4.1E-5
	LYMR	166	-0.30	1.2E-4	-0.35	3.3E-6
	MONR	166	0.06	0.45	0.24	1.7E-3
	NLR	166	0.30	1.0E-4	0.36	2.8E-6
	MCHC	166	-0.32	2.2E-5	-0.31	4.2E-5
	RDW-CV	166	0.33	1.5E-5	0.19	0.01
	RDW-SD	166	0.40	7.9E-8	0.34	8.3E-6

3.3 Association of hematological traits with lung lesions and *Mycoplasma* abundances.

A total of 7 hematological parameters showed significant correlations with lung lesions, among which RDW-SD ($r = 0.40$, P value = 7.9×10^{-8}), RDW-CV ($r = 0.33$, P value = 1.5×10^{-5}), NLR ($r = 0.29$, P value = 1.0×10^{-4}), and NEUR ($r = 0.21$, 7.3×10^{-3}) showed positive correlations, and LYM ($r = -0.34$, P value = 1.1

$\times 10^{-5}$), MCHC ($r = -0.32$, $P \text{ value} = 2.2 \times 10^{-5}$), and LYMR ($r = -0.30$, $P \text{ value} = 1.2 \times 10^{-4}$) showed negative correlations (**Table. 1**, Fig. 3E). The pigs with severe lung lesion had a average lymphocyte concentration of 8.69 ± 3.22 ($10^9/\text{L}$), which was significantly lower than the 13.74 ± 4.79 ($10^9/\text{L}$) in the top 10% of healthy individuals with lowest levels of lung lesions. This represented a 36.7% $((13.74 - 8.69)/13.74)$ decrease (Fig. 3F). Notably, we found that several of these hematological parameters significantly correlated with lung lesions, and also had significant and same direction of correlation with *Mycoplasma*. This included RDW-SD ($r = 0.40$, $P \text{ value} = 8.3 \times 10^{-6}$), NLR ($r = 0.36$, $P \text{ value} = 2.8 \times 10^{-6}$), NEUR ($r = 0.31$, $P \text{ value} = 4.1 \times 10^{-5}$), LYMR ($r = -0.35$, 3.3×10^{-5}), MCHC ($r = -0.31$, $P \text{ value} = 4.2 \times 10^{-5}$), and LYM ($r = -0.22$, $P \text{ value} = 5.0 \times 10^{-3}$). Additionally, *Mycoplasma* was positively correlated with NEU ($r = 0.33$, $P \text{ value} = 1.5 \times 10^{-5}$), MON ($r = 0.29$, $P \text{ value} = 1.5 \times 10^{-4}$), and MONR ($r = 0.24$, $P \text{ value} = 1.7 \times 10^{-3}$) (**Table. 1**).

3.4 Evidence of a nonlinear relationship between NEU and *Mycoplasma* abundances

Next, we use quadratic regression to assess the nonlinear relationship among traits. Notably, we obtained considerable enhancement in association significance between *Mycoplasma* levels and two hematological traits, NEU ($P \text{ value} = 2.6 \times 10^{-7}$) and NEUR ($P \text{ value} = 1.7 \times 10^{-8}$) based on quadratic regression compared to those of Pearson correlation analysis (Fig. 4A), suggesting existence of a nonlinear relationship between *Mycoplasma* levels with NEU and NEUR. In these two cases, we observed sharp increases of NEU and NEUR were occurred when the abundance of *Mycoplasma* in lung raised above the population average level (Fig. 4B-E).

4 Discussion

Respiratory diseases such as enzootic pneumonia cause great economic loss in pig production industry. The pathogenesis of some respiratory pathogens in pigs has been studied, the host immune response to infection and tissue lesions remain largely unclear and worth to be surveyed. In this study, we examined the association of various immune cell traits and hematological parameters with lung lesions and *Mycoplasma* levels. The identification of significant associations in this study would provide valuable information on the immune and hematological response of host to *Mycoplasma* infection and related lung lesion.

4.1 Immune and hematological traits positively correlated with lung lesion

We observed that pigs with more severe lung lesions had higher CD8+/CD3+, NEU, NEUR, NLR, RDW-CV, and RDW-SD. CD8 + T cells are a class of cytotoxic T cells which kill infected cells by secreting cytokines (TNF- α and IFN- γ), cytotoxic molecules (perforin and granzymes), and interacting with Fas/FasL (14). Increased CD8 + T cells in peripheral blood, lung tissue were reportedly associated with lung lesions in humans and pigs (15, 16). Further, a study in mice lacking the *CD8* gene exhibited a reduced

inflammatory response and did not develop lung disease after prolonged exposure to cigarette smoking (17). The excessive increase in the proportion of CD8 + in CD3 + T cells observed in the study, reflecting an imbalance of CD8 + T cell differentiation and severe inflammatory response, could contribute to development of lung lesion.

Neutrophils are thought to be the first line of defense against invading pathogens, which can trigger an inflammatory response which further prompts neutrophils to infiltrate tissues. There is evidence that higher levels of neutrophils are associated with lung lesions in pigs(18); this agrees with the positive correlation observed between lung lesions and neutrophil-related traits identified in this study. In humans, NLR is considered to be the biomarker of inflammation, which was positively correlated with the severity of COPD and predictor of mortality (6). The percentage of peripheral blood neutrophils was reported to be significantly increased in people with lung disease(19). Moreover, the lung cancer patients with higher NLR had poor overall survival(20).

RDW-CV (or RDW) and RDW-SD are measures of variation in red blood cell volume. The positive correlations of RDW-CV and RDW-SD with lung lesion observed here, suggested that the progression of lung lesion is accompanied by the increase in variation of red blood cell size. To our knowledge, no study in pigs has reported the association between RDW and lung lesions. However, the association of RDW with lung disease has been identified in humans. A study has found negative correlation of RDW with lung function(21). The evaluated RDW is reported to be related to chronic inflammation, which can alter erythropoiesis and promote erythrocyte membrane deformation (22). Higher RDW in individuals with severe lung lesions may promote the inflammation and further exacerbate the disease.

4.2 Immune and hematological traits negatively correlated with lung lesion

In this study, we found that CD4-CD8-/CD3+, CD3 + CD4-CD8-/PBMCs, CD14-CD16-/PBMCs, LYM, LYMR, and MCHC were negatively associated with lung lesions. As far as we know, this study is the first report of several immune associations in pigs. CD4-CD8- T cells also exist in rodents and humans, where they constitute about 1–5% of the CD3 + T cell population(23). CD4-CD8-CD3 + cells were known as regulatory T cell, as they were found to be involved in autoimmune diabetes and intracellular infection in humans(24). The CD4-CD8- T cell population was reported to be significantly reduced in severe pulmonary tuberculosis patients(25). CD14 and CD16 are monocyte markers in both pigs and humans. We reported a significant association of lung lesion with CD14-CD16- cell population, which encompassed different cells including B cells, T cells, NKs, DCs, further investigations are required to verify the involvement of these cells in lung lesion.

Among the parameters from routine blood test, lymphocytes are an important part of the immune system; reduced lymphocytes levels is usually associated with immunosuppression(26). An *in vitro* study showed that lipid-associated membrane proteins of *Mycoplasma* induced the apoptosis of lymphocytes and monocytes from PBMCs (27). We therefore hypothesize that the invasion of *Mycoplasma* may lead to the apoptosis of lymphocytes and immunosuppression of host.

MCHC, a measure of mean corpuscular hemoglobin concentration, was found to be negatively correlated with lung lesion. The decreased levels of MCHC could be linked to the deformation of red blood cells as indicated by the evaluated RDW-CV. A recent study in humans showed that MCHC levels were lower in a control (healthy) group than in a newly diagnosed COPD group (28). The process of hypoxia, induced by lower MCHC, may contribute to the lung tumor cell metastasis and poor prognosis (29). We postulate that the decline of MCHC may have contributed to the development of the disease by putting the animals in an anemic hypoxic state.

4.3 Immune and hematological traits associated with lung *Mycoplasma* levels

Previously, we found that *Mycoplasma* was the most abundant and only bacteria significantly associated with lung lesions in pigs (3). Similarly, we found that the pattern of correlations of *Mycoplasma* with six hematological parameters including RDW-SD, NLR, NEUR, LYMR, MCHC, and LYM was similar to the lung lesion correlations. These results agree with the finding that the percentage of neutrophils and lung damage appeared to be increased in pigs infected with *Mycoplasma* compared to the control group (30). *Mycoplasma pneumoniae* was also reported to be associated with the pathogenesis of asthma in humans (31); asthmatic children with *Mycoplasma* infection demonstrated a marked decline in T lymphocytes (32).

5 Conclusion

This study showed that the pigs from eight breed cross heterogeneous population reared under standardized housing conditions suffered lesion averagely covered 40% of lung, and the lung lesions were significantly linked to load of *Mycoplasma* in bronchoalveolar lavage fluid. Much more significant correlation of *Mycoplasma* with lung lesion than other genera of bacteria observed hereby supporting *Mycoplasma* is a main genera of bacteria that could lead to exacerbation of lung lesion in pigs. Through association analysis with immune cell and hematological traits, we showed that evaluated load of *Mycoplasma* and deterioration of lung lesions consistently induced significant changes of hematological status including the increase of RDW-SD, NLR, and NEUR, and decrease of LYMR, MCHC, and LYM in pigs, processes indicating the inflammation, immune suppression and hypoxia status of host, these results further support the comorbidity of *Mycoplasma* proliferation and lung lesion. Moreover, we found that lung lesions were positively associated with CD8+/CD3+ cell ratio, and negatively associated with CD4-CD8-/CD3+ cell ratio, indicating the balanced differentiation of CD4 and CD8 T cells is important to present or alleviate lung lesion. This study provides useful information that may be helpful to the diagnoses of relevant respiratory disease in pigs.

Abbreviations

WBC:White blood cell count; NEU:Neutrophil count; LYM:Lymphocyte count; MON:Monocyte count;EOS:Eosinophil count; BAS:Basophil count; NEUR:Neutrophil count percentage; LYMR:Lymphocyte

count percentage; MONR:Monocyte count percentage; EOSR:Eosinophil count percentage; BASR:Basophil count percentage; NLR:Neutrophil-to-lymphocyte ratio; EBR:Eosinophil-to-basophil ratio; RBC:Red blood cell count; HGB:Hemoglobin; HCT:Hematocrit; MCV:Mean corpuscular volume; MCH:Mean corpuscular hemoglobin; MCHC:Mean corpuscular hemoglobin concentration; RDW_CV:Coefficient of variation of red blood cell volume distribution width; RDW_SD:Standard deviation of red blood cell volume distribution width; PLT:Platelet count; MPV:Mean platelet volume; PDW:Platelet distribution width; PCT:Plateletcrit.

Declarations

Competing interest

The authors declare that they have no competing interest.

Availability of data and materials

Not applicable

Ethics approval and consent to participate

All of the procedures involving animals are in compliance with the care and use guidelines of experimental animals established by the Ministry of Science and Technology of China. The ethics committee of Jiangxi Agricultural University approved this study.

Consent for publication

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (31760657) and National modern agricultural technology system (CARS-35).

Author Contributions Statement

LH designed and organized the construction of pig heterogeneous population; BY supervised the experiments and data analysis in this study; HA determined the method for measuring pulmonary phenotypes; QZ and JL measured the phenotype of immune cells and performed data analysis; TH measured the lung lesions and microorganism levels; YZ collected samples and provided assistance in

data analysis; and WX contributed to blood routine tests. BY and QZ wrote the manuscript, and LH revised the manuscript. All authors read and approve the final manuscript.

Acknowledgments

We would like to express our gratitude to all colleagues in our laboratory for their contribution in animal breeding and sample collection.

References

1. Opriessnig T, Giménez-Lirola LG, Halbur PG. Polymicrobial respiratory disease in pigs. *Animal Health Research Reviews*. 2011;12(2):133–48.
2. Maes D, Verdonck M, Deluyker H, de Kruif A. Enzootic pneumonia in pigs. *Vet Q*. 1996;18(3):104–9.
3. Huang T, Zhang M, Tong X, Chen J, Yan G, Fang S, et al. Microbial communities in swine lungs and their association with lung lesions. *Microb Biotechnol*. 2019;12(2):289–304.
4. Fablet C, Marois-Crehan C, Simon G, Grasland B, Jestin A, Kobisch M, et al. Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: a cross-sectional study. *Vet Microbiol*. 2012;157(1–2):152–63.
5. Rankin LC, Artis D. Beyond Host Defense: Emerging Functions of the Immune System in Regulating Complex Tissue Physiology. *Cell*. 2018;173(3):554–67.
6. Xiong W, Xu M, Zhao Y, Wu X, Pudasaini B, Liu JM. Can we predict the prognosis of COPD with a routine blood test? *Int J Chron Obstruct Pulmon Dis*. 2017;12:615–25.
7. Shimazu T, Borjigin L, Katayama Y, Li M, Satoh T, Watanabe K, et al. Immunological characterization of peripheral blood leukocytes using vaccine for mycoplasmal pneumonia of swine (MPS) in swine line selected for resistance to MPS. *Anim Sci J*. 2013;84(10):683–92.
8. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 2010;26(19):2460–1.
9. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*. 2007;73(16):5261–7.
10. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol*. 2006;72(7):5069–72.
11. Karabasil N, Cobanovic N, Vucicevic I, Stajkovic S, Becskei Z, Forgach P, et al. Association of the severity of lung lesions with carcass and meat quality in slaughter pigs. *Acta Vet Hung*. 2017;65(3):354–65.
12. Team RC. R: A Language and Environment for Statistical Computing. 2013.
13. Fernandez-Serrano S, Dorca J, Coromines M, Carratala J, Gudiol F, Manresa F. Molecular inflammatory responses measured in blood of patients with severe community-acquired pneumonia.

Clin Diagn Lab Immunol. 2003;10(5):813–20.

14. Mittrucker HW, Visekruna A, Huber M. Heterogeneity in the differentiation and function of CD8(+) T cells. Arch Immunol Ther Exp (Warsz). 2014;62(6):449–58.
15. Yu DP, Han Y, Zhao QY, Liu ZD. CD3 + CD4 + and CD3 + CD8 + lymphocyte subgroups and their surface receptors NKG2D and NKG2A in patients with non-small cell lung cancer. Asian Pac J Cancer Prev. 2014;15(6):2685–8.
16. Freeman CM, Martinez FJ, Han MK, Washko GR Jr, McCubbrey AL, Chensue SW, et al. Lung CD8 + T cells in COPD have increased expression of bacterial TLRs. Respir Res. 2013;14:13.
17. Maeno T, Houghton AM, Quintero PA, Grumelli S, Owen CA, Shapiro SD. CD8 + T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice. J Immunol. 2007;178(12):8090–6.
18. Jolie R, Olson L, Backstrom L. Bronchoalveolar lavage cytology and hematology: a comparison between high and low health status pigs at three different ages. J Vet Diagn Invest. 2000;12(5):438–43.
19. Sahlander K, Larsson K, Palmberg L. Altered innate immune response in farmers and smokers. Innate Immun. 2010;16(1):27–38.
20. Guo D, Li M, Chen D, Jing W, Zhu H, Fu L, et al. Neutrophil-to-lymphocyte ratio is superior to platelet-to-lymphocyte ratio as a prognostic predictor in advanced non-small-cell lung cancer treated with first-line platinum-based chemotherapy. Future Oncol. 2019;15(6):625–35.
21. Grant BJ, Kudalkar DP, Muti P, McCann SE, Trevisan M, Freudenheim JL, et al. Relation between lung function and RBC distribution width in a population-based study. Chest. 2003;124(2):494–500.
22. Lippi G, Targher G, Montagnana M, Salvagno GL, Zoppini G, Guidi GC. Relation between red blood cell distribution width and inflammatory biomarkers in a large cohort of unselected outpatients. Arch Pathol Lab Med. 2009;133(4):628–32.
23. Juvet SC, Zhang L. Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. J Mol Cell Biol. 2012;4(1):48–58.
24. Hillhouse EE, Delisle JS, Lesage S. Immunoregulatory CD4(-)CD8(-) T cells as a potential therapeutic tool for transplantation, autoimmunity, and cancer. Front Immunol. 2013;4:6.
25. Pinheiro MB, Antonelli LR, Sathler-Avelar R, Vitelli-Avelar DM, Spindola-de-Miranda S, Guimaraes TM, et al. CD4-CD8-alpha-beta and gamma-delta T cells display inflammatory and regulatory potentials during human tuberculosis. PLoS One. 2012;7(12):e50923.
26. Teodorczyk-Injeyan JA, Sparkes BG, Mills GB, Peters WJ, Falk RE. Impairment of T cell activation in burn patients: a possible mechanism of thermal injury-induced immunosuppression. Clin Exp Immunol. 1986;65(3):570–81.
27. Bai F, Ni B, Liu M, Feng Z, Xiong Q, Shao G. Mycoplasma hyopneumoniae-derived lipid-associated membrane proteins induce inflammation and apoptosis in porcine peripheral blood mononuclear cells in vitro. Vet Microbiol. 2015;175(1):58–67.

28. Ugurlu E, Kilic-Toprak E, Can I, Kilic-Erkek O, Altinisik G, Bor-Kucukatay M. Impaired Hemorheology in Exacerbations of COPD. *Can Respir J*. 2017;2017:1286263.
29. Qu X, Zhang T, Ma H, Sui P, Du J. Lower mean corpuscular hemoglobin concentration is associated with unfavorable prognosis of resected lung cancer. *Future Oncol*. 2014;10(14):2149–59.
30. Zhang H, Lunney JK, Baker RB, Opriessnig T. Cytokine and chemokine mRNA expression profiles in tracheobronchial lymph nodes from pigs singularly infected or coinfectd with porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* (MHYO). *Vet Immunol Immunopathol*. 2011;140(1–2):152–8.
31. Hong SJ. The Role of *Mycoplasma pneumoniae* Infection in Asthma. *Allergy Asthma Immunol Res*. 2012;4(2):59–61.
32. Xin LH, Wang J, Wang Z, Cheng W, Zhang W. [Effect of *Mycoplasma pneumoniae* infection on function of T lymphocytes in bronchoalveolar lavage fluid of asthmatic children]. *Zhongguo Dang Dai Er Ke Za Zhi*. 2014;16(3):277–80.

Figures

Figure 3

Correlation of immune cell levels measured in arterial blood of individuals at age of 240 days with those measured at age of 120 days in venous blood. (A) boxplot showing similar phenotypic distribution of immune cells measured in arterial blood at age of 240 days and venous blood at age of 120 days. (B) examples of significant correlation of CD4+CD8+/CD3+ cell ratio and CD3-CD4-CD8+/LYMs cell ratio that were measured at 240 and 120 days in arterial and venous blood, respectively

Figure 4

Evidences of nonlinear associations between Mycoplasma levels and neutrophil related traits. (A) Comparison of association significance of Pearson correlation with quadratic regression in the assessment of association of levels of Mycoplasma with NEU and NEUR. (B-C) Scatter plot showing the nonlinear association between levels of Mycoplasma with NEU and NEUR. (D-E) The boxplot showing the distribution of Mycoplasma levels corresponding to individuals with different levels of NEU and NEUR grouped by five quantile intervals

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalTableS1.xlsx](#)
- [SupplementalTableS1.xlsx](#)