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Immune modulation of cholera toxin and *Bacillus subtilis* spore delivered intranasally to mouse

마우스 비강으로 전달된 콜레라 톡신과 바실러스 서브틸리스 포자의 면역 조절 작용

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Summary

In the respiratory system, chemokines, cytokines and antimicrobial peptides (AMPs) play important roles for regulation of immune response. AMPs can protect the foreign molecules and chemokines can recruit the leukocytes that can remove the pathogen. Cytokines can enhance the effect of AMPs and chemokines and activate various immune cells.

The mucosal adjuvant has been studied by many researchers. Cholera toxin (CT), lipopolysaccharide (LPS) and *Bacillus subtilis* spore were, also, examined for the development of mucosal adjuvant. However, the effects of these adjuvants have not been understood completely through the administration of intranasal route in the lung and bronchoalveolar lavage fluid (BALF).

In the present study, the effect of the mucosal adjuvants, CT, *B. subtilis* spore and LPS, was examined by the analysis on the expression of effector molecules and migration of leukocytes in the lung. The adjuvants, CT, *B. subtilis* spore or LPS, were administered through the intranasal route to mice maintained at SPF facility. Then lung, BALF and serum were taken from the mice at 6, 12, 24 and 72 hr and analyzed mRNA and protein levels of effector molecules. Furthermore, the migration of cells into BALF was examined through analysis of the surface marker using flow cytometer.

The results showed that mRNA of CCL2, CCL4 and CXCL10 were induced by spore or LPS in the lung. However, there are no differences on the mRNA expression of AMPs between the adjuvants. Spore and LPS promoted the expression and secretion of CCL2 and CCL4 proteins in the lung and BALF. In analysis of pro-inflammatory cytokines, spore and LPS dramatically induced the secretion of IL-1β, IL-6 and TNF-α in the lung and BALF. CT induced the increase of IL-6 in the serum. In analysis of surface marker, spore highly promoted the migration of Gr-1^{+}CD11b^{+} cells at 6 hr and F4/80^{+}CD11b^{+}Gr-1^{-} cells at 72 hr. LPS, also, induced the migration of Gr-1^{+}CD11b^{+} cells at 12 and 24 hr. In conclusion, *B. subtilis* spore can induce the chemokine and cytokine expression in the lung and BALF and enhance the migration of neutrophils into BALF.

Contents

List of Figures

List of Tables

List of Abbreviations

ALI: acute lung injury

AMPs: antimicrobial peptides

BALF: bronchoalveolar lavage fluid

B. subtilis: *Bacillus subtilis*

COPD: chronic obstructive pulmonary disease

CT: cholera toxin

GAGs: glycosaminoglycans

GALT: gut-associated lymphoid tissue

LPS: lipopolysaccharides

LTA: lipoteichoic acid

MPL: monophosphoryl lipid A

NALT: nasopharynx-associated lymphoid tissue

SAA: serum amyloid A

SLPI: secretory leukocyte peptidase inhibitor

TLR: toll-like receptor

TTFC: tetanus toxin fragment C

I. Introduction

Mucosal immunity in a lung is very important not only because the organ has a large surface area and but also is exposed continuously by external molecules entering through inhalation. The epithelial cells in the lung can recognize the foreign molecules by Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing proteins (NODs) and secrete the effector molecules such as cytokine, chemokine and antimicrobial peptides (AMPs) for a modulation of immune response [1]. Cytokines are the mediator of cell response and can regulate the immune activities against pathogens. In the lung, the cytokines can induce the immune response of leukocytes and promote the expression of chemokines for leukocyte recruitment into lung. AMPs are found on mucosal organ including lung and play an important role in innate immune response of respiratory system. The peptides can protect by directly killing pathogens, entering through inhalation. Especially, defensins have the antimicrobial activities against bacteria, viruses and fungi. AMPs have various functions such as recruitment of leukocyte, regulation of pulmonary surface tension, and inhibition of protease in the lung.

Chemokines are also found in respiratory tract. When foreign molecules are entering the respiratory tract of host, chemokines including CXCL8, are quickly induced and secreted by alveolar epithelial cells and local leukocytes. The chemokines can promote the migration of leukocytes and regulate the immune response. For instance, CCL2 binds to CCR2 and plays a pivotal role in immune response through the recruitment of monocytes and memory T cells [2, 3]. During inflammation, CCL2 is secreted by various cells including epithelial cells, endothelial cells, fibroblasts, and monocytes [4-7]. CCL4 belongs to a subfamily of CC chemokine and binds to CCR5. CCL4 promotes the recruitment of monocytes, immature dendritic cells, T cells, and NK cells[8]. Furthermore, pulmonary vascular smooth muscle cells treated with IL-1β, TNF, IL-4, IFN-γ or IL-10 could induce the release of CCL4 [8]. CXCL10 belongs to an ELR^- CXC chemokine family and can participate the recruitment process of CXCR3 expressing cells including activated T and B lymphocytes, natural killer (NK) cells, dendritic cells and macrophages. Also, CXCL10 is known to display antimicrobial activity [9].

Studies on mucosal vaccine have been conducted by many groups. Although it is feasible, oral vaccine has a big obstacle including a high acidic pH. Nasal vaccines have been studied to make up for such disadvantage. Many studies about lipopolysaccharide (LPS), and cholera toxin (CT) adjuvants are conducted, they are very harmful to apply to human. *Bacillus subtilis* spore can modulate the immune response [10, 11] and could be a potential mucosal adjuvant. To note that effector molecules induced by *B. subtilis* spore in the lung and bronchoalveolar lavage fluid (BALF) has not been completely understood.

In this study, the expression of effector molecules, chemokines, defensins, and AMPs was analyzed in the lung and BALF of mice administered with CT, *B. subtilis* spore or LPS via intranasal route. Furthermore, the infiltration of leukocytes was examined.

II. Review of Literature

1. Immune modulation in the lung

1.1. Recruitment of leukocytes in respiratory tract

Leukocyte migration from a blood stream to target site is one of the most critical processes in immune response, when host tissues are damaged by inflammation or injury. During inflammation, such as acute lung injury, it was revealed that neutrophils and macrophages are highly accumulated in airspaces of patients [12]. It is well known that leukocyte recruitment needs to a series of sequential stages, such as tethering, rolling, activation and firm adhesion, and diapedesis [13]. First, leukocytes in circulation contact with vascular endothelial cells, and initiate tethering and rolling by an interaction between selectins and selectin ligands. P- and E-selectins are expressed on surface of cytokines, such as tumor necrosis factor (TNF)-α, stimulated endothelial cells. However, the transient interaction between selectins and its ligands is not sufficient to stopping of leukocytes movement. Integrins are heterodimeric transmembrane glycoproteins existed on surface of leukocytes which are consisted of α and β subunits. The integrins, LFA-1, α4β1, and α4β7, can bind to the adhesion molecules on surface endothelial cells, ICAM-1, VCAM-1 and MadCAM-1, respectively. Chemokines are expressed by epithelial cells, endothelial cells, and leukocytes during the inflammatory responses. Secreted chemokines (exception of CXCL16 and CX3CL1) bind to glycosaminoglycans (GAGs) which are present on extracellular matrix of endothelial cells and have a highly level of negative charge [14]. Rolling of leukocytes by selectins- and integrins-interactions leads to chemokines in GAGs binding to chemokine receptors on leukocytes. G-protein signaling from chemokine receptors induces the affinity and avidity of integrins. These changes allow the leukocytes to firm adhesion and transmigrate across endothelial cells via paracellular or transcellular routes [15, 16].

1.2 Mucosal immunization via intranasal administration

Drugs, vaccines or adjuvants administered to mucosal immune organs have more benefits than injection drugs for the induction of mucosal responses. Mucosal drugs in general do not concern about spreading blood-borne infections by using contaminated needles. Furthermore, they are easy to use and one can do without a medical training [17].

Mucosal drugs can provoke the innate and adaptive immune responses at mucosal local sites as well as systemic body [18]. It has been reported that they generate and promote memory/effector B and T cell responses [19]. Potential delivery routes for efficient mucosal drugs are oral, nasal, rectal, sublingual and genital tracts. The oral and intranasal routes have been tried in human, but others are in trial using experimental animal models [20]. Oral vaccines together with proper adjuvant are known to induce immune responses in the gut-associated lymphoid tissue (GALT). Most licensed mucosal vaccines have developed for oral delivery, because of the simplicity of the administration. However, oral delivery has obstacles, such as degradation by gut environment and induction of mucosal tolerance. Treatment by intranasal vaccines promotes the immune responses in the nasopharynx-associated lymphoid tissue (NALT) and protects against external infection in the gastric mucosa, respiratory and genital tracts [21-24]. Intranasal administration could be an efficient route for drug delivery than oral route, because there are no conditions like digestive enzymes and low pH. Furthermore, it triggers the systemic immune response rather easily because of high vascularity under the nasal mucosa.

2. Effector molecules in the lung

2.1. Chemokines

Chemokine is a small protein, molecular weight ranging from 6-14 kDa, which possesses a chemotactic property for trafficking of immune cells, and regulatory activity on inflammation and immunity. Chemokine, also, involves in other biological processes, such as angiogenesis/angiostasis [25, 26], hematopoiesis [27], wound healing [28], malignancy [29], and in case of various infection [30]. Chemokine can be classified into four groups according to position of cysteine(s) and amino acid(s) in the NH terminus of the protein; (X)C, CC, CXC, CX3C, and the major chemokines is CC and CXC groups. CC chemokines have the adjacent cysteines, and their genes encode at 17q12 in human [31]. The chemokines bind to chemokine receptor, CCR. CXC chemokines have single amino acid residue between first two cysteines and bind to CXCR. CXC chemokines are further divided into two subgroups that contain ELR (glu-leu-arg) amino acid sequence or not at their NH-terminus, ELR+ and ELR-. Their genes are located at 4q 13.3 in human [31]. CXCL1 and CXCL2 have an ability of neutrophil chemoattractant and are secreted by myeloid cells during lung inflammation

[32]. CXCL15, also known as neutrophil chemoattractant, is secreted by bronchial epithelial cells [33, 34]. CX3C chemokines have three amino acid residues between first two cysteines, and (X)C chemokines lack one of the cysteines. CX3C and (X)C chemokines bind to CX3CR and XCR, respectively.

Chemokine receptors are a class A seven-transmembrane G-protein coupled receptors (GPCRs). Chemokine receptors are, also, divided into four groups (XCR, CCR, CXCR, and CX3CR) like the category of chemokines. When activated through binding with cognitive chemokine, the receptor induces the change from GDP to GTP. Heterotrimeric G-protein then dissociate into GTP-Gα subunit, and Gβγ subunits. These subunits transmit the signaling to downstream of pathways; regulation of PLC-β, PI3K, GRKs, MAPK, ion channels, adenylyl cyclases, *etc*. [35, 36].

Chemokines are related to many lung inflammatory diseases, such as acute lung injury (ALI), asthma and chronic obstructive pulmonary disease (COPD). ALI is a severe diffuse lung disease and have a feature of acute inflammatory process in the lung. In the bronchoalveolar lavage fluid (BALF) of ALI patients, CXCL1, CXCL5, CXCL8, and CCL2 are present in significant concentrations, which promotes the recruit of inflammatory cells including neutrophils and macrophages [37]. ALI patients have the high concentration of neutrophils in the bronchoalveolar lavage fluid (BALF) and the degree of neutrophil existence is associated with mortality [12]. Accumulation of neutrophils in ALI is a very important factor, because neutrophils and their products can injure host tissues under inflammation circumstance [38]. COPD, divided into two lung diseases: chronic bronchitis and emphysema, is related with an abnormal inflammatory response of the lung chronically and is participated by various inflammatory cells, such as macrophages, neutrophils, and $CD8^+$ T cells [39, 40]. CCL2, CXCL1, CXCL8, CXCL9, CXCL10, and CXCL11 are elevated in BALF, lung and airway of COPD patients and recruits the immune cells, monocytes, neutrophils and T cells [41, 42].

2.2. Anti-microbial peptides

Anti-microbial peptides (AMPs) play an important role in surface region of mucosal sites to deal with microbial invasion. Most AMPs have common features, such as small molecules, positive charges and an amphipathic structure. AMPs exert the antimicrobial activity through

a various AMP acting mechanism. In general, cationic AMPs, bound by electrostatic forces to the negative parts of the target membrane, include lipopolysaccharide (LPS), lipoteichoic acid (LTA), and lysylphosphatidylglycerol. Then, AMPs displaces the lipids in target membranes, alters the membrane structure, and creates a physical hole. Consequently, Contents of target leak out though the hole [43, 44]. However, the entire mechanisms of several AMPs are not clear.

More than 1200 AMPs have been discovered, and some AMPs, such as defensins, LL-37 (cathelicidin), lysozyme, lactoferrin, secretory leukocyte peptidase inhibitor (SLPI), human neutrophil peptides (HNPs), and acidic peptides, participate in airway defense and present in airway surface as a liquid phase [45].

Defensins, reported in 1985, are a small cationic molecules [46]. Defensins are divided in three groups: α-, β-, and θ-defensins. In general, α-defensins are secreted by neutrophils and airway epithelial cells during the inflammation response in the lung [47, 48]. α-defensin 1 has an antiviral properties against influenza A virus (WSN strain), herpes simplex virus, cytomegalovirus and vesicular stomatitis virus *in vitro* [49]. It also inhibited the viral replication and viral protein synthesis though the blocking PKC pathway after the invasion [50]. The β-defensins in both human and mouse play a role in protection of airway and lung [51-53]. Recombinant mouse β-defensin 2 (rmBD2) is shown to prevent influenza A virus (H1N1) infection by inhibiting the entry [54]. Human β-defensin 3 (HBD3) is produced by keratinocytes and lung epithelial cells and kill the Gram-positive bacteria *Staphylococcus aureus* through inhibiting the cell wall biosynthesis [55, 56]. HBD4 exhibited the antimicrobial activity against *Staphylococcus carnosus* and *Pseudomonas aeruginosa* [57].

Cathelicidin (LL-37) is a cationic, amphipathic antimicrobial peptide. LL-37 is secreted by epithelial cells of human lung, and have the antibacterial activities against *Escherichia coli*, *P. aeruginosa*, *E. faecalis*, and *S. aureus* [58]. It is known to inhibit neutrophil and airway epithelial cell apoptosis [59].

Lactoferrin and lysozyme are produced by serous cells of airway glands and exist at 1 to 10 μg/ml, and 10 μg/ml in airway lavage fluid. Lactoferrin and lysozyme kill the bacteria, such as a clinical strain of *S. aureus*, clinical and laboratory strains of *E. coli* and *P. aeruginosa*, when it is incubated with the bacteria for 3 hr [60].

SLPI is an inhibitor of serine proteases with two cysteine-rich whey acidic protein (WAP) domains. SLPI has a feature of antibacterial activity against *E. coli* and *S. aureus* [61]. It displays a property of antiviral activity because it block the internalization of human immunodeficiency virus type-1 (HIV-1) [62].

2.3. Cytokines

Cytokines are soluble proteins and peptides that transmit signals related to wide-ranging biological effects among cells. During infection, inflammatory cytokines are secreted by various cells recognizing external molecules and can modulate the immune response. The inflammatory cytokines of high concentration were found in the BALF and lung of patients with pulmonary disease.

IL-1β induces the production of IL-8, which plays an important role in recruitment of neutrophils, from monocytes, alveolar macrophages, endothelial cells, fibroblasts, epithelial cells and hepatoma cells [63]. Epithelial cell neutrophil activator (ENA-78) is secreted by IL-1β-stimulated human type II epithelial cell line (A549) [64]. Other chemokines, such as human monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory peptide-1α, are also induced by IL-1β [4, 65].

Type I interferons (IFNs) are produced via recognition of various pathogens, especially viruses, by macrophages and airway epithelial cells [66-68]. The type I IFNs interact with their receptors (IFNAR) located in the airway epithelium, resulting in induction of chemokines, such as CXCL10 [69, 70]. In addition, IFN-γ secreted by Th1 cells promotes the production of CXCR3 ligands, CXCL9, CXCL10 and CXCL11, in airway epithelial cells [71].

Tumor necrosis factor (TNF)-α is an important cytokine that is secreted by various cell types, such as epithelial cells, macrophages, T lymphocytes and airway smooth muscle cells. TNFR1 (TNF receptor) displays on the cell surface and induces CXCL8 expression when interacting with protein A from *S. aureus* [72, 73]. It enhances monocyte adhesion to lung epithelial cell via up regulation of ICAM-1 [74]. In human epithelial cells, TNF-α induces an expression of the MUC1 which is known to play an important role in *P. aeruginosa* infection [75].

IL-4 and IL-13 related with Th2 cells are produced by various cell types, such as Th2 lymphocytes, basophils, eosinophils and mast cells. IL-4 enhances the production of adhesion molecules and eotaxin and induce the isotype switching [76]. In addition, IL-13 can replace with IL-4, because of sharing the receptors [77]. IL-4 and IL-13, also, induce TSLP, GM-CSF and CCL20 in airway epithelial cells [78-80].

IL-17 (IL-17A and F) is produced by Th17 and γδ T cells. IL-17R signaling can protect against gram-negative bacterial infection, *Klebsiella pneumonia*, by enhancing G-CSF and MIP-2 expression [81]. Furthermore, in human bronchial epithelial and venous endothelial cells, IL-17 promotes the secretion of IL-8, leading to increase of neutrophil recruitment [82]. It is also known to induce the expression of MUC5AC and MUC5B in human tracheobronchial epithelial cells [83]. IL-17A can regulate antibody class-switching in B cells [84]. IL-17A is involved in the development of allergic asthma, thereby impacting on contraction of airway smooth muscle cells via NF-κB, RhoA and ROCK2 pathways [85].

3. The effect of mucosal adjuvant in lung

3.1. Cholera toxin

Cholera toxin (CT), secreted by *Vibrio cholera* is a 56 kDa oligomer, consists of two types of subunits, A for toxic-activity (CTA) and B for binding (CTB). Initially CTB binds to the ganglioside receptor (GM1) expressed on all nucleated cells, but higher on epithelial cells. CTA then reached the cytosol and binds to NAD and catalyze adenosine diphophate (ADP)-ribosylation of Gsα. The consequence of this signal pathway leads to the increase of cAMP, and the secretion of massive water and chloride ions.

CT is known to be a strong mucosal adjuvant. CT induces immunoglobulin G1 (IgG1), IgE and IgA against target pathogens through the action of Th2 type responses when mice were administrated orally together with vaccine candidate [86]. CT as a mucosal adjuvant also affects the induction of immunological memory in the intestinal lamina propria [87]. In addition, CT breaks the development of oral tolerance against itself and unrelated antigens treated together [88]. Although it has less adjuvanticity than whole CT, CTB (nontoxic subunit) is showed certain level of adjuvanticity. Recombinant CTB induced IgA levels when co-treated antigens, *Streptococcus mutans* through intranasal route [89].

Many groups have developed the mutant CT that retained the powerful adjuvant effect while minimize the toxicity. A double mutant CT, ADP-ribosyltransferase active center and COOH-terminal KDEL (E112K/KDEL), present the low toxicity and induce the antigen-specific antibody response to the degree of native CT [90]. Lycke's group created a fusion protein, CTA1-DD, which combined CTA1 and a dimer of the Ig binding D-region from *S. aureus* protein A. CTA1-DD showed dual functions, binding to specific Ig on B cells, and CTA, induction of ADP ribosylation. Interestingly, the fusion protein did not have the toxicity and bound to nervous tissue [91].

3.2. Lipopolysaccharide

Lipopolysaccharide (LPS) is an endotoxin that present on the cell membrane of gram-negative bacteria. The endotoxin is consisted of three parts; O-specific chain, core structure and lipid A. LPS binds to CD14 and the complex is recognized by Toll-like receptor 4 (TLR4) and MD-2. TLR4 activated by LPS-CD14 complex transmit the signal through the myeloid differentiation primary response protein 88 (MyD88)-dependent or -independent pathway. These signaling pathways result in the expression of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-β, via NF-κB or IRF 3/7 activation.

LPS enhances a secondary IgG response and induce the isotype-swiching from IgM to IgG [92]. Furthermore, LPS enhances the production of an antigen-specific IgM in mouse model [93]. LPS promotes the proliferation of T lymphocytes of mouse *in vitro* [94]. In a human study, T cells undergo proliferation and secrete the cytokines in presenting autologous monocytes when stimulated by LPS [95]. LPS can activate the antigen-experienced CD4 T cells without TCR signals [96]. LPS is also known to promote the differentiation of Th1 cell. When incubated with *Brucella abortus* LPS, human T cell express high IFN-γ *in vitro* [97]. Furthermore, Lipid A, a core part of LPS, inhibits the IL-4 from $CD4^+$ T cells, but do not affect the production of IFN-γ [98]. This phenomenon is not absolute because LPS can differentiate into Th2 cells dependent on doses, routes, or species of LPS. For instance, when a low dose of LPS administrated through intranasal in asthma model, Th2 response was elevated. However, Th1 response was promoted high LPS when administered through intranasal route [99]. It has been suggested that *E. coli* LPS induced the production of Th1 cytokines, on the other hand, *Porphyromonas gingivalis* LPS promote the production of Th2

cytokines in mouse model [100]. This difference could be explained that *P. gingivalis* LPS is known to activate the cells via TLR2 pathway [101]. LPS can differentiate not only Th1 or Th2 cells, but also Th17 and Treg cells. Naïve CD4 T cells can differentiate into Th17 cells in the circumstance, such as LPS-stimulated DC, TGF-β, IL-6, anti-CD3 and anti-CD28 coated bead [102]. LPS-treated monocyte induces the expression of Foxp3 transcription factor in $CD4^{+}CD25^{-}$ T cells via $COX2\text{-}PGE_2$ mechanism [103].

LPS appears to affect the T cell survival mechanism. TNF-α, induced by LPS stimulation, prevents T cell death driven by super-antigen, Staphylococcal enterotoxin A (SEA), without co-stimulatory molecules [104]. Bcl-3, NF-κB family, induces in T cell stimulated SEA with LPS and anti-CD40, and artificial Bcl-3 expression increase survival of the T cells in vitro and in vivo [105]. In addition, other molecules are also involved in LPS-stimulation, such as Bcl-6 and GSK-3β under the LPS stimulation modulate T cell survival [106, 107].

LPS cannot use as a human adjuvant, because of its extreme toxicity. Monophosphoryl lipid A (MPL) is a molecule derived from LPS and lacks the phosphate region of lipid A. MPL activates antigen presenting cells (APCs) (macrophage, dendritic cell, and B cell) and affects the development of Th1 and Th2 cells [108, 109]. MPL induces the clonal expansion of $CD4^{+}$ T cell about the same level of LPS effect, but unlikely LPS, has a low toxicity judged by serum amyloid A (SAA) production [110].

3.3. *Bacillus subtilis* spore

Sporulation is one of the various strategies of bacterial surviving. In harsh environment, bacteria conduct a mechanism called sporulation, the formation of spore for conserving their genome. Upon improvement of environmental condition, spores germinate and enter the vegetative state. Therefore, spore can resist and protect itself from extreme environment, such as, ultraviolet radiation (UV), chemicals, high temperature, dehydrate, and absence of nutrients. Spore coat which plays an important role in resistance of environmental stress can be divided into three layers, inner coat, out coat, and crust. Coat is consist of morphogenetic proteins, SpoIVA, SafA, CotE and CotX/Y/Z, and a number of other proteins, such as, YhaX, CotA, CotD, CotW, *etc* [111].

Many research groups have studied the efficient delivery system using *Bacillus subtilis* spores because they have advantageous features including heat-stable, non-pathogenic and

particulates (which facilitate antigen presenting processes efficiently by APCs), for applying as a mucosal adjuvant. To display a target molecule on surface of *B. subtilis* spores, one can construct the genes containing a spore surface protein and the target molecule. CotB (spore coat protein) - tetanus toxin fragment C (from *Clostridium tetani*, TTFC) fusion proteins displayed on surface of spores that induced TTFC specific immunoglobulins [112]. Recently, non-recombinant models, also, are introduced besides recombinant models. Yim *et al*. made the distinct *B. subtilis* which can express and retain a specific enzyme, NADPH-cytochrome p450. Also, in other enzyme-spore experiment, β-galactosidase of *Alicyclobacillus acidocaldarius* is adsorbed on *B. subtilis* spores in acidic circumstance (pH 4) without the falling of its activity [113]. Spores could induce immune responses and act as a mucosal adjuvant. Both recombinant and adsorbed spores with various antigens, for instances TTFC from *C. tetani*, $Cpa_{247-370}$ from *Clostridium perfringens*, and GST from *Shistosomiasis japonica*, are displayed to same patterns, which they induced the specific and protective immune responses in mouse [114]. It has been suggested that spores adsorbed by heat-labile toxin (LTB) of *E. coli* promote a Th1 biased, specific immune responses, when administrated through an intranasal route [115].

III. Materials and Methods

1) Reagents

Cholera toxin (CT) was purchased from List Biological Laboratories Inc. (Campbell, CA, USA) and *E. coli* (0111:B4) lipopolysaccharide (LPS) from Sigma-Aldrich (St. Louis, MO, USA). For analysis for surface staining, F4/80-APC (clone BM8) and GR-1-PERCP (clone RB6-8C5) were purchased from Biolegend (San Diego, CA, USA). Anti-mouse CD11b-PE-Cy7 (clone M1/70) were purchased from BD Biosciences (San Jose, CA, USA). Mouse CCL2/JE/MCP-1, CCL4/MIP-1β, CXCL10/IP-10/CRG-2, TNF-α, IL-1β, IL-6 and IFN-γ DuoSet ELISA kits were purchased from R&D systems (Minneapolis, MN, USA).

2) Preparation and isolation of *Bacillus subtilis* spore

Bacillus subtilis strain HB3 (National Culture Collection for Pathogen, Korea) was incubated in a agar plate containing 3% triptacase soy broth (TSB), 0.5% yeast extract (YE) and 1.5% BactoAgar (BD Biosciences) at 37°C for 9 hours. A single colony was picked and inoculated in the liquid media for 4-5 hours in the shaking incubator at 150 rpm, 37°C until the OD value reached between 0.45-0.60. The culture was transferred to sporulation media containing KCl, $MgSO_4 \cdot 7H_2O$, 1 M $Ca(NO_2)_3$, 0.01 M $MnCl_2$ and 1 mM $FeSO_4$ and incubated in the shaking incubator at 150 rpm and 37°C. After 48 hours, the cells were collected by centrifugation at 5516 *g* for 10 minutes. The cells were starved by adding cold distilled water and incubated at 4°C for 48 hours for the induction of sporulation, and washed every 24 hours. After the starvation, the cells were collected and heat-inactivated at 68°C for 45 minutes.

3) Approval for animal study

C57BL/6 female mice, aged 6-week old, were purchased from Orient Bio Inc. (Seongnam, Korea). The mice were anesthetized with intraperitoneal injection of mixture of Zoletil 50 (Virbac, Fort Worth, TX, USA) and Rompun (Bayer Korea, Ansan, Korea). Each mouse was injected with PBS, CT, LPS, or *Bacillus subtilis* spore through intranasal route. All mice were maintained at SPF facility, Seoul National University during the whole experimental period.

All the experiments using mouse were approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (Approval number: SNU-130527-5), Korea.

4) Isolation of serum, lung and bronchoalveolar lavage fluid (BALF)

A mice anesthetized by anesthetic mixture and then, heparinized capillary tube was injected to the blood vessels behind the eyeball. The blood run through the tube were taken and kept at room temperature, for 1 hour. After coagulation of the blood, the blood were centrifuged at 4°C, 3,000 rpm and the supernatants were collected. After conduct of the venous drainage, the trachea was exposed by incision using operating scissor. A catheter (BD Biosciences) was intubated in trachea, 600 μl PBS was injected, and then, BALF was isolated through suction. The thorax was dissected and opened. PBS containing heparin was perfused into the right ventricle for removing blood and the whole lung was collected.

5) Isolation of total lysate from lung

The lung was homogenized by tissue grinder and homogenizing stirrer HS-30E (DAIHAN Scientific Co., Wonju, Korea) in 1.5 ml RIPA buffer with protease inhibitor. After centrifugation at 4°C, 17,000 rpm for 10 minutes, the supernatants were taken for ELISA assay.

6) Isolation of total RNA from lung

Homogenization of the lung was conducted in 4 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA). The supernatants were taken after the centrifugation at 4°C, 12,000 *g* for 10 minutes and chloroform was added. Following centrifugation at 12,000 *g* for 15 minutes the aqueous phase containing RNA was transferred into a new tube. The sample was added an isopropyl alcohol and incubated for 10 minutes at room temperature. The RNA solution was centrifuged at 4°C, 12,000 x *g* for 10 minutes and the supernatant was discarded. The RNA pellet was washed with 75% ethyl alcohol and dissolved in RNase free-water (Sigma-Aldrich).

7) Reverse transcription polymerase chain reaction

The isolated RNA was analyzed by A260 measurements (NanoDrop® ND-1000 Spectrophotometer, GE Healthcare Bio-Science, Uppsala, Sweden). The 1 μl of total RNA was reverse transcribed into complementary DNA (cDNA) with MMLV reverse transcriptase (Life technologies, Carlsbad, CA, USA) and oligo dT (Bioneer, Daejeon, Korea) using PCR Thermal cycler (TAKARA Inc., Shiga, Japan). Then RT reaction mixture was used for PCR. The cDNA library was amplified with the primers, dNTP and Top polymerase (Bioneer) by the PCR Thermal cycler and My cycler (Bio-rad, Hercules, CA, USA). As shown in Table 1, sense and antisense oligonucleotide primers of the target molecules were synthesized by Bioneer (Korea). The PCR products were loaded on 2% agarose gel containing ethidium bromide (Sigma-Aldrich). The results were read by Gel-Doc EQ system and analyzed by Quantity one software (Bio-rad).

Table 1. The primer sequences used in the present study.

Peptides	Primer (5'→3')	AT	Size(bp)	Reference
Chemokine				
CXCL9	F: CCAGGGAACCCATTTCTCTCTT	62	169	NM_008599.4
	R: CCACACTTCCACTCGCAGAATT			
CXCL10	F: CCTCTCTCCATCACTCCCCTTT	62	242	NM_021274.2
	R: TCGCACCTCCACATAGCTTACA			
CXCL15	F: CCCGCGTTAGTCTGGTGTAT	53	227	NM_011339.2
	R: AACAGCCCATAGTGGAGTGG			
CCL2	F: AGGTCCCTGTCATGCTTCTG	64	249	NM_011333.3
	R: TCTGGACCCATTCCTTCTTG			
CCL4	F: GCAAACCTAACCCCGAGCAA	62	149	NM_013652.2
	R: TGCCGGGAGGTGTAAGAGAA			
CCL8	F: GGTGCTGAAAAGCTACGAGAGAAT	64	126	NM_021443.3
	R: TCCATGTACTCACTGACCCACTTC			
Defensin				
β-Defensin 1	F: TCTGCCTGGTCCTGAGTAATGA	56	120	NM_007843.3
	R: CCGTGTTCTCTGTTCCATTCC			
β-Defensin 2	F: CCACTCCAGCTGTTGGAAGTTTA	62	110	NM_010030.1
	R: CCTGGCAGAAGGAGGACAAAT			
β-Defensin 3	F: GCATTGGCAACACTCGTCAGA	62	85	NM_013756.2
	R: CGGGATCTTGGTCTTCTCTA			

β-Defensin 4	F: GCAGCCTTTACCCAAATTATC R: ACAATTGCCAATCTGTCGAA	56	102	NM_019728.4
β-Defensin 14	F: CCTACCAAAAACCCTCCGAAA R: TCGACCGCTATTAGAACATCGA	56	112	NM_183026.2
Surfactant				
Surfactant A1	F: CAGACTGCACTCTACGAGATCAAAC R: AGTTGACTGACTGCCCATTGG	62	121	NM_023134.4
Surfactant D	F: AAAGCGGGCTTCCAGACAGT R: TCGGCCATCAGGGAACAAT	62	122	NM_009160.2
Mucin				
Muc5ac	F: CCATGCAGAGTCCTCAGAACAA R: TTACTGGAAAGGCCCAAGCA	62	106	NM_010844.1
Muc5b	F: GCTGCTGTTACTCCTGTGAAAAAG R: TGACCTCTGTCTCACAGCCCTTA	40	96	NM_028801.2
Other antimicrobial peptides				
Lactoferrin	F: GCTGTTCAAGGAGTCTGCCATT R: GGCTATCACATCCTGCTGCTTT	62	127	D88510.1
lysozyme 2	F: ATGGAATGGCTGGCTACTATGG R: ACCAGTATCGGCTATTGATCTGA	56	150	NM_017372.3
SLPI	F: ACGGTGCTCCTTGCTCTG R: GTACGGCATTGTGGCTTCTC	62	134	NM_011414.3
House keeping				
β-actin	F: ATCACTATTGGCAACGAGCG R: TCAGCAATGCCTGGGTACAT	57	191	NM_007393.3

* AT: annealing temperature, ℃; F: forward; R: reverse

8) Real time - polymerase chain reaction

Amplification was performed with Power SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in Step One Plus Real-Time PCR System (Applied Biosystems) under the following conditions: pre-denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. Specificity of each primer pair was confirmed by melting curve analysis. All data were displayed by the comparative Ct ($2^{-\Delta\Delta Ct}$) method to quantify expression.

9) Analysis of surface marker

To measure of the various cell types through the lymphocytes recruitment, cell surface staining was conducted. BAL cells were isolated by centrifugation in BALF samples. The cells were stained with anti-mouse F4/80-APC, GR-1-PERCP, and CD11b-PE-Cy7 for 20 minutes at 4°C in dark room. The samples were washed with PBS at three times and then, fixed by 4% paraformaldehyde during overnight at 4°C in dark room. The samples were read by Canto II flow cytometer (BD Biosciences) and their data was analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

10) Measurement of chemokines and cytokines production

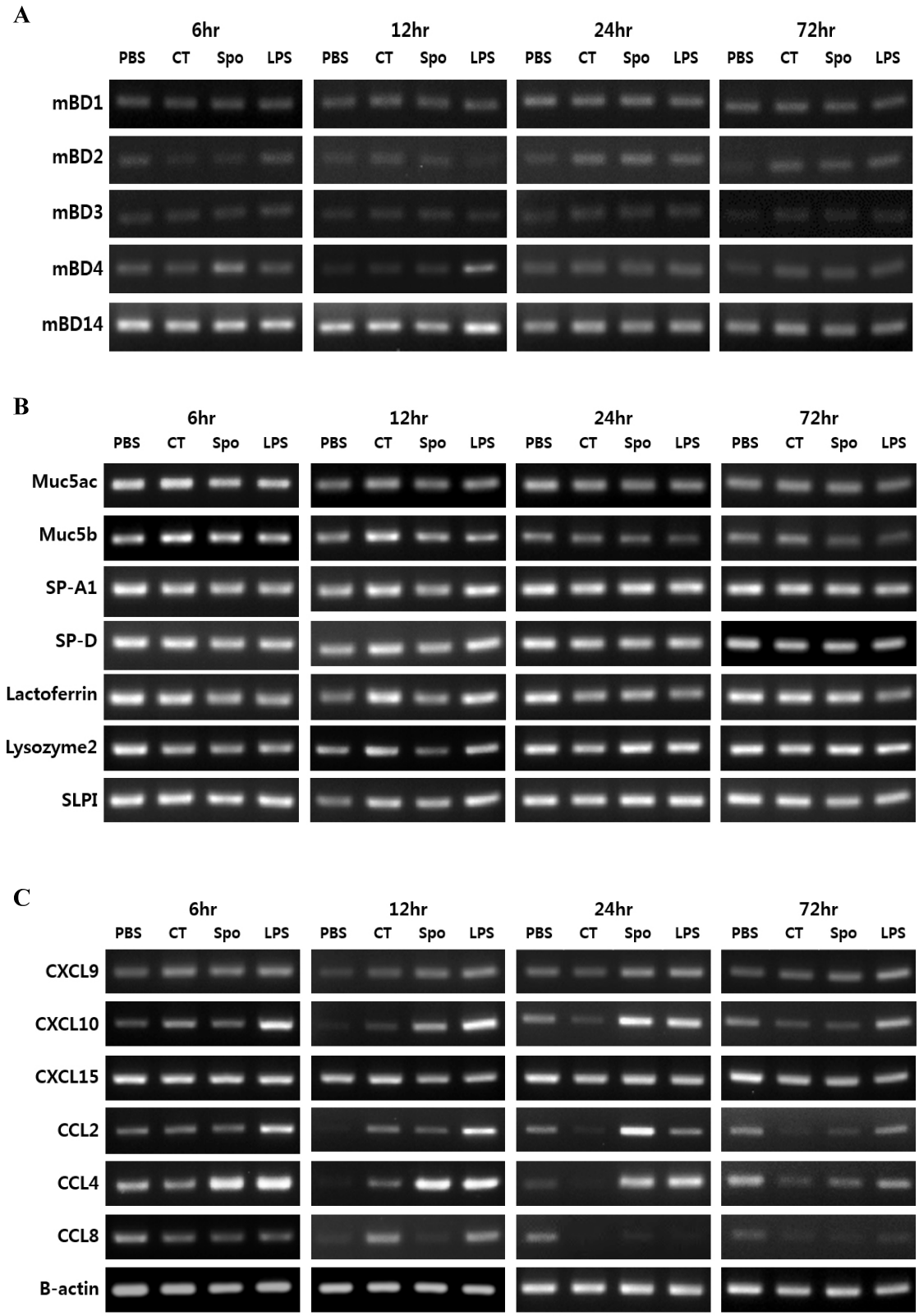
The production of chemokines and cytokines, mouse CCL2, CCL4, CXCL10, TNF-α, IL-1β, IL-6 and IFN-γ was measured by ELISA following the manufacturer's protocol. Briefly, the target chemokine capture antibody was coated in 96-well immune-plate (Nalge Nunc International, Penfield, NY, USA) for overnight at room temperature. The plate was washed for three times with washing buffer consisted of 0.05% tween® 20 in PBS and blocked with blocking buffer (1% BSA in PBS) for 1 hour. Then, series of standard molecule and samples were added into the plate and incubated for 2 hours. Detection antibody with biotin was added to the plate after washing process and incubated for 2 hours. Streptavidin-HRP responded to biotin was incubated for 20 minutes at room temperature in dark room. After the wash, substrate solution (TMB/E solution, Millipore, MA, USA) was added for 20 minutes and the response was stopped by adding 2N H_2SO_4. The chemokine quantity was analyzed at wavelength 450 nm by VERSA MAX microplate reader (Molecular Devices, Sunnyvale, CA, USA).

IV. Results and discussion

Adjuvants need to induce the antigen-specific immune response for the enhancement of vaccine efficacy. In intranasal administration, it is important to understand the immune activity in the lung in which the potential modulators include chemokines, cytokines, and antimicrobial peptides including mucin, surfactants and other peptides. In the present study, we examined the expression pattern of immune modulators in mice administered via intranasal route with cholera toxin (CT), *Bacillus subtilis* spore or lipopolysaccharide (LPS).

1) The mRNA of chemokines, CXCL10, CCL2 and CCL4, was remarkably increased in the whole lung of mouse treated with *B. subtilis* spore or LPS during the early phase of immune response.

To test the effect of adjuvants, the potential modulators in the lung were analyzed by RT-PCR assay. A mouse was administered with 20 μl of adjuvants, CT (1 μg), spore (1 x 10^9), or LPS (10 μg/mouse), through the intranasal route and then, mRNA expression of the whole lung was analyzed via PCR. AMPs (β-defensin1, 2, 3, 4, 14), mucin (muc5ac, muc5b), and SP-A1, SP-D, lactoferrin, lysozyme2 and SLPI, were minimally, if there were any, changed at 6, 12, 24 and 72 hr post treatment. (Fig. 1A, B) CXCL10, CCL2 and CCL4 mRNAs among the chemokines were increased in the lung of the mice treated with spore or LPS (Fig. 1C). Next, real time-PCR experiment was conducted for further analysis of expression degrees on CXCL10, CCL2 and CCL4. CCL2 and CCL4 mRNAs were enhanced after the treatment of LPS at 6 and 12 hr (Fig. 1D). It was noting that although lower than LPS, *B. subtilis* spore induced increase of these mRNAs. CXCL10 mRNA was highly induced by LPS treatment at 6 hr compared with other adjuvants. The mRNA expression of CXCL10, CCL2 and CCL4 was reduced gradually after 12 hr post treatment. Consistent with the PCR results, CT induced a little CCL2 mRNA, and no CCL4 and CXCL10 mRNA.


A
6hr
12hr
24hr
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PBS CT Spo LPS PBS CT Spo LPS PBS CT Spo LPS PBS CT Spo LPS
mBD1
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mBD4
mBD14
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Muc5ac
Muc5b
SP-A1
SP-D
Lactoferrin
Lysozyme2
SLPI
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CCL2
CCL4
CCL8
B-actin

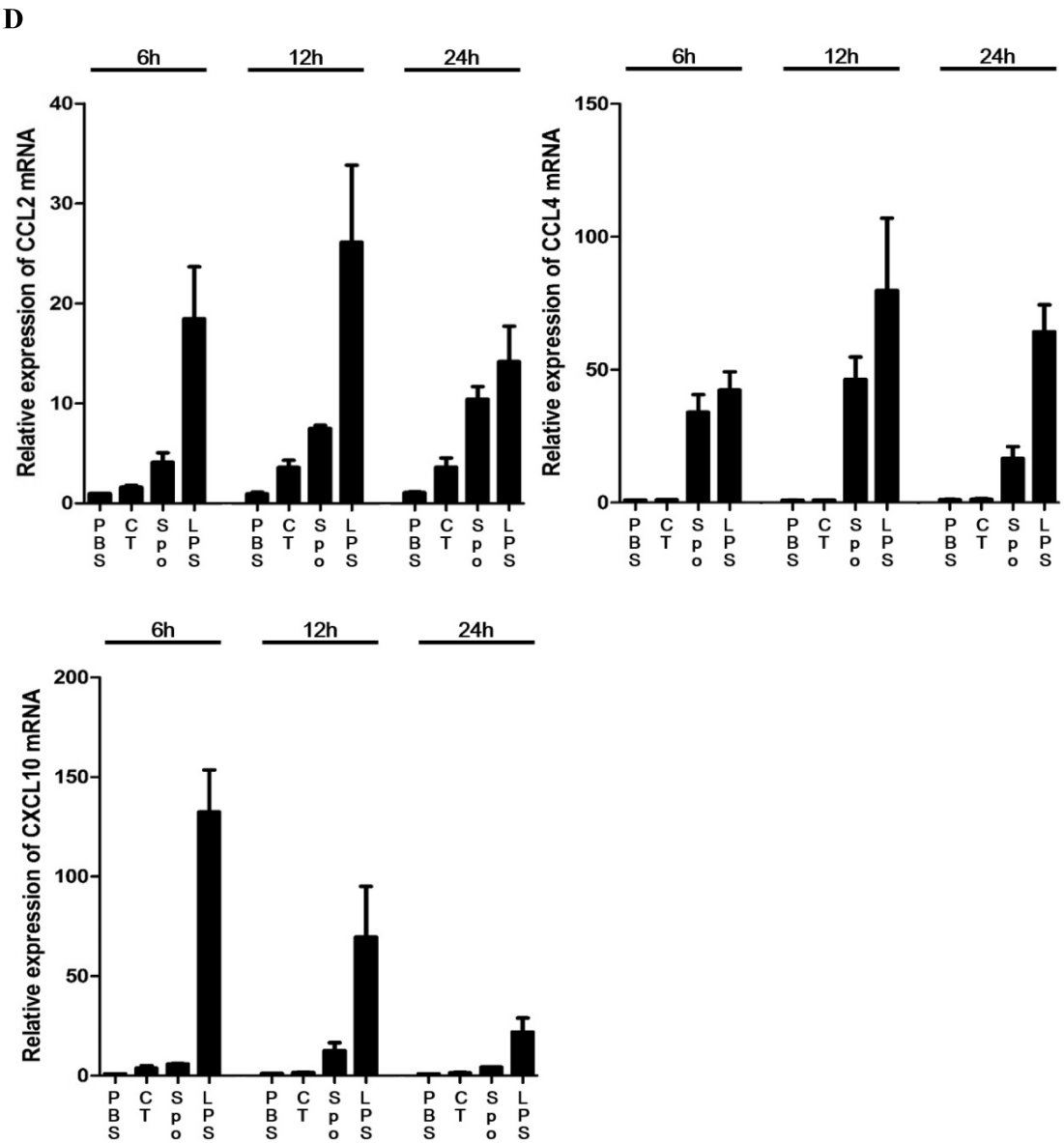


Fig. 1. The mRNA expression of the chemokines in whole lung of mouse administered with adjuvant via intranasal route. A mouse was administered with 20 μl of adjuvants, CT (1 μg), spore (1 x 10^9), or LPS (10 μg) via intranasal route. PBS treatment served as a control. Whole lung was taken at 6, 12, 24 and 72 hr. The mRNA expression of (A) defensins, (B) AMPs, and (C) chemokines were analyzed by RT (reverse transcription) – PCR and (D) CXCL10, CCL2 and CCL4 were analyzed by real time-PCR. Spo: spore.

2) *B. subtilis* spore and LPS enhanced the secretion of CCL2 and CCL4 proteins into BALF during the early immune response.

To further confirm the changes of the chemokines, CCL2, CCL4 and CXCL10 whose mRNAs was increased, the whole lung and BALF were examined for their protein expression by using ELISA. In the BALF, *B. subtilis* spore and LPS induced the secretion of CCL2 at 6 hr. (Fig 2A) and CCL4 at 24 hr (Fig. 2B). CXCL10 was minimally induced by the treatment with CT, spore and LPS (Fig. 2C).

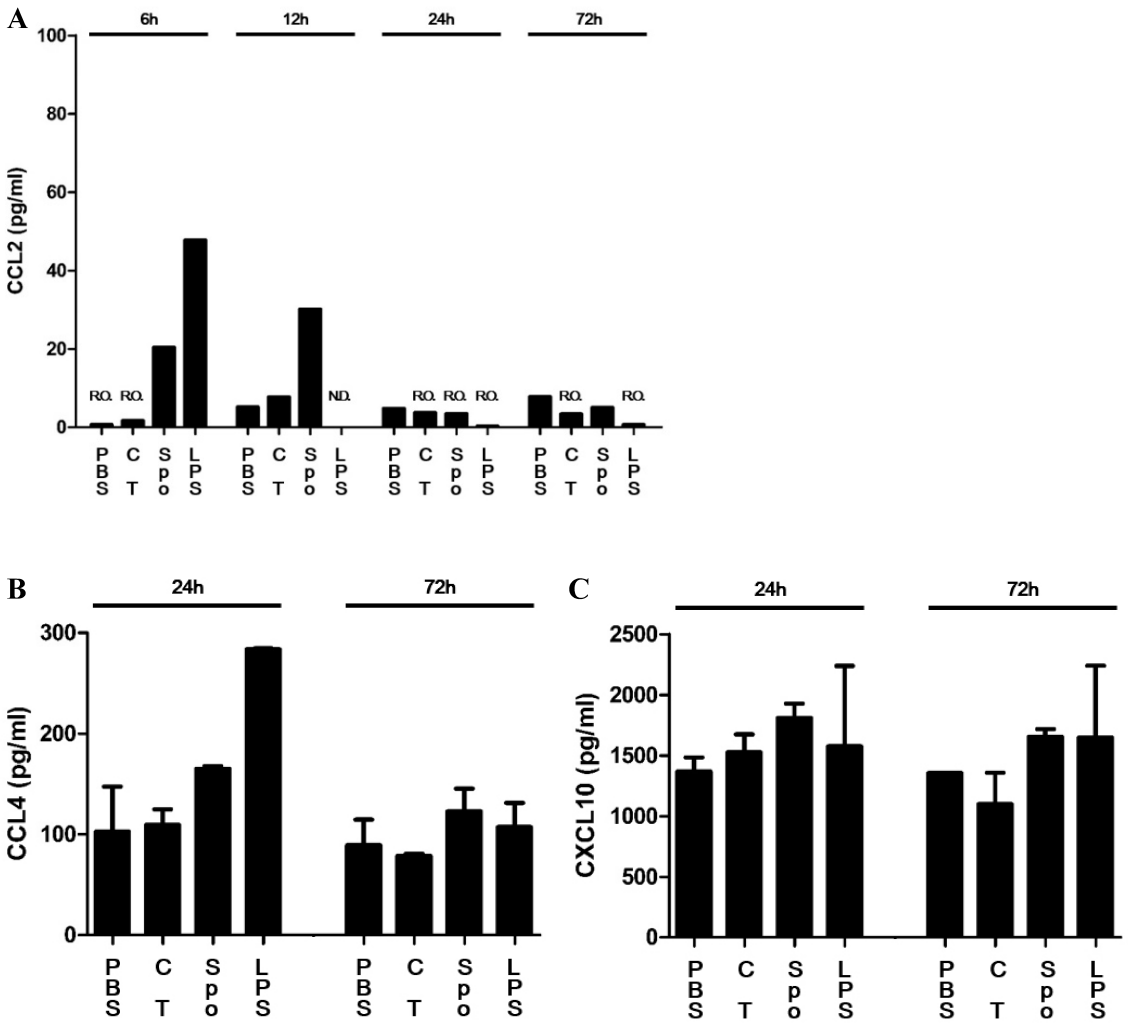
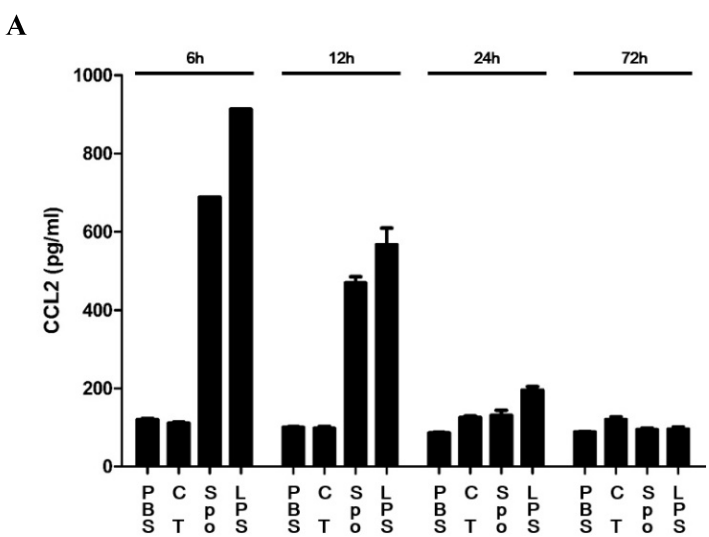


Fig. 2. CCL2, CCL4 and CXCL10 secretion in BALF of mouse administered with CT, *B. subtilis* spore or LPS through intranasal route. A mouse was administered with 20 μl of adjuvants, CT (1 μg), spore (1 x 10^9), or LPS (10 μg) via intranasal route. PBS (20 μl/mouse) treatment served as a control. (A) CCL2, (B) CCL4 and (C) CXCL10 proteins in the BALF taken from the mice administered with CT, *B. subtilis* spore or LPS were analyzed by ELISA assays. Spo: spore, R.O.: out of the range, N.D.: not detected.

3) CCL2 and CCL4 expression were induced by administration of *B. subtilis* spore and LPS in the lung.

The results showed that LPS dramatically induced the CCL2 and CCL4 expression at 6 and 12 hr in the lung. Interestingly, *B. subtilis* spore induced high expression of CCL2 alike LPS treatment despite the differences between the adjuvants in the mRNA expression (Fig. 4A, B). In the CXCL10 protein in the lung of the mice administered with the adjuvants, there was no difference between the treatments. The induction of CCL2 and CCL4 by LPS and spore was reduced after 12 hr and reached to the level of PBS treatment at 72 hr.



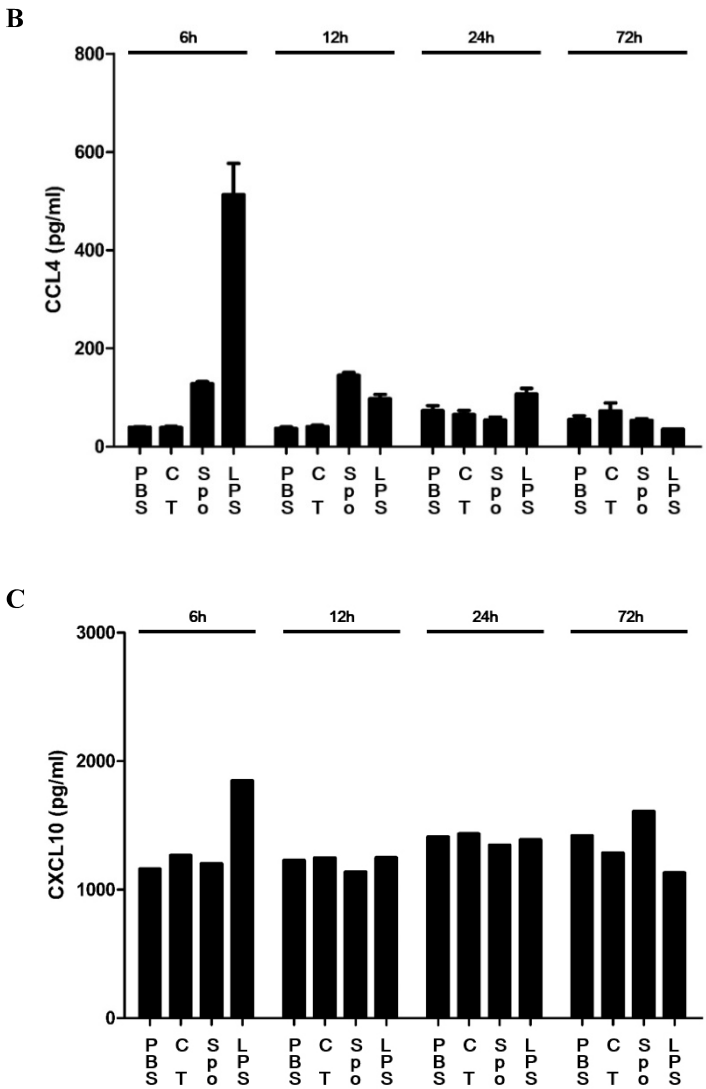


Fig. 3. CCL2, CCL4 and CXCL10 secretion in the lung of mouse when administered with CT, *B. subtilis* spore or LPS through intranasal route. A mouse was administered with 20 μl of adjuvants, CT (1 μg), spore (1 x 10^9), or LPS (10 μg) via intranasal route. PBS (20 μl/mouse) treatment served as a control. The whole lung was isolated at 6, 12, 24 and 72 hr and homogenized in RIPA buffer. (A) CCL2, (B) CCL4 and (C) CXCL10 proteins in the whole lung lysates were analyzed by ELISA. Spo: spore.

CCL2 mRNA and protein expression at 6 and 12 hr was observed in mice treated with LPS. It has been suggested that the induction of CCL2 in BALF from the mice administered with LPS via aerosolization is mediated with JNK signal pathway [116]. In the present study, *B. subtilis* spore treatment also induced the CCL2 expression in the lung. It is probable that CCL2 was induced through the TLR2 pathway [117].

The expression of CCL4 mRNA and protein was up regulated in the lung and BALF of mice administered with LPS or *B. subtilis* spore. It has been suggested that CCL4 can be induced by peptidoglycan (PGN) through toll-like receptor (TLR)2 signal pathway [118]. Although the concept must be proved by future studies it is probable that PGN in the *B. subtilis* also induce the CCL4 via TLR2 signaling.

The results in the present study showed that the intranasal delivery of LPS induced the CXCL10 mRNA expression in the lung. However, the increase in theCXCL10 protein was not observed in the lung from mice treated with CT, spore or LPS. It is well known that IFN-γ is one of the CXCL10 inducers. Therefore, it is most likely CXCL10 was not detected sue to lacking of IFN-γ expression in the current setting (Fig. 4D).

CCL2, CCL4 and CXCL10 induced by the adjuvants in the lung and BALF were modulated and returned to the basal levels after 24hr. It is speculated that the disappearance is related with D6 and DARC, non-signaling chemokine receptor-like molecules, for the modulation of lymphocyte recruitments.

4) *B. subtilis* spore and LPS induced the IL-1β expression in the lung.

Cytokines play important roles in immune response, as the other modulators in the respiratory system. To examined the expression of the cytokines, mice were administered with LPS, CT or spore and IL-1β, IL-6, TNF-α and IFN-γ were analyzed in the lung, BALF and serum. IL-1β, IL-6 and TNF-α expressions were increased by LPS treatment in the lung at 6 and 12 hr (Fig. 4). Surprisingly, *B. subtilis* spore powerfully promoted the production of IL-1β and the effect was continued at 12 hr. In the IL-6 and TNF-α expression, however, spore induced less than those of LPS. (Fig. 4A, B and C). The IL-1β production by treatment of CT was shown at 6, 12 and 24hr, but IL-6 and TNF-α proteins were not observed. IFN-γ proteins in the lung was not detected in all treatment of adjuvants.

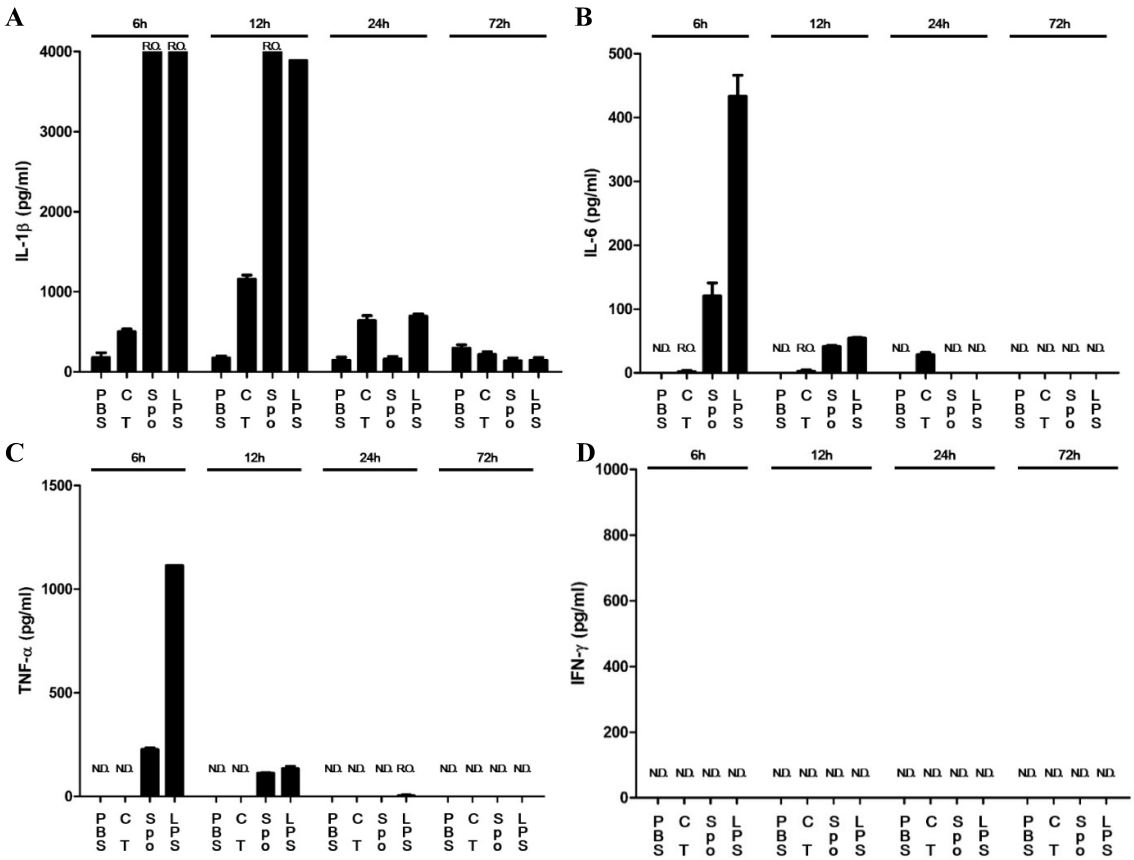


Fig. 4. IL-1β, IL-6, TNF-α, and IFN-γ secretion in the lung of mice administered with CT, *B. subtilis* spore or LPS. The mice were administered with 20 μl of adjuvants, CT (1 μg), spore (1 x 10^9), or LPS (10 μg) via intranasal route. PBS (20 μl/mouse) treatment served as a control. The whole lungs were isolated at 6, 12, 24 and 72 hr and homogenized in RIPA buffer. (A) IL-1β, (B) IL-6, (C) TNF-α and (D) IFN-γ proteins in the whole lung lysates were analyzed by ELISA. Spo: spore, R.O.: out of the range, N.D.: not detected.

5) *B. subtilis* spore and LPS induced the IL-6 and TNF-α secretion into BALF

B. subtilis spore induced the IL-1β secretion into BALF at 6 and 12 hr (Fig. 5A). Unlikely to the data in the lung, IL-6 and TNF-α secretion into BALF were strongly promoted by spore and LPS treatment at 6 and 12 hr (Fig. 5B, C). CT, also, induced the IL-6 secretion at 12 and 24 hr in a delayed passion compared to other adjuvants.

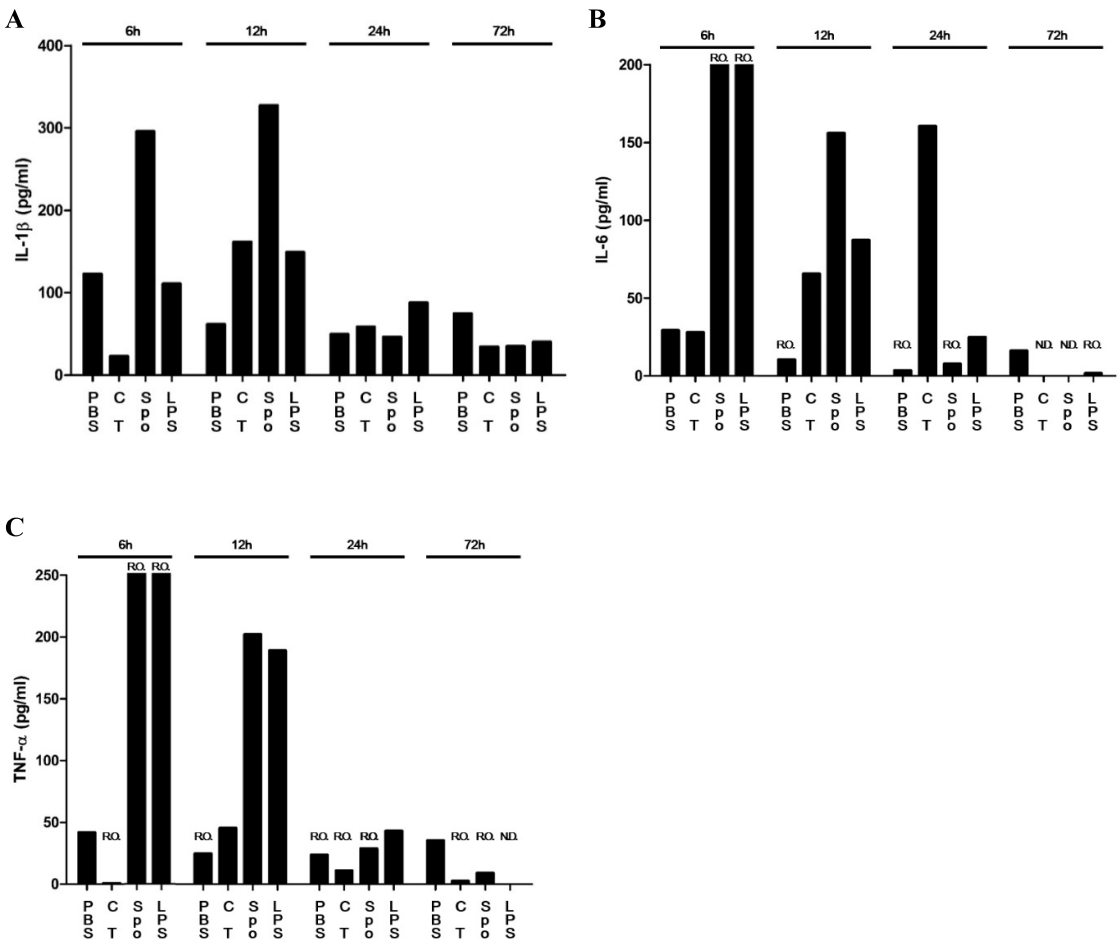


Fig. 5. IL-1β, IL-6 and TNF-α secretion in BALF of mice administered with CT, *B. subtilis* spore or LPS. The mice were administered with 20 μl of adjuvants, CT (1 μg), spore (1 x 10^9), or LPS (10 μg) via intranasal route. PBS (20 μl/mouse) treatment served as a control. The BALF were isolated at 6, 12, 24 and 72 hr and (A) IL-1β, (B) IL-6, (C) TNF-α and (D) IFN-γ proteins were analyzed by ELISA. Spo: spore, R.O.: out of the range, N.D.: not detected.

In the serum of the mice injected intranasally with the adjuvants, CT and LPS enhanced the secretion of IL-6 protein at 6 hr (Fig. 6B). IL-1β and TNF-α protein in the serum were not detected in all treatments (Fig. 6A and C).

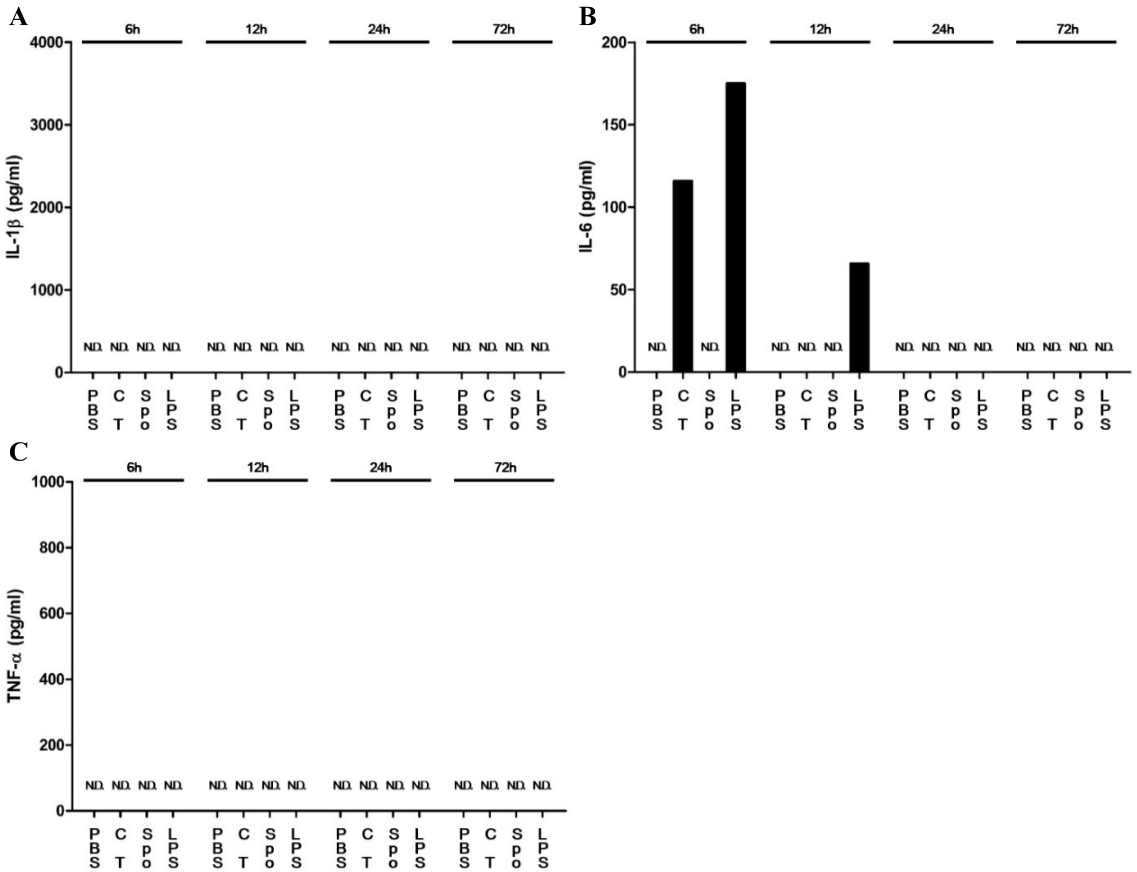


Fig. 6. IL-1β, IL-6 and TNF-α secretion in serum of mice administered with CT, *B. subtilis* spore or LPS. The mice were administered with 20 μl of adjuvants, CT (1 μg), spore (1 x 10^9), or LPS (10 μg) via intranasal route. PBS (20 μl/mouse) treatment served as a control. The blood samples were taken at 6, 12, 24 and 72 hr. After coagulation for 1hr, the serum was isolated by centrifugation. (A) IL-1β, (B) IL-6, and (C) TNF-α proteins in the serum were analyzed by ELISA. Spo: spore, N.D.: not detected.

The results showed that *B. subtilis* spore significantly induced the expression of cytokines, IL-1β, IL-6 and TNF-α. Cytokines can affect and amplify the expression of other cytokines and chemokines in the lung epithelial cells [119]. In the present study, *B. subtilis* spore and LPS highly induced IL-1β, IL-6 and TNF-α expression in the lung and BALF. It has been shown that *B. subtilis* B10 spores induced the expression of the cytokines in murine macrophages [10]. Intranasal administration of CT induced the secretion of IL-6 in BALF at

6, 12 and 24 hr and in the serum at 6 hr. It has been reported that CT can induce the expression of IL-6 in mouse bone marrow macrophages [120] and in rat peritoneal mast cells [121].

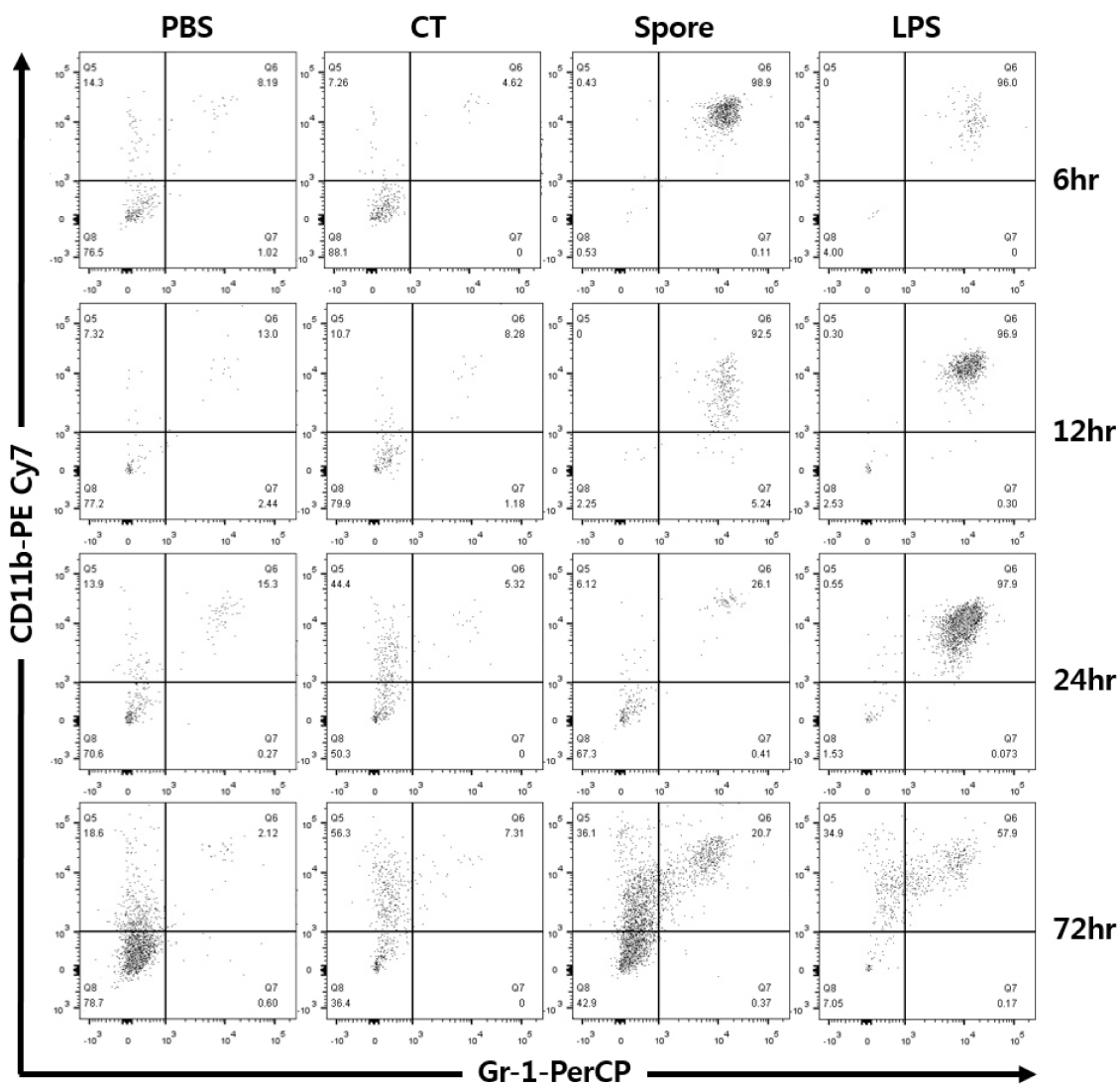
6) *B. subtilis* spore and LPS promoted the recruitment of Gr-1$^+$CD11b$^+$ cell into BALF in early phase

Neutrophils and monocyte/macrophages are recruitment to the lung from a pulmonary capillary blood vessel at first when entering of an external material. Then, they can remove the foreign objects or regulate the immune response via the secretion of cytokines, chemokines and AMPs. To confirm of the migration of neutrophils and monocyte/macrophages by the effect of adjuvants, BALF cells was analyzed by flow cytometry. The cells isolated the BALF were stained with Gr-1, CD11b and F4/80 antibodies. At 6hr, *B. subtilis* spore induced the recruitment of Gr-1$^+$CD11b$^+$ cells. The recruitment by the spore was reduced at 12 and 24hr, but was enhanced at 72hr again. The Gr-1$^+$CD11b$^+$ cell recruitment by LPS was increased gradually after 6hr and reached at a maximum at 24hr (Fig.7A). From the Gr-1$^-$CD11b$^+$ region of fig. 7, F4/80$^+$CD11b$^+$ cells were analyzed. *B. subtilis* spore enhanced the recruitment of F4/80$^+$CD11b$^+$Gr-1$^-$ cells more than CT and LPS treatment (Fig.7B). These data displayed that *B. subtilis* spore have more efficiency for the recruitment of neutrophils and monocyte/macrophages than CT and LPS. CXCL15, the chemokine for neutrophil migration in mouse, was not induced by the adjuvants in mRNA level (Fig. 1C). It have been reported that CCL2 requires other inflammatory molecules for the efficient recruitment of monocytes and lymphocytes [122]. CCL2, also, can induce the migration of neutrophils in acute and chronic inflammation [123, 124]. It suggest that the results in Fig.7 were related with the induction of CCL2 and cytokines by *B. subtilis* spore.

This study demonstrated the changes of effector molecules expression and leukocyte migration in the lung of mice administered with CT, *B. subtilis* spore and LPS. In the mRNA expression analysis of modulator, spore and LPS induced CCL2, CCL4 and CXCL10 mRNA in the lung but defensins and other AMPs were not induced by the adjuvants. Spore and LPS, also, enhanced the expression and secretion of CCL2 and CCL4 in the lung and BALF at 6 and 12 hr. In the analysis of cytokine protein, spore and LPS powerfully induced the expression IL-1β in the lung and the secretion of IL-6 and TNF-α into BALF in early phase.

In the analysis of surface marker in BALF cells, spore promote the migration of neutrophils and monocyte/macrophages more than other adjuvants. It suggest that these results can help to understanding of immune response by CT, *B. subtilis* spore and LPS in the lung. However, the pulmonary immune response by the adjuvants modified and derived from CT, *B. subtilis* spore and LPS have not understood completely and further studies need to confirm the effect and mechanism of the various adjuvants in respiratory system.

A



B

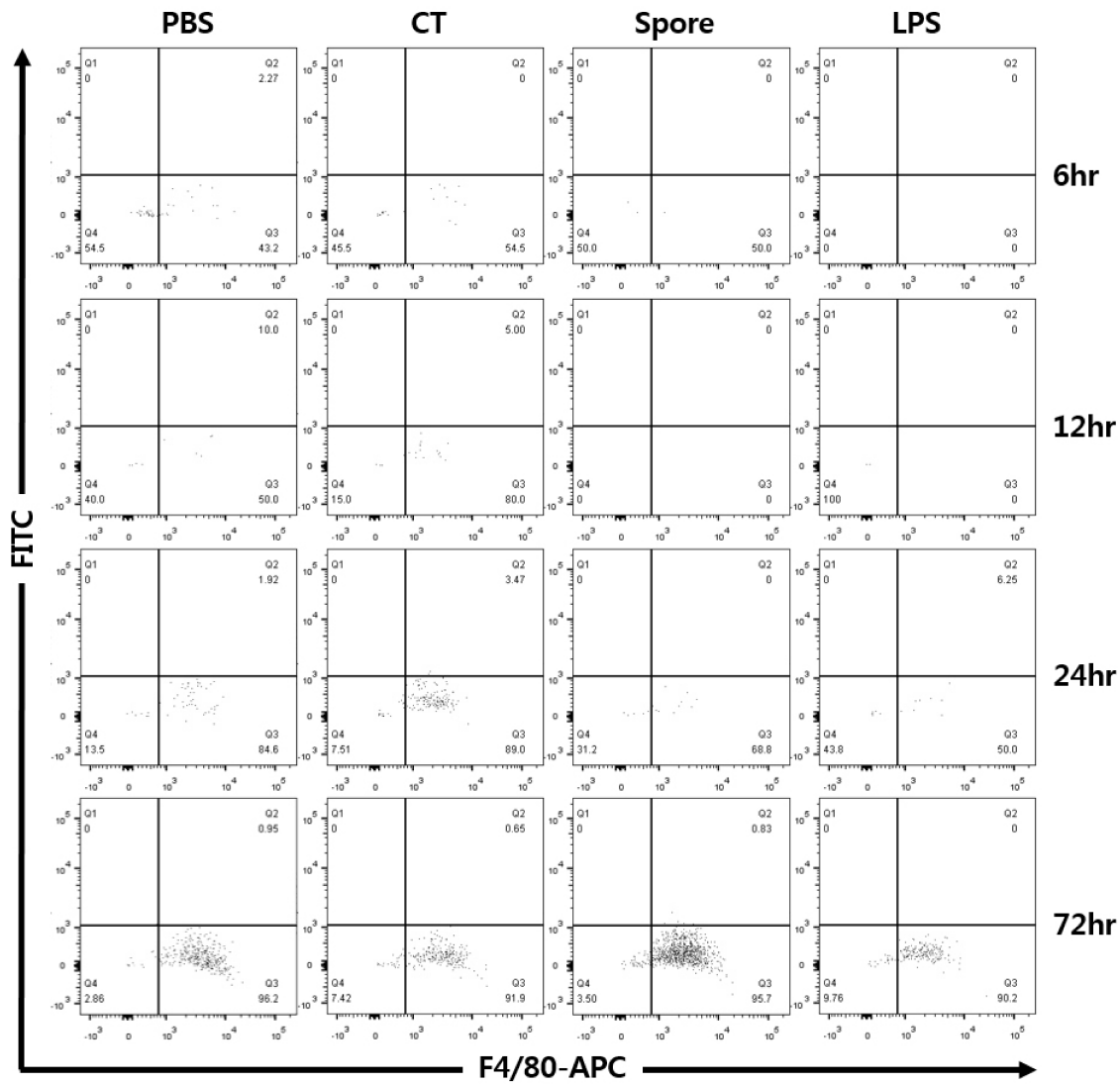


Fig. 7. The migration of leukocytes in BALF of mice administered with CT, *B. subtilis* spore or LPS. BALF of the mice administered with the adjuvants were taken at 6, 12, 24 and 72hr. The cells were isolated by centrifugation in BALF and stained with Gr-1-APC, F4/80-APC and CD11b-PECy7. Gr-1^{+}CD11b^{+} cells (A) and F4/80^{+}CD11b^{+}Gr-1^{-} cells (B) were analyzed by Canto II flow cytometer.

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VI. Summary in Korean

호흡기계에서 케모카인과 사이토카인, 항균 펩타이드들은 면역을 조절하는 중요한 역할을 한다. 항균 펩타이드들은 외부 물질들에 대해서 우선적으로 방어를 한다. 그리고 케모카인들은 외부물질들을 제거하기 위한 백혈구들이 호흡기관으로 유입하게 한다. 사이토카인은 이들 항균 펩타이드와 케모카인의 영향을 조절하거나 면역 세포들을 활성화 시킨다.

점막 백신 보조제는 오랫동안 많은 연구진들에 의해서 연구가 진행되어 왔다. 콜레라톡신과 리포폴리사카라이드, 바실러스 서브틸리스 포자 또한 보조제로써 연구 및 개발이 진행되었다. 하지만 비강내 투입된 보조제들이 숙주 내 폐와 다른 기관에서 미치는 영향에 대한 연구는 많이 미흡한 편이다.

본 연구에서 비강으로 투입된 점막 보조제들이 폐에서 미치는 영향을 대표적인 반응 물질인 케모카인과 항균펩타이드, 사이토카인 발현 및 백혈구의 유입 분석을 통해서 확인하였다. 각 보조제들을 비강내로 투입한 마우스들을 SPF 시설에서 유지하였다. 6, 12, 24, 72 시간에 맞추어 마우스들로부터 폐와 기관지폐포세척액을 채취하였다. 그리고 채취한 샘플들로부터 반응물질의 mRNA 와 단백질 발현양을 분석하였다. 추가적으로 유세포분석기를 이용하여 기관지폐포세척액에 포함된 세포들의 유입을 측정하였다. 실험 결과, 포자처리구와 리포폴리사카라이드 처리구에서 CCL2 와 CCL4, CXCL10 의 mRNA 의 발현이 유도가 되었다. 항균 펩타이드들의 경우 각 처리구 간에 발현 차이는 없었다. 포자와 리포폴리사카라이드는 폐와 기관지폐포세척액 지역으로 CCL2 와 CCL4 의 발현 및 분비를 촉진하였다. 사이토카인 분석에서 포자와 리포폴리사카라이드가 IL-1β와 IL-6, TNF-α를 폐와 기관지폐포세척액으로 급격히 발현 및 분비를 유도하는 것을 관찰하였다. 콜레라톡신은 혈액 내로 IL-6 의 분비를 유도하지만 다른 분자들은 변화가 없었다. 세포 표면표시 분석에서 포자는 6 시간 처리 후에는 $Gr\text{-}1^{+}CD11b^{+}$ 세포의 유입을 72 시간 처리 후에는 $F4/80^{+}CD11b^{+}Gr\text{-}1^{-}$ 세포의 유입을 매우 강하게 유도하였다. 리포폴리사카라이드 또한 12 시간 및 24 시간 처리 후에 $Gr\text{-}1^{+}CD11b^{+}$ 세포의

유입을 유도하였다. 본 연구에서 바실러스 서브틸리스 포자가 리포폴리사카라이드 처리구와 비슷한 수준으로 폐와 기관지폐포세척액 지역으로 케모카인과 사이토카인의 발현 및 분비를 유도하는 것을 관찰하였다. 또한, 포자가 리포폴리사카라이드보다 호중구가 기관지폐포 세척액 지역으로 더 빠르게 유입하도록 촉진하는 것을 확인하였다. 이러한 결과들은 선천성 면역반응내에서 바실러스 서브틸리스 포자가 독성이 강한 리포폴리사카라이드와 동등한 반응성을 보여주며, 또한 호중구를 매개로한 면역반응에서는 보다 효과적 유도할 수 있는 가능성을 나타내고 있다. 이를 토대로 바실러스 서브틸리스 포자에서 유래한 변형된 새로운 포자 보조제 개발에 기여할 것으로 기대된다.