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Rapid Onset of Innate Response, Cytokine Signaling and Humoral Immunity in Inactivated LPAI-H9N2-Vaccinated Broilers

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Abstract: The development of effective and innovative vaccination strategies is urgently needed to better control the spread and transmission of the low-pathogenic avian influenza H9N2 subtype (LPAI-H9N2) in poultry. In addition, the enhancement of innate immunity by some of these innovative inactivated vaccines has not yet been investigated. Here, an experiment was conducted in commercial broiler chickens to compare the immune response to two different inactivated H9N2 vaccines. For this, Group 1 (G1) broilers were vaccinated with vaccine 1 [Nobilis® H9N2-P (pathogen-associated molecular patterns—PAMP) technology], broilers in G2 were vaccinated with vaccine 2 [an inactivated whole H9N2 virus (IWV) autogenous oil emulsion vaccine], while birds in G3 were not vaccinated. The study lasted 34 days. Innate immune parameters (phagocytic activity, nitric oxide, and lysozyme), cytokine signaling (IL-1β, IL-6, IL-8), humoral immunity using the hemagglutination inhibition (HI) test, and the gene expressions of IFN-γ and TLR-21 were assessed. The results showed a significant increase in innate immunity and modulatory cytokines at 24–48 h after the vaccination of G1 broilers, with a continuous increase until the end of the experiment. In addition, a significant increase in geometric mean HI titers was observed in G1 at 11 days post-vaccination (dpv), and a significant ($p < 0.05$) upregulation of IFN-γ and TLR-21 was observed in the same group, G1, at 31 dpv compared to G2 and G3. Nobilis® H9N2-P may induce faster and stronger innate and active humoral immunity compared to another IWV, which may contribute to the protection of broilers against early H9N2 infections. However, challenge protection studies for several IWV vaccines, including PAMP-H9N2 against LPAI-H9N2, should be further evaluated in both specific pathogen-free (SPF) and commercial broilers.

Keywords: broiler chickens; cytokines; innate immunity; Nobilis® H9N2-P; TLR-21

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1. Introduction

The immune response of birds to pathogens is a potential determinant of disease prognosis. There are two main distinct branches of host immunity: the innate and the acquired response. Pattern recognition receptors (PRRs) on host cells are very important tools of the innate immune system, recognizing pathogens or danger-associated molecular patterns (PAMPs or DAMPs), including lipoproteins, polysaccharides, glycolipids, and nucleotides. Recognition initiates the immediate defense against pathogens and the harmonious interplay between innate and adaptive immunity to control the disease [1]. The

coordination of intracellular signaling pathways can result in the activation of microbicidal mechanisms, the release of cytokines and chemokines, and the production of co-stimulatory molecules required for antigen presentation and the activation of the acquired immune system [2,3].

It has been proposed that immune modulation targeting the innate system may prove more advantageous as induction is rapid and non-specific and offers therapeutic benefits, including adjuvant boosting effects with local and systemic protection involving numerous cellular immune targets [4]. The early response of the innate immune system in chickens within 1 week is characterized by the upregulation of genes associated with defense/pathogen response [5], inflammation [3,6], and the production and secretion of the cytokine IFN-γ [7].

Low-pathogenicity avian influenza H9N2 (LPAI-H9N2) is an enzootic virus causing significant economic losses to the poultry industry in the Middle East, Asia, and Africa. LPAI-H9N2 continues to diversify into multiple antigenically distinct lineages, which may further promote the emergence of potential pandemic strains that are capable of masking and facilitating HPAIV transmission in poultry. In addition, H9N2 infections are associated with significant morbidity and increased susceptibility to secondary viral or bacterial infections, resulting in respiratory and digestive disorders associated with high mortality [8,9]. Since the first report of LPAI-H9N2 was in quail (quail/V3413/2011) in Egypt in 2011, the virus has spread rapidly to several domestic birds in different locations [10]. Over 12 years, the genetic lineage of all LPAI H9N2 viruses detected was G1-like, with some antigenically distinct variants, and they continue to infect Egyptian poultry farms, causing significant economic losses [11]. As a result, many countries have had to introduce effective vaccination programs as a means of prevention and control. Oil emulsion-inactivated whole virus (IWV) vaccines for H9N2 have been widely used in poultry farms in Egypt and many other countries, including China, South Korea, Pakistan, Morocco, Iran, and the United Arab Emirates (UAE) [12–17]. However, the immunogenicity and efficacy of these vaccines remain questionable. Therefore, enhancing the immunostimulatory nature of H9N2 vaccine formulations is essential to counteract this virus, such as the incorporation of various adjuvants, and biological, or chemical substances that induce specific immune responses against specific pathogens when used in conjunction with vaccination [18].

Understanding the mechanism of the chicken immune response to LPAI-H9N2 is essential for the effective control of virus spread and vaccine development. LPAI-H9N2 virus infection in birds initially activates the innate immune response through pattern recognition receptors [19]. At the site of infection, the cytokines produced recruit innate immune and antigen-presenting cells, which subsequently transduce antigenic signals to adaptive immune cells (i.e., B and T cells) to induce specific humoral and cellular immunity that led to the clearance of the infected cells, and the virus, via antibody-mediated neutralization and cytotoxicity, respectively. In birds, phagocytosis by phagocytes (e.g., macrophages, dendritic cells, and heterophils) is essential for a variety of biological events, including tissue remodeling and the continuous clearance of dying cells. Furthermore, phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens and comprises a series of events, starting with the binding and recognition of particles by cell surface receptors, followed by the formation of actin-rich membrane extensions around the particle. The fusion of membrane extensions results in phagosome formation, which precedes phagosome maturation into a phagolysosome. Pathogens inside the phagolysosome are destroyed by a lowered pH, hydrolysis, and radical attack. These early events that are mediated by the innate immune system are critical for host survival [19]. Previously, Bastamy et al. [20] recorded greater immunomodulation in lysozyme-treated broilers compared to lysozyme-free broilers via raised cellular (opsonic activity through MΦ and phagocytic index), local (IgA), and humoral (HI titers for ND and HPAI-H5N1) immune responses.

In this way, recently, the H9N2-P vaccine was used to effectively enhance immunity, reducing the occurrence and control of H9N2 outbreaks. PAMPs are conserved small

molecular motifs within a class of microbes that are recognized by innate immune cells [19]. Therefore, in the present study, we performed a genetic and serological analysis of the innate and humoral immune responses in broiler chickens vaccinated with an inactivated low-pathogenic avian influenza virus subtype H9N2 produced by the innovative PAMP technology (Nobilis® H9N2-P) and another autogenous IWV H9N2 vaccine to fill the gap, whether the innovative inactivated vaccines were able to enhance innate immunity or not.

2. Materials and Methods

2.1. Ethical Approval

The study was approved by the Institutional Animal Care and Use Committee of Beni-Suef University (BSU-IACUC), Egypt. The ethical approval code was BSU-IACUC-022-444. Every effort was made to minimize bird suffering.

2.2. Experimental Study and Sampling

For the three groups (G1, G2, and G3) of commercial broiler chickens [Ross 308 of mixed sex], each one consisted of 100 birds reared with 10 replicates in 3 separate partitions. At 3 days old (DO), G1 was vaccinated subcutaneously (at a dose of 0.2 mL) using vaccine 1 [IWV, LPAI-H9N2 vaccine, strain A/CK/UAE/415/1999, prepared with the technology of PAMP (Nobilis® H9N2-P, MSD, Intervet Int., Boxmeer, The Netherlands) with antigenic content of 8 $\log_2$ hemagglutination units (HA) units], G2 was vaccinated with vaccine 2 [IWV, LPAI-H9N2 autogenous vaccine, strain A/CK/Egypt/S10490/2015 (produced by a local company, Cairo, Egypt) with antigenic content of 8 $\log_2$ HA units] and G3 was kept as the control and remained unvaccinated against H9N2. Routine vaccinations were given to all 3 groups against infectious bronchitis by spraying at the hatchery using the live H120 vaccine (Avishield® IB H120, Dechra, Shrewsbury, UK) then a live intermediate plus infectious bursal disease vaccine (Bursine plus®, Zoetis, Parsippany-Troy Hills, NJ, USA) via eye drops at 12 DO and a VG/GA strain of the Newcastle virus (Avinew®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) was given in drinking water at 16 DO.

2.3. Serum Sampling and Serological Indices of Immune Mediators

Serum samples were collected from the wing veins of 10 chickens at 12, 24, and 48 h post-vaccination (hpv) and 4-, 11-, 21-, and 31-days post-vaccination (dpv) with H9N2 for the detection of serological responses as follows:

2.3.1. Phagocytic Index Assay

The CytoSelect™ 96-well phagocytosis assay (red blood cell–substrate, Cell Biolabs Inc., San Diego, CA, USA) was used for the detection of the phagocytic index or opsonic activity assay in the collected serum samples according to Yu et al. [21].

2.3.2. Nitric Oxide (NO)

It was determined using the colorimetric assay kits (Elabscience®, Houston, TX, USA), which depend on the nitrate reduction test [22]. All procedures were applied according to manufacturer instructions, and the geometric mean titers (GMTs) were calculated.

2.3.3. Lysozyme (LYZ)

It was determined using lysozyme activity kits (Flourometric®; ab211113, Abcam, Cambridge, UK) at a wavelength of 360–455. All procedures were applied according to the manufacturer's instructions, and the GMTs were calculated [23].

2.3.4. Interleukin-1β (IL-1β) and Interleukin-8 (IL-8)

Interleukin levels were determined using highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) kits based on the principle of the double-antibody sandwich technology for chicken IL-1β and chicken IL-8 (Abbexa-Ltd®, Cambridge, UK). The optical density (OD) was measured spectrophotometrically at a 450 nm wavelength, and the GMTs

of IL-1β and IL-8 were calculated [24]. All procedures were applied according to the manufacturer's instructions.

2.3.5. Interleukin-6 (IL-6)

It was determined using Invitrogen® ELISA kits coated with a chicken IL-6-specific antibody (Life Technologies Ltd., Paisley, UK). The optical density was measured at a wavelength of 450 nm. The GMTs of IL-6 were calculated according to the manufacturer's instructions [25].

2.3.6. Humoral Immunity Evaluation Through Hemagglutination Inhibition Test

The hemagglutination inhibition (HI) titers against LPAI-H9N2 in the collected serum samples were determined using a standard H9N2 antigen (Royal GD, Deventer, The Netherlands) with hemagglutination titers of 8 $\log_2$. A two-fold serial dilution of serum against four hemagglutinating units of antigen was applied, and the geometric mean HI titers (GMTs) were calculated [26].

2.4. Gene Expression Analysis of Immune Cell Signaling (IFN-γ and TLR-21) Using Quantitative Reverse Transcriptase–Polymerase Chain Reaction (qRT-PCR)

Ten birds were sacrificed humanely through cervical dislocation after the intravenous injection of sodium pentobarbital with a dose of 50 mg/kg, and their spleens were collected at 31 dpv for gene expression. The collected spleens were homogenized in phosphate-buffer saline (pH 7.2) containing gentamycin (50 µg/mL) and mycostatin (1000 units/mL) in a 1:5 (*w*/*v*) dilution [26].

The total RNA was extracted by the RNeasy Mini Kit then SYBR® Green master mix kits were used in accordance with the manufacturer's instructions (QuantiTect, Qiagen, Germantown, MD, USA), in addition to specific primers for interferon-gamma (IFN-γ), foreword CAAGTCAAAGCCGCACATC and reverse CGCTGGATTCTCAAGTCGTT [27] and specific primers for Toll-like receptor-21 (TLR-21), forward CAAGAAGCAGCGGGAGAAG and reverse TCAGGATGCGGTTAAAGCG [28]. The thermocycling qRT-PCR conditions were 50 °C for 30 min, 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s and 40 s at 62 °C. Amplification curves and Ct values were determined by stratagene MX3005P software version 4.10. To quantify gene expression on the RNA of the different samples, the Ct of each sample was compared with the control G3 samples according to the "$2^{-\Delta\Delta Ct}$" method stated in [29].

2.5. Statistical Analysis

Graphpad Prism 5 was used to analyze all data using one-way ANOVA, and Tukey's post hoc test was used to determine the significant differences between groups at ($p < 0.05$).

3. Results

3.1. Detection of Phagocytic Activity Mediators (Nitric Oxide, Lysozyme, Interleukin-1β, Interleukin-6, Interleukin-8

This study indicated a gradual significant increase in the phagocytic activity of chickens in G1 (received vaccine 1) from 2 $\log_{10}$ at 48 hpv to 3, 5, and 6 and $\log_{10}$ at 4, 11, and 21 dpv, respectively. The phagocytic activity was associated with the detection of phagocytic mediators such as NO in the serum of chickens in G1 at significantly ($p < 0.05$) higher concentrations compared to G2 and G3. The GMTs of NO in serum of chickens in G1 ranged from 195 to 555.33 µmol/mL in comparison to 50–300 µmol/mL in chickens of G2 and G3 at 12 hpv to 31 dpv, respectively (Figure 1A). For LYZ, the GMTs quantified in the serum of chickens were significantly high in G1 ($p < 0.05$), starting from 122.67 to 682.33 ng/mL compared to 50–220 ng/mL in the other two groups at the same times (Figure 1B). The GMTs of IL-1β in G1 vaccinated chickens were 412–333 ng/mL (Figure 1C), and the GMTs of the proinflammatory IL-6 in G1 vaccinated chickens were 352.33–313.33 ng/mL during 31 dpv (Figure 1D). Also, the GMTs of IL-8 in G1 vaccinated chickens were 257.67–720 ng/mL com-

pared to 80–310 ng/mL in G2 vaccinated chickens and 80–260 ng/mL in G3 non-vaccinated chickens throughout the 31 dpv (Figure 1E).

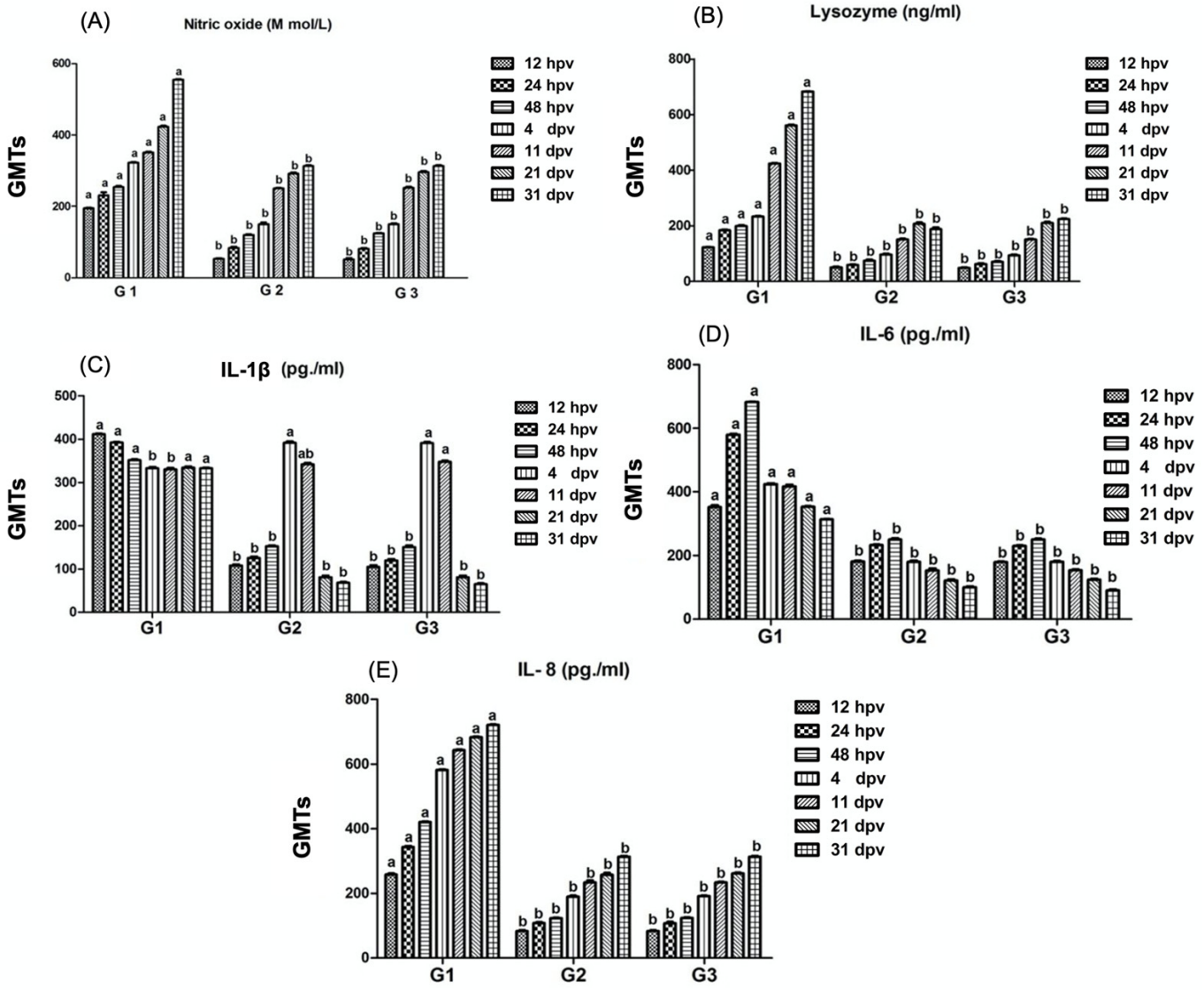

Figure 1. Results of geometric mean titers (GMTs) for NO (**A**), LYZ (**B**), IL-1β (**C**), IL-6 (**D**), and IL-8 (**E**) at 12, 24, and 48 hpv and 4, 11, 21, and 31 dpv with H9N2 (Group 1—H9 vaccine 1; Group 2—H9 vaccine 2; Group 3—non-vaccinated control). The significant differences ($p < 0.05$) between groups are referred as a, ab and b.

3.2. Detection of Humoral Immunity Evaluation Through Hemagglutination Inhibition Test

The geometric mean titer results of HI revealed a non-significant difference between all three groups at 12, 24, and 48 hpv and 4 dpv. The significance of difference began at 11 dpv with the highest titers ($p < 0.05$) at 6.7, 7, and 6.7 $\log_2$ in chickens from G1 followed by 4.3, 4.7, and 4.3 $\log_2$ in G2 in comparison to 3.3, 3.3, and 2.3 $\log_2$ in G3 (Figure 2).

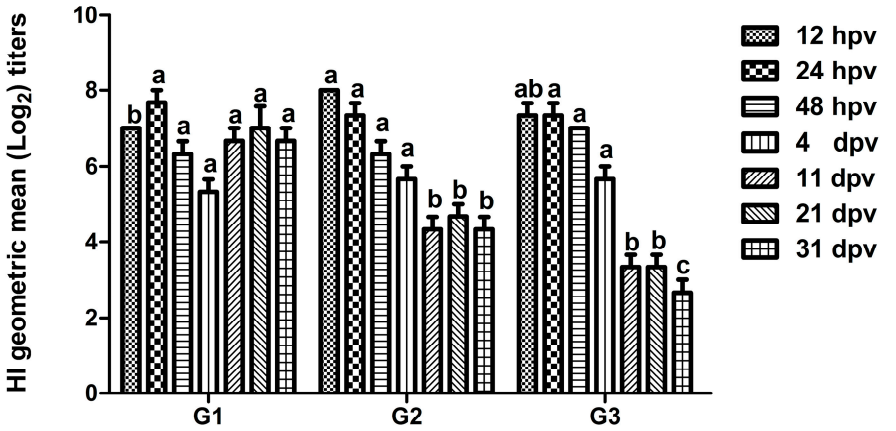

Figure 2. Geometric mean titer results of HI in serum samples of all chicken groups collected at 12, 24, and 48 hpv and 4, 11, 21, and 31 dpv with H9N2 (Group 1—H9 vaccine 1; Group 2—H9 vaccine 2; Group 3—non-vaccinated control). The significant differences ($p < 0.05$) between groups are referred as a, b, ab and c.

3.3. Results of Gene Expression of Immune Cells Signaling (IFN-γ and TLR-21)

Regarding the results of gene expression for interferon-gamma (IFN-γ) and Toll-like receptors-21 (TLR-21) in all splenic samples collected at 31 dpv, the detected Ct values of qRT-PCR for IFN-γ were 24.97, 35.1, and 35.9 (0.456 fold change), while the Ct values of TLR-21 were equal to 25.51, 38.17, and 38.31 (0.568 fold change) in G1, G2, and G3, respectively, which indicated significant ($p < 0.05$) lower Ct values for IFN-γ and TLR-21 in chickens from G1 compared to G2 and G3, indicating the significant ($p < 0.05$) upregulation of both genes in G1 (Figure 3A,B).

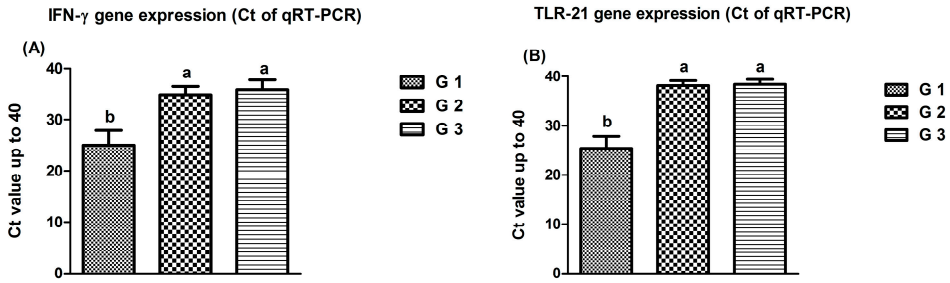

Figure 3. Results of IFN-γ (**A**) and TLR-21 (**B**) gene expressions (qRT-PCR) in splenic samples of all chicken groups collected at 31 dpv with H9N2 (Group 1—H9 vaccine 1; Group 2—H9 vaccine 2; Group 3—non-vaccinated control). The significant differences ($p < 0.05$) between groups are referred as a and b.

4. Discussion

LPAI-H9N2-inactivated whole virus vaccines have been widely used in poultry farms in several countries, such as Asia and Africa, including Egypt [12–14,17,30,31]. Although these inactivated H9N2 vaccines have been shown to be effective in reducing clinical disease and production losses, they fail to control the spread of the virus [32,33]. Consequently, there is a growing need to develop alternative vaccination strategies and systems for better control of LPAI-H9N2 spreading in poultry [34–36].

In this study, we investigated the innate and humoral immune response induced by imported water in an oil emulsion-inactivated vaccine against LPAI-H9N2 prepared in combination with PAMP technology (Nobilis® H9N2-P) in the broilers of G1 and an autogenous oil emulsion-inactivated H9N2 vaccine (locally prepared) in broilers from G2. The results showed a gradual significant increase in phagocytic activity, and phagocytic mediators such as NO, LYZ, proinflammatory cytokines (IL-1β and IL-6), and the chemoattractant cytokine

IL-8, which attracts and activates neutrophils in inflammatory regions, after vaccination in chickens from G1 compared to the vaccinated G2 and the non-vaccinated G3. Also, the gene expressions of IFN-γ and TLR-21 in spleen samples collected at 31 dpv were significantly up-regulated in G1 compared to G2 and G3. Previously, Shrestha et al. [37] reported a positive correlation between the improvement in the IFN-γ, IL-6, IL-1β, IL-4, and CxCLi2 mRNA expression levels in chicken splenocytes in vitro and the inhibition of virus shedding after the H9N2 challenge in specific pathogen-free (SPF) chickens when the HA antigen was fused to single-chain fragment variable (scFv) antibodies specific for the cluster of differentiation 83 (CD83) receptor. Also, Kaiser et al. [38] mentioned that the expression levels of the key inflammatory mediators or proinflammatory cytokines (IL-6 and IL-1β) are directly correlated with influenza virus replication as well as respiratory and systemic symptoms of the disease and concluded that IFN-γ is a potential inhibitor of influenza replication.

In contrast to adaptive immunity, innate immunity is immediately involved as the first line of defense against pathogens that infect birds, resulting in the limitation or mitigation of the infectious process. The recognition of PAMPs and DAMPs is mainly mediated by PRRs, which are mainly expressed on the surface or in the cytoplasm of macrophages, dendritic cells (DCs), monocytes, and non-immune cells (e.g., fibroblasts, endothelial and mucosal epithelial cells) [39]. Following the initial identification of the virus by innate receptors, a rapid stimulation of antiviral effector responses, such as IL-6, IL-1β, and TNF-a, from macrophages and monocytes [40], together with the secretion of type I IFNs, in particular DCs, thereby facilitated the stimulation of adaptive immunity [41,42], which is very important for viral clearance, although resistance to H9N2 viral invasion is mediated by innate immunity, and is a complex immune process involving multiple cell systems, including DCs, B, and T cells, macrophages, natural killer cells, and cytokines [19]. Although the effective enhancement of immunity in vaccine 1 could not only be attributed to PAMPs, there are several factors that contribute to a higher immune response between some inactivated vaccines, such as the potency, immunogenicity, and ability to interact with bird immunity to produce a higher antibody production of seed strains; a higher antigenic mass in the vaccine; and a type of oil or adjuvant [19]. Here, in this study, both viruses have the same antigenic mass of 8 $\log_2$ hemagglutination units (HA).

Cheng et al. [43] reported that the stimulator of IFN genes (STINGs) acts synergistically with melanoma differentiation-associated gene 5 (MDA5) to form the MDA5-STING-IFN-β pathway, conferring a potent antiviral state against RNA viruses (i.e., H9N2), complementing the absence of the retinoic acid-inducible gene I (RIG-I) in chickens. Furthermore, this STING activates both the NF-κB and IFN regulatory factor 7 (IRF-7) pathways to induce type I IFN and IFN-stimulated genes (ISGs). In addition, TLRs are considered one of the four major families of PRRs in chickens [44]. Wang et al. [45] observed an oviductal upregulation of site-specific TLR-3, TLR-7, TLR-21, and MDA5 in H9N2-infected laying hens. The results in this study confirm the upregulation of TLR-21 following significant H9N2P-HA antigen stimulation, especially in chickens of G1. Chicken TLR-21 is a molecule that enhances antibody-mediated responses as a single ligand against the H4N6 avian influenza virus in chickens, as previously shown by Singh et al. [46]. All these results highlight the importance of the innate immune response, especially during the first 2–3 weeks of life in broilers, as it is well documented that the adaptive immune system is not fully developed at hatching and that functional T- and B-cell responses are observed after approximately 2 to 3 weeks of life [47,48].

The serological response to H9N2 vaccination in broilers in this study was measured by the HI test and revealed a non-significant difference between all three groups from 12 h to 7 dpv, which confirmed the role of maternal-derived antibodies (MDAs) in delaying the immune response to the single vaccination with inactivated H9N2 vaccines [37]; however, a significant difference in GMTs began at 11 dpv in chickens from G1, earlier than G2, and continued to 31 dpv with highest significant ($p < 0.05$) in comparison to G2 and G3, indicating that MDA is more quickly overcome in chickens of G1, which could be

attributed to the triggering of humoral immunity through the stimulated innate and Th1 immune types. Interestingly, the GMTs of HI in both G1 and G2 were protective and more than 4 $\log_2$ [49], from 11 to 31 dpv, with significantly higher titers in chickens from G1 (6.7, 7 and 6.7 $\log_2$) than G2 (4.3, 4.7 and 4.3 $\log_2$). These significantly higher and earlier titers in chickens of G1 vaccinated broilers with PAMP-H9N2 eliminate the need for further vaccination in broilers as applied in some countries such as China, in which three vaccinations during 45 days of age are applied based on surveillance data collected from several poultry farms in order to overcome MDAs and to have high protective titers, as mentioned by Pan et al. [50]. Earlier findings recorded high immunogenicity and protection from a single administration of the oil-based-inactivated LPAI-H9N2 vaccine in specific pathogen-free chickens but not commercial broilers with high MDAs [16,51,52]. Recently, Elbestawy et al. [53] reported that the H9N2-P vaccination in broiler chickens received along with live Newcastle vaccines induced higher protection against the velogenic Newcastle disease genotype VII.1.1 challenge than the live vaccine alone as the birds exhibited milder lesions and a lower and shorter viral shedding rate. The current study was applied in broilers to simulate the field situation, and all three chicken groups received the same routine vaccinations except for H9N2; however, future studies will be applied in SPF chicks to exclude any possibility for cross-reactivity between the immune response and a routine vaccine.

5. Conclusions

The obtained results indicate the rapid and potent action of innate [phagocytic activity, nitric oxide, lysozyme, cytokine or interleukin signaling (IL-1β, IL-6, IL-8) and gene expression of IFN-γ and TLR-21] and humoral immunity in PAMP-H9N2-vaccinated compared to inactivated H9N2-vaccinated commercial broilers in G2 and non-vaccinated controls in G3. The current study demonstrates that immunogenicity and early high response to the whole H9N2 virus vaccine depends not only on the antigen and epitope similarity but also the innovative technology of vaccine manufacturing, such as pathogen-associated molecular patterns (PAMPs), which could play another important role. Further studies using these vaccines to investigate their protective efficacy against an early LPAI-H9N2 challenge should be applied in both SPF and commercial broilers.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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