

Modulation of systemic and mucosal immunity against an inactivated vaccine of Newcastle disease virus by oral co-administration of live attenuated *Salmonella enterica* serovar Typhimurium expressing chicken interleukin-18 and interferon- α

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ABSTRACT. Newcastle disease (ND) is a highly contagious disease of chickens causing significant economic losses worldwide. Due to limitations in the efficacy against currently circulating ND viruses, existing vaccination strategies require improvements, and incorporating immunomodulatory cytokines with existing vaccines might be a novel approach. Here, we investigated the systemic and mucosal immunomodulatory properties of oral co-administration of chicken interleukin-18 (chIL-18) and chicken interferon- α (chIFN- α) using attenuated *Salmonella enterica* serovar Typhimurium on an inactivated ND vaccine. Our results demonstrate that oral administration of *S. enterica* serovar Typhimurium expressing chIL-18 or chIFN- α provided enhanced systemic and mucosal immune responses, as determined by serum hemagglutination inhibition antibody and NDV Ag-specific IgG as well as NDV Ag-specific IgA in lung and duodenal lavages of chickens immunized with inactivated ND vaccine via the intramuscular or intranasal route. Notably, combined oral administration of *S. enterica* serovar Typhimurium expressing chIL-18 and chIFN- α significantly enhanced systemic and mucosal immunity in ND-vaccinated chickens, compared to single administration of *S. enterica* serovar Typhimurium expressing chIL-18 or chIFN- α . In addition, oral co-administration of *S. enterica* serovar Typhimurium expressing chIL-18 and chIFN- α provided enhanced NDV Ag-specific proliferation of peripheral blood mononuclear cells and Th1-biased cell-mediated immunity, compared to single administration of either construct. Therefore, our results provide valuable insight into the modulation of systemic and mucosal immunity by incorporation of immunomodulatory chIL-18 and chIFN- α using *Salmonella* vaccines into existing ND vaccines.

KEY WORDS: chicken interferon- α , chicken interleukin-18, mucosal immune response, Newcastle disease virus

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Newcastle disease (ND) is a notifiable disease to the Office International des Epizooties (OIE), as it causes biosecurity obstacles in the trade of avian products among countries [28]. This disease has a global distribution with a wide host range in which all orders of birds tested have been reported to be infected by NDV [20]. Current NDV vaccination programs include the use of either attenuated live-virus vaccines or inactivated vaccines to induce protective immunity while producing minimal adverse effects in birds. All vaccines and vaccination routes have their advantages and disadvantages, which have been reviewed previously [37]. Nevertheless, these vaccines induce better protection against viruses that were isolated in past epizootics, but do not effectively stop viral excretion, which is critical for controlling the spread of the disease [39]. Another limitation to their efficacy is that current vaccines do not provide better protection against

currently circulating viscerotropic virulent ND viruses [21, 39]. Therefore, research is emerging to develop improved vaccination strategies that can eliminate viral shedding upon infection with currently circulating virulent viruses in addition to reduce morbidity and mortality of chickens.

The use of chicken cytokines for disease prevention is becoming more feasible with the recent cloning and addressing the immunomodulatory and protective functions of a number of cytokines [19, 30, 31]. Chicken interferon- α (chIFN- α) belongs to the type I IFNs that play an essential role in the host antiviral response by stimulating the T-dependent lymphocyte system and inducing numerous IFN-stimulated genes (ISGs) [35]. Interleukin-18 (IL-18), originally known as interferon- γ (IFN- γ)-inducing factor, provides an important link between the innate and adaptive immune responses by promoting IFN- γ production and thereby inducing Th1 immune responses [1]. Thus, a combined oral administration of these two cytokines using a suitable delivery vector along with existing vaccines may be able to elicit better protection through an enhanced immunity to a specific pathogen. The ability of attenuated *Salmonella* vaccines to induce both cellular and humoral immunity in both mucosal and systemic compartments [8, 40] makes them good candidates to deliver heterologous antigens. These live attenuated *Salmonella*

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vaccines particularly mimic the natural infection of most mucosal pathogens, such as avian influenza virus (AIV) and NDV, which infect their host through mucosal surfaces. In our previous studies, we demonstrated that combined oral administration of attenuated *S. enterica* serovar Typhimurium (*Salmonella* Typhimurium) expressing chIL-18 and chIFN- α has enhanced protective efficacy against AIV H9N2 challenge [32] which was mounted through modulating systemic immune responses elicited by an AIV H9N2 killed vaccine [31]. Thus, it was anticipated that oral co-administration of attenuated *Salmonella* Typhimurium expressing chIL-18 and chIFN- α might enhance systemic as well as mucosal immune responses against NDV vaccine. Therefore, this study was designed to evaluate whether oral co-administration of *Salmonella* Typhimurium expressing chIL-18 and chIFN- α can modulate systemic and mucosal immune responses elicited by inactivated NDV (B1 strain) vaccine administered via the systemic or mucosal route.

MATERIALS AND METHODS

Ethics statement: Specific pathogen-free (SPF) White Leghorn layer chickens were purchased from OrientBio (Seongnam-Si, Korea) and reared on a formulated commercial feed with water provided *ad libitum* throughout the experimental period. The handling of animals in the study and specific experiments were performed in accordance with the requirements of the Animal Care and Ethics Committees of Chonbuk National University. The animal facility of Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Virus propagation and inactivation: NDV (B1 strain) used for the vaccination experiments was kindly provided by the National Veterinary Research and Quarantine Service of the Republic of Korea. The virus propagation was performed using 10-day-old embryonated eggs. Allantoic fluid was harvested 96 hr after inoculation of virus into the allantoic cavity of embryonated eggs in each case, and the infectious viral titer was determined using 10-day-old embryonated eggs. The inactivated vaccine was prepared by mixing 40% formalin and allantoic fluid with a titer of 2^9 of NDV in a ratio of 1:40, that is, formalin to virus. The preparation was kept at room temperature (24°C to 26°C) for 24 hr before use. The inactivation of the virus was confirmed through inoculation of embryonated eggs.

Construction of attenuated *Salmonella* Typhimurium expressing chIFN- α and chIL-18: Attenuated *Salmonella* Typhimurium expressing chIFN- α and chIL-18 (χ 8501/chIFN- α and χ 8501/chIL-18) was constructed by cloning chIFN- α and chIL-18 genes, as described previously [32]. Attenuated *Salmonella* Typhimurium χ 8501 (*hisG Acrp-28 Δ asdA16*) was used as the host bacteria to deliver the chIFN- α and chIL-18 proteins using the pYA3560 Asd⁺ and pYA3493 Asd⁺ plasmid vectors. pYA3560 Asd⁺ plasmid was derived from pYA3493 Asd⁺ plasmid by changing pBR ori gene (origin of replication of pBR322 plasmid) with p15A ori gene (origin of replication of p15A plasmid) to maintain stably in bacteria. Phosphate-buffered saline (PBS,

pH 7.4) containing 0.01% gelatin was used to resuspend the attenuated *Salmonella* bacteria that were concentrated by centrifugation at $7,000 \times g$ at 4°C for 5 min after growing in Luria-Bertani (LB) broth for 24 hr at 37°C.

Animal experimental designs for ND vaccination: Forty-eight SPF chickens (22-day-old) were divided randomly into five groups. The first group ($n=6$) was the negative control orally administered vehicle (PBS containing 0.01% gelatin) without *Salmonella* Typhimurium expressing chIFN- α or chIL-18. The second group ($n=6$) was orally administered *Salmonella* Typhimurium harboring the pYA3560 vector (10^9 cfu/chicken) as a control for the empty pYA3560 vector. The remaining three groups ($n=12$ per group), each comprised of two replications ($n=6$ per replication) for the two different doses, were orally administered either *Salmonella* Typhimurium expressing chIL-18 (10^9 and 10^{11} cfu/chicken) or chIFN- α (10^9 and 10^{11} cfu/chicken) or both in combination (10^9 and 10^{11} cfu/chicken). The 25-day-old chickens from all groups, except the negative control, were vaccinated intramuscularly (i.m.) with formalin inactivated NDV (B1 strain) vaccine ($10^{6.0}$ EID₅₀ per dose) 3 days after treatment. Chickens receiving a primary vaccination were boosted using the same protocol 7 days later. Another experiment was performed using the same experimental design with the exception of vaccinating chickens with NDV (B1 strain) via the intranasal (i.n.) route ($10^{8.0}$ EID₅₀ per dose).

Sample collection: Blood samples (3.0 ml per chicken) were collected 7 days after the primary vaccination and 7 and 14 days after booster vaccinations and allowed to clot at 37°C for 2 hr prior to collect serum. Serum was separated by centrifugation and stored at -20°C before use. Peripheral blood mononuclear cells (PBMCs) were prepared from the blood of vaccinated chickens using OptiPrep™ (13.8% iodixanol) 14 days post-booster vaccination, according to the manufacturer's instructions (Axis-Shield, Oslo, Norway). To evaluate mucosal immunity, chickens were euthanized 14 days post-booster vaccination, and lung and duodenal lavages were collected. Lung lavages were collected as described previously [15] using a special lung lavage device consisted of 15 cm of 1/16 in internal diameter, 1/8 in outer diameter TygonJ tubing (Fisher Scientific, Norcross, GA, U.S.A.) inserted 1 cm into a second 15 cm TygonJ tubing (1/8 in internal diameter and 3/16 in outer diameter) that was attached to a syringe containing 10 ml lavage solution (1 M Tris/glycine buffer with 0.25% Tween 20, pH 7 to 8). The narrow diameter tubing was inserted down the trachea to the syringe; air was withdrawn from the lungs through the syringe, and the lavage fluid was administered slowly into the lung and then withdrawn. Blood-tinged samples were routinely discarded. Processing of the lung lavage sample entailed centrifugation for 5 min at $12,000 \times g$ and freezing the supernatants at -20°C until use for the assay. Duodenal lavages were derived from 5 cm duodenum by lavage with 5 ml cold 1% BSA/PBS containing 0.1 mg/ml soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, U.S.A.).

Hemagglutination inhibition (HI) assay: HI test was performed using NDV B1 antigen to determine NDV specific HI antibody titers in the sera of vaccinated chickens follow-

ing previously described method [30]. The geometric means of serum HI antibody titers obtained from each group were defined as the reciprocal logarithm in a base of 2 of the highest serum dilutions that completely inhibited agglutination.

Enzyme-linked immunosorbent assay (ELISA) for detecting NDV-specific systemic and mucosal antibodies: A standard ELISA was used to determine NDV (B1 strain)-specific antibody levels in serum, lung and duodenal lavage fluids [total immunoglobulin G (IgG), and lung and duodenal IgA]. Briefly, ELISA plates were coated overnight at 4°C with partially purified NDV (B1 strain) antigen in 0.1 M bicarbonate buffer (pH 9.6) and goat anti-chicken IgG or goat anti-chicken IgA (Bethyl Laboratories, Inc., Montgomery, TX, U.S.A.) in standard wells. The plates were washed three times with PBS-0.05% Tween 20 and blocked with 3% nonfat-dehydrated milk. The sera and lavage samples were initially diluted 50-fold and 20-fold, respectively, followed by a two-fold serial dilution, incubated for 2 hr at 37°C and then incubated with either horseradish peroxidase-conjugated goat anti-chicken IgG or horseradish peroxidase-conjugated goat anti-chicken IgA (Bethyl Laboratories) for 1 hr. Prior standardization of sample (sera and lavage) dilution was done before starting the final experiment. The color was developed by adding a suitable substrate (11 mg of 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid in a mixture of 25 ml of 0.1 M citric acid, 25 ml of 0.1 M sodium phosphate and 10 μ l of H₂O₂). The concentration of NDV (B1 strain)-specific antibodies was determined using a Spectra MAX340 automated ELISA reader and the SOFTmax Pro4.3 program (Molecular Devices, Sunnyvale, CA, U.S.A.).

Cell proliferation assay to determine NDV-specific proliferation of PBMCs: In both the experiments, antigen-specific proliferation of PBMCs was assessed by measuring viable cell ATP bioluminescence as described previously [33]. Briefly, PBMCs were prepared and cultured together with stimulator cells at three different ratios. Enriched autologous PBMCs (10⁶ cells/ml) that had been pulsed with ultraviolet (UV)-inactivated NDV B1 antigen (2.5 \times 10² HA units/ml) were used as stimulator cells. Following 72 hr incubation, the proliferated cells were evaluated using a ViaLight[®] Cell proliferation assay kit (Cambrex Bio Science, Rockland, ME, U.S.A.) according to the manufacturer's instructions. PBMC stimulators that were not pulsed with UV-inactivated NDV B1 antigen were used as the negative control.

Real-time PCR to determine the mRNA expression of IFN- α and IL-4 in NDV B1 antigen-stimulated PBMCs: To determine mRNA expression levels of chicken IFN- γ (chIFN- γ) and IL-4 (chIL-4) in PBMCs stimulated with specific NDV B1 antigen, real-time PCR analysis was used using a One-Step SYBR[®] qRT-PCR reagent kit (Takara, Otsu, Japan) as described previously [33]. In brief, total RNAs were extracted from the stimulated PBMCs using the total RNA extraction kits (iNtRON Biotech, Inc., Daejeon, Korea) according to the manufacturer's instructions, and RNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA, U.S.A.). Reverse transcription and real-time PCR amplification of targeted genes were carried out with

each 75 ng total RNA as a template in 50 ml of the reaction mixture using specific primer pairs, reaction conditions and temperature cycles, as described previously (Rahman *et al.*, 2011). Each sample was subjected to real-time PCR in duplicate, and the mean Ct value of the duplicates was used for subsequent analysis. The Ct values of the target genes were normalized (Δ Ct) to an average Ct value of a house keeping gene GAPDH, and the relative expression of each target gene was calculated as 2^{- Δ Ct}. Data were analyzed using CFX96[™] manager software version 1.6 (Bio-Rad, Hercules, CA, U.S.A.).

Statistical analysis: All data are expressed as mean \pm standard error, and significant differences were analyzed by the unpaired two-tailed Student's *t*-test for two groups and analysis of variance (ANOVA) and post-hoc tests for multiple mean comparisons. A *P*-value < 0.05 was considered significant.

RESULTS

Oral co-administration of *Salmonella Typhimurium* expressing chIL-18 and chIFN- α enhances systemic immune responses induced by inactivated ND vaccine administered via both systemic and mucosal routes: In order to examine systemic humoral immune responses in NDV (B1 strain)-vaccinated chickens with or without oral co-administration of *Salmonella Typhimurium* expressing chIL-18 and chIFN- α , NDV Ag-specific HI antibody titers and IgG levels in sera were determined at 7 days after primary vaccination and 7 and 14 days after booster vaccination. Significantly enhanced HI antibody titers were observed at all three time points in sera of both χ 8501/chIL-18 and χ 8501/chIFN- α -administered chickens at both doses and both vaccination trials, compared to that of χ 8501 (pYA3560)-treated chickens (Fig. 1). Notably, the combined oral administration of χ 8501/chIFN- α and χ 8501/chIL-18 resulted in significantly enhanced HI antibody titers in sera of ND-vaccinated chickens at both doses and both vaccination trials, compared to those in single administration of χ 8501/chIFN- α or χ 8501/chIL-18. Serum IgG level specific for NDV Ag was determined by standard ELISA. Similar to the HI antibody titers, NDV Ag-specific IgG levels were significantly enhanced in the sera of chickens that received χ 8501/chIL-18 or χ 8501/chIFN- α or both, compared to that of χ 8501 (pYA3560)-treated chickens at both doses and both vaccination trials (Fig. 2). Combined oral administration of χ 8501/chIFN- α and χ 8501/chIL-18 (10¹¹ cfu/chicken) resulted in significantly enhanced IgG levels in the sera of ND-vaccinated chickens during both vaccination trials, compared to single administration of χ 8501/chIFN- α or χ 8501/chIL-18. Therefore, these results indicate that oral co-administration of *Salmonella Typhimurium* expressing chIL-18 and chIFN- α produces enhanced systemic humoral immune responses against an ND vaccine administered via systemic or mucosal route.

Oral co-administration of *Salmonella Typhimurium* expressing chIL-18 and chIFN- α enhances mucosal immune responses induced by inactivated ND vaccine administered via both systemic and mucosal routes: To evaluate mucosal

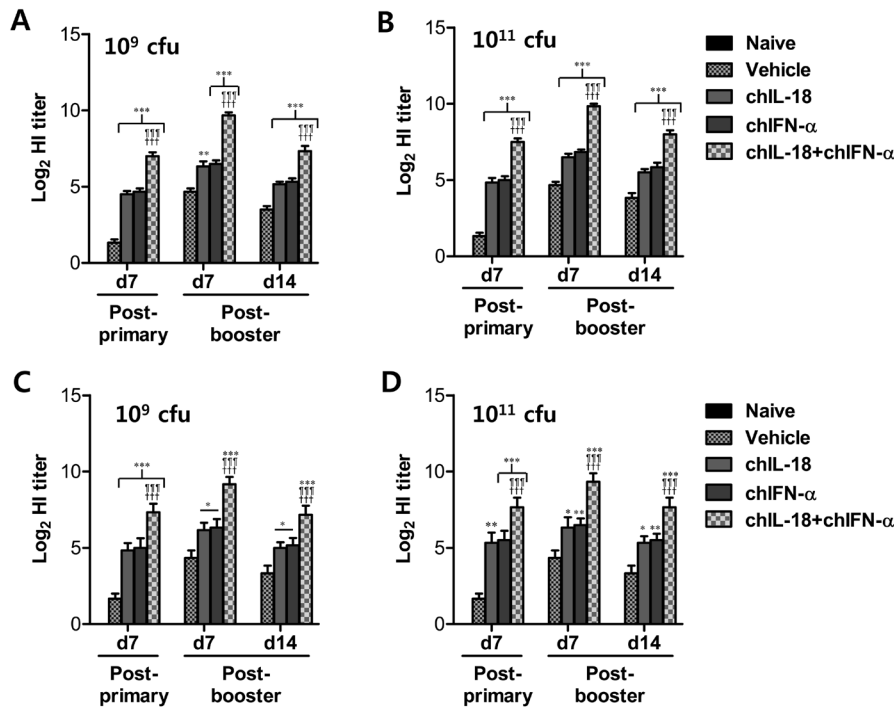


Fig. 1. Serum NDV-specific HI titers in chickens immunized via systemic and mucosal routes following co-administration of live attenuated *Salmonella* Typhimurium expressing chIFN- α and chIL-18. Serum samples were collected from immunized chickens 7 days after primary vaccination and 7 & 14 days after booster vaccination and subjected to the HI test. (A and B) HI antibody titers in chickens immunized with inactivated NDV vaccine via i.m. route. (C and D) HI antibody titers in chickens immunized with inactivated NDV vaccine via i.n. route. Data are expressed as reciprocal log₂ of the geometric average and standard error of HI titers obtained from six chickens per group. * P <0.05; ** P <0.01; *** P <0.001 compared to vehicle group that was treated with control bacteria. **** P <0.001 compared to chIL18 and ††† P <0.001 compared to chIFN- α .

humoral immune responses, mucosal IgA levels specific for NDV Ag in lung and duodenal lavages were measured by standard ELISA. Significantly enhanced NDV Ag-specific IgA levels were detected in the lung and duodenal lavages of chickens that received χ 8501/chIL-18 or χ 8501/chIFN- α or both, compared to that of χ 8501 (pYA3560)-treated chickens at both doses and both vaccination trials (Fig. 3). Combined oral administration of χ 8501/chIFN- α and χ 8501/chIL-18 (10^{11} cfu/chicken) resulted in significantly enhanced NDV Ag-specific IgA levels in both lung and duodenal lavages of ND-vaccinated chickens at both vaccination trials, compared to single administration of χ 8501/chIL-18 or χ 8501/chIFN- α . Taken together, these results indicate that oral co-administration of *Salmonella* Typhimurium expressing chIL-18 and chIFN- α stimulates enhanced mucosal humoral immune responses against an ND vaccine, along with systemic immune responses.

Oral co-administration of Salmonella Typhimurium expressing chIL-18 and chIFN- α enhances Th1-biased immunity against inactivated ND vaccine administered via both systemic and mucosal routes: To evaluate cellular immune responses, NDV-antigen specific proliferation of PBMCs prepared from immunized chickens was measured. PBMCs of chickens that received χ 8501/chIFN- α or χ 8501/chIL-18 (10^9 and 10^{11} cfu each per chicken) orally prior to

ND vaccination via the i.m. route had significantly enhanced proliferation upon NDV Ag-specific stimulation, compared to PBMCs from chickens that received χ 8501/pYA3560 (vehicle) (Fig. 4A and 4B). In particular, oral co-administration of χ 8501/chIFN- α and χ 8501/chIL-18 produced markedly enhanced proliferation of PBMCs upon NDV Ag-specific stimulation, compared to that of a single administration of χ 8501/chIL-18 or χ 8501/chIFN- α . In addition, the mRNA expression levels of IFN- γ and IL-4 in PBMCs were determined by real-time PCR following stimulation with NDV Ag. Both IFN- γ and IL-4 mRNA levels in PBMCs prepared from chickens that received a single administration of χ 8501/chIFN- α or χ 8501/chIL-18 (10^9 and 10^{11} cfu) were significantly enhanced, compared to chickens that received *Salmonella* Typhimurium harboring the empty pYA3560 vector. A markedly enhanced effect on IFN- γ and IL-4 mRNA expression in PBMCs prepared from χ 8501/chIFN- α plus χ 8501/chIL-18-co-administered chickens was also observed (Fig. 4C and 4D). More importantly, IFN- γ mRNA expression was more significantly up-regulated than IL-4 mRNA following a single administration of χ 8501/chIFN- α or χ 8501/chIL-18, and co-administration of χ 8501/chIFN- α and χ 8501/chIL-18 induced more up-regulated levels of IFN- γ mRNA than single administration of the constructs. Similar cell-mediated immune responses were observed in

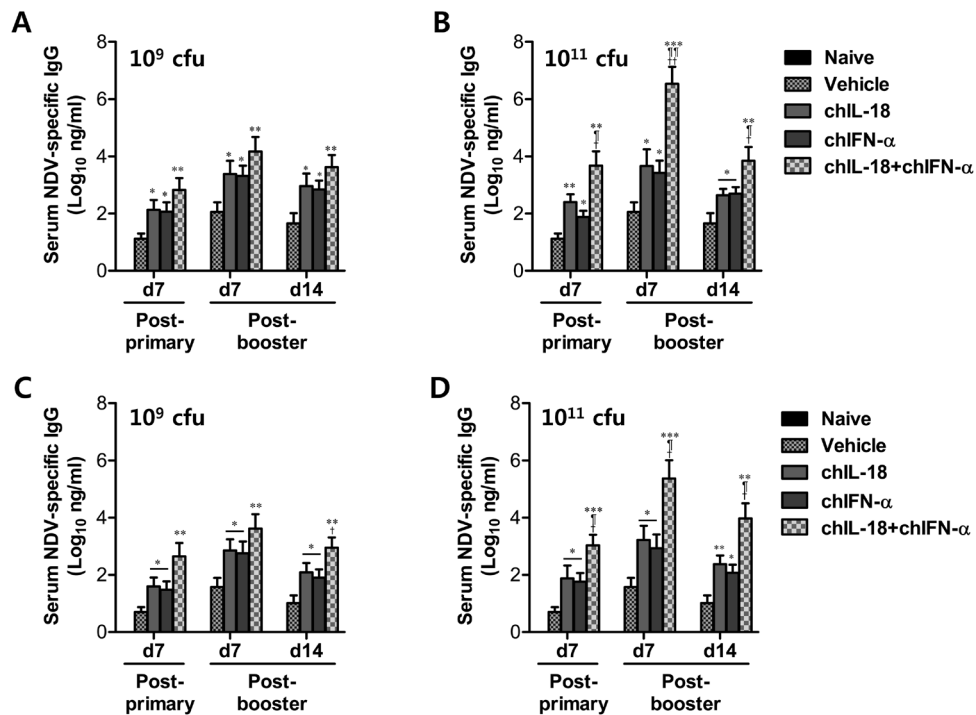


Fig. 2. Serum NDV-specific IgG levels in chickens immunized via the systemic and mucosal routes following oral co-administration of live attenuated *Salmonella* Typhimurium expressing chIFN- α and chIL-18. (A and B) IgG levels in chickens immunized with inactivated NDV vaccine via i.m. route. (C and D) IgG levels in chickens immunized with inactivated NDV vaccine via i.n. route. Data represent the mean and standard error derived from six chickens per group. * P <0.05; ** P <0.01; *** P <0.001 compared to vehicle group that was treated with control bacteria. † P <0.05; ‡ P <0.01 compared to chIL18 and * P <0.05; ** P <0.01 compared to chIFN- α .

chickens that were orally administered χ 8501/chIL-18 or χ 8501/chIFN- α or both and immunized with inactivated NDV (B1 strain) vaccine via i.n route (Fig 5A–5D). Taken together, these results indicate that oral co-administration of *Salmonella* Typhimurium expressing chIFN- α and chIL-18 induces enhanced Th1-biased immunity against inactivated ND vaccine administered via both systemic and mucosal routes, compared to single administration of *Salmonella* Typhimurium expressing chIL-18 or chIFN- α .

DISCUSSION

With increasing interest in using chicken cytokines as natural alternatives to existing disease control strategies in poultry production systems, it is important to understand the role of chicken cytokines in modulating the host immune system. Enhanced immunomodulation using two or more cytokines with additive or synergistic effects is of spark concern to the end [23, 31]. In the present study, we demonstrated that oral co-administration of live attenuated *Salmonella* Typhimurium expressing chIL-18 and chIFN- α greatly modulated both systemic and mucosal immunity in chickens immunized with inactivated NDV (B1 strain) vaccine via both systemic and mucosal routes, as evident by elevated serum HI antibody titer, serum IgG levels, mucosal IgA levels and enhanced Th1 biased cell-mediated immunity.

Similar to other members of the type I IFN family, chIFN- α performs its antiviral, anti-proliferative and immunomodulatory functions [4, 31–33] through binding receptors (IFNAR1 and IFNAR2) located on the membrane surface of most cells [12] and subsequently induces ISG transcription within cells through downstream signaling via the JAK-STAT pathway. On the other hand, IFN- γ , the only type II IFN, is a signature cytokine of Th1-associated response produced primarily by T lymphocytes (Th1) and natural killer (NK) cells, and IL-18 induces its production. Although IFN- γ is said to give the immune system a Th1 bias, there are reports about its enhancing effect on the humoral response in chickens. Recent reports confirm that cells produce IL-18, which acts synergistically with IFN- α and enhances IFN- γ synthesis after macrophages are infected with influenza virus [27]. Therefore, it is conceivable that oral co-administration of live attenuated *Salmonella* Typhimurium expressing chIL-18 and chIFN- α modulates systemic as well as mucosal immune responses induced by inactivated ND vaccine administered via either a systemic or a mucosal route. Our results are supported by our previous findings that combined oral administration of *Salmonella* Typhimurium expressing chIFN- α and chIL-18 modulates both systemic humoral and Th1-biased cell mediated immunity elicited by an inactivated H9N2 LPAI vaccine [31]. Furthermore, recombinant chIL-18 potentiates both the humoral and cell-

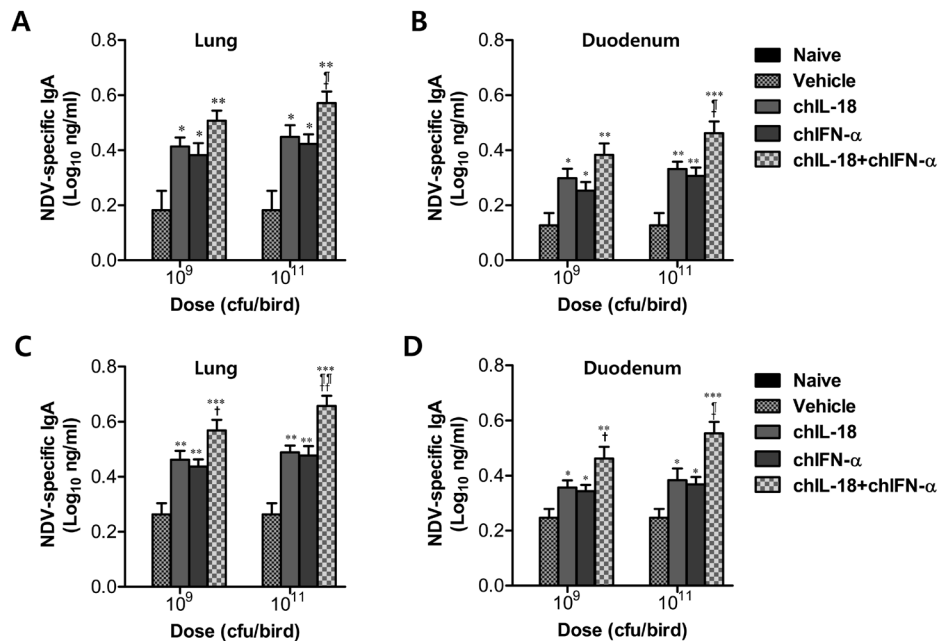


Fig. 3. NDV-specific IgA levels in lung and duodenal lavages of chickens immunized via the systemic and mucosal routes following oral co-administration of live attenuated *Salmonella* Typhimurium expressing chIFN- α and chIL-18. (A and B) IgA levels in lung (A) and duodenal (B) lavages of chickens 14 days after booster vaccination via i.m. route. (C and D) IgA levels in lung (C) and duodenal (D) lavages of chickens 14 days after booster vaccination via i.n. route. Data represent the mean and standard error derived from six chickens per group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to vehicle group that was treated with control bacteria. † $P < 0.05$; †† $P < 0.01$ compared to chIL-18 and ††† $P < 0.05$; †††† $P < 0.01$ compared to chIFN- α .

mediated immune responses against NDV vaccine [11, 30, 38] which also supports our findings.

Vaccination using non-virulent NDV strains protects susceptible birds against disease, producing an antibody response either locally, systemically or both. Several studies have reported that application of attenuated live virus on mucosa induces both systemic and local immunity, whereas parenteral immunization with inactivated vaccine generally induces systemic immunity [9, 16, 24]. In our present study, we detected significantly enhanced serum HI antibody titers and serum IgG levels as well as mucosal IgA levels in duodenal and lung lavages of chickens that received *Salmonella* Typhimurium expressing either chIFN- α or chIL-18 or both along with inactivated NDV (B1 strain) vaccines, compared to chickens that received only inactivated NDV vaccine via a systemic or mucosal route. We believe that NDV-specific mucosal IgA antibodies are produced locally, as IgA secreting cells are detected in the trachea, lungs and the lamina propria of the duodenum, cecal tonsils and cecum of chicken from the 2nd week of life [13, 17] and are suggested to be the prime source of IgA in lung and digestive lavages. It is well established that upon invading mucosal surfaces, *Salmonella* bacteria are capable of surviving and replicating inside antigen-presenting cells (dendritic cells and macrophages) [36], which facilitate the continual processing and presentation of foreign antigens that they are carrying to the immune system; thereby, provoking mucosal as well as systemic immune responses [10]. The immunomodulatory roles of con-

tinuously expressed chIFN- α or chIL-18 by live attenuated *Salmonella* Typhimurium might be the cause of achieving enhanced systemic humoral and mucosal immunity in NDV-vaccinated chickens that received *Salmonella* Typhimurium expressing either chIFN- α or chIL-18. At mucosal compartments, expected B cell immunity comprises mainly secretory IgA, whereas the serum IgG immune response is expected in the systemic compartments [8, 25, 40]. Additionally, live attenuated *Salmonella* bacteria are immunogenic to the host immune system as they are capable of inducing host non-specific innate immunity [26]; thus, influencing the nature of specific adaptive immunity to specific vaccine antigens.

Both mucosal and systemic immunity play a role in the protective immune response against NDV. Locally produced IgA antibodies are an essential part of the antigen-specific defense against mucosal pathogens, such as NDV. In particular, local immunity is provided within the upper respiratory and intestinal tract of chickens by IgA production after a vaccination [7]. The effectiveness of IgA is attributed to its ability to achieve both immune protection and pathogen exclusion in a non-inflammatory manner [6]. Immune exclusion involves blocking access of the pathogenic organism to the mucosa to minimize inflammatory immune responses. Local antibodies on the mucosal surface of the respiratory tract play an important role in not only protecting challenged birds but also limit of primary replication and shedding of virus at the portal of entry. Although IgA is the chief antibody responsible for humoral protection on mucosal

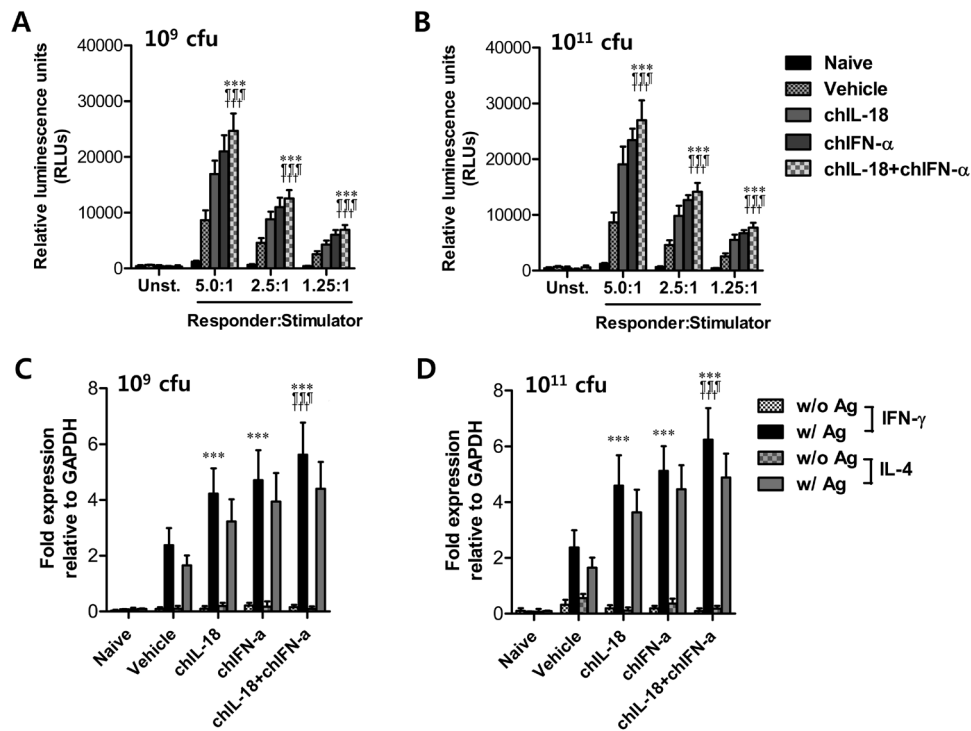


Fig. 4. Enhanced Th1-biased immunity in chickens immunized via the systemic route following oral co-administration of *Salmonella Typhimurium* expressing chIFN- α and chIL-18. (A and B) NDV antigen-specific proliferation of PBMCs. PBMCs were collected 14 days after booster vaccination and used for NDV Ag-specific stimulation. PBMCs that were not stimulated with Ag (Unst.) were also used for a negative control. (C and D) Expression of IFN- γ and IL-4 mRNA by PBMCs following stimulation with specific NDV (B1 strain) antigen. PBMCs were incubated in the presence (w/ Ag) or absence (w/o Ag) of NDV Ag, and RNAs extracted from PBMCs were used for real-time qRT-PCR to determine IFN- γ and IL-4 mRNA. Data represent the mean and standard error of IFN- γ and IL-4 expression obtained from six chickens per group, after normalization to GAPDH. *** $P < 0.001$ compared to vehicle group that was treated with control bacteria. **** $P < 0.001$ compared to chIL-18-treated chickens. ††† $P < 0.001$ compared to chIFN- α -treated chickens.

surfaces, serum-derived IgG responses neutralize replicating viruses and prevent further spread of the infection [5]. Although we could not perform challenge infections with virulent NDV due to limitations with Bio-safety Level 3 (BSL3) facility at our institute, reports have suggested that high levels of both systemic and mucosal antibodies may be associated with protection against NDV challenge [29, 34]. Moreover, Th1-biased cell-mediated immune responses can also control pathogenic infection in both the mucosal and systemic compartments [31–33]. Additionally, it has been documented that T cells produced at one mucosal surface are capable of homing and offering protection at other mucosal surfaces [18, 22]. This is one of the key advantages of mucosal vaccines and can therefore be potentially used as vaccines for NDV, which is also a mucosal pathogen.

As the *Salmonella* bacteria used in this study were devoid of the aspartate β -semialdehyde dehydrogenase (Asd) gene, which is essential for a balanced-lethal host-vector system, they may have been sufficiently attenuated in their capacity to cause acute diseases in chickens and are genetically stable [14]. Accordingly, attenuated *Salmonella* vaccine expressing chIFN- α and chIL-18 produced no apparent side effects during the examination period. However, successful and prolonged

colonization of *Salmonella Typhimurium* expressing chIFN- α and chIL-18 may be necessary to accomplish control of infectious diseases in chickens effectively with a *Salmonella* delivery system. According to previous findings, *Salmonella Typhimurium* can persist in adult chickens for at least 3 weeks and in younger chickens up to 7 weeks [2, 3], but may be cleared ultimately. Therefore, it is believed that *Salmonella* bacteria used for cytokine delivery can persist in chickens for 3–7 weeks, depending on chicken age and can provide continuous long-term protection against virus infection.

Based on our findings, we conclude that oral co-administration of live attenuated *Salmonella Typhimurium* expressing chIL-18 and chIFN- α greatly modulate both systemic and mucosal immunity in chickens immunized with inactivated NDV (B1 strain) vaccine via either a systemic or a mucosal route. Therefore, incorporation of naturally occurring chicken cytokines like chIFN- α and chIL-18 with commercially available inactivated vaccines using attenuated *Salmonella Typhimurium* may be considered to generate effective immunization strategies against currently circulating heterologous virulent Newcastle disease virus strains in chickens.

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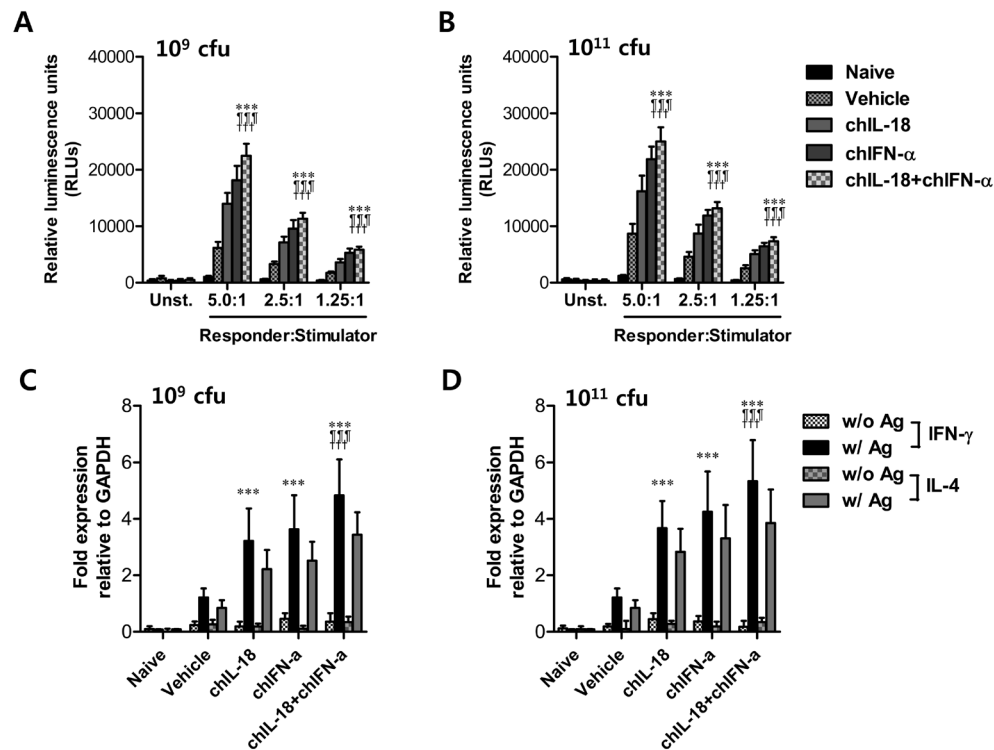


Fig. 5. Enhanced Th1-biased immunity in chickens immunized via the mucosal route following oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN- α and chIL-18. (A and B) NDV antigen-specific proliferation of PBMCs. PBMCs were collected 14 days after booster vaccination and used for NDV Ag-specific stimulation. PBMCs that were not stimulated with Ag (Unst.) were also used for a negative control. (C and D) Expression of IFN- γ and IL-4 mRNA by PBMCs following stimulation with specific NDV (B1 strain) antigen. PBMCs were incubated in the presence (w/ Ag) or absence (w/o Ag) of NDV Ag, and RNAs extracted from PBMCs were used for real-time qRT-PCR to determine IFN- γ and IL-4 mRNA. Data represent the mean and standard error of IFN- γ and IL-4 expression levels obtained from six chickens per group, after normalization to GAPDH. *** P <0.001 compared to vehicle group that was treated with control bacteria. ††† P <0.001 compared to chIL-18-treated chickens. †††† P <0.001 compared to chIFN- α -treated chickens.

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