



Exosomes derived from avian influenza virus-infected chickens modulate host immune responses

Chaeun Kim^a, Thi Hoai Phan^a, Anh Duc Truong^b, Yeong Ho Hong^{a,*}

^a Department of Animal Science and Technology, Chung-Ang University, Anseong 17546, Republic of Korea

^b Department of Biochemistry and Immunology, National Institute of Veterinary Research, 86 Truong Chinh, Dong Da, Hanoi 100000, Vietnam

ARTICLE INFO

Keywords:

Exosome
Avian influenza virus
Cytokine
Viral component
Chicken

ABSTRACT

Exosomes are emerging as key mediators of host–pathogen interactions, particularly as carriers of viral components during infection. This study aims to examine the immunomodulatory effects of serum-derived exosomes from Brown Leghorn chickens infected with low pathogenic avian influenza virus (LPAIV) or highly pathogenic avian influenza virus (HPAIV). These exosomes (CTRL-EXO [noninfected], LPAIV-EXO, and HPAIV-EXO) were intramuscularly injected into naïve chickens, after which tissues and serum were collected. Cytokine gene expression in immune-related tissues (lung, spleen, and trachea) was quantified through reverse transcription-quantitative polymerase chain reaction to evaluate the immune response. Unlike the lung and trachea, the spleen showed the strongest immune response following exosome injection, associated with elevated antiviral cytokines and interferons in the AIV-exosome group. In parallel, these exosomes were applied to chicken macrophage HD11 cells to determine cellular uptake and cytokine expression using reverse transcription-quantitative polymerase chain reaction. Furthermore, immunocytochemistry was performed to detect exosome-delivered viral nucleoprotein and nonstructural protein 1 proteins in HD11 cells. LPAIV-EXO induced the strongest immune activation, evidenced by increased cytokine expression and immunochemical detection of viral proteins. Collectively, these findings indicate that AIV-derived exosomes modulate host immune responses in vivo and in vitro, underscoring their potential in immune regulation and vaccine development.

Introduction

Avian influenza virus (AIV) remains a major threat to global poultry production and public health. AIV are classified into subtypes according to the antigenicity of their hemagglutinin (HA) and neuraminidase proteins. Among the HA subtypes, H5, H7, and H9 are the most frequently detected in wild birds. (Shi et al., 2023). The predominant subtypes, H5 and H7, are typically identified as low pathogenic avian influenza virus (LPAIV) with few or no clinical signs but can mutate into highly pathogenic avian influenza virus (HPAIV), causing severe illness and high mortality in galliformes (Luczo et al., 2015). AIV outbreaks have caused devastating poultry losses, with millions of birds reported dead. Additionally, studies report AIV infections in non-avian species, including pigs (Meseko et al., 2018), dairy cattle (Garg et al., 2025), and humans (Subbarao et al., 1998), underscoring the need for rigorous

control measures against this zoonotic disease.

Exosomes are small (30 to 200 nm) membrane-bound vesicles released by all cell types that transport biomolecules—including proteins, lipids, mRNAs, miRNAs, and other noncoding RNAs—between cells (Bobrie et al., 2011; Wang et al., 2022). They are key mediators of intercellular communication in health and disease (Gurung et al., 2021). Exosomal cargo mirrors the molecular profile of the originating cell; consequently, exosomes from virus-infected cells can transfer viral proteins and RNA to recipient cells (Bedford et al., 2020; Hong et al., 2022; Peng et al., 2023). These features indicate that exosomes may critically influence immune responses (Dreux et al., 2012a; Luong et al., 2021a). However, studies investigating exosomes derived from AIV-infected chickens, especially their immunomodulatory functions in the host, remain limited. Studies show that exosomes derived from virus-infected cells can modulate or trigger immune responses,

Abbreviation: AIV, Avian influenza virus; HPAIV, Highly pathogenic AIV; LPAIV, Low pathogenic AIV; RT-qPCR, Reverse transcription-quantitative polymerase chain reaction; Days-post infection, dpi; HA, Hemagglutinin; NA, Neuraminidase.

* Corresponding author at: Department of Animal Science and Technology, Chung-Ang University, Anseong 17546, Republic of Korea.

E-mail addresses: kchea77@naver.com (C. Kim), phanhoai271@gmail.com (T.H. Phan), truonganhduc84@gmail.com (A.D. Truong), yhong@cau.ac.kr (Y.H. Hong).

<https://doi.org/10.1016/j.psj.2026.106386>

Received 1 August 2025; Accepted 3 January 2026

Available online 3 January 2026

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highlighting their potential as immunoregulatory mediators. For instance, exosomes released by influenza A virus-infected A549 cells suppress immune responses in neighboring uninfected cells by delivering viral mRNA or immunosuppressive proteins, thereby promoting viral replication (Zabrodska et al., 2022). Similarly, exosomes from Theiler's murine encephalomyelitis virus-infected microglia deliver viral RNA to bystander central nervous system cells, triggering neuroinflammation through innate immune receptors and increasing interferon, cytokine, and chemokine expression (Luong et al., 2021b). A previous study shows that exosomes derived from HPAIV H5N1-infected chickens trigger antiviral responses in multiple chicken immune cells, including macrophages, fibroblasts, and T and B cell lines, by activating type I interferons, pro-inflammatory cytokines, and immune signaling pathways (Hong et al., 2022). However, although exosome-mediated delivery of viral proteins is linked to antiviral activity, these findings are restricted to in vitro analyses, and the mechanisms underlying exosome function in vivo remain unclear.

Therefore, this study aims to assess the immunomodulatory potential of exosomes derived from LPAIV- and HPAIV-infected chickens by transferring them to naïve chickens or cultured immune cells. Exosomes were intramuscularly administered to naïve chickens to determine systemic immune responses in vivo. We also examined whether the injected exosomes altered the content of re-isolated exosomes, including viral components. In parallel, exosome uptake and immunological effects were examined in a chicken macrophage cell line.

Additionally, by combining in vivo and in vitro approaches, this study aims to clarify how virus-associated exosomes modulate host immunity. Understanding their immunomodulatory role could support the development of extracellular vesicle-based diagnostics and vaccines.

Materials and methods

Animal experiment and exosome injection

In total, 21 4-week-old specific-pathogen-free Brown Leghorn chickens were employed in animal experiments conducted in a biosafety level 2 plus facility at the Department of Biochemistry and Immunology, National Institute of Veterinary Research, Vietnam.

Three groups of seven chickens—each noninfected, LPAIV-infected, and HPAIV-infected—were used. The LPAIV group was administered 200 µL intranasal inoculation of allantoic fluid containing 1 × 10⁴ 50% egg infectious dose (EID₅₀) (Huprikar et al., 1980) of A/Chicken/Vietnam/NA11/2021 (H5N6). The HPAIV group was similarly challenged with the same dose of A/Chicken/Vietnam/NA01/2019 (H5N1). Blood samples for exosome extraction were collected from the wing vein of chickens 3 days post-infection.

Exosomes were isolated from noninfected (CTRL-EXO), LPAIV-infected (LPAIV-EXO), and HPAIV-infected (HPAIV-EXO) chickens and injected into 30 naïve chickens (Table 1). Six uninjected chickens served as negative controls (NC). Each exosome group included eight chickens: four were administered 100 µg, and four were administered 200 µg of exosomes via intramuscular injection. At 3 days post-infection, blood was collected for exosome isolation from CTRL-EXO-, LPAIV-EXO-, and HPAIV-EXO-injected chickens. Finally, the spleen, lungs, and trachea were harvested from all four groups (NC, CTRL-EXO, LPAIV-EXO, and HPAIV-EXO). All animal experiments complied with the World Organization for Animal Health (OIE) guidelines. Figure 1A schematically illustrates all experimental procedures. This study was conducted in accordance with institutional guidelines for the care and ethical use of laboratory animals under a protocol approved by the Ministry of Agriculture and Rural Development, Vietnam (TCVN 8402:2010/TCVN 8400-26:2014).

Exosome isolation and characterization

Exosomes from CTRL-EXO, LPAIV-EXO, and HPAIV-EXO serum were

Table 1
Number of chickens used for exosome injection.

Chicken No	Exosome injection	¹ Day 1	Day 2	Day 3
1	Control-exo (100 µg)-1			
2	Control-exo (100 µg)-2			
3	Control-exo (100 µg)-3			
4	Control-exo (100 µg)-4			
5	Control-exo (200 µg)-1			
6	Control-exo (200 µg)-2			
7	Control-exo (200 µg)-3			
8	Control-exo (200 µg)-4			
9	² LPAI-exo (100 µg)-1			
10	LPAI-exo (100 µg)-2			
11	LPAI-exo (100 µg)-3			
12	LPAI-exo (100 µg)-4			
13	LPAI-exo (200 µg)-1			
14	LPAI-exo (200 µg)-2			
15	LPAI-exo (200 µg)-3			
16	LPAI-exo (200 µg)-4			
17	HPAI-exo (100 µg)-1			
18	HPAI-exo (100 µg)-2			
19	HPAI-exo (100 µg)-3			
20	HPAI-exo (100 µg)-4			
21	HPAI-exo (200 µg)-1			
22	HPAI-exo (200 µg)-2			
23	HPAI-exo (200 µg)-3			
24	HPAI-exo (200 µg)-4			
25	Negative control			
26	Negative control			
27	Negative control			
28	Negative control			
29	Negative control			
30	Negative control			

¹ A slash (/) in Day 1 columns for numbers 12 and 15 individuals indicates the death of the animal.

² Abbreviations: LPAI, low pathogenic avian influenza; HPAI, highly pathogenic avian influenza; exo, exosomes

isolated and characterized for size and marker expression, as previously described (Kim et al., 2025). Influenza A virus nucleoprotein (NP; #PA5-32242, Thermo Fisher Scientific, Waltham, MA, USA) and nonstructural protein 1 (NS1; #GTX125990, GeneTex, Irvine, CA, USA) were detected using western blotting following established protocols (Hong et al., 2021).

RNA extraction from exosomes and tissues and detection of viral RNA

Total exosomal RNA was extracted using TRIzol reagent (Thermo Fisher Scientific), following the protocol of the manufacturer. cDNA was synthesized from 2 µg of RNA using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific). H5 was amplified with DreamTaq polymerase (Thermo Fisher Scientific) with primers listed in Table 2, following the recommended procedure (Chaharaein et al., 2009). PCR products were separated on a 2% agarose gel stained with Dyne STAR (Dyne Bio, Seongnam, South Korea), an ethidium bromide alternative.

Total RNA was extracted from lung, spleen, and trachea tissues through homogenization with a tissue grinder motor (Taeshin Bio Science, Korea). Following homogenization, 1 mL of TRIzol reagent (Thermo Fisher Scientific) was added. Phase separation by adding 200 µL of chloroform, followed by RNA precipitation with 500 µL of isopropanol. Finally, the RNA pellet was washed with 75% ethanol.

Western blotting

Approximately 40 µg of exosome samples were loaded onto a 15% SDS gel for electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Invitrogen). The membrane was blocked with 5% skim milk in phosphate-buffered saline containing 0.05% Tween-20 (PBST; Sigma-Aldrich, St. Louis, MO, USA) and

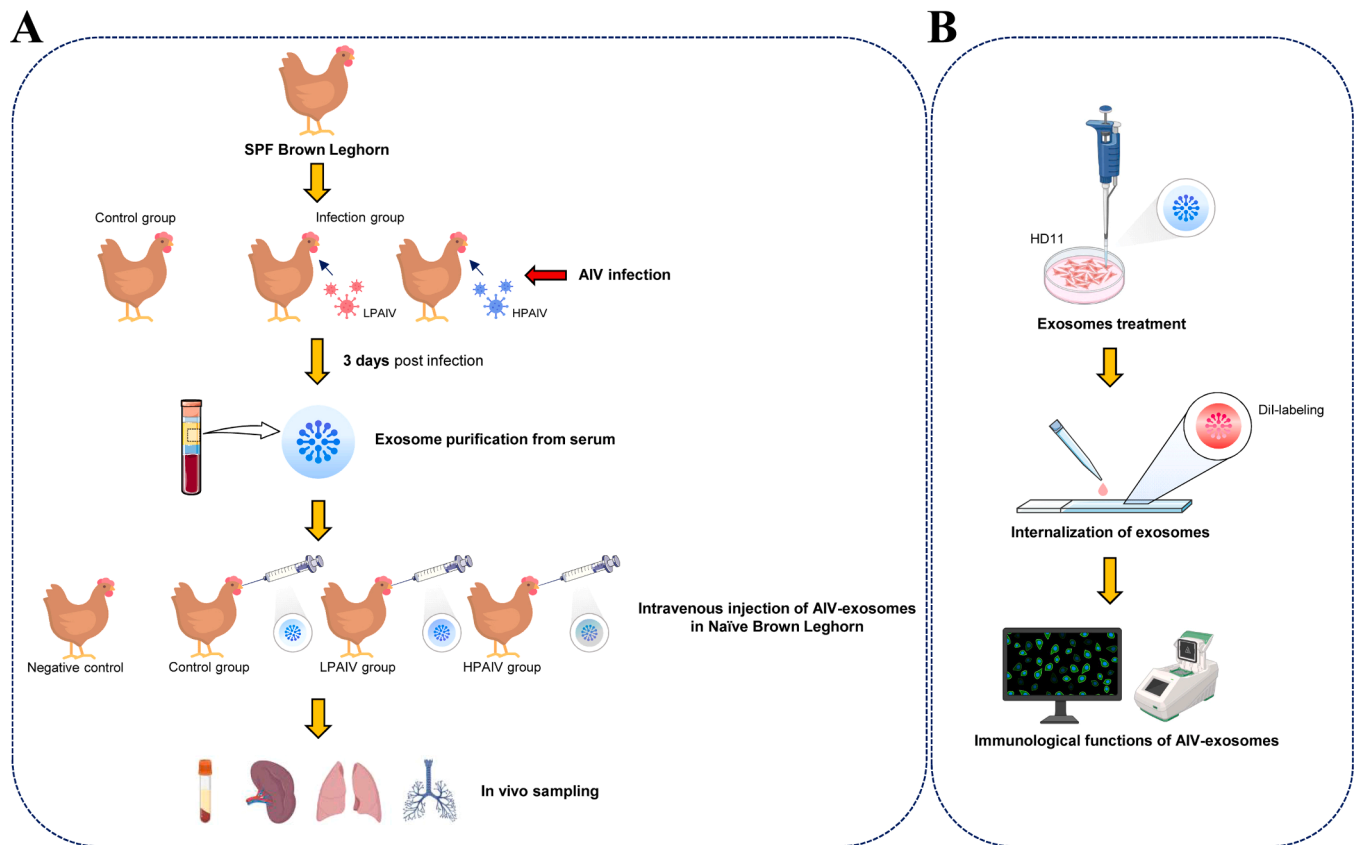


Fig. 1. Experimental workflow. (A) Animal experiments: chickens were divided into three groups (control, LPAIV, and HPAIV). Exosomes were extracted from CTRL-EXO, LPAIV-EXO, and HPAIV-EXO chickens and subsequently injected into naïve chickens. (B) Cell experiments: CTRL-EXO, LPAIV-EXO, and HPAIV-EXO exosomes were labeled with DiI and incubated with HD11 cells to assess exosome internalization.

Table 2
Primer sequences for RT-qPCR.

Genes	Sequences (5'→3')	Accession number
H5	F: ACAAGCTCTATCAAAACCCAAAC R: TACCCATACCAACCATCTACCAT	(Chaharaein et al., 2009)
IFN- α	F: GAGCAATGCTTGGACAGCAG R: GAGGTGTGGATGTGCAGGA	GU119896.1
IFN- β	F: CTGCCCCACACAAGACGTG R: TGTTTTGGAGTGTGTGGGCT	NM_001024836.1
IFN- γ	F: AACAACTTCTCTGATGGCGT R: TGAAGAGTTCATTCGCGGCT	NM_205149.1
IL-1 β	F: TGCCTGCAGAGAAGCCTCG R: CTCGCGACAGTTTGGTCAT	NM_204524.1
IL-4	F: TTGTTTGGGAGAGCCAGCA R: GACATGGTGCCTTGAGGGAG	NM_001007079.1
IL-6	F: GCAGGACGAGATGTGCAAGA R: ATTTCTCTCTCGTGAAGCCG	NM_204628.1
IL-8	F: GGCTTGCTAGGGGAATGA R: AGCTGACTCTGACTAGGAACTGT	NM_205498.1
IL-10	F: GCTTCTACACAGATGAGGTCC R: TCCGTTCTCATCATCTTC	NM_001004414.4
IL-18	F: GGAATGGCATGCCTTTTG R: ATTTTCCTGCTCTTCTCA	NM_204608.1
TNF α	F: CGCTCAGAACGACGTCAA R: GTCGTCCACACCAACGAG	(Rohde et al., 2018)
¹ GAPDH	F: TGCTGCCAGAACATCATCC R: ACGGCAGGTCAGGTCAACAA	NM_204305

¹ Abbreviations: RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

incubated overnight at 4°C with primary antibodies against Influenza A NP Polyclonal Antibody (#PA5-32242, Thermo Fisher Scientific) or Influenza A virus NS1 (#GTX125990, Irvine, CA, USA), diluted in PBST

containing 2% skim milk. After washing with PBST, the membrane was incubated for 1 h at room temperature (RT) of 23°C with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Thermo Fisher Scientific) diluted in 2% skim milk. Protein bands were detected using the Western Lightning Plus ECL substrate (Thermo Fisher Scientific).

Cell culture and exosome treatment

The chicken HD11 macrophage cell line (Klasing et al., 1987) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific), 100 IU/mL penicillin, and 100 µg/mL streptomycin. HD11 cells (8×10^5 cells/well) were seeded in 12-well plates (SPL Life Sciences, Pocheon, Korea) and incubated overnight at 41°C in a humidified 5% CO₂ incubator. The medium was then replaced with exosome-depleted RPMI 1640 containing 10% exosome-depleted FBS (#EXO-FBSHI-250A-1; System Biosciences, Palo Alto, CA) and 100 IU/mL penicillin with 100 µg/mL streptomycin. Cells were treated with 50 µg/mL exosomes (CTRL-EXO, LPAIV-EXO, and HPAIV-EXO) for 4 h. Total RNA was extracted from culture supernatants using TRIzol reagent (Thermo Fisher Scientific) following the protocol of the manufacturer. cDNA was synthesized from 2 µg of RNA using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) following the instructions of the manufacturer.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Oligonucleotide primers for reverse transcription quantitative real-time PCR (RT-qPCR) were designed with Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and synthesized by Genotech

(Daejeon, South Korea; Table 2). RT-qPCR was performed on a CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA) using a SYBR Green PCR assay, and cytokine expression was validated using Dyne qPCR 2X PreMix (Dyne Bio). Amplification was performed for 40 cycles at 95°C, 56°C to 60°C, and 72°C for 30, 30, and 30 s, respectively. GAPDH served as the housekeeping gene, and relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak et al., 2001). All RT-qPCR analyses were performed in triplicate.

Cellular uptake of CTRL-EXO, LPAIV-EXO, and HPAIV-EXO

To assess exosome internalization (CTRL-EXO, LPAIV-EXO, and HPAIV-EXO), exosomes were labeled with DiI (#D3911; Sigma-Aldrich) and incubated with HD11 cells cultured in Nunc™ Lab-Tek™ Chamber Slides (#177380; Thermo Fisher Scientific). Exosomes (50 µg/mL) were diluted in 200 µL PBS and 2 µL of 1 mM DiI (in methanol) and incubated for 2 h at RT, protected from light. After three centrifugation cycles (18,000 × g, 20 min), the supernatant was discarded, and the pellet was washed with 200 µL PBS. DiI-labeled exosomes were added to HD11 cells cultured in exosome-free medium and incubated for 12 h at 41°C with 5% CO₂. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at RT in the dark and stained with DAPI (#P6966; Thermo Fisher Scientific) for 5 min. Fluorescence images were captured using an EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific; Fig. 1B).

Immunocytochemistry

HD11 cells (6×10^5 cells/well) were cultured in exosome-free complete RPMI 1640 in Nunc™ Lab-Tek™ Chamber Slides (#177380; Thermo Fisher Scientific) for 4 h at 41°C (5% CO₂), then treated with exosomes (50 µg/mL; CTRL-EXO, LPAIV-EXO, and HPAIV-EXO) or left untreated (NC) for 24 h.

For viral NP detection, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at RT and permeabilized with ice-cold methanol (Sigma-Aldrich) for 10 min at −20°C. For viral NS1 detection, cells were fixed with 100% methanol (Sigma-Aldrich) for 5 min at RT and permeabilized with ice-cold methanol for 10 min at −20°C. After overnight incubation at 4°C with primary antibodies (NP, NS1), cells were incubated with Alexa Fluor® 488-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h at RT. Nuclei were stained with DAPI for 5 min. Immunostained slides were imaged using a confocal microscope (LSM800, Carl Zeiss, Germany) and analyzed with ZEN v2.6 software.

Statistical analyses

Statistical analyses were performed using SPSS software (IBM SPSS Statistics 28.0 for Windows; Chicago, IL, USA). Data are presented as mean ± SEM (N = 3). Differences among groups were assessed using one-way ANOVA followed by Tukey's post hoc multiple-comparison test. A $P < 0.05$ was considered statistically significant. Error bars represent fold changes from three technical replicates.

Results

Detection of viral components in exosomes

To identify viral particles in exosomes, PCR and western blot analyses were performed on exosomes isolated from CTRL-EXO, LPAIV-EXO, and HPAIV-EXO chicken serum. H5 AIV RNA was detected in lanes 2, 3, 5, 6, and 10 (Fig. 2A). Spleen samples injected with AIV exosomes (lanes 2 and 3) exhibited stronger H5 RNA amplification with LPAIV-EXO than with HPAIV-EXO. Conversely, exosomes alone (lanes 5 and 6) showed stronger H5 signals with HPAIV-EXO than with LPAIV-EXO. Among exosomes isolated from chickens injected with CTRL-EXO, LPAIV-EXO, HPAIV-EXO, or left uninjected (lanes 7 to 10), the H5 RNA band was detected only in the HPAIV-EXO sample (lane 10).

Figure 2B shows that exosomes contained influenza A NP and NS1 during infection. Only LPAIV-EXO showed a clear NP band, whereas HPAIV-EXO exhibited a weaker band. NS1 was analyzed under identical conditions, but reliable results were not obtained (data not shown).

Immune responses induced in chicken tissues through AIV exosomes injection

To examine exosome-mediated immune responses in vivo, naïve chickens were intramuscularly injected with exosomes from NC, CTRL-EXO, LPAIV-EXO, and HPAIV-EXO groups. Cytokine expression was measured using RT-qPCR (Fig. 3).

In the lungs of AIV-exosome-injected chickens, IFN-β, IFN-γ, and IL-10 showed similar expression patterns and were most highly upregulated in the CTRL-EXO group (2.73-, 3.44-, and 2.26-fold increases, respectively). No significant differences were observed between CTRL-EXO and HPAIV-EXO, although HPAIV-EXO showed the second-highest expression for these cytokines. Compared with NC, IL-4 expression increased 1.12-fold in LPAIV-EXO and decreased by 0.19- and 0.18-fold in CTRL-EXO and HPAIV-EXO, respectively, although the difference between NC and LPAIV-EXO was not statistically significant.

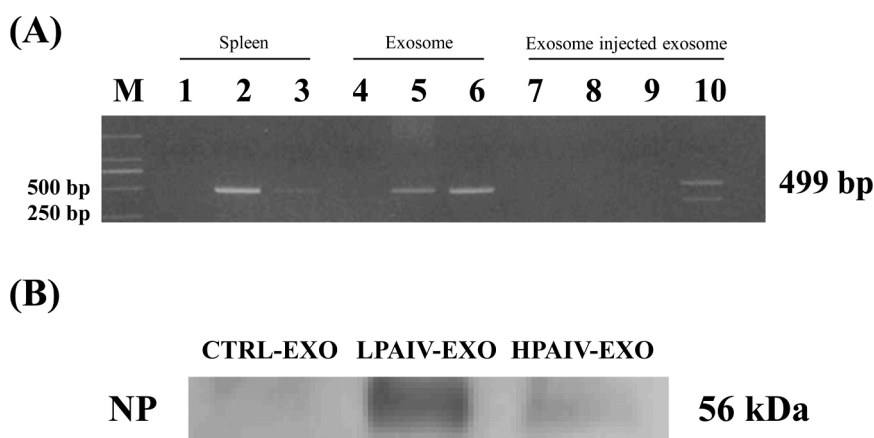


Fig. 2. (A) RT-qPCR detection of H5 avian influenza viral RNA in spleen, exosomes, and exosome-injected chicken samples. Lanes 1 to 3 (spleen): 1, control; 2, LPAIV; 3, HPAIV. Lanes 4 to 6 (exosomes): 4, CTRL-EXO; 5, LPAIV-EXO; 6, HPAIV-EXO. Lanes 7 to 10 (exosome-injected chickens): 7, NC; 8, CTRL-EXO; 9, LPAIV-EXO; 10, HPAIV-EXO. Lane M indicates a 100-bp DNA ladder. (B) Western blot analysis of viral NP in exosomes. Exosome samples were transferred to membranes and probed with an influenza A NP polyclonal antibody, followed by a horseradish peroxidase-conjugated anti-rabbit secondary antibody.

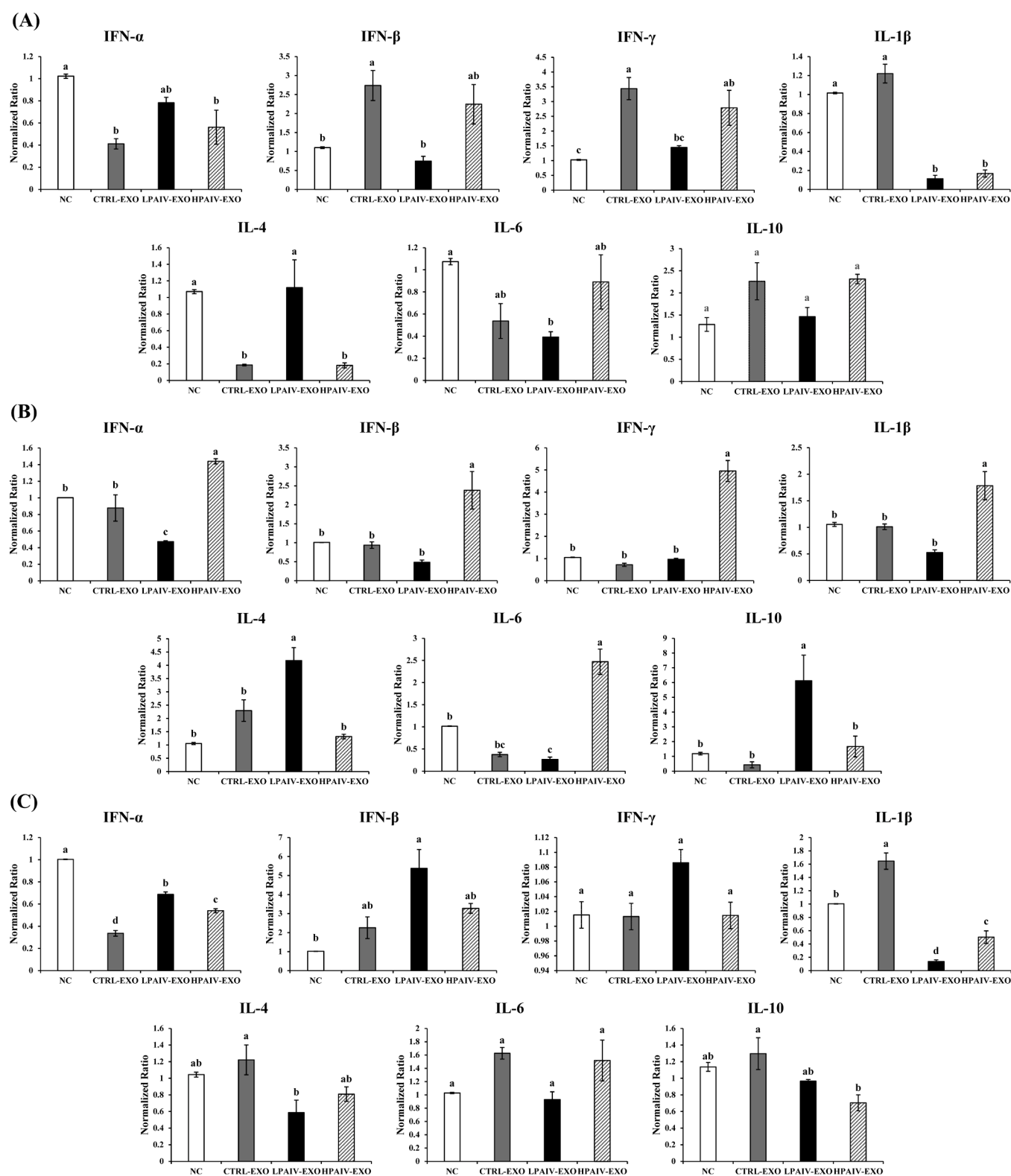


Fig. 3. Cytokine expression in exosome-injected chicken (A) lung, (B) spleen, and (C) trachea tissues using RT-qPCR. NC represents samples from non-exosome-injected chickens, whereas CTRL-EXO, LPAIV-EXO, and HPAIV-EXO represent samples from chickens injected with the corresponding exosome preparations. Chickens were administered a 200 μ g exosome dose, and tissues were collected at 3 dpi. Different lowercase letters indicate statistically significant differences ($p < 0.05$) as determined using one-way analysis of variance followed by Tukey's multiple comparison test. Data values are presented as mean $\pm$ SEM ($n = 3$), normalized to GAPDH from three independent experiments.

IFN- α showed a similar expression pattern to that of IL-4. IL-6 expression decreased by 0.54-, 0.39-, and 0.89-fold in CTRL-EXO, LPAIV-EXO, and HPAIV-EXO, respectively. IL-1 β was significantly downregulated in the LPAIV-EXO and HPAIV-EXO groups (Fig. 3A).

Cytokine expression was highest in the spleens of AIV-exosome-injected chickens (Fig. 3B). IFN- α , IFN- β , IFN- γ , IL-1 β , and IL-6 were most strongly upregulated in the HPAIV-EXO group, with fold increases of 1.44, 2.38, 4.96, 1.78, and 2.48, respectively. In contrast, the anti-inflammatory cytokines IL-4 and IL-10 were most upregulated in the LPAIV-EXO group, with 4.18- and 6.13-fold increases, respectively.

IL-1 β expression in the trachea mirrored the downregulation observed in the lungs after AIV exosome injection (Fig. 3C). IFN- α exhibited a similar pattern, whereas IL-6 levels did not differ significantly among groups. IFN- β and IFN- γ were most highly expressed in the LPAIV-EXO group, whereas IL-4 and IL-10 showed the lowest expression in the LPAIV-EXO and HPAIV-EXO groups, respectively. Overall, cytokine expression suggests that the spleen mounts a stronger immune response than that of the lung or trachea.

Cellular uptake of AIV exosomes in chicken immune cell lines

To determine whether exosomes from AIV-infected chickens transmit viral particles and induce immune responses, HD11 cells were treated with 50 μ g/mL exosomes. DiI-labeled CTRL-EXO, LPAIV-EXO, and HPAIV-EXO were internalized into the HD11 cell cytoplasm (Fig. 4).

HD11 macrophage cell treated with AIV exosomes induced cytokine expression

To assess exosome-induced immune responses, HD11 cells were treated with 50 μ g/mL exosomes for 4 h. Subsequently, mRNA levels of type I (IFN- α , IFN- β) and type II (IFN- γ) interferons, pro-inflammatory cytokines (IL-1 β , IL-18, and tumor necrosis factor α [TNF- α]), anti-inflammatory cytokines (IL-4), and chemokine IL-8 (CXCL8) were measured. The LPAIV-EXO groups had the highest expression (Fig. 5), with no significant difference between CTRL-EXO and HPAIV-EXO. These findings suggest that LPAIV-EXO elicits a stronger immune response than that of HPAIV-EXO, likely due to differences in viral components delivered via exosomes.

Viral proteins were delivered via AIV exosomes in chicken macrophage cell lines

Immunocytochemistry was used to confirm NP and NS1 in AIV-EXO-treated cells (LPAIV-EXO and HPAIV-EXO), indicating successful

exosome-mediated viral delivery. NP signals were stronger in the LPAIV-EXO group than in the HPAIV-EXO group (Fig. 6A). NS1 was detected in LPAIV-EXO but weakly expressed in HPAIV-EXO, with nuclear localization observed (Fig. 6B). These findings suggest a stronger immune response in the LPAIV-EXO group, consistent with RT-qPCR cytokine profiles in exosome-treated HD11 cells (Fig. 6). They also indicate that NP is expressed more strongly and consistently than NS1, and that LPAIV-EXO exerts a greater immunomodulatory effect than that of HPAIV-EXO.

Discussion

Exosomes are gaining interest as biomarkers and diagnostic tools because they reflect physiological and pathological states of their cellular origin. However, their role in immune regulation—particularly in chickens at the whole-animal level—remains underexplored. This study investigated whether exosomes derived from LPAIV- or HPAIV-infected chickens can modulate immune responses in recipient hosts and immune cells. Bedford et al. report that airway exosomes in bronchoalveolar lavage fluid from influenza-infected mice transferred viral proteins (HA, neuraminidase, NP, M1, and NS1) to uninfected mice and activated antigen-presenting cells (Bedford et al., 2020). Exosome-induced pulmonary inflammation is associated with cytokine release, type I and II interferon induction, and neutrophil recruitment (Bedford et al., 2020). Viral RNA in exosomes is typically recognized by Toll-like receptors (TLR), particularly TLR7, which detects viral RNA in endosomal compartments after exposure (Blasius et al., 2010; Kawai et al., 2011). For instance, exosomes from Human immunodeficiency virus type-1-infected macrophages induce pro-inflammatory cytokine production via TLR recognition of their RNA cargo (Sampey et al., 2016). This mechanism allows host cells to package viral RNA into exosomes, thereby modulating innate immunity and influencing pathogenesis in nearby uninfected cells.

Our findings indicate that exosomes from virus-infected chickens can transmit viral components, including H5 viral RNA, as observed in exosomes from AIV-EXO-injected chickens (Fig. 2). These findings suggest that exosomes deliver viral genetic material, not merely act as intracellular signaling messengers, implicating them in viral infection pathways. Moreover, the findings indicate that viral components may influence the cellular state of other organisms without viral infection, though the precise role of exosomes in viral transmission requires further investigation.

We examined cytokine expression in the lung, spleen, and trachea of naïve chickens after exosome injection. The spleen showed robust immune activation, especially following HPAIV-EXO treatment (Fig. 3B).

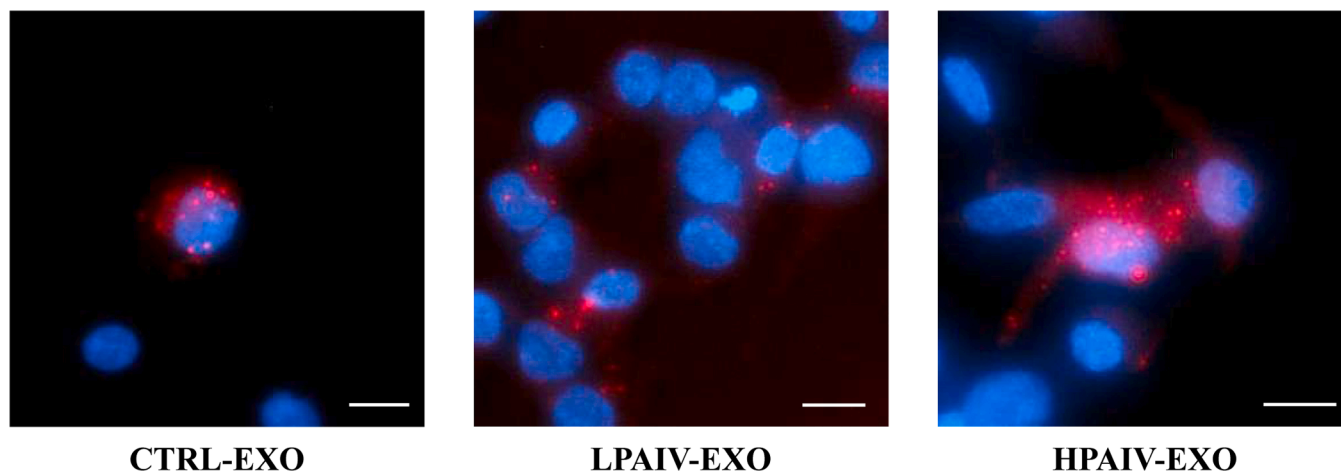


Fig. 4. Internalization of DiI-labeled exosomes purified from the serum of CTRL-EXO, LPAIV-EXO, and HPAIV-EXO chickens following incubation with HD11 cells. Cell nuclei were stained with DAPI (blue), and DiI-labeled exosomes are shown in red. Scale bar = 10 μ m.

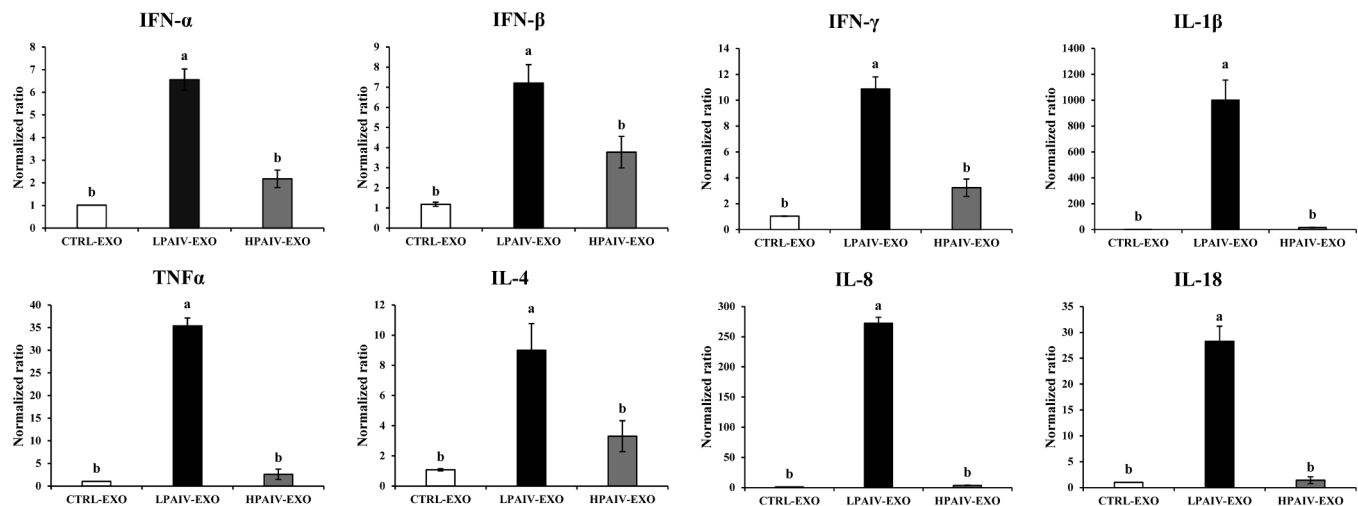


Fig. 5. RT-qPCR analysis of cytokine expression in chicken macrophage cells treated with 50 µg/mL CTRL-EXO, LPAIV-EXO, and HPAIV-EXO and incubated for 4 h. Data are presented as mean ± SEM from three independent experiments (n = 3), with expression normalized to GAPDH. Different lowercase letters above the error bars indicate statistically significant differences ($p < 0.05$) as determined through one-way analysis of variance followed by Tukey's multiple comparison test.

HPAIV-EXO significantly upregulated IFN-α, IFN-β, IFN-γ, IL-1β, and IL-6. Type I and II interferons are key antiviral defenses, with IFN-β and IFN-α subtypes inducing antiviral molecules encoded by IFN-stimulated genes (Lee et al., 2018; McNab et al., 2015). IL-6, a pleiotropic cytokine, is produced in response to tissue damage and viral infection, modulating host immune responses (Velazquez-Salinas et al., 2019). Along with TNF-α and IL-1 (including IL-1β), IL-6 ranks among the most important cytokines in viral infections. IL-1β, an inflammatory plasma marker, was upregulated in cells treated with AIV H7N9-derived HA protein (Slaats et al., 2016; Zhang et al., 2015), indicating splenic immune cells respond strongly to HPAIV-derived exosomal cargo. Anti-inflammatory cytokines IL-4 and IL-10 were markedly upregulated in the LPAIV-EXO group. These cytokines primarily mediate the Th2 response and regulate inflammatory balance during acute viral infection, protecting host tissues (Rojas et al., 2017; van den Broek et al., 2000). Overall, pro-inflammatory cytokines (IL-1β, IL-6) and antiviral interferons (IFN-α, IFN-β, and IFN-γ) were expressed at higher levels in HPAIV-EXO than in LPAIV-EXO. Mo et al. (2024) reports higher pro-inflammatory gene expression in HPAIV-infected bone marrow-derived dendritic cells than in LPAIV-infected cells. This suggests that HPAIV-EXO carries cytokine-rich cargo that elicits an immune response similar to direct HPAIV infection. Conversely, anti-inflammatory cytokines (IL-4 and IL-10) were elevated in LPAIV-EXO, suggesting that cytokine responses vary with pathogenicity. Unlike HPAIV, which triggers a strong inflammatory response and severe symptoms due to high mortality, LPAIV appears to promote immune homeostasis.

Although uninfected exosomes were not expected to modulate immunity, some cytokines differed between NC and CTRL-EXO. This suggests that exosomes from uninfected hosts may either carry bioactive molecules that elicit minor immune effects or function as foreign particles that induce mild immune activation. Alternatively, variation may result from injection or subtle physiological differences in donor chickens.

Exosomes carrying viral RNA and proteins act as pathogen-associated molecular patterns, triggering innate immunity via pattern recognition receptors such as TLR, RIG-I, MDA5, and NLR (Dreux et al., 2012b; Kawai et al., 2009). Our findings indicate that the AIV-derived exosomes exert tissue-specific immunomodulatory effects dependent on viral pathogenicity. HPAIV-EXO strongly stimulates systemic immune responses in lymphoid organs such as the spleen while limiting inflammation in mucosal tissues, including the lung and trachea. Although derived from AIV infection, these exosomes elicit weaker immune responses than the virus itself. However, in the spleen—the

second largest lymphoid tissue rich in immune cells (Lewis et al., 2019)—both AIV-EXOs strongly activated immune responses. These findings suggest that, unlike primary respiratory organs (lungs and trachea), viral molecules delivered via exosomes act as viral antigens that preferentially activate immune organs.

Furthermore, immune responses may differ based on organ type. The lungs, as primary respiratory and mucosal immune organs, protect the host by eliminating pathogens and foreign particles via the mucosal barrier and epithelial cells (Gopallawa et al., 2023; Sato et al., 2012). Thus, intramuscularly injected exosomes may be recognized as foreign substances. Unlike the lungs and trachea—mucosa-associated lymphoid tissues directly exposed to external antigens—the spleen efficiently detects circulating exosomes and activates a systemic immune response via innate immune cells.

Accumulating evidence shows that exosomes derived from virus-infected cells play a key role in orchestrating cellular immune responses. For example, respiratory syncytial virus infection triggered an immune response in human monocytes and airway epithelial cells by delivering viral components via exosomes, leading to cytokine and chemokine release (Chahar et al., 2018). Similarly, plasma vesicles from SARS-CoV-2-infected patients activate peripheral immune cells, with viral dsRNA recognized by TLR3 inducing IL-6, IL-8, and TNF-α production, contributing to the cytokine storm (Chen et al., 2022).

Our study confirmed exosome-mediated immune responses by showing elevated cytokine levels in exosome-treated chicken macrophages. Type I (IFN-α, IFN-β) and type II (IFN-γ) interferons, pro-inflammatory cytokines (IL-1β, IL-18), anti-inflammatory cytokine IL-4, TNF-α, and chemokine IL-8 were detected, with especially high levels in LPAIV-EXO-treated cells (Fig. 5). TNF-α, produced by macrophages and monocytes, acts early to limit immunopathology during H1N1 influenza infection (Mohd Zawawi et al., 2023; Savenkova et al., 2024). IL-8, a CXC chemokine, activates neutrophils by binding to CXCR1 and CXCR2 and, along with IL-6, is highly expressed in patients infected with SARS-CoV-2 (Cesta et al., 2022; Lee et al., 2021; Zhu et al., 2021; Zimmermann et al., 2011). IL-18, the “IFN-γ-inducing factor,” is an IL-1 family cytokine that regulates innate and adaptive immunity by activating macrophages synergistically with IL-12 (Ihim et al., 2022; Munder et al., 1998).

In Fig. 6, NP and NS1 showed stronger signals in LPAIV-EXO than in HPAIV-EXO, consistent with the western blot results (Fig. 2B). Similarly, RT-qPCR revealed higher cytokine expression in LPAIV-EXO, supporting its stronger immunomodulatory effect.

However, NP was more strongly expressed than NS1 in LPAIV-EXO

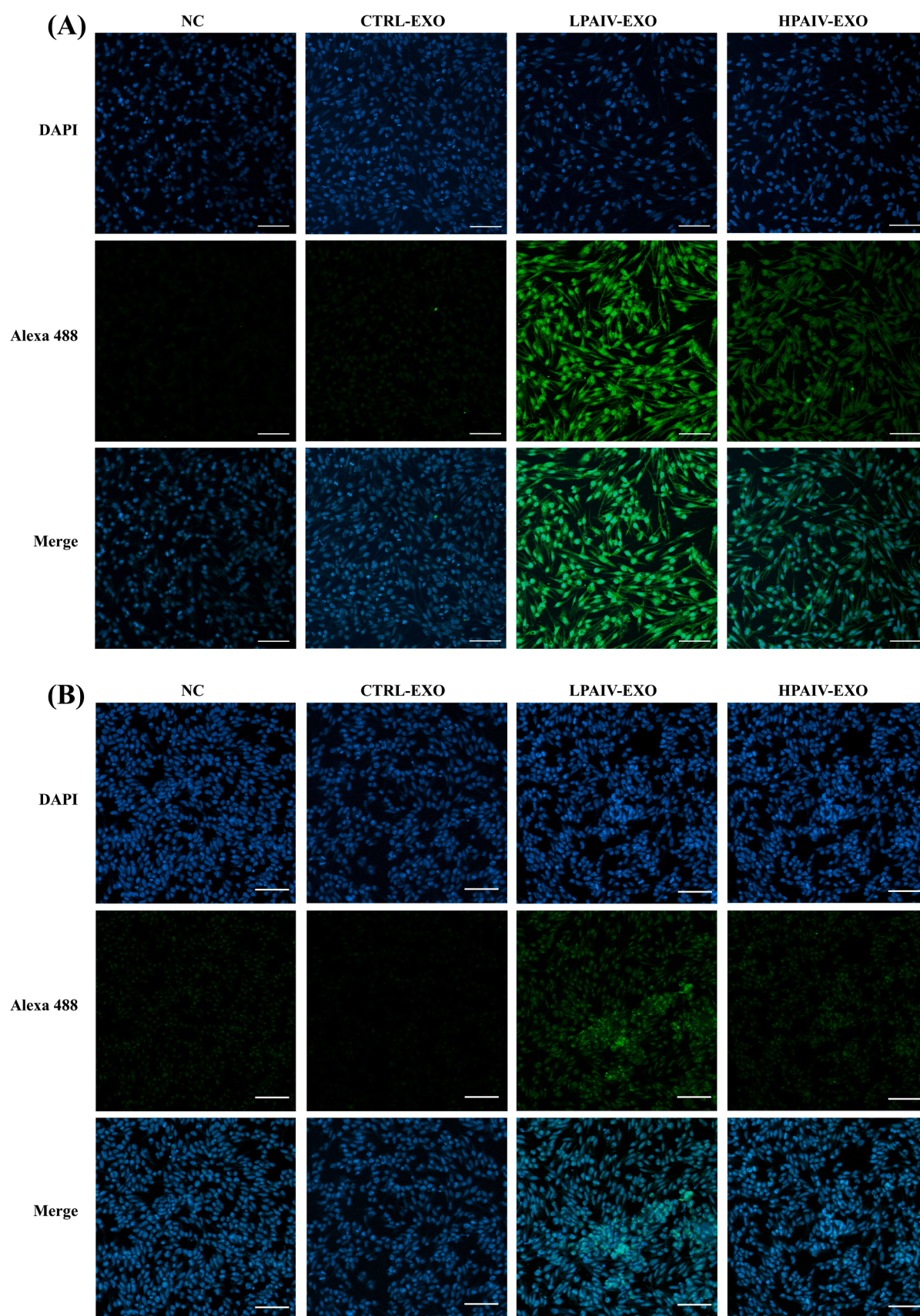


Fig. 6. Confocal immunocytochemistry of viral NP and NS1 in HD11 macrophage cells. HD11 cells were incubated with 50 $\mu\text{g}/\text{mL}$ CTRL-EXO, LPAIV-EXO, and HPAIV-EXO or NC for 24 h. Immunodetection was performed using primary antibodies against (A) NP and (B) NS1, followed by Alexa Fluor®488-conjugated secondary antibodies (green). Cell nuclei were stained with DAPI (blue). Scale bar = 50 μm .

and HPAIV-EXO, indicating that NP is an essential structural protein with “high and sustained” expression, whereas NS1, a functional protein, shows “low and early” expression, regulating host immunity (Turrell et al., 2013; Yang et al., 2023). Therefore, NP and NS1 proteins are believed to differ within exosomes.

These findings suggest that stronger viral protein signals in LPAIV-EXO drive elevated cytokine expression. NP, secreted by influenza virus-infected cells, is essential for viral replication and induces pro-inflammatory cytokines IL-1 β and IL-6 through TLR4 and NLRP3 inflammasome activation (Eisfeld et al., 2015; Kim et al., 2022). NS1 counters host antiviral responses by suppressing type I interferons and blocking RIG-I signaling pathways during influenza A virus infection (Koliopoulos et al., 2018; Zhang et al., 2022). However, NS1 in exosomes may modulate immune activation rather than fully suppress it, depending on the balance between immune evasion and recognition in recipient cells. For instance, Dengue virus-derived NS1 induces pro-inflammatory cytokine production in human dendritic cells (Alayli et al., 2016). Upon AIV-EXO treatment, NS1 localized to the nucleus in HD11 cells (Fig. 6B), consistent with previous studies showing that the influenza A H3N2 subtype virus NS1 protein contains a C-terminal NLS2/NoLS domain facilitating nuclear import and interaction with proteins such as nucleolin and fibrillarin (Melén et al., 2012).

Variations in exosomal NP and NS1 levels directly affect the host immune response. NP-rich exosomes typically stimulate immunity, whereas NS1-rich exosomes suppress it (Jiang et al., 2020). These immune effects always depend not only on the content of NP and NS1, but also on (i) the substances contained in the exosome (viral mRNA/-miRNA/host regulatory factors), (ii) the cell/host (chicken vs. mammal), and (iii) the viral strain (e.g., H5N1 vs. H9N2) (Zabrodska et al., 2022; Hong et al., 2022; Kim et al., 2025). Immunocytochemistry revealed higher NP and NS1 levels in HD11 cells treated with LPAIV-EXO than in cells treated with HPAIV-EXO, suggesting that elevated NP and NS1 in LPAIV-EXO may boost immune responses, including cytokine expression.

A study reports that exosomal miRNAs from LPAIV- or HPAIV-infected chickens are associated with the MAPK signaling pathway, a key regulator of host immunity. Additionally, we detected viral RNA and proteins in the exosomes, suggesting that viral molecules and small non-coding RNAs, such as miRNAs, contribute to the immune response. Since miRNAs regulate host gene expression via immune-related pathways, their delivery via exosomes—whether viral or host-derived miRNAs—may further modulate immune activation (Chandan et al., 2020; Vu et al., 2023a; Vu et al., 2023b). Therefore, the immunostimulatory effects observed in this study likely arise from the combined actions of viral proteins, RNA, and regulatory noncoding RNAs carried within exosomes.

In summary, this study shows that exosomes from AIV-infected chickens carry viral RNA and proteins and modulate host immune responses in vivo and in vitro. Furthermore, these exosomes can independently induce immune responses in naïve chickens without direct viral infection. This finding indicates the functional role of exosomes in actively modulating immune responses in living hosts. HPAIV-derived exosomes triggered a strong splenic immune response, whereas LPAIV-EXO strongly stimulated HD11 cells. These findings suggest that splenic immune homeostasis and cell interactions may limit excessive inflammation, while macrophages directly activate immune genes in response to exosomal viral proteins, including NP and NS1. Overall, these findings indicate that exosomal immunomodulatory effects depend on cargo and the specific immune environment. Therefore, further research is needed to clarify how viral components engage innate immune receptors to modulate immunity. Understanding these processes will enhance knowledge of viral pathogenesis and inform the development of exosome-based diagnostics, vaccines, and therapeutic strategies in the poultry industry.

Consent for publication

Not applicable.

Funding

This work was supported by the National Research Foundation grant (NRF-2021R1A2C2005236) of the Republic of Korea and the Chung-Ang University Young Scientist Scholarship in 2025.

Conflict of interest

The authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

Chaeun Kim: Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Thi Hoai Phan:** Validation, Methodology, Investigation. **Anh Duc Truong:** Resources, Methodology, Investigation, Conceptualization. **Yeong Ho Hong:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors thank the Department of Biochemistry and Immunology Laboratory of the National Institute of Veterinary Research, Vietnam, for conducting the animal experiments. Immunocytochemistry analysis was performed at the BT research facility center, Chung-Ang University.

References

- Alayli, F., Scholle, F., 2016. Dengue virus NS1 enhances viral replication and pro-inflammatory cytokine production in human dendritic cells. *Virology* 496, 227–236. <https://doi.org/10.1016/j.virol.2016.06.008>.
- Bedford, J.G., Infusini, G., Dagley, L.F., Villalon-Letelier, F., Zheng, M.Z.M., Bennett-Wood, V., Reading, P.C., Wakim, L.M., 2020. Airway exosomes released during influenza virus infection serve as a key component of the antiviral innate immune response. *Front. Immunol.* 11, 887. <https://doi.org/10.3389/fimmu.2020.00887>.
- Blasius, A.L., Beutler, B., 2010. Intracellular toll-like receptors. *Immunity* 32, 305–315. <https://doi.org/10.1016/j.immuni.2010.03.012>.
- Bobrie, A., Colombo, M., Raposo, G., Thery, C., 2011. Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic* 12, 1659–1668. <https://doi.org/10.1111/j.1600-0854.2011.01225.x>.
- Cesta, M.C., Zippoli, M., Marsiglia, C., Gavioli, E.M., Mantelli, F., Allegritti, M., Balk, R.A., 2022. The role of interleukin-8 in lung inflammation and injury: implications for the management of COVID-19 and hyperinflammatory acute Respiratory distress Syndrome. *Front. Pharmacol.* 12, 808797. <https://doi.org/10.3389/fphar.2021.808797>.
- Chahar, H.S., Corsello, T., Kudlicki, A.S., Komaravelli, N., Casola, A., 2018. Respiratory syncytial virus infection changes cargo composition of exosome released from Airway Epithelial Cells. *Sci. Rep.* 8, 387. <https://doi.org/10.1038/s41598-017-18672-5>.
- Chaharaein, B., Omar, A.R., Aini, I., Yusoff, K., Hassan, S.S., 2009. Detection of H5, H7 and H9 subtypes of avian influenza viruses by multiplex reverse transcription-polymerase chain reaction. *Microbiol. Res.* 164, 174–179. <https://doi.org/10.1016/j.micres.2007.01.001>.
- Chandan, K., Gupta, M., Sarwat, M., 2020. Role of host and pathogen-derived MicroRNAs in immune regulation during infectious and inflammatory diseases. *Front. Immunol.* 10, 3081. <https://doi.org/10.3389/fimmu.2019.03081>.
- Chen, L., Chen, R., Yao, M., Feng, Z., Yuan, G., Ye, F., Nguyen, K., Karn, J., McComsey, G.A., McIntyre, T.M., Jin, G., 2022. COVID-19 plasma exosomes promote proinflammatory immune responses in peripheral blood mononuclear cells. *Sci. Rep.* 12, 21779. <https://doi.org/10.1038/s41598-022-26457-8>.
- Dreux, M., Garaigorta, U., Boyd, B., Decembre, E., Chung, J., Whitten-Bauer, C., Wieland, S., Chisari, F.V., 2012a. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. *Cell Host Microbe* 12, 558–570. <https://doi.org/10.1016/j.chom.2012.08.010>.
- Dreux, M., Garaigorta, U., Boyd, B., Decembre, E., Chung, J., Whitten-Bauer, C., Wieland, S., Chisari, F.V., 2012b. Short-range exosomal transfer of viral RNA from

- infected cells to plasmacytoid dendritic cells triggers innate immunity. *Cell Host Microbe*. 12, 558–570. <https://doi.org/10.1016/j.chom.2012.08.010>.
- Eisfeld, A.J., Neumann, G., Kawaoka, Y., 2015. At the centre: influenza A virus ribonucleoproteins. *Nat. Rev. Microbiol.* 13, 28–41. <https://doi.org/10.1038/nrmicro3367>.
- Garg, S., Reinhart, K., Couture, A., Kniss, K., Davis, C.T., Kirby, M.K., Murray, E.L., Zhu, S., Kraushaar, V., Wadford, D.A., Drehoff, C., Kohnen, A., Owen, M., Morse, J., Eckel, S., Goswitz, J., Turabelidze, G., Krager, S., Unutzer, A., Gonzales, E.R., Hamid, C.Abdul, Ellington, S., Mellis, A.M., Budd, A., Barnes, J.R., Biggerstaff, M., Jhung, M.A., Richmond-Crum, M., Burns, E., Shimabukuro, T.T., Uyeki, T.M., Dugan, V.G., Reed, C., Olsen, S.J., 2025. Highly pathogenic Avian Influenza A (H5N1) virus infections in humans. *N. Engl. J. Med* 392, 843–854. <https://doi.org/10.1056/NEJMoa2414610>.
- Gopallawa, I., Dehinwal, R., Bhatia, V., Gujar, V., Chirmule, N., 2023. A four-part guide to lung immunology: invasion, inflammation, immunity, and intervention. *Front. Immunol.* 14, 1119564. <https://doi.org/10.3389/fimmu.2023.1119564>.
- Gurung, S., Perocheau, D., Touramanidou, L., Baruteau, J., 2021. The exosome journey: from biogenesis to uptake and intracellular signalling. *Cell Commun. Signal.* 19, 47. <https://doi.org/10.1186/s12964-021-00730-1>.
- Hong, Y., Lee, J., Vu, T.H., Lee, S., Lillehoj, H.S., Hong, Y.H., 2021. Immunomodulatory effects of poly(I:C)-stimulated exosomes derived from chicken macrophages. *Poult. Sci.* 100, 101247. <https://doi.org/10.1016/j.psj.2021.101247>.
- Hong, Y., Truong, A.D., Vu, T.H., Lee, S., Heo, J., Kang, S., Lillehoj, H.S., Hong, Y.H., 2022. Exosomes from H5N1 avian influenza virus-infected chickens regulate antiviral immune responses of chicken immune cells. *Dev. Comp. Immunol.* 130, 104368. <https://doi.org/10.1016/j.dci.2022.104368>.
- Huprikar, J., Rabinowitz, S., 1980. A simplified plaque assay for influenza viruses in Madin-Darby kidney (MDCK) cells. *J. Virol. Methods* 1, 117–120. [https://doi.org/10.1016/0166-0934\(80\)90020-8](https://doi.org/10.1016/0166-0934(80)90020-8).
- Ihim, S.A., Abubakar, S.D., Zian, Z., Sasaki, T., Saffarioun, M., Maleknia, S., Azizi, G., 2022. Interleukin-18 cytokine in immunity, inflammation, and autoimmunity: biological role in induction, regulation, and treatment. *Front. Immunol.* 13, 919973. <https://doi.org/10.3389/fimmu.2022.919973>.
- Jiang, Y., Cai, X., Yao, J., Guo, H., Yin, L., Leung, W., Xu, C., 2020. Role of extracellular vesicles in Influenza virus infection. *Front. Cell Infect. Microbiol.* 10, 366. <https://doi.org/10.3389/fcimb.2020.00366>.
- Kawai, T., Akira, S., 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int. Immunol.* 21, 317–337. <https://doi.org/10.1093/intimm/dxp017>.
- Kawai, T., Akira, S., 2011. Toll-like receptors and their crosstalk with other Innate receptors in. *Infect. Immun.* 79, 637–650. <https://doi.org/10.1016/j.immuni.2011.05.006>.
- Kim, C.-U., Jeong, Y.-J., Lee, P., Lee, M.-S., Park, J.-H., Kim, Y.-S., Kim, D.-J., 2022. Extracellular nucleoprotein exacerbates influenza virus pathogenesis by activating Toll-like receptor 4 and the NLRP3 inflammasome. *Cell. Mol. Immunol.* 19, 715–725. <https://doi.org/10.1038/s41423-022-00862-5>.
- Kim, C., Phan, T.H., Vu, T.H., Truong, A.D., Lillehoj, H.S., Hong, Y.H., 2025. Comparison of exosomal miRNA profiling in highly pathogenic and low pathogenic avian influenza virus-infected chickens. *J. Vet. Sci.* 26 (5), e64. <https://doi.org/10.4142/jvs.25077>.
- Klasing, K.C., Peng, R.K., 1987. Influence of cell sources, stimulating agents, and incubation conditions on release of interleukin-1 from chicken macrophages. *Dev. Comp. Immunol.* 11, 385–394. [https://doi.org/10.1016/0145-305x\(87\)90082-6](https://doi.org/10.1016/0145-305x(87)90082-6).
- Koliopoulos, M.G., Lethier, M., van der Veen, A.G., Haubrich, K., Hennig, J., Kowalinski, E., Stevens, R.V., Martin, S.R., Reis e Sousa, C., Cusack, S., Rittinger, K., 2018. Molecular mechanism of influenza A NS1-mediated TRIM25 recognition and inhibition. *Nat. Commun.* 9, 1820. <https://doi.org/10.1038/s41467-018-04214-8>.
- Lee, A.J., Ashkar, A.A., 2018. The dual nature of type I and type II interferons. *Front. Immunol.* 9, 2061. <https://doi.org/10.3389/fimmu.2018.02061>.
- Lee, J., Choi, J.A., Ju, H.-H., Kim, J.-E., Paik, S.-Y., Rao, P.V., 2021. Role of MCP-1 and IL-8 in viral anterior uveitis, and contractility and fibrogenic activity of trabecular meshwork cells. *Sci. Rep.* 11, 14950. <https://doi.org/10.1038/s41598-021-94391-2>.
- Lewis, S.M., Williams, A., Eisenbarth, S.C., 2019. Structure and function of the immune system in the spleen. *Sci. Immunol.* 4. <https://doi.org/10.1126/sciimmunol.aau6085>.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 25, 402–408. <https://doi.org/10.1006/meth.2001.1262>.
- Luczo, J.M., Stambas, J., Durr, P.A., Michalski, W.P., Bingham, J., 2015. Molecular pathogenesis of H5 highly pathogenic avian influenza: the role of the haemagglutinin cleavage site motif. *Rev. Med. Virol.* 25, 406–430. <https://doi.org/10.1002/rmv.1846>.
- Luong, N., Olson, J.K., 2021a. Exosomes secreted by microglia during virus infection in the Central nervous system activate an inflammatory response in bystander cells. *Front. Cell Dev. Biol.* 9, 661935. <https://doi.org/10.3389/fcell.2021.661935>.
- Luong, N., Olson, J.K., 2021b. Exosomes secreted by microglia during virus infection in the Central nervous system activate an inflammatory response in bystander cells. *Front. Cell Dev. Biol.* 9, 661935. <https://doi.org/10.3389/fcell.2021.661935>.
- McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A., 2015. Type I interferons in infectious disease. *Nat. Rev. Immunol.* 15, 87–103. <https://doi.org/10.1038/nri3787>.
- Melén, K., Tynell, J., Fagerlund, R., Roussel, P., Hernandez-Verdun, D., Julkunen, I., 2012. Influenza A H3N2 subtype virus NS1 protein targets into the nucleus and binds primarily via its C-terminal NLS2/NoLS to nucleolin and fibrillarin. *Virol. J.* 9, 167. <https://doi.org/10.1186/1743-422X-9-167>.
- Meseko, C., Globig, A., Ijomanta, J., Joannis, T., Nwosuh, C., Shamaki, D., Harder, T., Hoffman, D., Pohlmann, A., Beer, M., Mettenleiter, T., Starick, E., 2018. Evidence of exposure of domestic pigs to highly pathogenic Avian Influenza H5N1 in. *Nigeria. Sci. Rep.* 8, 5900. <https://doi.org/10.1038/s41598-018-24371-6>.
- Mo, J., Segovia, K., Chrzastek, K., Briggs, K., Kapczynski, D.R., 2024. Morphologic characterization and cytokine response of chicken bone-marrow derived dendritic cells to infection with high and low pathogenic avian influenza virus. *Front. Immunol.* 15, 1374838. <https://doi.org/10.3389/fimmu.2024.1374838>.
- Mohd Zawawi, Z., Kalyanasundram, J., Mohd Zain, R., Thayan, R., Basri, D.F., Yap, W.B., 2023. Prospective roles of tumor necrosis factor-α (TNF-α) in COVID-19: prognosis, therapeutic and management. *Int. J. Mol. Sci.* 24, 6142. <https://doi.org/10.3390/ijms24076142>.
- Munder, M., Mallo, M., Eichmann, K., Modolell, M., 1998. Murine macrophages secrete interferon γ upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. *J. Exp. Med.* 187, 2103–2108. <https://doi.org/10.1084/jem.187.12.2103>.
- Peng, Y., Yang, Y., Li, Y., Shi, T., Luan, Y., Yin, C., 2023. Exosome and virus infection. *Front. Immunol.* 14, 1154217. <https://doi.org/10.3389/fimmu.2023.1154217>.
- Rohde, F., Schusser, B., Hron, T., Farkašová, H., Plachý, J., Härtle, S., Hejnar, J., Elleder, D., Kaspers, B., 2018. Characterization of chicken tumor necrosis factor-α, a long missed. *Cytokine Birds. Front. Immunol.* 9, 605. <https://doi.org/10.3389/fimmu.2018.00605>.
- Rojas, J.M., Avia, M., Martín, V., Sevilla, N., 2017. IL-10: A multifunctional cytokine in viral infections. *J. Immunol. Res.* 2017, 6104054. <https://doi.org/10.1155/2017/6104054>.
- Sampey, G.C., Saifuddin, M., Schwab, A., Barclay, R., Punya, S., Chung, M.C., Hakami, R. M., Zadeh, M.A., Lepene, B., Klase, Z.A., El-Hage, N., Young, M., Jordanski, S., Kashanchi, F., 2016. Exosomes from HIV-1-infected cells stimulate production of pro-inflammatory cytokines through trans-activating response (TAR) RNA. *J. Biol. Chem.* 291, 1251–1266. <https://doi.org/10.1074/jbc.M115.662171>.
- Sato, S., Kiyono, H., 2012. The mucosal immune system of the respiratory tract. *Curr. Opin. Virol.* 2, 225–232. <https://doi.org/10.1016/j.coviro.2012.03.009>.
- Savenkova, D.A., Gudymo, A.S., Korablev, A.N., Taranov, O.S., Bazovkina, D.V., Danilchenko, N.V., Perfiljeva, O.N., Ivleva, E.K., Moiseeva, A.A., Bulanovich, Y.A., Roshchina, E.V., Serova, I.A., Battulin, N.R., Kulikova, E.A., Yudkin, D.V., 2024. Knockout of the Tnfr gene decreases influenza virus-induced histological reactions in laboratory mice. *Int. J. Mol. Sci.* 25, 1156. <https://doi.org/10.3390/ijms25021156>.
- Shi, J., Zeng, X., Cui, P., Yan, C., Chen, H., 2023. Alarming situation of emerging H5 and H7 avian influenza and effective control strategies. *Emerg. Microbes Infect.* 12, 2155072. <https://doi.org/10.1080/22221751.2022.2155072>.
- Slaats, J., Ten Oever, J., van de Veerdonk, F.L., Netea, M.G., 2016. IL-1β/IL-6/CRP and IL-18/ferritin: distinct inflammatory programs in infections. *PLoS Pathog* 12, e1005973. <https://doi.org/10.1371/journal.ppat.1005973>.
- Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K., Cox, N., 1998. Characterization of an Avian Influenza A (H5N1) virus isolated from a child with a fatal Respiratory illness. *Science* 279, 393–396. <https://doi.org/10.1126/science.279.5349.393>.
- Turrell, L., Lyall, J.W., Tiley, L.S., Fodor, E., Vreede, F.T., 2013. The role and assembly mechanism of nucleoprotein in influenza A virus ribonucleoprotein complexes. *Nat. Commun.* 4, 1591. <https://doi.org/10.1038/ncomms2589>.
- Van Den Broek, M., Bachmann, M.F., Köhler, G., Barner, M., Escher, R.D., Zinkernagel, R., Kopf, M., 2000. IL-4 and IL-10 antagonize IL-12-mediated protection against acute vaccinia virus infection with a limited role of IFN-γ and nitric oxide synthetase 21. *J. Immunol* 164, 371–378. <https://doi.org/10.4049/jimmunol.164.1.371>.
- Velazquez-Salinas, L., Verdugo-Rodriguez, A., Rodriguez, L.L., Borca, M.V., 2019. The role of interleukin 6 during viral infections. *Front. Microbiol.* 10, 1057. <https://doi.org/10.3389/fmicb.2019.01057>.
- Vu, T.H., Heo, J., Kang, S., Kim, C., Lillehoj, H.S., Hong, Y.H., 2023a. Chicken miR-26a-5p modulates MDAs during highly pathogenic avian influenza virus infection. *Dev. Comp. Immunol.* 149, 104921. <https://doi.org/10.1016/j.dci.2023.104921>.
- Vu, T.H., Hong, Y., Heo, J., Kang, S., Lillehoj, H.S., Hong, Y.H., 2023b. Chicken miR-148a-3p regulates immune responses against AIV by targeting the MAPK signalling pathway and IFN-gamma. *Vet. Res.* 54, 110. <https://doi.org/10.1186/s13567-023-01240-3>.
- Wang, X., Tian, L., Lu, J., Ng, I.O.-L., 2022. Exosomes and cancer - diagnostic and prognostic biomarkers and therapeutic vehicle. *Oncogenesis* 11, 54. <https://doi.org/10.1038/s41389-022-00431-5>.
- Yang, Q., Elz, A.E., Panis, M., Liu, T., Nilsson-Payant, B.E., Blanco-Melo, D., 2023. Modulation of Influenza A virus NS1 expression reveals prioritization of host response antagonism at single-cell resolution. *Front. Microbiol.* 9 (14), 1267078. <https://doi.org/10.3389/fmicb.2023.1267078>.
- Zabrodska, Y., Plotnikova, M., Gavrilova, N., Lozhkov, A., Klotchenko, S., Kiselev, A., Burdakova, V., Ramsay, E., Purvinsh, L., Egorova, M., Vysochinskaya, V., Baranovskaya, I., Brodskaya, A., Povalikhin, R., Vasin, A., 2022. Exosomes released by influenza-virus-infected cells carry factors capable of suppressing immune defense genes in naive cells. *Viruses* 14, 2690. <https://doi.org/10.3390/v14122690>.
- Zhang, Q., Zhang, X., Lei, X., Wang, H., Jiang, J., Wang, Y., Bi, K., Diao, H., 2022. Influenza A virus NS1 protein hijacks YAP/TAZ to suppress TLR3-mediated innate immune response. *PLoS Pathog* 18, e1010505. <https://doi.org/10.1371/journal.ppat.1010505>.
- Zhang, S., Gu, D., Ouyang, X., Xie, W., 2015. Proinflammatory effects of the hemagglutinin protein of the avian influenza A (H7N9) virus and

- microRNA-mediated homeostasis response in THP-1 cells. *Mol. Med. Rep.* 12, 6241–6246. <https://doi.org/10.3892/mmr.2015.4142>.
- Zhu, Q.C., Li, S., Yuan, L.X., Chen, R.A., Liu, D.X., Fung, T.S., 2021. Induction of the proinflammatory chemokine interleukin-8 is regulated by integrated stress response and AP-1 Family proteins activated during coronavirus infection. *Int. J. Mol. Sci.* 22, 5646. <https://doi.org/10.3390/ijms22115646>.
- Zimmermann, H.W., Seidler, S., Gassler, N., Nattermann, J., Luedde, T., Trautwein, C., Tacke, F., 2011. Interleukin-8 is activated in patients with chronic liver diseases and associated with hepatic macrophage accumulation in human liver fibrosis. *PLoS One* 6, e21381. <https://doi.org/10.1371/journal.pone.0021381>.