A Thesis for the degree of Doctor of Philosophy

The activation mechanism of Immune cells by *Bordetella bronchiseptica*-derived substances and their application study

Department of Veterinary Medicine

GRADUATE SCHOOL
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List of Abbreviations

5-FU	5-Fluorouracil
APC	allophycocyanin
B. bronchiseptica	*Bordetella bronchiseptica*
Bb-LPS	*Bordetella bronchiseptica*-derived LPS
CCK-8	cell counting kit-8
DCs	dendritic cells
DOX	doxorubicin
O26-LPS	*E. coli* O26:B6-derived LPS
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage-colony stimulating factor
IL	interleukin
LPS	lipopolysaccharide
NO	nitric oxide
O.D.	optical density
PI	propidium iodide
R-LPS	rough LPS
S-LPS	smooth LPS
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
sBb	sonicated *Bordetella bronchiseptica*
ibTLR2	TLR2 signaling inhibitors
ibTLR4	TLR4 signaling inhibitors
TLR	toll-like receptor
TNF	tumor necrosis factor
VCR	vincristine

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Part 1

Immunostimulatory mechanism of *Bordetella bronchiseptica*-derived substances on dendritic cells and the structural analysis

1. Abstract

Using bacterial-derived lipopolysaccharide (LPS) as an adjuvant (used to boost vaccine immunogenicity) is hampered by side effects, including toxicity and pyrogenicity, necessitating the development of a safer and more effective adjuvant. I have previously confirmed that sonicated *Bordetella bronchiseptica* (sBb) increases the antigen-presenting capability of dendritic cells (DCs). Moreover, I demonstrated that the *Bordetella bronchiseptica*-derived LPS (Bb-LPS) is safer than *E. coli* O26:B6-derived LPS (O26-LPS). In this study, I aimed to understand the structural characteristics of LPS and lipid A extracted from *B. bronchiseptica* and its effects on DCs. The chemical structural differences and the biological effects of Bb-LPS and O26-LPS on DCs are shown, and the profiles of the two LPS types differ. I also investigated the mechanism of action of sBbs. TNF-α production in DCs and the expression of immune-related surface markers were measured following treatment with TLR2 and 4 signaling inhibitors (ibTLR 2 and 4) TL2-C29 and CLI-095. In DCs treated with sBb and O26-LPS, ibTLR4 significantly reduced TNF- production and MHC class II and CD86 expression. These findings suggest that sBb and O26-LPS mainly induce a TLR4-dependent immune response. Taken together, this study revealed that sBb activates DCs specifically through TLR4 signaling, leading to improved antigen-presenting capability of DCs taken together. This suggests that sBb and Bb-LPS may be used as cost-effective and safe adjuvant candidates to develop vaccines against *B. bronchiseptica*–related or other diseases.

Keywords: *Bordetella bronchiseptica*; lipopolysaccharide; lipid A; vaccine adjuvant; dendritic cells

2. Introduction

Bordetella bronchiseptica (*B. bronchiseptica*) is a rod-shaped gram-negative bacterial pathogen with a broad host range that infects animals, including pigs, dogs, and rabbits (El Garch et al., 2016). Unlike *B. pertussis* and *B. parapertussis*, which cause pertussis in humans, *B. bronchiseptica* has relatively low frequency of infection in humans (Yacoub et al., 2014). *B. bronchiseptica* is responsible for atrophic rhinitis in pigs, kennel cough in dogs, and snuffles in rabbits (Mattoo et al., 2005). Rather than causing the disease alone, it collaborates with *Pasteurella multocida* to cause atrophic rhinitis in pigs. Several *B. bronchiseptica* vaccines protect against severe diseases; however, their efficacy and safety are inadequate (Yevsa et al., 2013). Therefore, immune responses to *B. bronchiseptica* recombinant protein have been investigated and found to have antigenicity and adjuvant function, causing humoral and cell-mediated immune responses (Yount et al., 2019). Furthermore, sonicated *B. bronchiseptica* bacterin was found to stimulate the production of *Mycoplasma hyopneumoniae* antigen-specific IgG and can activate the antigen-presenting capacity of dendritic cells (DCs) (Lee et al., 2020; Yim et al., 2017).

A vaccine adjuvant is a component that is added to a vaccine antigen to elicit a strong immune response, resulting in increased vaccine effectiveness and reduced vaccine dosage and production costs (Vogel, 1998). Mineral salts, oil emulsions, immune-stimulating complexes, liposomes, cytokines, and bacterial derivatives are examples of vaccine adjuvants in use or development (Sivakumar et al., 2011). Bacterial toxins, lipopolysaccharides (LPS), and DNA have all been studied as adjuvants of bacterial derivatives. Among them, LPS, a major polysaccharide of the outer membrane of

gram-negative bacteria, contributes to bacteria-host interaction and increases the immune response through Toll-like receptor (TLR) activation (Audibert et al., 1993). LPS with the active lipid A moiety can be a potent mucosal adjuvant; nevertheless, it is too toxic for vaccines. Therefore, AS04, containing 3-O-desacyl-40-monophosphoryl lipid A (MPL), was developed (Pichichero, 2008). By repeated hydrolysis, a portion of *Salmonella minnesota*–derived LPS, the (R)-3-hydroxytetradecanoyl group, and 1-phosphate were removed (Schneerson et al., 1991). MPL, like LPS, stimulates the production of cytokines but is a hundred times less toxic (Ulrich et al., 1988).

DCs are potent antigen-presenting cells that connect the innate and adaptive immune systems. DCs uptake, process, and present antigens to T cells activating naïve T cells and enhancing the immune response. The adjuvant has the important property of improving the antigen-presenting capability of DCs and inducing potent and long-lasting immunity. Therefore, determining whether an adjuvant can improve the activation and antigen presentation abilities of DCs is crucial for determining its efficacy.

E. coli-derived LPS has been extensively studied for its structure, function, and toxicity (Goldstein et al., 1992; Wu et al., 2013). However, few studies on *B. bronchiseptica*–derived substances have been conducted, particularly regarding the immune response to LPS and lipid A. Therefore, this study aimed to investigate the structural characteristics and effect of LPS and lipid A extracted from *B. bronchiseptica* on DCs. In addition, the mechanism of action of sonicated *B. bronchiseptica* was also investigated.

3. Materials and Methods

3.1. Bacteria

B. bronchiseptica CNUB1B5, derived from a measles-infected dog, was obtained from the Laboratory of Microbiology, College of Veterinary Medicine at Jeju National University. Next-generation sequencing was used to identify this strain. Bacteria were cultured in Luria-Bertani (LB) medium at 36°C. The cultured bacteria were sonicated three times for 30 s at 60% amplitude.

3.2. Bb-LPS / O26-LPS & Lipid A separation

Using an LPS extraction kit, crude LPS was extracted from *B. bronchiseptica* and identified by next-generation sequencing (Intron Biotechnology Inc., Sungnam, Korea). This procedure was performed according to the manufacturer's instructions. *Escherichia coli* O26:B6 (O26-LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). First, lipid A was isolated from LPS as previously described (Hwang et al., 2016). Briefly, 1% acetic acid was mixed with 3 mg of dried LPS and heated at 100°C for 90 min to hydrolyze. Next, 400 μL chloroform and 200 μL methanol were added and mixed after cooling at room temperature. Next, centrifugation was performed to obtain hydrophobic lipid A, and 200 μL of the lower layer (chloroform) was transferred to a new tube and dried at room temperature. Finally, dried lipid A was dissolved in 300 μL dimethyl sulfoxide (DMSO).

3.3.Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

All LPS and lipid A were dissolved in a digestion buffer containing 50 mM Tris pH 6.8, 2 mM EDTA, 1% SDS, 0.5% 2-mercaptoethanol, and 0.001% bromophenol blue

(Tsai, 1986). The samples in the digestion buffer were then heated at 100°C for 5 min. Next, the samples were fractionated on SDS-polyacrylamide gels containing 14% and 15% acrylamide for LPS and lipid A, respectively. Finally, electrophoresis was performed at 140 V for approximately 2 h, or until the tracking, dye reached the end of the gel.

3.4.Silver staining of Gels

Silver staining procedure was performed as previously described (Tsai, 1986). The samples were fixed overnight in a polyacrylamide gel in 40% ethanol-5% acetic acid and oxidized for 10 min with 0.7% periodic acid in 40% ethanol-5% acetic acid. The gel was washed thrice with deionized water for 20 min. The gel was stained for 10 min with 0.2 N ammonium hydroxide-0.02 N sodium hydroxide solution containing 0.8% silver nitrate and visualized in 0.02% formaldehyde-0.005% citric acid.

3.5. Generation of Bone marrow-derived DCs

DCs were cultured from bone marrow cells obtained from C57BL/6 mice femurs and tibias. The bone marrow cells were flushed out, depleting erythrocytes. The cells were cultured in RPMI1640 medium supplemented with 5% fetal bovine serum, 100 units/mL penicillin/streptomycin, and 2 mM L-glutamine, containing 10 ng/mL recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF; Peprotech, Cranbury, NJ, USA) and recombinant murine interleukin (IL)-4 (Peprotech). Every 2 d, the culture medium was replaced with fresh medium containing GM-CSF and IL-4. Non-adherent cells were removed on the 2nd and 4th days of culture, and the precursor cells of DCs attached to the bottom were cultured to remove lymphocytes and granulocytes. DCs were collected on days six and eight of

the experiment.

3.6. Cytokine measurement

DCs were treated with Bb-LPS, O26-LPS, Bb-Lipid A, and O26-Lipid A in 6-well plates to measure cytokine production. To inhibit TLR signaling, DCs were incubated for 2 h with 100 μM TLR2 signaling inhibitor (ibTLR2, TL2-C29; InvivoGen, San Diego, CA, USA) or 2 μM TLR4 signaling inhibitor (ibTLR4, CLI-095; InvivoGen), followed by treatment with sBb and O26-LPS. Tumor necrosis factor (TNF)-alpha and IL-12 p40 enzyme-linked immunosorbent assay (ELISA) kits (all from Invitrogen, Carlsbad, CA, USA) were used to measure cytokine production according to the instructions of the manufacturer. A microplate reader was used to determine the optical density was measured at 450 nm (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA).

3.7. Flow cytometry analysis

Flow cytometry was used to measure the expression of immune-related surface markers in DCs. DCs were treated with Bb-LPS, O26-LPS, Bb-Lipid A, or O26-Lipid A in 6-well plates. For TLR inhibition, DCs were incubated for 2 h with 100 μM ibTLR2 or 2 μM ibTLR4, followed by treatment with sBb or O26-LPS. Cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse MHC class II ($I\text{-}A^b$) antibody and allophycocyanin (APC)-labeled anti-mouse CD86 antibody (BioLegend, San Diego, CA, USA). CytoFLEX LX was used to analyze all the cells (Beckman Coulter, Brea, CA, USA).

3.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard deviation. The analysis of variance and multiple comparison tests were used to analyze the results. Statistical significance was set at $p < 0.05$.

4. Results

4.1. Analysis of LPS and lipid A profile by silver staining

A total of 1 μg and 5 μg of Bb-LPS and *E. coli* O26:B6 were loaded into a 14% SDS-polyacrylamide gel (Fig. 1), and 5 μL and 10 μL of lipid A isolated from both LPS were loaded into 15% SDS polyacrylamide (Fig. 2) followed by silver staining. The ladder patterns of LPS obtained from the different bacteria differed noticeably. The Smooth LPS pattern containing the O-antigen of O26-LPS showed a wide distribution of O-antigen size. In contrast, Bb-LPS showed a simple O-antigen pattern with a lower molecular weight than that of O26-LPS. In addition, the pattern of Rough LPS containing core and lipid A revealed that both LPS had similar molecular weights; however, the amount of Bb-LPS was obviously lesser. The molecular weight of lipid A was similar and had a very low molecular weight near bromophenol blue, with a molecular weight of approximately 0.6 kDa. The amount of lipid A extracted using the same method with the same amount of LPS also varied.

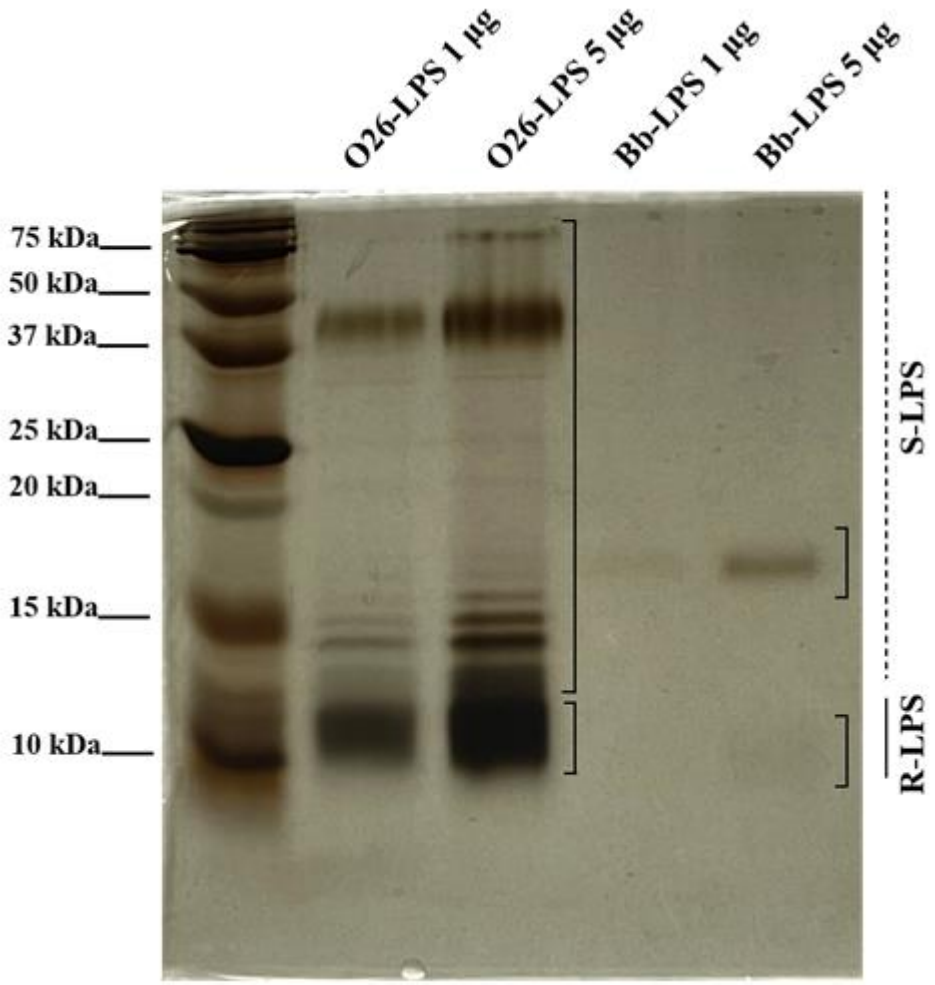


Fig. 1. Analysis of LPS profile by silver staining. A total of 1 μg and 5 μg LPS from *E. coli* and *B. bronchiseptica* dissolved in digestion buffer and loaded in a gel containing 14% acrylamide. The ladder pattern of LPS band, which is characteristic of Smooth LPS (S-LPS) containing O-antigen and Rough LPS (R-LPS) containing Core and lipid A at the bottom was shown.

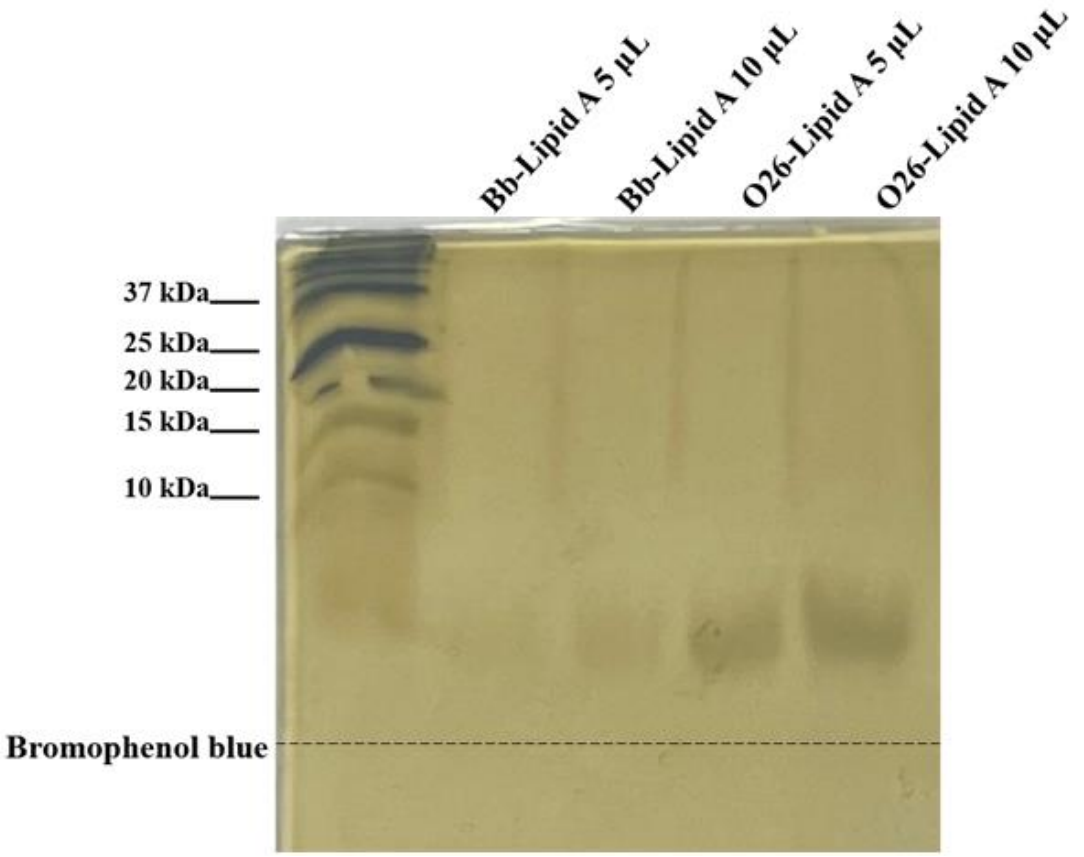


Fig. 2. Analysis of lipid A profile by silver staining. A total of 5 μL and 10 μL Lipid A from *E. coli* and *B. bronchiseptica* dissolved in digestion buffer and loaded in a gel containing 14% acrylamide. The samples were fractionated by SDS-PAGE followed by silver staining. The patterns are visible just above the bromophenol blue line.

4.2. Cytokine production of DCs treated with LPS, and Lipid A extracted from B. bronchiseptica

I measured TNF-α and IL-12 production using ELISA to determine the extent to which LPS and lipid A extracted from *B. bronchiseptica* affected cytokine production by DCs (Fig. 3). Bb-LPS significantly increased ($p < 0.0001$) TNF-α and IL-12 production at all concentrations, which was less than the same concentration of O26-LPS. Conversely, DCs treated with Bb-Lipid A produced almost no TNF-α, and produced as much IL-12 as the control group.

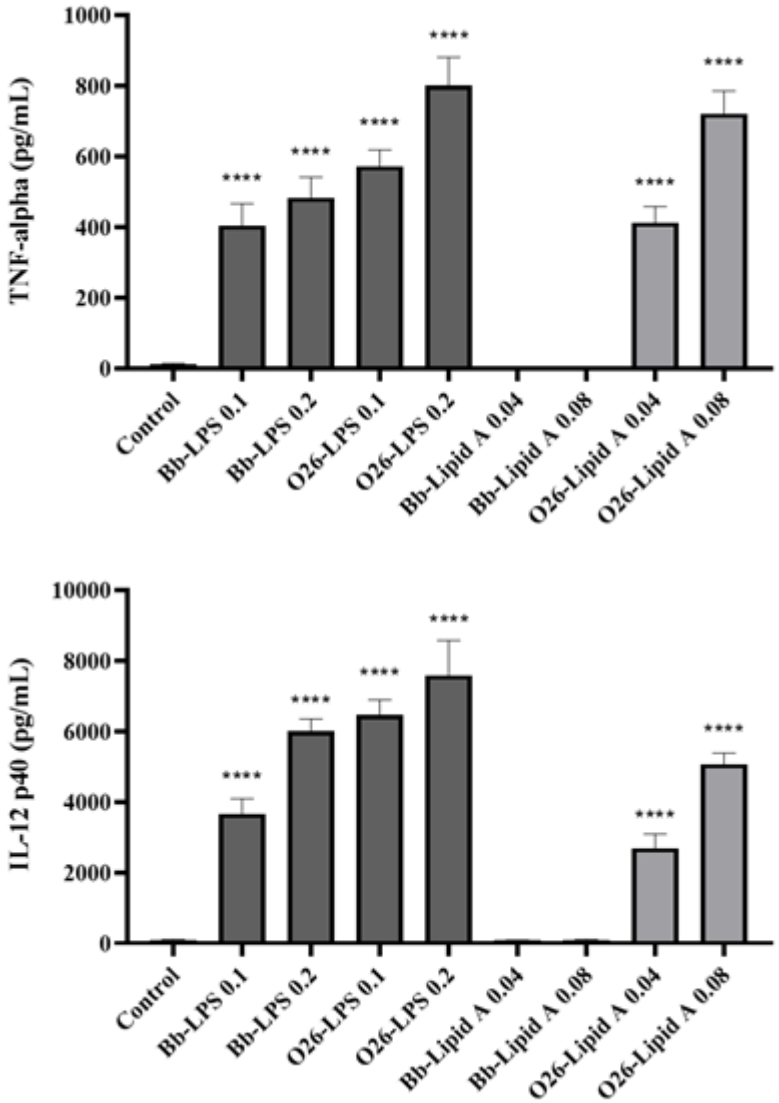
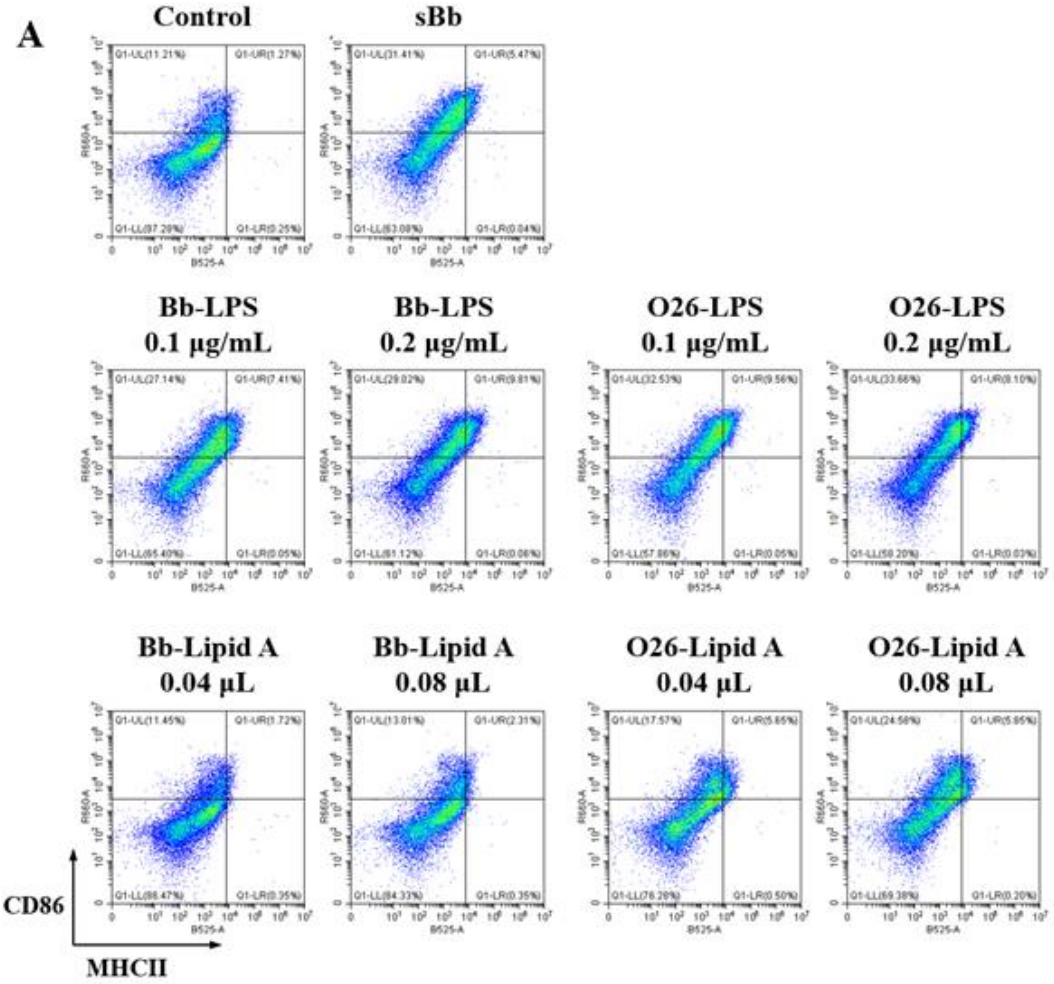


Fig. 3. LPS and Lipid A extracted from *B. bronchiseptica* reduced the cytokine production of DCs. DCs were treated with 0.1 μg and 0.2 μg of Bb-LPS and O26-LPS and 0.04 μL and 0.08 μL of Bb-lipid A and O26-lipid A. After 3 d, the supernatant of cells was harvested and used for ELISA. Data are presented as mean ± SD. **** represents $p < 0.0001$ compared to the control group.

4.3. Analysis of the expression of immune-related surface markers on dendritic cells treated with LPS and Lipid A extracted from B. bronchiseptica

Bb-LPS and O26-LPS enhanced MHC Class II expression up to 1.7-fold and 1.4-fold, respectively, and CD86 expression by 3.7-fold and 4.8-fold, compared to the control group (Fig. 4). However, Bb-Lipid A did not affect MHC class II expression and slightly increased CD86 expression, whereas the same amount of O26-Lipid A increased the expression of both markers on DCs.



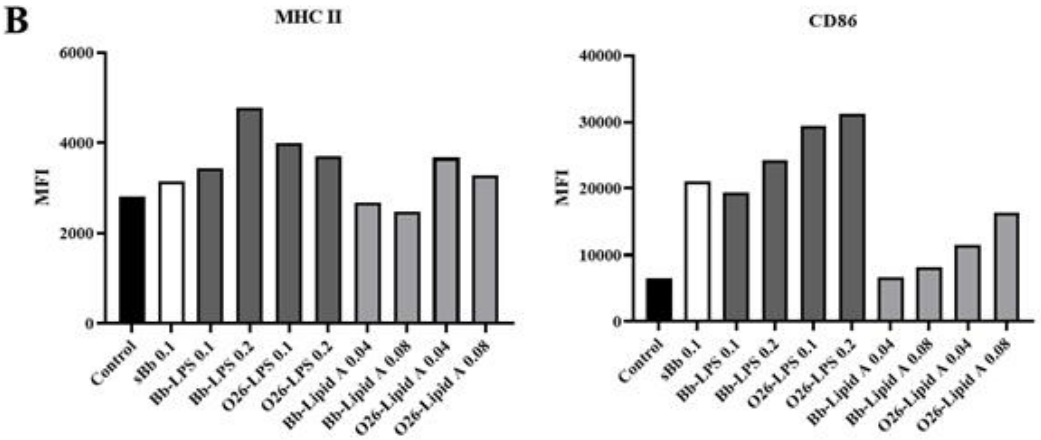


Fig. 4. Expression of immune-related surface markers on DCs treated with LPS, and Lipid A extracted from *B. bronchiseptica*. DCs were treated with 0.1 μg and 0.2 μg of LPS and 0.04 μL and 0.08 μL of Lipid A for 3 d. After treatment, DCs were stained with FITC-labeled anti-mouse MHC class II ($I\text{-}A^b$) and APC-labeled anti-mouse CD86 antibodies. (A) shows MHC class II/CD86 dot plots of cells. (B) shows the mean fluorescence intensity of cells.

4.4. TLR4 signaling inhibitor reduced sBb-induced TNF-alpha production in DCs

To confirm the pathway by which sBb increases cytokine production by DCs, I measured TNF-α production by DCs treated with TLR 2 and 4 signaling inhibitors using ELISA (Fig. 5). TLR 2 or 4 signaling inhibitors did not affect control DCs, whereas sBb and O26-LPS significantly reduced TNF-α production in DCs. Furthermore, ibTLR4 reduced TNF production more than ibTLR2, and the production was comparable to that of control DCs.

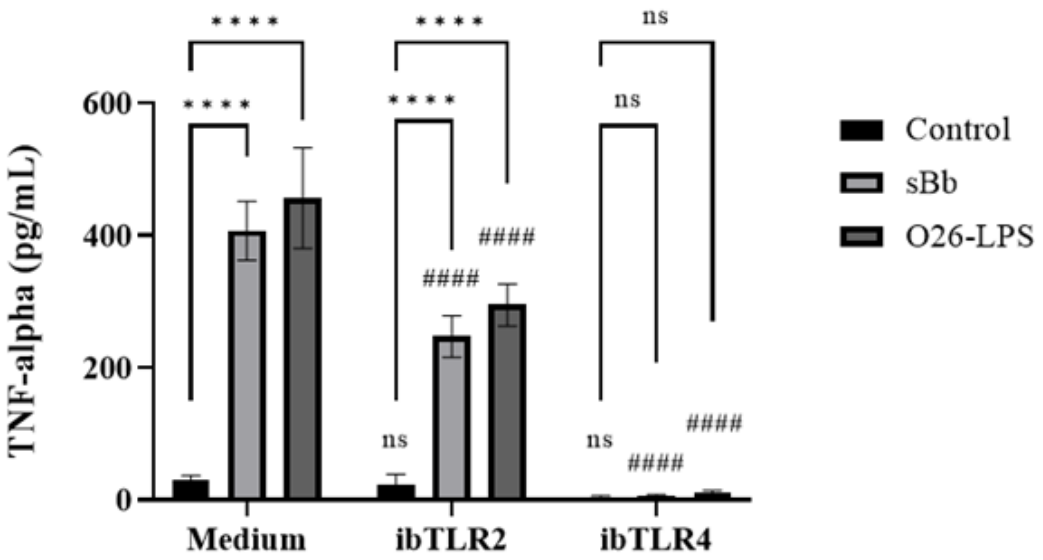


Fig. 5. TLR4 signaling inhibitor reduced sBb-induced TNF-alpha production in DCs. DCs were pre-treated with 100 μM ibTLR2 or 2 μM ibTLR4 for 2 hr. Then, DCs were treated with 0.1 μg/mL sBb and O26-LPS for 3 d. After treatment, the supernatant of cells was harvested and used for ELISA. Data are presented as mean ± SD. **** represents $p < 0.0001$ compared to the control group. ns, no significance.

4.5. TLR4 signaling inhibitor impeded sBb-induced surface marker expression on DCs

To investigate the mechanism by which sBb enhances surface marker expression on DCs, I analyzed the MHC class II and CD86 expression on sBb-treated DCs after a 2 h pre-incubation with a TLR signaling inhibitor. ibTLR4 reduced MHC class II and CD86 expression levels in sBb-treated DCs by 23% and 21%, respectively, compared to those in sBb-treated DCs without inhibitors, whereas ibTLR2 did not (Fig. 6). Furthermore, O26-LPS-treated DCs were influenced by ibTLR2 and ibTLR4. MHC class II and CD86 expression decreased by 10% with ibTLR2 and 31% with ibTLR4, respectively. These findings indicated that TLR4 signaling was required for sBb-induced surface marker expression in DCs.

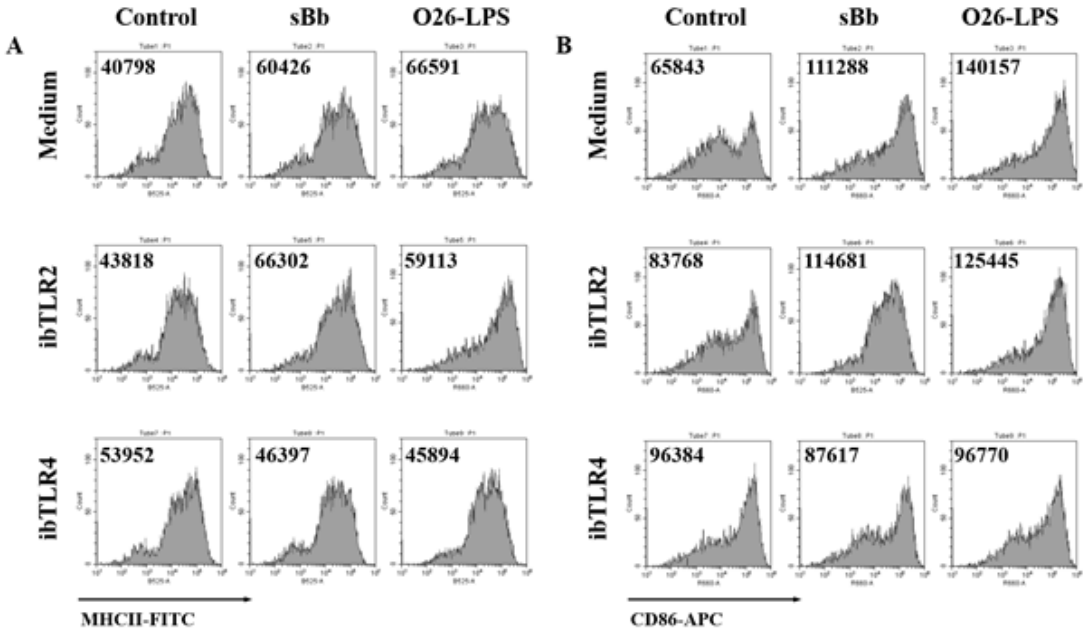


Fig. 6. TLR4 signaling inhibitor impeded sBb-induced surface marker expression on DCs. DCs were pre-treated with 100 μM ibTLR2 or 2 μM ibTLR4 for 2 hr. Then, DCs were treated with 0.1 μg/mL sBb and O26-LPS for 3 d. After treatment, DCs were stained with FITC-labeled anti-mouse MHC class II ($I\text{-}A^b$) and APC-labeled anti-mouse CD86 antibodies. The numbers in the upper left of the histograms indicate the mean fluorescence intensity of MHC class II (A) and CD86 (B).

5. Discussion

B. bronchiseptica is a gram-negative bacterium that causes infection in several animals. A vaccine for this pathogen is required because of a severe disease outbreak caused by a combination of *B. bronchiseptica* and other pathogens. Unfortunately, no effective vaccine against *B. bronchiseptica* infection has been developed to date. Therefore, the protective effects of various virulence factors and proteins in *B. bronchiseptica* have been studied. Furthermore, a previous study found that Bordetella colonization factor A has antigenic and adjuvant functions. This study revealed that sonicated *B. bronchiseptica* bacterin could be used as an adjuvant to enhance the antigen-presenting ability of DCs.

LPS is a major component of the outer membrane of Gram-negative bacteria. LPS protects bacteria from external chemical attacks and plays an important role in bacteria-host interaction by modulating immune responses (Bertani et al., 2018). LPS consists of three structural components: the O-antigen, core oligosaccharide, and lipid A. The O-antigen is a polysaccharide structure composed of oligosaccharide repeat units attached to a core oligosaccharide. Serotyping of gram-negative bacteria is possible due to the diversity of the O-antigen (Hong et al., 2014). The core structure typically consists of an inner core with a 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and heptose residues and an outer core with a tri-hexose backbone modified with various side branches (Heinrichs et al., 1998). Lipid A, located in the innermost part of LPS, consists of two glucosamines with acyl chains attached, with one phosphate added to each glucosamine (Raetz et al., 2002). Through a pathogen-associated molecular pattern, LPS can elicit potent innate immune responses. In particular, lipid

A is crucial in eliciting an inflammatory immune response, binding to the TLR4-MD2 complex by interacting with the positively charged residue of TLR4 (Park et al., 2009). Lipid A has also been reported to have adjuvant effects (Alving, 1993).

Furthermore, MPL, a form of lipid A in which the (R)-3-hydroxytetradecanoyl group and 1-phosphate have been removed, is being used as an adjuvant (Schneerson et al., 1991). Using SDS-PAGE and silver staining to investigate the profile of LPS isolated from *B. bronchiseptica* and *E. coli*, I confirmed that smooth LPS, including the O-antigen of the two LPS, showed a distinct difference, supporting the fact that O-antigen has the greatest diversity in LPS structure. Many studies have found that Lipid A is a conserved part of the LPS structure that varies depending on the strain and environmental changes. Lipid A of *E. coli* and *B. bronchiseptica* shows various acylation patterns and modified backbones not only between bacteria but also within each strain (Bouchez et al., 2017; Casabuono et al., 2019; Mangoni et al., 2008). Moreover, differences in acylation patterns of lipid A influence its immune stimulation (Li et al., 2013). The specific structural difference between Lipid A of *B. bronchiseptica* CNUB1B5 and *E. coli* O26:B6 is unknown; however, the band of Bb-Lipid A is closer to that of bromophenol blue, indicating the molecular weight of Bb-Lipid A is smaller than that of O26-Lipid A (Fig. 2).

E. coli-derived LPS and lipid A stimulated cytokine production and MHC class II and CD86 expression in DCs, whereas *B. bronchiseptica*-derived LPS had a smaller effect, and Bb-lipid A had no effect (Fig. 3 and 4). The polysaccharide of LPS has antigenicity, and it is assumed that difference in cytokine production and immune-related surface marker profiles of Bb-LPS and O26-LPS was caused by the difference between O-

antigen and Lipid A (Fig. 1 and 2). According to previous studies (Lee et al., 2020) and the above findings, Bb-LPS can activate and enhance the antigen presentation capability of DCs, whereas Bb-Lipid A exerts a lesser effect than O26-lipid A. This suggests that Bb-LPS can be used as an adjuvant, whereas other Lipid A requires MPL modification owing to lipid A toxicity. Using Bb-LPS as an adjuvant is expected to reduce the number of steps involved in manufacturing vaccine adjuvants, resulting in cost savings.

In a previous study, I revealed that sBb could be used as an adjunct candidate for vaccine production by improving the antigen presentation capability of DCs and are safer than *E. coli*-LPS. TLR signaling inhibitors were used to identify the activation pathway of DCs to determine the mechanism by which sBb activates them. DCs treated with sBb, and O26-LPS showed a significant decrease in TNF-α production and MHC class II and CD86 expression by ibTLR4. These findings suggest that sBb and O26-LPS mainly induce a TLR4-dependent immune response. Given that the positive control O26-LPS is a well-known TLR4 agonist, and ibTLR2 and ibTLR4 inhibit the MyD88-dependent pathway (Komegae et al., 2013), the decrease in cytokine production by ibTLR2 suggests that sBb and O26-LPS activate DCs through TLR2 and TLR4 or that ibTLR2 may disrupt some part of TLR4 signaling.

These findings reveal that sBb primarily activates DCs through TLR4 signaling, leading to improved antigen-presenting capability of DCs taken together. This suggests that *B. bronchiseptica*-derived LPS can be used as an inexpensive and safe adjuvant for developing vaccines against *B. bronchiseptica*–related or other diseases. However, further studies are needed to evaluate the adjuvant effects of sBb and *B.*

bronchiseptica-derived LPS *in vivo*.

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PART 2

The protective effects of sonicated *Bordetella bronchiseptica* bacterin on the immunosuppression of spleen cells induced by anticancer drugs (5-Fluorouracil, doxorubicin, and vincristine)

1. Abstract

For decades, 5-fluorouracil, doxorubicin, and vincristine have been used as chemotherapeutic drugs to treat patients with various cancers, such as breast cancer and lymphoma, and their effects on cancer have been well-documented. However, these anticancer drugs cause fatal side effects, including immunosuppression. This study aimed to investigate whether sonicated *Bordetella bronchiseptica* bacterin (sBb) can attenuate the immunosuppression of spleen cells caused by these chemotherapeutic agents and which subsets of spleen cells were affected. sBb increased the metabolic activity of spleen cells treated with the three anticancer drugs. Cell death analysis using Annexin V/propidium iodide revealed that sBb significantly decreased spleen cell death. Flow cytometry was used to examine spleen cell subsets using a surface marker-specific antibody. In addition, sBb increased nitric oxide production in anticancer drug-related spleen cells ($p < 0.0001$). Despite the pharmacological effects of anticancer drugs, many patients suffer from the fatal side effects of immunosuppression. These findings add to my understanding of the protective effects of sBb on anticancer drug-treated spleen cells.

Keywords: anticancer drugs; spleen cells; immunosuppression; *Bordetella bronchiseptica*; cytokine production

2. Introduction

5-Fluorouracil (5-FU) is an antimetabolic anticancer drug that is primarily used to treat solid tumors such as colorectal, gastric, and breast cancer (Sethy et al., 2021). Doxorubicin (DOX) is an anthracycline anticancer drug that interrupts type II topoisomerase inhibitors (Carvalho et al., 2009), and vincristine (VCR) is a vinca alkaloid anticancer drug that inhibits microtubule polymerization (Dumontet et al., 2010). These two medications are typically used to treat various cancers, particularly lymphoma (Lewis et al., 2020). However, chemotherapy with anticancer drugs reduces circulating lymphocytes and, in severe cases, causes treatment-induced lymphopenia, which affects the survival rate of patients (Ray-Coquard et al., 2009).

Bordetella bronchiseptica (*B. bronchiseptica*) is a rod-shaped gram-negative bacterium that causes respiratory infections in various animals. However, it rarely infects humans and is closely related to Bordetella pertussis (B. pertussis), which causes pertussis in humans (Gupta et al., 2019; Parkhill et al., 2003). Studies have shown that B. pertussis activates mouse dendritic cells (DCs) and macrophages through TLR2 and has a strong immunostimulatory and adjuvant effect (Dunne et al., 2015).

Mice injected with *B. bronchiseptica* were found to show a proliferative response and an induced T helper 1 cell response (Gueirard et al., 1996). A previous study found that *B. bronchiseptica* antigen-stimulated B lymphocytes by increasing the production of *Mycoplasma hyopneumoniae*-specific IgG, activating bone marrow cells, and improving immune memory response (Yim et al., 2017). In addition, I showed that *B. bronchiseptica* bacterin could be used as an adjuvant vaccine candidate to boost DC

antigen-presenting capability (Lee et al., 2020).

This study aimed to investigate whether *B. bronchiseptica* can protect spleen cells against anticancer drugs to overcome immunosuppression caused by cancer chemotherapy.

3. Materials and Methods

3.1. Cell lines

EL-4 cells (Murine T lymphoblast cell line) were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin, and 2 mM L-glutamine.

3.2. Animals and reagents

BALB/c mice were purchased from Samtaco Bio Korea (Osan, Korea) and housed in an animal facility at Jeju National University. Mice aged 8– 12 weeks were used in the experiments and all animal experiments were performed in accordance with the Institutional Guidelines for Animal Use and Care of Jeju National University (2021-0047). 5-FU, DOX, and VCR were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.3. Preparation of B. bronchiseptica

B. bronchiseptica bacterin was prepared using *B. bronchiseptica* CNUB1B5, which was identified using next-generation sequencing. *B. bronchiseptica* CNUB1B5, derived from a measles-infected dog, was obtained from the Laboratory of Microbiology, College of Veterinary Medicine, Jeju National University. Bacteria were cultured in Luria-Bertani (LB) medium at 36°C. The cultured bacteria were sonicated three times for 30 s at 60% amplitude. The protein content of sonicated *B. bronchiseptica* bacterin (sBb) was determined using the Bradford assay. Cells were treated with bacterin at a concentration of 1 μg/mL, which is the same as the concentration of lipopolysaccharide (LPS) derived from *E. coli* O26 (Sigma-Aldrich),

to activate spleen cells (Kim et al., 2016).

3.4. Preparation of spleen cells

Spleen cells were prepared according to previously established experimental methods in my laboratory (Kim and Joo, 2016). First, the spleen was harvested and physically mashed to obtain the cells. After lysing erythrocytes with ACK lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), the cells were filtered through a cell strainer (sieve size, 70 μm). Spleen cells were cultured in RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, 10 mM HEPES buffer, and 55-μM 2-mercaptoethanol at a concentration of 1×10^6 cells/mL.

3.5. Measurement of the cellular metabolic activity

Spleen cells and EL-4 cells were treated with anticancer drugs (5-FU, DOX, and VCR) and sBb for 3 d to measure cell metabolic activity. Spleen cells and EL-4 cells were cultured at 1×10^6 and 1×10^5 cells/mL concentrations, respectively. Cell counting kit-8 (CCK-8; Dojindo, Japan) solution was added at a concentration of 10 μL/well and incubated for 4 h. Optical density was measured at 450 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, USA).

3.6. Cytokine measurement

To measure the production of interleukin (IL), -2, and IL-4 in spleen cells, the cells were treated with anticancer drugs (5-FU, DOX, and VCR) and sBb for 3 d. Cytokine production was measured using enzyme-linked immunosorbent assay (ELISA) kits (Thermo Fisher Scientific) according to the manufacturer's instruction. Optical density

was measured at 450 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific).

3.7. Nitric oxide production measurement

Nitric oxide (NO) production in spleen cells was detected using Griess reagent (0.1% N-1-naphthylethylenediamine, 1% sulfanilamide, and 5% H3PO4). Spleen cells were treated with anticancer drugs (5-FU, DOX, and VCR) at 0.2 μg/mL and sBb at 1 μg/mL. Following treatment, the supernatant was mixed with the Griess reagent in a 1:1 ratio and incubated for 10 min. Nitrite levels were calculated using a sodium nitrite standard curve.

3.8. Determination of apoptotic cells

To examine spleen cell apoptosis were stained using Annexin V-fluorescein isothiocyanate (FITC; BD Biosciences; San Jose, CA, USA) and propidium iodide (PI; Sigma-Aldrich) solutions. The spleen cells were treated with anticancer drugs (5-FU, DOX, and VCR) and sBb in 24-well plates for 24 h. After treatment, the cells were harvested and washed with 1 x Annexin binding buffer (Thermo Fisher Scientific, USA). Subsequently, the cells were stained with 1 μL annexin V-FITC and 1.5 μg/mL PI solution for 10 min in the dark. The stained cells were analyzed using an LSRFortessa flow cytometer (BD Biosciences) and the FlowJo software (BD Biosciences).

3.9. Analysis of lymphocyte subsets

To analyze the population of major lymphocyte subsets in spleen cells, cells were treated with 1 μL of primary antibody (Biotin-labeled CD4, CD8, CD19 antibodies;

BD Biosciences) at 4°C for 30 min. Subsequently, cells were stained for 30 min at 4°C with 0.25 μL of secondary antibody (allophycocyanin-streptavidin; BioLegend, San Diego, CA, USA). The stained cells were analyzed using an LSRFortessa flow cytometer (BD Biosciences) and FlowJo software (BD Biosciences).

3.10. Statistical analysis

GraphPad Prism software was used for statistical analysis (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard deviation. The results were analyzed using a two-way analysis of variance and Sidak multiple comparison test (sBb-treated group vs. non-sBb-treated group). Statistical significance was set at $p < 0.05$.

4. Results

4.1. Effect of sBb in metabolic activity of spleen cells and EL-4 cells treated with anticancer drugs

A CCK-8 assay was performed to determine whether sBb affected the metabolic activity of anticancer drug-treated spleen cells and EL-4 cells (Fig. 1). At a concentration of 0–1 μg/mL in the 5-FU- and VCR-treated groups and 0–0.2 μg/mL in the DOX-treated group, sBb significantly increased the metabolic activity of spleen cells. There was no significant difference between the treatment groups at a 5 g/mL concentration. However, metabolic activity decreased significantly from 1 μg/mL in the 5-FU treated group to 0.2 μg/mL in the DOX and VCR treated groups in the case of EL-4 cells. There was no increase in the metabolic activity of sBb at any concentration.

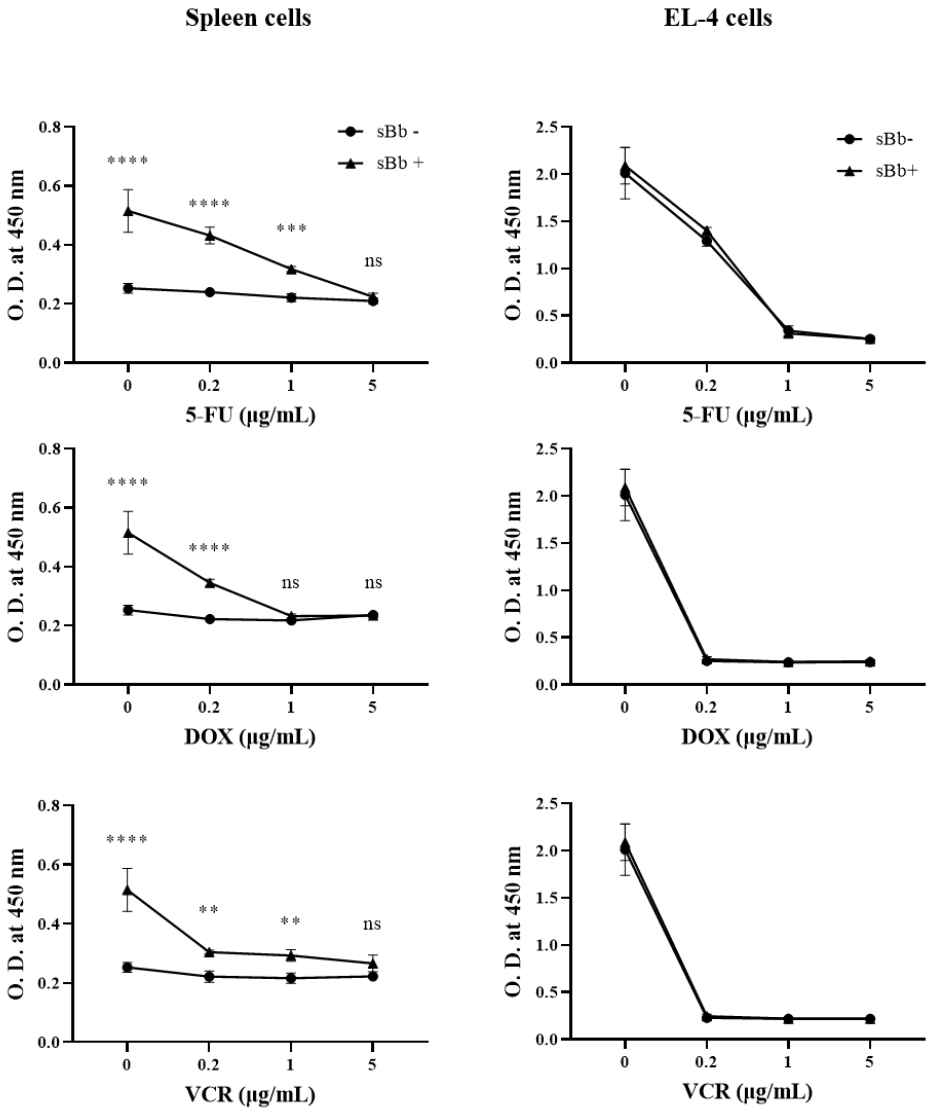

Fig. 1. sBb increases the metabolic activity of chemotherapeutic agent-treated spleen cells; however, not EL-4 cells. Spleen cells and EL-4 cells were cultured in a 96-well culture plate at a concentration of 1 x 10^6 cells/mL, 1 x 10^5 cells/mL, respectively, and treated with 0–5 μg/mL 5-FU, DOX, and VCR and 1 μg/mL sBb for 3 d. The cells were incubated with CCK-8 solution for the last 4 hr, and the optical density (O.D.) was measured at 450 nm using a microplate reader. Data are presented as mean ± SD. **, **** represents $p< 0.01$, 0.001, 0.0001, respectively, compared to the group not treated with sBb.

4.2. sBb increases the cytokine production in spleen cells treated with anticancer drugs

The production of IL-2 and IL-4 was measured using ELISA to investigate the effect of sBb on cytokine production in spleen cells treated with anticancer drugs (Fig. 2). The three anticancer drugs were treated at a concentration of 0.2 μg/mL, while sBb was treated at a concentration of 1 μg/mL. Except for IL-2 production in the VCR-treated group, sBb significantly increased IL-2 and IL-4 production in all experimental groups. However, IL-2 production was increased the most in the DOX-treated group, while IL-4 production was increased the most in the 5-FU-treated group.

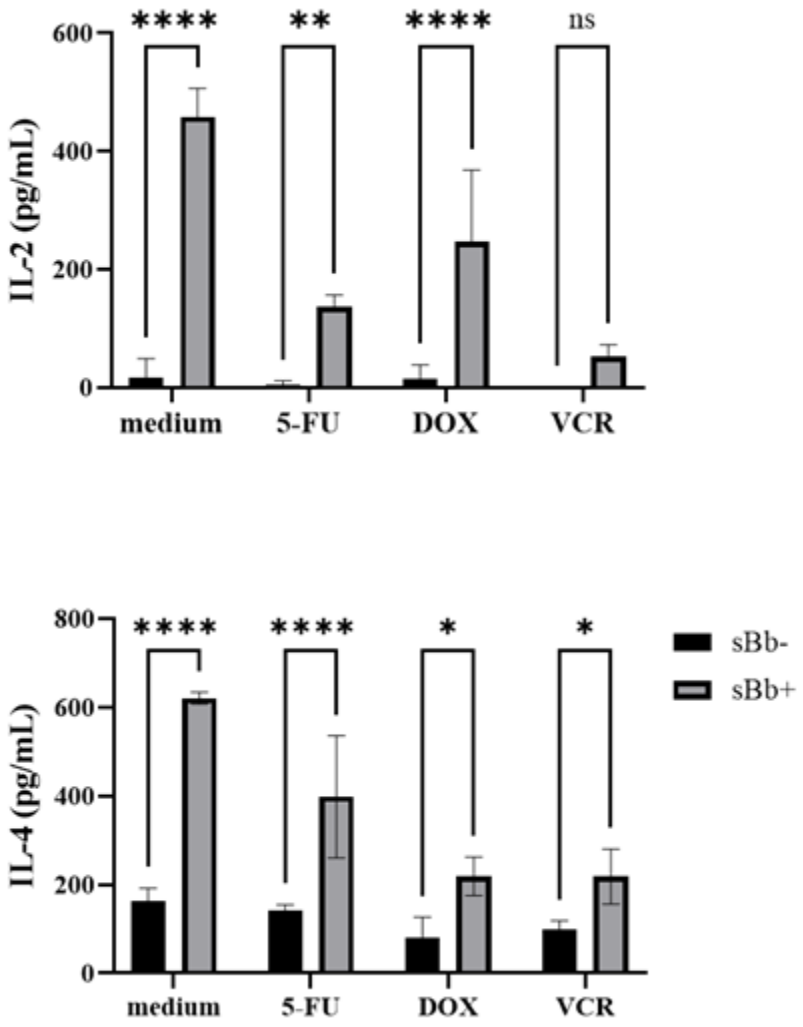

Fig. 2. Effect of sBb on the production of IL-2 and IL-4 in spleen cells. Spleen cells were treated with 0.2 μg/mL 5-FU, DOX, and VCR and 1 μg/mL sBb for 3 d. The supernatant was used for ELISA. The optical density was measured at 450 nm by using a microplate reader. Data are presented as mean ± SD. *, **, **** represents $p< 0.05$, 0.01, 0.0001, respectively, compared to the group not treated with sBb.

4.3. sBb increases NO production in spleen cells

The NO production was measured to determine whether sBb increased the activity of chemotherapy-treated spleen cells (Fig. 3). When sBb was not treated, NO production in spleen cells treated with the three anticancer drugs was not significantly different from that in the control group. However, sBb increased NO production significantly in all treatment groups, and the difference was statistically significant ($p < 0.0001$).

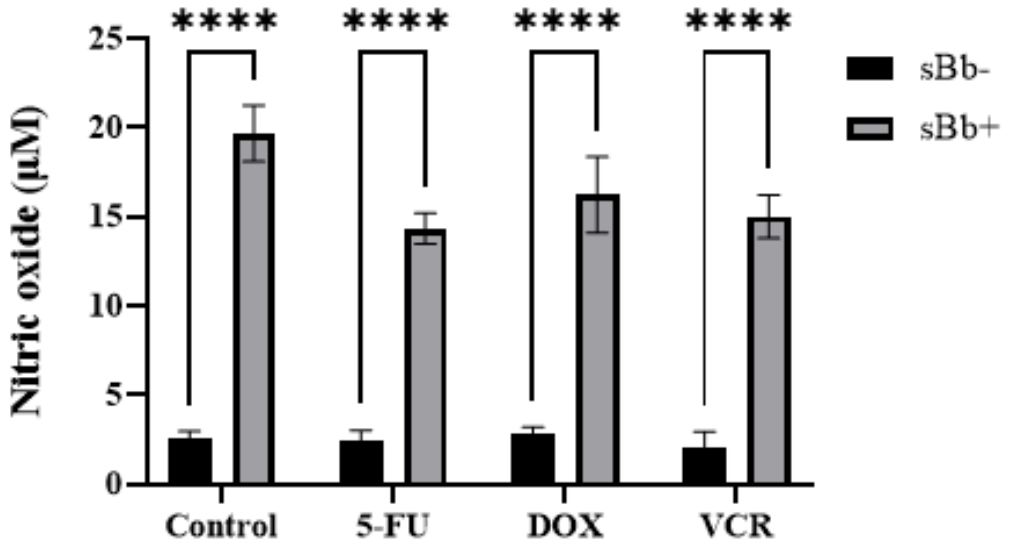

Fig. 3. sBb increases NO production in chemotherapeutic agent-treated spleen cells. Spleen cells were treated with 0.2 μg/mL 5-FU, DOX, and VCR and 1 μg/mL sBb. NO production was determined by NO assay as described in "Materials and Methods".

4.4. Effects of sBb on the apoptosis of spleen cells treated with anticancer drugs

To determine whether sBb reduces apoptosis of spleen cells caused by anticancer drugs, spleen cells were treated with 0.2 μg/mL and 1 μg/mL anticancer drugs that were effective in the metabolic activity analysis. The percentage of viable cells, cells in early/late apoptosis, and necrosis were all determined using Annexin V-FITC and PI staining (Fig. 4). sBb increased the ratio of viable cells (annexin V-/PI-) in the group treated with 1 μg/mL 5-FU, DOX, and VCR, by 8.1%, 7.5%, and 7.8%, respectively. Conversely, the percentage of apoptotic (Annexin V+) cells decreased by 10.6%, 8.0%, and 9.1%, respectively. In contrast, the ratio of necrosis (annexin V-/PI+) slightly increased by 2.5% for 5-FU, 0.5% for DOX, and 1.2% for VCR, respectively.

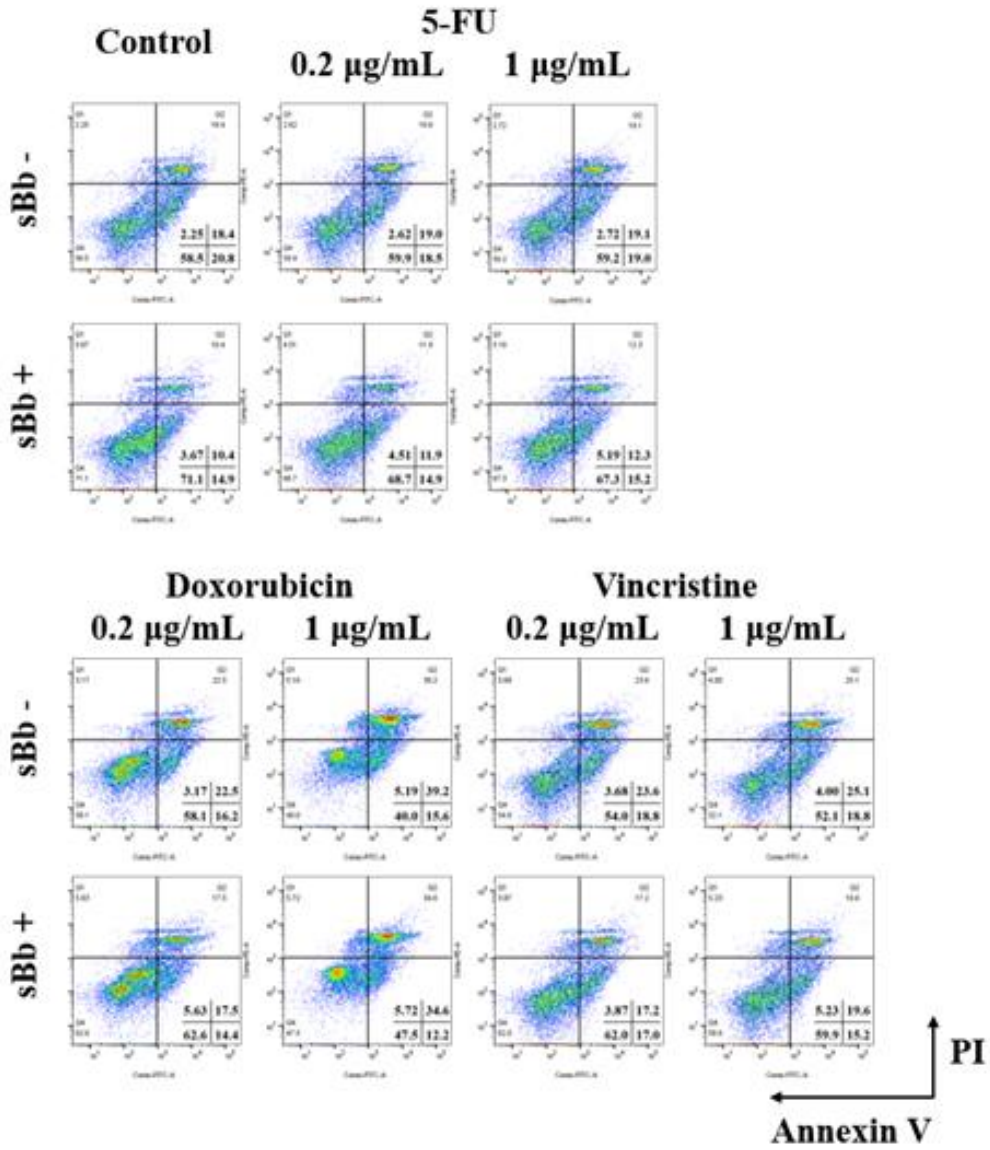

Fig. 4. sBb reduces the cell death of spleen cells caused by chemotherapeutic agents. The cell death of spleen cells was analyzed by staining with Annexin V-FITC and PI, followed by flow cytometric analysis. Quadrants indicate the percentage of the cell populations: viable cells (lower left), early apoptotic cells (lower right), late apoptotic cells (upper right), and necrotic cells (upper left).

4.5. Alteration in lymphocyte subsets in spleen cells treated with sBb

Next, I investigated whether sBb alters the lymphocyte subset (Fig. 5). When sBb was not treated, the anticancer drugs did not affect the subset compared to the control group. When sBb was used alone, the proportion of $CD4^+$ T cells decreased by 5%, while the proportions of $CD8^+$ T cells and $CD19^+$ B cells increased by 2% and 4%, respectively, compared to the control group that was not treated with sBb. However, when the anticancer drug and sBb were combined, the ratio of $CD4^+$ T cells and $CD8^+$ T cells increased, whereas the $CD19^+$ B cells decreased by 14% and 11% in the DOX and VCR-treated groups, respectively, compared to the control group with sBb treatment alone.

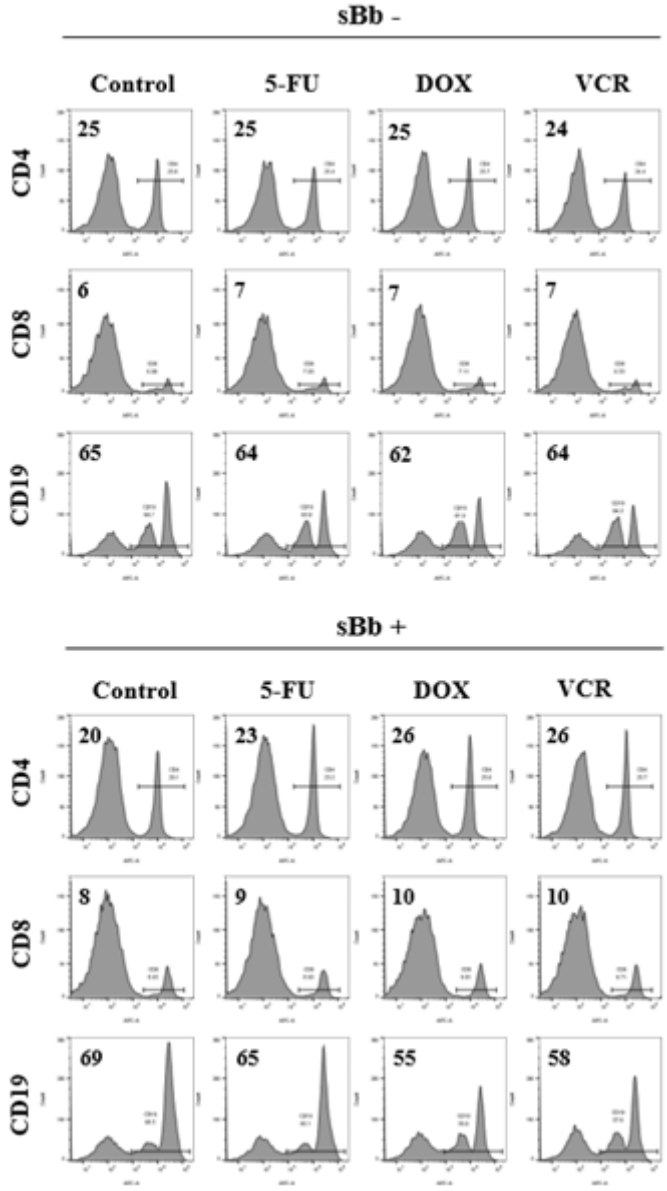

Fig. 5. Effect of sBb on the population of major lymphocyte subsets in spleen cells. Lymphocyte subsets were detected by flow cytometric analysis. A representative histogram set was presented from three independent experiments with consistency. The number in histograms indicates the percentage of $CD4^+$, $CD8^+$, and $CD19^+$ T cells in spleen cells.

5. Discussion

Several types of anticancer drugs have been developed and used; however, their side effects remain unclear. In particular, the reduction in circulating lymphocytes caused by myelosuppression significantly increases the risk of infection and affects the survival rate of patients with cancers (Ray-Coquard et al., 2009). In a previous study, *B. bronchiseptica* inoculation induced the proliferation of mouse spleen cells and affected lymphocyte function by activating bone marrow cells and enhancing the immune memory effect (Gueirard et al., 1996).

sBb significantly increased the metabolic activity of spleen cells treated with anticancer drugs, whereas in EL-4 cells, there was no increase by sBb (Fig. 1). However, the extent of the increase caused by sBb decreased as the concentration of the anticancer drug increased. These findings revealed that sBb increased the metabolic activity of spleen cells without affecting the metabolic activity of EL-4 cells, indicating that the influence of sBb is a cell-specific effect. NO production analysis revealed a similar trend to that of the CCK-8 assay.

sBb increased IL-2 and IL-4 production (Fig. 2) and reduced the rate of cell death caused by anticancer drugs, thereby increasing the proportion of viable cells (Fig. 4). IL-2 deprivation from proliferating T cells not only inhibits cell growth but also induces cell death through apoptosis (Röpke et al., 1996), and IL-4 is involved in the activation, differentiation, and death of T lymphocytes (Riou et al., 2006). It is assumed that the increase in IL-2 and IL-4 production in spleen cells by sBb led to a decrease in cell death. The ratio of $CD4^+$ cells increased in the anticancer drug + sBb treatment group, while the ratio of $CD19^+$ cells decreased compared to that in the group treated

with sBb alone. Given the increase in IL-2 and IL-4 levels, it is assumed that sBb increased $CD4^+$ and $CD8^+$ cell survival, leading to a relative decrease in the ratio of $CD19^+$ cells. These findings reveal that sBb has a protective effect on spleen cells against anticancer drug-induced damage when used in combination. Moreover, based on several studies that B7/CD28-mediated stimulation of T cells is effective in tumor therapy and as a vaccine adjuvant (Runyon et al., 2001; Sturmhoefel et al., 1999), sBb shows the possibility of inducing effective tumor immunogenicity through the CD28 pathway. However, since the response of spleen cells to sBb depends on the anticancer drug and lymphocyte subtypes, further studies are needed to determine which combination of sBb and the anticancer drug is most effective.

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Abstract in Korean

Bordetella bronchiseptica 유래물질의 면역세포 활성 기전 및 응용 연구

(지도교수 : 주 홍 구)

이 유 정

제주대학교 일반대학원 수의학과

백신 어쥬번트는 면역원성이 약한 백신 항원과 함께 사용하여 백신의 효력을 높여주는 물질이다. 개발된 많은 백신 어쥬번트 중, 세균 유래 LPS는 독성 및 발열 등의 부작용으로 인해 더욱 안전하고 효과적인 어쥬번트의 개발이 필요한 실정이다. 이전 연구에서, 사균처리된 *Bordetella bronchiseptica* (*B. bronchiseptica*) 박테린 (sBb)이 면역 반응을 시작하는 데 필수적인 수지상세포의 항원 제시 능력을 증가시키며 *B. bronchiseptica* 유래 LPS (Bb-LPS)의 면역 자극 효과가 *E. coli* O26:B6 (O26-LPS)와 정성적 및 정량적 측면에서 다르며 더 안전하다는 것을 확인했다.

본 연구에서는 *B. bronchiseptica*에서 추출한 LPS와 lipid A의 구조적 특성 및 수지상세포에 미치는 영향을 파악하고자 하였다. Bb-LPS와 lipid A는 화학적 구조의 차이를 보였으며 수지상세포의 사이토카인 및 면역 관련 표면 마커 발현을 증가시키는 효과의 차이를 보였다. 특히, Bb-LPS가 수지상세포를 활성시키는 데 반해 *B.*

bronchiseptica 유래 lipid A는 그 영향이 미미함을 알 수 있었다. 또한 sBb의 작용 메커니즘을 파악하기 위해 TLR2 및 TLR4 신호 억제제 (ibTLR2와 ibTLR4)를 수지상세포에 처리한 후 TNF-α 생산량 및 면역 관련 표면 마커의 발현을 측정했다. sBb를 처리한 수지상세포에서 ibTLR4가 TNF-α 생산량 및 면역 관련 표면 마커의 발현을 감소시켰으며, 이를 통해 sBb가 주로 TLR4를 통한 면역 반응을 유도함을 알 수 있다. 이는 TLR4를 통해 수지상세포를 활성화한 sBb는 그 자체와 유래 LPS는 면역 자극 효과를 지니면서도, lipid A로 인한 부작용이 적으며 추가적인 변형 없이도 백신 어쥬번트로 사용될 수 있음을 보여준다.

더 나아가 sBb의 면역세포 활성 효과를 통해 항암제로 인한 비장세포의 면역 억제를 줄일 수 있는지 확인해 보았다. sBb는 세 가지의 항암제 (5-Fluorouracil, doxorubicin, vincristine)를 처리한 비장세포의 대사활성도를 증가시켰으며, 이는 암세포에는 적용이 되지 않는 세포 특이적 효과였다. sBb는 비장세포의 사멸 (Annexin V+ 세포)을 감소시켰으며, 림프구의 분화 및 생존 관련 사이토카인인 IL-2와 IL-4의 생산량도 증가시켰다. 림프구 아형 분석 결과, sBb를 항암제와 같이 처리했을 때, $CD4^+$ 세포의 비율이 증가했다. 이는 sBb가 비장세포의 생존 및 분화, 활성을 통해 항암제로 인한 면역 억제를 줄여줄 수 있음을 보여준다.

결론적으로, 본 연구는 sBb 및 Bb-LPS가 *B. bronchiseptica* 관련 또는 기타 질병에 대한 백신을 개발하는 데 있어 경제적이고 안전한 어쥬번트 후보로 사용될 수 있을 뿐만 아니라 항암 치료에서의 면역보호제로도 응용될 수 있음을 시사한다.

주요어: *Bordetella bronchiseptica*, 수지상세포, 백신 어쥬번트, 비장세포, 면역 억제