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Article

Primary infection of *Chlamydia psittaci* triggers invasion of H9N2 avian influenza virus by impairing functions of chicken macrophages

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Simple Summary: *Chlamydia psittaci*, an obligate intracellular gram-negative bacterium and economically relevant pathogen in poultry and pet birds, could cause psittacosis/ornithosis, and is also a human pathogen causing atypical pneumonia after zoonotic transmission. H9N2 influenza virus, a low pathogenic avian influenza viruses subtype, has become endemic in different types of domestic poultry in lots of countries, resulting in great economic loss due to reduced egg production or high mortality associated with co-infection with other pathogens. These two pathogens are easily mixed with other pathogens to aggravate the disease, and *C. psittaci* and H9N2 often cause mixed infection. Co-infection of *C. psittaci* with H9N2 commonly induces severe pneumonia and high mortality in SPF chickens. According to previous studies, we postulated that *C. psittaci* infection may beneficial for the replication of H9N2 in HD11. Consequently, in this study, we clarify the pathogenic mechanism of coinfection with *C. psittaci* and H9N2 in the chicken macrophage cell line HD11, which is the first study of the coinfection of *C. psittaci* and H9N2 *in vitro*.

Abstract: We investigated the effect regarding coinfection of *C. psittaci* and H9N2 on HD11 cells, expecting to find the potential pathogenesis of the coinfection induced airsacculitis. HD11 cells were infected with *C. psittaci* and/or H9N2 in different orders, and effects of the coinfection on iNOS expression and activity, NO synthesis, cell phagocytosis, and cytokines in HD11 cells were determined. Results showed that coinfection of *C. psittaci* and H9N2 significantly aggravated the mortality of HD11 cells compared to one pathogen alone. Furthermore, *C. psittaci* infection increased the replication of H9N2 in HD11 cells, whereas decreased the iNOS, enzyme activity, and NO of HD11 cells with H9N2 infection. Additionally, H9N2 also increased the pathogen loads of *C. psittaci* in HD11 cells. We also found that *C. psittaci* infection alone significantly decreased the phagocytosis of HD11 cells, compared to H9N2 alone. What's more, *C. psittaci* infection increased type Th2 cytokines IL-6 and IL-10 of HD11 cells with H9N2 infection. All the above data indicated that primary *C. psittaci* infection is able to aggravate H9N2 invasion by down-regulating functions of HD11 cells.

Keywords: *Chlamydia psittaci*; H9N2 avian influenza virus; Coinfection; HD11 cells; immune function

1. Introduction

Chlamydia psittaci, an obligate intracellular gram-negative bacterium and economically relevant pathogen in poultry and pet bird, could cause psittacosis/ornithosis, and is also a human pathogen causing atypical pneumonia after zoonotic transmission [1]. During the period from 1890 to 1930, human psittacosis outbreak in Europe, North and South America, all of which can be attributed to contacting with the sick birds [2]. Later, thanks to the improved knowledge on etiology & epidemiology and antimicrobials, large outbreaks became rare exceptions. However, what is still not to be ignored is that chlamydiosis is still widespread and represents as a major factor of economic loss in the poultry industry, as well as a permanent risk for zoonotic transmission to human [3]. Apart from overt clinical manifestations, latent *C. psittaci* infection can cause recurrent and chronicity diseases which take an adverse effect on the production performance of animals. Recently, *C. psittaci* prevalence in birds has been reported around the world [4-7].

C. psittaci has caused respiratory and systemic infections in birds and continues for a long time under unfavorable conditions, and chlamydia parrot and other pathogens can cause mixed infection in birds and poultry [8]. A great number of reports have indicated that virus and bacteria often act synergistically in causing diseases in humans or animals. In recent years, with the continuous expansion of the poultry industry, the incidence of various diseases has increased, and the mixed infection of Chlamydia and other pathogens seriously affects the diagnosis and prevention of poultry disease, resulting in great economic loss to poultry industry.

H9 subtype of *avian influenza virus* (AIV) is one of the subtypes most frequently circulation in domestic chickens [9]. H9N2 influenza virus, a low pathogenic avian influenza viruses' subtype, has become endemic in different types of domestic poultry in lots of countries, resulting in great economic loss due to reduced egg production or high mortality associated with co-infection with other pathogens [10]. In addition, more human infection with H9N2 has been reported since 2014. Some researchers showed that poultry workers had an overall H9N2 sero-prevalence of 1.87 % and a sero-incidence of 8.78/1,000 person-years, which is significantly higher than those of H7N9 and H5N1 [11]. H9N2 is important due to its widespread circulation in domestic poultry, especially in the presence of other co-infecting pathogens. For example, co-infection with E. coli and H9N2 could cause more serious synergistic pathogenic effects, indicating the role of both pathogens as complicating factors in poultry infections [12,13].

Because *C. psittaci* and H9N2 is not highly pathogenic, the extent of infection in poultry and humans is likely to remain underappreciated. However, these two pathogens are easily mixed with other pathogens to aggravate the disease. More importantly, *C. psittaci* and H9N2 often cause mixed infection in clinic. Our previous study found that *C. psittaci* infection increases the mortality of avian influenza virus H9N2 by suppressing host immune response [14]. In addition, co-infection of *C. psittaci* with H9N2, ORT and *Aspergillus fumigatus* commonly induces severe pneumonia and high mortality in SPF chickens, explaining why severe avian airsacculitis is prevalent in winter season in northern China [15].

Macrophages play critical roles in innate and adaptive immunity against chlamydial infections. Depletion of macrophages from mice prior to infection with *Chlamydia muridarum* and *C. psittaci* results in increased morbidity and pathogen burden [16,17]. Macrophage activities may not contribute to pathogen clearance because *C. psittaci* is able to survive and deliver to other tissue by using macrophage as a vehicle [18]. Macrophage activated by IFN-γ and lipopolysaccharide (LPS) or other microbial PAMPs is associated with increased mortality of macrophages, nitric oxide (NO)

production, secretions of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines [19]. Our previous studies on co-infection of *C. psittaci* and H9N2 are often based on animal models. According to previous studies, we postulated that *C. psittaci* infection may beneficial for the replication of H9N2 in HD11 cells. Consequently, in this study, we clarify the pathogenic mechanism of co-infection with *C. psittaci* and H9N2 in the chicken macrophage cell line HD11, which is the first study of the co-infection of *C. psittaci* and H9N2 *in vitro*.

2. Materials and Methods

2.1. Cells and Virus.

The chicken macrophage HD11 cells were kindly provided by Prof. Jian Qiao (College of Veterinary Medicine, China Agricultural University, Beijing, China). HD11 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, USA) at pH 7.2, and were kept at 37 °C with 5% CO_2. The Buffalo Green Monkey (BGM) cell-adapted *C. psittaci* 6BC strain used in the current study was housed in our laboratory. *C. psittaci* 6BC standard strain were kindly provided by Prof. Yimou Wu (Institute of Pathogenic Biology, University of South China, Hengyang, Hunan Province, China) and the AIV H9N2/chicken/Shandong/2011 was isolated from broilers as described previously [20]. Susceptibility of HD11 cells to *C. psittaci* and H9N2 was measured by morphological changes, growth curves using 50% tissue culture infective doses ($TCID_{50}$) and indirect immunofluorescence assay (IFA).

2.2. Virus and Bacteria Titration.

H9N2 AIV (H9N2/chicken/Shandong/2011) chicken embryo allantoic fluid was diluted 1000 times, SPF chicken embryos (9-11-day old) were inoculated with 0.2 milliliters, incubated in 37 °C incubators, after 24 hour (h) abandoned the dead chicken embryos and incubated for 48h in the thermostat. After overnight refrigeration (4 °C), the allantoic fluids were collected and pooled, and titrated, under aseptic conditions for HA activity (HA titer was $\geqslant$ 7 log2), and save at -80 °C. TCID50 were applied to HD11 cells to determine virus titers as described previously [21]. HD11 cells were cultured in 96-well plates, and ten-fold dilutions of the virus were prepared in DMEM supplemented with 2% FBS. Cultured cells were infected with the virus and then observed daily for cytopathic effects (CPE). The final virus titers as calculated by Reed-Muench method were $10^{3.86}$ $TCID_{50}$/mL.

For *C. psittaci*, BGM cells were seeded on round coverslips and cultured in growth medium consisting of minimal essential medium supplemented with 5% fetal calf serum (FCS). Ten-fold dilutions of the inoculum were centrifuged at 3400 g for 1 h at 37°C and incubated for 2 h at 37°C. The growth medium was subsequently replaced by serum-free medium. After 48 h of incubation, the cells were fixed in absolute methanol for 10 min. Chlamydial inclusions were detected by direct immunofluorescence using a monoclonal antibody conjugated to fluorescein isothiocyanate (FITC), diluted 1:5 in phosphate buffered saline (PBS), pH 7.4 (Imagen Chlamydia, Oxoid, France). The number of inclusion-forming units (IFU) per mL was assessed by counting the total number of inclusions on the whole coverslip of a countable inoculum dilution. A final titer of 4.05×10^8 IFU/mL was determined for the inoculum.

2.3. Experimental design.

HD11 cells were cultured in DMEM with 10 % FBS and seed in 6 well cell culture plate at the concentration of 1×10^6 per well and kept at 37 °C with 5 % CO_2 for 24 h. The *C. psittaci* + H9N2 group cells were infected simultaneously *C. psittaci* and H9N2. The *C. psittaci*/H9N2 group cells were infected *C. psittaci* first and then received H9N2 24 h later. The H9N2/*C. psittaci* group cells were infected H9N2 first and then received *C. psittaci* 24 h later. The *C. psittaci* and H9N2 group only infected *C. psittaci* or H9N2, respectively. The infection doses of the two pathogens were both 1 multiplicity of infection (MOI).

2.4. Quantitative real-time reverse transcription PCR.

Total RNA was isolated using TRIzol agent (TransGen Biotech, Beijing, China), and each RNA sample was reverse-transcribed to complementary DNA (cDNA) by PrimeScript™ RT Reagent Kit (Takara, Dalian, Liaoning Province, China) and the oligo dT primer was used. cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The sets of primer pairs of two pathogens and iNOS genes are listed in Table 1 and the primer pairs of cytokines refer to the primers used. For qRT-PCR reactions, the 25 μL reaction mixture included 2 μL cDNA, 12.5 μL SYBR Premix Ex TaqTM II (Takara), 1.0 μl of forward and 1.0 μL of reverse primer and 8.5 μL RNAase-free water (Takara, Japan). Reaction conditions were 95 °C for 3 minute (min) followed by 44 cycles of 95 °C for 10 s, the specific melting temperature (Tm) of a primer pair for 30 s, and then 95 °C for 10 s, then 72 °C for 10 s, using a Bio-Rad IQ5 Thermal Cycler (Bio-Rad). GAPDH was selected as a reference gene. The expression fold changes were calculated using the $2^{-\triangle\triangle Ct}$ method.

Table 1. Sequences of chicken primer pairs used for quantitative real-time polymerase chain reaction (qRT-PCR).

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
pmpD	GGATCCAATGTGTTGATTTCT	TCAAACAGCCCCACCTGTA
C. psittaci	GTCAGCTATAACGCCGTG	CCAACTCCCATGATGTGACG
H9N2 AIV	CTGGAATCTGAGGGAACTTACAAA	GAAGGCAGCAAACCCCATT
iNOS	AGGCCAAACATCCTGGAGGTC	TCATAGAGACGCTGCTGCCAG
GAPDH	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC

2.5. Determination of cell mortality.

HD11 cell line is one of the most common used cell models for investigation of the chickens' immunologic functions, and its motality can reflect the immune function lesion by pathogens. The cell mortality was detected by CellTox™ Green Cytotoxicity Assay Kit (Promega, WI, Wisconsin, USA). The detection system uses a proprietary asymmetric cyanine dye, which is blocked by living cells but can stain DNA of dead cells. When the dye binds with DNA, it will emit fluorescence. Therefore, the fluorescence signal produced by the dye bound to the dead cell DNA is directly proportional to the cytotoxicity. We operate strictly according to the protocol, adding 50 μL cells with the concentration of 1×10^5/mL to the 96 well cell culture plate. Then add 100 μL CellTox™ Green reagent, after incubation for 15 min, it was put into a fluorescence enzyme labelling instrument. After 1 min of simple oscillation, the fluorescence signal value was detected at 485-500 nm Ex/520-530 nm Em wavelength.

2.6. Determination of iNOS activity.

The activated macrophages can release some immune mediums to control infection and kill pathogens, such as nitric oxide synthase (iNOS) and nitric oxide (NO). then, the iNOS activity was detected by NOS Activity Assay Kit (BioVision, SMB, Milpitas, CA, USA). Briefly, 100-200 μL cold NOS Assay Buffer containing protease inhibitor cocktail was added to fresh cells ($2\text{-}5\times10^6$) and homogenize to disrupt the cells. The tissue or cell was centrifuged homogenate at 10,000 ×*g*, 4 °C for 10 min. Then, the clarified supernatant was transfered to a fresh pre-chilled tube & keep on ice, and then the protein concentration was measured. The NOS activity was assayed immediately using the lysates. Total 30-60 μL (200-400 μg protein) of cell/tissue homogenate or purified protein was added into desired wells in a 96-well plate. For Positive Control, 5-10 μL of diluted NOS enzyme (1:20) was added into desired well (s). The total volume of samples and positive control wells were 60 μL/well with NOS assay buffer. Enough reaction mix for the number of wells was prepared (Standards, Positive Control and sample) for analysis. Then, the mix was incubated at 37°C for 1 h. After incubation, 90 μL of NOS Assay Buffer was added into Standard, Positive Control, and sample wells and subsequently 5μL of the enhancer was added into each well. After incubating at room temperature for 10 min, 50μL of Griess Reagent 1 and 50μL of Griess Reagent 2 were added

into the Standard, Positive Control and sample wells. After incubating for 10 min, the samples were determined by using a microplate reader under 540 nm. The nitrite Standard Cure was plotted and the iNOS activity was calculated according to the formula: sample iNOS activity = nitrite amount in sample well from Standard Curve/ (reaction time × amount of protein) = pmol/min/μg = mU/mg.

2.7. Nitrite quantification.

Besides iNOS, the nitric oxide (NO) levels were also determined by colorimetric assay based on the Griess reaction (Beyotime, Haimen, Jiangsu Province, China), using sodium nitrite standards. Briefly, 100 μL of cell-free pretreated supernatant was mixed with 100 μL of Griess reagent, and after 10 minutes, absorbance was measured at 540 nm. Using a standard curve, the absorbance of the samples was converted to micromolar NO.

2.8. Detection of cell phagocytosis.

The phagocytosis of macrophages was detected by Fluorescein-labeled Escherichia coli K-12 BioParticles of Vybrant™ Phagocytosis Assay Kit (ThermoFisher, Waltham, MA, USA). Briefly, 100 μL of the prepared fluorescent BioParticle suspension was added to all the negative control, positive control and experimental wells and incubate for 2h. The BioParticle was removed and then 100 μL of the prepared trypan blue suspension was added to all of the wells immediately. The mixture solution was incubated for 1 minute at room temperature after adding the trypan blue. The experimental and control wells of the microplate were determined in the fluorescence plate reader using 480 nm excitation, 520nm emission and the appropriate sensitivity settings. Then, the net phagocytosis and the response to the phagocytosis effector agent were calculated. First, the average fluorescence intensity of a group of negative-control wells from that of a group of positive-control wells was subtracted to yield the Net Positive Reading. Second, the average fluorescence intensity of a group of negative-control wells from that of a group of identical experimental wells was subtracted to obtain the Net Experimental Reading. The phagocytosis response to the effector can then be expressed as follows: % Effect = Net Experimental Reading/Net Positive Reading ×100%.

2.9. Quantitative secretions of cytokines by ELISA kits

For cytokines determination, roughly 200 μl aliquots of each sample were used to measure the cytokines IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α with the commercial ELISA kits (Kingfisher Biotech Inc, USA).

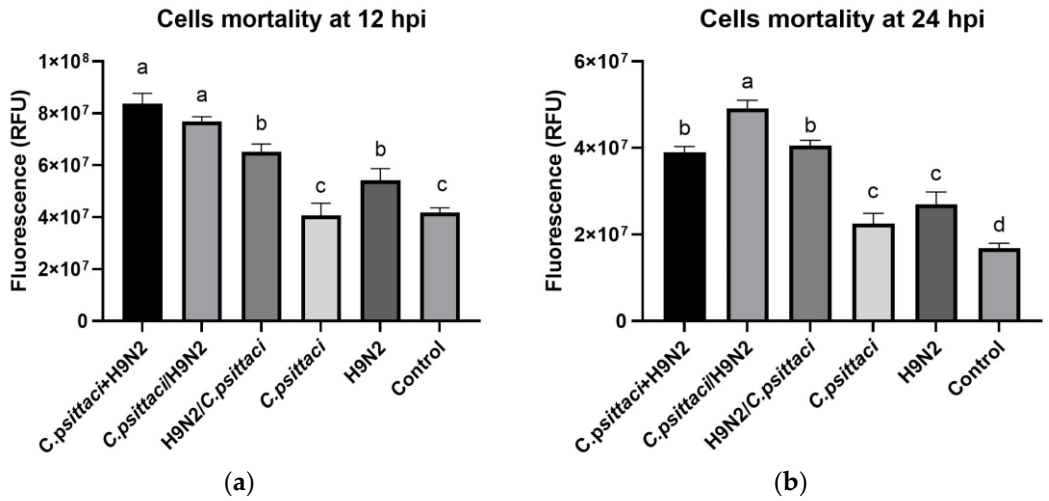
2.10. Statistical analysis.

The data are presented as averages ± standard deviations (SDs), as indicated. Statistical comparisons were made by using one-way ANOVA following by with the LSD post-hoc test between different groups with the IBM SPSS Statistics 25.0 software, property of IBM Corp. ©Copyright IBM Corporation and its licensors 1989, 2017. Statistically highly significant differences were judged as $P < 0.01$ and statistically significant differences were judged as $P < 0.05$.

3. Results

3.1. Coinfection with C. psittaci and H9N2 aggravated mortality of macrophages

To study the effect of coinfection with *C. psittaci* and H9N2 on the mortality of HD11 cells, we used fluorescent dye to detect cell membrane integrity to determine cell death rate. The data showed that *C. psittaci* primary infection and H9N2 secondary inoculation induced significantly increasing the mortality of HD11 cells as compared to H9N2 alone or *C. psittaci* alone at 12, and 24 hpi ($P<0.01$). At the same time, primary infection with H9N2 and then following infection with *C. psittaci* also induced higher the mortality of HD11 cells than *C. psittaci* alone did from 12 to 24 hpi. In addition, no statistical difference of HD11 mortality was found between *C. psittaci* alone and H9N2 alone at 24 hpi (Figure. 1).

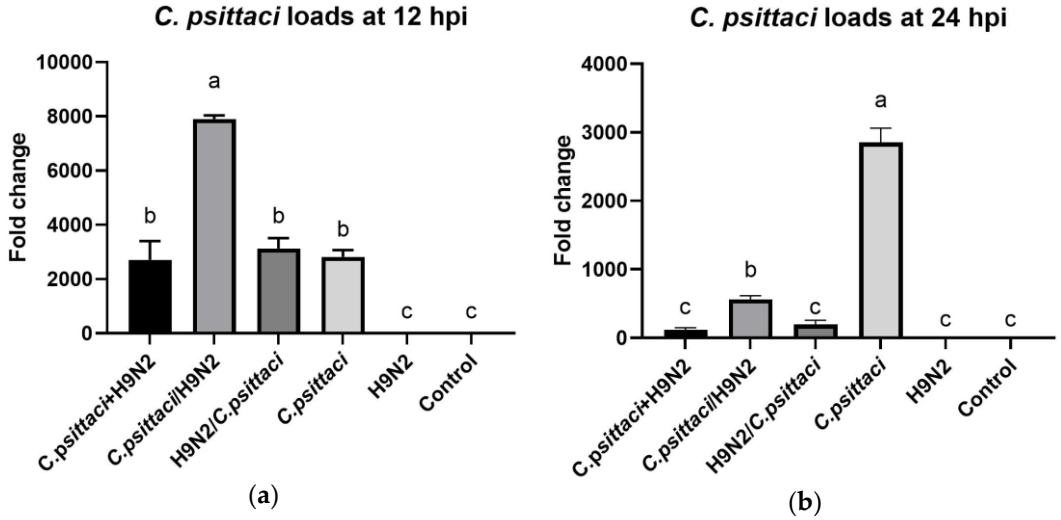


(a) (b)

Figure 1. Effect of coinfection on cell mortality. The cell mortality of HD11 cells were tested after *C. psittaci* and/or H9N2 infection. (a) The cell mortality of HD11 cells were tested at 12 hpi after *C. psittaci* and/or H9N2 infection. (b) The cell mortality of HD11 cells were tested at 24 hpi after *C. psittaci* and/or H9N2 infection. Data shown represent the mean ± SD, and the error bars represent standard deviations from four independent experiments. One-way ANOVA with the LSD post-hoc test was used for statistical analysis of the experimental and control groups, the data with different little letters in same column show significant difference.

3.2. Coinfection with C. psittaci and H9N2 promoted H9N2 loads

To study the effect of coinfection with *C. psittaci* and H9N2 on the pathogen loads of HD11 cells, we detected the replication levels of *C. psittaci* and H9N2 by RT-PCR in HD11 cells at 12, and 24 hpi. The data showed that primary inoculation with *C. psittaci* and secondary infection with H9N2 aggravated higher *C. psittaci* loads at 12 hpi ($P<0.01$) (Figure 2a), but no significant difference was found at 24 hpi (Figure 2b). As for the replication level of H9N2, the secondary infection with *C. psittaci* induced significantly increase of H9N2 loads at 12 hpi ($P<0.05$) (Figure 2c), but there's no difference at 24 hpi ($P>0.05$) (Figure 2d).



(a) (b)

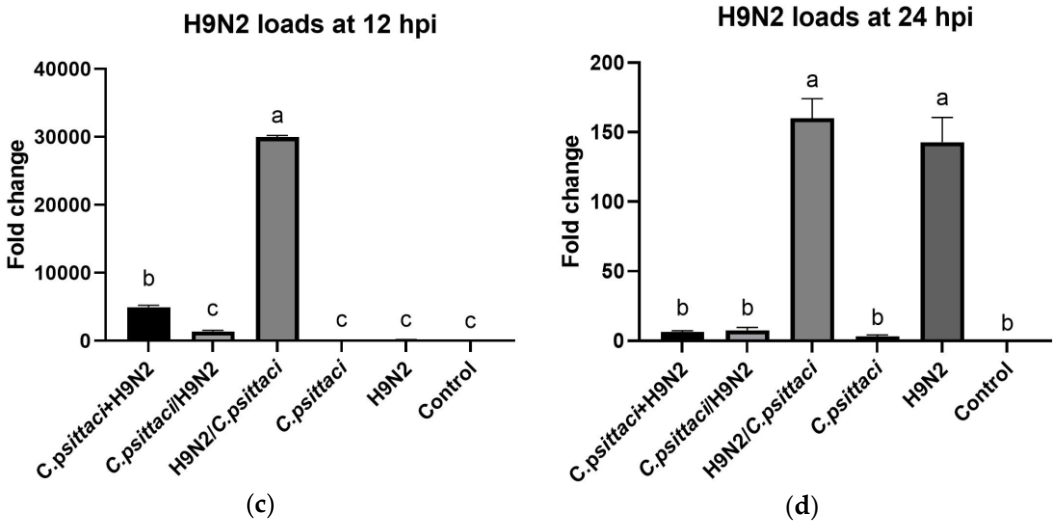
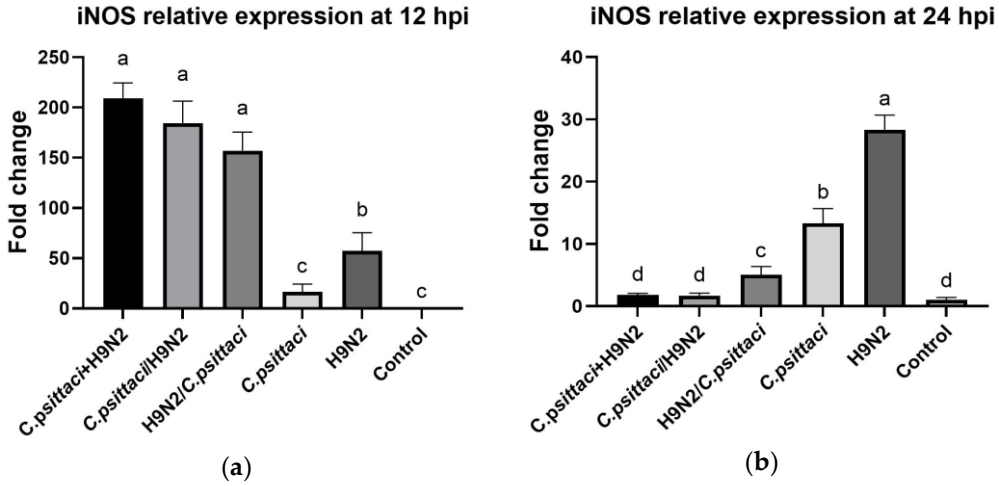


Figure 2. Effect of coinfection on pathogen loads. The pathogen loads of HD11 cells were tested at 12 and 24 hpi after *C. psittaci* and/or H9N2 infection. *C. psittaci* loads of HD11 cells were tested at 12 (**a**), and 24 (**b**) hpi after *C. psittaci* and/or H9N2 infection. H9N2 loads of HD11 cells were tested at 12 (**c**), and 24 (**d**) hpi after *C. psittaci* and/or H9N2 infection. The data shown represent the mean ± SD, and the error bars represent standard deviations from four independent experiments. One-way ANOVA with the LSD post-hoc test was used for statistical analysis of the experimental and control groups, the data with different little letters in same column show significant difference.

3.3. Coinfection with C. psittaci and H9N2 downregulated iNOS activity of HD11 cells

To study the effect of coinfection with *C. psittaci* and H9N2 on the iNOS activity of HD11 cells, we detected the relative expression level of iNOS mRNA by RT-PCR and the enzyme activity by test kits. The data showed that all coinfection groups induced higher iNOS expression levels and enzyme activity as compared to *C. psittaci* alone did at 12 hpi (Figure. 3a and 3c). However, both iNOS and its activity were reduced significantly as compared to those of H9N2 alone at 24 hpi ($P < 0.01$) (Figure. 3b and 3d). As for iNOS activity, higher activity was found between *C. psittaci*/H9N2 group and *C. psittaci* group and lower activity was found at 24 hpi. In comparison with *C. psittaci* alone, iNOS activity was significantly increased in the H9N2 group and maintained highly levels at 24 hpi ($P<0.01$) (Figure. 3c and 3d).



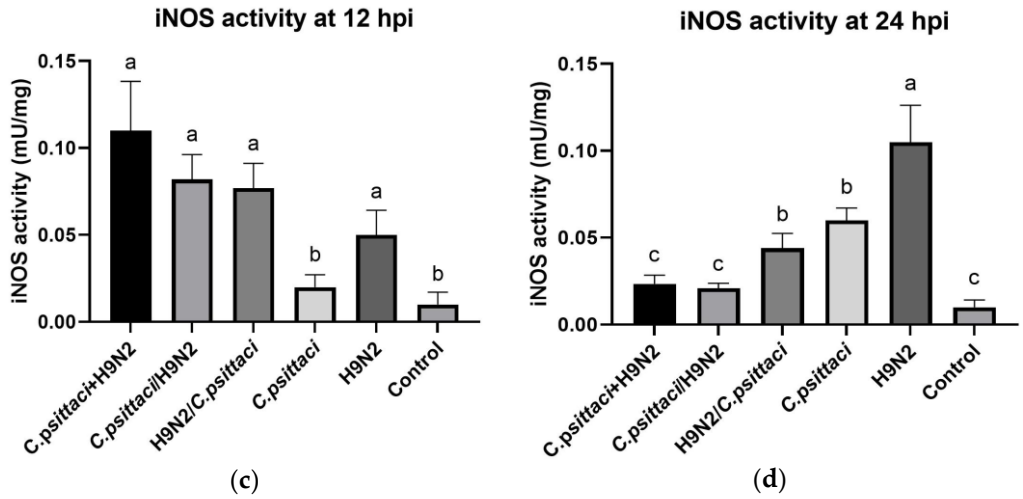


Figure 3. Effect of coinfection on iNOS expression and activity. The iNOS expression and activity of HD11 cells were tested after *C. psittaci* and/or H9N2 infection. The iNOS expression of HD11 cells were tested at 12 (A), and 24 (B) hpi after *C. psittaci* and/or H9N2 infection. The iNOS activity of HD11 cells were tested at 12 (C), and 24 (D) hpi after *C. psittaci* and/or H9N2 infection. Data shown represent the mean ± SD, and the error bars represent standard deviations from four independent experiments. One-way ANOVA with the LSD post-hoc test was used for statistical analysis of the experimental and control groups, the data with different little letters in same column show significant difference.

3.4. Coinfection with C. psittaci and H9N2 reduced NO production of HD11 cells

To study the effect of coinfection with *C. psittaci* and H9N2 on the NO concentration of HD11 cells, we detected the NO synthesized and released level of HD11 cells by total nitric oxide detection kit. NO production displayed significant elevations post combination with H9N2+*C. psittaci*, or H9N2/*C. psittaci* or *C. psittaci*/H9N2 group as compared to that of H9N2 alone or *C. psittaci* alone at 12 hpi ($P<0.01$) (Figure 4a). Later on, NO productions were reduced significantly in three coinfection groups as compared to H9N2 group at 24 hpi ($P<0.01$) (Figure 4b). As for statistical difference, there was no significance among three coinfection groups and the *C. psittaci* group at 24 hpi.

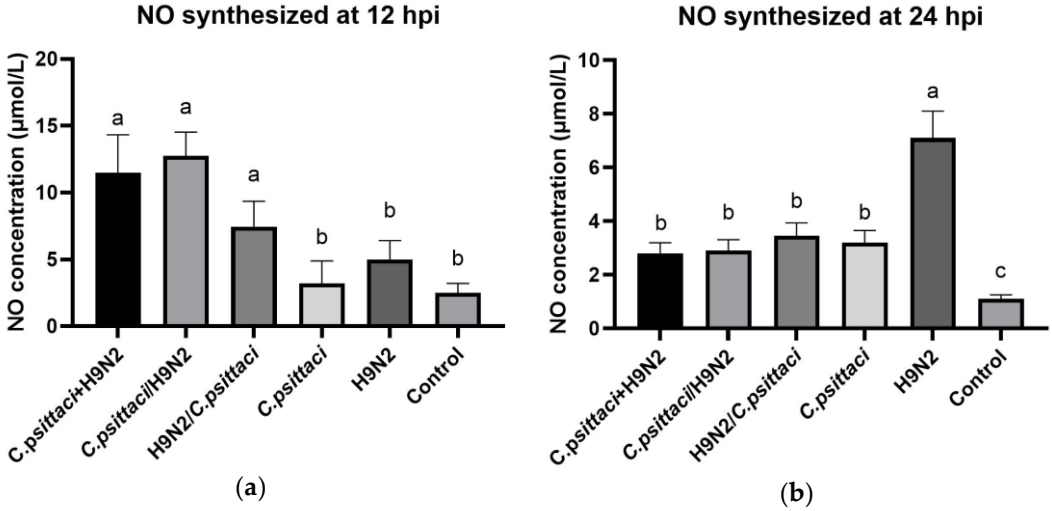


Figure 4. Effect of coinfection on NO Synthesis. The NO synthesis of HD11 cells were tested after C. psittaci and/or H9N2 infection. (a) The NO synthesis of HD11 cells were tested at 12 hpi after C. psittaci and/or H9N2 infection. (b) The NO synthesis of HD11 cells were tested at 24 hpi after C. psittaci and/or H9N2 infection. Data shown represent the mean ± SD, and the error bars represent standard deviations from four independent experiments. One-way ANOVA with the LSD post-hoc

test was used for statistical analysis of the experimental and control groups, the data with different little letters in same column show significant difference.

3.5. Coinfection with C. psittaci and H9N2 downregulated phagocytosis of HD11 cells

The phagocytosis of HD11 cells was determined by fluorescent microspheres kit. At 12 hpi, phagocytic activity was reduced significantly in *C. psittaci* alone compare to H9N2 alone ($P<0.05$) (Figure 5a). No significant difference was found among the H9N2 + *C. psittaci* group and the H9N2/*C. psittaci* group as compared to that of H9N2 alone. Post treatment at 24 hpi, three coinfection groups and the *C. psittaci* group induced lower phagocytosis than the H9N2 group did ($P<0.05$) (Figure 5b). However, no significant difference was found among the H9N2 + *C. psittaci* group, the H9N2/*C. psittaci* group, the *C. psittaci*/H9N2 group, and the *C. psittaci* group.

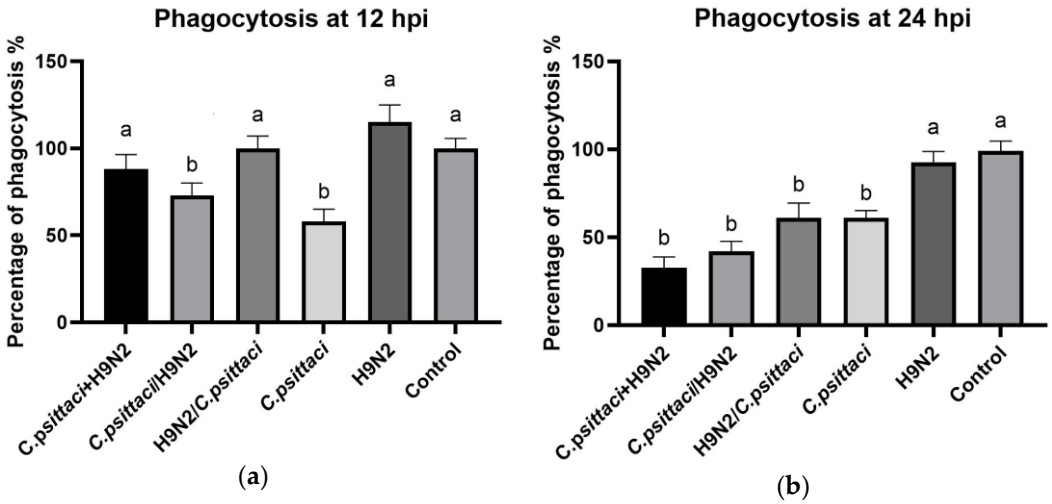


Figure 5. Effect of coinfection on cell phagocytosis. The cell phagocytosis of HD11 cells were tested after *C. psittaci* and/or H9N2 infection. (A) The cell phagocytosis of HD11 cells were tested at 12 hpi after *C. psittaci* and/or H9N2 infection. (B) The cell phagocytosis of HD11 cells were tested at 24 hpi after *C. psittaci* and/or H9N2 infection. Data shown represent the mean ± SD, and the error bars represent standard deviations from four independent experiments. One-way ANOVA with the LSD post-hoc test was used for statistical analysis of the experimental and control groups, the data with different little letters in same column show significant difference.

3.6. Coinfection with C. psittaci and H9N2 polarized Th2 cytokines of HD cells

The levles of cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α of HD11 cells were detected ELISA. At 12 hpi, IL-4 and IL-10 were increased gradually while IL-6 were upregulated dramatically in three coinfection groups compared to those of H9N2 alone or *C. psittaci* alone ($P<0.01$) (Figure 6a, 6c, 6d & 6e). Later on, IL-4 expressions were increased greatly in three coinfection groups, the *C. psittaci* group and H9N2 group, while IL-10 expressions were elevated significantly in three coinfection groups as compared to those of the H9N2 group and the *C. psittaci* group ($P<0.05$) (Figure 6c & 6e).

Regarding type Th1 cytokines, both IL-1β, IL-2, and TNF-α expressions were increased significantly in the H9N2 + *C. psittaci* group and the H9N2/*C. psittaci* group as compared to those of the H9N2/*C. psittaci* group and the *C. psittaci*/H9N2 group ($P<0.01$) while significant increase was found in the H9N2/*C. psittaci* group as compared to other two coinfection groups at 12 hpi ($P<0.01$) (Figure 6a, 6b, 6f & 6g). After treatment at 24 hpi, significant increase of TNF-α and IFN-γ expressions were found in the H9N2+*C. psittaci* group and the H9N2/*C. psittaci* group as compared to other coinfection groups ($P<0.05$).

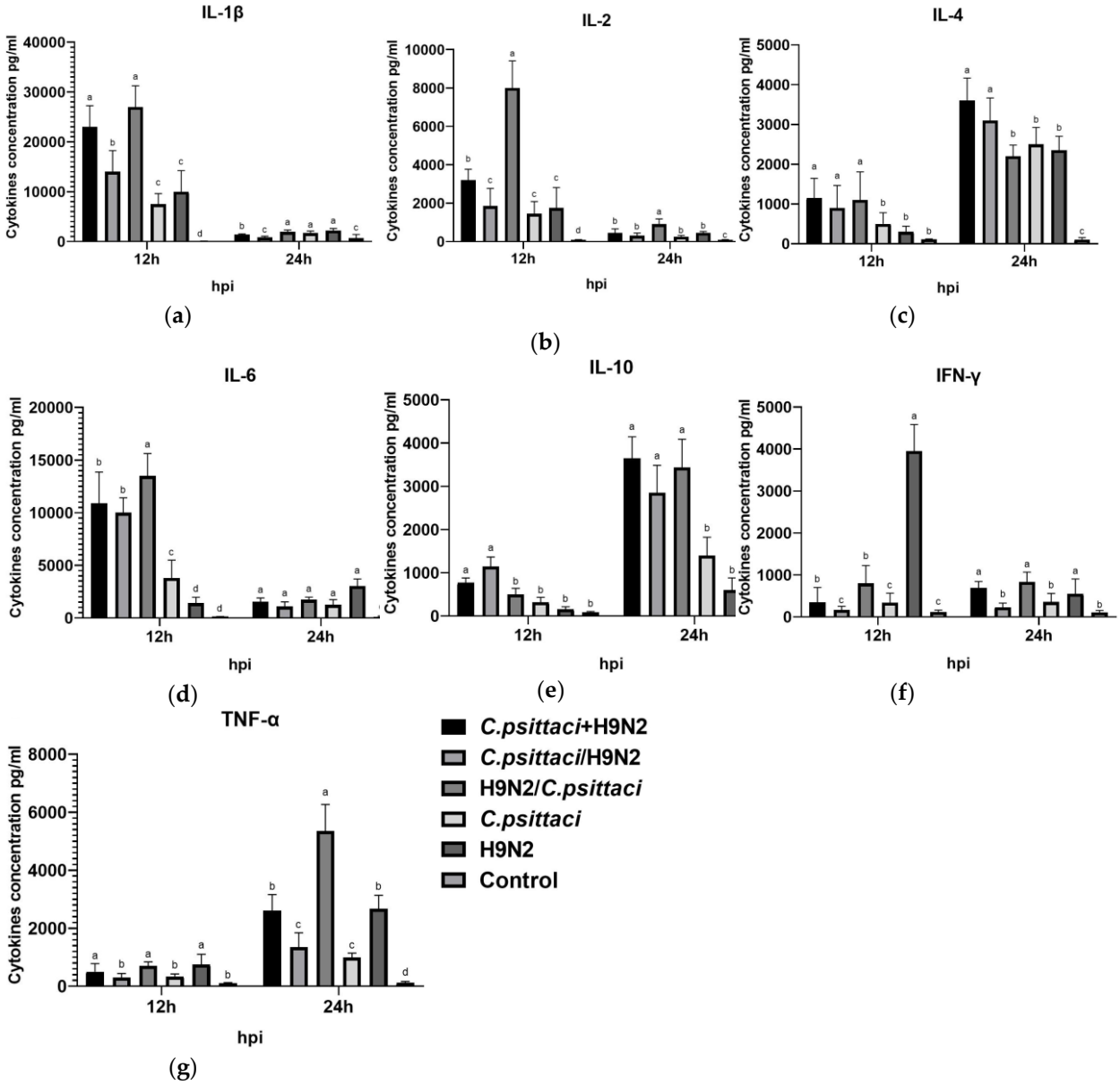


Figure 6. Effect of coinfection on cell cytokines. The levels of pro-/anti-inflammatory cytokines of HD11 cells were tested at 12, and 24 HPI after *C. psittaci* and H9N2 infection. The levels of (A) IL-1β, (B) IL-2, (C) IL-4, (D) IL-6, (E) IL-10, (F) IFN-γ, and (G) TNF-α of HD11 cells were tested after *C. psittaci* and H9N2 infection. The data shown represent the mean ± SD, and the error bars represent standard deviations from four independent experiments. One-way ANOVA with the LSD post-hoc test was used for statistical analysis of the experimental and control groups, the data with different little letters in same column show significant difference.

4. Discussion

In the present study, we investigated the influence of chicken macrophage immune function in response to co-infection with *C. psittaci* and/or H9N2 avian influenza virus. Our major finding was that co-infection with *C. psittaci* and H9N2 can significantly aggravate the mortality of HD11 cells compared to pathogen infection alone. In addition, infection with *C. psittaci* can increase the replication of H9N2 in HD11 cells, but infection with H9N2 did not increase the pathogen loads of *C. psittaci*. Moreover, infection with *C. psittaci* can significantly decrease iNOS expression level and enzyme activity as well as NO concentration of HD11 cells by H9N2 infection. In other words, H9N2 stimulated HD11 cells to increase more iNOS and NO compared with *C. psittaci*. In addition, infection with *C. psittaci* or H9N2 did not decrease the phagocytosis of HD11 cells by another

pathogen infection. But, we found that *C. psittaci* infection alone can significantly decrease the phagocytosis of HD11 cells compared to H9N2 infection alone. Furthermore, infection with *C. psittaci* can increase the mRNA expression of type Th2 cytokines IL-6 and IL-10 in HD11 cells by H9N2 infection. All the above data support our hypothesis that a primary *C. psittaci* infection can aggravate infection by H9N2 by influence the function of HD11 cells.

In our previous study, we established a SPF chicken animal model with co-infection of *C. psittaci* and H9N2, and found that *C. psittaci* infection increased the mortality of H9N2 by inhibiting humoral immunity and cellular immunity as well as altering Th1/Th2 balance ultimately weakens the immune system of the body [14]. In conclusion, we first reported that a primary *C. psittaci* infection may lead to immune suppression in vivo which will increase susceptibility to H9N2. It suggested that we should consider a primary infection by *C. psittaci* in any respiratory disease and should eradicate it during treatment of avian respiratory disease. In addition, other studies have also reported the role of *C. psittaci* and H9N2 in the pathogenesis of co-infection. Co-infection is a common infection of two or more than two pathogens to the same host. There is a common infection in human and animal infections. For example, in 1918, the human influenza virus pandemic, almost all cases of death were caused by bacteria mixed infection, and the study found that the addition of bacteria could greatly increase the risk of death [24]. Common infections of malaria and helminth are common in less developed countries [25]. In pig breeding industry, the mixed infection of porcine reproductive and respiratory syndrome virus and porcine circovirus type 2 virus is very serious in large-scale pig farms [26]. Mixed infection is more common in poultry industry, poultry disease survey results show that most of the deaths caused by low pathogenic avian influenza virus are attributed to infection of bacteria [27]. Since 2007, the outbreak of avian airbag inflammation is the result of mixed infection of various pathogens [28].Macrophage is the most important cell type in natural immune response, and plays a crucial role in the immunologic functions of host. Our previous studies revealed that coinfection of *C. psittaci* and H9N2 are often based on animal models that are studied in vivo, and according to previous studies, we postulate that *C. psittaci* infection may beneficial for the replication of H9N2 in macrophages of chicken. HD11 cell line is one of the most common used cell models for investigation of the chickens' immunologic functions, and its motality can reflect the immune function lesion by pathogens. Consequently, in our study, we further found that infection with *C. psittaci* and H9N2 can increase the mortality of HD11 cells caused by another pathogen. In addition, the activated macrophages can release some immune mediums to control infection and kill pathogens, such as nitric oxide synthase (iNOS), nitric oxide (NO), and reactive oxygen species (ROS). Our present results showed that infection with *C. psittaci* will increase the replication of H9N2, and decrease the iNOS, NO level, as well as increase the IL-6 and IL-10 expression of HD11 cells by H9N2 infection. Meanwhile, *C. psittaci* infection alone can decrease the phagocytosis of HD11 cells.

Macrophages play an important role in innate immunity, such as phagocytosis, antiviral infection and enhanced immune regulation. Pathogens infect the body and monocytes after infection of the main target cells. After entering macrophages, Chlamydia can escape immune surveillance and transport to the whole body with macrophages, when macrophages act as the delivery system for Chlamydia. Therefore, it is necessary to study how the macrophages play a role in the host's immune response to the pathogen and the pathogenesis of the pathogen after the study of Chlamydia and H9N2 infection. We studied the changes of iNOS gene and its activity, which involved in the reaction of L-arginine decomposition into NO and L- citrulline. Endotoxin or cytokines, such as LPS and IFN-γ, can induce chicken macrophages to produce iNOS and further produce nitric oxide [25]. Our research shows that infection with *C. psittaci* can significantly decrease iNOS expression level and enzyme activity as well as NO concentration of HD11 cells by H9N2 infection. It's not surprising that *C. psittaci* infection alone can significantly decrease the iNOS and NO level of HD11 cells compared to H9N2 infection alone. This furthermore shows the negative effect of *C. psittaci* on the iNOS-NO pathway. Monocyte macrophage system has the function of phagocytosis and killing of pathogens and tumor cells directly. It also plays an important role in antigen processing and immune-regulation. It is important to detect the

phagocytic function of macrophage to judge the function of macrophage and to understand the specific and non-characteristic immune state of the body. In our study, we found that infection with *C. psittaci* or H9N2 did not decrease the phagocytosis of HD11 cells by another pathogen infection. But, *C. psittaci* infection alone can significantly decrease the phagocytosis of HD11 cells compared to H9N2 infection alone and furthermore show the negative effect of *C. psittaci* on the phagocytosis of HD11 cells. Cytokines secreted by macrophages are also important components of their immune regulation, such as TNF-α, IL-1, IL-2, IL-4, IL-5, IL-6, and IL-10, *etc* [29-32]. Here, we have detected several pro- and anti- inflammatory cytokines in our present investigations. IL-6 can promote inflammation as a pro-inflammatory factor, but also has anti-inflammatory effects. It is reported that IL-6 can change the differentiation of monocytes from dendritic cells to macrophages and inhibit the differentiation of Th1 by up regulation of SOCS1, thus regulating the balance of Th1/Th2 [30]. IL-10, a kind of Th2 - type cytokine, is an anti-inflammatory cytokine and inhibits Th1 differentiation, which is synergistic with IL-4 and IL-5 produced by Th2 cells. In our result, the levles of IL-4 and IL-10 were increased significantly in the coinfection groups while IFN-γ maintained same levels, suggesting that Chlamydial infection mediated the polarization of Th1/Th2 to the Th2 direction. The dominant role of Th2 cytokines will lead to hamper immune function, so that pathogens can evade immune surveillance and immune attacks. The Th2 polarization was consisted with our previous data in vivo, implying that immune suppression was aggravated post artificial infection with H9N2 and *C. psittaci* [15].

5. Conclusions

In conclusion, co-infection often increases disease severity in both humans and animals. Understanding the mechanisms and effects of co-infection will improve the understanding of the common pathogenicity mechanism. Since 2007, severe respiratory infections and avian air-sacculitis in chickens in many parts of China have been the result of co-infection with bacteria and viruses. The role of viral-bacterial co-infection in animal-to-human transmission of infectious agents has not received sufficient attention and should be emphasized in future investigations. We have established an SPF chicken model of coinfection of *C. psittaci* and H9N2 in vivo. At this point, it is eager to continue to study cell models of coinfection of *C. psittaci* and H9N2 in vitro. In this study, we did the work and found that infection with *C. psittaci* will increase the replication of H9N2, decrease the iNOS-NO pathway, and raise the IL-6 and IL-10 expression of HD11 cells by H9N2 infection. At the same time, *C. psittaci* infection can also reduce the phagocytosis of macrophage. Similar to the results of previous animal models, we found C. psittaci can reduce the function of immune cells in many ways, thus aggravating the susceptibility of H9N2. In addition, the further findings of this study are not only *C. psittaci* aggravates H9N2 infection, but H9N2 also can increase the mortality caused by *C. psittaci* infection. Whether this is beneficial to the infection and spread of *C. psittaci* remain to be confirmed. These findings suggest that we should consider the primary and latent infection of *C. psittaci* in respiratory disease and should eradicate *C. psittaci* during treatment.

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