Journal of Natural Science Review

Vol. 2, Special Issue, 2024
https://kujnsr.com
e-ISSN: 3006-7804

# In-ovo Delivery of Lipopolysaccharide Induces Protective Antiviral Responses Against Infectious Laryngotracheitis Virus Encountered Post-Hatch

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## ABSTRACT

Infectious laryngotracheitis virus (ILTV) is an avian respiratory virus that causes significant economic losses to the worldwide poultry industry. To address the limitations of current control measures against ILTV infections, it is crucial to develop novel measures that can complement or replace the existing control measures. The in-depth understanding of toll-like receptor (TLR)ligand interaction and activation mechanisms has facilitated the development of more specific synthetic ligands with therapeutic potentials capable of enhancing host immune responses against various infections. As a key ligand for activating the TLR4 signaling pathway, lipopolysaccharide (LPS) triggers immune responses against numerous pathogens. Previous research has demonstrated that the intramuscular injection of LPS in chickens can induce antiviral responses against low pathogenic avian influenza virus (AIV) infections. This study evaluated the potential of *in-ovo* delivery of LPS to stimulate innate antiviral responses against ILTV infection in post-hatch chickens. The results showed that *in-ovo* delivery of LPS at embryo day (ED)-18 significantly reduced mortality, clinical symptoms, and virus excretion in chickens infected with ILTV at day 1 post-hatch, which was associated with the expansion of the macrophage population in the lungs. This study provides a detailed understanding of the mechanisms underlying the antiviral responses elicited against ILTV infection following *in-ovo* delivery of LPS in chickens.

## ARTICLE INFO

**Article history:**
Received: Dec 23, 2023
Revised: July 19, 2024
Accepted: Nov 10, 2024

**Keywords:**
Antiviral; In-ovo; Lipopolysaccharide; Toll-like receptor; TLR4; Infectious laryngotracheitis virus

**To cite this article:** Sarjoon, M., Abdul-Cader, M., Contreras, A. P., & Abdul-Careem, M. F. (2024). In-ovo Delivery of Lipopolysaccharide Induces Protective Antiviral Responses Against Infectious Laryngotracheitis Virus Encountered Post-Hatch. *Journal of Natural Science Review*, *2*(Special Issue), 565–580. https://doi.org/10.62810/jnsr.v2iSpecial.Issue.159

**Link to this article:** https://kujnsr.com/JNSR/article/view/159



## Introduction

Infectious laryngotracheitis virus (ILTV) is one of the avian respiratory viruses causing significant economic losses to the poultry industry in Canada and worldwide. Control measures for ILTV include biosecurity measures and vaccination. Although various vaccines are available, they may not protect chickens fully. Due to the limitations inherent in existing

control strategies for ILTV infections, it is imperative to develop innovative measures to complement or potentially replace the current control methods.

The innate immune responses are the first line of host-defense mechanisms and play a crucial role against invading pathogens. Innate immunity elicits broader, non-specific early responses and contains various immune cells, including macrophages. Macrophages are among the first cells to encounter invading pathogens and trigger immune reactions through phagocytosis, antigen presentation, and the production of immune mediators (Abdul-Cader, Ahmed-Hassan, et al., 2017; Amarasinghe et al., 2017; Haddadi et al., 2013). They can recognize and engulf pathogens through toll-like receptors (TLR) (Akira, 2003; Medzhitov & Janeway, 1997; Meylan, Tschopp, & Karin, 2006).

TLRs are the well-studied receptor family (Akira, 2003; Medzhitov & Janeway, 1997; Meylan et al., 2006) that can identify the existence of particular molecular structures, known as pathogen-associated molecular patterns (PAMP)s, from a wide range of invading pathogens (Arpaia & Barton, 2011; Galiana-Arnoux & Imler, 2006; Haynes et al., 2001; Lester & Li, 2014). Several TLRs, including TLR3, TLR7, TLR8, and TLR21, have been identified as playing a crucial role in the recognition of viral genetic materials within endosomal compartments and activating distinct signaling pathways that result in the secretion of antiviral interferon (IFN)s and cytokines (Mogensen & Paludan, 2001). While TLR2 and TLR4 are primarily known for recognizing bacterial surface PAMPs, there is evidence to suggest that they are also linked to antiviral responses through the recognition of viral structural and nonstructural proteins (Bieback et al., 2002; Ge et al., 2013; Kurt-Jones et al., 2000; Mogensen & Paludan, 2005).

A comprehensive understanding of the TLR-ligand interaction and activation mechanisms has paved the way for the development of more specific synthetic ligands with therapeutic potential (Jain et al., 2002; Krieg, 2008; Liang et al., 2011; Vollmer & Krieg, 2009). These synthetic ligands can be utilized to enhance host immune responses against various infections (Ho, Fontoura, Ruiz, Steinman, & Garren, 2003; Hung et al., 2011; Kandimalla et al., 2003; Kitagaki, Jain, Businga, Hussain, & Kline, 2002; Vollmer & Krieg, 2009). Different types of synthetic TLR ligands can be activated in chickens to induce a selective antiviral response. For example, the TLR7 ligand, ssRNA, or its synthetic analogs, such as resiquimod, has been shown to induce antiviral activity against very virulent infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV) infections (Annamalai et al., 2016; De Silva Senapathi et al., 2020). Similarly, *in-ovo* treatment with the TLR21 ligand, cytosine guanine (CpG) DNA, significantly increases the recruitment of macrophages in the chicken lungs, leading to the inhibition of AIV replication in a nitric oxide (NO)-dependent manner (Abdul-Cader, Ahmed-Hassan, et al., 2017).

The cell wall of Gram-negative bacteria contains lipopolysaccharide (LPS), which interacts with TLR4 in chickens (Beutler, 2000). The LPS-TLR4 interaction has been found to initiate protective antiviral responses against several viruses, such as herpes simplex virus (HSV)-2 and influenza virus (Ashkar, Mossman, Coombes, Gyles, & Mackenzie, 2008; Shinya

et al., 2012). In avian species, TLR4-mediated antiviral responses have been observed both *in-vivo* against Newcastle disease virus (NDV) and avian influenza virus (AIV) (Barjesteh, Brisbin, Behboudi, Nagy, & Sharif, 2015; Kannaki, Priyanka, & Reddy, 2019) and *in-vitro* against ILTV and Marek's disease virus (MDV) (Ahmed-Hassan, Abdul-Cader, Sabry, Hamza, & Abdul-Careem, 2018; Bavananthasivam, Kulkarni, Read, & Sharif, 2018; Haddadi et al., 2013). In addition, LPS-TLR4 mediated signaling has been shown to produce NO, interleukin (IL)-1β, and type 1 IFNs (Ahmed-Hassan, Abdul-Cader, Sabry, et al., 2018; Bavananthasivam, Alkie, Matsuyama-Kato, Hodgins, & Sharif, 2017; He, Genovese, Nisbet, & Kogut, 2006). Studies have shown that intramuscular injection of LPS in chickens induces antiviral responses against low pathogenic AIV infection (St Paul et al., 2012). Additionally, it was observed that the stimulation of macrophages with LPS led to an enhanced production of NO, which resulted in antiviral responses against ILTV infection *in vitro* (Ahmed-Hassan et al., 2018; Haddadi et al., 2013). However, the ability of *in-ovo*-delivered LPS to elicit antiviral responses against ILTV infection in chickens post-hatch remains unknown. The objective of this study was 1) to determine whether *in-ovo* delivery of LPS can stimulate innate antiviral activity against ILTV infection in chickens post-hatch and 2) to investigate the capability of *in-ovo* treated LPS to elicit macrophage responses in the lungs post-hatch.

## Materials and Methods

### *Animals*

The chickens, embryos, and specific pathogen-free (SPF) eggs used in the experiment were approved by the Veterinary Science Animal Care Committee and Health Science Animal Care Committee. The SPF eggs were sourced from the Canadian Food Inspection Agency in Ottawa, Canada. Subsequently, they were placed in digital incubators (Rcom Pro 20 and 50, Kingsuromax 20, and Rcom MARU Deluxe max, manufactured by Autoelex Co., Ltd., GimHae, GyeongNam, Korea) and incubated until hatching at the Health Research Innovation Center, University of Calgary. The temperature was set at 37.2°C, and relative humidity was at 60% for the first 18 days, then raised to 37.6°C and 70% relative humidity for the last 3 days of incubation (Haddadi et al., 2015). The eggs were examined through candling at embryo day (ED)-11 during incubation to determine their fertility. Only fertile eggs were selected for the experiments and treated *in-ovo* at ED-18, as described below. The collection of chicken tissue samples adhered to the protocols approved by the institutional animal care committees. Before taking samples, the chickens were humanely euthanized using cervical dislocation after being given an overdose of isoflurane anesthesia.

### *Virus and TLR ligand*

The ILTV strain (N-71851) used in the experiments was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) (Thapa, Abdul-Cader, et al., 2015; Thapa, Nagy, & Abdul-Careem, 2015). The virus was initially propagated in fertile SPF chicken eggs between ED-9 to ED-11 and then titrated by performing a plaque assay on leghorn chicken hepatocellular carcinoma (LMH) cells (ATCC, Manassas, Virginia, USA) (Haddadi et al., 2013)

to determine the number of live viral particles in the harvest. The LPS (a TLR4 ligand) used in the experiments was sourced from *Escherichia coli* (O127:B8) and was purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

### *Experimental design*

**Determination of whether *in-ovo* treatment of LPS induces antiviral responses against ILTV when chickens are exposed to the virus post-hatch.** During incubation at ED-18, fertilized chicken eggs were divided into two groups randomly. One group was treated *in-ovo* with 40µg of LPS diluted in 200µl of sterile phosphate-buffered saline (PBS) (n=6), while the other group was treated with 200µl of sterile PBS as a control (n=6). The eggs continued to incubate until hatched, and both groups were infected with ILTV (3x104 plaque-forming units (PFU)/chicken) intratracheally on day 1 post-hatch. To facilitate the intratracheal ILTV infection, chickens were anesthetized with isoflurane, and the tongue was gently pulled outward and downward to expose the glottis and the laryngeal opening. The challenged chickens were observed for 12 days post-infection (dpi), and clinical scores were documented utilizing a scale previously described (0 indicating absence of clinical signs, 1 indicating droopy wings/ruffled feathers, huddling together/depression or inactivity with a lowered head, loss of body weight, or a mild increase in respiration, 2 indicating a moderate increase in respiration, and 3 indicating severe increase in respiration or gasping) (Abdul-Cader, Amarasinghe, et al., 2018; Thapa, Abdul-Cader, et al., 2015). The chickens were euthanized if a bird reached a cumulative score of 5 (human endpoint) or the experimental endpoint (Abdul-Cader, Amarasinghe et al., 2018). Oropharyngeal and cloacal swabs were collected at 5 dpi, and the ILTV genome loads were quantified using real-time-polymerase chain reaction (RT-PCR) as described elsewhere in the text.

**Determination of whether *in-ovo* treatment of LPS induces expansion of macrophage population in the lungs.** During incubation at ED-18, fertilized chicken eggs were divided into two groups randomly. One group was treated *in-ovo* with 40µg of LPS diluted in 200µl of sterile PBS (n=6), while the other group was treated with 200µl of sterile PBS as a control (n=6). The eggs continued to incubate until hatching. On the first day post-hatch, the chickens were euthanized, and one lung was collected, preserved in optimum cutting temperature (OCT) compound (Tissue-Tek®, Sakura Finetek USA inc, Torrance CA, USA) at -80°C, and sectioned for immunofluorescent staining as described in the section entitled "Immunofluorescent Assay."

### *Experimental Techniques*

**In-ovo treatment technique.** The *in-ovo* delivery method refers to administering a substance or treatment directly into the egg through the shell before hatching. This technique is widely used in the poultry industry to provide nutrition, vaccines, and other treatments to developing chickens during incubation. The delivery of a TLR ligand *in-ovo* has been shown to induce early immune responses compared to post-hatch treatment (Abdul-Cader et al., 2017; Abdul-Cader et al., 2017; Wakenell et al., 2002). In this study, we performed the *in-ovo*

delivery of treatment substances at ED-18, as described previously (Abdul-Cader, Ahmed-Hassan, et al., 2017; Guo, Giambrone, Wu, & Dormitorio, 2003; Wakenell et al., 2002). Suitable fertile eggs, free from cracks or damage, were selected, and their outer surface was disinfected with 70% ethanol to reduce the risk of contamination. Using a 21-gauge needle, a small hole was made at the broad end of the eggshell, and the entire length of a sterile 23-gauge needle (1 inch in length) was inserted perpendicular to the hole to deliver the treatment substance directly into the amniotic cavity. Following the procedure, the punctured holes were closed with lacquer, and the treated eggs were returned to the incubator for incubation until hatching. The eggs were monitored for hatchability, and any eggs that failed to hatch or any day-old chickens with abnormal development were excluded from the experiments.

**Immunofluorescent assay.** Using an indirect immunofluorescent assay, the study analyzed lung tissues collected from day 1 chickens for macrophage numbers. The tissues preserved in OCT were sectioned with a thickness of 5 µm and prepared for staining as previously described (Abdul-Cader, Amarasinghe et al., 2018; Abdul-Cader, De Silva Senapathi, Ahmed-Hassan, Sharif, & Abdul-Careem, 2019; De Silva Senapathi et al., 2018). Briefly, the sectioned slides were incubated with a blocking buffer containing 5% goat serum in tris-buffered saline (TBS) buffer (Trizma base: 20 mM, NaCl: 138 mM, and distilled water, pH 7.6) for 30 mins. An unlabeled mouse monoclonal antibody specific for chicken macrophages, KUL01 (Southern Biotech, Birmingham, AL, USA), was used at a dilution of 1:200 in blocking buffer and incubated for 30 mins. A secondary antibody, DyLight® 550 conjugated goat anti-mouse IgG (H+L) (Bethyl Laboratories Inc., Montgomery TX, USA), was used at a dilution of 1:500 in blocking buffer and incubated for 1 hr. The slides were washed twice with TBS-T buffer (TBS with 0.1% Tween 20) and once with PBS following each step. All incubations were taken place at ambient temperature within a humidified chamber. Subsequently, the slides were affixed with coverslips using a mounting solution containing 4′, 6-Diamidine-2′-phenylindole dihydrochloride (DAPI, Vectashield, Vector Laboratories Inc., Burlingame, CA, USA) and the coverslips were secured with lacquer.

**Data analyses.** The number of macrophages in each lung sample was quantified by capturing 5 areas with the highest DyLight® 550 fluorescent signals and corresponding DAPI-stained nuclei areas under 40X magnification. The fluorescent signals were analyzed using the Image-J software (National Institute of Health, Bethesda, Maryland, USA). They expressed a percentage of positive signals for DyLight® 550 relative to the total areas, as estimated by nuclear staining with DAPI (Abdul-Cader, Ahmed-Hassan et al., 2017; Abdul-Cader, Amarasinghe et al., 2017; Abdul-Cader et al., 2019).

The student's *t*-test (GraphPad Prism Software 5, La Jolla, CA, USA) was used to identify differences between the two groups. In addition, the Log-rank test was used to identify differences in survival percentage between groups. Before analysis, each dataset underwent an outlier test using the Grubbs' test (GraphPad Software Inc., La Jolla, CA, USA). The $P \leq 0.05$ was considered statistically significant.

## Results

### *In-ovo-delivered LPS induces protective host responses against ILTV encountered post-hatch and is associated with decreased ILTV shedding.*

In light of our findings that *in-ovo* treatment with CpG DNA leads to protective host responses against ILTV post-hatch (Abdul-Cader, Amarasinghe, et al., 2017), we investigated whether *in-ovo* delivery of LPS could elicit similar protection. When we delivered either LPS or PBS (control) *in-ovo* at ED-18 to two separate groups of embryonated eggs, followed by intratracheal injection of ILTV at day 1 post-hatch, we found that *in-ovo*-delivered LPS significantly reduced: 1) mortality (P<0.05, Figure 1a), 2) clinical scores at 6-8 dpi (P<0.05, Figure 1b) and 3) cloacal and oropharyngeal excretion of ILTV genome at 5 dpi (P<0.05, Figure 1c and 1d) compared to the PBS control group.

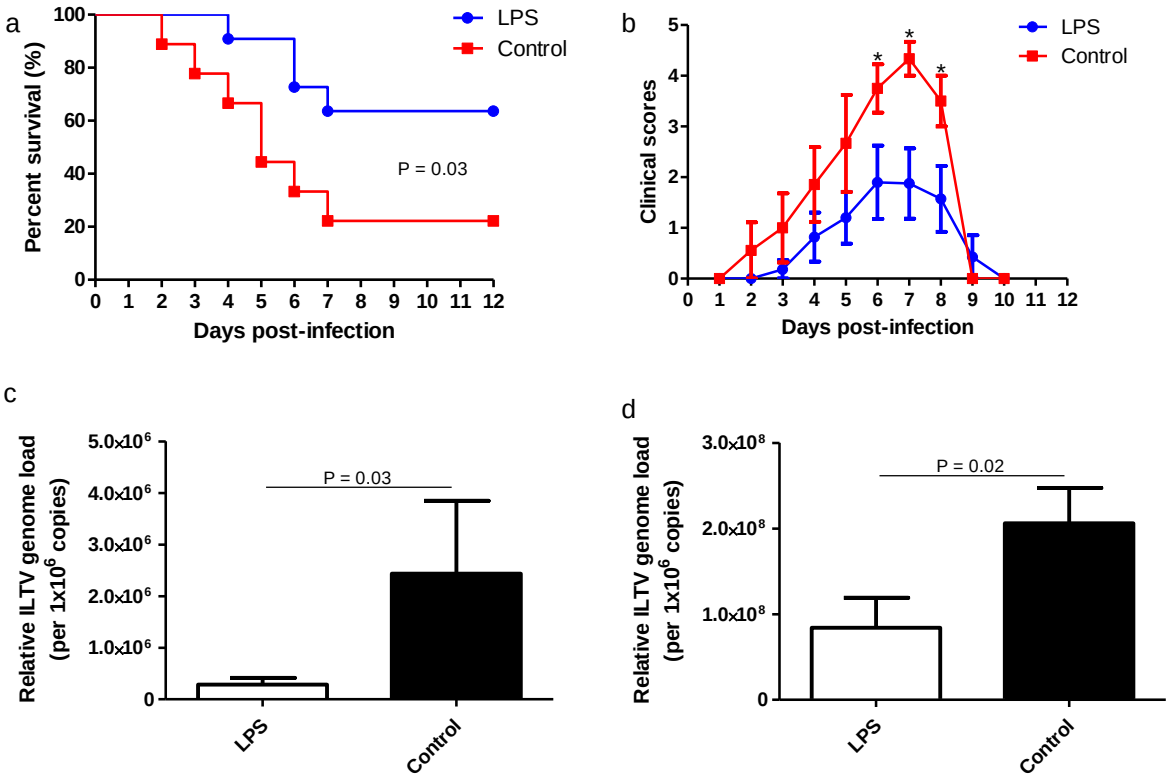


*Figure 1. In-ovo delivery of LPS induces protection against ILTV encountered post-hatch associated with decreased ILTV shedding*

SPF eggs were treated *in-ovo* with either LPS or PBS at ED-18 and infected with ILTV intratracheally at day 1 post-hatch. The chickens were monitored for 12 days, and cloacal and oropharyngeal swabs were collected for analysis at 5 dpi. The a) survival percentage, b) clinical score, c) ILTV genome expression in cloacal swabs, and d) ILTV genome expression in oropharyngeal swabs were evaluated. The Log-rank test was used to determine the group differences in survival percentage, the two-way ANOVA was used to identify differences in clinical score data, and the student's *t*-test was used to determine differences in genome expression. The differences were considered significant at P< 0.05.

### *In-ovo delivery of LPS increases macrophage numbers in lungs post-hatch*

Since we observed that *in-ovo*-delivered LPS induces an antiviral response against ILTV post-hatch, then the expansion of the macrophage population in the lungs at day 1 post-hatch was investigated as a potential underlying mechanism. We found that *in-ovo* treatment of LPS at ED-18 significantly increased the number of macrophages in the lungs post-hatch compared to the PBS control group (P<0.05, Figure 2).

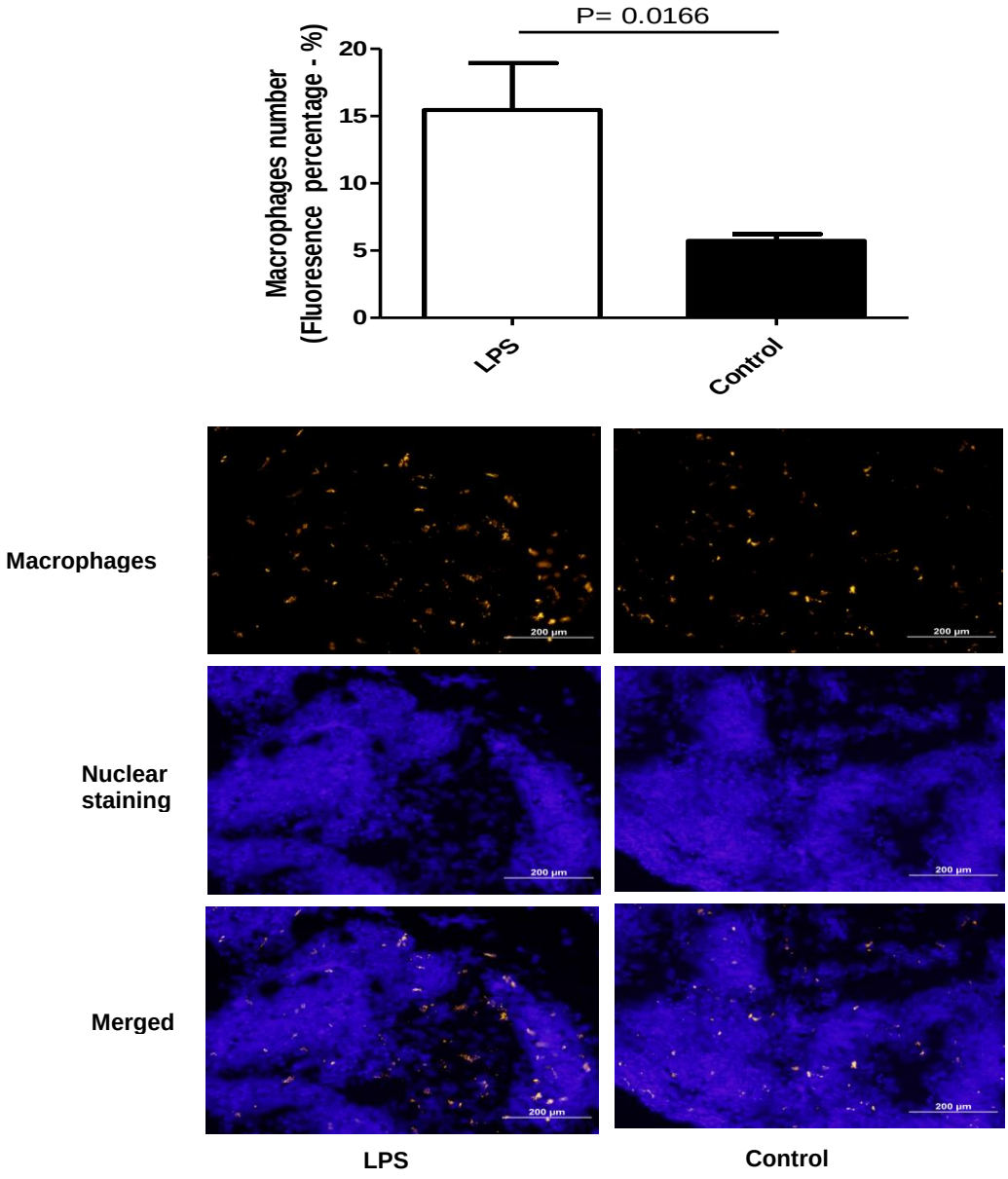


*Figure 2. In-ovo treated LPS increases macrophage population in lungs post-hatch*

SPF eggs were treated *in-ovo* with either LPS or PBS at ED-18 and then incubated until hatching. On the first day post-hatch, the lung tissues were sampled, preserved in OCT, sectioned, and then subjected to an immunofluorescent analysis utilizing a mouse monoclonal antibody specific for chicken macrophages (KUL01, indicated by red signals) along with DAPI (blue signals) for nuclear staining. The results are presented, including the

quantitative data and representative immunofluorescent figures. A Student's t-test was performed to determine group differences, which were considered significant at $P< 0.05$.

## Discussion

The current study presents key findings which are of great significance. The results show that *in-ovo* treatment with LPS can elicit protective antiviral responses against ILTV encountered post-hatch, as evidenced by increased survival rate, reduced viral shedding, and decreased clinical disease. This suggests that *in-ovo* LPS treatment could be a viable strategy for protecting chickens from ILTV infection. In addition, we found that the LPS-mediated antiviral response was positively correlated with the expansion of the macrophage population in the lungs, indicating the crucial role of macrophages in the host's defense against ILTV infections.

The *in-ovo* delivery is a standard method of hatchery vaccination in the poultry industry. It is considered a humane and efficient alternative to traditional methods of treating chickens after hatching. Studies have shown that *in-ovo* treatment with TLR ligands can activate innate immunity in developing chicken embryos, thus protecting subsequent viral infection post-hatch. For example, *in-ovo* treatment with TLR3 ligand, ds RNA, has been shown to protect chickens from AIV (H4N6) (Ahmed-Hassan, Abdul-Cader, Ahmed Sabry, et al., 2018; Ahmed-Hassan et al., 2017). Similarly, *in-ovo* treatment with TLR7 ligands protects IBDV and IBV (Annamalai et al., 2016; De Silva Senapathi et al., 2020; Matoo et al., 2018). Furthermore, *in-ovo* treatment with TLR3 and TLR21 ligands has exhibited antiviral effects against NDV and AIV, respectively (Abdul-Cader, Ahmed-Hassan et al., 2017; Cheng et al., 2014). However, it was unclear whether *in-ovo* treatment with the TLR4 ligand, LPS, activates antiviral responses against ILTV infection. Previous studies from our group have demonstrated that *in-ovo* treatment with TLR7 and TLR21 ligands, ssRNA and CpG DNA, respectively, enhances antiviral responses against ILTV encountered post-hatch (Abdul-Cader, Amarasinghe, et al., 2018; Abdul-Cader et al., 2019). Considering these previous findings, this study was designed to evaluate the efficacy of *in-ovo* LPS treatment against ILTV. The results in this study show that *in-ovo* treatment with the bacterial ligand, LPS, generates protective antiviral responses in chickens post-hatch, particularly against ILTV infection, as evidenced by reduced mortality, clinical disease, and the virus shedding at 5 dpi. This suggests that *in-ovo* LPS treatment may be a promising strategy for protecting chickens from this highly contagious virus that causes severe respiratory disease and significant economic losses to the poultry industry. However, it is essential to determine whether the reduction in ILTV shedding observed at 5 dpi is maintained throughout the infection and to explore the long-term efficacy of *in-ovo* LPS treatment in reducing ILTV shedding and protecting chickens from infection.

Meanwhile, following our previous findings that *in-ovo* ssRNA- or CpG DNA-mediated antiviral response against ILTV infection in chickens is characterized by the expansion of macrophage population in the lungs post-hatch (Abdul-Cader, Amarasinghe, et al., 2018;

Abdul-Cader et al., 2019), we sought to determine whether *in-ovo* delivery of LPS would result in a similar pattern of expansion of macrophages. Our results in this study, similar to our previous findings, show that the LPS-mediated antiviral response against ILTV infection was correlated with an expansion of the macrophage population in the lungs post-hatch.

As the first immune cells to encounter a virus upon infection, macrophages play a central role in antiviral immune responses in chickens. Although this study did not investigate the exact mechanism by which macrophages mediate antiviral responses, numerous previous studies have demonstrated that macrophages contribute to antiviral responses against invading viruses through multiple mechanisms. Firstly, macrophages can recognize and engulf the virus, effectively neutralizing it (Fujimoto, Pan, Takizawa, & Nakanishi, 2000). Once inside the macrophage, the virus can be degraded, and its components, including viral antigens, can be displayed on the surface of the macrophage for presentation to other immune cells, such as T cells (Unanue, 1984). This stimulates a more specific and targeted adaptive immune response against the virus, leading to the formation of memory cells. Secondly, macrophages secrete various cytokines, such as type 1 IFNs and IL-1β, which help in coordinating the immune response against the virus (Alkie et al., 2017; Lavric, Bencina, Kothlow, Kaspers, & Narat, 2007). Some of these cytokines, such as type I IFNs, may contribute to antiviral responses directly by inhibiting the replication of various viruses, including coronavirus, paramyxovirus, birna virus, AIV, and herpes viruses (Ahmed-Hassan et al., 2017; Ahmed-Hassan, Abdul-Cader, Sabry, et al., 2018; Haasbach, Droebner, Vogel, & Planz, 2011; Jarosinski, Jia, Sekellick, Marcus, & Schat, 2001; Meng et al., 2011; Mo, Cao, & Lim, 2001; Pei, Sekellick, Marcus, Choi, & Collisson, 2001). Other cytokines, such as tumor necrosis factor (TNF), IL-1β, and IL-12, help to recruit other immune cells, such as natural killer (NK) cells, neutrophils, and T cells, to the site of infection (Berbert et al., 2021; Morris & Ley, 2004; Pilaro et al., 1994; Presicce et al., 2015; Rider et al., 2011; Wang et al., 2022). Thirdly, another pro-inflammatory molecule macrophages produce is inducible NO synthase (iNOS). This enzyme facilitates the production of a potent antiviral free radical molecule, NO (Setta, Barrow, Kaiser, & Jones, 2012). Previous studies have shown that NO produced by macrophages plays a significant role in the antiviral response against avian viruses like AIV and ILTV (Abdul-Cader, Ahmed-Hassan, et al., 2017; Abdul-Cader, Amarasinghe, & Abdul-Careem, 2016; Abdul-Cader et al., 2019; Abdul-Cader, De Silva Senapathi, Nagy, Sharif, & Abdul-Careem, 2018; Ahmed-Hassan, Abdul-Cader, Sabry, et al., 2018; Haddadi et al., 2013). However, further studies are required to fully understand the exact antiviral mechanisms of activated macrophages following *in-ovo* delivery of LPS against ILTV infection in chickens.

## Conclusion

In conclusion, the study's results suggest that *in-ovo* LPS treatment can lead to a protective antiviral response against ILTV, as evidenced by reduced mortality, viral shedding, and clinical disease. The study also revealed that the LPS-mediated antiviral response was positively correlated with the expansion of the macrophage population in the lungs. However, additional research is needed to fully understand the exact mechanisms of LPS-

mediated antiviral response. Additionally, the long-term efficacy and safety of this approach should be evaluated. Overall, this study's findings contribute to avian virology and may pave the way for developing new strategies for controlling ILTV infection in chickens.

**Conflict of Interest:** The author(s) declared no conflict of interest.

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