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Original article

Integrated analysis of differential gene expression profiles in porcine alveolar macrophages induced by *Mycoplasma hyopneumoniae* strain 232

G. Wang, J. Li, Y. Liu

College of Veterinary Medicine, Hunan Agricultural University, Changsha 410128, China

Correspondence to: Y. Liu, e-mail: liuyisong@hunau.edu.cn

Abstract

Porcine alveolar macrophages (PAMs) can resist infection caused by *Mycoplasma hyopneumoniae* (Mhp) through phagocytosis. However, it is unknown what gene expression changes occur in PAM after Mhp stimulation. Therefore, the differential gene expression (DGE) profiling technique was employed to analyze differentially expressed genes in PAMs infected with Mhp strain 232. Eighty-six and 889 differentially expressed (DE) genes were identified in PAMs at 12 hours post-infection (hpi) and 24 hpi. Using Gene Ontology (GO) analysis, DE genes were involved in 54 (12 hpi) and 128 (24 hpi) GO enrichment items. Using Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, DE genes were involved in 101 (12 hpi) and 250 (24 hpi) KEGG enrichment items. Using Ingenuity Pathway Analysis (IPA), DE genes were connected, forming 25 internally interacting subnetworks. STRING analysis revealed 131 proteins encoded by DE genes involved in network interactions. Five novel genes were closely related to clinical symptoms and pathological changes of *Mycoplasma hyopneumoniae*. This is the first study to investigate PAM transcriptional responses to *Mhp* infection using the DGE profiling technique.

Keywords: differential gene expression profiles, *Mycoplasma hyopneumoniae*, porcine alveolar macrophage

Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is an agriculturally significant swine respiratory pathogen of mycoplasma pneumoniae of swine (MPS), which causes substantial economic losses in almost all countries (Xu et al. 2021, Biebaut et al. 2021). Numerous previous studies have shown that *M. hyopneumoniae* infection can lead to changes in PAM function; These previous studies used various methods such as real-time fluorescence quantitative polymerase chain reaction (qPCR), enzyme-linked immunosorbent assay (ELISA) and high-throughput cDNA microarray assays (Li et al. 2015, Yang et al. 2021). *M. hyopneumoniae* infection can also lead to increased production of cytokines, including Toll-like receptor (TLR) 2, TLR4, interleukin (IL)1α, IL1β, IL8, IL10, and tumor necrosis factor (TNF)α (Li et al. 2019, Tonni et al. 2021, Liu et al. 2022).

Interactions between *M. hyopneumoniae* and PAMs have been reported in the literature (Deeney et al. 2019, Mei et al. 2022). High throughput cDNA microarray assays were employed to evaluate the host responses of PAMs to *M. hyopneumoniae* field strain XLW-1 infection. A total of 1033 and 1235 differentially expressed genes were identified in PAMs in response to exposure to *M. hyopneumoniae* at 6 hours and 15 hours post infection, respectively (Bin et al. 2014). The detailed interaction mechanisms between other *M. hyopneumoniae* strains and PAMs has not been elucidated. To study the molecular mechanisms underlying the host response to pathogenic microorganisms, digital gene expression profiling techniques have been widely used in recent years (Zhang et al. 2022, Zhou et al. 2023). Digital gene expression profiling technique is one of the essential tools for functional genomics research, which aims to detect all differentially expressed gene sequences (Li et al. 2023). Compared to the gene chip and the high-throughput cDNA microarray, the DGE profiling technique is inexpensive, efficient, and short. It is therefore widely used to analyze the differential gene expression of tissues and cells against pathogenic microbial infections (Alfonseca et al. 2016, Jaiswal et al. 2016). In this study, the DGE profiling technique was used to detect the differential expression profile of PAMs induced by *M. hyopneumoniae* strain 232, which provided new clues and ideas to further clarify the pathogenesis and clinical symptoms caused by *M. hyopneumoniae* infection through *M. hyopneumoniae*-induced PAM.

Materials and Methods

M. hyopneumoniae and PAMs

M. hyopneumoniae strain 232 was presented to us as a gift from researcher Shao Guoqing, Jiangsu Academy of Agricultural Sciences. This strain was isolated from an infected pig, the genome of which is composed of 892,758 bp and has an average G + C content of 28.6 mol%. There are 692 predicted protein coding sequences, the average protein size is 388 amino acids, and the mean coding density is 91%. Unlike other mycoplasmas, *M. hyopneumoniae* contains few genes with tandem repeat sequences that could be involved in phase switching or antigenic variation. Thus, it is not clear how *M. hyopneumoniae* evades the immune response and establishes a chronic infection (Minion et al. 2004).

PAMs were prepared as described previously (Muneta et al. 2008). Five 4-week-old piglets, serologically negative for *M. hyopneumoniae*, Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2), were anesthetized and sacrificed by bloodletting. The sacrifice of the five piglets was approved by the Biomedical Ethics Committee of Hunan Agricultural University, approval number 2022-34. Five samples (bronchoalveolar lavage fluid) isolated from five lungs were detected for *M. hyopneumoniae*, PRRSV, PCV2, Pseudorabies virus (PRV), and classical swine fever virus (CSFV) by polymerase chain reaction (PCR) according to the literature (Wang et al. 2013). The steps are described briefly as follows. After the piglets were slaughtered, the whole lungs were washed with sterile PBS. Fifty mL of sterile PBS was infused from the trachea. After the lungs were gently rubbed for 5 min, the lavage fluid was recovered. The recovered porcine alveolar lavage fluid was gently blown with a straw and filtered with sterile gauze to collect all the filtrate. The filtrate was centrifuged at 1500 r/min for 5 min, the supernatant was removed, and the precipitate was resuspended in 10 mL of sterile PBS. More washes were carried out as above and porcine alveolar macrophages were obtained.

After isolating and purifying a negative sample for the 5 pathogenic microorganisms, PAMs were grown and maintained in RPMI-1640 medium containing 10% (v/v) FBS from GIBCO (Invitrogen) at 37°C with 5% CO_2.

Experimental design

Mhp and PAM were incubated in the test group (6 bottles): an appropriate amount of Mhp (3×10^7CCU) was centrifuged to remove the supernatant and added to an equal volume of 1640 culture fluid. According to the optimal incubation ratio (3:1, data not shown), Mhp

and PAM were mixed as 10 mL/bottle; in the control group (6 bottles), 1640 medium and PAM were mixed evenly as 10 mL/ bottle. A total of 12 bottles of the mixture were cultured at 37°C and 5% CO_2. After 12 h of incubation, 6 bottles (3 test bottles and 3 control bottles) were removed by mixing the PAMs from 3 test bottles into one sample named PAM12S; the PAMs from 3 control bottles were combined into one sample named PAM12D. After 24 h of incubation, the remaining 6 bottles (3 test bottles and 3 control bottles) were removed by mixing the PAMs from 3 test bottles into one sample named PAM24S; the PAMs from 3 control bottles were combined into one sample named PAM24D. All four samples were immediately sent to Beijing Novogene Info Tech Ltd (Beijing, China PR) for RNA preparation, RNA library preparation and analysis of differential gene expression profiles using Digital Gene Expression Profiling technique.

RNA preparation

Total RNA was extracted from the PAM of each sample using Trizol (Invitrogen) and then monitored for degradation and contamination on 1% agarose gels. RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using the Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit in the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation and analysis of differential gene expression profiles

Libraries were generated using an NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Library quality was assessed on an Agilent Bioanalyzer 2100 system. Library preparations were sequenced on the Illumina Hiseq 2000/2500 platform, and 100 bp/50bp single-end reads were generated. For gene expression analysis, the number of expressed tags was calculated and then normalized to RPKM (Reads Per Kilobase of exon model per Million mapped reads) (Mortazavi et al. 2008).

Analysis of DE gene data

Differential expression analysis of two conditions/ groups (two biological replicates per condition) was performed using the DESeq R package (1.10.1). DESeq provides statistical routines for determining differential expression in digital gene expression data using a negative binomial distribution-based model. The resulting P-values were adjusted using Benjamini and Hochberg's approach to control for false discovery rates. Genes with an adjusted P-value < 0.05 found by DESeq were assigned as differentially expressed. Differential expression analysis was performed for both conditions using the DEGSeq R package (1.12.0). P-values were adjusted using the Benjamini and Hochberg method. The corrected P-value of 0.05 and log2 (Fold change) of 1 was set as the threshold for significantly differential expression. The numbers of differentially expressed genes in "PAM12 vs. PAM12D" and "PAM24S vs. PAM24D" and the numbers of differentially expressed genes common between "PAM12 vs. PAM12D" and "PAM24S vs. PAM24D" were statistically analyzed.

GO (http://www.geneontology.org/) is a shared database that classifies gene annotations into three major categories: biological processes, molecular functions, and cellular components. GO enrichment analysis of differentially expressed genes was implemented by the GOseq R package, which corrects for gene length bias. GO terms with a corrected P-value < 0.05 were considered significantly enriched by differentially expressed genes.

KEGG is a database resource for understanding high-level functions and utilities of biological systems such as cells, organisms, and ecosystems from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS software to test the statistical enrichment of differentially expressed genes in KEGG pathways.

PPI analysis of differentially expressed genes was based on the STRING database, which is known and predicted for Protein-Protein interactions. For species existing in the database, we constructed the networks by extracting the target gene list from the database; otherwise, Blastx (v2.2.28) was used to align the target gene sequences with the selected reference protein sequences, and the networks were then built according to the known interaction of the chosen reference species. The Gi numbers of all significantly regulated genes were imported into IPA software (www.ingenuity.com) for bioinformatics analysis based on published reports and databases such as Gene Ontology and Uniport.

QPCR analysis of chemokine expression

To validate chemokine mRNA transcript levels in PAMs infected with *M. hyopneumoniae* using the DGE profiling technique, qRT-PCRs were used to detect mRNA levels of 4 chemokines (TRL4, IL1β, IL8 and MHCII). The specific primers used in qPCRs are listed in Table 1. QPCRs were performed in tripli-

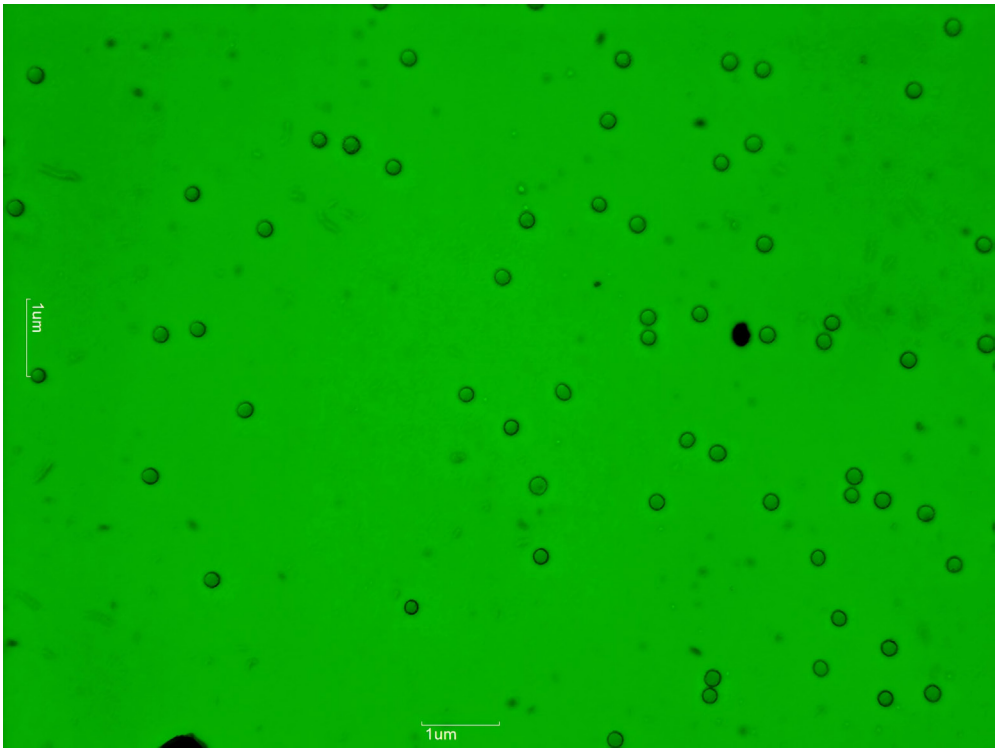

Fig. 1. Purified porcine alveolar macrophages (PAMs) before 2 hours of culture. The PBST method obtains a clear outline of the PAM, most of them are round, cell size is different, the larger the size of the mature PAM, the smaller for the immature PAM and lymphocytes, etc. After 2 hours of culture, the adherent cells were washed away, and the fresh medium was added. The purified PAM was round, and full of cytoplasm.

Table 1. QRT- PCR primers for verification of differentiated expressed genes in this study.

Name	Sequence	PCR production (bp)	document
MHCII	F:AACCTCCTGGTCTGCTCT R:CGACCCCACTCATCATCT	272	Mo 2005
IL1β	F:GCCCTGTACCCCAACTGGTA R:CCAGGAAGACGGGCTTTTG	61	Moue et al. 2008
IL8	F:GCTCTCTGTGAGGCTGCAGTT R:TTTATGCACTGGCATCGAAGTT	62	Moue et al. 2008
TLR4	F:CGAGGCCGTCATTAGT R:ACAAAGGCGTCATAGGT	144	Moue et al. 2008
β-actin	F:CATCACCATCGGCAACGA R:GCGTAGAGGTCCTTCCTGATGT	144	Moue et al. 2008

cate for all reactions using the SYBR green detection system and the ABI 7500 real-time PCR system (ABI, USA). Relative standard curves of target and endogenous control primer pairs were performed to verify comparable PCR efficiencies, and once established, the comparative (2- delta- delta) Ct method was applied.

Results

PAM isolation and acculturation

The concentration of isolated PAM was 5×10^8 cells per milliliter. The profiles of these PAMs are clear and the appearance of these PAMs is mainly circular (Fig. 1).

Statistical analysis of differentially expressed genes

After filtering out the low-quality data, the results are shown in Table 2. The error rate in the sequencing data was controlled within the permissible range, and the GC content was uniformly distributed, with Q20 and Q30 above 90%, indicating a sequencing data satisfying the analysis requirements. According to the statistical results, there were 86 differentially expressed genes at 12 hpi (PAM12S vs. PAM12D), of which 49 expressions were up-regulated and 37 expressions were down-regulated (Fig.2A). There were 889 differentially expressed genes at 24 hpi (PAM24S vs. PAM24D) (Fig.2B), of which 517 expressions were up- regulated

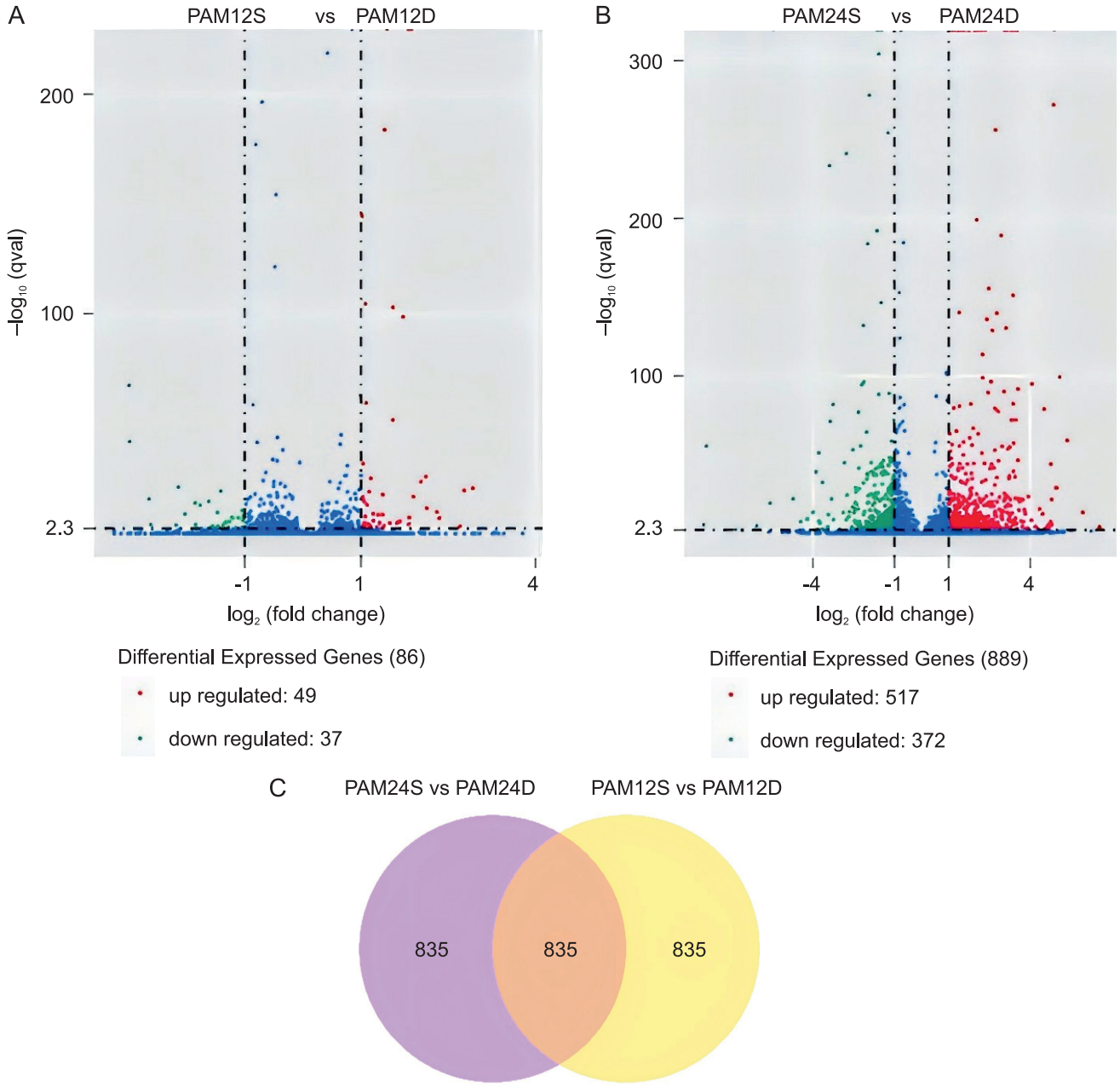

Fig. 2. The genes expression clustering map of PAM induced by Mhp at 12 hpi (A) and at 24 hpi (B) and the Venn Figure of DEGs between of Mhp induced PAM between 12 hours and 24 hours (C). Red: Up- regulated gene; Blue: Down- regulated gen

Table 2. Quality data schedule of 4 PAMs samples induced by *M. hyopneumoniae.*

Sample name	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
PAM12S	9752202	9682591	0.48G	0.01	98.84	96.21	52.29
PAM12D	12727490	12646691	0.63G	0.01	98.64	95.69	51.57
PAM24S	15574112	15474629	0.77G	0.01	98.67	95.76	52.04
PAM24D	14822416	14739185	0.74G	0.01	98.65	95.70	52.29

and 372 expressions were down-regulated. There were 54 differentially expressed genes common between "PAM12 vs. PAM12D" and "PAM24S vs. PAM24D" (Fig.2C).

Analysis of DE genes using GO

All DE genes were analyzed by GO analysis. Of all 86 DE genes at 12 hpi, 78 DE genes were annotated on the gene ontology database (PAM12S vs. PAM12D).

Table 3. Most notable enrichment items in differential expression (Top 10) (PAM12S vs PAM12D).

Num.	GO accession No.	Name of enrichment items	P value	Number of DEGs	Number of all genes	Term type
1	GO:0002376	immune system process	1.04E-10	27	1034	biological process
2	GO:0006954	inflammatory response	8.37E-08	13	228	biological process
3	GO:0006955	immune response	1.91E-07	17	521	biological process
4	GO:0005125	cytokine activity	2.89E-07	10	152	molecular function
5	GO:0006952	defense response	4.14E-07	16	500	biological process
6	GO:0009611	response to wounding	9.34E-07	15	393	biological process
7	GO:0048583	regulation of response to stimulus	5.28E-06	27	1664	biological process
8	GO:0005615	extracellular space	1.02E-05	14	473	cellular component
9	GO:0005126	cytokine receptor binding	2.44E-05	9	181	molecular function
10	GO:0009966	regulation of signal transduction	3.92E-05	23	1348	biological process

Table 4. Most notablest enrichment items of differential expression (Top 10) (PAM24S vs PAM24D).

Num.	GO accession No.	Name of Enrichment items	P value	Number of DEGs	Number of all genes	Term type
1	GO:0002376	immune system process	1.23E-16	118	1034	biological process
2	GO:0006955	immune system process	9.27E-15	73	521	biological process
3	GO:0005737	cytoplasm	7.86E-09	353	5437	cellular component
4	GO:0006952	defense response	2.29E-08	57	500	biological process
5	GO:0006950	response to stress	2.50E-07	128	1502	biological process
6	GO:0045321	leukocyte activation	2.50E-07	48	365	biological process
7	GO:0001775	cell activation	4.43E-07	52	422	biological process
8	GO:0002682	regulation of immune system process	5.06E-07	58	510	biological process
9	GO:1901700	response to oxygen-containing compound	6.64E-07	55	456	biological process
10	GO:0042981	regulation of apoptotic process	6.64E-07	76	747	biological process

Classification analysis showed significant differences in the enrichment functions of the 78 differentially expressed genes ($p<0.05$). Gene expression differed significantly between the top 10 enrichment functions, with the highest level shown in Table 3. The most significant difference in enrichment function expression was in the immune system (p=1.04E-10), inflammatory reaction (p=8.37E-08) and immune response (p=1.91E-07). The results indicate that Mhp, 12 h after PAM stimulation, mainly affects the immune process and inflammatory reaction to PAM. GO enrichment analysis revealed that there were 889 differentially expressed genes, 812 of which could be annotated in the GO database (PAM24D vs. PAM24D). These 812 genes were differentially expressed in 128 ($p<0.05$) GO enrichments. Table 4 lists the expression of the most significant differences between the first 10 enrichment functions. As can be seen from Table 4, at 24 hpi, differentially expressed genes were mainly concentrated in immune system processes (p=1.23E-16), immune response (p=9.27E-15) and cytoplasm (p=7.86E-09).

Analysis of DE genes by KEGG pathway

To gain insight into the different functional groups associated with MPS at 12 hpi and 24 hpi respectively, pathway mapping of DE genes was performed according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database.

At 12 hpi, all DE genes were mainly clustered in the following pathways: Toll-like receptor signaling pathway, TNF signaling pathway, Cytokine-cytokine receptor interaction, Antigen processing and presentation, and T-cell receptor signaling pathway. At 24 hpi, the main pathways were the Toll-like receptor signaling pathway, the TNF signaling pathway, the Cytokine-

Table 5. Differentially expressed gene analysis on KEGG library in this study (PAM 12S vs PAM 12D).

Pathway name	Number of DEG	Gene
Toll-like receptor signaling pathway	5	TRIF, OPN, TNF-α, IL-1β, MIP-1α, TRL4
TNF signaling pathway	5	CXC11, CXC12, CXC13, CSF2, IL-1
Cytokine-cytokine receptor inte raction	8	CCL3, CSF2, TNF, IL-1β, TNFSF10, CXCL2, LOC100525528, AMCF-II
Antigen processing and presentation	3	HSP70.2, HSPA2, TNF-α
T cell receptor signaling pathway	3	SLP-76, RASGRP1, GM-CSF, TNF-α
Endocytosis	2	HSP70.2, HSPA1

Table 6. Differentially expressed genes analysis base on KEGG in PAMs related closely to *M. hyopneumoniae* infection at 12 hpi and at 24 hpi in this study.

Pathway name	Number	Gene
At 12 hpi		
Toll-like receptor signaling pathway	5	TRIF, OPN,TNF-α, IL-1β, MIP-1α, TLR4
TNF signaling pathway	5	CXC11, CXC12, CXC13, CSF2, IL-1
Cytokine-cytokine receptor inte raction	8	CCL3, CSF2, TNF, IL-1β, TNFSF10, CXCL2, LOC100525528, AMCF-II
Antigen processing and presentation	3	HSP70.2, HSPA2, TNF-α
T cell receptor signaling pathway	3	SLP-76, RASGRP1, GM-CSF, TNF-α
At 24 hpi		
Toll-like receptor signaling pathway	10	TRIF, OPN, IL-1β, MIP-1α, MEK1/2, IRF5, AP-1, IL-8, RANTES, TLR4
TNF signaling pathway	16	cIAP1/2, MEK1, Cc15, Cxc11, Cxc12, Cxc13, Csf2, FAS, IL1β, BCL3, TNFaip3, FOS, MmP9, Icam1, Vcam1, RIP3
Cytokine-cytokine receptor inte raction	20	IL8, CCL3, CCL5, CCL8, IL13RA1, CSF2, CSF2RA, CSF2RB, IL10RA, FAS, TNFSF14, VEGFA, IL1-α, IL1-β, ACVR1B, TGFβ1, IL8RB, CCR7, TNFSF10, SF21
Antigen processing and presentation	6	MHCII, HSP70.2, HSPA2, SLA-5, SLA-6, HSPA8
T cell receptor signaling pathway	9	FYN, CBL, SLP-76, RASGRP1, MAK2P1, FPS, C-JUN, GM-CSF, CD45
Endocytosis	22	TGFβ1, Cb1, LDLR, E3Ligase (CBLB), E3Ligase (SMURF1), DAB2, HSPA2, HSP70.2, AGAP3, ASAP1, ARAP1, Smurf2, Smad7, Rab11, PLD, GPCR HSPA8

-cytokine receptor interaction, the Antigen processing and presentation, the T-cell receptor signaling pathway, the Endocytosis (Table 5). Differently expressed genes were closely related to MPS at 12 hpi and 24 hpi, respectively, as shown in Table 6.

Analysis of DE genes by IPA analysis

IPA was used to determine if altered genes could be mapped to specific functional networks and generate several biological pathways of interest that may be common to viral infections. IPA analysis identified 889 DE genes forming 25 networks (networks 1-25) at 24 hpi (Fig. 3). Network 7 and Network 15 were closely related to *Mhp*-infection. Some DE genes were found to be closely related to *Mhp*-infection, including IL1, HSP70, HSP90, CSF2, CCL5, FAS, IRF5, TLR4, MHC I, MHC II, IL12, TCR, CCL3, and HLA-DMB. Among the above factors, three cytokines (IL1, FAS and MHC II) closely related to *Mhp*-infection were selected for further analysis. In the Fas network, up-regulated cytokines (IL1) promoted lymphocyte infiltration and hyperplasia. CSF2 expression was up-regulated by regulating Fas expression, which further up-regulated

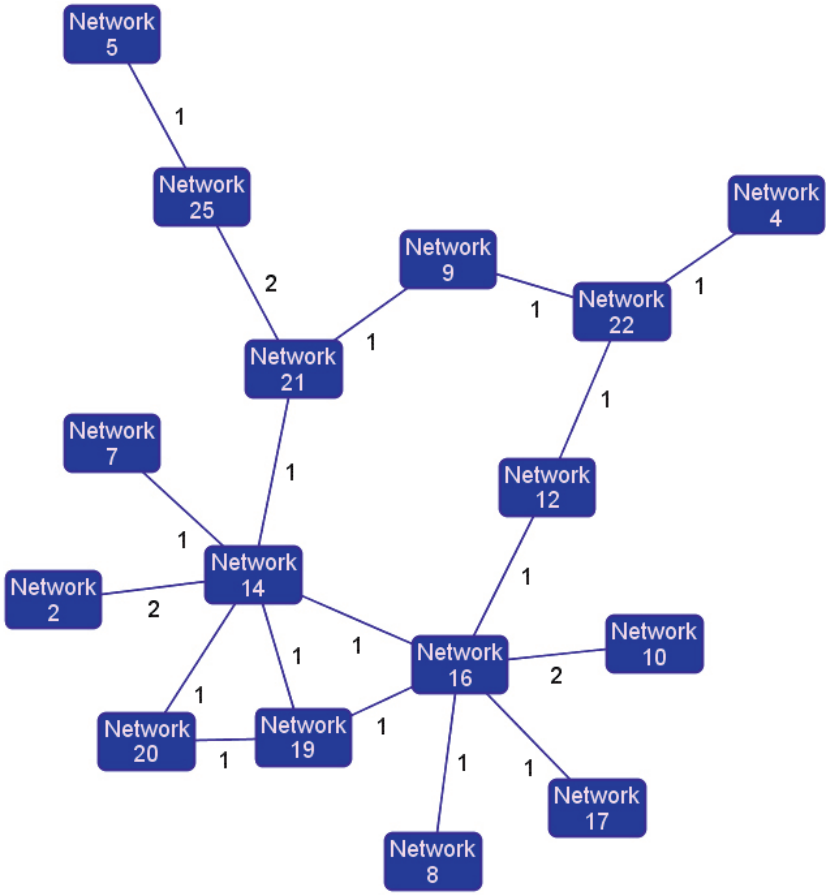

Fig. 3. Twenty-one specific functional networks, each of which contained 9 or more "focus" proteins (genes that were significantly up- or down-regulated). Each box contains an arbitrary network number. The numbers between two networks represent the amount of overlapped proteins.

the expression of interstitial pneumonia and *M. hyopneumoniae,* closely related to other cytokines CCL5 (the formation of a chronic inflammation and HSP70-related PAM) and cellular immune function. In IL1 supplements, the expression of IL increased the expression of CCL5, HSP70 and CSF2. Down-regulated expression of TRL4 could affect the expression of MHC II, which in turn affected the upregulation of SLA-DMB expression. In MHC II, down-regulated expression of TRL4 affected the expression of MHC II, which further contributed to changes in SLA-DMB expression. These factors were closely related to clinical symptoms and pathologic changes caused by *M. hyopneumoniae* infection. These factors interact and, together with other factors, promote the formation of clinical symptoms and pathological changes caused by M. hyopneumoniae infection.

STRING analysis of the relationships among DE genes

These relationships were analyzed using STRING to predict the network of proteins encoded by DE genes (von et al. 2005). At 24 hpi, the results showed that of 889 DE genes (PAM24S vs PAM 24 D), 131 genes (81 up-regulated genes and 50 down-regulated expression genes) were identified in the STRING database. Some genes were associated with each other to form a STRING sub-network, participating in many signaling pathways. These genes are IL1β (3.3064), Myc (1.8596), P21 (1.0248), AP1 (3.3105), CCL5 (1.8966), CCL8 (4.5513), IL8 (1.543), CCLX (4.0646), CCL3 (2.5266), AMCF II (2.0690), CCR7 (-1.6383), CXCR (-2.4636), and CXCL2 (2.326). Based on STRING analysis, many proteins (e.g., AP1, CCL5, IL8, CCR7 and CXCR) were named as key proteins, linking to other proteins. However, many proteins cannot be linked to other proteins, so their functions are unrelated or unknown.

Novel differentially expressed genes associated with MPS

A total of 86 genes at 12 hpi and 889 genes at 24 hpi were identified as differentially expressed genes. By performing IPA analysis and protein interaction STRING analysis on the above genes, combined with published literature, Many novel differentially

Table 7. Detection result of qPCR compared to DEGs in this study.

Name	[A]ΔΔct	[B]$2^{-\Delta\Delta ct}$	[C]DGE Value
MHCII	- 0.9945	1.9923	2.5971
IL1β	- 2.4839	5.5940	9.8929
IL8	- 0.287	1.2201	2.9140
TLR4	1.8539	0.2766	0.4605

note: [A] $\Delta\Delta Ct = (C_t$ value of one gene in test group – C_t value of β-actin gene C_t value in test group) – (C_t value of one gene in control group – C_t value of β-actin gene C_t value in test group)

[B] $2^{-\Delta\Delta ct}$ (qPCR Value): $2^{-\Delta\Delta ct} \geq 2$ indicated up-regulated, $0.5 < 2^{-\Delta\Delta ct} < 2$ indicated not significant regulated, $2^{-\Delta\Delta ct} \leq 0.5$ indicated down-regulated.

[C] DGE Value: DGE Value ≥2 indicated up-regulated, 0.5 < DGE Value < 2 indicated not significant regulated, DGE Value ≤0.5 indicated down-regulated.

expressed genes involved in the interaction between PAM and *M. hyopneumoniae* were identified. Among these genes, CCL3, CCL5, CCL8, TGFβ1 and MHC I, which are closely related to the clinical symptoms and pathological changes of MPS, suggest that these genes may be related to the pathogenesis of infection and pathogenesis.

QPCR validation of DE genes

To confirm the statistical significance of our findings, qPCRs were analyzed for the four DE genes. Four pairs of primers were used to detect the four DE genes, and the melt curves of the primers were qualified. The results showed that all four DE genes could be used for qRT-PCR detection. The comparative detection results using qPCR with the digital gene expression profiling technique show that the overall change trend of the 4 genes (up- or down-regulated) is consistent (Table 7), which indicates that the results of digital gene expression profiling are reliable.

Discussion

Gene chip is gradually showing its advantages in mapping gene expression and looking for some target genes and functional genes. Gene chip technology provides a powerful means for researchers to search for differentially expressed genes within the whole genome. However, gene chips can produce an abundance complex information. There is a difficulty for gene chip technology to interpret such a large amount of information in order to explore the biological characteristics and rules contained in diseases. Bioinformatics technology is an effective way to solve this difficulty (Wang. 2015). As a bioinformatics technology, differential expression analysis is the most common step in expression profiling data analysis. Its purpose is to identify genes with significantly different expression values in two different groups of samples, and genes with significantly different expression values are called differentially expressed genes (Xue et al. 2023).

The present study aimed to identify DE genes involved in *M. hyopneumoniae*-infected PAM. This study reports for the first time an investigation of transcriptional responses of PAM to *M. hyopneumoniae* infection using the digital gene expression profiling technique. 889 DE genes were identified as significantly differentially expressed in PAMs infected by *M. hyopneumoniae* at 24 hpi. From GO ontology analysis, KEGG pathway analysis, IPA analysis and STRING analysis, these DE genes were shown to belong to a variety of functional categories and signaling pathways. To better understand the role of differential genes in PAM against Mhp infection, it is important to analyze the number and function of differentially expressed genes.

Many DE genes were involved in the inflammatory response in PAMs at 24 hpi, including IL1β, IL8, TGFβ1, CCL3, CCL5, CCL8, CXC11, CXC12, CXC13, CSF2, etc. Ten GE genes were involved in the Toll-like receptor signaling pathway, including TRIF (2.2826), OPN (4.8623), IL1β (3.3064), MIP-1α (2.5266), MEK1/2 (1.4542), IRF5 (1.022), AP1 (3.3105), IL8 (1.543), RANTES (1.8966) and TLR4 (-1.1188). Furthermore, 12 DE genes were involved in the PPAR signaling pathway, 15 in the TNF signaling pathway, 13 in the NF-kappa B signaling pathway, 19 in the chemokine signaling pathway, and 3 in the RIG-I-like receptor signaling pathway. These results reflect the upstream signal cascades that may lead to the secretion of inflammatory cytokines and chemokines.

PAM, being an important immune cell, is responsible for phagocytosis, antigen processing and secretion of cytokines. When PAMs recognize Mhp, they initiate the process of endocytosis. When they are expressed on the surface of PAM, some phagocytic receptors can induce cytoskeletal changes that lead to the phagocytic function. Endocytosis is an important mechanism used

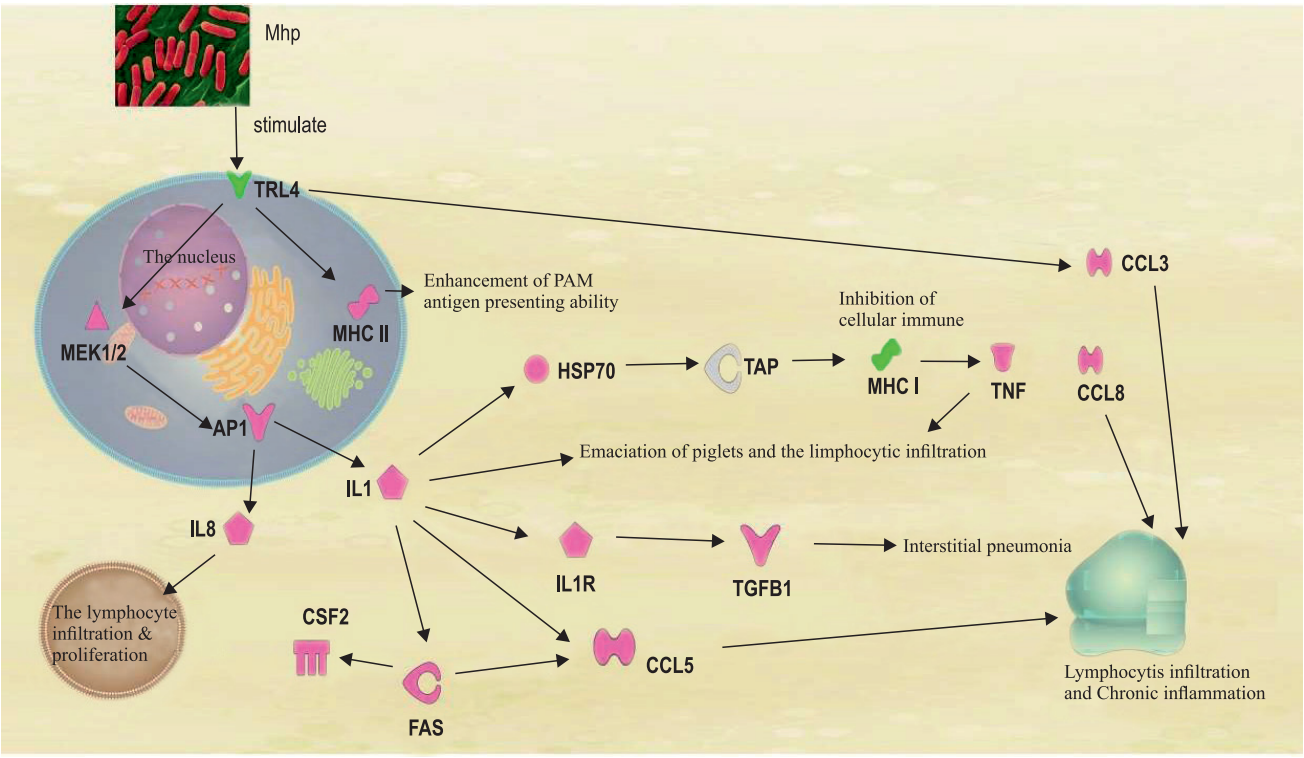

Fig. 4. No legend

by PAM to fight against various pathogenic microorganisms and apoptotic cells. Analysis of differentially expressed genes in PAMs at 24 hpi shows that a large set of DE genes are involved in the endocytosis process. TGFβ1 (1.5411) controls the proliferation and differentiation of stromal cells and the production of extracellular matrix (Venkatesan et al. 2014). There are steps in the TGF TGFβ/Smad signaling pathway conduction: binding of TGFβto TGF receptors (TGFβR) can activate R-Smad (receptor-associated Smad) and transmit the signal to the cytoplasm. Smurf2 is a regulatory protein of Smad7 that blocks TGFβ signaling (Wang et al. 2013). In this study, Smurf2 (1.5186) and Smad7 (1.4537) in PAMs at 24 hpi may prevent further TGFβ signals from entering the nucleus of PAM cells. Therefore, it can be speculated that the TGFβ signaling pathway is not the main signaling pathway.

In PAMs at 24 hpi, many genes associated with the processing of exogenous antigen presentation were up-regulated, such as MHCII (1.3769), HSP70.2 (1.8836) and HSPA2 (3.5052). This may be an instinctive reaction to the stimulation of external antigens by PAM and other APC cells. HC class I molecules are mainly involved in the processing of endogenous antigen presentation. The expression of MHC I determines the ability of PAM to present and process endogenous antigens. Surprisingly, in the present study, there was a decrease in the expression of MHC I, such as SLA-5 (-1.1517) and SLA-6 (-1.1492). *Mhp*-infection can weaken the cellular immunity of the PRRSV vaccine (Park et al. 2014), which may be achieved by decreasing the expression of MHC I.

Cytokines and chemokines play significant roles in host-pathogen interactions, including clearance of invading pathogenic microorganisms, as well as the initiation, progression and resolution of inflammation. In this study, 20 genes encoding cytokines were differentially expressed in PAMs at 24 hpi, and four of them were closely related to the clinical symptoms and pathological changes caused by *M. hyopneumoniae* infection, These four genes being CCL3 (2.5266), CCL5 (1.8966), CCL8 (4.5513) and TGFβ1 (1.5411). CCL3 strongly adsorbs mononuclear cells. CCL5 (RANTES) is an important chemokine that preferentially attracts T cells, eosinophils, eosinophils and mast cells (Hosokawa et al. 2016). It can be inferred that PAM upon Mhp stimulation is expressed mainly by increasing the expression of CCL3, CCL5 and CCL8 as well as the uptake of chemotactic infections by lymphocytes monocytes, eosinophils, and basophils. Inflammation plays an important role, with lymphocyte infiltration causing specific pathological changes caused by *M. hyopneumoniae* infection in bronchial bronchioles and lymphocytes perivascular infiltration of mononuclear cells (Barbara et al. 2008, Leal et al. 2020).

After PAMs were infected by *M. hyopneumoniae*, 86 genes at 12 hpi and 889 genes at 24 hpi were identified as differentially expressed genes. Using IPA analysis and protein interaction STRING analysis of these genes, combined with relevant published literature, five

DE genes involved in Mhp-PAM interactions, including CCL3, CCL5, CCL8, TGFβ1 and MHCI, were identified for the first time in this study. These five DE genes were found to be closely related to the clinical symptoms and pathological changes caused by *M. hyopneumoniae* infection, suggesting the possible association of these five genes with the infection and pathogenic mechanism of *M. hyopneumoniae*.

Some were involved in many signaling pathways (Fig. 4). After invading the pig respiratory tract, Mhp accumulates mainly on the bronchial surface and then enters the mucosal layer of the lymph space, causing bronchial inflammation, which in turn causes lobular pneumonia and fusion pneumonia. IL-1 and IL-8 can release histamine from mast cells and eosinophils. Histamine release induces micro-artery diastole, reduced precapillary resistance and enhanced venule permeability, which results in blood leukocytes, lymphocytes, monocytes, neutrophils and plasma protein extravasation (Ning et al. 2020). IL8 and CCL5 can be chemotaxis of lymphocyte infiltration CCL5 up-regulated expression, which allows CD8+ T lymphocytes to infiltrate from the blood to the prostate epithelial cell, forming benign prostatic hyperplasia and then lymphatic follicles (Artuc et al. 2011). CCL5 allows macrophages and immune cells (lymphocytes) to move to the site of the lesion, further resulting in the formation of lymphocyte proliferation and lymph follicle formation. This movement and infiltration can be inhibited by antagonists of CCR5 (CCL5 receptor). These reports suggest that CCL5 plays a crucial role in the activation process of lymphocytes to the site of inflammation. Lymphocyte infiltration at the site of MPS inflammatory lesions and further formation of lymph follicles are typical pathological changes of MPS (Zong et al. 2022). Based on the results of the above literature and the present study, the formation of the above typical pathological changes was closely related to the up-regulated expression of IL1, IL8, CCL3, CCL5 and CCL8 in the following processes. Mhp infection caused bronchial inflammation, pneumonia and fusion pneumonia. After the occurrence of inflammation, PAM accumulates to the site of inflammation and is activated to further secrete Pro-inflammatory cell factor such as IL-1 (3.3064), which induces inflammation of the body. After invading the body, Mhp can selectively induce specific B cells to produce IgE antibodies, which combine with mast cells and put the body in a sensitized state. When the antigen is re-invaded into the body, mast cells and eosinophils release histamine, which enhances capillary permeability. After the exudation of blood lymphocytes outside the blood vessels, the infiltrating lymphocytes accumulate and infiltrate into the inflammatory lesion site (around the bronchus) under the chemotactic effect of chemokines such as CCL3 (2.5266), CCL5 (1.8966) and CCL8 (4.5513).

In conclusion, this is the first study to evaluate the different gene expression profiles of *M. hyopneumoniae-infected* PAMs using the digital gene expression profiling technique. In this study, a total of 889 differentially expressed genes were shown to be present in PAMs at 24 hpi. These DE genes are involved in inflammatory response, cell death and survival, immune response, defense response and signal transduction regulation. This study lays the foundation for the pathogenesis of MPS, helps to clarify the molecular mechanism of *M. hyopneumoniae* effect on PAM function, and provides a new foundation to further elucidate the promotion of MPS formation by *M. hyopneumoniae* through *M. hyopneumoniae*.

Ethics approval and consent to participate

The animal tests were approved by the Biomedical Ethics Committee of Hunan Agricultural University, approval number 2022-34.

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References

Alfonseca E, Hernández R, Gutiérrez A **(2016)** *Mycobacterium bovis*-infected macrophages from resistant and susceptible cattle exhibited a differential pro-inflammatory gene expression profile depending on strain virulence. Vet Immunol Immunopathol 176: 34-43.

Artuc M, Guhl S, Babina M, Unger T, Steckelings M, Zuberbier T **(2011)** Mast cell-derived TNF-α and histamine modify IL-6 and IL-8 expression and release from cutaneous tumor cells. Exp Dermatol 20: 1020-1022.

Barbara ES, Jeffery JZ, Sylvie David JT **(2008)** Diseases of swine, 9th ed., China Agriculture Press, China.

Biebaut E, Beuckelaere L, Boyen F, Haesebrouck F, Gomez C, Devriendt B, Maes D **(2021)** Transfer of *Mycoplasma hyopneumoniae*-specific cell mediated immunity to neonatal piglets. Vet Res 52: 96.

Bin L, Luping D, Bing S, Zhengyu Y, Maojun L, Zhixin F, Yanna W, Haiyan W, Guoqing S, Kongwang H **(2014)** Transcription analysis of the porcine alveolar macrophage response to *Mycoplasma hyopneumoniae*. PLoS One 9: e101968.

Deeney S, Maglennon A, Chapat L, Crussard S, Jolivet E, Rycroft N **(2019)** *Mycoplasma hyopneumoniae* evades phagocytic uptake by porcine alveolar macrophages in vitro. Vet Res 50: 51.

Hosokawa Y, Hosokawa I, Shindo S, Ohta Y, Ozaki K, Matsuo T **(2016)** Alkannin inhibits CCL3 and CCL5 production in human periodontal ligament cells. Cell Biol Int 40: 1380-1385.

Jaiswal S, Sahoo K, Ryan D, Das K, Chakraborty E, Mohakud K, Suar M **(2016)** Altered virulence potential of Salmonella Enteritidis cultured in different foods: A cumulative effect of differential gene expression and immunomodulation. Int J Food Microbiol 230: 64-72.

Leal A, Paes A, Zaha A, Ferreira B **(2020)** Pathogenicity & virulence of *Mycoplasma hyopneumoniae*. Virulence 11: 1600- 1622.

Li B, Du L, Xu X, Sun B, Yu Z, Feng Z, Liu M, Wei Y, Wang H, Shao G, He K **(2015)** Transcription analysis on response of porcine alveolar macrophages to co-infection of the highly pathogenic porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*. Virus Res 196: 60- 69.

Li X, Zhang YK, Yin B, Liang JB, Jiang F, Wu WX **(2019)** Toll-Like Receptor 2 (TLR2) and TLR4 Mediate the IgA Immune Response Induced by *Mycoplasma hyopneumoniae*. Infect Immun 88: e00697-19.

Li S, Wu Z, Ma Y, Zhu Y, Feng Z, Zhu Z, Qiu Y, Mao S **(2023)** Differential Gene Expression Profiles and Pathways Highlight the Role of Osteoimmunology in Neurofibromatosis Type 1-Related Dystrophic Scoliosis with Osteopenia. Spine (Phila Pa 1976) 48: 1588-1598.

Liu W, Jiang P, Yang K, Song Q, Yuan F, Liu Z, Gao T, Zhou D, Guo R, Li C, Sun P, Tian Y **(2022)** *Mycoplasma hyopneumoniae* Infection Activates the NOD1 Signaling Pathway to Modulate Inflammation. Front Cell Infect Microbiol 12: 927840.

Mei X, Wang J, Zhang C, Zhu J, Liu B, Xie Q, Yuan T, Wu Y, Chen R, Xie X, Wei Y, Wang L, Shao G, Xiong Q, Xu Y, Feng Z, Zhang Z **(2023)** Apigenin suppresses mycoplasma--induced alveolar macrophages necroptosis via enhancing the methylation of TNF-α promoter by PPARγ-Uhrf1 axis. Phytomedicine 108: 154504.

Minion FC, Lefkowitz EJ, Madsen ML, Cleary BJ, Swartzell SM, Mahairas GG **(2004)** The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. J Bacteriol 186: 7123-7133.

Mo ML **(2005)** Effect of PRRSV infection on processing and presentation function of porcine pulmonary alveolar macrophage for exogenous antigen. PhD dissertation. www: cnki.net

Moue M, Tohno M, Shimazu T, Kido T, Aso H, Saito T, Kitazawa H **(2008)** Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. Biochim biophys acta 1780: 134-144.

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B **(2008)** Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5: 621-628.

Muneta Y, Minagawa Y, Shimoji Y, Ogawa Y, Hikono H, Mori Y **(2008)** Immune response of gnotobiotic piglets against *Mycoplasma hyopneumoniae*. J Vet Med Sci 70: 1065-1070.

Ning Y, Ding H **(2020)** Interaction between *Mycoplasma hyopneumoniae* and host-a review. Sheng Wu Gong Cheng Xue Bao 36: 1741-1753.

Park SJ, Seo HW, Park C, Chae C **(2014)** Interaction between single-dose *Mycoplasma hyopneumoniae* and *porcine reproductive and respiratory syndrome virus* vaccines on dually infected pigs. Res Vet Sci 96: 516-522.

Tonni M, Boniotti MB, Gasparrini S, Guarneri F, Formenti N, Pieters M, Pasquali P, Alborali GL **(2021)** Genomic variability of *Mycoplasma hyopneumoniae* within pig lung lobes. Porcine Health Manag 7: 14.

Venkatesan N, Tsuchiya K, Kolb M, Farkas L, Bourhim M, Ouzzine M, Ludwig MS **(2014)** Glycosyltransferases and glycosaminoglycans in bleomycin and transforming growth factor-β1-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 50: 583-594.

Von MC, Jensen LJ, Snel B, Hooper SD, Krupp M, Foglierini M, Jouffre N, Huynen MA, Bork P **(2005)** STRING: known and predicted protein-protein associations, integrated and transferred across organisms. Nucleic acids research 33 (Database issue): D433- D437.

Wang C, Song X, Li Y, Han F, Gao S, Wang X, Xie S, Lv C **(2013)** Low-dose paclitaxel ameliorates pulmonary fibrosis by suppressing TGF-β1/Smad3 pathway via miR-140 upregulation. PLoS One 8: e70725.

Wang GP, Liu FL, Liu J, Shi BH **(2013)** Porcine alveolar macrophages were isolated and cultured from slaughtered pig lungs. Pi g production 2: 123-124. www: cnki.net

Wang M **(2015)** Differentially expressed genes profiling in ESCC and effect of HPGD on the carcinogenesis of ESCC. Capital Medical University. PhD dissertation. www: cnki.net

Xu M, Zheng J, Hu S, Wang G **(2021)** Expression and immunogenicity study of a novel mhp183 gene fragment of *Mycoplasma hyopneumoniae*. Pol J Vet Sci 24: 553-561.

Xue ZF **(2023)** Tumor signature identification and analysis based on gene expression profile. Jiangxi University of Science and Technology. PhD dissertation. www: cnki.net

Yang X, Xing F, Wang L, Zhao W, Fu Y, Tu F, Li B, Fang X, Ren S **(2021)** Effect of Pregnane X Receptor on CYP3A29 Expression in Porcine Alveolar Macrophages during *Mycoplasma hyopneumonia*e Infection. Animals (Basel) 11: 349.

Zhang Y, Zhang T, Jia J, Jin C, Li Y **(2022)** Analysis of differential gene expression profiles uncovers mechanisms of Xuesaitong injection against cerebral ischemia-reperfusion injury. Phytomedicine 103: 154224.

Zhou H, Yan F, Hao F, Ye H, Yue M, Woeste K, Zhao P, Zhang S **(2023)** Pan-genome and expression profiles related to disease resistance and fatty acid biosynthesis in eastern black walnut (Juglans nigra). Horticulture Research 10: uhad015.

Zong B, Zhu Y, Liu M, Wang X, Chen H, Zhang Y, Tan C **(2022)** Characteristics of *Mycoplasma hyopneumoniae* Strain ES-2 Isolated from Chinese Native Black Pig Lungs. Front Vet Sci 9: 883416.