Modulation of Low Pathogenic Avian Influenza Using Attenuated *Salmonella* Vaccine Chicken Interferon-α and Interleukin-18

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By

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Dedication

Dedicated to my beloved parents, who have raised me to be the person I am today and to my lovely wife and kids, who offered me unconditional love & support throughout the course of my study.

Acknowledgement

At first all praises are due to the Almighty Allah, The Omnipotent, Omnipresent and Omniscient who enable me to complete the dissertation. It deems a proud privilege to express my deepest sense of gratitude, indebtedness, profound regards and sincere appreciation to my reverend supervisor, Dr. Seong Kug Eo, whose sage advice, insightful criticisms, and patient encouragement aided me in innumerable ways. I appreciate his vast knowledge and skill in many arenas, and his assistance in writing this dissertation which have on occasion made me green with envy. He quickly became for me the role model of a successful researcher in the field. He provided me with direction, technical support and became more of a mentor and friend, than a professor. I am in doubt that I will ever be able to convey my appreciation fully, but I owe him my eternal gratitude. It's my pleasure to show my gratitude towards the other members of thesis evaluation committee for their critical reviewing and valuable comments which obviously improve my thesis quality. Special appreciation also goes out to my dear lab members who has helped me immensely in lab work and provided me a friendly environment in the lab. I wish to thank my friends and others with whom I have interacted throughout my research. I cannot end without thanking my family, on whose constant encouragement and love I have relied throughout my time at the academy. By the fear of leaving someone out, I say thank you very much to all. Lastly but not leastly, I express my gratitude to the funding agencies for financial assistance without which this research would not have been possible.

Abstract

Control of avian diseases using attenuated *Salmonella enteric* serovar Typhimurium expressing chicken interferon-α and interleukin-18. Disease outbreak is a major problem in intensive livestock animals and existing control measures include combined use of vaccines, antibiotics and chemicals. However, extensive use of antibiotics and chemicals in food animals like chicken has resulted in environmental and human health hazards particularly with regard to the emergence of drug-resistance bacteria in the food chain. Cytokines play pivotal roles as natural mediators and regulators of the immune response and therefore may offer exciting new alternatives to the conventional therapies. The utilization of cytokines in poultry disease prevention is becoming more promising with the growing list of new cytokines.

Chapter 1: A brief review was made on chicken cytokines to know the recent updates and problems & prospects of the therapeutic usages of chicken cytokines in disease prevention. Based on the recent literature review results, it is pointed out that use of chicken cytokines in disease prevention might be promising if cost-effective large scale production of cytokines using effective delivery system could be ensured. Additionally, a synergistic immunomodulation and thereby, an enhanced protective immune response might be generated against vaccine antigens or pathogens by combined use of two or more cytokines with synergy functions. According to recent literature review, both chicken interferon-α (chIFN-α) and chicken interleukin-18(chIL-18) have strong antiviral properties *in vitro* and *in vivo* and these two cytokines may act synergistically to modulate chicken immune responses against vaccine or viral antigens and may provide enhanced protection against viral insult. In order to provide valuable insights into these issues, several experiments were designed and performed that were described in chapter 2, 3 & 4.

Chapter 2: We designed an experiment to evaluate the antiviral properties of recombinant chIFN-α against AIV H9N2 using *Salmonella enterica* serovar Typhimuirum as delivery vector. SPF chickens were intra-tracheally infected with AIV H9N2 (01310) ($10^{10.83}$ EID_{50}/chicken) 3 days after oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α. According to our results, oral administration of single dose of attenuated *Salmonella enterica* serovar Typhimuirum expressing chIFN-α alleviated clinical signs and histopathological changes caused by respiratory infection with AIV H9N2 and reduced the excretion of virus in cloacal swab samples. Similarly, chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α showed inhibited replication of AIV H9N2 in several different tissues including trachea, lung, cecal tonsil, and brain. Furthermore, immune responses specific for challenged AIV H9N2 were enhanced in chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α, as determined by hemagglutination inhibition assay of sera, proliferation and IFN-γ and interleukin-4 expression by AIV H9N2 antigen-stimulated peripheral blood mononuclear cells and splenocytes.

Chapter 3: We provide valuable insight into the combined use of chicken cytokines in disease prevention. We investigated the antiviral efficacy of oral co-administration of chicken interferon-α (chIFN-α) and interleukin-18 (chIL-18) using attenuated *Salmonella enterica* serovar Typhimurium in chickens infected with avian influenza virus (AIV) H9N2. Our results demonstrate that oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 displayed significantly enhanced alleviation of the clinical signs of chickens caused by respiratory infection with AIV H9N2, when compared to chickens orally administered *S. enterica* serovar Typhimurium expressing either chIFN-α or chIL-18, as determined by mortality, clinical severity, and feed and water uptakes. This enhancement of antiviral immunity was further confirmed by reduced rectal shedding and replication of AIV H9N2 in several different tissues of challenged chickens including trachea, lung, cecal tonsil, and brain. Furthermore, oral co-administration of chIFN-α and chIL-18 more efficiently modulated the immune responses of chickens against AIV H9N2 through enhancing both humoral and Th1-biased cell mediated immunity, compared to single administration of constructs. Therefore, our results suggest that the combined administration of chIFN-α and chIL-18 using attenuated *S. enterica* serovar Typhimurium as an oral carrier of two chicken cytokines provides a useful means for controlling respiratory disease caused by AIV H9N2 infection.

Chapter 4: We evaluated the combined Immunomodulatory functions of chicken interferon-α (chIFN-α) and interleukin-18 (chIL-18) in vaccination with inactivated H9N2 LPAI vaccine. Our results revealed that oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 prior to vaccination with inactivated AI H9N2 vaccine modulated the immune responses of chickens against vaccine antigen through more enhanced both humoral and Th1-biased cell-mediated immunity, compared to the chickens that received oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18 alone. To further test the protective efficacy of this improved vaccination regimen, immunized chickens were intra-tracheally challenged with high dose of AIV H9N2 7 days after booster vaccination. Combined administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 showed markedly enhanced protection compared to single administration of construct, as determined by mortality, clinical severity, and feed and water intakes. This enhancement of protective immunity was further confirmed by reduced rectal shedding and replication of AIV H9N2 in different tissues of challenged chickens. Our results provide a useful value of combined administration of chIFN-α and chIL-18 using *Salmonella* vaccine strain to generate an effective immunization strategy in chickens against LPAI H9N2.

Overall, our results suggest that attenuated Asd-negative *S. enterica* serovar Typhimurium may be useful for single or combined oral administration of chIFN-α and chIL-18 with enhanced functions in order to provide a useful means for controlling respiratory disease caused by AIV H9N2 infection. To improve the practical use of the attenuated *Salmonella* strain as oral carrier of cytokines, the number of times being administered and its schedule should be addressed further.

Chapter 1

Literature Review

Introduction

Disease outbreak is a major problem in intensive livestock animals and existing control measures include combined use of vaccines, antibiotics and chemicals. However, extensive use of antibiotics and chemicals in food animals like chicken has resulted in environmental and human health hazards[1-2] particularly with regard to the emergence of drug-resistance bacteria in the food chain [3] that appear impervious to even the most powerful antimicrobial agents. The imminent banning of antibiotic usage in livestock feed has intensified the search for environmentally-friendly alternative methods to control diseases. Cytokines play pivotal roles as natural mediators and regulators of the immune response [4-5] and therefore may offer exciting new alternatives to the conventional therapies. The utilization of cytokines in poultry disease prevention is becoming more promising with the growing list of new cytokines [6], due to the availability of the chicken whole genome sequence [7-8] and those of other avian species [9] together with the post-genomic approach [8,10-11]. Recent developments in recombinant DNA technologies and gene delivery vectors provide realistic approach in the use of recombinant cytokines as natural alternatives to disease prevention. However, several critical constraints have to be circumventing like cost-effective large scale production, effective delivery, protein stability and bio-activity in vivo, if recombinant cytokines are to be used as commercial therapeutics. Combined use of cytokines with synergy function needs to be further explored that may provide more powerful approach to disease prevention in future.

Existing Disease Control Strategies in Livestock Animals and their Limitations

The importance of livestock in the agricultural sector has been emphasized in a number of FAO publications. Poultry is an important component of livestock animals and the sheer size of poultry industry is staggering worldwide. Chicken meat represents approximately 40% of all meat consumed and is a US $150 billion per annum retail market, as well as supporting a multi-billion dollar poultry health market [5]. Chickens are reared under intensive conditions which are conducive to infection by opportunistic pathogens. This is especially critical during the first weeks of life, a time at which the immune system has not yet fully matured [12] and when levels of maternal antibody are declining. A major problem faced by all intensive livestock industries, such as the poultry industry, is loss of productivity due to disease outbreaks, therefore effective control measures are required in order to maintain the health status of these animals.

Existing control measures against various infectious diseases includes the combined use of vaccines, antibiotics and chemicals. Vaccines can provide long term immunity and thereby confer specific protection against a particular pathogen following immunization. Usually the most effective form of vaccination is immunization with a live (attenuated) organism, however, the possibility of reassortment between vaccine strains and field isolates [13] and of mutations from low-pathogenic to highly pathogenic organisms [14] has imposed serious safety concerns about using live (attenuated) organisms as animal vaccines. Vaccination with killed organisms or recombinant antigens is generally less immunogenic and requires the use of powerful adjuvants in order to develop a long-term protective immune response. Unfortunately, most commercial adjuvants are oil-based and their use can result in adverse local reactions resulting in downgrading of meat quality.

In contrast to vaccines, antibiotics provide short-term, broad-spectrum protection with growth promoting activity [15] but it requires continuous supplementation as feed additives. In fact, up to half of the world's production of antibiotics is used in agriculture. The extensive use of antibiotics, particularly those that are used in human medicine, has resulted in serious human health concerns. The emergence of drug resistant bacteria in humans has in many cases been traced back to animals [16], indicating that transfer has occurred through the food chain. The World Health Organization (WHO) has recently urged meat producers to stop using the same antibiotics that are used in humans or those which select for cross-resistance, however, these are usually the most effective in controlling disease. Furthermore, the WHO has strongly recommended the development and use of alternative, environmentally-friendly methods to control diseases. Similarly, the long term use of chemicals, such as coccidiostats, results in emergence of resistant strains of pathogens such as *Eimeria* [17], increases environmental contamination and results in the deposition of residues in meat products. Some European countries have already banned the use of antibiotic and chemical feed additives in food production animals and more plans to follow suit. Livestock industries now acknowledge that withdrawal of antimicrobials from feed is inevitable and we must now develop strategies to cope with this.

Cytokines and their Usages as Natural Alternative in Disease Prevention

Cytokines and their role in immune system

Over the past four decades, the perception of the immune system has changed from a self-contained, specialized cell and organ system to one of a diverse and dynamic system regulating not only host defenses, but also the repair and physiological homeostasis of the host [18]. Controlling these diverse physiological mechanisms locally and systemically are soluble peptide molecules, collectively called cytokines. Cytokines are soluble, low molecular weight polypeptides and glycopeptides produced by a broad range of cell types of hematopoietic and nonhematopoietic origin that have suppressive or enhancive effects on cellular proliferation, differentiation, activation, and motility [19-20]. These molecules are not only produced by diverse cell types, but are also pivotal for communication between varieties of cells. Cytokines, for the most part, are not constitutively secreted, but are produced in response to stimulation by infectious agents or their derived products (for example, endotoxin), inflammatory mediators, mechanical injuries,

and cytokines themselves. As cell regulators, cytokines bind with high affinity to ligand-specific receptors on the surface of target cells and generate intracellular signal transduction and messenger pathways. Because of these high-affinity receptors, cytokines are very potent effector molecules working in picomolar to fentamolar concentrations [21-22]. Cytokines play a crucial role in controlling the immune system. They determine both the type and extent of an immune response that is generated following infection with a pathogen or after vaccination. Depending on the combination of cytokines produced, a protective immune response can be generated as either an antibody-mediated (Th2) response or a cell-mediated (Th1) response [23]. Cytokines therefore represent excellent candidates as naturally occurring therapeutics as well as vaccine adjuvants [24].

Chicken cytokines and their therapeutic potentials in disease prevention

The efficacy of cytokine therapy has already been demonstrated in several human and animal studies [25-27]. Studies on the therapeutic usages of cytokines in poultry are becoming more feasible with the recent cloning of a number of avian cytokine genes [6]. Before going to review on the recent update of chicken cytokines and their therapeutic applications, it is better to give a short description on the recent updates on the chicken's immune response.

Recent updates on the chicken's immune response: The past decade has seen a revolution in our understanding of the immune response to infection and disease in avian species that becomes possible due the availability of genome sequences of several model species such as chicken [7-8], turkey [http://www.tc.umn.edu/~reedx054/Turkeygenome.htm] and duck [http://pre.ensembl.org/Anas_platyrhynchos/Info/]. The crucial role and specificity of the innate immune response in driving and controlling adaptive immune responses to a particular pathogen is now beginning to be understood. The roles of the effector cells of the innate immune response such as natural killer cells, neutrophils (heterophils in the chicken) and other lymphocyte subsets (γδ T cells) and interactions between these and antigen-presenting cells, particularly dendritic cells (DCs), are also better characterized. Another major advance is in our understanding of the regulation of adaptive immune responses in biomedical model species (such as man and mouse), particularly in the repertoire of CD4 T-cell subsets, which has expanded beyond the original Th1/Th2 paradigm [28] to include regulatory subsets (e.g. Treg, Th3, Tr1) [29] and other effector subsets: Th17 [30] and Th9 [31-32]. It remains to be explored whether all of these CD4 T-cell subsets are present in the chicken, although recent studies demonstrated that the Th1/Th2 paradigm applies in the chicken [33-34]. In the chicken, as in biomedical model species, Th1 cytokine responses (IFN-γ, IL-12 and IL-18) predominate in responses to infection with intracellular pathogens, and Th2 cytokine responses (IL-4, IL-13, IL-19) predominate in responses to infection with extracellular pathogens.

Our recent understanding of these cellular subsets and their responses to infection and immunity in chicken lags behind that in biomedical model species and, to a degree, in other farm animal species (such as cow, horse and pig). However, the availability of genome sequences for avian species coupled with the post-genomic technologies have allowed the identification of the repertoires of immune molecules present in these species, and facilitates the rapid development of necessary reagents to study their functions. Based on the recent research findings, it is becoming clear that immune responses in avian species fit broadly to the biomedical species blueprint, but that differences do occur in the detail. However, the basic principles of innate immune responses driving appropriate adaptive immune responses to clear initial infection and develop immunological memory remains constant for all vertebrate species so far studied, including the chicken.

Chicken cytokines: a growing list: Generally, the cytokine families have smaller numbers of genes in chickens compared to mammals. However, recent revolution of genome sequences of avian species including chicken extends our ability to identify a number of cytokine genes in chickens and other avian species with a growing list of new cytokines (Table 1). A number of laboratories involved in avian cytokine research have recently formed the Avian Cytokine Group (ACG) in order to facilitate the exchange of basic information and reagents for research purposes. A web site has been developed; for further information on the ACG see: www.ah.csiro.au/AvianCytokines/. Cytokines have been classified into a number of groups based on their activity and the cells they are produced by or act upon. These groups include: interferons (IFN), interleukins (IL), transforming growth factors (TGF), tumour necrosis factors (TNF), colony-stimulating factors and the smaller chemokines. An arbitrary list of all chicken cytokines identified so far is made according to the above mentioned classification (Table 1). Recent identification of several cytokine receptor genes in the chicken genome raises the intriguing prospect that still more cytokines remain to be found.

Chicken cytokines in disease prevention: The utilization of cytokines in poultry is becoming more feasible with the recent cloning of a number of avian cytokines. Since the chicken's immune system and its response to disease and vaccination is similar to that of mammals, it offers an attractive model system with which to study the effectiveness of cytokine therapy in the control of disease in intensive livestock. Recently several prokaryotic and eukaryotic expression systems are available for the production of recombinant cytokines that make it possible to extensively study the therapeutic application of different chicken cytokines. In this section, I'll provide an insight into the function and roles of the currently studied chicken cytokines (under several broad headings for better description) that they play in disease prevention with special emphasis on chicken interferon alpha (chIFN α) and chicken interleukin 18 (chIL-18).

Table 1: An arbitrary list of chicken cytokine repertoire.

Group/Family		Cytokine
Interferons	Type I	IFN-α, IFN-β, IFN-κ, IFN-ω.
	Type II	IFN-γ
	Type III	Single IFN-λ gene
Interleukins	IL-1 family	4 members (IL-1β, IL-1RN, IL-18, IL-1F5)
	IL-10 family	4 members (IL-10, IL-19, IL-22, IL-26)
	IL-12 family	2 members (IL-12, IL-23)
	IL-17 family	5 members (IL-17A-D, IL-17F)
	T cell proliferative	3 members (IL-2, IL-15, IL-21)
	Th2 family	3 members (IL-4, IL-5, IL-13)
	Others	IL-3, IL-6, IL-7, IL-9, IL-11, IL-34
Transforming growth Factors		3 members
Tumor Necrosis factors		11 members
Colony-stimulating factors		3 members
Chemokines	XCL	1 member
	CCL	14 members
	CXCL	8 members
	CX3CL	1 member

I. **Interferons:** Interferons (IFNs) are glycoproteins produced by immune cells and have antiviral, anti-tumoral, and immunoregulatory effects. They were first reported by Isaacs & Lindenmann [35] who, in 1957, discovered that chick cells infected by influenza virus produced IFNs that mediate the transfer of a virus-resistant active state against both homologous and heterologous viruses. Actually, IFNs were so named due to their anti-viral properties. Although IFNs were first reported in poultry, researches on poultry IFNs were very few and progress was slow. Chicken and other poultry IFN systems are similar to mammalian IFN systems. According to the origin and physico-chemical property of IFNs, they can be grouped into three types: Type I, Type II and Type III.

   A. **Type I interferons:** Type I IFNs in chicken so far identified includes: IFN- α, IFN-β, IFN-κ, IFN-ω of which IFN- α, IFN-β have well defined anti-viral activity.

      a. **Chicken interferon-α (chIFN-α):** chIFN-α plays an essential role in the host antiviral response through stimulating T-dependent lymphocyte system and induction of numerous IFN-stimulated genes (ISGs) [36]. There is evidence that chIFN-α administered by oral ingestion or intravenous injection inhibits many epidemic avian viruses, such as infectious bronchitis virus (IBV) [37], infectious bursal disease virus (IBDV) [38], Newcastle disease virus (NDV) [39], and AIV [39-40]. Recently it has been reported that oral administration of recombinant chIFN-α have the capability of protecting chickens from AIV H9N2 challenge in SPF chickens which provides a new option in the prevention and therapy of AIV H9N2 infection [40].

   B. **Type II interferons:** IFN-γ belongs to Type II interferon. As with mammalian IFN-γ, native chicken IFN-γ has potent macrophage activating factor activity that is heat and pH-labile [41]. Recombinant chicken IFN-γ expressed from *E. coli* or COS cells were poor antiviral agents but strongly stimulated NO secretion and expression of MHC class II in macrophages [42]. However, baculovirus-derived recombinant chicken IFN-γ, as well as stimulating macrophages, also had antiviral activity [43]. Chicken IFN-γ additionally has adjuvant and growth promoting property [43-44] and thus is probably a more suitable recombinant for studies into the function of avian IFN-γ. Again similarly to mammals, chicken type I and type II IFN act synergistically [45], both in terms of antiviral activity and in their ability to activate macrophages.

   C. **Type III interferons:** Only single IFN-λ gene has recently been identified in chicken and its properties and functions are still unknown.

II. **Interleukins:** Till date, a total of 23 interleukin genes have been identified in chicken genome that are listed in Table 1 under different families based on their structural and functional characteristics. Receptor genes present in the genome suggested the likely presence of two other interleukin genes (IL-11 and IL-13). Although a number of interleukin genes are identified in chickens,

only few of them are well characterized in terms of their structure and function. Their functions and therapeutic potentials are discussed below:

A. **IL-1b:** In the chicken a cDNA encoding the chicken homologue of mammalian IL-1b was recently cloned by expression screening [46]. It is a proinflammatory cytokine. In protozoal infections of chickens, expression of IL-1b mRNA in the gut has been shown to increase 80-fold seven days after *Eimeria tenella* infection [47]. An increase, but to a lesser degree, was also found following *E. maxima* infection. Infection models have also been used to determine activity following viral and bacterial infections in the chicken [48-49]. IL-1 activity was increased in macrophage supernatants from birds suffering from poult enteritis and mortality syndrome (PEMS) [48]. Conversely, following *Salmonella enterica* invasion in an in vitro cell culture system, IL-1b mRNA expression was generally decreased [49]. However, it should be noted that IL-1b mRNA levels do not necessarily reflect release of biologically active protein.

B. **IL-6:** IL-6 is a multifunctional cytokine produced by a number of cell types and is involved in acute-phase responses, immune regulation and haematopoesis [50]. Recombinant chicken IL-6 induced proliferation of the IL-6-dependent murine hybridoma cell line 7TD1, and when injected into chickens, it induced an increase in serum corticosterone levels indicating induction of acute phase activity. IL-6 activity has been found in several infectious diseases of chickens. IL-6 is produced during both murine and chicken Eimeria infections [51], and IL-6 activity, similarly to IL-1, was increased in macrophage supernatants from birds suffering from PEMS [48]. Interestingly the induction of an IL-6 response may play a major role in the nature of the response to different serovars of *Salmonella enterica* in chickens [49]. Invasion of chicken cells by serovars *S. Typhimurium* or *S. Enteritidis* results in an 8-fold increase of IL-6 mRNA determined by quantitative RT-PCR. Such activity *in vivo* would induce a strong inflammatory and immune response, limiting these serovars mainly to the gut and preventing development of systemic disease. In contrast, invasion by the avian specific serovar *S. Gallinarum*, does not lead to an increase of IL-6 mRNA. This would result in little or no inflammation or induction of an immune response, allowing invasion to take place almost by 'stealth', subsequently allowing development of the systemic disease fowl typhoid.

C. **IL-18:** Interleukin-18 (IL-18), originally known as interferon-γ (IFN-γ)-inducing factor, was initially found in Kupffer cells of mice sequentially treated with Propionibacterium acnes and lipopolysaccharides (LPS) in 1995 [52]. IL-18 shares properties with IL-12 and both cytokines act synergistically to promote IFN-γ production, which plays an important role in inducing Th1 immune responses [53]; thus IL-18 provides an important link between the innate and adaptive immune responses. IL-18 is synthesized as a full length precursor molecule which is then cleaved by caspase-1 (IL-1β converting enzyme) into a bioactive cytokine which is the mature form of IL-18. Only this mature IL-18 rather than the full length form of IL-18 is biologically active in mammals [54-55]. Numerous studies have ensured that mammalian IL-18 has been characterized in great detail [53]. However, the properties and application of chicken IL-18 in disease prevention still remains largely uninvestigated as of yet. The few studies of chicken IL-18 that have been conducted have yielded that the predicted protein sequence of complete chicken IL-18 cDNA bears only around 30% amino acid identity with mammalian IL-18 [56], and the bacterially expressed chicken IL-18 is capable of inducing both the synthesis of chicken IFN-γ in cultured primary chicken spleen cells and the proliferation of CD4+ T cells [56-57]. In addition, the purified *Escherichia coli*-expressed recombinant chicken IL-18 significantly enhanced antibody responses to *Clostridium perfringens* α-toxoid and Newcastle disease (ND) virus antigens, to a degree comparable to the aluminum gel or Miglyol/chitosan adjuvants used in vaccination of specific pathogen-free (SPF) chickens [58]. Chicken IL-18 cDNA linked with recombinant encoding sequences of H5-H7 avian influenza virus (AIV) in a fowl pox-based DNA vaccine (rFPV-H5-H7-IL18) successfully induces complete protection (100%) in SPF chicken after challenge with H5 AIV, and lymphocyte proliferation induced by the rFPV-H5-H7-IL18 is significantly higher than that induced by rFPV-H5-H7 alone [59]. Chicken IL-18 also has the ability to act as a potent adjuvant in avian vaccines [60].

D. **IL-2:** IL-2 is a Th1 cytokine that lead primarily to the activation of macrophages and the development of a cell-mediated immune response. In birds, both chicken and turkey IL-2 have been described [61]. Functionally, recombinant chicken IL-2 activates γδ T cells [62]. In an experimental *Eimeria* infection high levels of both γδ T cells and expression of IL-2 mRNA were found in the gut of chickens [62].

E. **IL-4, IL-5 and IL-13:** They are recently identified three important members of Th2 cytokine family in chicken. Detailed information on their functional roles in chicken immune system is still unavailable and therefore, Th1/Th2 paradigm in chicken is not fully clear.

III. **Transforming growth factor beta (TGFb):** TGF-b is a Th3 cytokine. As in mammals, three forms of TGF-b have been cloned from chickens: TGF-b4 (equivalent to mammalian TGF-b1) [63], TGF-b2 [64] and TGF-b3 [65]. The expression of TGF-b in the chicken thymus

may regulate the ability of immature thymocytes to progress through the cell cycle and differentiate into mature CD3+ (a receptor found on T lymphocytes) thymocytes [66]. TGF-b4 mRNA expression has been shown to increase in the caecal tonsils, spleen and duodenum following *E. acervulina* infection [67], presumably as part of an anti-inflammatory response.

IV. **Chemokines:** IL-8 is a member of a group of small structurally related cytokines that have chemotactic activity for specific leukocyte types and are termed chemokines [68]. In the chicken a number of CXC and CC chemokines have been identified [69-71]. The chicken chemokine IL8/CAF appears to be the equivalent of mammalian IL-8 in the chicken. IL8/CAF has been shown to play a role in wound healing [72], and can initiate the wound-healing cascade *in vivo* [73]. It is also chemotactic for chicken peripheral blood mononuclear cells and motogenic for fibroblasts [74]. Marek's Disease Virus has been shown to encode a CXC chemokine, which has been described in the literature as an IL-8 homologue (vIL-8) [75].

Possibility of synergy function between chicken interferon alpha (chIFNa) and chicken interleukin 18 (chIL-18): Based on the immunobiological mechanisms, the combined effects of two or more cytokines might be antagonistic, additive, or synergistic [76]. The enhanced effect of cytokine combinations has been shown empirically, based on their biological mechanisms. Therefore, a synergistic immunomodulation and thereby, an enhanced protective immune response can be generated against vaccine antigens or pathogens as either an antibody-mediated (Th2) response or a cell-mediated (Th1) response by combined use of two or more cytokines with synergy functions [77-79]. Both chicken IFN-α and chicken IFN-γ have potent antiviral activities [36,43]. Chicken IFN-γ plays a role in modulation of the immune cells, in addition to its antiviral activity [80]. Again similarly to mammals, chicken type I and type II IFN act synergistically [45] both in terms of antiviral activity and in their ability to activate macrophages. On the other hand, chicken IL-18, which was initially identified as IFN-γ-inducing factor, provides an important link between the innate and adaptive immunity through the induction of IFN-γ [81]. Also, the significance of chicken IL-18 in the development of antiviral immune responses has been shown in several viral infections like H5-H7 avian influenza virus (AIV) infection. It has been demonstrated that after infection of macrophages with influenza virus, cells produce IL-18, which acts synergistically with IFN-α and enhances IFN-γ synthesis [82]. Therefore, it is possible that chicken IFN-α and IL-18 might have synergistic antiviral and Immunomodulatory activity in combination which is addressed in the present study.

Constraints in the Use of Chicken Cytokines for Disease Prevention

The ability of chicken cytokines to combat infection and enhance vaccine efficacy makes them excellent candidates as therapeutic agents and vaccine adjuvant. However, if recombinant cytokines are to be used as commercial therapeutics, particularly in the poultry industry, there are several critical constraints that have to be considered in order for a product to be considered cost-effective. The protein must be able to be produced on a commercial scale (multi-billion doses per annum), easily purified, inexpensive to produce, stable upon storage, effective delivery and retain bio-activity in vivo. Recent developments in gene delivery vectors and DNA vaccination technologies now provide realistic alternatives. Several prokaryotic and eukaryotic expression systems, as well as virus-based systems are available for the production of recombinant cytokines; each has their particular advantages as well as disadvantages. Various avian cytokines have been successfully expressed in E. coli. This system allows large amounts of biologically active recombinant protein to be produced, which can be purified by relatively simple procedures. Disadvantages of this system are that these type of proteins are non-glycosylated and may not be folded in authentic native forms, thereby having less than optimal specific activities or reduced half-lives in vivo. Live attenuated *Salmonella* vaccine strains are also used as gene delivery vector detailed review on which is made separately in next section. Cytokines have also been expressed in a variety of eukaryotic systems, including COS, CHO, and yeast. Viral vector technology has allowed a variety of cytokines to be administered and expressed in animals [83]. This provides a simple, effective and inexpensive commercial delivery method via feed, water or aerosol. These new generation delivery mechanisms also allow the administration of single or multiple cytokines, in combination with antigen(s) or antigen-cytokine fusion proteins. The choice of particular viruses will allow antigen and cytokine targeting to specific sites such as gut, thereby allowing more appropriate types of immune responses to be generated. Available virus-based systems include baculovirus, tobacco mosaic virus (TMV), fowlpox virus and fowl adenovirus [83]. In general, viruses are very efficient gene-transfer vehicles; however, significant limitations are inherent to their use. Viral vectors may provoke mutagenesis and carcinogenesis. Repeated administration of a viral vector induces an immune response which abolishes the transgene expression. Moreover, antibiotic resistance selection markers are needed to maintain plasmids.

The Attenuated *S. enterica* Serovar Typhimurium strain x8501: an Excellent Tool for Gene Delivery

Live attenuated Salmonella vaccine strains have been used as carriers of heterologous antigen(s) from bacteria, viruses and parasites [84]. Following oral administration, *Salmonella* has been shown to be capable of stimulating systemic antibody and cell-mediated immunity [85-86]. Conventionally, a *Salmonella* vaccine strain contains a plasmid-based expression vector, which encodes the heterologous antigen(s) of interest, and an antibiotic-resistance selection marker that is used, after addition of

the corresponding antibiotic, for plasmid maintenance. The use of such *Salmonella* strains has been discouraged because of concerns over safety regarding use in humans, and because of concerns regarding cost-effectiveness, as it is necessary to produce large quantities of antibiotics by large-scale fermentation for production of the bacteria as inoculates [87-88].

The attenuated *S. enterica* serovar Typhimurium strain x8501 harbours deletion mutations in cya and crp, defective in the synthesis of the adenylate cyclase and cyclic AMP receptor, and asd, which encodes the aspartate β-semialdehyde dehydrogenase (Asd), an essential enzyme for cell-wall biosynthesis [89]. This Asd auxotrophic mutant was unable to grow in complex medium without supplementation with diaminopimelic acid (DAP), a bacteria amino acid not found in eukaryotes, but, after trans-complementation with an Asd+ plasmid, the mutant's growth was restored [90]. Hence, only Asd+ plasmid-carrying cells can grow in DAP-free medium, making the Asd-*Salmonella* strain dependent on the plasmid maintenance, owing to the balanced lethal relationship between vector and host systems [91]. Recently, several multicopy, stable Asd+ antigen-expressing vectors (eg. pAY3493, pAY3560) has been specially designed to express recombinant protein antigens by means of the fusion of the β-lactamase signal sequence in an Asd-*Salmonella* vaccine strain [92] that makes it an unique gene delivery vector. Recently, we showed that oral administration of attenuated *S. enterica* serovar Typhimurium harboring gene could effectively express biologically active swine interferon-α protein in piglets that could alleviate the clinical severity induced by the transmissible gastroenteritis virus [25]. Successful delivery of chicken cytokine genes using this vector will circumvent all the barriers in using chicken cytokines as natural alternatives in disease prevention in future.

Chapter 2

Oral Administration of Live Attenuated *Salmonella Enterica* Serovar Typhimurium Expressing Chicken Interferon-α Alleviates Clinical Signs Caused by Respiratory Infection with Avian Influenza Virus H9N2

Abstract

Low pathogenic avian influenza (LPAI) H9N2 has attracted considerable attention due to severe commercial losses in the poultry industry. Furthermore, avian influenza virus (AIV) H9N2-infected chickens can be a reservoir for viral transmission to mammals including pigs and humans, complicating control of viral mutants. Chicken interferon-alpha (chIFN-α) may be useful as an exogenous antiviral agent to control AIV H9N2 infection. However, a superior vehicle for administration of chIFN-α is needed because of challenges of protein stability, production cost, and labor associated with mass administration. Presently, oral administration of single dose of attenuated *Salmonella enterica* serovar typhimurium expressing chIFN-α alleviated clinical signs and histopathological changes caused by respiratory infection with AIV H9N2 and reduced the excretion of virus in cloacal swab samples. Similarly, chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α showed inhibited replication of AIV H9N2 in several different tissues including trachea, lung, cecal tonsil, and brain. Furthermore, immune responses specific for challenged AIV H9N2 were enhanced in chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α, as determined by hemagglutination inhibition assay of sera, proliferation and IFN-γ and interleukin-4 expression by AIV H9N2 antigen-stimulated peripheral blood mononuclear cells and splenocytes. Therefore, oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α can successfully control clinical signs caused by respiratory infection with AIV H9N2, which provides valuable insight into the use of attenuated *Salmonella* vaccine as an oral delivery system of chIFN-α to prevent AIV H9N2 respiratory tract replication.

Introduction

Avian influenza viruses (AIV) H9N2 have become panzootic in Asia during the past decade and have been isolated from terrestrial poultry worldwide [93-96]. Since the 1996 outbreak of low pathogenic avian influenza (LPAI) H9N2 [97] the virus has become endemic in Korea, especially in layer farms [98,99] and has attracted considerable attention due to its rapid spread across Korean chicken farms with enhanced clinical severity and increasing mortality (up to 65% for leghorn layer chickens)[100]. Of additional concern, AIV H9N2-infected chickens can serve as reservoir host and transmit the virus to mammals such as pigs and humans, hampering the control of viral mutants [101-104]. Moreover, AIV H9N2-infected chickens are vulnerable to secondary infection by pathogenic microbes, which may consequently cause severe commercial loss. Although immunization with vaccines is not complete, it is one of the most promising control measures for the LPAI H9N2 to date. Modified live virus (MLV) vaccines have been used in many countries to control AIV, since vaccination with inactivated AIV is usually efficacious but requires individual administration and more time to develop protective immunity. However, the possibility of reassortment between vaccine viruses and field isolates and of mutations from low-pathogenic to highly pathogenic viruses may create serious safety concerns about using MLV strains as poultry vaccines. Therefore, prior stimulation of the immune system using some immunomodulators followed by vaccination with inactivated vaccines may be needed to confer better protective immunity within short time and may be promising in controlling LPAI H9N2.

The World Health Organization (WHO) has urged meat producers to use environmentally-friendly alternative methods to control disease. Cytokines, as natural mediators of the immune response, are an alternative to conventional therapeutics. The efficacy of cytokine therapy has been demonstrated in several human and animal studies [25,26,105-106]. The utilization of chicken cytokines is becoming more feasible with the recent cloning of a number of cytokine genes, since the chicken's immune system is similar to that of mammals. Chicken interferon-alpha (chIFN-α) belongs to type I IFNs and plays an essential role in the host antiviral response through the stimulation of T-dependent lymphocyte system and induction of numerous IFN-stimulated genes (ISGs) [107-109]. There is evidence that chIFN-α administered by oral ingestion or intravenous injection inhibits many epidemic avian viruses, such as infectious bronchitis virus (IBV) [37], infectious bursal disease virus (IBDV) [38], Newcastle disease virus (NDV) and AIV [39-40]. Recently, it was reported that the oral administration of a recombinant chIFN-α protein can protect specific pathogen-free (SPF) chickens from AIV H9N2 challenge [40], which provides a new option in the prevention and therapy of AIV H9N2 infection. However, the mass administration of chicken cytokines to control poultry diseases is limited by cost, labor, time, and protein instability. Therefore, it is necessary to develop an effective delivery system for the mass administration of chicken cytokines to overcome these limitations.

To this end, our previous study reported that an attenuated aspartate β-semialdehyde dehydrogenase (Asd)-negative *Salmonella enterica* serovar Typhimurium strain devoid of antibiotic resistance genes could be an effective delivery system for the mass administration of cytokines without the need for antibiotic selection [25]. Furthermore, cytokines produced by *S. enterica* serovar Typhimurium may be able to provide immunomodulatory functions to both mucosal and systemic sites, because orally administered *S. enterica* serovar Typhimurium can colonize the secondary lymphoid and nonlymphoid tissues including the lymph node, spleen, and liver, as well as the gut-associated lymphoid tissues (Peyer's patch) [110]. The specific aims of the present study were to provide insight into the values of attenuated *S. enterica* serovar Typhimurium as a carrier for chIFN-α protein to control LPAI H9N2 in chickens. Oral administration of single dose of *S. enterica* serovar Typhimurium expressing chIFN-α alleviated clinical signs caused by respiratory infection with AIV H9N2 and reduced the excretion of AIV H9N2. Furthermore, specific immune responses against AIV H9N2 challenge in chickens orally administered *S. enterica* serovar Typhimurium expressing chIFN-α were evaluated.

Materials and Methods

Animals and ethics statement

SPF leghorn layer (white) chickens were obtained from Jinan Baizhun Biologic Inspection, China, and were reared with formulated commercial feed and water provided *ad libitum* throughout the whole experimental period. All experimental procedures and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committees of Chonbuk National University. The animal facility of the Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Cells and viruses

Low pathogenic avian influenza A virus (AIV) H9N2 strain, A/Chicken/Korea/01310/2001 (01310), was a kind gift from the National Veterinary Research and Quarantine Service of the Republic of Korea [111]. AIV H9N2 (01310) was propagated by inoculating in the allantoic cavity of 10-day-old embryonated eggs and allantoic fluid was harvested 96 h after inoculation. Virus in the allantoic fluid was titrated using a standard hemagglutination test [112] and the infectious viral titer was determined by using 10-day-old embryonated eggs, as previously described [113].

Bacterial strains, plasmid, media, and growth conditions

Escherichia coli χ6212 ($F^-\lambda^-\Phi 80\Delta$ (*lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ*asdA4*) [91] was used as the host strain for construction of the Asd+ vectors. Attenuated *S. enterica* serovar Typhimurium χ8501 (*hisG Δcrp-28 ΔasdA16*), which was kindly provided by Dr. H.Y. Kang (Pusan National University, Korea) [92], was used for the delivery of swIFN-α proteins. 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 [92]. *E. coli* and *S. enterica* serovar Typhimurium cultures were grown at 37 °C in Lennox broth [114], Luria-Bertani (LB) broth or on LB agar [115]. Diaminopimelic acid (DAP; Sigma-Aldrich, St. Louis, MO) was added (50μg/ml) to induce the growth of Asd-negative bacteria [91]. Phosphate-buffered saline (PBS, pH 7.4) containing 0.01% gelatin (BSG) was used for the resuspension of *Salmonella* vaccines that were concentrated by centrifugation at 7000 × *g*, 4 °C for 5min.

Construction of attenuated S. enterica serovar Typhimurium expressing chIFN-α

Total RNA was extracted from chicken splenocytes that were previously stimulated with lipopolysaccharde (LPS, 20μg/ml) for 48h after resuspending cells (10^7 cells/ml) in complete RPMI medium, and employed to amplify chIFN-α gene with reverse transcription-polymerase chain reaction (RT-PCR) using specific primer pairs corresponding to chIFN-α 6 nucleotide sequences (DQ026259.1) (Table 2). The PCR products were then inserted into pGEMT vector (Promega, Madison, WI) and the chIFN-α gene was sequenced to identify an open reading frame. Subsequently, the pGEMT vector encoded with chIFN-α was digested with *Eco*RI and *Hin*dIII, after which the released fragment containing the chIFN-α gene was subcloned into the *Eco*RI and *Hin*dIII sites of pYA3560 expression vector using *E. coli* χ6212 hosts grown in the presence of DAP. The positive colonies of *E. coli* χ6212 harboring pYA3560 were selected in the absence of DAP. To construct *Salmonella* vaccine expressing chIFN-α protein, *S. enterica* serovar Typhimurium χ8501 (1×10^8 cfu) washed extensively with sterilized ice-cold WB (10% ultra pure glycerol, 90% distilled water; v/v) were mixed with 10 pg to 0.1μg of chIFN-α-encoding pYA3560 plasmid DNA on ice in a 0.2cm cuvette and electroporated using a Bio-Rad Gene pulser at 12.5 kV/cm (2.5 kV, 25μF and 200 Ω; Bio-Rad, Hercules, CA). The bacteria were then removed from the cuvette into a sterile culture tube containing 1ml of LB broth medium and incubated with moderate shaking for 60 min at 37 °C. The transformed culture (100μl) was then plated onto LB agar in the absence of DAP. Finally, colonies of the attenuated *S. enterica* serovar Typhimurium harboring pYA3560 vector (χ8501/chIFN-α) were cultured and stored after confirmation of the coding sequences.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

The expression of chIFN-α protein by *S. enterica* serovar Typhimurium harboring pYA3560 encoded with chIFN-(χ8501/chIFN-α) was identified by immunoblot following gel separation of prepared protein by SDS-PAGE. For the preparation of protein samples, *Salmonella* cultured for 12,18, and 24h were resuspended in 4ml of 20mM Tris-HCl (pH 8.6) and then disrupted by two passages through a French pressure cell (American Instrument, Silver Spring, MD). Cell lysates were centrifuged at 7000 ×*g*, 4 °C for 6 min to remove unbroken cells and the supernatant fraction was used for protein samples of cell lysates. The original culture supernatants were filtered (0.22μm-pore-size filter) and proteins that had been secreted into the supernatants were precipitated with 10% trichloroacetic acid (TCA) at 4 °C for 1h. Prepared protein samples were boiled for 5 min and then separated by SDS-PAGE. The protein bands were visualized by staining with 0.1% Coomassie brilliant blue R-250 solution (Elpis-biotech, Deajeon, Korea). For immunoblotting, the resolved proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked with a blocking buffer consisting of PBS containing 3% skim milk and 0.5% Tween 20, and incubated with mouse monoclonal antibody specific for chIFN-α (Serotec, Raleigh, NC) and then with a peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Southern Biotech, Birmingham, AL). Immunoreactive bands were detected by the addition of chemiluminescence dye using a WEST-one™ Western Blot Detection System (iNtRON, Seongnam-Si, Korea) in the presence of H_2O_2.

Table 2: Primers for PCR amplification of chIFN-α, AIH9, IFN-γ, IL-4 and GAPDH.

Target Gene		Primer Sequence (5'-3')	Accession No.	Reference
chIFN-aa	F	ATG GCT GTG CCT GCA AGC CCA	DQ026259.1	-
	R	CTA AGT GCG CGT GTT GCC TGT		
AIH9	F	CTA CTG TTG GGA GGA AGA GAA TGG T	AF461510.1	[116]
	R	TGG GCG TCT TGA ATA GGG TAA		
IFN-γ	F	CAA AGC CGC ACA TCA AAC A	X99774	[117]
	R	TTT CAC CTT CTT CAC GCC ATC		
IL-4	F	GAG AGG TTT CCT GCG TCA AG	FJ907790.1	[118]
	R	TGG TGG AAG AAG GTA CGT AGG		
GAPDH	F	AGA ACA TCA TCC CAG CGT CC	X01578	[117]
	R	CGG CAG GTC AGG TCA ACA		

[a]The primer pair specific for chIFN-α gene was designed using chIFN-α 6 nucleotide sequences (Genebank accession number DQ026259.1), and the sequences of the two primers were checked using the NCBI Blast Software.

Antiviral activity of culture supernatant of S. enterica serovar Typhimurium expressing chIFN-α

To measure the antiviral activity of culture supernatant of *S. enterica* serovar Typhimurium expressing chIFN-α against AIV H9N2, we used 10-day-old embryonated eggs. The culture supernatants of χ8501/chIFN-α were inoculated into the allantoic cavity of embryonated eggs with the different dose based on protein amount of culture supernatants (10 eggs per dose of culture supernatant), and the inoculated eggs were infected with AIV H9N2 (4 or 40 hemagglutinin (HA) units, 5 eggs per group) 24h later. Following 4-days incubation, the titer of propagated virus in allantoic fluids was determined by standard hemagglutination assay.

Animal experimental design for AIV H9N2 challenge

White leghorn, 18-day-old SPF chickens were divided randomly into four groups (n=5 per group). The first group was a negative control that was orally administered vehicle (PBS containing 0.01% gelatin) without *S. enterica* serovar Typhimurium expressing chIFN-α. The second group was orally administered *S. enterica* serovar Typhimurium harboring pYA3560 vector (10^9 cfu/chicken) as a control of the empty pYA3560 vector. The remaining two groups were orally administered two different doses of *S. enterica* serovar Typhimurium expressing chIFN-α (10^9 and 10^{11} cfu/chicken). Oral administration was given only once and performed by instilling the resuspended bacteria (1ml/chicken) into esophagus using flexible gavage feeding needle (Fine Science Tools, North Vancouver, British Columbia, Canada) after withholding feed for 4h. Subsequently, each chicken was intra-tracheally infected with AIV H9N2 (01310) ($10^{10.83}$ EID_{50}/chicken) 3 days after treatment. Following administration of *S. enterica* serovar Typhimurium expressing chIFN-α and challenge, chickens were observed daily for clinical signs and mortality throughout the duration of the experiment. The clinical signs were scored daily as follows: 0, no sign; 1, slight depression; 2, moderate depression + reduced movement + reduced food/water intake (anorexia); 3, moderate respiratory distress (sinusitis, cough); 4, severe respiratory distress (sinusitis, severe cough) +diarrhea; 5, death. Average feed and water intake was determined daily for 9 days after challenge. Cloacal swab samples were collected at 0, 1, 3, 5, 7, and 9 days post-infection (p.i.). Another experiment was carried out with same experimental setup except that the experiment was carried out for up to 14 days p.i. to collect additional samples for histopathological study and determination of virus amount in tissues. Furthermore, peripheral blood mononuclear cells (PBMC) and splenocytes were isolated to determine cell proliferation, and IFN-γ and IL-4 mRNA expression upon AIV H9N2 antigen-specific stimulation.

Histopathological examinations

Lung and tracheal tissues were harvested from chickens challenged with AIV H9N2 (01310) at 5 days p.i. to detect the histopathological changes [116]. Immediately after collection, tissue samples were fixed with 10% formaldehyde-containing PBS, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and then examined for pathological changes by light microscopy using a model BX51 microscope (Olympus, Tokyo, Japan).

Real-time quantitative RT-PCR (qRT-PCR) analysis

Real-time qRT-PCR was employed to determine the amount of AI H9N2 virus in cloacal swab samples or the tissues using a CFX96™ real-time PCR detection system (Bio-Rad). Total RNA was extracted from cloacal swab samples and tissue samples (trachea, lung, brain, cecal tonsil, spleen, and kidney) using viral RNA extraction and total RNA extraction kits (iNtRON), respectively, according to the manufacturer's instructions. The extracted RNA was subjected to real-time qRT-PCR using a One-Step SYBR® qRT-PCR reagent kit (Takara, Shiga, Japan) and primers specific for the AIV H9 gene (Table 2). Following reverse-

transcription of the viral RNA at 45 °C for 30 min, the resulting cDNAs were used for real-time PCR amplification [117-118]. PCR amplification was conducted by subjecting reaction mixtures to initial denaturation at 95 °C for 5min, followed by 40 cycles of denaturation at 94 °C for 15 s and annealing/elongation at 58 °C for 30s. A standard curve was generated by plotting threshold cycle values against serially diluted plasmid DNA encoding the AIV H9 protein. After the reaction cycle was completed the temperature was increased from 50 °C to 95 °C at a rate of 0.2 °C /15s and the fluorescence was measured every 5 s to construct a melting curve that was used to confirm the authenticity of the amplified products. A control sample that contained no template RNA was run with each assay, and qRT-PCR data for AIV H9 amounts in the tissues was normalized using the commonly used reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2). All determinations were performed by data from wells evaluated in duplicate to ensure reproducibility. The copy number of the experimental samples was determined by interpolating the threshold cycle values using the standard curve. All data were analyzed using the CFX96™ manager software version 1.6 (Bio-Rad).

Hemagglutination inhibition (HI) assay

To determine the HI titer of the sera samples collected from experimental chickens, the HI tests were performed with AIV H9N2 (01310) using a standard method [119]. The geometric mean of serum HI titers obtained from each group was defined as the reciprocal logarithm in a base of 2 of the highest serum dilution completely inhibiting agglutination.

AIV H9N2-specific proliferation

AIV H9N2 antigen-specific proliferation of PBMCs and splenocytes were assessed by measuring the viable cell ATP bioluminescence [120]. Briefly, PBMCs and splenocytes were prepared as previously described [121], and cultured together with stimulator cells at three different ratios. Enriched APCs (10^6 cells/ml) that had been pulsed with ultraviolet (UV)-inactivated AIV H9N2 antigen (2.5×10^2 HA units/ml) were used as stimulator cells. Following 72h incubation, replicate cultures were transferred to V-bottom 96-well culture trays, which were centrifuged to collect the cells. The proliferated cells were then evaluated using a Vialight® Cell proliferation assay kit (Cambrex Bio Science, Rockland, ME) according to the manufacturer's instructions.

Expression of IFN-α and IL-4 by PBMCs and splenocytes following stimulation with AI H9N2 antigen

The mRNA expression levels of IFN-γ and IL-4 in PBMC and splenocytes were determined by real-time qRT-PCR using a CFX96™ real-time PCR detection system (Bio-Rad) following stimulation with AIV H9N2 antigen. Prepared PBMCs and splenocytes were stimulated with the stimulator cells (UV-inactivated AIV H9N2-pulsed APCs) for 72h. Total RNAs were extracted from the harvested cells using the total RNA extraction kits (iNtRON) according to the manufacturer's instructions and RNA concentrations were measured using a NanoDrop 2000 spectophotometer (Thermo Fisher Scientific, Pittsburgh, PA). The extracted RNA was subjected to real-time qRT-PCR using a One-Step SYBR® qRT-PCR reagent kit (Takara) and primers specific for the IFN-γ and IL-4 genes (Table 1). RT and real-time PCR amplification of targeted genes were carried out with each 75 ng total RNA as a template in 50μl of the reaction mixture with same reaction conditions and temperature cycles, as described above. The relative expression values of IFN-γ and IL-4 were normalized using the commonly used reference gene GAPDH. The fold change in relative gene-expression levels was calculated and all data were analyzed using the CFX96™ manager software version 1.6 (Bio-Rad).

Statistical analysis

Where specified, the data were analyzed for statistical significance using an unpaired two-tailed Student's *t*-test. A *p*-value <0.05 was considered significant.

Results

Construction of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and identification of chIFN-α expression

To test the efficacy of oral administration of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α to provide protection against AIV H9N2 infection, we constructed attenuated *S. enterica* serovar Typhimurium expressing chIFN-α. Initially, a 582-bp DNA fragment of the chIFN-α gene was amplified by RT-PCR using total RNA extracted from LPS-stimulated splenocytes (Figure 1A), and subcloned into the *Eco*RI and *Hin*dIII sites of pYA3560 vector that was used for the expression of foreign protein in *S. enterica* serovar Typhimurium (Figure 1B). Subsequently, pYA3560 encoding chIFN-α vector was transformed into attenuated *S. enterica* serovar Typhimurium χ8501 host by electroporation. The positive colonies of *S. enterica* serovar Typhimurium χ8501 harboring pYA3560 (χ8501/chIFN-α) were selected in the absence of DAP, and the in-frame fusion of the chIFN-α with the β-lactamase signal sequence was confirmed by nucleotide sequencing (data not shown). To identify the expression of chIFN-α protein by the constructed χ8501/chIFN-α, TCA-precipitated culture supernatants and bacterial cell lysates prepared at different incubation time points (12, 18, and 24h) were subjected to SDS-PAGE and Western blot. Attenuated *S. enterica* serovar Typhimurium harboring pYA3560 (vector alone) cultured for 18 h was used as a negative control. The expression of chIFN-α from *S. enterica* serovar Typhimurium harboring chIFN-α–encoding pYA3560 plasmid DNA (χ8501/chIFN-α) was detectable as early as 12h post-incubation, and gradually increased and saturated in the culture supernatants and cell lysates within 24h incubation (Figure 1C). Therefore, these results indicate that the attenuated *S. enterica* serovar Typhimurium harboring chIFN-α-encoding

pYA3560 (χ8501/chIFN-α) successfully expressed chIFN-α protein, which was actively secreted into the culture media instead of resulting from nonspecific membrane leaking or cell death by lysis.

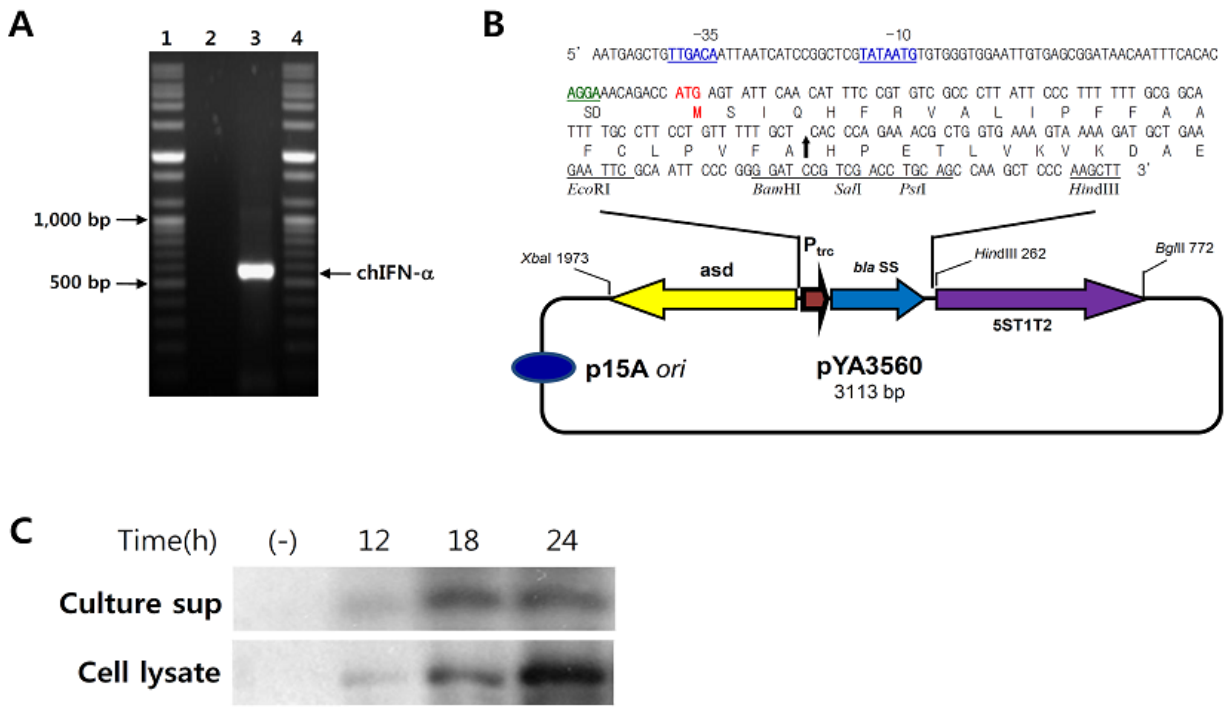

Figure 1: Construction of live attenuated *S. enterica* serovar Typhimurium expressing chIFN-α.

Antiviral activity of *S. enterica* serovar Typhimurium expressing chIFN-α against AIV H9N2

To estimate the antiviral activity of culture supernatants that included secreted chIFN-α, we used 10-day embryonated eggs that were previously inoculated by culture supernatants of χ8501/chIFN-α following filtration through a 0.22μm-pore size filter. The culture supernatants were inoculated into the allantoic cavity with different doses, based on total protein, and subsequently infected with 4 or 40 HA units of AIV H9N2 (01310). When the quantity of the propagated AIV H9N2 was determined by standard hemagglutination assay 4 days later, the growth of inoculated AIV H9N2 was reduced and inhibited in a dose-dependent manner (Figure 2). Therefore, this result indicates that biologically active chIFN-α proteins were successfully secreted from χ8501/swIFN-α into culture supernatants.

Alleviation of clinical signs and pathological changes by attenuated *S. enterica* serovar Typhimurium expressing chIFN-α in chickens infected with AIV H9N2

To determine if χ8501/chIFN-α could modulate the clinical signs caused by respiratory infection with AIV H9N2, we examined the mortality and severity of clinical signs in chickens that were administered χ8501/chIFN-α followed by respiratory infection with AIV H9N2. Chickens were intra-tracheally infected with AIV H9N2 strain ($10^{10.83}$ EID_{50}/chicken) 3 days after administration of χ8501/chIFN-α, and subsequently observed daily to record mortality and clinical severity. Mortality was evident at 4-5 days p.i., with the highest mortality rate (60%) evident in chickens that did not receive χ8501/chIFN-α. However, administration of χ8501/chIFN-α (10^9 and 10^{11}cfu) prior to AIV H9N2 infection significantly reduced mortality (20%) (Figure 3A). Also, when the severity of clinical signs caused by respiratory infection with AIV H9N2 was scored, clinical signs appeared 2 days p.i., and the severity of clinical signs peaked at 4-7 days p.i. (Figure 3B). Chickens that received *S. enterica* serovar Typhimurium harboring chIFN-α-encoded pYA3560 (10^9 and 10^{11} cfu) showed significant alleviation of clinical severity when compared to the group (vehicle) that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector. Furthermore, feed and water intakes were recorded daily after AIV H9N2 challenge of χ8501/chIFN-α-administered chickens. The average intake of feed (Figure 3C) and water (Figure 3D) improved in chickens that received χ8501/chIFN-α (10^9 and 10^{11} cfu), compared to chickens that received χ8501 harboring empty pYA3560 vector. Overall, these results indicate that oral administration of χ8501/chIFN-α could reduce mortality and alleviate clinical signs induced by respiratory infection with AIV H9N2.

Furthermore, to confirm the alleviation of AIV H9N2-induced clinical signs in chickens that received *S. enterica* serovar Typhimurium expressing chIFN-α (10^9 and 10^{11} cfu), we examined the histopathological changes in lung and tracheal tissues of chickens 5 days after AIV H9N2 infection. Chickens that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector displayed severe broncho-alveolar pneumonia by AIV H9N2 challenge, as manifested by hyperemia with infiltration of mononuclear inflammatory cells in bronchi and lung parenchyma resulting in destruction of lung alveoli (Figure 4A). Tracheitis in the vehicle-treated group was also noted, as characterized by infiltration of mononuclear inflammatory cells in the lamina propria of trachea and sloughing of lining epithelial cells with loss of cilia (Figure 4B). However, chickens that received χ8501/chIFN-α (10^9 and 10^{11} cfu) prior to AIV H9N2 challenge showed apparently normal lung and trachea, which were comparable to that of naïve chickens that received only PBS without treatment and AIV H9N2 challenge (Figure 4A & 4B). These results indicate that oral administration of χ8501/chIFN-α could provide protection against assaults of lung and tracheal tissues by respiratory infection with AIV H9N2.

Reduction of AIV H9N2 excretion by attenuated *S. enterica* serovar Typhimurium expressing chIFN-α

To evaluate the effect of the oral administration of χ8501/chIFN-α on virus shedding from AIV H9N2-infected chickens, the amount of virus in cloacal swabs was determined by real-time qRT-PCR at 0, 1, 3, 5, 7 and 9 days post-challenge. Virus shedding was detected from 1 day after AIV H9N2 infection and peaked at 5 days p.i. (Figure 5A). However, chickens that received χ8501/chIFN-α (10^9 and 10^{11} cfu) had significantly lower peak levels of virus shedding at 5 days p.i., compared to chickens that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector. Additionally, the amount of virus in different tissues (trachea, lung, brain, cecal tonsil, spleen, and kidney) of AIV H9N2-infected chickens was determined at 4 and 7 days p.i. As expected, the amount of AIV H9N2 in different tissues of chickens that received χ8501/chIFN-α was significantly lower than those of groups treated with *S. enterica* serovar Typhimurium harboring empty pYA3560 vector (Figure 5B). Taken together, these results indicate that oral administration of χ8501/chIFN-α could alleviate clinical signs induced by AIV H9N2 infection through reduction of virus replication in tissues.

Immune responses of chIFN-a-expressing Salmonella-administered chicks against AIV H9N2

We next examined the adaptive immune response in AIV H9N2-infected chickens with or without the oral administration of χ8501/chIFN-α, to better understand the protective role of χ8501/chIFN-α in respiratory infection with AIV H9N2. We determined the HI antibody titers in sera samples collected at 7 days p.i. Significantly enhanced HI antibody levels were observed in the sera of χ8501/chIFN-α-administered chickens, compared to that of χ8501 (pYA3560)-treated chickens (Figure 6A). To evaluate the cellular immune responses, PBMCs and splenocytes were prepared from AIV H9N2-challenged chickens at 14 days p.i. and stimulated with UV-inactivated AIV H9N2 antigen-pulsed APCs. PBMCs and splenocytes of chickens that received χ8501/chIFN-α (10^9 and 10^{11} cfu) orally prior to AIV H9N2 infection displayed significantly enhanced proliferation upon AIV H9N2 antigen-specific stimulation, compared to the chickens (vehicle) that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector (Figure 6B). Furthermore, the mRNA expression levels of IFN-γ and IL-4 in PBMCs and splenocytes were determined by real-time qRT-PCR following stimulation with AIV H9N2. Both IFN-γ and IL-4 mRNA levels in PBMCs and splenocytes prepared from χ8501/chIFN-α-treated chickens (10^9 and 10^{11} cfu) were significantly enhanced, compared to the chickens that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector. In particular, the expression of IFN-γ mRNA was more significantly up-regulated than IL-4 mRNA in all cases, indicating that the Th1-biased immunity was mounted by oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α. Taken together, our results indicate that oral administration of χ8501/chIFN-α prior to AIV H9N2 infection could effectively induce enhanced humoral and cell-mediated immunity in chickens.

Discussion

AIV H9N2 has attracted considerable attention due to severe commercial losses in poultry industry. Furthermore, since H9N2-infected chickens can serve as reservoir host and transmit the virus to mammals such as pigs and humans [100-101], control of viral mutants is becoming increasingly difficult. Therefore, chIFN-α may be clinically useful as an exogenous antiviral agent to boost host innate immunity responses for controlling low-pathogenicity AIV infection. Although it was identified that recombinant chIFN-α protein can protect SPF chickens from AIV H9N2 challenge following oral administration, a more superior delivery vehicle for oral administration of chIFN-α is needed due to protein instability, production costs, and labor associated with mass administration. Here, we provide valuable insight into the use of attenuated *Salmonella* bacteria as an oral delivery system of chIFN-α that can be used for mass administration, thereby overcoming the cost and production issues. Presently, the oral administration of a single dose of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α alleviated clinical signs and pathological changes caused by respiratory infection with AIV H9N2 and reduced the excretion of AIV H9N2 in cloacal swab samples. Similarly, chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α showed inhibited replication of AIV H9N2 in several different tissues including trachea, lung, brain, cecal tonsil, spleen, and kidney. Furthermore, immune responses specific for AIV H9N2 were enhanced in chickens

administered *S. enterica* serovar Typhimurium expressing chIFN-α, as evaluated by HI assay, and the proliferation and IFN-γ and IL-4 expression of AIV H9N2 antigen-stimulated PBMC and splenocytes. Therefore, these results indicate that chIFN-α expressed from attenuated *S. enterica* serovar Typhimurium can successfully control respiratory disease caused by AIV H9N2 infection by inhibiting *in vivo* replication of virus following oral administration, by which may block transmission of virus to neighboring chickens as well as mammals.

Cytokines, as natural mediators of the innate and adaptive immune responses, may be an excellent alternative to conventional therapeutics such as treatment with antibiotics. Indeed, cytokine therapy has been shown to be effective in livestock and poultry. The use of chicken cytokines is becoming more feasible with the recent cloning of a number of cytokine genes, since the chicken's immune system is similar to that of mammals [6]. However, the main obstacles in the practical use of chicken cytokines for prevention and/or therapeutic of avian diseases are the lack of suitably cost-effective production and delivery systems for mass administration. To overcome these obstacles, our group previously showed that oral administration of attenuated *S. enterica* serovar Typhimuirum expressing swine interferon-α could be used to control intestinal diseases caused by intestinal infection of piglets with virus. The present study demonstrates that oral administration of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α can modulate clinical signs and pathological changes caused by respiratory infection with AIV H9N2. The collective data imply that oral administration of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α can control disease caused by viral infection via a different route (i.e., respiratory route). Conceivably, it is assumed that chIFN-α secreted from attenuated *Salmonella* bacteria may be able to affect responses throughout the host body since *Salmonella* bacteria can colonize the gut-associated lymphoid tissue (Peyer's patch) as well as visceral non-lymphoid and lymphoid tissues (liver, lymph nodes, and spleen). In support of this view, chickens that received oral administration of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α showed enhanced humoral and cellular immune responses specific for AIV H9N2 antigen, as detected in PBMCs and spleen (Figure 6). Therefore, it is possible that attenuated *S. enterica* serovar Typhimurium expressing chIFN-α may be used as delivery for innate modulator to enhance immune responses against several vaccines following oral administration. The immunomodulatory functions of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α are currently being investigated using inactivated AIV H9N2 vaccine.

The biological activity of chIFN-α that are encoded in recombinant *Salmonella* bacteria can be elicited by binding to type I IFN-α/β receptor on target cells through soluble forms secreted from the cells. Therefore, the stable maintenance of chIFN-α-encoding plasmid DNA in *Salmonella* vaccines during the *in vivo* colonization process and secretion of chIFN-α from bacteria are required. For the former issue, we used Asd+ plasmid DNA (pYA3560) that are retained *in vivo* in *Salmonella* vaccine strains devoid of the asd gene, as an essential factor for a balanced-lethal host-vector system [91-92]. A signal sequence plus an additional 12 amino acids of mature β-lactamase are required to translocate β-lactamase through the cytoplasmic membrane of gram-negative bacteria [122-123]. Thus, fusion of a protein to the β-lactamase signal peptide is expected to promote the secretion of the fusion protein into the bacterial periplasm [123-124]. For the latter issue, the presently-constructed pYA3560 plasmid DNA was designed to use for the periplasmic secretion of chIFN-α by the *Salmonella* vaccine. We reasoned that chIFN-α attached to the β-lactamase signal peptide should be secreted into the culture of attenuated *Salmonella* bacteria. Indeed, a significant amount of chIFN-α protein was secreted into the culture supernatant, as detected by Western blot (Figure 1C). Appropriately, the culture supernatant of attenuated *Salmonella* vaccine containing chIFN-α-encoded pYA3560 vector showed antiviral activity against AIV H9N2 inoculated in embryonated eggs (Figure 2), which indicates that biologically intact chIFN-α protein existedin the culture supernatants.

The primary target cells for AIV infection and replication are ciliated epithelial cells. However, AIV can also infect macrophages and dendritic cells [125-126]. In avian species, intestinal epithelia are also targets of infection and, in the later stage of infection, mononuclear cells become involved [127-128]. Influenza A virus causes NS1-mediated suppression of selected genes involved in IFN and IFN-inducible gene expression [129], and induction of a weak chemokine expression in human lung epithelial cells [130], which enable the virus to replicate before the host inflammatory and antiviral responses are activated. Protection of chickens from AIV H9N2 requires early induction of type I IFNs, especially IFN-α, which have direct antiviral effects mediated by IFN-α-induced antiviral proteins such as the Mx1, 2',5'-oligoadenylate synthetase (OAS) that confers an antiviral state to cell. IFN-α pretreatment also enhances chemokine and cytokine production through the activation of IRF and NF-κB transcription factors, which have major roles in recruiting leukocytes to the site of inflammation and activating innate immune responses [130]. Therefore, it is possible that oral administration of attenuated *Salmonella* bacteria expressing chIFN-α could effectively stimulate host innate and adaptive immune responses before the establishment of infection, thereby preventing virus replication in host tissues and effectively alleviating the clinical severity of AIV H9N2 infection.

Attenuated *S. enterica* serovar Typhimurium is a well-characterized vaccine strain available to livestock industry for the prevention of salmonellosis. This registered attenuated *Salmonella* strain has the potential for heterologous protein delivery in livestock vaccination [131]. Furthermore, since

the *Salmonella* bacteria used in this study were devoid of the asd gene that is essential for a balanced-lethal host-vector system, they may have been sufficiently attenuated in their capacity to cause acute diseases in chickens. Indeed, all chickens orally administered *S. enterica* serovar Typhimurium expressing chIFN-α did not display any signs of disease for a 15-day monitoring period (data not shown). However, to accomplish the effective results for controlling infectious diseases in chickens by the *Salmonella* delivery system, the successful and prolonged colonization of *S. enterica* serovar Typhimurium expressing chIFN-α may be needed. It is anticipated that the periods of colonization of *S. enterica* serovar Typhimurium expressing chIFN-α may depend upon the age of animals at administration [132-134], although the persistence and distribution of *S. enterica* serovar Typhimurium were not determined in this study. Conceivably, it is possible that *S. enterica* serovar Typhimurium expressing chIFN-α may persist in younger chickens for a longer time. To improve the practical use of the attenuated *Salmonella* strain as oral carrier of cytokines, the number of times being administered and its schedule should be addressed further. In conclusion, our results suggest that oral administration of single dose of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α provides a useful means for controlling respiratory disease caused by AIV H9N2 infection.

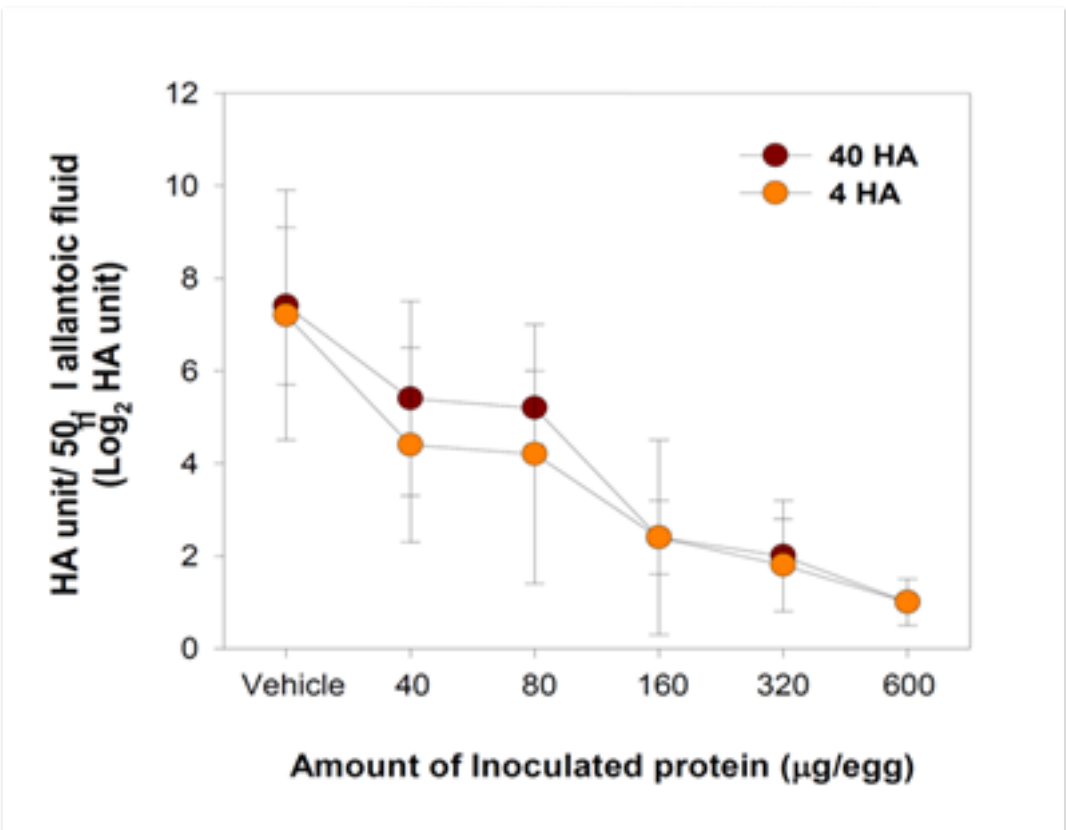

Figure 2: Antiviral activity of culture fluid obtained from *S. enterica* serovar Typhimurium expressing chIFN-α against AIV H9N2.

We recommend that attenuated *S. enterica* serovar Typhimurium may be used as an effective delivery system of chIFN-α and other chicken cytokines which may circumvent the obstacles in the use of chicken cytokines in disease prevention. Oral administration of chIFN-α in chickens at their early age using attenuated *S. enterica* serovar Typhimurium may stimulate their immune system to mount better protective immunity upon exposure to respiratory viruses like AIV H9N2 at their critical stage and therefore, may represent an alternative strategy in disease prevention.

Figure 1: Construction of live attenuated *S. enterica* serovar Typhimurium expressing chIFN-α. (A) The chIFN-α gene amplified by RT-PCR. Total RNA extracted from LPS-stimulated splenocytes was employed to amplify the chIFN-α gene using specific primer pair. The arrow in agarose gel image indicates amplified chIFN-α gene. Lanes 1 and 4, size marker; 2, naïve splenocytes; 3, LPS-stimulated splenocytes (B) Diagram of periplasmic secretion Asd+ vector pYA3560. A DNA fragment encoding the β-lactamase signal sequence and 12 amino acid residues of the N terminus of mature β-lactamase of plasmid pBR322

was positioned under the control of the P_{trc} promoter. The map of pYA3560 and the nucleotide sequences of the P_{trc} promoter region, β-lactamase signal sequence (*bla* SS) and multicloning sites are shown. The P_{trc} sequences for -35, -10 (RNA polymerase-binding site) and Shine-Dalgarno box (SD, ribosomal binding site) are indicated by blue and green boldface, and the translocation start codon (ATG) is in red boldface. An arrow within the sequence indicates the signal peptidase cleavage site. Unique restriction enzyme sites in the multicloning site are indicated. P15A *ori* represents origin of replication of p15A plasmid, and 5ST1T2 is a transcriptional terminator. (C) Identification of chIFN-α expression by immunoblot analysis. The expression of chIFN-α protein was detected by immunoblotting with chIFN-α-specific monoclonal antibody in TCA-precipitated culture supernatants (sup) and cell lysates of attenuated *S. enterica* serovar Typhimurium harboring pYA3560/chIFN-α (χ8501/chIFN-α) that were incubated for 12, 18, and 24h. Attenuated *S. enterica* serovar Typhimurium carrying empty pYA3560 vector cultured for 18 h was used as a negative control.

Figure 2: Antiviral activity of culture fluid obtained from *S. enterica* serovar Typhimurium expressing chIFN-α against AIV H9N2. The culture supernatants of *S. enterica* serovar Typhimurium expressing chIFN-α were inoculated into allantoic cavity of embryonated eggs, based on protein amount of culture supernatant (10eggs per dose of culture supernatant), and the inoculated eggs were subsequently infected with AIV H9N2 (4 or 40 HA units, 5 eggs per group). Following 4 days incubation, the virus titers in allantoic fluids were determined by hemagglutination assay. The data was expressed by reciprocal log 2 of the geometric average and SD of HA units/50μl of allantoic fluid.

Figure 3: Alleviation of clinical signs by attenuated *S. enterica* serovar Typhimurium expressing chIFN-α in AIV H9N2-infected chickens. (A) Mortality of AIV H9N2-infected chickens. Three days following oral administration of χ8501/chIFN-α (10^9 or 10^{11} cfu/bird), groups of chickens (n=5) were intra-tracheally infected with AIV H9N2 ($10^{10.83}$ EID_{50}/bird). The graph shows the proportion of surviving chickens on different days p.i. (B) Clinical severity of AIV H9N2-infected chickens. Chickens administered χ8501/chIFN-α was infected with AIV H9N2 virus, after which the clinical severity was scored daily. *p<0.05; **p<0.01 compared between vehicle-treated and χ8501/chIFN-α (10^9 or 10^{11} cfu/bird)-treated groups (C and D) Feed and water intake of AIV H9N2-infected chickens. Feed and water intake were recorded daily after AIV H9N2 challenge of χ8501/chIFN-α-administered chickens. Data show the average of feed (C) and water (D) intake obtained from five chickens per group.

Figure 4: Histopathological lesions in lung and trachea of AIV H9N2-infected chickens. Groups of chickens were intra-tracheally infected with AIV H9N2 ($10^{10.83}$ EID_{50}/bird) 3 days following oral administration of χ8501/chIFN-α (10^9 or 10^{11} cfu per bird). The histopathological lesions in lung (A) and trachea (B) were examined by euthanizing chickens 4 days p.i. The histopathological pictures are representative of sections derived from four chickens per group (H & E, ×100).

Figure 5: Reduction of virus shedding and replication in AIV H9N2-challenged chickens by *S. enterica* serovar Typhimurium expressing chIFN-α. (A) Virus shedding of χ8501/chIFN-α-treated chickens after AIH9N2 challenge. Groups of chickens treated with 8501/chIFN-α were intratracheally infected with AIV H9N2 3 days later, and cloacal swab samples were taken at 0, 1, 3, 5, 7 and 9 days post-challenge. The amounts of AIV H9N2 in swab samples were determined by real-time qRT-PCR specific for hemagglutinin protein of AIV H9N2. Data represent the average and SD of five chickens per group. (B) The amount of virus in tissues of AIH9N2-infected chickens. Chickens treated with χ8501/chIFN-α were euthanized 4 and 7 days after AIV H9N2 virus challenge. Total RNAs extracted from tissues (trachea, lung, brain, cecal tonsil, spleen, and kidney) were used for real-time qRT-PCR analysis to determine AIV H9N2 amount. Data show the average and SD of AIV H9 fold expression obtained from five chickens per group, after normalized to GAPDH. *p<0.05; **p<0.01; ***p<0.001 compared between vehicle-treated and χ8501/chIFN-α (10^9 or 10^{11} cfu/bird)-treated groups.

Figure 6: Immune responses of AIV H9N2-challenged chickens following administration of *S. enterica* serovar Typhimurium expressing chIFN-α. (A) Serum HI antibody titers in AIV H9N2-challenged chickens. Serum samples were collected from χ8501/chIFN-α-administered chickens 7 days after AIV H9N2 challenge and subjected to HI test. Data was expressed as reciprocal log2 of the geometric average and SD of HI antibody titers obtained from five chickens per group. (B) AIV H9N2 antigen-specific proliferation of PBMCs and splenocytes prepared from challenged chickens. PBMCs and splenocytes were prepared from AIV H9N2-challenged chickens 14 days p.i., and stimulated with enriched APCs that had been pulsed with inactivated AIV H9N2 antigen. AIV H9N2 antigen-specific proliferation of PBMC and splenocytes were assessed by measuring the viable cell ATP bioluminescence following 72 h incubation. (C) The expression of IFN-γ and IL-4 in PBMCs and splenocytes following stimulation of AIV H9N2 antigen. Total RNAs were extracted from PBMC and splenocytes stimulated with AIV H9N2 antigen for 72 h, and used for real-time qRT-PCR to determine the expression of IFN- and IL-4. Data show the average and SD of IFN-γ and IL-4 expression obtained from five chickens per group, after normalized to GAPDH. *p<0.05; **p<0.01; ***p<0.001 compared between vehicle-treated and χ8501/chIFN-α (10^9 or 10^{11} cfu/bird)-treated groups

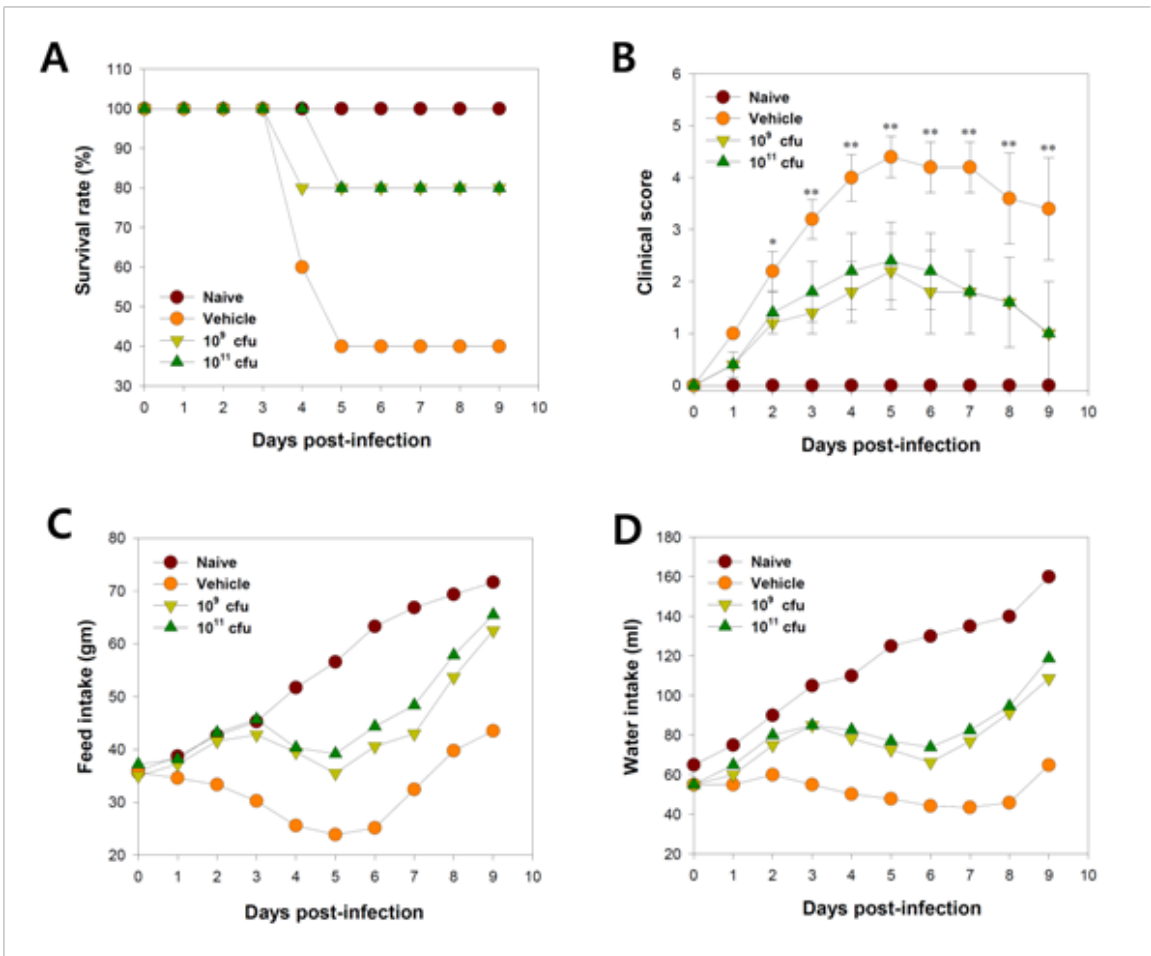

Figure 3: Alleviation of clinical signs by attenuated *S. enterica* serovar Typhimurium expressing chIFN-α in AIV H9N2-infected chickens.

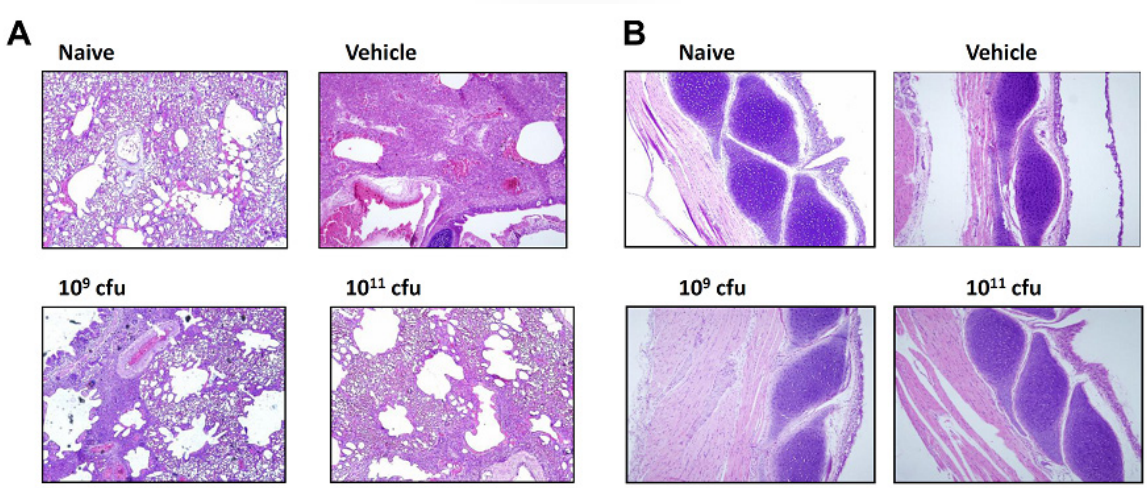

Figure 4: Histopathological lesions in lung and trachea of AIV H9N2-infected chickens.

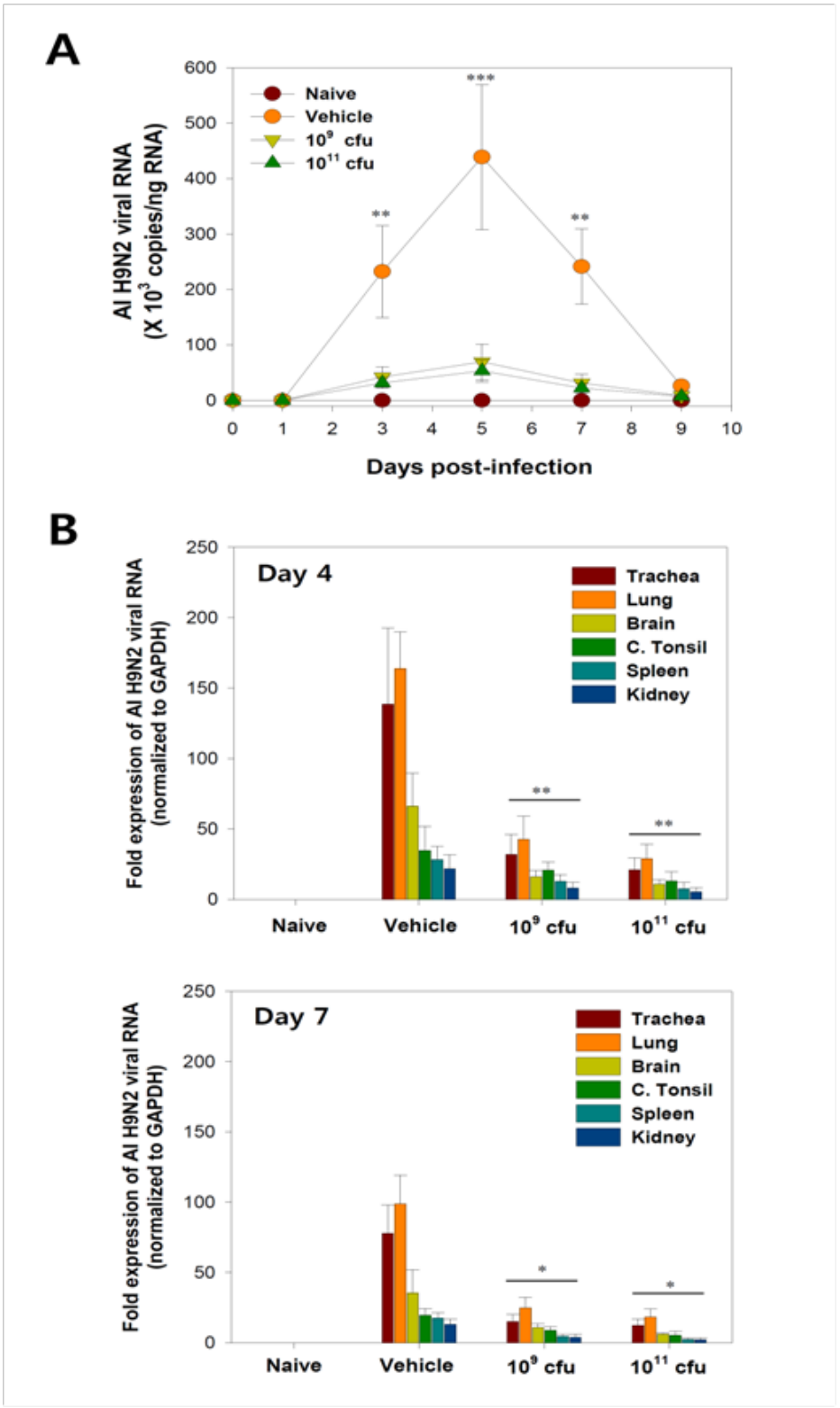

Figure 5: Reduction of virus shedding and replication in AIV H9N2-challenged chickens by *S. enterica* serovar Typhimurium expressing chIFN-α.

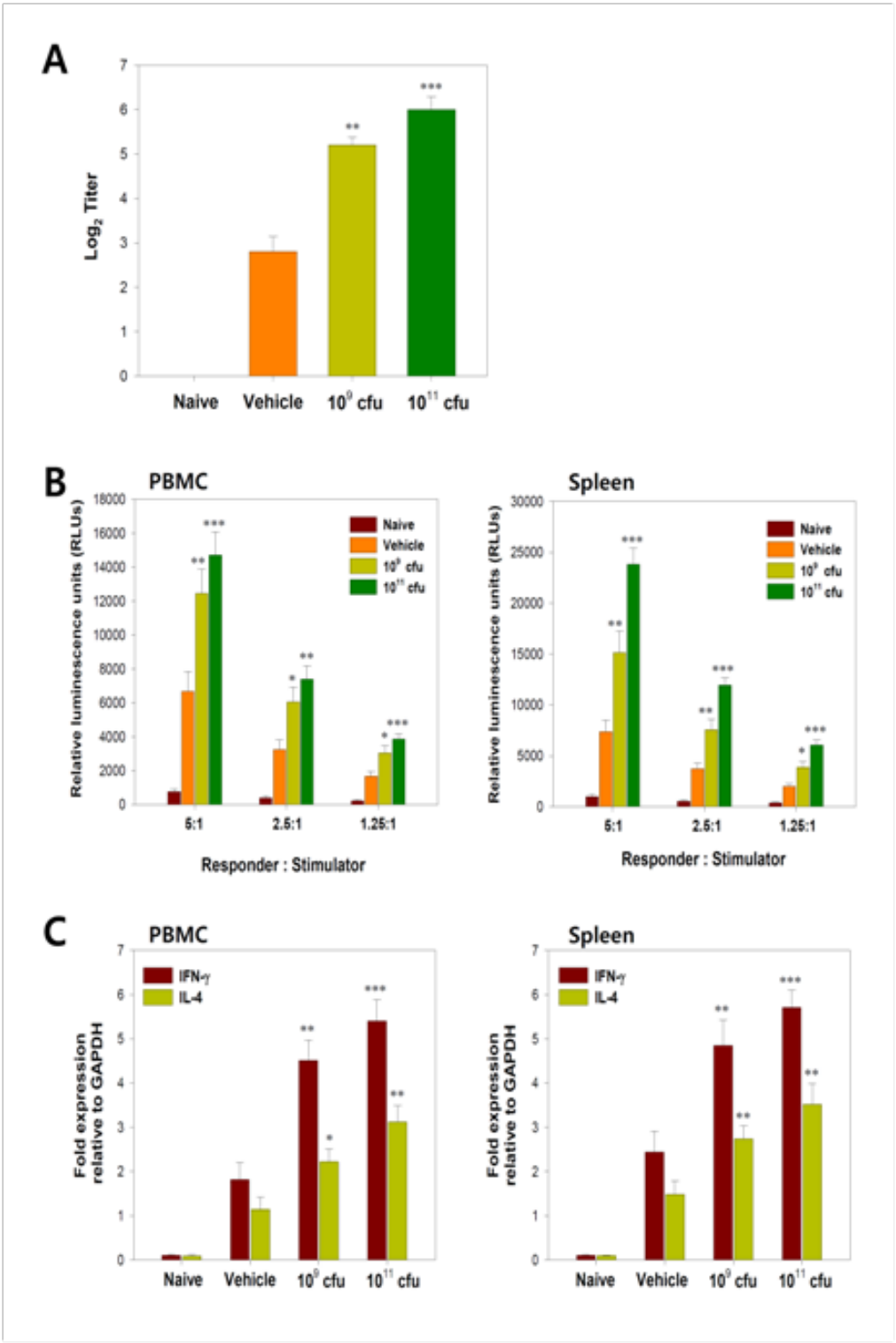

Figure 6: Immune responses of AIV H9N2-challenged chickens following administration of *S. enterica* serovar Typhimurium expressing chIFN-α.

Chapter 3

Oral co-administration of live attenuated Salmonella enterica serovar Typhimurium expressing chicken interferon-α and interleukin-18 induced enhanced alleviation of clinical signs caused by infection with low pathogenic avian influenza virus H9N2

Abstract

Cytokines may represent most exciting new candidates of naturally occurring environmental friendly alternatives over existing conventional disease control strategies. Combined use of cytokines has shown synergistic and/or additive effects in controlling several viral infections of livestock animals. However, little is known about the practical use of chicken cytokines in combinations to control avian diseases, due to lack of cost effective suitable production and delivery systems for mass administration. In order to provide valuable insight into the combined use of chicken cytokines in disease prevention, we investigated the antiviral efficacy of oral co-administration of chicken interferon-α (chIFN-α) and interleukin-18 (chIL-18) using attenuated *Salmonella enterica* serovar Typhimurium in chickens infected with avian influenza virus (AIV) H9N2. Our results demonstrate that oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 displayed significantly enhanced alleviation of the clinical signs of chickens caused by respiratory infection with AIV H9N2, when compared to chickens orally administered *S. enterica* serovar Typhimurium expressing either chIFN-α or chIL-18, as determined by mortality, clinical severity, and feed and water uptakes. This enhancement of antiviral immunity was further confirmed by reduced rectal shedding and replication of AIV H9N2 in several different tissues of challenged chickens including trachea, lung, cecal tonsil, and brain. Furthermore, oral co-administration of chIFN-α and chIL-18 more efficiently modulated the immune responses of chickens against AIV H9N2 through enhancing both humoral and Th1-biased cell mediated immunity, compared to single administration of constructs. Therefore, our results suggest that the combined administration of chIFN-α and chIL-18 using attenuated *S. enterica* serovar Typhimurium as an oral carrier of two chicken cytokines provides a useful means for controlling respiratory disease caused by AIV H9N2 infection.

Introduction

The World Health Organization (WHO) has now advised meat producers to use environmentally-friendly alternative methods to control livestock and poultry diseases after performing an extensive review on the use of in-feed antibiotics and chemicals, a usual practice over 50 years in disease prevention and control. Immunization with vaccines is considered as the most promising control measure for infectious diseases to date although vaccines can confer specific protection against a particular pathogen following immunization and several limitations in using both live and inactivated vaccines needs to be circumvented yet. Cytokines, as natural mediators of the innate and adaptive immune responses might be an excellent alternative to conventional disease prevention strategies. Cytokines determine both the type and extent of an immune response that is generated following infection with a pathogen or after vaccination, thereby playing a crucial role in controlling the immune response. Depending on the combination of cytokines produced, a protective immune response can be generated as either an antibody-mediated (Th2) response or a cell-mediated (Th1) response [77-79].The use of chicken cytokines is becoming more promising with the recent cloning of many new cytokine genes, since the chicken's immune system is similar to that of mammals [6].

The potential effectiveness of cytokine combinations has been addressed factually, based upon mechanisms determining the nature of innate and acquired immunity [77-79]. Likewise, the enhanced combined effects of cytokines in antiviral response has been described in several infectious diseases of livestock animals such as foot-and-mouth disease (FMD) [135], transmissible gastroenteritis (TGE) [136], porcine reproductive and respiratory syndrome (PRRS) [137] and Pseudorabies [138]. However, little is known about the combined use of chicken cytokines in disease prevention. Chicken interferon-α (chIFN-α) is a type I IFN that plays essential roles in the host antiviral responses through stimulating T-dependent lymphocyte system and inducing a number of IFN-stimulated genes (ISGs) through triggering the Janus-activated kinases (JAKs)-signal transducer and activators of transcription (STAT) 1/2 pathway [36]. Accordingly, there have many reports that administration of chIFN-α inhibits important epidemic avian viruses, such as infectious bronchitis virus (IBV) [37], infectious bursal disease virus (IBDV) [38], Newcastle disease virus (NDV) [39], and avian influenza virus (AIV) [139]. Interleukin-18 (IL-18) is originally known as potent interferon-γ (IFN-γ)-inducing factor (IGIF) which shares properties with IL-12. It acts synergistically with IL-12 to promote IFN-γ production, which plays an important role in inducing Th1 immune responses [53], thus IL-18 provides an important link between the innate and adaptive immune responses. Numerous studies have ensured that mammalian IL-18 has been characterized in great detail [53]. However, the properties and application of chicken IL-18 in disease prevention still remains largely uninvestigated as of yet. The few studies of chicken IL-18 that have been conducted have showed that recombinant fowl pox vaccine expressing chIL-18 and surface glycoprotein H5-H7 of avian influenza (rFPV-H5-H7-IL18) successfully induced complete protection (100%) in SPF chicken after challenge with H5 AIV, and lymphocyte proliferation induced by the rFPV-H5-H7-IL18 was significantly higher than that induced by rFPV-H5-H7 alone [59]. In our previous study, we reported that oral co-administration of attenuated *Salmonella enterica* serovar Typhimurium expressing swine interferon-α and interleukin-18 could provide enhanced protection in piglets against infection with TGEV [136] Therefore, it is assumed that both chIFN-α and chIL-18 may have great values for use in combination in chicken as an excellent natural alternative of disease prevention. However, the practical use of chicken cytokines either singly or in combination to control poultry diseases is limited particularly by cost, labor, and time, as well as protein stability. Therefore, it is necessary to develop an effective delivery system for the mass administration of chicken cytokines to overcome these limitations.

To this end, our previous studies reported that attenuated aspartate β-semialdehyde dehydrogenase (Asd)-negative *Salmonella enterica* serovar Typhimurium devoid of antibiotic resistance genes might be used as an effective delivery system for the mass administration of both mammalian and non-mammalian cytokines and other proteins without the need for antibiotic selection [25,136,139-140]. Additionally, cytokines produced by *S. enterica* serovar Typhimurium may be able to provide protective functions at both mucosal and systemic sites, as the bacteria after oral administration colonize the secondary lymphoid and nonlymphoid tissues including the lymph node, spleen, and liver as well as the gut-associated lymphoid tissues [90,110]. Based on the recent information on immunobiological activities of chIFN-α and IL-18, we speculated that combined use of chIFNa and chIL-18 may have more enhanced protective function in viral infections. In the present study, we investigated the antiviral efficacy of oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 in chickens using respiratory infection with AIV H9N2, one of the serious global problems not only for poultry industry but also for other animals including human. The oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 induced more enhanced alleviation of AIV H9N2-induced clinical signs in chickens, compared to the chickens that received single administration of *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18. Furthermore, oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN- and chIL-18 markedly inhibited both viral shedding and replication in tissues that might be mediated by providing Th1-biased protective immunity against avian influenza H9N2 virus.

Materials and Methods

Animals and ethics statement

SPF leghorn layer (white) chickens were obtained from Jinan Baizhun Biologic Inspection, China, and reared with formulated commercial feed and water provided ad libitum throughout the whole experimental period. All experimental procedures and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committees of Chonbuk National University. The animal facility of the Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Cells and viruses

Low pathogenic avian influenza A virus (LPAIV) H9N2 strain, A/Chicken/Korea/01310/2001 (01310), which has been described previously [111], was a kind gift from the National Veterinary Research and Quarantine Service of the Republic of Korea and used for challenge infection to chickens. The AIV H9N2 (01310) was propagated by inoculating in the allantoic cavity of 10-day-old embryonated eggs and allantoic fluid was harvested 96 h after inoculation. Virus in the allantoic fluid was titrated using a standard hemagglutination test [112] and the infectious viral titer was determined by using 10-day-old embryonated eggs, as previously described [141].

Bacterial strains, plasmid, media, and growth conditions

Escherichia coli χ6212 (F$^-$λ$^-$Φ80Δ (lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4) [91] was used as the host strain for construction of the Asd+ plasmid vectors encoding chIFN-α or chIL-18. Attenuated *S. enterica* serovar Typhimurium χ8501 (hisG Δcrp-28 ΔasdA16), which was kindly provided by Dr. HY. Kang (Pusan National University, Korea) [92], was used for host bacteria to deliver chIFN-α and chIL-18 proteins. 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 [116]. *E. coli* and *S. enterica* serovar Typhimurium cultures were grown at 37 °C in Lennox broth, Luria-Bertani (LB) broth or on LB agar. Diaminopimelic acid (DAP; Sigma-Aldrich, St. Louis, MO) was added (50μg/ml) to induce the growth of Asd-negative bacteria [91]. Phosphate-buffered saline (PBS, pH 7.4) containing 0.01% gelatin (BSG) was used for the resuspension of Salmonella bacteria that were concentrated by centrifugation at 7000 × g, 4 °C for 5min.

Construction of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18

Total RNA was extracted from splenocytes that were previously stimulated with lipopolysaccharde (LPS, 20μg/ml) for 48h after resuspending cells (10^7 cells/ml) in complete RPMI medium, and employed to amplify the chIFN-α chIL-18 genes with reverse transcription-polymerase chain reaction (RT-PCR) using specific primer pairs (Table 3). The PCR products were then inserted into pGEMT vector (Promega, Madison, WI) and the chIFN-α and chIL-18 genes were sequenced to confirm the authenticity of insert sequences. Subsequently, chIFN-α and chIL-18 genes were sub cloned into the pYA3560 and pYA3493 plasmid vectors, respectively. The pGEMT vectors encoded with chIFN-α and chIL-18 genes were digested with *EcoRI* and *HindIII*, after which the released fragments containing the chIFN-α and chIL-18 genes were inserted into the same restriction sites of pYA3560 and pYA3493 plasmid vectors using E. coli χ6212 hosts grown in the presence of DAP. The positive colonies of *E. coli* χ6212 harboring either chIFN-α-encoding pYA3560 or chIL-18-encoding pYA3493 vectors were selected in the absence of DAP. To construct attenuated *S. enterica* serovar Typhimurium expressing either chIFN-α or chIL-18, *S. enterica* serovar Typhimurium χ8501 (1×10^8 cfu) washed extensively with sterilized ice-cold WB (10% 10% ultra pure glycerol, 90% distilled water, v/v) were mixed with 10 pg to 0.1μg of either chIFN-α-encoding pYA3560 or chIL-18-encoding pYA3493 plasmid DNA on ice in a 0.2 cm cuvette and electroporated using a Bio-Rad Gene pulser at 12.5 kV/cm (2.5 kV, 25μF and 200 Ω; Bio-Rad, Hercules, CA), respectively [116]. The bacteria were then removed

from the cuvette into sterile culture tubes containing 1ml of LB broth medium and incubated with moderate shaking for 60 min at 37 °C. The transformed cultures (each 100µl) were then plated onto LB agar plates in the absence of DAP. Finally, colonies of the attenuated *S. enterica* serovar Typhimurium harboring either chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) vector were cultured and stored after confirmation of the coding sequences.

Table 3: Primers for PCR amplification of chIFN-α, chIL-18, AIH9, IFN-γ, IL-4 and GAPDH.

Target Gene		Primer Sequence (5'-3')	Accession No.	Reference
chIFN-aa	F	ATGGCTGTGCCTGCAAGCCCA	DQ026259.1	-
	R	CTAAGTGCGCGTGTTGCCTGT		
chIL-18b	Fc	GAATTCGCCTTTTGTAAGGATAAAACT	HM854281.1	-
	Rc	AAGCTTTCAGTGATGGTGATGGTGATG TAGGTTGTGCCTTTC		
AIH9	F	CTACTGTTGGGAGGAAGAGAATGGT	AF461510.1	[116]
	R	TGGGCGTCTTGAATAGGGTAA		
IFN-γ	F	CAAAGCCGCACATCAAACA	X99774	[117]
	R	TTTCACCTTCTTCACGCCATC		
IL-4	F	GAGAGGTTTCCTGCGTCAAG	FJ907790.1	[118]
	R	TGGTGGAAGAAGGTACGTAGG		
GAPDH	F	AGAACATCATCCCAGCGTCC	X01578	[117]
	R	CGGCAGGTCAGGTCAACA		

[a]The primer pair specific for chIFN-α gene was designed using chIFN-α 6 nucleotide sequences (Genebank accession number DQ026259.1), and the sequences of the two primers were checked using the NCBI Blast Software.

[b]The primer pair specific for chIL-18 gene was designed using chIL-18 nucleotide sequences (Genebank accession number HM854281.1), and the sequences of the two primers were checked using the NCBI Blast Software.

[c]The forward and reverse primers specific for chIL-18 gene contain *EcoR* I and *Hind* III restriction sites as indicated by the underline. Reverse primer also contains 6xHis-Tag sequences indicated as boldface.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses

The expression of chIFN-α and chIL-18 proteins by *S. enterica* serovar Typhimurium harboring either chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) plasmid was identified by immunoblot following gel separation of prepared proteins by SDS-PAGE. For the preparation of protein samples, *Salmonella* bacteria cultured for 12, 18, and 24h were resuspended in 4 ml of 20mM Tris-HCl (pH 8.6) and then disrupted by two passages through a French pressure cell (American Instrument, Silver Spring, MD). Cell lysates were centrifuged at 7000×g, 4 °C for 6 min to remove unbroken cells and the supernatant fraction was used for protein samples of cell lysates. The original culture supernatants were filtered (0.22µm-pore-size filter) and proteins that had been secreted into the supernatants were precipitated with 10% trichloroacetic acid (TCA) at 4 °C for 1h. Prepared protein samples were boiled for 5 min and then separated by SDS-PAGE. The protein bands were visualized by staining with 0.1% Coomassie brilliant blue R-250 solution (Elpis-biotech, Deajeon, Korea). For immunoblotting, the resolved proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked with a blocking buffer consisting of PBS containing 3% skim milk and 0.5% Tween 20, and then incubated with chIFN-α monoclonal antibody (Serotec, Raleigh, NC) and His_6-Tag antibody (Novagen, Madison, WI) to detect chIFN-α and 6×histidine tagged-chIL-18, respectively. Following 1.5h incubation, a peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Southern Biotech, Birmingham, AL) was added. Immunoreactive bands were detected by the addition of chemiluminescence dye using a WEST-one™ Western Blot Detection System (iNtRON, Seongnam-Si, Korea) in the presence of H_2O_2.

Animal experimental designs for AIV H9N2 vaccination and challenge

A total of 40 SPF chickens (18 days old) were divided randomly into five groups. The first group (n=5) was a negative control that was orally administered vehicle (PBS containing 0.01% gelatin) without *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18. The second group (n=5) was orally administered *S. enterica* serovar Typhimurium harboring pYA3560 vector (10^9 cfu/chicken) as

a control of the empty pYA3560 vector. The remaining three groups (n=10 per group), each comprising two replications (n=5 per replication) for two different doses, were orally administered either *S. enterica* serovar Typhimurium expressing chIFN-α (10^9 and 10^{11} cfu/chicken) or chIL-18 (10^9 and 10^{11} cfu/chicken) or both in a combination (each 10^9 and 10^{11} cfu/chicken). Oral administration was performed by instilling the resuspended bacteria (1 ml/chicken) into esophagus using flexible gavage feeding needle (Fine Science Tools, North Vancouver, British Columbia, Canada) after withholding feed for 4h. Three days after treatment, chickens of all groups, except negative control group, were intra-tracheally infected with AIV H9N2 (01310) ($10^{10.83}$ EID_{50}/chicken) [117,118] [26,27]. Following challenge, chickens were observed daily for clinical signs and mortality throughout the duration of the experiment. The clinical signs were scored daily as follows: 0, no sign; 1, slight depression; 2, moderate depression + reduced movement + reduced food/water intake (anorexia); 3, moderate respiratory distress (sinusitis, cough); 4, severe respiratory distress (sinusitis, severe cough)+diarrhea; 5, death. Average feed and water intake was determined daily for 9 days after challenge. Cloacal swab samples were collected at 0, 1, 3, 5, 7, and 9 days post-infection (p.i.). Another experiment was carried out with same experimental setup except that the experiment was carried out for up to 14 days p.i. to collect additional samples for determination of virus amount in tissues. Furthermore, peripheral blood mononuclear cells (PBMC) and splenocytes were isolated to determine cell proliferation, and IFN-γ and IL-4 mRNA expression upon AIV H9N2 antigen-specific stimulation.

Real-time quantitative RT-PCR (qRT-PCR) analysis

Real-time qRT-PCR was employed to determine the amount of AI H9N2 virus in cloacal swab samples or the tissues using a CFX96™ real-time PCR detection system (Bio-Rad). Total RNA was extracted from cloacal swab samples and tissue samples (trachea, lung, brain, cecal tonsil, spleen, and kidney) using viral RNA extraction and total RNA extraction kits (iNtRON), respectively, according to the manufacturer's instructions. The extracted RNA was subjected to real-time qRT-PCR using a One-Step SYBR® qRT-PCR reagent kit (Takara, Shiga, Japan) and primers specific for the AIV H9 gene (Table 3). Following reverse-transcription of the viral RNA at 45 °C for 30min, the resulting cDNAs were used for real-time PCR amplification. PCR amplification was conducted by subjecting reaction mixtures to initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 15 s and annealing/elongation at 58 °C for 30s. A standard curve was generated by plotting threshold cycle values against serially diluted plasmid DNA encoding the AIV H9 protein. After the reaction cycle was completed the temperature was increased from 50 °C to 95 °C at a rate of 0.2 °C /15 s and the fluorescence was measured every 5 s to construct a melting curve that was used to confirm the authenticity of the amplified products. A control sample that contained no template RNA was run with each assay, and qRT-PCR data for AIV H9 amounts in the tissues was normalized using the commonly used reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 3). All determinations were performed by data from wells evaluated in duplicate to ensure reproducibility. The copy number of the experimental samples was determined by interpolating the threshold cycle values using the standard curve. All data were analyzed using the CFX96™ manager software version 1.6 (Bio-Rad).

Hemagglutination inhibition (HI) assay

To determine the HI titer of the sera samples collected from experimental chickens, the HI tests were performed with AIV H9N2 (01310) using a standard method [119]. The geometric mean of serum HI titers obtained from each group was defined as the reciprocal logarithm in a base of 2 of the highest serum dilution completely inhibiting agglutination.

AIV H9N2-specific proliferation

AIV H9N2 antigen-specific proliferation of PBMCs and splenocytes were assessed by measuring the viable cell ATP bioluminescence [120]. Briefly, PBMCs and splenocytes were prepared, as previously described [121], and cultured together with stimulator cells at three different ratios. Enriched APCs (10^6 cells/ml) that had been pulsed with ultraviolet (UV)-inactivated AIV H9N2 antigen (2.5×10^2 HA units/ml) were used as stimulator cells. Following 72h incubation, replicate cultures were transferred to V-bottom 96-well culture trays, which were centrifuged to collect the cells. The proliferated cells were then evaluated using a Vialight® Cell proliferation assay kit (Cambrex Bio Science, Rockland, ME) according to the manufacturer's instructions.

Expression of IFN-α and IL-4 by PBMCs and splenocytes following stimulation with AI H9N2 antigen

The mRNA expression levels of IFN-γ and IL-4 in PBMC and splenocytes were determined by real-time qRT-PCR using a CFX96™ real-time PCR detection system (Bio-Rad) following stimulation with AIV H9N2 antigen. Prepared PBMCs and splenocytes were stimulated with the stimulator cells (UV-inactivated AIV H9N2-pulsed APCs) for 72h. Total RNAs were extracted from the harvested cells using the total RNA extraction kits (iNtRON) according to the manufacturer's instructions and RNA concentrations were measured using a NanoDrop 2000 spectophotometer (Thermo Fisher Scientific, Pittsburgh, PA). The extracted RNA was subjected to real-time qRT-PCR using a One-Step SYBR® qRT-PCR reagent kit (Takara) and primers specific for the IFN-γ and IL-4 genes (Table 3). RT and real-time PCR amplification of targeted genes were carried out with each 75 ng total RNA as a template in 50µl of the reaction

mixture with same reaction conditions and temperature cycles, as described above. The relative expression values of IFN-γ and IL-4 were normalized using the commonly used reference gene GAPDH. The fold change in relative gene-expression levels was calculated and all data were analyzed using the CFX96™ manager software version 1.6 (Bio-Rad).

Statistical analysis

Where specified, the data were analyzed for statistical significance using an unpaired two-tailed Student's t-test. A p-value < 0.05 was considered significant.

Results

Construction of attenuated *S. enterica* serovar Typhimurium expressing chIFN-a and chIL-18

In order to evaluate the effect of oral co-administration of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 against respiratory infection with AIV H9N2, we primarily constructed attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. The DNA fragments of chIFN-α (582 bp) and chIL-18 (510 bp) amplified by RT-PCR (Figure 7A) were subcloned into the EcoRI and HindIII sites of pYA3560 and pYA3493 plasmids that were used for the expression of chIFN-α and chIL-18 in *S. enterica* serovar Typhimurium, respectively (Figure 7B). Subsequently, chIFN-α-encoding pYA3560 and chIL-18-encoding pYA3493 vectors were transformed into attenuated *S. enterica* serovar Typhimurium χ8501 host by electroporation and positive colonies of *S. enterica* serovar Typhimurium χ8501 harboring chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) were selected in the absence of DAP. The in-frame fusion of the chIFN-α and chIL-18 with the β-lactamase signal sequence was confirmed by nucleotide sequencing (data not shown). To identify the expression of chIFN-α and chIL-18 proteins by transformed *S. enterica* serovar Typhimurium, TCA-precipitated culture supernatants and bacterial cell lysates prepared at different incubation time points (12, 18, and 24h) were subjected to SDS-PAGE and immunoblot analysis. Attenuated *S. enterica* serovar Typhimurium harboring the empty vector pYA3560 (χ8501/pYA3560) or pYA3493 (χ8501/ pYA3493) cultured for 18 h was used as a negative control. The expression of chIFN-α and chIL-18 proteins from *S. enterica* serovar Typhimurium harboring chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) was detectable as early as 12h post-incubation, and gradually increased and saturated in the culture supernatants and cell lysates within 24 h-incubation (Figure 7C). Furthermore, the biological activity of secreted chIFN-α protein in culture supernatants was evaluated by in ovo antiviral activity against AIV H9N2, as previously described [136]. Also, chIL-18 secreted from χ8501/chIL-18 was shown to induce nitric oxide (NO) production by HD-11 cells measured by Griess assay [142] indicating IFN-γ release (data not shown). Therefore, these results indicate that the attenuated *S. enterica* serovar Typhimurium harboring chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) successfully expressed bioactive chIFN-α and chIL-18 proteins, which were actively secreted into the culture media instead of resulting from nonspecific membrane leaking or cell death by lysis.

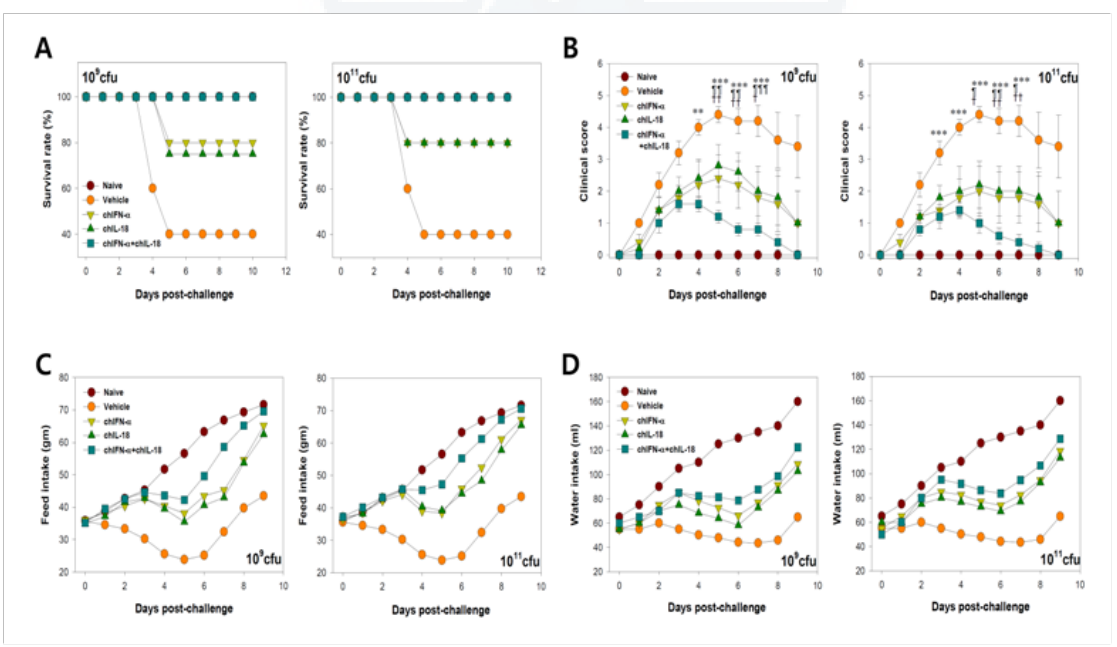

Figure 8: Enhanced alleviation of clinical signs by oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 in AIV H9N2-challenged chickens.

Enhanced alleviation of AIV H9N2-caused clinical signs by co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18

To investigate the protective efficacy of oral co-administration of S. enteric serovar Typhimurium expressing chIFN-α and chIL-18 against AIV H9N2 infection, SPF chickens (18-days old) were orally administered χ8501/chIFN-α or χ8501/chIL-18 or both in combined suspension (10^9 and 10^{11}cfu) followed by intra-tracheal infection with AIV H9N2 (01310) ($10^{10.83}$ EID_{50}/chicken) 3days after treatment. Following challenge, chickens were observed daily to record mortality and clinical severity throughout the duration of the experiment. The results revealed that the mortality showed between 4-5 days p.i., and the chickens (vehicle group) that received empty vector (χ8501/ pYA3560) showed the highest mortality (60%). Single administration of *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18 (10^9 and 10^{11} cfu) could reduce the mortality to around 20%. Notably, their combined administration at any dose (10^9 and 10^{11}cfu) could effectively protect all chickens from respiratory infection with AIV H9N2 (Figure 8A). Additionally, when the severity of clinical signs caused by AIV H9N2 challenge infection was scored, clinical signs appeared 2days p.i., and the severity of clinical signs peaked at 4-7 days p.i. (Figure 8B). The chickens that received single administration of either χ8501/chIFN-α or χ8501/chIL-18 (10^9 and 10^{11}cfu) showed significant alleviation of clinical severity, when compared to the vehicle group that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector. Furthermore, the co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 displayed more enhanced alleviation of clinical severity caused by respiratory infection with AIV H9N2, compared to groups that received single administration of *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18. Also, feed and water intakes were recorded daily after AIV H9N2 challenge of chickens' co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. The results showed that average feed (Figure 8C) and water (Figure 8D) intakes were improved in chickens that received either χ8501/chIFN-α or χ8501/chIL-18 (10^9 and 10^{11} cfu), compared to vehicle group that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector, and enhanced improvement was noticed in chickens co-administered χ8501/chIFN-α and χ8501/chIL-18. Overall, these results indicate that oral co-administration of *S. enterica* serovar Typhimuirum expressing chIFN-α and chIL-18 could markedly reduce mortality and alleviate clinical signs caused by respiratory infection with AIV H9N2. Further, the chickens that received χ8501/chIFN-α and χ8501/chIL-18 either singly or in combination (10^9 and 10^{11}cfu) before AIV H9N2 challenge showed normal lung and trachea, compared to vehicle group that received only *S. enterica* serovar Typhimurium expressing empty pYA3560 vector (data not shown). Taken together, these results indicate that oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 could provide complete protection against assaults of lung and tracheal tissues by infection with AIV H9N2.

Reduction of AIV H9N2 shedding and replication in chickens

To evaluate the effect of oral co-administration of χ8501/chIFN-α and χ8501/chIL-18 on virus shedding from AIV H9N2-infected chickens, the amount of virus in cloacal swab was determined by real-time qRT-PCR at 0, 1, 3, 5, 7 and 9 days post-challenge. Virus shedding was detected from 3 day after AIV H9N2 infection and peaked at 5 days p.i. (Figure 9A). However, the chickens that received χ8501/chIFN-α or χ8501/chIL-18 or both (10^9 and 10^{11}cfu) had significantly lower peaked levels of virus shedding at 3-7 days p.i., compared to chickens that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector. Additionally, the amount of virus in different tissues (trachea, lung, brain, cecal toncil, spleen, and kidney) of AIV H9N2-infected chickens was determined at 4 (Figure 9B) and (Figure 9C) days p.i. As expected, the amount of AIV H9N2 in different tissues of chickens that received χ8501/chIFN-α or χ8501/chIL-18 (10^9 and 10^{11} cfu) was significantly lower at both time points than those of groups treated with *S. enterica* serovar Typhimurium harboring empty pYA3560 vector (Figure 9B & 9C). Further, the oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN- and chIL-18 significantly reduced the amount of AIV H9N2 in different tissues, compared to group that received single administration of *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18. Taken altogether, these results indicate that oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 could alleviate clinical signs caused by respiratory infection with AIV H9N2 through enhanced reduction of virus replication in tissues.

Immune responses of chickens administered with *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 against challenged AIV H9N2

In order to better understand the protective role of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 in AIV H9N2 infection, we next examined how the adaptive immunity was responded in AIV H9N2-infected chickens with or without oral co-administration of χ8501/chIFN-α and χ8501/chIL-18. When the HI antibody titers in sera samples collected 7 days p.i. were determined, significantly enhanced HI antibody levels were observed in sera of chickens administered *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18 (10^9 and 10^{11} cfu), compared to that of chickens treated with *S. enterica* serovar Typhimurium harboring empty pYA3560 vector (Figure 10). Notably, combined oral administration of χ8501/chIFN-α and χ8501/chIL-18 showed significantly enhanced HI antibody titers in sera of AIV H9N2-infected chickens at both the doses, compared to single administration of either χ8501/chIFN-α or χ8501/chIL-18. Therefore, these results indicate that co-administration of *S. enterica* serovar Typhimurium expressing chIL-18 and chIFN-α displayed more enhanced humoral immune responses upon AIV H9N2 challenge.

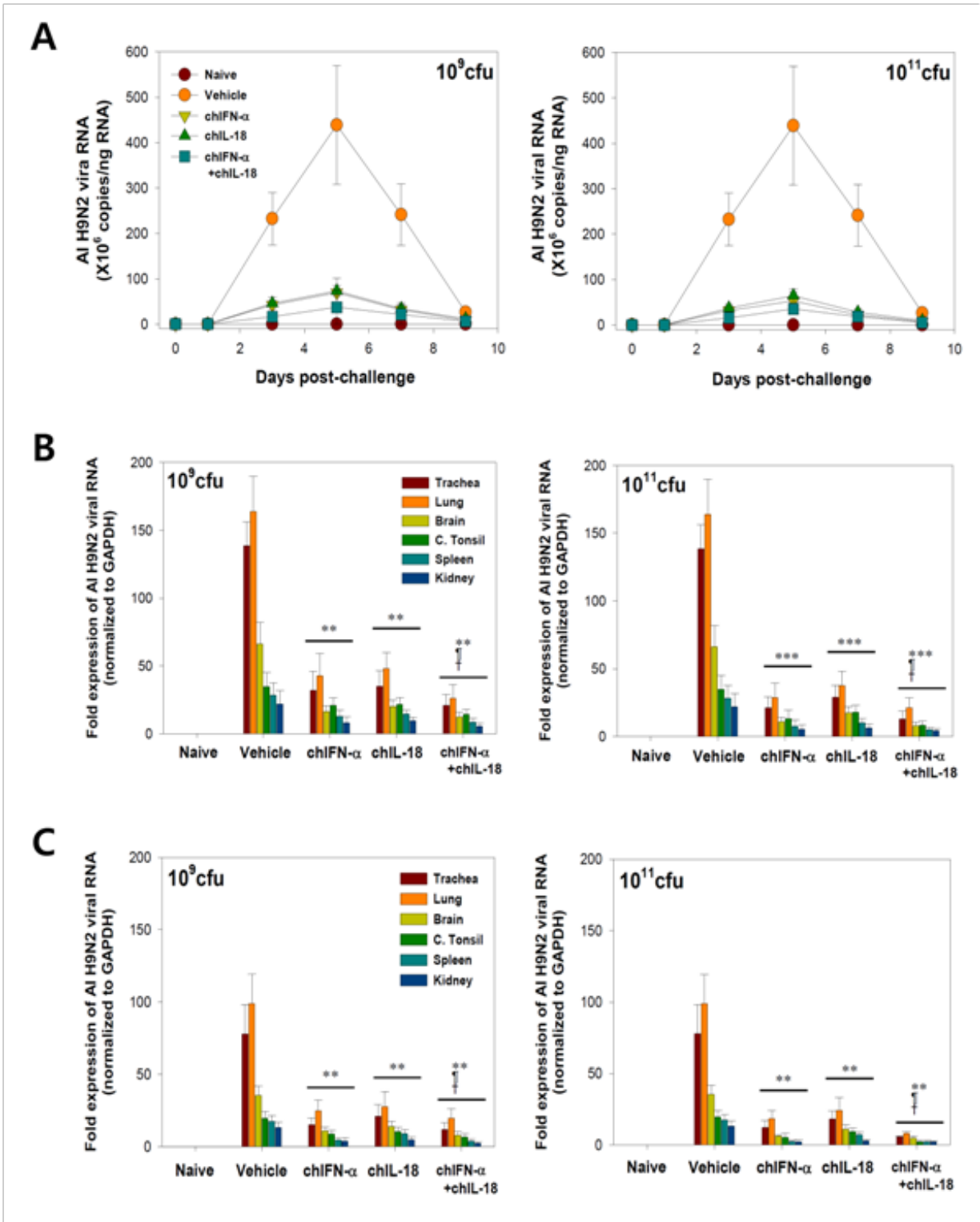

Figure 9: AIV H9N2 shedding and amount in tissues of AIV H9N2-challenged chickens co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18.

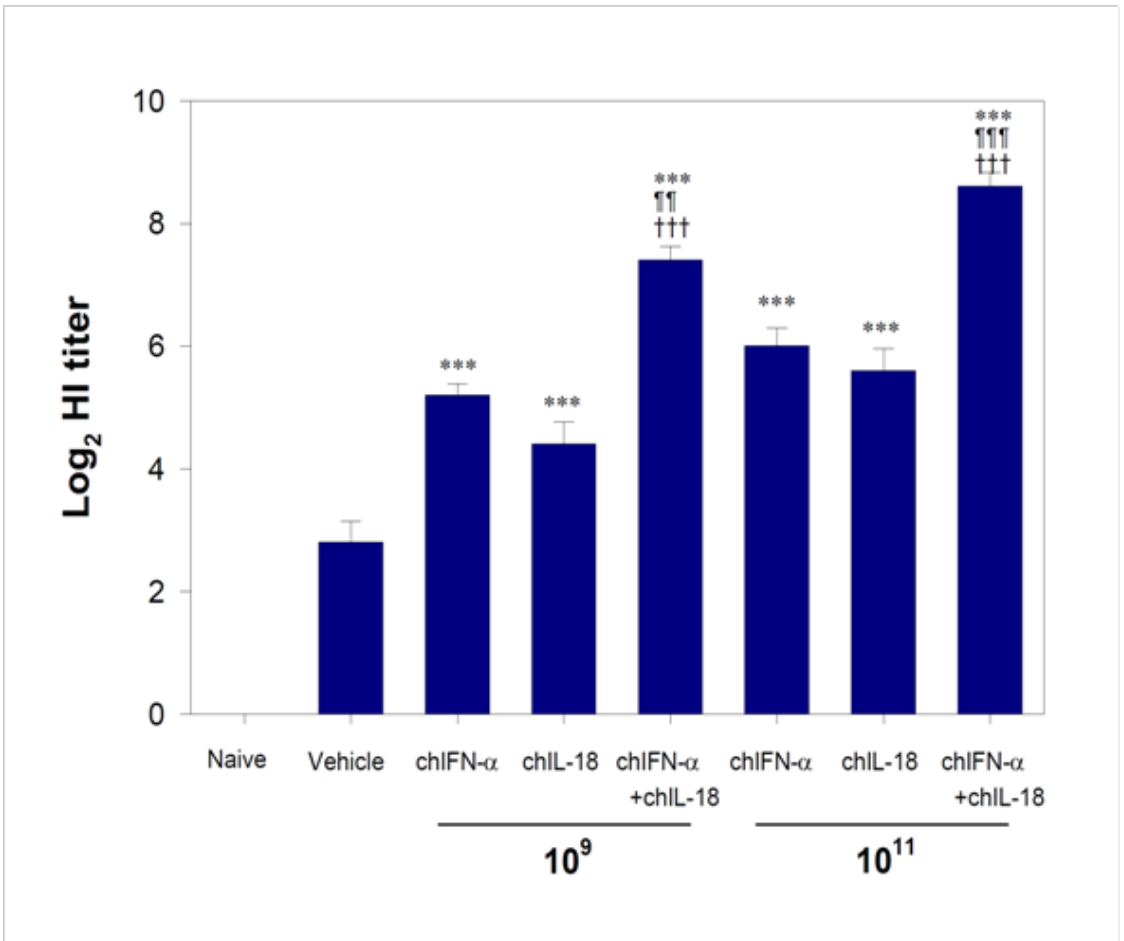

Figure 10: Serum HI antibody titers of chickens challenged by AIV H9N2 following oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18.

To evaluate the cellular immune responses, PBMCs and splenocytes were prepared from AIV H9N2-challenged chickens 14 days p.i. and stimulated with UV-inactivated AIV H9N2 antigen-pulsed PBMCs. PBMCs and splenocytes of chickens that received χ8501/chIFN-α or χ8501/chIL-18 (10^9 and 10^{11} cfu) orally prior to AIV H9N2 infection were found to show significantly enhanced proliferation upon AIV H9N2 antigen-specific stimulation, compared to the chickens (vehicle) that received χ8501/pYA3560 (Figure 11A) In particular, the oral co-administration of χ8501/chIFN-α and χ8501/chIL-18 before AIV H9N2 infection showed more enhanced proliferation of PBMC and splenocytes upon AIV H9N2 antigen-specific stimulation than single administration of χ8501/chIFN-α or χ8501/chIL-18. Furthermore, the mRNA expression levels of IFN-γ and IL-4 in PBMC and splenocytes were determined by real-time qRT-PCR following stimulation with AIV H9N2 antigen. Both IFN-γ and IL-4 mRNA levels in PBMCs and splenocytes prepared from chickens that received single administration of χ8501/chIFN-α or χ8501/chIL-18 (10^9 and 10^{11} cfu) were significantly enhanced, compared to the chickens that received empty vector (χ8501/ pYA3560) and more markedly enhanced effect in IFN-γ and IL-4 mRNA expression of PBMCs and splenocytes prepared from χ8501/chIFN-α plus χ8501/chIL-18-co-administered chickens is also observed (Figure 11B). In particular, the expression of IFN-γ mRNA was more significantly up-regulated than IL-4 mRNA in both single administration of χ8501/chIFN-α or χ8501/chIL-18 and co-administration of χ8501/chIFN-α and χ8501/chIL-18 with more enhanced up-regulation in later which indicates Th1-biasness. Taken altogether, our results indicate that oral co-administration of *S. enterica* serovar Typhimuirum expressing chIFN-a and chIL-18 could induce more enhanced Th1-biased cellular immunity against AIV H9N2 infection in chickens, which could effectively reduce virus replication within the host tissues and as a result provide complete protection against AIV H9N2 infection.

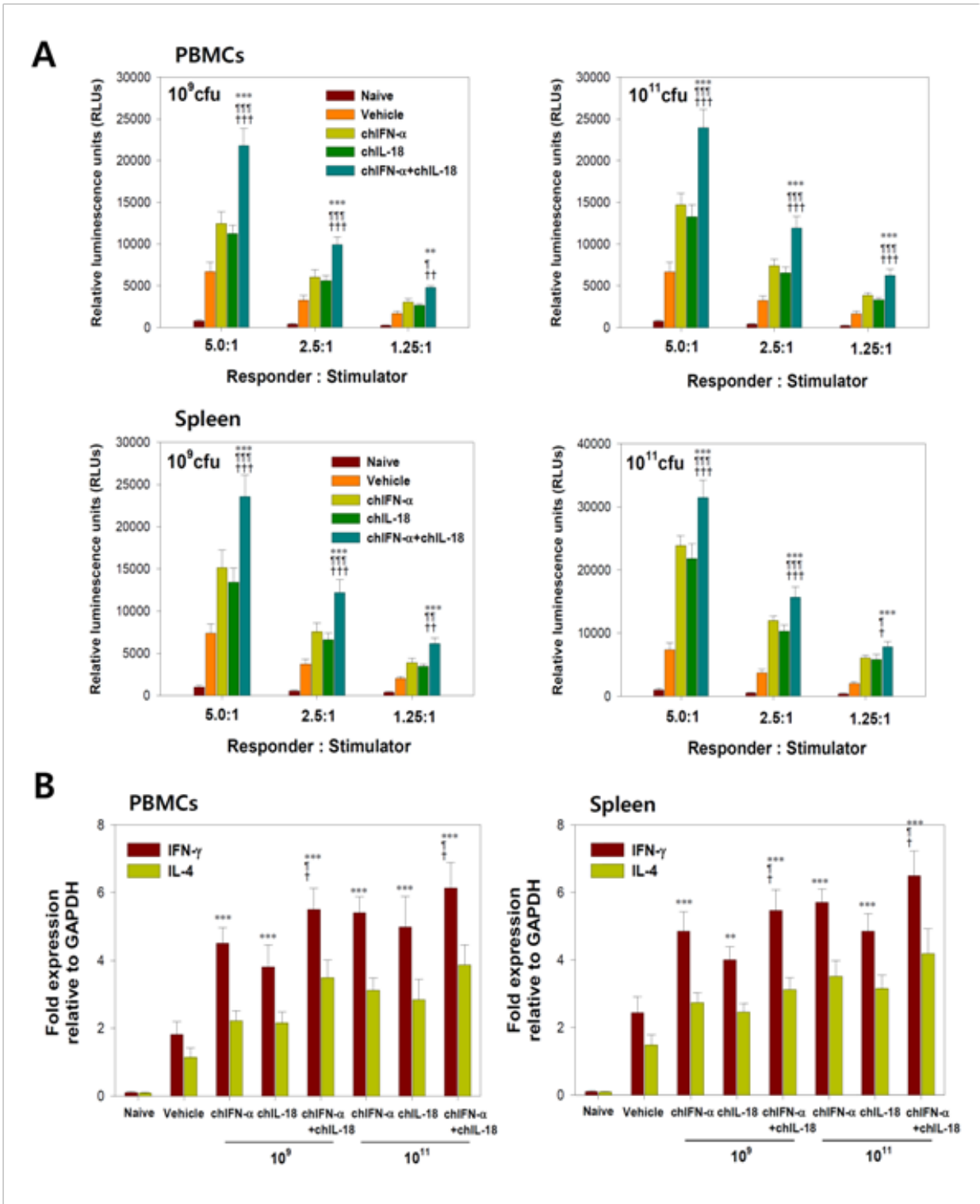

Figure 11: AIV H9N2-specific cell-mediated immunity of challenged chickens following oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18.

Discussion

Cytokines play critical role in host defense and inflammatory responses by providing a regulatory bridge between innate and adaptive immunity. They ascertain both the type and extent of an immune response that is generated after an infection with a pathogen or after immunization. The combined effects of two or more cytokines may be antagonistic, additive, or synergistic based on the immunobiological mechanisms [76]. Therefore, it is possible to generate a markedly enhanced protective immunity against a viral pathogen by the combined use of two or more cytokines with synergic and additive functions [77-79]. Cytokines therefore represent most exciting new candidates of naturally occurring environmental friendly therapeutics over existing conventional control measures of combined use of vaccines, antibiotics and chemicals. Preventive or therapeutic usage of chicken cytokines is becoming more promising day by day with the recent cloning of a number of avian cytokine genes. In the present study, oral co-administration of chIFN-α and chIL-18 using attenuated *S. enterica* serovar Typhimurium as a carrier of the two cytokines displayed significantly enhanced alleviation of clinical signs caused by respiratory infection with AIV H9N2, compared to the chickens orally administered *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18 alone. Furthermore, such enhanced alleviation of clinical signs was considered by modulating viral replication in tissues and the immune responses of chickens against challenged AI H9N2 virus. Therefore, our results provided valuable insights into oral co-administration of chIFN-α and chIL-18 using attenuated *S. enterica* serovar Typhimurium to control AI H9N2.

Combined use of two or more cytokines has shown enhanced effect empirically, based on their biological mechanisms. Type I IFNs (IFN-α and IFN-β) rapidly induced by viral infection or other stimuli have been known to show strong antiviral activity. The binding of type I IFNs to their cognate receptors results in the rapid phosphorylation and activation of receptor-associated JAKs, Tyk2, and Jak1, and subsequent transcription factor STAT1/2, which induces the expression of master regulator genes such as OAS, RNase L, Mx1, and PKR to confer antiviral state in cells [40]. Alternatively, IFN-γ, the only type II IFN, is an important cytokine produced primarily by T lymphocytes (Th1) and NK cells that plays a pivotal role in macrophage activation and modulation of the immune response, in addition to its antiviral activity [143]. The antiviral effect of IFN-γ is shown by direct (intracellular, NO secretion) or indirect mechanism that involves activation of effector cells of the immune system [144]. Based on recent reports, chicken type I and type II IFNs have been shown to act synergistically [45], both in terms of antiviral activity and in their ability to activate macrophages. IL-18 provides an important link between the innate and adaptive immunity through the induction of IFN-γ secretion [53]. Additionally, chicken IL-18 has been shown to play significant role in inducing antiviral immune responses against several viral infections like H5-H7 avian influenza virus (AIV) infection [59]. It has been shown that after infection of macrophages with influenza virus, cells produce IL-18, which with IFN-α synergistically enhances IFN-γ synthesis [82]. Therefore, it is expedient that chicken IFN-α and IL-18 in combination may have enhanced antiviral activity; however, a practical evaluation of their combined antiviral function has not yet been addressed to the best of our knowledge. It is possible that type II IFN-γ induced by IL-18 may act synergistically with type I IFN and induce enhanced alleviation of the clinical signs of AIV H9N2 infection. Furthermore, our results are supported by the finding that recombinant fowl pox vaccine encoding sequences of H5-H7 avian influenza virus (AIV) and chIL-18 (rFPV-H5-H7-IL18) had successfully induced complete protection in SPF chicken after challenge with H5 AIV [59]. Therefore, the present data demonstrate the valuable use of combined administration of type I IFN and IL-18 in controlling respiratory diseases caused by AIV H9N2.

It is considered that the primary target cells for AIV infection and replication are ciliated epithelial cells. However, AIV was shown to infect macrophages and dendritic cells [125-126]. Also, intestinal epithelia may be targets of infection in avian species and, in the later stage of infection, mononuclear cells may become involved [127-128]. It has been reported that influenza A virus causes NS1-mediated suppression of selected genes involved in IFN and IFN-inducible gene expression [130], and induction of a weak chemokine expression in human lung epithelial cells [130], thereby facilitating viral replication before the host inflammatory and antiviral responses are activated. Protection of chickens from AIV H9N2 requires early stimulation of immune systems by immune-modulatory cytokines like chIFN-α and chIL-18. Therefore, it is possible that oral co-administration of attenuated *Salmonella* bacteria expressing chIFN-α and chIL-18 could effectively stimulate host innate and adaptive immune responses before the establishment of infection, thereby preventing virus replication in host tissues and effectively alleviating the clinical severity of AIV H9N2 infection.

The practical use of cytokine proteins in livestock and poultry is limited, due to cost, labor, and time, as well as protein stability associated with mass administration. Therefore, a suitable delivery vector is required for mass administration of cytokines to control diseases in poultry industry. Our previous reports demonstrated the value of attenuated *Salmonella* vaccine in the oral delivery of immunomodulatory cytokines. Compared to genetically modified *Lactococcus lactis* (food-grade lactic acid bacteria) that has been considered as a candidate vehicle of biologically active molecules [145], live attenuated *S. enterica* serovar Typhimurium χ8501 can colonize gut-associated lymphoid tissue and visceral non-lymphoid and lymphoid tissues following oral administration, and subsequently stimulate local and systemic immune responses [99,100]. Furthermore, the *Salmonella* bacteria used in this study were devoid of the asd gene essential for a balanced-lethal host-vector system and are therefore

believed to be sufficiently attenuated in their capacity to cause acute diseases in chickens. Indeed, all chickens orally administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 did not manifest any clinical signs of disease for a monitoring period of 15 days (data not shown). However, the successful and prolonged colonization of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 may be required for the effective results for controlling infectious diseases in chickens by the *Salmonella* delivery system. According to previous findings, the clearance time of *S. enterica* serovar Typhimurium in adult chicken is more than three weeks which is more extended in younger chickens, up to 7 weeks [133-134]. Therefore, it is believed that the *Salmonella* bacteria used for cytokine delivery can persist in chicken body for prolonged period and can provide continuous long term protection against virus infection through secretion of encoded recombinant cytokines, although the persistence and distribution of *S. enterica* serovar Typhimurium were not determined in this study. To improve the practical use of the attenuated *Salmonella* strain as oral carrier of cytokines, the number of times being administered and its schedule should be addressed further.

Conclusion

In conclusion, we have demonstrated for the first time to the best of our knowledge that combined oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 could provide complete protection to the chickens against high dose AI H9N2 virus challenge through providing enhanced Th1-biased protective immunity against the challenge virus. Our results suggest that attenuated Asd-negative *S. enterica* serovar Typhimurium may be useful for the combined oral administration of two or more cytokines in order to provide a useful means for controlling respiratory viral infections of poultry in future.

Figure 7: Construction of live attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (A) The chIFN-α and chIL-18 genes amplified by RT-PCR. Total RNAs extracted from LPS-stimulated splenocytes were subjected to amplify the chIFN-α and chIL-18 genes using specific primer pairs. Amplified chIFN-α and chIL-18 genes are indicated by arrows in the agarose gel image. Lane M, size marker; 1 and 3, naïve splenocytes; 2 and 4, LPS-stimulated splenocytes. (B) Diagram of periplasmic secretion Asd+ vector pYA3560 and pYA3493. 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). A DNA fragment encoding the β-lactamase signal sequence and 12 amino acid residues of the N terminus of mature β-lactamase of plasmid pBR322 was positioned under the control of the P_{trc} promoter. The map of pYA series vectors (pYA3560 and pYA3493) and the nucleotide sequences of the P_{trc} promoter region, β-lactamase signal sequence (*bla* SS) and multicloning sites are shown. The P_{trc} sequences for -35, -10 (RNA polymerase-binding site) and Shine-Dalgarno box (SD, ribosomal binding site) are indicated by blue and green boldface, and the translocation start codon (ATG) is in red boldface. An arrow within the sequence indicates the signal peptidase cleavage site. Unique restriction enzyme sites in the multicloning site are indicated, and 5ST1T2 is a transcriptional terminator. (C) Identification of chIFN-α and chIL-18 expression from constructed *S. enterica* serovar Typhimurium by immunoblot analysis. The chIFN-α protein expressed by χ8501/chIFN-α and the chIL-18 protein expressed by χ8501/chIL-18 were detected from both TCA-precipitated culture supernatants (sup) and cell lysates 12, 18 and 24 h post-incubation by immunoblotting with chIFN-α-specific monoclonal antibody and 6xHis-Tag antibody respectively. Attenuated *S. enterica* serovar Typhimurium carrying empty vector pYA3560 (χ8501/ pYA3560) or pYA3493 (χ8501/ pYA3493) cultured for 18 h was used as a negative control.

Figure 8: Enhanced alleviation of clinical signs by oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 in AIV H9N2-challenged chickens. (A) Mortality of AIV H9N2-challenged chickens. Chickens received oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (10^9 or 10^{11} cfu/bird) were intra-tracheally infected with AIV H9N2 ($10^{10.83}$ EID_{50}/bird) and mortality was recorded up to 9 days p.i. The graphs show the proportion of surviving chickens on different days p.i. at two different doses. (B) Alleviation of clinical severity in AIV H9N2-infected chickens. Chickens were infected with AIV H9N2 3 days after co-administration of *S. enterica* serovar Typhimruium expressing chIFN-α and chIL-18 at two different doses (10^9 or 10^{11} cfu/chicken) and the clinical severity was scored daily. $^{**}p<0.01$; $^{***}p<0.001$ compared co-administered group to vehicle group that was treated with control bacteria. $^{¶}p<0.05$; $^{¶¶}p<0.01$; $^{¶¶¶}p<0.001$ compared co-administered group to chIFN-α-treated group. $^{†}p<0.05$; $^{††}p<0.01$ compared co-administered group to chIL-18-treated group. (C and D) Feed and water intakes of AIV H9N2-infected chickens. Feed and water intakes were recorded daily after AIV H9N2 challenge of chickens co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. Data show the average of feed (C) and water (D) intakes obtained from five chickens per group.

Figure 9: AIV H9N2 shedding and amount in tissues of AIV H9N2-challenged chickens co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. (A) Virus shedding from AIV H9N2-infected chickens. Cloacal swab samples of chickens co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (10^9 or 10^{11} cfu/chicken) were taken at alternative days and the amounts of AIV H9N2 were determined by real-time qRT-PCR using primers specific for hemagglutinin protein of AIV H9N2 following total RNA extraction. Data represent the average and SEM of five chickens per group. (B and C) The amount of virus in tissues of AIV H9N2-infected chickens. Chickens that received co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (10^9 or 10^{11} cfu per chicken) were euthanized 4 and 7 days after AIV H9N2 challenge. Total RNAs extracted from tissues

(trachea, lung, brain, cecal toncil, spleen, and kidney) were subjected to real-time qRT-PCR to determine AIV H9N2 amounts at 4 days p.i. (B) and 7 days p.i. (C). Data show the average and SEM of AIV H9 fold expression obtained from four chickens per group, after normalized to GAPDH. $^{**}p<0.01$; $^{***}p<0.001$ compared to vehicle group that was treated with control bacteria. $^{¶}p<0.05$ compared to chIFN-α-treated group. $^{†}p<0.05$ compared to chIL-18-treated group.

Figure 10: Serum HI antibody titers of chickens challenged by AIV H9N2 following oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. Groups of chickens co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (10^9 or 10^{11} cfu/chicken) were intra-tracheally infected with AIV H9N2 ($10^{10.83}$ EID_{50}/bird) three days later. Serum samples were collected from chickens 7 days after AIV H9N2 challenge and subjected to HI test to measure HI antibody titers. Data was expressed as reciprocal log2 of the geometric average and SEM of HI antibody titers obtained from five chickens per group. $^{***}p<0.001$ compared to vehicle group that was treated with control bacteria. $^{¶¶}p<0.01$; $^{¶¶¶}p<0.001$compared to chIFN-α-treated group. $^{†††}p<0.001$ compared to chIL-18-treated group.

Figure 11: AIV H9N2-specific cell-mediated immunity of challenged chickens following oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. (A) AIV H9N2 antigen-specific proliferation of PBMCs and splenocytes prepared from AIV H9N2-infected chickens. PBMCs and splenocytes obtained from AIV H9N2-challenged chickens 14 days p.i. were stimulated with enriched APCs that had been pulsed with inactivated AIV H9N2 antigen. AIV H9N2 antigen-specific proliferation of PBMCs and splenocytes was assessed by measuring the viable cell ATP bioluminescence following 72h-incubation. Data show the average and SEM of viable cell ATP bioluminescence of PBMCs and splenocytes obtained from five chickens per group. (B) The expression of IFN-γ and IL-4 mRNAs in PBMCs and splenocytes upon stimulation with AIV H9N2 antigen. Total RNAs were extracted from PBMCs and splenocytes after stimulation with AIV H9N2 antigen for 72h and used for real-time qRT-PCR to determine the expression of IFN-γ and IL-4. Data show the average and SEM of IFN-γ and IL-4 expression obtained from five chickens per group, after normalized to GAPDH. $^{**}p<0.01$; $^{***}p<0.001$ compared to vehicle group that was treated with control bacteria. $^{¶}p<0.05$; $^{¶¶}p<0.01$; $^{¶¶¶}p<0.001$compared to chIFN-α-treated group. $^{†}p<0.05$; $^{††}p<0.01$; $^{†††}p<0.001$ compared to chIL-18-treated group.

Chapter 4

Enhancement of Th1-Biased Protective Immunity Against Inactivated Avian Influenza H9N2 Vaccine Via Oral Co-Administration of Live Attenuated *Salmonella Enterica* Serovar Typhimurium Expressing Chicken Interleukin-18 and Interferon-α

Abstract

Control of currently circulating reasserted low-pathogenicity avian influenza (LPAI) H9N2 is a major concern for both animal and human health. Thus, an improved H9N2 LPAI vaccination strategy is particularly needed to induce complete immunity in chickens against H9N2 LPAI virus strains. Cytokines play a crucial role in mounting both the type and extent of an immune response that is generated following infection with a pathogen or after vaccination. To improve the efficacy of inactivated H9N2 LPAI vaccine, attenuated *Salmonella enterica* serovar Typhimurium was used for oral co-administration of chicken interferon-α (chIFN-α) and interleukin-18 (chIL-18) as natural, environmentally friendly immunomodulators. The oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 prior to vaccination with inactivated AI H9N2 vaccine modulated the immune responses of chickens against vaccine antigen through more enhanced both humoral and Th1-biased cell-mediated immunity, compared to the chickens that received oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18 alone. To further test the protective efficacy of this improved vaccination regimen, immunized chickens were intra-tracheally challenged with high dose of AIV H9N2 7 days after booster vaccination. Combined administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 showed markedly enhanced protection compared to single administration of construct, as determined by mortality, clinical severity, and feed and water intakes. This enhancement of protective immunity was further confirmed by reduced rectal shedding and replication of AIV H9N2 in different tissues of challenged chickens. Our results provide a useful value of combined administration of chIFN-α and chIL-18 using *Salmonella* vaccine strain to generate an effective immunization strategy in chickens against LPAI H9N2.

Introduction

Avian influenza viruses (AIV) of the H9N2 subtype are classified as low-pathogenicity viruses both by molecular characterization and by pathotyping. This subtype has attracted great concerns among low-pathogenicity avian influenza (LPAI) viruses due to its wide host range including chickens, quail, turkeys, ducks, geese, pigs, and humans [101]. Two cases of human infection with LPAI H9N2 viruses were reported in Hong Kong in 1999 as the first avian-to-human transmission, and another case was reported in 2003 [146,147]. Antigenic and genetic analysis of these viruses showed that the six internal genes had a novel reassortant [146] which were similar to those of the high-pathogenicity avian influenza (HPAI) H5N1 viruses [148]. The prevalence of LPAI in poultry may provide opportunities for the generation of H9N2 reassortants associated with human disease [95]. Thus, circulation of H9N2 viruses in poultry not only causes industrial loss, but also poses a potential threat to human health. The first outbreak of LPAI in Korea was caused by influenza A/Chicken/Kor/MS96/96 (H9N2) viruses in 1996. The virus has become endemic in Korea since 2000, especially in layer farms, and has gained considerable attention due to its rapid spread across Korean chicken farms with enhanced clinical severity and severe economic losses. Initially, vaccines for subtypes of AIV, including H9N2 LPAI, were prohibited in Korea because they interfered in the discrimination of naturally infected birds from vaccinated birds. Since vaccination is a promising control measure for H9N2 LPAI, the Korean animal health authorities has changed their control policy from 2004 and permitted the use of inactivated vaccine derived from a selected single vaccine strain (01310 CE3) to simplify the antigenic variation of the H9N2 LPAI virus which is circulating in the country [149-150]. Inactivated oil adjuvant H9N2 LPAI vaccine prepared from selected vaccine strain (01310 CE3) is highly immunogenic and protective in laboratory trials using specific pathogen-free (SPF) chickens [111]. Although the inactivated vaccine can prevent clinical disease and reduce viral shedding in field condition, they cannot prevent vaccinated poultry from becoming infected and from shedding wild viruses in farm [151]. Moreover, the recent H9N2 Korean isolates show altered antigenic and pathogenic characteristics in chickens as compared to isolates from 1996 to mid-2003 due to their continuous evolvement by reassortment of their internal genes with other subtypes, which makes the existing vaccination strategy inefficient [96,99]. Therefore, improved vaccination strategy is urgently required to control H9N2 LPAI outbreaks in poultry farms and enhancement of immune response using some natural immunomodulators along with inactivated H9N2 LPAI vaccine may be the best approach to control the reassorted H9N2 isolates.

Cytokines are the natural mediators of the innate and adaptive immune responses which play a crucial role in controlling the immune system. The use of chicken cytokines is becoming more feasible with the recent cloning of a number of cytokine genes [6], since the chicken's immune system is similar to that of mammals. Although recent studies on avian cytokines identified a number of cytokines having immunomodulatory and antiviral properties against several viral infections, little is known about the combined use of chicken cytokines in disease prevention. Chicken interferon-α (chIFN-α) belongs to type I IFNs and plays an essential role in the host antiviral response through stimulating T-dependent lymphocyte system and induction of numerous IFN-stimulated genes (ISGs) through the interaction with specific type I receptor complexes and triggering of the Janus-activated kinases (JAKs)-signal transducer and activators of transcription (STAT) 1/2 pathway [36]. Accordingly, there is evidence that chIFN-α administered by oral ingestion or intravenous injection inhibits many epidemic avian viruses, including AIV [37-40]. Interleukin-18 (IL-18), originally known as potent interferon-γ (IFN-γ)-inducing factor (IGIF), shares properties with IL-12 and both cytokines act synergistically to promote IFN-γ production, which plays an important role in inducing Th1 immune responses [53]; thus IL-18 provides an important link between the innate and adaptive

immune responses. Recent studies on chicken IL-18 (chIL-18) showed that chIL-18 cDNA linked with recombinant encoding sequences of H5-H7 AIV in a fowl pox-based DNA vaccine (rFPV-H5-H7-IL18) successfully induced complete protection (100%) in SPF chicken after challenge with H5 AIV, and lymphocyte proliferation induced by the rFPV-H5-H7-IL18 is significantly higher than that induced by rFPV-H5-H7 alone [59]. Therefore, both chIFN-α and chIL-18 have great values for use either singly or in combination in disease prevention in chickens. However, the practical mass administration of chicken cytokines to control poultry diseases is particularly limited by a cost effective delivery system. To this end, our previous study reported that attenuated aspartate β-semialdehyde dehydrogenase (Asd)-negative *Salmonella enterica* serovar Typhimurium devoid of antibiotic resistance genes could be an effective delivery system for the mass administration of chicken cytokines without the need for antibiotic selection [139]. Furthermore, cytokines produced by *S. enterica* serovar Typhimurium may be able to provide immunomodulatory functions to both mucosal and systemic sites, because orally administered *S. enterica* serovar Typhimurium can colonize the secondary lymphoid and nonlymphoid tissues including the lymph node, spleen, and liver as well as the gut-associated lymphoid tissues (Peyer's patch) [90,110].

Based on recent review on immunobiological activities of type I IFN and IL-18, it is hypothesized that combined use of chIFN-α and chIL-18 may have enhanced immunomodulatory function and therefore, combined oral administration of these two cytokines using attenuated *S. enterica* serovar Typhimurium prior to vaccination with inactivated H9N2 LPAI vaccine may confer enhanced protective immunity against AIV H9N2. The present study was designed to test this hypothesis. According to our findings, oral co-administration of live attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 markedly enhanced Th1-biased protective immunity against inactivated avian influenza H9N2 vaccine. Therefore, a useful value of combined administration of chIL-18 and chIFN-α using attenuated *S. enterica* serovar Typhimurium in inactivated H9N2 LPAI vaccination is discussed herein.

Materials and Methods

Animals and ethics statement

SPF leghorn layer (white) chickens were obtained from Jinan Baizhun Biologic Inspection, China, and reared with formulated commercial feed and water provided *ad libitum* throughout the whole experimental period. All experimental procedures and animal management procedures were undertaken in accordance with the requirements of the Animal Care and Ethics Committees of Chonbuk National University. The animal facility of the Chonbuk National University is fully accredited by the National Association of Laboratory Animal Care.

Cells and viruses

Low pathogenic avian influenza A virus (LPAIV) H9N2 strain, A/Chicken/Korea/01310/2001 (01310), which has been described previously, was a kind gift from the National Veterinary Research and Quarantine Service of the Republic of Korea and used for challenge infection to chickens. The AIV H9N2 (01310) was propagated by inoculating in the allantoic cavity of 10-day-old embryonated eggs and allantoic fluid was harvested 96 h after inoculation. Virus in the allantoic fluid was titrated using a standard hemagglutination test [112] and the infectious viral titer was determined by using 10-day-old embryonated eggs, as previously described [114].

Bacterial strains, plasmid, media, and growth conditions

Escherichia coli χ6212 ($F^-\lambda^-\Phi 80\Delta$ (*lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1* Δ*asdA4*) [91] was used as the host strain for construction of the Asd+ plasmid vectors encoding chIFN-α or chIL-18. Attenuated *S. enterica* serovar Typhimurium χ8501 (*hisG Δcrp-28 ΔasdA16*), which was kindly provided by Dr. HY. Kang (Pusan National University, Korea) [92], was used for host bacteria to deliver chIFN-α and chIL-18 proteins. 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 [92]. *E. coli* and *S. enterica* serovar Typhimurium cultures were grown at 37 °C in Lennox broth, Luria-Bertani (LB) broth or on LB agar. Diaminopimelic acid (DAP; Sigma-Aldrich, St. Louis, MO) was added (50μg/ml) to induce the growth of Asd-negative bacteria [91]. Phosphate-buffered saline (PBS, pH 7.4) containing 0.01% gelatin (BSG) was used for the resuspension of *Salmonella* bacteria that were concentrated by centrifugation at 7000 × *g*, 4 °C for 5min.

Construction of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18

Total RNA was extracted from splenocytes that were previously stimulated with lipopolysaccharde (LPS, 20μg/ml) for 48h after resuspending cells (10^7 cells/ml) in complete RPMI medium, and employed to amplify the chIFN-α chIL-18 genes with reverse transcription-polymerase chain reaction (RT-PCR) using specific primer pairs (Table 3). The PCR products were then inserted into pGEMT vector (Promega, Madison, WI) and the chIFN-α and chIL-18 genes were sequenced to confirm the authenticity of insert sequences. Subsequently, chIFN-α and chIL-18 genes were sub cloned into the pYA3560 and pYA3493 plasmid vectors, respectively. The pGEMT vectors encoded with chIFN-α and chIL-18 genes were digested with *EcoRI* and *HindIII*, after which the released fragments containing the chIFN-α and chIL-18 genes were inserted into the same restriction sites of pYA3560 and pYA3493 plasmid vectors using *E. coli* χ6212 hosts grown in the presence of DAP. The positive colonies of *E. coli* χ6212 harboring either chIFN-α-encoding pYA3560 or chIL-18-encoding pYA3493 vectors were selected in the absence of DAP. To construct attenuated *S. enterica* serovar Typhimurium expressing either chIFN-α or

chIL-18, *S. enterica* serovar Typhimurium χ8501 (1×10^8 cfu) washed extensively with sterilized ice-cold WB (10% 10% ultra pure glycerol, 90% distilled water, v/v) were mixed with 10 pg to 0.1μg of either chIFN-α-encoding pYA3560 or chIL-18-encoding pYA3493 plasmid DNA on ice in a 0.2cm cuvette and electroporated using a Bio-Rad Gene pulser at 12.5kV/cm (2.5 kV, 25μF and 200 Ω; Bio-Rad, Hercules, CA), respectively. The bacteria were then removed from the cuvette into sterile culture tubes containing 1ml of LB broth medium and incubated with moderate shaking for 60 min at 37 °C. The transformed cultures (each 100μl) were then plated onto LB agar plates in the absence of DAP. Finally, colonies of the attenuated *S. enterica* serovar Typhimurium harboring either chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) vector were cultured and stored after confirmation of the coding sequences.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses

The expression of chIFN-α and chIL-18 proteins by *S. enterica* serovar Typhimurium harboring either chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) plasmid was identified by immunoblot following gel separation of prepared proteins by SDS-PAGE. For the preparation of protein samples, *Salmonella* bacteria cultured for 12, 18, and 24h were resuspended in 4 ml of 20mM Tris-HCl (pH 8.6) and then disrupted by two passages through a French pressure cell (American Instrument, Silver Spring, MD). Cell lysates were centrifuged at 7000 ×*g*, 4 °C for 6 min to remove unbroken cells and the supernatant fraction was used for protein samples of cell lysates. The original culture supernatants were filtered (0.22μm-pore-size filter) and proteins that had been secreted into the supernatants were precipitated with 10% trichloroacetic acid (TCA) at 4 °C for 1h. Prepared protein samples were boiled for 5min and then separated by SDS-PAGE. The protein bands were visualized by staining with 0.1% Coomassie brilliant blue R-250 solution (Elpis-biotech, Deajeon, Korea). For immunoblotting, the resolved proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were then blocked with a blocking buffer consisting of PBS containing 3% skim milk and 0.5% Tween 20, and then incubated with chIFN-α monoclonal antibody (Serotec, Raleigh, NC) and His_6-Tag antibody (Novagen, Madison, WI) to detect chIFN-α and 6×histidine tagged-chIL-18, respectively. Following 1.5h incubation, a peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Southern Biotech, Birmingham, AL) was added. Immunoreactive bands were detected by the addition of chemiluminescence dye using a WEST-one™ Western Blot Detection System (iNtRON, Seongnam-Si, Korea) in the presence of H_2O_2.

Animal experimental designs for AIV H9N2 vaccination and challenge

A total of 40 SPF chickens (32 days old) were divided randomly into five groups. The first group (*n*=5) was a negative control that was orally administered vehicle (PBS containing 0.01% gelatin) without *S. enterica* serovar Typhimurium expressing chIFN-α or chIL-18. The second group (*n*=5) was orally administered *S. enterica* serovar Typhimurium harboring pYA3560 vector (10^9 cfu/chicken) as a control of the empty pYA3560 vector. The remaining three groups (*n*=10 per group), each comprising two replications (*n*=5 per replication) for two different doses, were orally administered either *S. enterica* serovar Typhimurium expressing chIFN-α (10^9 and 10^{11} cfu/chicken) or chIL-18 (10^9 and 10^{11} cfu/chicken) or both in a combination (each 10^9 and 10^{11} cfu/chicken). Oral administration was performed by instilling the resuspended bacteria (1ml/chicken) into esophagus using flexible gavage feeding needle (Fine Science Tools, North Vancouver, British Columbia, Canada) after withholding feed for 4h. Three days after treatment, chickens of all groups, except negative control group, were vaccinated intramuscularly (i.m.) with AIV H9N2 inactivated vaccine (PoulShot® Flu H9N2; JoongAng Vaccine Inc., Daejeon, Korea) at 35 days old with recommended dose. Primarily vaccinated chickens were boosted by using the same protocol 7 days later. Blood samples were collected 7 days after primary vaccination and 7 & 14 days after booster vaccination followed by sera separation. Peripheral blood mononuclear cells (PBMC) were enriched from blood of vaccinated chickens using OptiPrepTM (13.8% iodixanol) 14 days post-booster vaccination, according to manufacturer's instruction (Axis-Shield, Oslo, Norway). To evaluate the protective immunity of AI H9N2 vaccine in chickens co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18, SPF chickens (7-days old) were vaccinated by the same protocol and intra-tracheally challenged with AIV H9N2 (01310) ($10^{10.83}$ EID_{50}/chicken) 7 days after booster vaccination i.e. at 24 days old. Following challenge, chickens were observed daily for clinical signs and mortality throughout the duration of the experiment. The clinical signs were scored daily as follows: 0, no sign; 1, slight depression; 2, moderate depression + reduced movement + reduced food/water intake (anorexia); 3, moderate respiratory distress (sinusitis, cough); 4, severe respiratory distress (sinusitis, severe cough) + diarrhea; 5, death. Average feed and water intake was determined daily for 9 days after challenge. Cloacal swab samples were collected at 0, 1, 3, 5, 7, and 9 days post-infection (p.i.). Another experiment was carried out with same experimental setup to collect additional samples for determination of virus amount in tissues.

Hemagglutination inhibition (HI) assay

To determine the HI titers of the sera samples collected from vaccinated chickens, the HI tests were performed with AIV H9N2 (01310) using a standard method [119]. The geometric mean of serum HI titers obtained from each group was defined as the reciprocal logarithm in a base of 2 of the highest serum dilution completely inhibiting agglutination.

AIV H9N2-specific proliferation

AIV H9N2 antigen-specific proliferation of PBMCs was

assessed by measuring the viable cell ATP bioluminescence [120]. Briefly, PBMCs (responder) were prepared from vaccinated chickens as previously described [121], and cultured together with stimulator cells at three different ratios. PBMCs (10^6 cells/ml) that had been pulsed with ultraviolet (UV)-inactivated AIV H9N2 antigen (2.5×10^2 HA units/ml) following enriching from naïve chickens were used as stimulator cells. Following 72h incubation, replicate cultures were transferred to V-bottom 96-well culture trays, which were centrifuged to collect the cells. The proliferated cells were then evaluated using a Vialight® Cell proliferation assay kit (Cambrex Bio Science, Rockland, ME) according to the manufacturer's instructions.

Expression of IFN-α and IL-4 by PBMCs following stimulation with AIV H9N2 antigen

The mRNA expression levels of IFN-γ and IL-4 in PBMCs were determined by real-time qRT-PCR using a CFX96™ real-time PCR detection system (Bio-Rad) following stimulation with AIV H9N2 antigen. Total RNAs were extracted from 72-h stimulated PBMCs using the total RNA extraction kits (iNtRON) according to the manufacturer's instructions and RNA concentrations were measured using a NanoDrop 2000 spectophotometer (Thermo Fisher Scientific, Pittsburgh, PA). The extracted RNA was subjected to real-time qRT-PCR using a One-Step SYBR® qRT-PCR reagent kit (Takara) and primers specific for the IFN-γ and IL-4 genes (Table 3). RT and real-time PCR amplification of targeted genes were carried out with each 75 ng total RNA as a template in 50μl of the reaction mixture. Following reverse-transcription of the total RNA at 45°C for 30 min, the resulting cDNAs were used for real-time PCR amplification. PCR amplification was conducted by subjecting reaction mixtures to initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 15 s and annealing/elongation at 58 °C for 30s. The relative expression values of IFN-γ and IL-4 were normalized using the commonly used reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 3). The fold change in relative gene-expression levels was calculated and all data were analyzed using the CFX96™ manager software version 1.6 (Bio-Rad).

Real-time quantitative RT-PCR (qRT-PCR) analysis

Real-time qRT-PCR was employed to determine the amount of AIV H9N2 in cloacal swab samples or in tissues of AIV H9N2-challenged chickens using a CFX96™ real-time PCR detection system (Bio-Rad). Viral and total RNAs were extracted from cloacal swab samples and tissue samples (trachea, lung, brain, cecal tonsil, spleen, and kidney) using viral RNA extraction and total RNA extraction kits (iNtRON), respectively, according to the manufacturer's instructions. The extracted RNAs were then subjected to real-time qRT-PCR using a One-Step SYBR® qRT-PCR reagent kit (Takara, Shiga, Japan) and primers specific for the AIV H9 gene (Table 3). RT and real-time PCR amplification of targeted genes were carried out with the same reaction conditions and temperature cycles, as described above. A standard curve was generated by plotting threshold cycle values against serially diluted plasmid DNA encoding the AIV H9 protein. After the reaction cycle was completed the temperature was increased from 50 °C to 95 °C at a rate of 0.2 °C /15 s and the fluorescence was measured every 5 s to construct a melting curve that was used to confirm the authenticity of the amplified products. A control sample that contained no template RNA was run with each assay, and qRT-PCR data for AIV H9 amounts in the tissues was normalized using the commonly used reference gene GAPDH (Table 3). All determinations were performed by data from wells evaluated in duplicate to ensure reproducibility. The copy number of the experimental samples was determined by interpolating the threshold cycle values using the standard curve. All data were analyzed using the CFX96™ manager software version 1.6 (Bio-Rad).

Statistical analysis

Where specified, the data were analyzed for statistical significance using an unpaired two-tailed Student's *t*-test. A p-value < 0.05 was considered significant.

Results

Construction of attenuated *S. enterica* serovar Typhimurium expressing chIFN-a and chIL-18

To test the immunomodulatory functions of oral co-administration of attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 against AIV H9N2 vaccine antigen, we constructed attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. Initially, the DNA fragments of chIFN-α (582 bp) and chIL-18 (510 bp) were amplified by RT-PCR using total RNA extracted from LPS-stimulated splenocytes (Figure 7A), and subcloned into the *Eco*RI and *Hin*dIII sites of pYA3560 and pYA3493 plasmids that were used for the expression of chIFN-α and chIL-18 in *S. enterica* serovar Typhimurium, respectively (Figure 7B). Subsequently, chIFN-α-encoding pYA3560 and chIL-18-encoding pYA3493 vectors were transformed into attenuated *S. enterica* serovar Typhimurium χ8501 host by electroporation and positive colonies of *S. enterica* serovar Typhimurium χ8501 harboring chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/ chIL-18) were selected in the absence of DAP. The in-frame fusion of the chIFN-α and chIL-18 with the β-lactamase signal sequence was confirmed by nucleotide sequencing (data not shown). To identify the expression of chIFN-α and chIL-18 proteins by transformed *S. enterica* serovar Typhimurium, TCA-precipitated culture supernatants and bacterial cell lysates prepared at different incubation time points (12, 18, and 24h) were subjected to SDS-PAGE and immunoblot analysis. Attenuated *S. enterica* serovar Typhimurium harboring the empty vector pYA3560 (χ8501/ pYA3560) or pYA3493 (χ8501/ pYA3493) cultured for 18 h was used as a negative control. The expression of chIFN-α and chIL-18 proteins from *S. enterica* serovar Typhimurium harboring chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) was detectable as early as 12 h post-incubation, and gradually

increased and saturated in the culture supernatants and cell lysates within 24h-incubation (Figure 7C). Furthermore, the biological activity of secreted chIFN-α protein in culture supernatants was evaluated by in ovo antiviral activity against AIV H9N2, as previously described [139]. Also, chIL-18 secreted from χ8501/chIL-18 was shown to induce nitric oxide (NO) production by HD-11 cells measured by Griess assay indicating IFN-γ release (data not shown). Therefore, these results indicate that the attenuated *S. enterica* serovar Typhimurium harboring chIFN-α-encoding pYA3560 (χ8501/chIFN-α) or chIL-18-encoding pYA3493 (χ8501/chIL-18) successfully expressed bioactive chIFN-α and chIL-18 proteins, which were actively secreted into the culture media instead of resulting from nonspecific membrane leaking or cell death by lysis.

Enhancement of humoral immune responses against AI vaccine by co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18

In order to examine how the humoral immunity was responded in AIV H9N2-vaccinated chickens with or without oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18, groups of chickens (*n*=5) treated with χ8501/chIFN-α or χ8501/chIL-18 or both at two different doses (10^9 and 10^{11} cfu/chicken) were vaccinated two times with AIV H9N2 inactivated vaccine. Sera samples were collected 7 days after primary vaccination and 7 & 14 days after booster vaccination and HI antibody titers were determined. Results revealed that significantly enhanced HI antibody levels were observed at all three time points in sera of both χ8501/chIFN-α- and χ8501/chIL-18-administered chickens at both the doses, compared to that of χ8501 (pYA3560)-treated chickens (Figure 12). Notably, combined oral administration of χ8501/chIFN-α and χ8501/chIL-18 showed significantly enhanced HI antibody titers in sera of AI-vaccinated chickens at both the doses, compared to administration of *S. enterica* serova Typhimurium expressing chIL-18 or chIFN-α alone. Therefore, this result indicates that co-administration of *S. enterica* serovar Typhimurium expressing chIL-18 and chIFN-α displayed more enhanced humoral immune responses against AI vaccine.

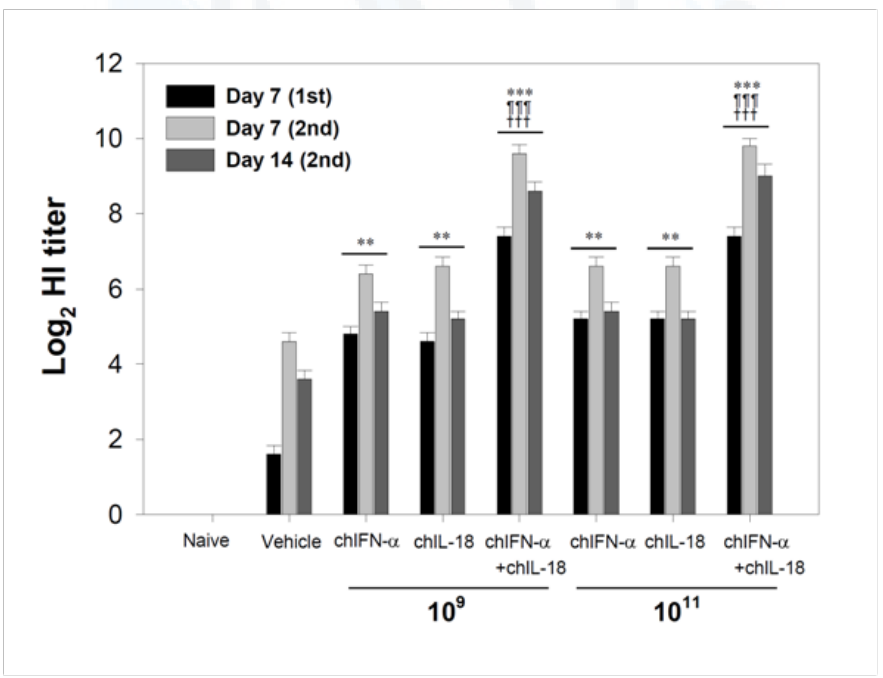

Figure 12: Serum HI antibody titers of inactivated AIV-vaccinated chickens following co-administration of live attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18.

Co-administration of *S. enterica* serovar Typhimurium expressing chIFN-a and chIL-18 induces enhanced Th1-biased immunity against AI vaccine

To evaluate the cellular immune responses, PBMCs were prepared from AIV H9N2-vaccinated chickens that received oral co-administration of χ8501/chIFN-α and χ8501/chIL-18. PBMCs were prepared 14 days after booster vaccination and subjected to stimulation with UV-inactivated AIV H9N2 antigen-pulsed PBMCs to determine antigen specific proliferation. PBMCs of chickens that received χ8501/chIFN-α or χ8501/chIL-18 (each 10^9 and 10^{11} cfu per chicken) orally prior to AI vaccination were found to show

significantly enhanced proliferation upon AIV H9N2 antigen-specific stimulation, compared to the chickens (vehicle) that received χ8501/pYA3560 (Figure 13A). In particular, the oral co-administration of χ8501/chIFN-α and χ8501/chIL-18 showed more markedly enhanced proliferation of PBMC upon AIV H9N2 antigen-specific stimulation than single administration of χ8501/chIFN-α or χ8501/chIL-18 alone. Furthermore, the mRNA expression levels of IFN-γ and IL-4 in PBMC were determined by real-time qRT-PCR following stimulation with AIV H9N2 antigen. Both IFN-γ and IL-4 mRNA levels in PBMCs prepared from chickens that received single administration of χ8501/chIFN-α or χ8501/chIL-18 (10^9 and 10^{11} cfu) were significantly enhanced, compared to the chickens that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector and more markedly enhanced effect in IFN-γ and IL-4 mRNA expression of PBMCs prepared from χ8501/chIFN-α plus χ8501/chIL-18 co-administered chickens is also noticed (Figure 13B). More importantly, the expression of IFN-γ mRNA was more significantly up-regulated than IL-4 mRNA in single administration of χ8501/chIFN-α or χ8501/chIL-18 and co-administration of χ8501/chIFN-α and χ8501/chIL-18 induced more enhanced upregulation of IFN-γ mRNA than single administration of construct. Taken altogether, our results indicate that oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-a and chIL-18 induces more enhanced Th1-biased immunity against AI vaccine, compared to chickens that received single administration of *S. enterica* serovar Typhimurium expressing chIL-18 or chIFN-α.

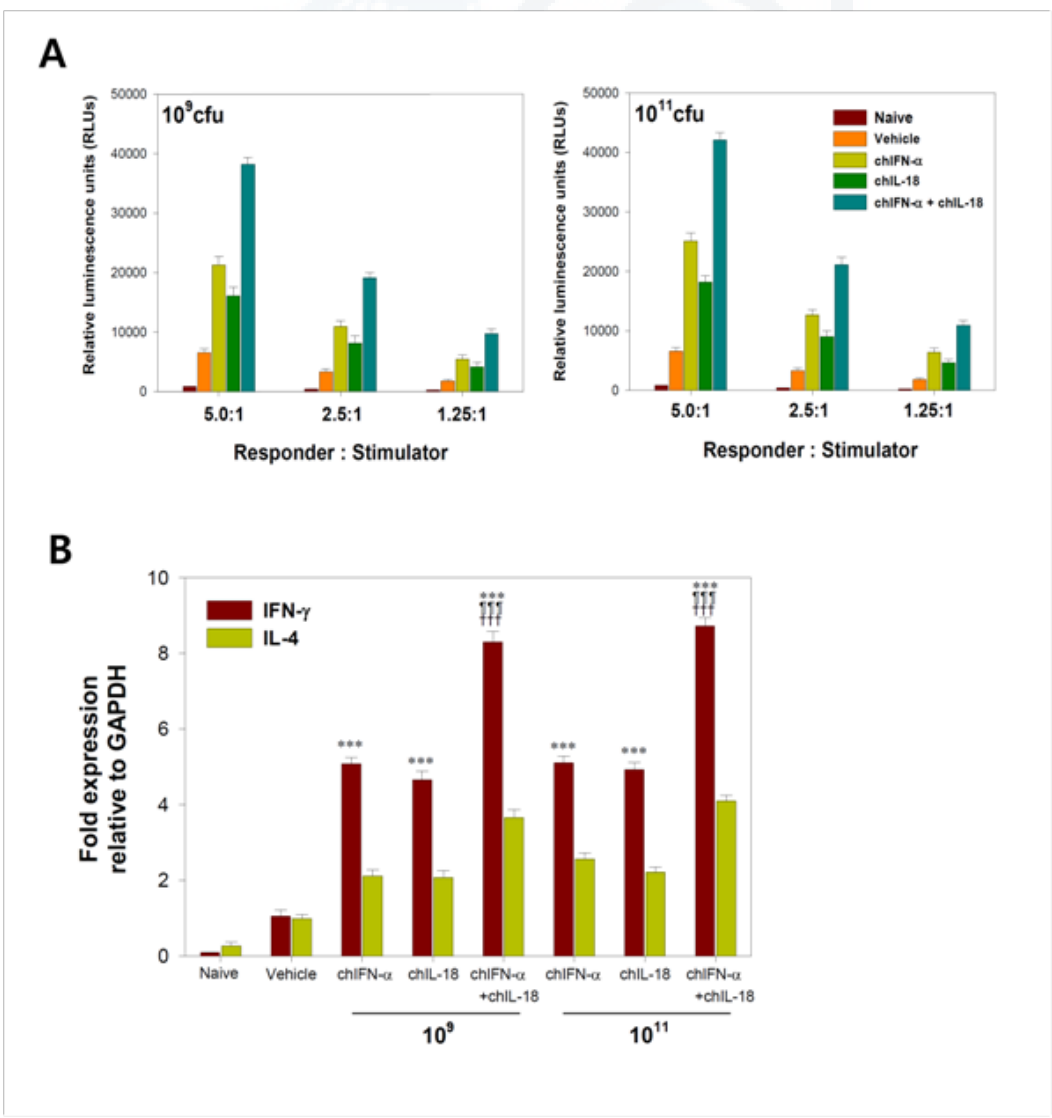

Figure 13: Enhanced Th1-biased immunity in chickens that received the co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18.

Enhanced protective immunity of AI vaccine by co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18

After addressing the enhanced immune-modulatory functions of orally co-administered χ8501/chIFN-α and χ8501/chIL-18 in AI-vaccinated chickens, we next focused our study to evaluate the protective immunity of AI H9N2 vaccine in chickens co-administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. To this end, SPF chickens (7-days old) co-administered *S. enterica* serovar Typhimurium expressing chIFN- and chIL-18 were vaccinated twice at 10 and 17-days old and then intra-tracheally challenged with AIV H9N2 (01310) ($10^{10.83}$ EID_{50}/chicken) 7 days after booster vaccination. Following challenge, chickens were observed daily to record mortality and clinical severity throughout the duration of the experiment. The results revealed that the mortality showed between 4 and 6 days p.i., and the chickens that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector without AI-vaccine showed highest mortality (50%). Vaccination with inactivated AI vaccine could reduce the mortality to 25% and oral administration of χ8501/chIFN-α or χ8501/chIL-18 (10^9 cfu) prior to AI vaccination could reduce it to 12.5%. However, single administration of either χ8501/chIFN-α or χ8501/chIL-18 at higher dose (10^{11} cfu) or their combined administration at any doses (10^9 and 10^{11} cfu) could effectively protect all vaccinated chickens from AIV H9N2 challenge infection (Figure 14A). Also, when the severity of clinical signs caused by AIV H9N2 challenge infection was scored, clinical signs appeared 2 days p.i., and the severity of clinical signs peaked at 4-7 days p.i. (Figure 14B). The chickens that received single or combined administration of χ8501/chIFN-α and χ8501/chIL-18 (**(**10^9 and 10^{11} cfu) before vaccination showed significant alleviation of clinical severity during the whole course of clinical infection, when compared to the group (vehicle) that received *S. enterica* serovar Typhimurium harboring empty pYA3560 vector. Furthermore, feed and water intakes were recorded daily after AIV H9N2 challenge of AI-vaccinated chickens that received oral co-administration of χ8501/chIFN-α and χ8501/chIL-18 (**(**10^9 and 10^{11} cfu) before vaccination. The results showed that average feed (Figure 14C) and water (Figure 14D) intakes were improved in chickens that received AI vaccine, compared to chickens that received only empty vector (χ8501/ pYA3560) without AI vaccine. In particular, average feed and water intakes were improved more when the chickens received χ8501/chIFN-α and χ8501/chIL-18 either singly or in combination (10^9 and 10^{11} cfu) before vaccination with better result in later. Overall, these results indicate that oral co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 prior to AI vaccination could markedly reduce mortality and alleviate clinical signs induced by infection with AIV H9N2. Further, when we examined histopathological changes in lung and tracheal tissues of chickens 5 days after AIV H9N2 challenge infection, the chickens that received χ8501/chIFN-α and χ8501/chIL-18 either singly or in combination (10^9 and 10^{11} cfu) before vaccination showed apparently normal lung and trachea, compared to chickens that received only empty vector without AI vaccine (Data not shown). These results indicate that oral co-administration of *S. enterica* serovar Typhimuirum expressing chIFN-α and chIL-18 along with AI vaccine could provide complete protection against assaults of lung and tracheal tissues by infection with AIV H9N2.

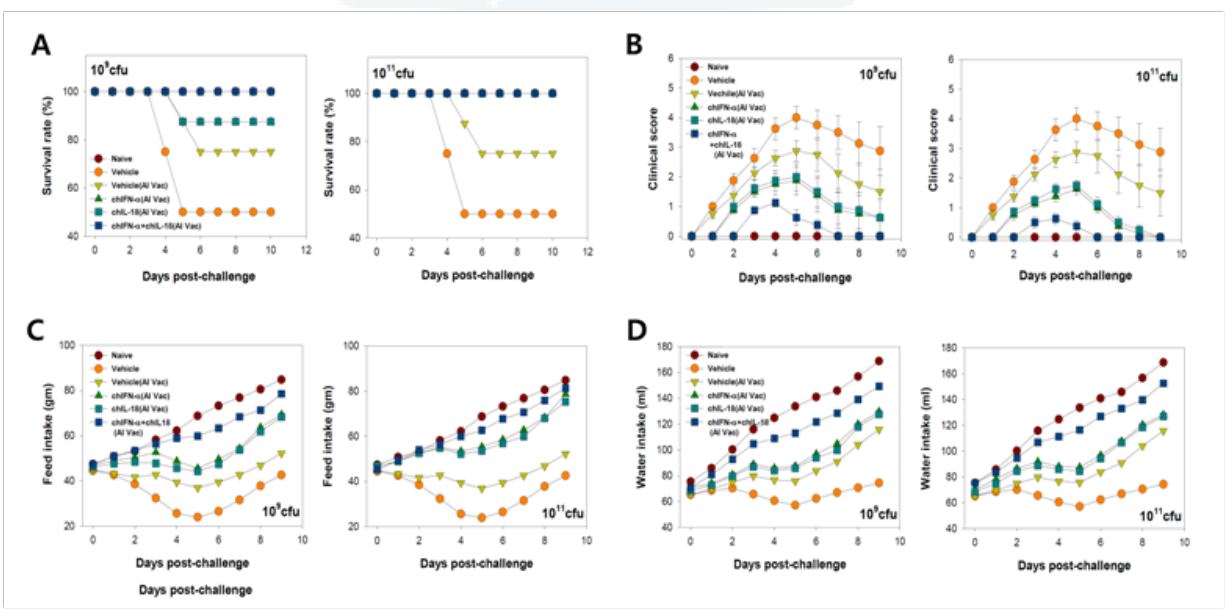

Figure 14: Enhanced protective immunity of inactivated AI H9N2 vaccine by co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18.

Reduction of AIV H9N2 shedding and replication in vaccinated chickens by the co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18

To evaluate the effect of oral co-administration of χ8501/ chIFN-α and χ8501/chIL-18 on virus shedding from AIV H9N2-infected chickens that received cytokine treatment and AI vaccine before infection, the amount of virus in cloacal swab was determined by real-time qRT-PCR at 0, 1, 3, 5, 7 and 9 days post-challenge. Virus shedding was detected from 3 day after AIV H9N2 infection and peaked at 5 days p.i. (Figure 15A). However, the chickens that received χ8501/chIFN-α or χ8501/chIL-18 or both (10^9 and 10^{11} cfu) before AI-vaccine had significantly lower peaked levels of virus shedding at 3, 5, 7 and 9 days p.i., with better effect in combined treatment, compared to the chickens that received only vehicle (χ8501/ pYA3560) or vehicle plus AI vaccine. Significant lower peaked levels of virus shedding was also detected in AIV H9N2-infected chickens at 5 and 7 days p.i., that received vehicle plus AI vaccine, compared to chickens that received only vehicle (χ8501/ pYA3560). Additionally, the amount of virus in different tissues (trachea, lung, brain, cecal toncil, spleen, and kidney) of AIV H9N2-infected chickens was determined at 4 and 7 days p.i. As expected, the amount of AIV H9N2 in different tissues of chickens that received χ8501/chIFN-α or χ8501/ chIL-18 or both (10^9 and 10^{11} cfu) prior to AI vaccination was significantly lower both at 4 days p.i. (Figure 15B) and 7 days p.i. (Figure 15C), compared to the chickens that received vehicle (χ8501/ pYA3560) only. It was noted that only AI vaccine could also significantly reduce the virus amounts in different tissues, compared to the chickens that received only vehicle (χ8501/ pYA3560) but significant differences existed when compared between vehicle plus AI vaccine and cytokines (chIFN-α or chIL-18 or both) plus AI-vaccine, which indicates that cytokine treatment before vaccination provided better protection than only vaccination. Taken altogether, these results indicate that oral co-administration of *S. enterica* serovar Typhimuirum expressing chIFN-α and chIL-18 along with AI-vaccine could alleviate clinical signs induced by AIV H9N2 infection through reduction of virus replication in tissues.

Discussion

Vaccination is the major tool for the prevention and control of H9N2 LPAI in poultry industry. However, while the conventional killed vaccines against H9N2 AIV can eliminate clinical signs of illness, they do not completely prevent infection and virus shedding [152]. Therefore, an improved H9N2 LPAI vaccine and/or vaccination strategy is particularly needed to induce complete immunity in chickens against H9N2 LPAI virus strains.

Cytokines play a crucial role in host defense and inflammatory responses by providing a regulatory bridge between innate and adaptive immunity, thereby controlling the immune system. They determine both the type and extent of an immune response that is generated following infection with a pathogen or after vaccination. Based on the immunobiological mechanisms, the combined effects of two or more cytokines might be antagonistic, additive, or synergistic [76]. Therefore, synergistic and additive immunomodulation and thereby, an enhanced protective immune response can be generated against vaccine antigens as either an antibody-mediated or a cell-mediated response by combined use of two or more cytokines [77-78]. In the present study, oral co-administration of chIFN-α and chIL-18 using attenuated *S. enterica* serovar Typhimurium as a carrier of the two cytokines, modulated the immune responses of chickens against inactivated H9N2 LPAI vaccine antigen through more enhanced both humoral and Th1-biased cell mediated immunity than single administration of constructs, thereby conferring complete protection against a high dose ($10^{10.83}$ EID_{50}/ chicken) homologous virus challenge. This enhancement of protective immunity was further confirmed by reduced rectal shedding and replication of AIV H9N2 in different tissues of challenged chickens. Thus, we claim that modulation of immune response elicited by commercially available, inactivated H9N2 LPAI vaccine through combined use of chIFN-α and chIL-18 may be a novel approach to induce complete immunity in chickens against H9N2 LPAI virus strains.

The enhanced effect of cytokine combinations has been shown empirically, based on their biological mechanisms. IFN-α and β (type I IFN) rapidly induced by viral infection and/or a series of events have well-defined strong antiviral activity along with immunoregulatory functions. The binding of type I IFNs to type I IFN receptor complexes results in the rapid phosphorylation and activation of receptor-associated JAKs, Tyk2, and Jak1, and subsequent transcription factor STAT1/2, which induces the expression of OAS, RNase L, Mx1, and PKR genes that confer the antiviral state in cells [40]. Alternatively, IFN-γ, the only type II IFN, is a multifunctional cytokine produced primarily by T lymphocytes (Th1) and NK cells. IFN-γ plays a vital role in macrophage activation and modulation of the immune system, in addition to its antiviral activity [143]. The antiviral effect of IFN-γ may be direct (intracellular, NO secretion) or indirect, involving activation of effector cells of the immune system [144]. Similar to mammals, chicken type I and type II IFN act synergistically [45], both in terms of antiviral activity and in their ability to activate macrophages. IL-18, which was initially identified as potent IGIF, provides an important link between the innate and adaptive immunity through the induction of IFN-γ [81]. It is an important cytokine in the initiation of Th1-type cell mediated immune responses. Also, the significance of chicken IL-18 in the development of antiviral immune responses has been shown in several viral infections like H5-H7 avian influenza virus (AIV) infection. It has been demonstrated that after infection of macrophages with influenza virus, cells produce IL-18, which acts synergistically with IFN-α and enhances IFN-γ synthesis [82]. Therefore, it is possible that chIFN-α and chIL-18 might have enhanced immunomodulatory function in combination;

however, a practical assessment of their combined function in immune modulation has not yet been addressed. It is conceivable that type II IFN-γ produced by IL-18 exposure might induce enhanced alleviation of the clinical signs of AIV H9N2 infection and modulate immunity, along with type I IFN. Furthermore, our results are supported by the finding that chicken IL-18 cDNA linked with recombinant encoding sequences of H5-H7 AIV in a fowl pox-based DNA vaccine (rFPV-H5-H7-IL18) successfully induces complete protection (100%) in SPF chicken after challenge with H5 AIV, and lymphocyte proliferation induced by the rFPV-H5-H7-IL18 is significantly higher than that induced by rFPV-H5-H7 alone [59]. Therefore, the present data for the first time provides valuable insight into the use of combined administration of type I IFN and IL-18, known as potent IGIF, in controlling viral infection in poultry industry.

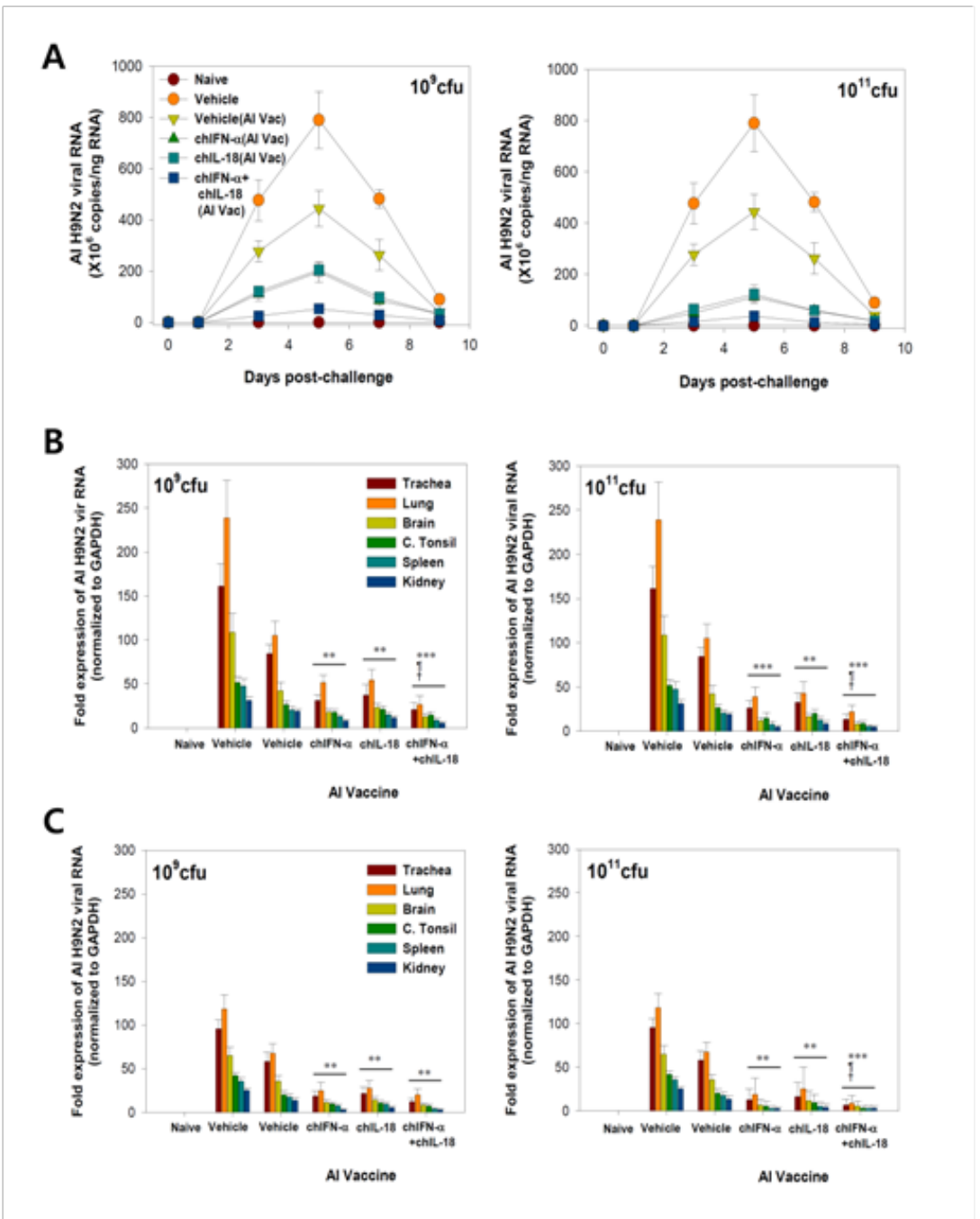

Figure 15: Reduction of virus shedding and replication in AIV H9N2-challenged chickens that received the co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 followed by AIV H9N2 vaccination.

The primary target cells for AIV infection and replication are ciliated epithelial cells. However, AIV can also infect macrophages and dendritic cells [125,126]. In avian species, intestinal epithelia are also targets of infection and, in the later stage of infection, mononuclear cells become involved. Influenza A virus causes NS1-mediated suppression of selected genes involved in IFN [127-128] and IFN-inducible gene expression [130], and induction of a weak chemokine expression in human lung epithelial cells, which enable the virus to replicate before the host inflammatory and antiviral responses are activated. Thus, complete protection of chickens from AIV H9N2 requires early stimulation of immune systems by immune-modulatory cytokines like chIFN-α and chIL-18. Therefore, it is possible that oral co-administration of attenuated *Salmonella* bacteria expressing chIFN-α and chIL-18 prior to vaccination with inactivated H9N2 LPAI could effectively modulate host innate and adaptive immune responses, thereby providing complete protection against AIV H9N2 challenge.

There are a few obstacles for the practical use of mass administration of cytokine proteins in livestock and poultry, such as cost, labor, and time, as well as protein stability. Establishment of a suitable delivery vector is of prime importance to make sure the use of cytokines in disease prevention. Our previous report [139] and present study demonstrated the value of attenuated *Salmonella* vaccine in the oral delivery of Immunomodulatory cytokines. Live attenuated *S. enterica* serovar Typhimurium χ8501 can colonize gut-associated lymphoid tissue and visceral non-lymphoid and lymphoid tissues following oral administration, and subsequently stimulate local and systemic immune responses [90,110]. Furthermore, since the *Salmonella* bacteria used in this study were devoid of the asd gene that is essential for a balanced-lethal host-vector system, they may have been sufficiently attenuated in their capacity to cause acute diseases in chickens. Indeed, all chickens orally administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 did not display any signs of disease for a 15-day monitoring period (data not shown). However, to accomplish the effective results for controlling infectious diseases in chickens by the *Salmonella* delivery system, the successful and prolonged colonization of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 might be needed. According to previous findings, *S. enterica* serovar Typhimurium can persist in adult chicken for at least three weeks which is more prolonged in younger chickens, up to 7 weeks [133-134]. Therefore, it is believed that the *Salmonella* bacteria used for cytokine delivery can persist in chicken body for prolonged period and can provide continuous long term protection against virus infection.

In conclusion, we have demonstrated in this study that modulation of immune response elicited by commercially available, inactivated H9N2 LPAI vaccine through combined oral administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 can completely protect the immunized chickens from high dose homologous virus challenge. The results suggest that naturally occurring Immunomodulatory cytokines like chIFN-α and chIL-18 can be combined with commercially available inactivated vaccines to generate an effective immunization strategy in chickens. It will be interesting to assess the protective efficacy of this immunization strategy against challenge with currently circulating heterologous virus strains in future studies.

Figure 7: Construction of live attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. (A) The chIFN-α and chIL-18 genes amplified by RT-PCR. Total RNAs extracted from LPS-stimulated splenocytes were subjected to amplify the chIFN-α and chIL-18 genes using specific primer pairs. Amplified chIFN-α and chIL-18 genes are indicated by arrows in the agarose gel image. Lane M, size marker; 1 and 3, naïve splenocytes; 2 and 4, LPS-stimulated splenocytes. (B) Diagram of periplasmic secretion Asd+ vector pYA3560 and pYA3493. 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). A DNA fragment encoding the β-lactamase signal sequence and 12 amino acid residues of the N terminus of mature β-lactamase of plasmid pBR322 was positioned under the control of the P_{trc} promoter. The map of pYA series vectors (pYA3560 and pYA3493) and the nucleotide sequences of the P_{trc} promoter region, β-lactamase signal sequence (*bla* SS) and multicloning sites are shown. The P_{trc} sequences for -35, -10 (RNA polymerase-binding site) and Shine-Dalgarno box (SD, ribosomal binding site) are indicated by blue and green boldface, and the translocation start codon (ATG) is in red boldface. An arrow within the sequence indicates the signal peptidase cleavage site. Unique restriction enzyme sites in the multicloning site are indicated, and 5ST1T2 is a transcriptional terminator. (C) Identification of chIFN-α and chIL-18 expression from constructed *S. enterica* serovar Typhimurium by immunoblot analysis. The chIFN-α protein expressed by χ8501/chIFN-α and the chIL-18 protein expressed by χ8501/chIL-18 were detected from both TCA-precipitated culture supernatants (sup) and cell lysates by immunoblotting with chIFN-α-specific monoclonal antibody and 6×His-Tag antibody after 12, 18, 24h- incubation, respectively. Attenuated *S. enterica* serovar Typhimurium carrying empty vector pYA3560 (χ8501/ pYA3560) or pYA3493 (χ8501/ pYA3493) cultured for 18 h was used as a negative control.

Figure 12: Serum HI antibody titers of inactivated AIV-vaccinated chickens following co-administration of live attenuated *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. Groups of chickens were administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (10^9 and 10^{11} cfu/chicken) and vaccinated with inactivated AIV H9N2 3 days later. The vaccination was performed by same protocol two times at 7-days interval. Serum samples collected from chickens of all groups 7 days after primary vaccination and 7 & 14 days after booster vaccination were subjected to HI test. Data were expressed as reciprocal log2 of the geometric average and SEM of

HI titers obtained from five chickens per group. **p<0.01; ***p<0.001 compared to vehicle group that was treated with control bacteria. ¶¶¶p<0.001 compared to chIFN-α-treated chickenss. †††p<0.001 compared to chIL-18-treated chickens.

Figure 13: Enhanced Th1-biased immunity in chickens that received the co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. (A) AIV H9N2 antigen-specific proliferation of PBMCs. Groups of chickens were administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (10^9 and 10^{11} cfu/chicken) and vaccinated with inactivated AIV H9N2 3 days later. The vaccination was performed by same protocol two times at 7-days interval. PBMCs (responder) were prepared from chickens 14days after booster vaccination, and subsequently stimulated with naïve PBMCs (stimulator) that had been pulsed with inactivated AIV H9N2 antigen. AIV H9N2 antigen-specific proliferation of PBMCs was assessed by measuring the viable cell ATP bioluminescence following 72h-incubation. (B) The expression of IFN-γ and IL-4 mRNAs by PBMCs following stimulation with AIV H9N2 antigen. Total RNAs were extracted from PBMCs stimulated with AIV H9N2 antigen for 72h, and subjected to real-time qRT-PCR to determine the expression of IFN-γ and IL-4. Data show the average and SEM of IFN-γ and IL-4 mRNA expression normalized to GAPDH (*n*=5). ***p<0.001 compared to vehicle group that was treated with control bacteria. ¶¶¶p<0.001 compared to chIFN-α-treated chickens. †††p<0.001 compared to chIL-18-treated chickens.

Figure 14: Enhanced protective immunity of inactivated AI H9N2 vaccine by co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18. (A) Mortality of AIV H9N2-challenged chickens. Groups of chickens were administered *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 (10^9 and 10^{11} cfu/chicken) and vaccinated with inactivated AIV H9N2 3 days later. The vaccination was performed by same protocol two times at 7-days interval. Seven days after booster vaccination, chickens were intra-tracheally infected with AIV H9N2 ($10^{10.83}$ EID_{50}/bird). The graphs show the proportion of surviving chickens on different days p.i. (B) Clinical severity of AIV H9N2-challenged chickens. Chickens immunized with inactivated AIV H9N2 vaccine were challenged with AIV H9N2 virus, after which the clinical severity was scored daily. (C and D) Feed and water intake of AIV H9N2-challenged chickens. Feed and water intakes were recorded daily after AIV H9N2 challenge of inactivated AIV H9N2-vaccinated chickens. Data show the average of feed (C) and water (D) intakes obtained from eight chickens per group.

Figure 15: Reduction of virus shedding and replication in AIV H9N2-challenged chickens that received the co-administration of *S. enterica* serovar Typhimurium expressing chIFN-α and chIL-18 followed by AIV H9N2 vaccination. (A) Virus shedding of vaccinated chickens after AIV H9N2 challenge. Groups of chickens that received the co-administration of *S. enterica* serovar Typhimurium expressing chIFN- and chIL-18 (10^9 and 10^{11} cfu/chicken) followed by inactivated AIV H9N2 vaccination were intra-tracheally challenged with AIV H9N2 ($10^{10.83}$ EID_{50}/bird). The amounts of AIV H9N2 in cloacal swab samples taken at the indicated dates post-challenge were determined by real-time qRT-PCR using primers specific for hemagglutinin protein of AIV H9N2. Data represent the average and SEM of five chickens per group. (B and C) The amount of virus in tissues of AIV H9N2-challenged chickens. Groups of chickens that received the co-administration of *S. enterica* serovar Typhimurium expressing chIFN- and chIL-18 (10^9 and 10^{11} cfu/chicken) followed by inactivated AIV H9N2 vaccination were euthanized 4 (B) and 7 days (C) after AIV H9N2 challenge. Real-time qRT-PCR using total RNAs extracted from tissues (trachea, lung, brain, cecal toncil, spleen, and kidney) was conducted to determine AIV H9N2 amounts. Data show the average and SEM of AIV H9 fold expression obtained from four chickens per group, normalized to GAPDH. **p<0.01; ***p<0.001 compared to vehicle. ¶p<0.05 compared to chIFN-α-treated group †p<0.05 compared to chIL-18-treated chickens.

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