A Thesis

for the Degree of Master of Science

Bacillus subtilis spore as an adjuvant in chicken treated with inactivated avian influenza virus, H9N2

불활화 조류 인플루엔자 바이러스, H9N2 를 처리한 닭에서 *Bacillus subtilis* 스포어의 면역증강제로서 역할

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이 논문을 농학석사 학위논문으로 제출함

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Summary

Bacillus subtilis, a rod-shaped gram-positive and endospore-forming bacterium, is a nonpathogenic species that has been used in a probiotic formulation for both animals and humans. *B. subtilis* spore is also known as a particulate adjuvant and has remarkable heat resistance properties which can remain dormant to survive under harsh environmental conditions. The objective of the present study was to elucidate the effect of *B. subtilis* spore as an adjuvant in chicken treated with inactivated avian influenza virus, H9N2 via intramuscular administration. Herein, the adjuvanticity of *B. subtilis* spore in chicken was demonstrated by enhancing H9N2 virus-specific IgG responses. *B. subtilis* spore enhanced proportion of B cells and innate cell population in splenocytes from chicken treated with both inactivated H9N2 and *B. subtilis* spore. Furthermore, the treatment induced significantly high expression of BAFF, CD40, and their receptors coincident with increase of pro-inflammatory cytokines, IL-1β and IL-6 when compared with chicken treated with inactivated H9N2 only. Then, total splenocytes from chicken immunized with inactivated H9N2 in the presence or absence of *B. subtilis* spore were re-stimulated with inactivated H9N2 *in vitro*. The results showed that the extent of antigen specific $CD4^+$ and $CD8^+$ T cell proliferation was higher in group co-administered with inactivated H9N2 and *B. subtilis* spore. Taken together, these data demonstrate that *B. subtilis* spore, as an adjuvant, enhance both H9N2 virus-specific IgG response and $CD4^+$ and $CD8^+$ T cell proliferation, with increased mRNA level of BAFF, BAFF receptor, CD40, CD40L, and pro-inflammatory cytokines. This

unique approach to vaccination in chicken together with *B. subtilis* spore as an adjuvant, indicates a significant role in protection against avian influenza virus.

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List of Abbreviations

7AAD	7-Aminoactinomycin D
ACK	Ammonium-chloride-potassium
AIV	Avian influenza virus
APC	Antigen presenting cells
BAFF	B cell activating factor in TNF family
BAFF-R	B cell activating factor receptor
BCMA	B cell maturation antigen
B. subtilis	*Bacillus subtilis*
CD	Clusters of differentiation
CD40L	CD40 ligand
CFU	Colony forming unit
Con	Control
CTV	CellTrace™ Violet
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and drug administration
GRAS	Generally regarded as safe
HA	Hemagglutinin
HAU	Hemagglutination units
HI	Hemagglutination inhibition

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.m.	Intramuscular
LPAI	Low pathogenic avian influenza
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
NA	Neuraminidase
PBS	Phosphate buffered saline
PG	Peptidoglycan
RNA	Ribonucleic acid
RT	Room temperature
TACI	Transmembrane activator and calcium-modulating cytophilin ligand interactor
TCR	T cell receptor
TSB	Trypticase soy broth
YE	Yeast extract

I. Review of Literature

1. Avian influenza virus H9N2

1.1 Characteristics and symptoms

H9N2 subtype avian influenza virus (AIV) belongs to the influenza virus A genus of Orthomyxoviridae family, and composed of eight pieces of segmented negative-sense, single-stranded RNA that encode at least ten proteins including two surface glycoproteins (hemagglutinin [HA] and neuraminidase [NA]) [1, 2].

AIV is classified into subtypes on the basis of two glycoproteins on the surface of the virus, HA and NA. There are 16 different HA subtypes and 9 different NA subtypes, making 144 combinations possible [3, 4]. H9N2 subtype viruses are classified as low pathogenic avian influenza (LPAI) based on molecular characteristics of the virus and the relatively low ability of the virus to cause disease and mortality in chicken [5]. Poultry infected with the H9N2 virus show no signs of disease or only exhibit mild illness, such as ruffled feathers egg production drop [2]. The spread of AIV has resulted in significant economic losses due to reduced egg production rate and high mortality associated with co-infection with other respiratory pathogens [6-8]. Among the 144 possible combinations of HA and NA proteins in avian influenza virus, the H9N2 subtype is thought to become one the most prevalent LPAI viruses in the domestic poultry industry [2]. However, the H9N2 subtype virus is concerned with human-like receptor specificity [2, 9], and avian-to-human transmissibility is raising public health concerns [10, 11].

1.2 H9N2 vaccine in poultry

For H9N2, which subtype has zoonotic potential LPAI virus, oil based inactivated H9N2 LPAI vaccine was employed in the chicken industry for preventing H9N2 infection worldwide [2]. In order to control the endemic situation, the oil based inactivated H9N2 LPAI vaccine has been administered to chicken. Adjuvants including oil emulsion have shown improved humoral immune efficacy in avian influenza protection, such as in H9N2 [12]. However, oil-adjuvanted vaccines have a weak cell-mediated immune response after revaccination, while it has strong humoral immune response [13].

2. Characteristics of Bacillus subtilis

2.1 B. subtilis spore as an adjuvant

B. subtilis is a gram-positive, nonpathogenic, and endospore forming bacteria that have been used as a biotechnological application [14, 15]. *B. subtilis* spore, which is considered as a Generally Regarded as Safe (GRAS) molecule by the Food and Drug Administration (FDA), is a robust bio-particle that displays adjuvant effects on immune responses after administration [16]. Adjuvants are essential for enhancing the adaptive immune response to vaccine antigens, and one of the most critical roles is increasing antibody response against target antigen (Figure 1). They are defined as any substance that applied to accelerate, prolong, or enhance antigen-specific immune responses when used together with specific vaccine antigens. The adjuvant *B. subtilis* spores have a safety record based on the worldwide commercial use of its spores as probiotics for humans and animals for the oral prophylaxis of gastrointestinal disorders [17]. They have been successfully employed as vector for the mucosal or systemic delivery of

vaccine antigens [18, 19]. Although recent studies demonstrated that *B. subtilis* spore have been shown to induce strong adjuvant effects after co-administration with vaccine antigens, there is a difficulty in explaining the adjuvant properties of *B. subtilis* spore as an effective immune enhancer.

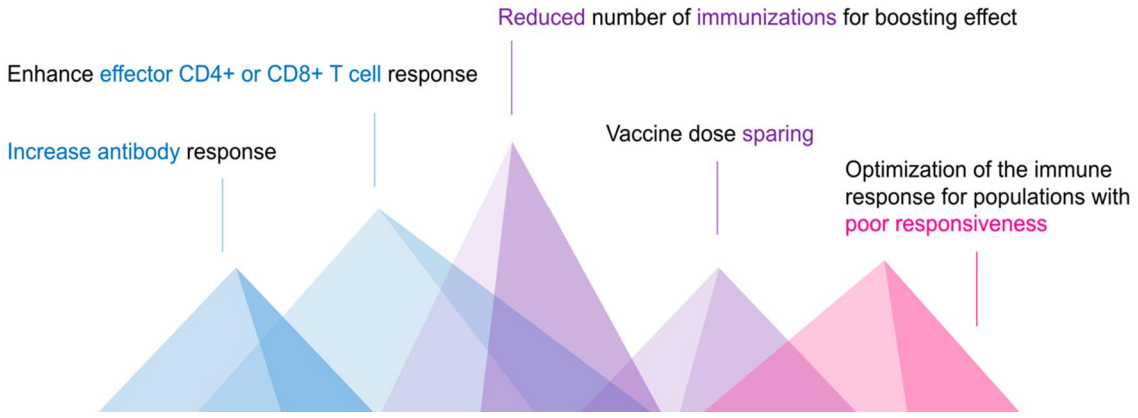

Figure 1. Essential role of adjuvant. Adjuvants are essential component in vaccine formulation for enhancing the adaptive immune responses against target antigen. The critical roles of adjuvants are increasing antibody responses, enhancing effector T cell responses, reducing the frequency of immunizations for boosting effect, vaccine dose sparing, and optimization of the immune responses for populations with poor responsiveness.

2.2 Cell cycle of B. subtilis

The bacterial cell cycle incorporates three important, discontinuous processes. The period between the cell division and initiation of chromosome replication, which is a stage required for replication, and the period between end of the replication and completion of division [20]. Many of genes and proteins involved in these three

processes have been characterized previously. As shown in Figure 2, the life cycle of spore-forming bacteria, *B. subtilis* consists of three different physiological processes, vegetative growth, sporulation and germination [21]. Sporulation in *B. subtilis* is intimately tied to the cell cycle, and the entrance into the sporulation stage is mediated by alteration in cell division and chromosome partitioning [22, 23]. The cell cycle is an integral part of development and entrance into sporulation is modulated by signals that transmit the status of DNA integrity, chromosome replication and segregation. In addition, *B. subtilis* modifies cell division and DNA segregation to establish cell type specific gene expression during sporulation [23, 24].

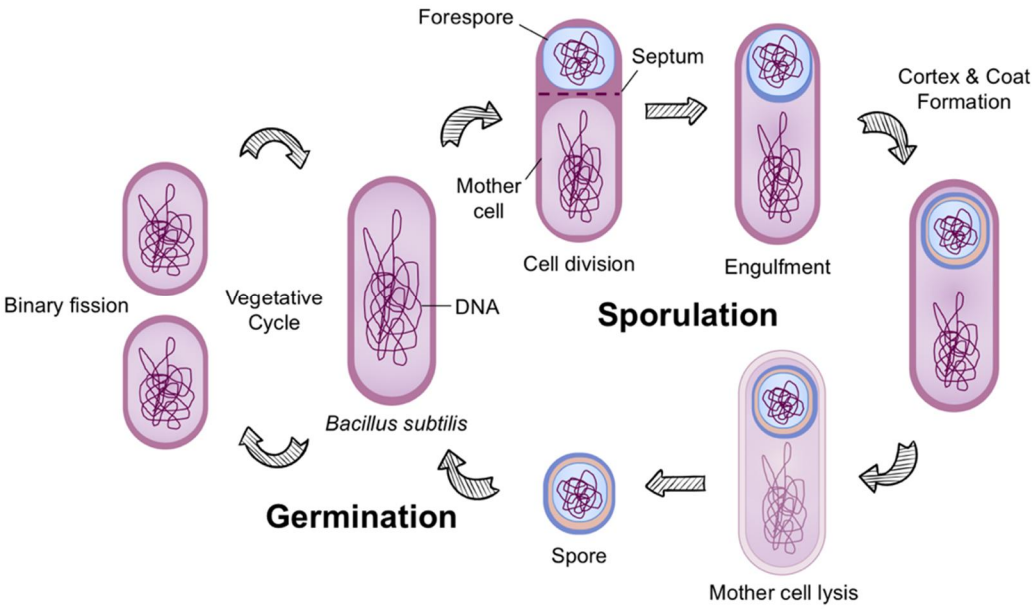

Figure 2. The sporulation and germination cycle of *B. subtilis*. Sporulation begins at the stationary phase when nutrients are depleted, and *B. subtilis* spore is able to germinate rapidly when nutrients become available again. Early morphological event of

sporulation is the formation of septum which is asymmetrically placed in the *B. subtilis*, which divides the bacteria into the mother cell and forespore compartments, and the smaller compartment becomes a spore.

2.3 Vegetative growth

Vegetative growth is characterized by binary symmetric fission cell growth that occurs when nutrients are available [21]. Chromosome replication occurs in company with cell growth to ensure that: at each origin, replication initiated once and only once per division cycle; at least one round of replication is completed and nucleoids have segregated before the completion of cell division; and there are sufficient nutrients to support these processes [20]. This initiation of chromosome replication cycle is tied to the vegetative cell division cycle, the bacterial chromosome maintains a specific orientation throughout most of the cell cycle and that at least some of the steps in chromosome partitioning are active processes [23].

2.4 Sporulation and germination in B. subtilis

B. subtilis can divide symmetrically into two daughter cells with binary fission which is resistant to environmental factors such as heat, radiation, and desiccation which can be last in the environment for long periods of time [25]. Although the sporulation of *B. subtilis* is induced by multiple environmental signals, such as nutrient deprivation, high mineral composition, neutral pH, temperature, and high cell density, the developmental stage of sporulation is not initiated immediately when growth slows due to these environmental limitations [21, 26]. A variety of alternative response strategies can occur in that stage, including the activation of flagellar motility to seek new nutrient sources

by chemotaxis, secretion of hydrolytic enzymes to scavenge polysaccharides and extracellular proteins, or induction of uptake exogenous DNA for consumption, with the occasional side-effect that new genetic information is stably integrated [25, 26]. Therefore, sporulation is the last determined response to starvation, at the times of nutritional stress, allowing the *B. subtilis* to persist in the environment until conditions become resistant [27]. The transcription factor, Spo0A plays an important role in initiating sporulation and the activity of Spo0A depends on phosphorylation [28]. Spo0A is phosphorylated on a highly conserved aspartate residue by histidine sensor kinases, which have catalytic mechanisms including KinA, KinB, and KinC. Then the Spo0A lies at the series of phosphor-relay, and the phosphor-relay culminates in the activated transcription factor Spo0A-P, which initiates the sporulation developmental program [26]. The sporulation inducing signal is not fully identified yet, but the recent results suggest the strategies by which *B. subtilis* integrates diverse signal in the decision-making process [19, 26]. In addition, the germination of *B. subtilis* spore, which was turned into dormant and robust form, is the first crucial step in the return of spores to vegetative growth, and is induced by nutrient and a variety of non-nutrient agents [29].

2.5 Structure of B. subtilis spore

The morphological process of sporulation in dormant *B. subtilis* spore is driven by a temporally and spatially regulated program of gene expression. The *B. subtilis* spore coat is an initial barrier to play a role in spore resistance, especially in preventing the access of peptidoglycan (PG)-lytic enzyme lysosome, which would otherwise have access to the spore cortex, and also likely plays role in spore resistance to some

chemicals, particularly oxidizing agents, such as hydrogen peroxide, chlorine dioxide, hypochlorite, and ozone [30-32]. Spore coat is predominantly composed of protein contains minor (6%) carbohydrate components, and the protein fraction of spore coat represents about 50-80% of the total spore protein [21]. The soluble fraction of spore coat for about 70% of the total spore protein, and the rest 30% insoluble coat proteins are characterized by high cysteine contents, and the formation of disulfide cross-links likely contributes to their insoluble compartment. Cross-linked materials in spore coat are likely associated to the chemical and mechanical resistance of the spore [21, 30, 33, 34]. So far successful heterologous protein display was reported for the use of three outer and one inner spore coat proteins as carriers: CotB, CotC, CotG, and OxdD respectively [35-40]. The selection of these spore coat proteins as carriers was based not only on their location but also their relative abundance [40]. The coat surface may present peculiar structures that appear to form when the spore volume decreases, during dehydration, and disappear when the spores swell during germination and hydration [21, 41, 42]. It suggests that the *B. subtilis* spore coat is a dynamic structure that may sense the external compartment through active enzyme lysosomes present on their surface, and adapt to changes in the spore without compromising the structural and biochemical integrity [21, 30, 43]. *B. subtilis* spore consists of multiple concentric shells encasing dehydrated genetic materials at the core. The spore cortex is a loosely cross-linked peptidoglycan layer, which is surrounding the core [43]. The *B. subtilis* spore cortex is covered by a multilayered protein coat, formed by an inner membrane which is opposed to the cortex, and an outer layer [35]. Surrounding the inner membrane is the germ cell wall, consisting of peptidoglycan, with a structure most likely identical to that of peptidoglycan in a growing cell wall [21]. The core is located in the center of the *B.*

subtilis spore and contains the DNA, RNA, ribosomes and majority of spore enzymes [21, 44]. Spore resistance to wet or heat is determined largely by the water content of spore core, and the lower core water content gives more wet and heat-resistant spores [44]. The humoral immune response of mouse induced by *B. subtilis* spore is already elucidated [45], but the adjuvant effects on immune responses induced in chicken remained to be determined.

II. Introduction

One of the best ways to improve the immunogenicity of relatively weak antigens is to identify new vaccine adjuvants [46]. Adjuvants, descends from the Latin word *adjuvare*, which means serving to help or assist [47]. In the context of vaccines, they are defined as components capable of enhancing antigen-specific immune response [48]. An immunologic adjuvant is defined as some material that acts to boost, prolong, or enhance antigen-specific immune responses when used in combination with a vaccine [49]. It can provoke humoral and cell-mediated immune responses, which are essential for protection against pathogenic antigens.

Bacillus subtilis spores have been shown to act as particulate vaccine adjuvants, increasing the antibody and T cell responses to a co-administered antigen [45]. More specifically, *B. subtilis* is a gram-positive bacterium, which is a nonpathogenic species that has been used as probiotic formulations for both animals and humans as consumption or feed products [50]. Indeed, they are classified by the Food and Drug Administration (FDA) in as Generally Regarded as Safe (GRAS) microorganism. *B. subtilis* is an endospore forming bacterium that can differentiate into a life form of dormant and robust spores when starved of nutrients to survive harsh environmental conditions [46]. Sporulation initiates when the DNA segregation completed, it occurs concurrently with the invagination of membrane asymmetrically by forming a polar septum near one pole of the cell [21, 51]. Then the forespore, which is the immature stage of spore is surrounded by a double membrane of the mother cell, and it mediates the development of the forespore into the spore [21]. However, upon return of nutrients

to the environment of the dormant spore retains the capacity to immediately convert to vegetative cell growth, which called stage of germination [30]. In the previous research with mice, *B. subtilis* spores increase the humoral effect of antigen-specific antibody responses to a co-administered soluble antigen and enhance cell-mediated antigen-specific responses, both at systemic and mucosal sites [18, 45]. Moreover, *B. subtilis* spore-induced cross presentation in response to co-administered antigen, suggesting that *B. subtilis* spores instruct a diverse adaptive immune response against the antigen [45]. The present evidences demonstrate that *B. subtilis* spores display adjuvant effects when co-administered with virus vaccines [18].

It is important to note that only a simple efficacy study has been performed with vaccine adjuvant in the poultry field, so I examined *B. subtilis* spore works as an adjuvant based on the induction of cellular immune response in chicken. In the present study, the adjuvanticity and efficacy of *B. subtilis* spore was investigated in chickens, challenged with formaldehyde-inactivated avian influenza virus H9N2.

III. Materials and methods

Chickens

Fertile eggs from White Leghorn chickens, were provided by University Animal Farm, College of Agriculture and Life Sciences, Seoul National University (Pyeongchang, Korea). The eggs were incubated at 37.5 - 38 ℃ incubator (Rcom, Korea) for 21 d. Care room maintained at 23-25°C with 40% of humidity under a positive pressure. Hatched chickens were raised in conventional conditions and were allowed free access to food and water. The experiment was approved by Institutional Animal Care and Use Committee of Seoul National University (IACUC No., SNU-150327-2-1).

Preparation and isolation of Bacillus subtilis spore

Bacillus subtilis strain HB3 (National Culture Collection for Pathogen, Korea) was spread in an agar plate containing 3% Trypticase soy broth (TSB), 0.5% Yeast extract (YE) and 1.5% Bacto Agar (all from BD Biosciences, San Diego, USA) and incubated at 37°C for 9 h. One colony was randomly picked and inoculated in 25 ml of 3% TSB and 0.5% YE liquid media. Then, it was incubated for 5 h in the shacking incubator (BioFree, BF50SIR, Korea) at 37°C until the OD value reached between 0.45 - 0.6. For sporulation, the culture was transferred to 500 ml of the autoclaved 3% TSB and 0.5% YE media which containing 5 ml of 10% KCI, 5 ml of 1.2% $MgSO_4.7H_20$ (pH 7.6), 0.5 ml of 1 M $Ca(NO_2)_3$, 0.01 M $MnCl_2$, and 1 mM $FeSO_4$. The culture was incubated at 37°C for 48 h in shaking incubator. The cells were collected by centrifugation at 5516 g for 10 min, re-suspended in distilled water, and incubated at 4°C for 48 h on the rocker. Then, the cells were sonicated at 35% amplitude (1 watt) for 90 seconds with 0.5 second pulse. Spore was loaded on the layers of 35%, 25%, 15% OptiPrep Density gradient

(Sigma-Aldrich, Missouri, USA) were centrifuged at 10,000 g for 40 min at 25°C without break for the purification. The *B. subtilis* spore was washed and re-suspended in distilled water.

Virus

Thirty-two hemagglutination units (HAU) of H9N2 (from Prof. Jae Hong Kim, College of Veterinary Science, Seoul National University) was inactivated with formalin at 37°C at a final concentration of 0.1%. H9N2 (A/Chicken/Korea/ 01310/2001, CE20 strain) inactivated oil vaccine was purchased from KBNP, INC. (Anyang, Korea).

Immunization schedule

One-week-old White Leghorn chickens were immunized with PBS, *B. subtilis* spore, inactivated H9N2, or both *B. subtilis* spore and inactivated H9N2 in a volume of 200 μl. Randomly selected chickens were allotted into four different groups as following; PBS as a negative control, $2x10^9$ CFU of *B. subtilis* spore alone, inactivated H9N2 alone, or inactivated H9N2 together with *B. subtilis* spore. Immunization regimen comprised two doses via intramuscular (i.m.) route at 7 and 14 days old, respectively. Blood samples were collected on 7 and 14 days after the last immunization from wing vein to analyze the antigen-specific antibody responses in serum. Spleens were taken and used for in vitro T cell receptor (TCR) stimulation and flow cytometric analysis.

Serum antibody detection

Antigen-specific IgG in serum was analyzed by ELISA. For H9N2-specific IgG, 100 μl/well of formalin inactivated H9N2 influenza A virus was coated onto a 96-well microplate (Nunc, Roskilde, Denmark) overnight at 4°C. Serially diluted sera along with controls were incubated for 2 h at room temperature, followed by a 1 h incubation with 100 μl of rabbit anti-chicken IgG conjugated with HRP (Bethyl, Alabama, USA)

(1:50,000 dilution). After incubation for 1 h at room temperature, TMB (Millipore, Darmstadt, Germany) was added until color developed, and then the reaction was stopped by the addition of 50 μl of 2 N H_2SO_4. Absorbance was measured at 450 nm using an ELISA microplate reader (Molecular Device, Sunnyvale, USA) and the amount of total IgG was determined from the standard curve.

Hemagglutination inhibition (HI) assay

Hemagglutination inhibition (HI) titer was determined by using chicken erythrocytes which were collected with Alsever's solution (Sigma-Aldrich, Missouri, USA) from 3- to 4-week-old chickens. Serially 2-fold diluted serum (25 μl/well) from each group of chickens was incubated with 25 μl/well of H9N2 virus in V-bottom 96-well plate (EMD, Darmstadt, Germany) for 30 min at room temperature. Chicken erythrocytes (50 μl/well) were added and incubated for 30 min at RT. Then, it was analyzed to distinguish agglutinated from non-agglutinated wells, of which the highest dilution showing clear red dot was determined as HI titer.

In vitro TCR stimulation

Splenocytes from 3-week-old chicken were stained with anti-chicken CD3 antibody followed by the incubation with anti-mouse IgG bead (Miltenyi Biotec, California, USA) for 20 min. $CD3^+$ T cells were isolated by MACS magnetic bead sorter (Miltenyi Biotec, California, USA) and stained with 1 μM CellTrace™ Violet (CTV) dye (Invitrogen, California, USA) for 25 min at 37°C. And then, the cells were washed with pre-warmed complete media. $CD3^+$ T cells stained with CTV were cultured in anti-chicken CD3 and CD28 antibodies-coated 96 flat-bottom plates (Thermo Scientific Nunc, New York, USA) for 2 d. The cells were stained with anti-chicken CD4, CD8 and proliferative activity was determined by flow cytometry (FACS Canto II, BD Biosciences) and analyzed using FlowJo software (Tree star, California, USA).

Single cell dissociation

Spleen was collected, minced and filtered through a 70 µm nylon cell strainer (BD Falcon, California, USA) to obtain a single cell suspension. The splenocytes were, then, suspended in 5 ml RPMI 1640 containing heat-inactivated 5% (vol/vol) FBS and 1% (vol/vol) antibiotics/antimycotic solution (all from Invitrogen) and centrifuged at 300 *g* for 3 min at 4°C. Then, the pellet was treated with 1 ml ACK lysing buffer (Gibco, New York, USA) incubated for 3 min at room temperature, and centrifuged at 300 *g* for 3 min at 4°C. The pellet was washed and re-suspended with media and filtered through 70-µm strainer.

Flow cytometric analysis

Single-cell suspension of total splenocytes was stained for 20 min at 4°C in the dark with the combination of following fluorochrome-conjugated monoclonal antibodies; anti-chicken CD3-PACBLU (clone CT-3), CD4-FITC (clone CT-4), CD8α-PE (clone CT-8), Bu-1-Alexa Fluor® 647 (clone AV20), Monocyte/Macrophage-PE (clone KUL01) from Southern Biotechnology (Birmingham, Alabama, USA). 7AAD-PerCP-Cy5.5 antibody was purchased from Biosciences (San Diego, California, USA). After the staining, the cells were washed and the expression of surface markers was measured by flow cytometry (FACS Canto II, BD Biosciences). All the flow cytometric data were analyzed using FlowJo software (Tree star, California, USA).

Cell purification by magnetic beads

Splenocytes were stained with mouse anti-chicken monocyte/macrophage (clone KUL01) antibody, purchased from Southern Biotechnology (Birmingham, Alabama, USA). After washing with MACS buffer (PBS containing 0.5% BSA and 2 mM EDTA), the cells were incubated with anti-mouse IgG microbeads (Miltenyi Biotec, California,

USA) for 15 min in the dark and centrifuged at 300 *g* for 10 min at 4°C. Then, the cell suspension was separated on a MACS LS column which is placed in the magnetic field of a MACS Separator (Miltenyi Biotec, California, USA). The magnetic fraction of positively selected cells was used in the mRNA experiments.

RNA extraction and cDNA synthesis

Total RNA was extracted from splenocytes using NucleoZOL (MACHERY-NAGEL, Duren, Germany) according to the manufacturer's instruction. Briefly, single cells of the splenocytes were treated with 1 ml of NucleoZOL per 5 x 10^6 cells. Total RNA was isolated by the addition of 400 µl of RNase-free water (Sigma-Aldrich, St Louis, USA) followed by centrifugation at 12,000 *g* for 15 min. The 500 µl of aqueous phase was transferred into a new tube and 500 µl of isopropanol was added. Then, the samples were incubated for 10 min at room temperature for RNA precipitation and centrifuged at 12,000 *g* for 10 min. RNA pellet was obtained as at the bottom of the tube after washed with 75% ethanol, air dried for 5-10 min, and then re-suspended with RNase-free water. RNA concentration was quantified with NanoDrop (Amersham Biosciences, Piscataway, USA) at A260. Subsequently, 500 ng of purified RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen, California, USA) according to the manufacturer's instruction.

Real time quantitative PCR

The real-time quantitative PCR was performed on cDNA using a StepOne Plus real-time PCR system (Applied Biosystems, California, USA). SYBR® Green PCR Master Mix was used according to manufacturer's specification (Applied Biosystems,

California, USA). The PCR reaction was carried out in a 96-well reaction plate with 10 µl SYBR® green PCR master mix, 0.5 µl primers, 1-2 µl cDNA template and 7-8 µl nuclease-free H_2O. Each reaction involved a pre-incubation at 95°C for 10 min, followed by 45 thermal cycles at 95°C for 15 s, 55°C for 30 s and elongation at 72°C for 30 s. Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Target gene expression was normalized to β-actin mRNA level. Primers were designed using the NCBI Primer-BLAST and synthesized from Bioneer Inc. (Daejeon, Korea). Primer sequences used for real time quantitative PCR are shown in Table 1.

Statistical Analysis

All data were expressed as means ± standard deviation (SD). For comparison of means between two groups, the data were analyzed using two-tailed paired Student's *t*-test and considered statistically significant when *P*-value was less than 0.05. For multiple group comparison, one-way ANOVA followed by a Friedman test. All statistical analyses were performed using GraphPad Prism 5 version 5.01 (GraphPad Software, Inc., San Diego, USA).

Table 1. Primer sequences used for real time quantitative PCR.

Target gene	Primer sequence	Product size (bp)
β-actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	120
BAFF	F: CACGTCATCCAGCAGAAGGAT R: ACAAGAGGACAGGAGCATTGC	120
BAFF-R	F: CCTGGCCCCACCATAAGG R: CATTACAGTCTCTCCTCACCCATACA	120
CD40	F: TGCACACCCTGTGAGAATGGT R: CGTTGCGTTTCCCTGTCTCTT	120
CD40L	F: TGAAGTGGATGACGACGAGCTA R: TGGTGCAGAAGVTGAVTTGTG	120
TACI	F: GGCTCCTCATCCCAGTTCCT R: TTGTGCGTGAAGAAAGCTCTGT	120
IL-1β	F: GCTCTACATGTCGTGTGTGATGAG R: TGTCGATGTCCCGCATGA	120
IL-4	F: AACATGCGTCAGCTCCTGAAT R: TCTGCTAGGAACTTCTCCATTGAA	120
IL-6	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG	120
IL-15	F: TAGGAAGCATGATGTACGGAACAT R: TTTTTGCTGTTGTGGAATTCAACT	120
IL-17	F: GCTGCAGCAAGAAAAGGAAGA R: GCCGTATCACCTTCCCATGT	120
IFN-γ	F: AACCTTCCTGATGGCGTGAA R: GCTTTGCGCTGGATTCTCAA	120

IV. Results

1) B. subtilis spore enhances H9N2 virus-specific IgG responses

B. subtilis spore is known as a particulate adjuvant for enhancing immune responses when co-administered with antigen [52, 53]. The non-pathogenic status of *B. subtilis* spore could provide efficient adjuvant activity for induction of systemic antibody responses to tetanus toxoid fragment C as a model antigen, when formulated as an admixture [45]. *B. subtilis* spores have the innate ability to enhance priming of antibody responses to co-inoculated antigen, so I sought to determine if *B. subtilis* spore could provide efficient adjuvant effect for induction of H9N2-specific antibody responses in chicken.

In the present study, to determine if *B. subtilis* spore could boosts immune antibody responses to co-administrated with inactivated H9N2 virus, I examined the H9N2 virus-specific responses in chicken intramuscularly immunized with inactivated H9N2 with or without *B. subtilis* spore and represented the immunization schedule (Figure 1A). All the chickens immunized differently were observed for 28 d from hatching, but none of those chickens showed any abnormality, signs of ill health or died within the period. Also, the body weight changes showed no significant differences among groups (Figure 1B). Blood samples were taken at 7 and 14 days post second immunization, and the antigen-specific IgG antibody levels in blood serum were measured. Serum H9N2 virus-specific IgG expression in chicken administered with inactivated H9N2 and *B. subtilis* spore was significantly higher than other groups at 3 weeks (Figure 2A) and 4 weeks (Figure 2B). To note that levels of the IgG in groups treated with inactivated

H9N2 + *B. subtilis* spore was significantly higher than those of H9N2 treated. Subsequently, to investigate the adjuvanticity of *B. subtilis* spore, sera from different groups were analyzed by HI assay to measure the functional antibody responses against H9N2 virus. The results showed that the HI titer of inactivated H9N2 and *B. subtilis* spore co-administered group was significantly higher than the group with inactivated H9N2 only, while *B. subtilis* spore or control group showed no changes (Figure 3A and 3B). Collectively, these results suggested that *B. subtilis* spore acts as an adjuvant in chicken treated with inactivated avian influenza virus, H9N2.

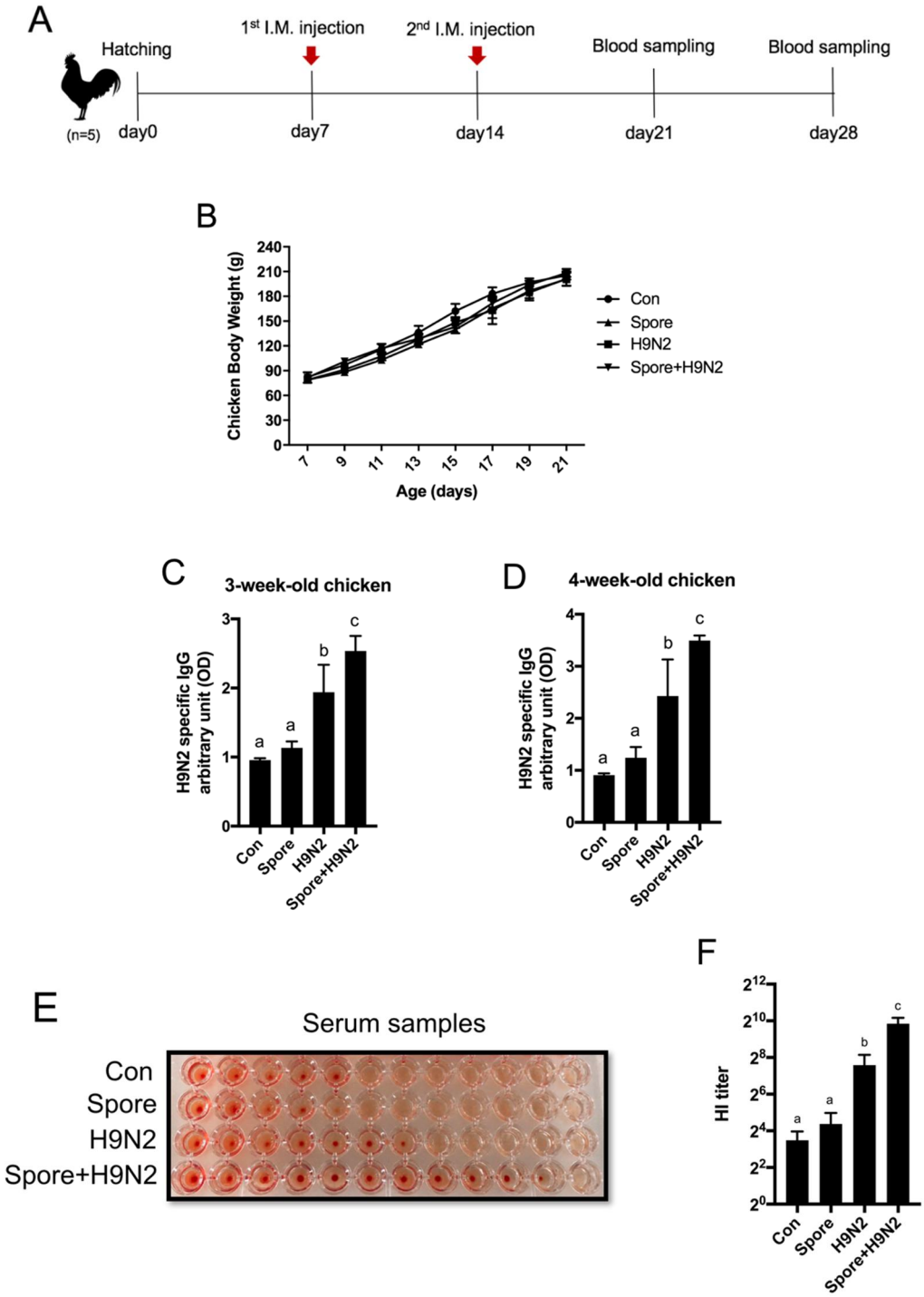
A
Hatching
1st I.M. injection
2nd I.M. injection
Blood sampling
Blood sampling
(n=5)
day0
day7
day14
day21
day28
B
Chicken Body Weight (g)
Age (days)
Con
Spore
H9N2
Spore+H9N2
C
3-week-old chicken
H9N2 specific IgG arbitrary unit (OD)
D
4-week-old chicken
H9N2 specific IgG arbitrary unit (OD)
E
Serum samples
Con
Spore
H9N2
Spore+H9N2
F
HI titer

Figure 1. Antigen-specific IgG response and HI titer in chickens with intramuscular administration of inactivated H9N2 and/or *B. subtilis* spore.

Randomly selected chickens were allotted into four different groups, and body weights were monitored in the immunized chickens after i.m. administration with inactivated H9N2 and/or *B. subtilis* spore. (A) One-week-old White Leghorn chickens were administered with PBS as a negative control, $2x10^9$ CFU of *B. subtilis* spore, or inactivated H9N2 together with *B. subtilis* spore. (B) Body weight changes were monitored every other day during the whole experimental periods (till 21-day-old). Blood was collected from wing vein after one and two weeks after the second immunization. The antigen-specific IgG antibody response in serum was measured and expressed as arbitrary unit on (C) 3-wk-old and (D) 4-wk-old chicken. Blood serum was also collected to examine HI titers. (E) In the highest dilution of the serum as described in the Materials and Methods, (F) mean HI titers of sera against H9N2 virus was examined and expressed. To determine the significance, one-way ANOVA followed by a Friedman test corrected by Dunn's multiple comparison test was performed from three independent experiments from twelve different chickens. Data are expressed as the mean values ± SD. Different letters on each group denote a significant difference at $P \leq 0.05$.

2) Changes of B cells and monocytes/macrophages subsets in chicken administered with inactivated H9N2 and/or B. subtilis spore

Next, I investigated the role and effect of *B. subtilis* spore, as an adjuvant, on the kinetics of immune cell responses in the spleen from chicken administered with inactivated H9N2 and/or *B. subtilis* spore.

Total cell number of splenocytes showed no significant changes among the treatment groups (Figure 2A). The percentage (Figure 2B) and absolute number (Figure 2C) of KUL01^{+} monocytes/macrophages population in chickens treated with inactivated H9N2 and *B. subtilis* spore were significantly ($P \leq 0.05$) increased compared to those of inactivated H9N2-administered group. In Bu-1^{+} B cell population, percentage (Figure 2D) and absolute number (Figure 2E) of chickens administered with *B. subtilis* spore, or inactivated H9N2 together with *B. subtilis* spore were significantly ($P \leq 0.05$) high when compared to control or inactivated H9N2-administered group. These results suggested that the population change of B cells and monocytes/macrophages was dependent on *B. subtilis* spore, rather than antigen. These results suggested that the induction of virus-specific immune cell population through i.m. administration of inactivated H9N2 and *B. subtilis* spore seemingly contribute to the protection against H9N2 virus.

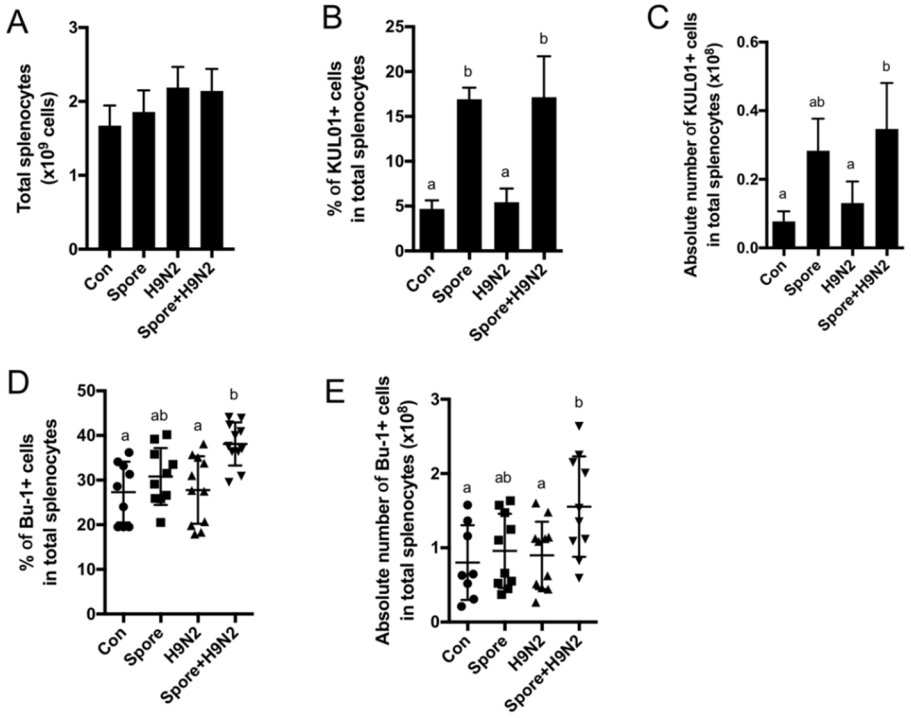

Figure 2. Splenic B cells and innate immune cells were increased in chicken administered with inactivated H9N2 and *B. subtilis* spore.

Chickens, 7 days old, were administered twice with inactivated H9N2 and/or *B. subtilis* spore in one-week interval. (A) Spleens were taken and examined for total cell number. Splenocytes were stained with proper combination of anti-chicken CD3, Bu-1, KUL01, and Annexin V/7AAD antibodies and flow cytometry analysis was performed. To note that Annexin $V^{-}7AAD^{-}$ cells were pre-gated in order to exclude dying and dead cells (data not shown). The (B) percentage and (C) absolute number of Bu-1^{+} B cells population, and (D) percentage and (E) absolute number of $KUL01^{+}$ monocytes/macrophages population was analyzed using flow cytometry. Data are expressed as the mean values ± SD.

3) Co-administration of inactivated H9N2 and B. subtilis spore increase the innate cell and B cell population compared to oil vaccine

Since inactivated H9N2 and/or *B. subtilis* spore showed the increase of KUL01$^+$ monocytes/macrophages and B cells (Figure 2), I decided to compare the results with commercially available H9N2 oil vaccine. To test whether *B. subtilis* spore could efficiently induces specific cell population against a co-administered antigen compared to oil adjuvant, chickens were immunized i.m. with H9N2 oil vaccine or inactivated H9N2 and *B. subtilis* spores together.

Both H9N2 oil vaccine and inactivated H9N2 + *B. subtilis* spore groups showed a significantly increased percentage of KUL01$^+$ monocytes/macrophages populations compared to the control (Figure 3A). Intriguingly, the absolute number of KUL01$^+$ monocytes/macrophages population in the inactivated H9N2 and *B. subtilis* spore co-administered group showed a significant increase when compared to H9N2 oil vaccine group (Figure 3B). Both percentage (Figure 3C) and absolute number (Figure 3D) of Bu-1$^+$ B cell population in chicken administered with inactivated H9N2 and *B. subtilis* spore are significantly higher than other groups. These results suggested that *B. subtilis* spore is a more effective adjuvant to augment monocytes/macrophages and B cell populations than oil emulsion in chicken.

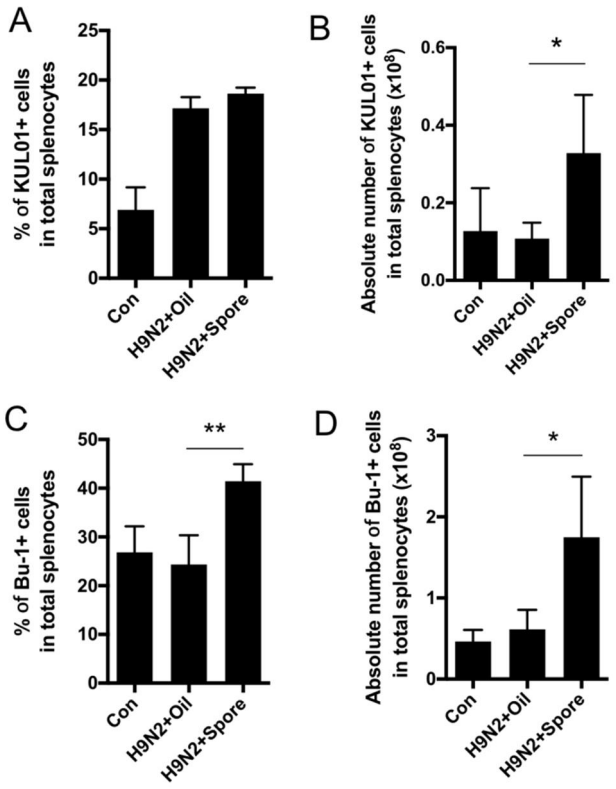

Figure 3. *B. subtilis* spore, as an adjuvant, effectively induced splenic monocytes/macrophages and B cell population.

B. subtilis spores induced innate cell and B cell population efficiently in the chicken spleen. Chickens, 7 days old, were administered twice in 7-day interval with PBS, H9N2 oil vaccine or inactivated H9N2 with *B. subtilis* spore. At one week post second immunization, splenocytes were stained with anti-Bu-1 and anti-KUL01 antibodies. Then, the (A) percentage and (B) absolute number of $KUL01^+$ monocytes/macrophages, and the (C) percentage and (D) absolute number of $Bu\text{-}1^+$ B cells were examined by using flow cytometry. Data are expressed as the mean values ± SD.

4) T cell population in splenocytes was increased in chickens co-administered with inactivated H9N2 and B. subtilis spore

It has become increasingly clear that for various vaccines containing adjuvant to be maximally effective, T cell responses need to be enhanced [45]. To examine the change of cell population, I investigated total percentage and absolute number of $CD4^+$ and $CD8^+$ T cells responses of splenocytes, after i.m. administration with H9N2 oil vaccine, or inactivated H9N2 and/or *B. subtilis* spore. There were no significant differences in H9N2 virus-specific $CD4^+$ T cells among the groups in percentage (Figure 4A) and absolute number (Figure 4B). On the other hand, the percentage (Figure 4C) and absolute number (Figure 4D) of H9N2 virus-specific $CD8^+$ T cells were significantly increased in chickens administered with inactivated H9N2 and *B. subtilis* spore compared to the other groups. Administration of *B. subtilis* spore as an adjuvant effectively induced monocytes/macrophages and B cells populations in spleen compared to H9N2 oil vaccine, and I also observed increased T cell population in splenocytes, which from the chicken administered with inactivated H9N2 and *B. subtilis* spore as an adjuvant. These findings clearly demonstrated that systemic co-administration of inactivated H9N2 and *B. subtilis* spore can induce the $CD8^+$ T cell proliferation.

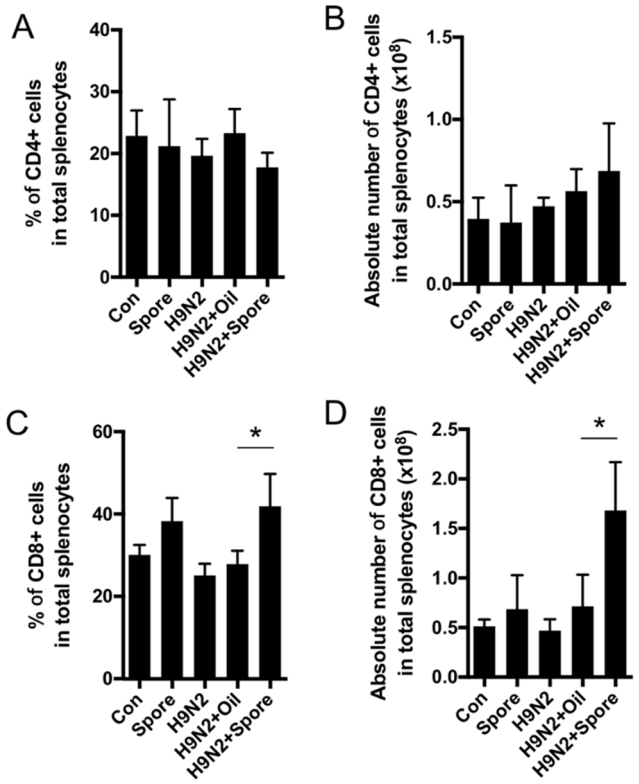

Figure 4. Changes of T cell population in chickens administered with inactivated H9N2 and *B. subtilis* spore.

B. subtilis spore, as an adjuvant, induces $CD8^+$ T cell population efficiently in the chicken spleen. Chickens were administered twice in 7-day interval with PBS, H9N2 oil vaccine or inactivated H9N2 with *B. subtilis* spore. At one week after the last treatment, spleens were taken and splenocytes were stained with anti-CD3, CD4 and -CD8 antibodies. Then, the (A) percentage and (B) absolute number of $CD4^+$ T cell population, and the (C) percentage and (D) absolute number of $CD8^+$ T cell population were examined by using flow cytometry. Data are expressed as the mean values ± SD.

5) Induction of pro-inflammatory cytokines, IL-1β and IL-6, by B. subtilis spore in splenocytes and monocytes/macrophages

B. subtilis spore induces a strong pro-inflammatory response, characterized by significantly augmented IFN-γ-producing T cells, which is a Th1 immune response [54, 55]. In the previous study, *B. subtilis* spore induced activation of APCs coincident with recruitment of pro-inflammatory cells, which is in accordance with the immunomodulatory effects of *B. subtilis* spore for vaccines [18]. APCs can respond to foreign organisms, initiating an intracellular signaling pathway that leads to the production of pro-inflammatory cytokines and co-stimulatory molecules for linking innate and adaptive immunities [14].

To determine the change of pro-inflammatory cytokine expression, splenocytes were stimulated with inactivated H9N2 and/or *B. subtilis* spore. The mRNA expression of pro-inflammatory cytokines, IL-1β (Figure 5A) and IL-6 (Figure 5B) were examined. Significant increase of IL-1β (Figure 5A) and IL-6 (Figure 5B) was found in splenocytes stimulated with inactivated H9N2 and *B. subtilis* spore together or *B. subtilis* spore alone. Subsequently, monocytes/macrophages, purified from splenocytes were stimulated with inactivated H9N2 and/or *B. subtilis* spore. IL-1β (Figure 5C) and IL-6 (Figure 5D) were significantly increased in the group stimulated with inactivated H9N2 and *B. subtilis* spore or *B. subtilis* spore alone. These results suggested that the adjuvant effect of *B. subtilis* spore enhanced the expression of pro-inflammatory cytokines in total splenocytes and monocytes/macrophages stimulated with inactivated H9N2.

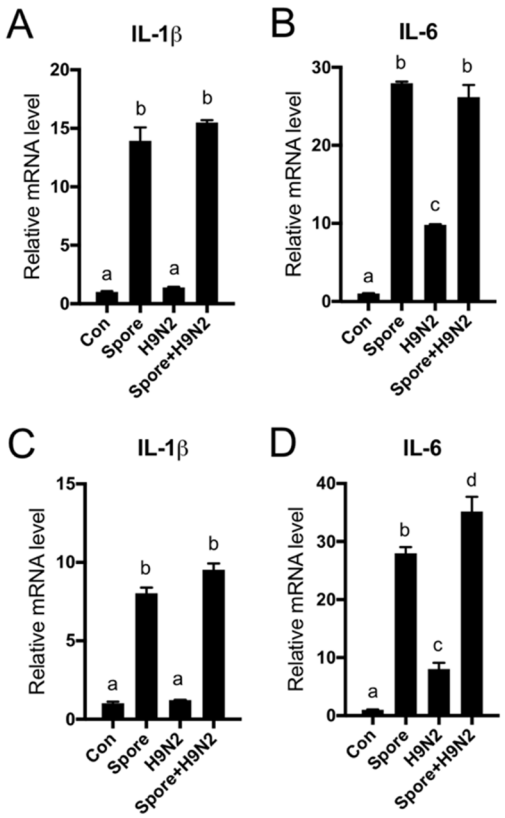

Figure 5. The mRNA expression of pro-inflammatory cytokines, IL-1β and IL-6 in chicken splenocytes and monocytes/macrophages stimulated with inactivated H9N2 and *B. subtilis* spore.

B. subtilis spore induced pro-inflammatory cytokine in total splenocytes and monocytes/macrophages. Total splenocytes and monocytes/macrophages were isolated from 3-week-old chicken, and stimulated with inactivated H9N2 and/or *B. subtilis* spore for 3h. The mRNA expression levels of (A, C) IL-1β and (B, D) IL-6 in (A, B) splenocytes, and (C, D) in monocytes/macrophages subsets were examined by using qRT-PCR. Data are expressed as the mean values ± SD. Different letters on each group denote a significant difference at $P \leq 0.05$.

6) Induction of BAFF and CD40L expression in splenocytes treated with B. subtilis spore as an adjuvant

It has been identified the avian homologue of B cell activating factor of the TNF family (BAFF) as a potentially major regulator of B cell homeostasis in the chicken [56]. BAFF is a member of the tumor necrosis factor ligand superfamily of cytokines and is a major regulator of B cell survival, differentiation, proliferation, activation and immunoglobulin class-switching [57-60]. BAFF interacts with three receptors: BAFF receptor or BR3 (BAFF-R), Transmembrane activator and calcium-modulating cytophilin ligand interactor (TACI), and B cell maturation antigen (BCMA), the latter expressed on peripheral B cells [60-62]. In the chicken genome, functional homologs for BAFF-R and TACI have been identified and characterized, but the gene encoding the third BAFF-binding receptor, BCMA homolog turns out to be disrupted [63]. The critical role of BAFF and its synergic effect with IL-4 and BAFF-R on the production of IgG and IgA antibodies has been reported [60, 64-66].

To examine the expression of B cell proliferation-related genes, splenocytes were treated with inactivated H9N2 and/or *B. subtilis* spore and mRNA expression of BAFF, BAFF receptor (BAFF-R), and Transmembrane activator and calcium-modulating cytophilin ligand interactor (TACI), responsible for the proliferation and survival signal in B cells [56, 67], were assessed. CD40 and CD40 ligand are also known to induce B cell proliferation and antibody production [68]. The mRNA expression levels of BAFF (Figure 8A), BAFF-R (Figure 6B), TACI (Figure 6C), CD40 (Figure 6D) and CD40L (Figure 6E) were higher in the group stimulated with inactivated H9N2 and *B. subtilis* spore than other groups. It was noting that spore treatment alone appeared to induce CD40 expression. Taken together, these results indicated that the expression level of

BAFF, BAFF-R, TACI, CD40 and CD40L, which are regulator of B cell survival signal, demonstrated that co-stimulation of inactivated H9N2 and *B. subtilis* spore induced the capability for the proliferation and differentiation in B cells compared to inactivated H9N2 only. These data showed that the adjuvant effects of *B. subtilis* spore upregulated the secretion level of genes about B cell survival regulators.

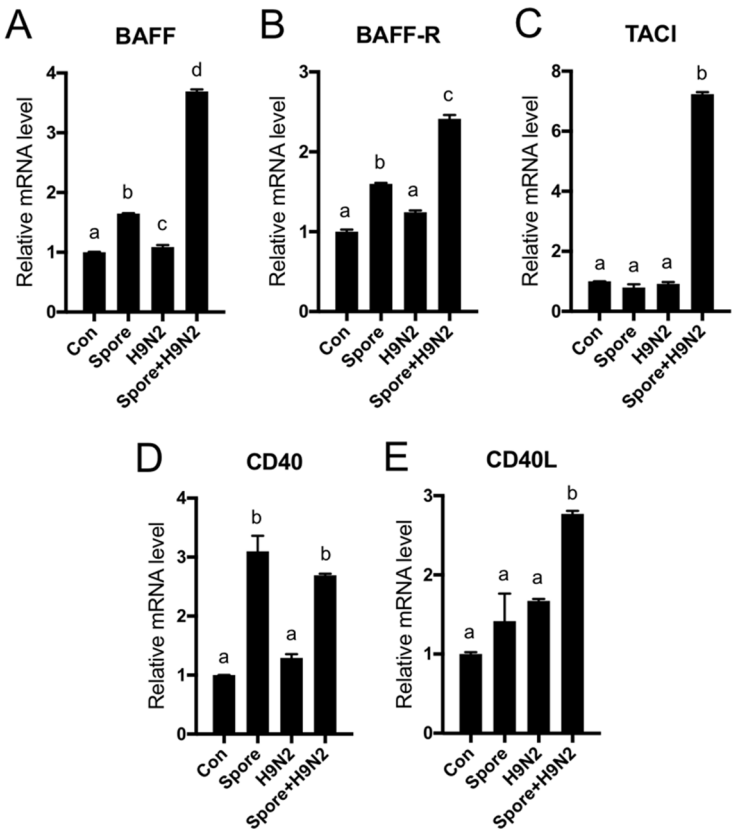

Figure 6. The mRNA expression of B cell proliferation/survival-related genes in splenocytes treated with inactivated H9N2 and/or *B. subtilis* spore.

Co-stimulation of inactivated H9N2 and *B. subtilis* spore secreted higher expression level of B cell proliferation-related genes in total splenocytes. Spleen was isolated from 3-week-old chicken, and single cells from splenocytes were stimulated with inactivated H9N2 and/or *B. subtilis* spore for 3h. The mRNA expression levels of (A) BAFF, (B) BAFF-R, (C) TACI, (D) CD40, (E) CD40L in splenocytes were examined by using qRT-PCR. Data are expressed as the mean values ± SD. Different letters on each group denote a significant difference at $P \leq 0.05$.

7) Induction of IL-4 and IL-15 expression in splenocytes treated with B. subtilis spore

A number of cytokines, the most potent being IL-4 and IL-15, have been reported to augment B cell proliferation and differentiation [69, 70]. IL-15 is necessary to stimulate the secretion of antibodies by APRIL-activated B cells, and IgG and IgA production is further enhanced by IL-15, which is an inducer of antibody secretion produced by APCs and T cells [65]. IL-15 plays a key role in the differentiation of APRIL-stimulated B cells to plasmacytoid cells, co-stimulates antibody production in CD40-activated B cells [71, 72].

To investigate whether IL-4 and IL-15 are involved in chicken B cell activity, splenocytes were stimulated with inactivated H9N2 and/or *B. subtilis* spore, and then, mRNA expression of IL-4 and IL-15 was assessed. In the group stimulated with inactivated H9N2 and *B. subtilis* spore, or *B. subtilis* spore, the expression of IL-4 (Figure 7A) and IL-15 (Figure 7B) was higher than those of control or inactivated H9N2, suggesting conceivable correlation between *B. subtilis* spore and B cell proliferation-associated mediators, IL-4 and IL-15. Next, splenocytes were stimulated with recombinant chicken IL-4 or IL-15, and then analyzed B cell population. The percentage of B cells in the group stimulated with IL-4 (Figure 7C) or IL-15 (Figure 7D) was higher than control group. It was noting that combination of recombinant chicken IL-4 and IL-15 did not enhance the B cell proportions synergistically (data not shown). Taken together, IL-4 and IL-15 regulate differentiation and proliferation of chicken B cells and could be an important factor in B cell responses in chicken.

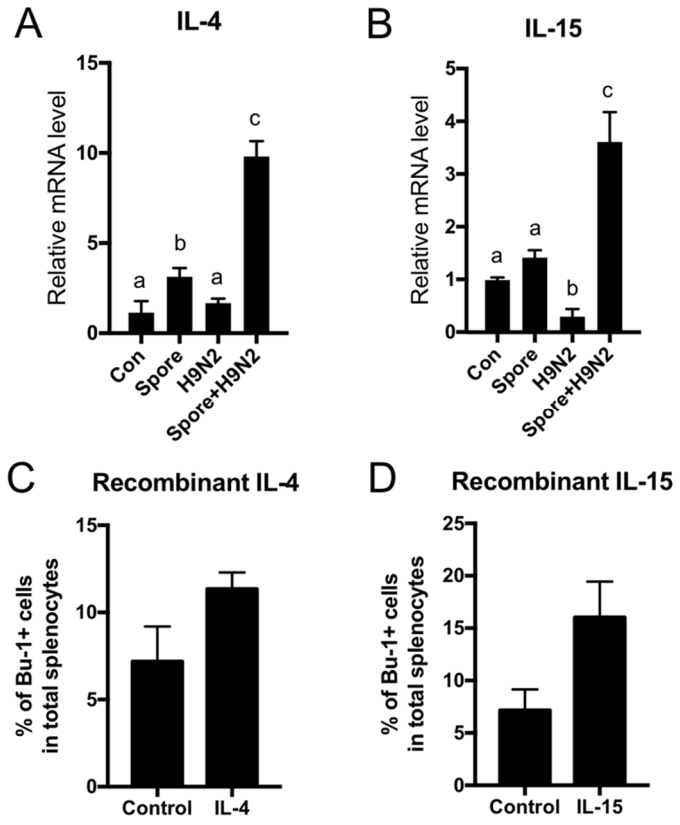

Figure 7. Effect of IL-4 and IL-15 in chicken B cells.

Total splenocytes were stimulated with inactivated H9N2 and/or *B. subtilis* spore for 3h, and then the mRNA expression of (A) IL-4 and (B) IL-15 was examined by using qRT-PCR. Splenocytes were stimulated with recombinant chicken (C) IL-4 or (D) IL-15 for 48 h. Then, the cells, stained with anti-Bu-1 antibodies, were analyzed for the proportion of B cells by using flow cytometry. Data are expressed as the mean values ± SD. Different letters on each group denote a significant difference at $P \leq 0.05$.

8) B. subtilis spore promotes CD4$^+$ and CD8$^+$ T cell proliferation

Next, I conducted TCR stimulation *in vitro* and attempted to identify what signals are induced for T cell proliferation in APCs stimulated with inactivated H9N2 and *B. subtilis* spore. It is known that the interactions are taking place at the immunological synapse, at which T cells are licensed by antigen-bearing APCs [73]. To compare the effect of cytokines by using inactivated H9N2 and/or *B. subtilis* spore, T cell receptor signals were normalized by using anti-CD3 and -CD28 antibodies (Figure 8A). For compensating the broad TCR signaling stage, the supernatant of splenocytes or monocytes/macrophages stimulated with inactivated H9N2 and/or *B. subtilis* spore was added to T cells [74]. Flow cytometry analysis showed a strong CD4$^+$ T cell proliferation in the group stimulated with inactivated H9N2 and *B. subtilis* spore together or *B. subtilis* spore only (Figure 8B). This CD4$^+$ T cell proliferative population also showed high division index score (Figure 8C). In splenocytes, CD4$^+$ T cell proliferation was more or less equally prominent in all treatment groups when compared to control. When monocytes/macrophages were used, CD4$^+$ T cell proliferation was observed in groups stimulated with inactivated H9N2 and *B. subtilis* spore together, and *B. subtilis* spore only. These results demonstrated that the impact of *B. subtilis* spore is leaning towards CD4$^+$ T cell activation.

Subsequently, flow cytometry analysis on CD8$^+$ T cells was performed in the same manner, and then, the results showed a strong CD8$^+$ T cell proliferation (Figure 8D) as well as division index scores (Figure 8E) in the group stimulated with inactivated H9N2 and *B. subtilis* spore together or *B. subtilis* spore only. These results demonstrated that *B. subtilis* spore have a positive effect on CD8$^+$ T cell activation.

Expression of IFN- γ in all treatment groups was higher than that of control group. It was noting that level of IFN- γ in the cells stimulated with inactivated H9N2 and *B. subtilis* spore was significantly higher than other groups (Figure 8F). In contrast, the expression of IL-4 was relatively low in all groups compared to IFN-γ (Figure 8G). Collectively, *in vitro* TCR stimulation could confirm that the *B. subtilis* spore, as an adjuvant, is more likely to induce Th1-polarized response.

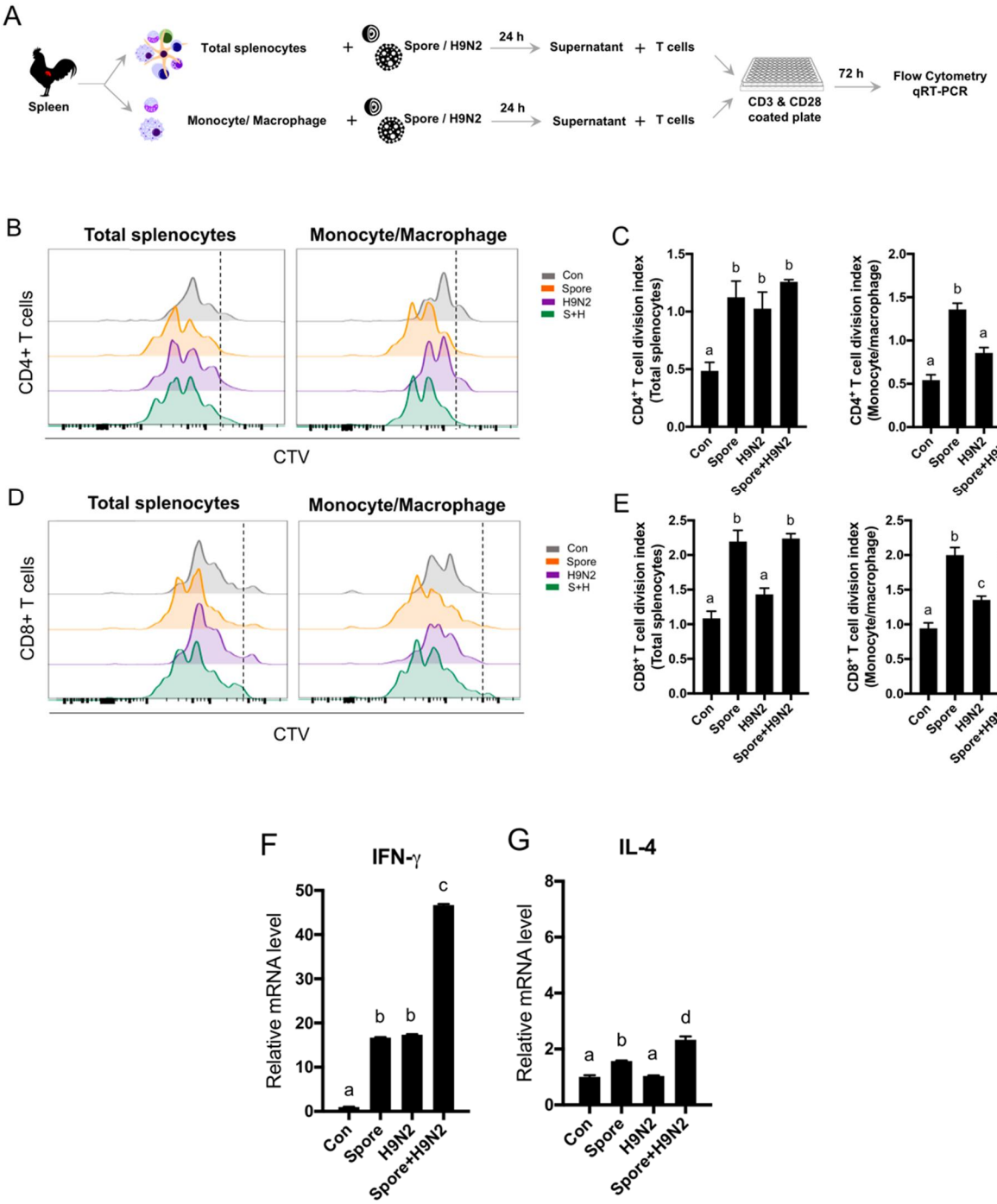
A
Spleen
Total splenocytes
Spore / H9N2
24 h
Supernatant + T cells
Monocyte/ Macrophage
Spore / H9N2
24 h
Supernatant + T cells
CD3 & CD28 coated plate
72 h
Flow Cytometry qRT-PCR
B
Total splenocytes
Monocyte/Macrophage
CD4+ T cells
CTV
Con
Spore
H9N2
S+H
C
CD4+ T cell division index (Total splenocytes)
CD4+ T cell division index (Monocyte/macrophage)
Con
Spore
H9N2
Spore+H9N2
D
Total splenocytes
Monocyte/Macrophage
CD8+ T cells
CTV
Con
Spore
H9N2
S+H
E
CD8+ T cell division index (Total splenocytes)
CD8+ T cell division index (Monocyte/macrophage)
Con
Spore
H9N2
Spore+H9N2
F
IFN-γ
Relative mRNA level
Con
Spore
H9N2
Spore+H9N2
G
IL-4
Relative mRNA level
Con
Spore
H9N2
Spore+H9N2

Figure 8. $CD4^+$ and $CD8^+$ T cell proliferation and cytokine expression in chicken splenocytes treated with H9N2 or *B. subtilis* spore together with TCR stimulation.

(A) The experimental scheme for T cell proliferation and cytokine expression in chicken splenocytes treated with inactivated H9N2 and/or *B. subtilis* spore coincident with TCR stimulation is indicated. CTV-labeled T cells were cultured into anti-CD3 and -CD28 antibody-coated plates with supernatants from splenocytes or monocytes/macrophages stimulated with inactivated H9N2 and/or *B. subtilis* spore for 72 h, and analyzed by flow cytometry or qRT-PCR. (B) The proliferative population of (B and C) $CD4^+$ or (D and E) $CD8^+$ T cells was measured by (B and D) CTV histograms and (C and E) division index scores. Furthermore, mRNA expression of (F) IFN-γ and (G) IL-4 was examined. Data are expressed as the mean values ± SD. Different letters on each group denote a significant difference at $P \leq 0.05$.

9) B. subtilis spore, as an adjuvant, promotes Th1 and Th17 cytokine expression

The goal of vaccination is to induce a specific immune response coincident with a long-term protection against target infectious antigen. Immuno-stimulating activity may result in a general upregulation of the entire immune system, while most common results in upregulation of certain cytokines [75]. T cell response need to be enhanced for the long-term protection, and I investigated T cell proliferative responses of total splenocytes re-stimulated with inactivated H9N2 virus after i.m. administration with H9N2 oil vaccine or inactivated H9N2 virus together with/without *B. subtilis* spore (Figure 9A). To investigate the H9N2 virus-specific T cell activities, the T cells were dissociated and analyzed for the expression level of IFN-γ IL-4, and IL-17. Spleen cells from chicken administered with inactivated H9N2 alone failed to induce proliferative response dominated by IFN-γ IL-4, or IL-17. Both IFN-γ (Figure 9B) and IL-17 (Figure 9D), however, were significantly increased in chickens administered with inactivated H9N2 and *B. subtilis* spore together, as an adjuvant. Otherwise, the level of IL-4 was relatively low in all groups (Figure 9C).

On the other hand, the expression of IFN-γ and IL-17 in the group administered with H9N2 oil vaccine was significantly low when compared to those of group administered with inactivated H9N2 and *B. subtilis* spore together. These results demonstrated that *B. subtilis* spore, as an adjuvant, promote Th1- and Th17-driven immune responses rather than Th2 response.

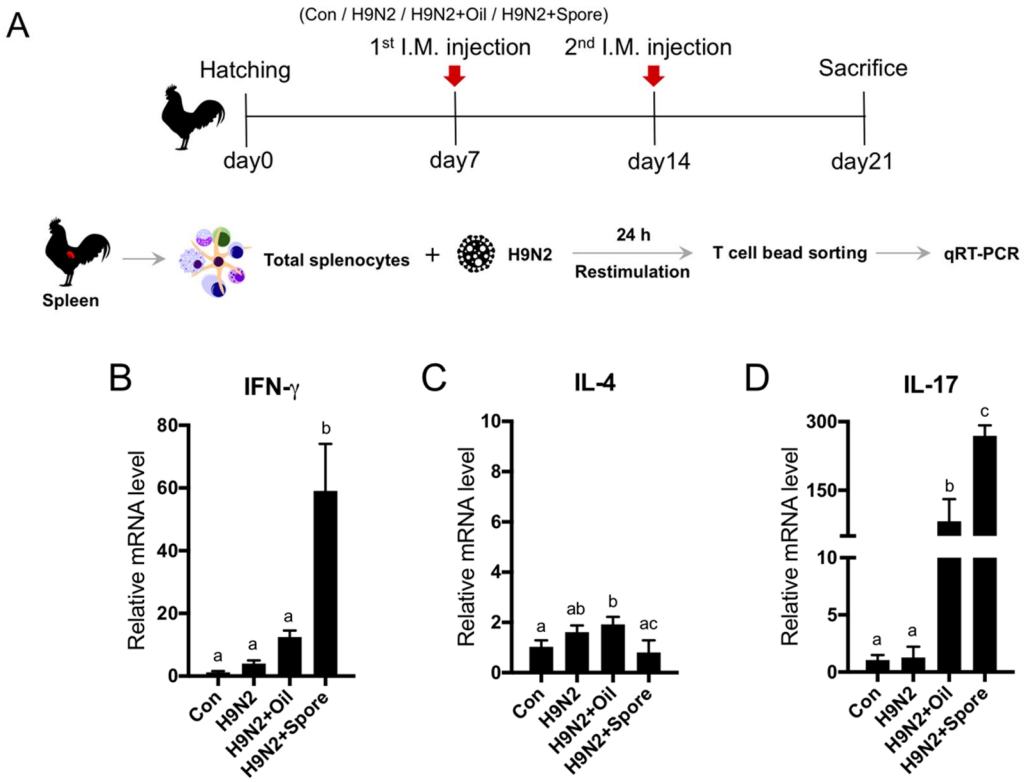

Figure 9. mRNA expression of helper T cell cytokines in chickens treated with H9N2 vaccine.

H9N2 virus-specific T cells express (A) The experimental scheme is shown. Briefly, chickens were administered with H9N2 oil vaccine (commercially available), inactivated H9N2 or inactivated H9N2 and *B. subtilis* spore, twice at one-week-interval. Seven days after the last administration, splenocytes were re-stimulated with inactivated H9N2 for 24 h, and then, T cells were isolated by magnetic bead sorting, and mRNA expression of (B) IFN-γ (C) IL-4, and (D) IL-17 was examined by qRT-PCR. Data are expressed as the mean values ± SD. Different letters on each group denote a significant difference at $P \leq 0.05$.

V. Discussion

In the present study, I attempted to demonstrate that *B. subtilis* spore acts as a potential particulate vaccine adjuvant, which is able to synergistically assign protection against the avian influenza virus H9N2 in chicken. Adjuvants are considered to be an essential component of most inactivated virus vaccines in field [76]. The elucidation of action mechanisms of adjuvant has contributed greatly to make a selection for the appropriate adjuvant as an integral component in a rational approach to modern vaccine formulation [49, 77]. Although adjuvants have diverse action mechanisms [48], the exact roles are not well clarified yet, especially in chickens. Adjuvants can act directly or indirectly with antigen presenting cells (APCs) such as macrophages or dendritic cells [49, 78].

Bacillus subtilis is a non-pathogenic and Generally Regarded as Safe (GRAS) microorganism. Among the various species of vaccine adjuvants, spores from *B. subtilis* is known to able to enhance antigen-specific antibody response and augment polyvalent antigen-specific $CD4^+$ and $CD8^+$ T cell dependent effector responses [45, 79]. Public awareness and need for the development of more safe and effective avian influenza vaccines containing new types of adjuvants is steadily increasing [80]. Despite the problems encountered in the development of poultry vaccines, especially against avian influenza virus [75], not much progress has been made in relation to adjuvant. The current studies demonstrated that *B. subtilis* spores may be a viable vaccine-adjuvant for influenza vaccine with regard to safety and efficacy that is in need for further empirical investigation [81]. In the present study, I explored the ability of *B. subtilis* spore work

as an adjuvant to influence the diversity of immune responses induced by inactivated avian influenza virus H9N2 in chicken. Specifically, I attempted to elucidate the mechanism for the intrinsic induction of humoral and cell-mediated immune responses in chickens co-administered inactivated H9N2 with *B. subtilis* spore, as an adjuvant.

Notable findings in the present study are as following; Firstly, inactivated H9N2 and *B. subtilis* spore increased H9N2 virus-specific IgG in chicken. The current approach of systemic administration of *B. subtilis* spore as an adjuvant is considered in a tenable position on poultry field. Function of adjuvants is enhancing or boosting the potency, longevity, and quality of antigen specific immune responses, which could induce a minimal or no toxicity coincident with long-lasting immune responses on their own [82, 83]. Adjuvants and their delivery systems have been approved for clinical trial testing or components of licensed vaccines, oil adjuvant is one of the formulations of vaccine adjuvants that has been shown to have a robust safety and efficacy profiles with inducing antibodies against influenza viruses [80, 84]. However, oil adjuvant is insufficient for inducing T cell responses, the novel adjuvant and formulation of vaccine adjuvants are essentially required [85]. Additional advantage of *B. subtilis* spore as an adjuvant to H9N2 vaccines could be reduction of the amount of vaccine or number of immunizations required to optimized immune response until generating full protection in recipients [86, 87].

Secondly, the percentage and absolute number of B cells, monocytes/macrophages, and $CD8^+$ T cells in total splenocytes were significantly increased in the group administered with inactivated H9N2 and *B. subtilis* spore.

Thirdly, $CD4^+$ and $CD8^+$ T cell proliferative population and division index score in total splenocytes and monocytes/macrophages subsets were significantly higher in the

group administered with inactivated H9N2 and *B. subtilis* spores together, compared to inactivated H9N2 only.

Unlike H9N2 oil vaccine, *B. subtilis* spore could induce T cell immune responses in the present study. This is in agree with the previous study that its ability to increase the level of functional hemagglutination activity and $CD8^+$ T cell responses [85]. Furthermore, total splenocytes co-stimulated with inactivated H9N2 and *B. subtilis* spores indicated higher expression of IFN-γ than inactivated H9N2 only, and confirmed that the *B. subtilis* spore is more likely to instruct Th1 immune response rather than Th2 to H9N2 vaccine. IFN-γ and IL-17 in H9N2 virus-specific T cells were expressed significantly higher in the group stimulated with inactivated H9N2 and *B. subtilis* spores together than other groups. A further research would be required to validate and identify the antigen-specific T cells licensing APCs to cross-prime exogenous antigen *in vivo* [88, 89].

While virus neutralizing antibody responses are outermost significance in the preventing virus infection, the antigen specific T cells through their cytolytic and cytokine secreting ability prevent virus spread and aid virus clearance, thereby decreasing disease severity and facilitating recovery [90, 91]. In order to improve the induction of immune responses to poorly immunogenic avian influenza virus strains, *B. subtilis* spores would be potential adjuvants able to confer protection to avian influenza virus. The adjuvanticity of *B. subtilis* spore drives an effective T cell response, which can be increased in intensity by repeated antigen administration when *B. subtilis* spores are partially associated with B cell responses to inactivated H9N2. T cell-dependent antibody responses require the activation of B cells by T cell responses that respond to the same antigen [92]. Therefore, I undertook the present study to evaluate the

immunomodulatory effect of the adjuvant *B. subtilis* spores on the systemic immune response against H9N2. *B. subtilis* spores showed substantial effects on the antigen-specific antibody response as well as cell-mediated immune response. While enhancing antigen-specific T cell responses is an essential requirement of a vaccine adjuvant, the ability of inducing Th1 and Th17 response is also important for the protection [84, 93]. In this study, the secretion of IFN-γ and IL-17 in the H9N2 virus-specific T cells from chickens administered with inactivated H9N2 and *B. subtilis* spores together enhanced more than inactivated H9N2 or H9N2 oil vaccine.

The present study showed that *B. subtilis* spores could induce the expression of pro-inflammatory cytokines, IL-1β and IL-6, in the total splenocytes or monocytes/macrophages stimulated with inactivated H9N2 and *B. subtilis* spores or *B. subtilis* spores alone. It is known that *B. subtilis* spores can induce APC activation and production of pro-inflammatory cytokines, which play a critical role in controlling and eliminating invading pathogens [14, 94]. The synergy effect observed in co-stimulation of inactivated H9N2 and *B. subtilis* spores, compared to *B. subtilis* spores or inactivated H9N2 only, on pro-inflammatory cytokine expression in chicken splenocytes. The innate immune response of producing inflammatory cytokines and other pro-inflammatory mediators are critical for controlling pathogenic infections, and it has been demonstrated that chickens with increased innate immune responses which have a greater resistance to infectious pathogen [94, 95]. The microbial recognition by the innate immune responses with activation of intracellular signaling pathways that initiate cellular immune processes, the production of pro-inflammatory cytokines is required for antigen presentation to the adaptive immune responses [95, 96]. It has been suggested

that high expression of pro-inflammatory cytokines upon *B. subtilis* spore stimulation would have been associated with better resistance to H9N2 infection in chicken.

Taken together, the current study demonstrated that *B. subtilis* spore could be a viable and novel vaccine adjuvant for avian influenza virus in chicken, with regard to effectively induced humoral and cellular immune responses more potent than those induced with oil adjuvant.

VI. Literature Cited

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VII. Summary in Korean

Bacillus subtilis 는 막대 모양의 그람 양성 세균이자 포자 형성 세균으로, 사람과 동물에서 생균제 형태로 사용이 되는 비 병원성 세균이다. *B. subtilis* 로부터 형성된 내생포자는 미립자 면역증강제로도 알려져 있는 동시에 내열성 또한 가지고 있어, 영양분이 고갈된 혹독한 환경 조건에서도 생존이 가능하도록 휴면 상태를 유지할 수 있는 특징을 가지고 있다. 따라서, 본 연구에서는 비활성화 된 조류 인플루엔자 바이러스 H9N2 및 *B. subtilis* 내생포자를 근육 내 투여 시킨 닭으로부터 *B. subtilis* 내생포자의 면역증강 능력 및 그 영향을 밝히고자 하였다. 닭에서 *B. subtilis* 내생포자의 항원 보조제로써의 효과는 H9N2 바이러스에 특이적인 IgG 의 반응을 증진시킴으로써 입증되었다. 비활성화 된 조류 인플루엔자 바이러스 H9N2 와 *B. subtilis* 내생포자를 함께 처리하였을 때, 비장 내 B 세포와 monocyte/macrophage 의 군집이 증가하는 현상을 확인하였다. 또한, 비활성화 된 조류 인플루엔자 바이러스 H9N2 를 단독으로 처리한 그룹에 비하여 바이러스와 *B. subtilis* 내생포자를 함께 처리해준 그룹에서 염증성 사이토카인 (pro-inflammatory cytokines) 및 B 세포의 증식과 분화에 관련된 유전자의 발현이 증가된 것을 확인하였다. 항원에 특이적인 T 세포 반응을 관찰하였을 때, H9N2 특이적인 $CD4^+$ 및 $CD8^+$ T 세포 발현 정도가 H9N2 바이러스와 *B. subtilis* 내생포자를 면역증강제로써 함께 처리해준 그룹에서 가장 높게 증가한 현상을 보여주었으며, H9N2 바이러스 특이적인 T 세포로부터 Th1, Th17 사이토카인의 발현이 증가하는 현상을 관찰했다. 종합적으로, 닭에서 *B. subtilis* 내생포자는 항원에 특이적인 항체 반응 및 T 세포의 발현 정도를 토대로 불활화

조류독감바이러스인 H9N2 에 대하여 효과적으로 면역증강 능력을 보여주었으며, 이에 체액성 면역반응 및 세포성 면역 반응 유도 능력을 증명함으로서, 조류에서의 인플루엔자 바이러스에 대한 방어 전략 및 효과적인 면역증강제로써 *B. subtilis* 내생포자의 이용 가능성을 시사한다.