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이학석사 학위논문

**Immunostimulatory activity of gamma-
irradiation-killed *Streptococcus pneumoniae*
whole cell in human bronchial epithelial cells**

감마선 조사 사멸 폐렴구균에 의한
사람 기관지 상피세포의 면역활성

2018년 8월

서울대학교 대학원

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좌민용

ABSTRACT

Immunostimulatory activity of gamma-irradiation-killed *Streptococcus pneumoniae* whole cell in human bronchial epithelial cells

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Objectives

Streptococcus pneumoniae is a major respiratory pathogen that can cause pneumonia, meningitis, and otitis media. Although capsular polysaccharide-based vaccines are commercially available, there is a need for broad-spectrum, serotype-independent, and cost-effective vaccines. Recently, an intranasal vaccine formulated with gamma-irradiated nonencapsulated *S. pneumoniae* whole cells has been introduced and its efficacy and immunogenicity is under investigation. Since innate immunity influences the subsequent adaptive immune responses, the present study investigated

the immunostimulatory activity of gamma-irradiated *S. pneumoniae* (r-SP) in the human bronchial epithelial cells by comparing with that of heat-inactivated *S. pneumoniae* (h-SP) or formalin-inactivated *S. pneumoniae* (f-SP).

Methods

To investigate the efficacy of inactivated whole-cell vaccines, nonencapsulated *S. pneumoniae* TIGR4 strain was generated and subsequently inactivated by gamma-irradiation, heat, or formalin. Human bronchial epithelial cells, BEAS-2B, were stimulated with r-SP, h-SP, or f-SP. The mRNA expression and protein secretion of interleukin (IL)-6 and IL-8 were determined using reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The mRNA expression of CCL5, CCL28, and CXCL10 was analyzed by real-time RT-PCR. Cell viability was determined by trypan blue staining and annexin V/PI staining. Toll-like receptor (TLR) 2 and TLR4 activation was determined using Chinese hamster ovary (CHO) cells overexpressing human CD14 and TLR2 (CHO/CD14/TLR2 cells) or TLR4 (CHO/CD14/TLR4 cells) by analyzing CD25 expression with flow cytometry. Phosphorylation of mitogen-activated protein (MAP) kinases and TLR2 induction were analyzed by western blot assay. To determine the intracellular signaling pathway involving MAP kinases, the cells were pretreated with U0126, SP600125, or SB203580 followed by stimulation with inactivated *S. pneumoniae*, and the expression of IL-6 and IL-8 was detected by

ELISA. Each inactivated *S. pneumoniae* was separated into soluble and insoluble components by centrifugation. Soluble components separated from each inactivated *S. pneumoniae* were detected by silver staining and Coomassie blue staining.

Results

When BEAS-2B cells were stimulated with inactivated *S. pneumoniae*, r-SP potently induced IL-6 and IL-8 at both mRNA and protein levels in a dose- and time-dependent manner compared with h-SP or f-SP, but not other chemokines, CCL5, CCL28, and CXCL10. Stimulation with inactivated *S. pneumoniae* followed by analyzing CD25 expression on CHO/CD14/TLR2 cells demonstrated that r-SP induced activation of TLR2, whereas h-SP or f-SP showed a minimal activation. In addition, CHO/CD14/TLR4 cells showed that only r-SP activated TLR4, while h-SP or f-SP did not. On the other hand, TLR2 synthesis was not induced by r-SP in BEAS-2B cells. Moreover, r-SP potently phosphorylated ERK, JNK, and p38 kinase, whereas h-SP and f-SP poorly phosphorylated them. The potent expression of IL-6 and IL-8 induced by r-SP was diminished by inhibitors for ERK, JNK, or p38 kinase, suggesting that r-SP induced IL-6 and IL-8 expression through the activation of MAP kinases. Remarkably, when r-SP was further treated with heat or formalin, there was a decrease in the expression of IL-6 and IL-8 and activation of TLR2 and TLR4. Of note, soluble components of r-SP potently induced IL-6 and IL-8 expression as much as whole r-SP compared with insoluble components of r-SP. r-SP potently released a

large amount of different soluble components compared with h-SP or f-SP.

Conclusions

The present study demonstrated that r-SP potently stimulates the human respiratory epithelial cells to produce the cytokines IL-6 and IL-8 via MAP kinase pathway by activating TLR2, which might influence the induction of adaptive immune responses and enhance vaccine efficacy.

Keywords: *Streptococcus pneumoniae*, gamma-irradiation, BEAS-2B, vaccine, innate immunity

Student Number: 2016-26545

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Chapter I. Introduction

Streptococcus pneumoniae is a Gram-positive, non-motile, and facultative anaerobic bacterium [1]. It infects humans through the respiratory tract and causes serious diseases such as pneumonia, sepsis, and meningitis [2]. Notably, these pneumococcal diseases are estimated to cause approximately two million deaths and cost hundreds of billions of dollars per year [3]. Of the many virulence factors of *S. pneumoniae*, the capsular polysaccharides are the major virulence factor and the main target of pneumococcal vaccines [4]. So far, 97 different *S. pneumoniae* serotypes have been identified based on capsular polysaccharide structures [5]. One of the first commercialized pneumococcal vaccines is formulated with capsular polysaccharides purified from 23 prevalent serotypes of *S. pneumoniae* which can effectively induce protective immunity largely in adults [6]. However, since capsular polysaccharides induce T cell-independent immune responses, the development of long-term memory is difficult and the vaccine shows weak immunogenicity in elderly people and children under two years of age [7]. To overcome these limitations, pneumococcal conjugate vaccines were developed by conjugating protein carriers to several capsular polysaccharides [8]. These vaccines led to T cell responses and increased immunogenicity, but still have limitations such as serotype coverage and high cost of manufacturing [9].

Among the manufacturing methods, inactivated whole-cell vaccines could potentially overcome the limitations of the commercially available pneumococcal vaccines. Whole-cell vaccines are considered to be serotype-independent and cost-

effective [10]. However, bacterial inactivation methods, usually heat or formalin treatment, can cause physical damage to the molecular structures of bacterial key epitopes [11, 12]. Thus, an alternative inactivation method that could maintain most of the antigens of live bacteria needs to be developed. Gamma-irradiation is a type of electromagnetic radiation caused by the radioactive decay of atomic nuclei [13]. When bacteria are gamma-irradiated, gamma-irradiation, which mainly targets bacterial nucleic acids, causes less damage to surface antigenic proteins [14]. Therefore, gamma-irradiation might be one of the promising methods to prepare inactivated whole-cell vaccines.

We previously have shown that mucosal immunization can induce both mucosal and systemic immunity, whereas immunization by non-mucosal routes induce systemic immunity with marginal mucosal immunity [15]. Mucosal tissues are sites of intense immunological activity [16]. The respiratory epithelium, one of these mucosal tissues, plays an important role in pulmonary innate immune responses [17]. Thus, if *S. pneumoniae* vaccines are administered intranasally, immune responses would be initiated in the respiratory epithelium, and allow effective prevention of the infection at the mucosal site [18]. The stimulated respiratory epithelium is known to secrete various soluble mediators which are involved in innate immune responses [19]. Therefore, it plays a leading role in activating innate immune responses against mucosal vaccines, by secreting cytokines and chemokines.

Among various cytokines and chemokines, IL-6 and IL-8 play important roles in adaptive immune responses. IL-6 activates the differentiation of B cells into antibody-secreting cells and promotes both expansion and activation of T cells [20]. Furthermore, IL-6 is an important cytokine that effectively induces Th17 responses *in vivo* which is an essential cell type for clearance of *S. pneumoniae* in the upper respiratory tract [21]. IL-8 induces the recruitment of T cells which is crucial for cell-mediated immunity [22, 23]. Moreover, IL-8 activates $\gamma\delta$ T cells, which are the predominant cell type in newborns, who require pneumococcal vaccination [24]. In addition, $\gamma\delta$ T cells have been suggested to play an important role in pneumococcal vaccine-mediated protection against *S. pneumoniae* infection [25]. Since IL-6 and IL-8 would be important in pneumococcal vaccine responses, in this study, we investigated the immunostimulatory activity of gamma-irradiated *S. pneumoniae* in a human bronchial epithelial cell line, BEAS-2B, by comparing with heat- or formalin-inactivated *S. pneumoniae*.

Chapter II. Materials and Methods

2.1. Reagents and chemicals

Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium were purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA) and HyClone, respectively. Tryptic soy broth (TSB), Todd-Hewitt broth (THB) and Bacto™ agar were from BD Biosciences (San Diego, CA, USA). FITC-conjugated annexin V, Propidium Iodide (PI), and APC-conjugated anti-CD25 monoclonal antibodies were obtained from BioLegend (San Diego, CA, USA). annexin V-binding buffer were from BD Biosciences. Rabbit polyclonal antibodies specific to ERK, phospho-ERK (p-ERK), JNK, phospho-JNK (p-JNK), p38 kinase, and phospho-p38 kinase (p-p38) were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse polyclonal antibodies specific to TLR2 and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG were obtained from Southern Biotech (Birmingham, AL, USA). An ERK inhibitor, U0126, a JNK inhibitor, SP600125, a p38 kinase inhibitor, SB203580 were purchased from Calbiochem (La Jolla, CA, USA).

2.2. Generation of nonencapsulated pneumococcal strain

Nonencapsulated *S. pneumoniae* TIGR4 strain was generated as previously described [26]. Briefly, the upstream flanking region of *cps2B* was amplified by

polymerase chain reaction (PCR) using primers Cps4BKO-UpF (5'-AAC TCG AGT GGA TAT CAA TTA CTA T-3') and Cps2BKO-UpR (5'-TTA AGC TTT CAT CTA CCC TCC ATC-3') followed by digestion with *Xho*I and *Hind*III. The downstream flanking region was amplified by PCR using primers Cps4CKO-DnF (5'-AAG AAT TCT GGT AAA AGA CTA CCG TG-3') and Cps2CKO-DnR (5'-TTG AAT TCT ATT TCA ACT TAC CCA AG-3') followed by digestion with *Eco*RI. PCR fragments were ligated into multiple cloning site of pE326 [27]. Then, pE326 containing flanking regions of *cps2B* (pKO-CPS2B) were introduced into TIGR4 by natural transformation as described previously [28]. Deletion of capsular polysaccharides was confirmed by ELISA as described previously [29].

2.3. Preparation of gamma-irradiated *S. pneumoniae* (r-SP), heat-inactivated *S. pneumoniae* (h-SP), and formalin-inactivated *S. pneumoniae* (f-SP)

Nonencapsulated *S. pneumoniae* TIGR4 strain was cultured in trypticase soy broth (TSB) at 37°C to mid-log phase. After suspension with phosphate-buffered saline (PBS), bacterial suspension was spotted on TSB agar plate and further incubated at 37°C for 2 days. The number of CFU was measured by triplicate. Bacterial cells were irradiated with 4 kGy of gamma-irradiation using cobalt-60 gamma-ray irradiator (AECL, IR-79, Nordion) for preparation of r-SP. Formalin- and heat-inactivation was performed as described previously [30]. Briefly, for preparing f-SP, the bacterial cells were treated with 0.2% formaldehyde in PBS for 4 h. Then, the cells were washed twice with PBS to remove residual formaldehyde. To prepare h-SP, the cells

were incubated at 65°C for 2 h. Bacterial inactivation was ascertained by plating on Todd Hewitt agar containing 5% yeast extract at 37°C for 3 days. No colonies were observed (data not shown). The preparation of each inactivated *S. pneumoniae* was illustrated on Fig 1.

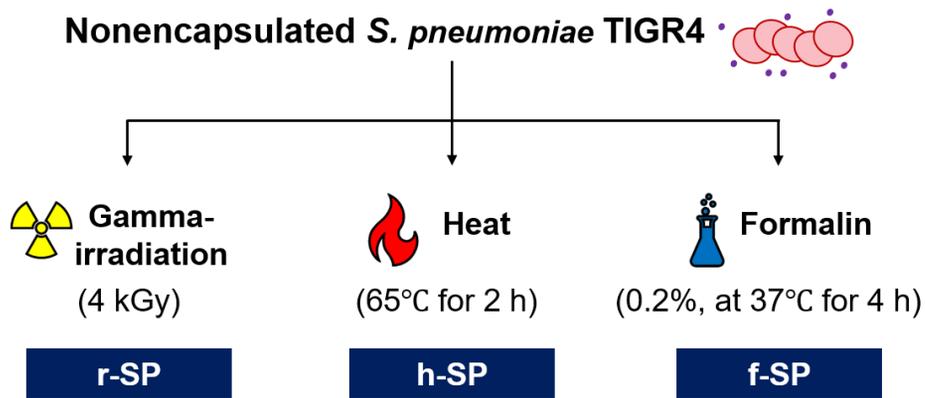


Figure 1. Preparation of r-SP, h-SP, or f-SP. Nonencapsulated *S. pneumoniae* TIGR4 cells were suspended in PBS and used to prepare each vaccine candidate. The bacterial cells were irradiated with 4 kGy of gamma-irradiation to prepare r-SP. To prepare h-SP, the bacterial cells were incubated at 65°C for 2 h. The bacterial cells were treated with 0.2% formalin at 37°C for 4 h to prepare f-SP.

2.4. Cell culture

BEAS-2B cells were purchased from the American Type Culture Collection (ATCC) (Mannas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were NF-κB reporter cell lines, which express CD25 on the cell membrane proportionally to the activation of TLR2 or TLR4, as previously described [31]. The cells were cultured in Ham's F-12 medium containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mg/ml G418 (Invitrogen, Carlsbad, CA, USA), and 0.4 mg/ml hygromycin B (Invitrogen) at 37°C in a humidified incubator with 5% CO₂.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from BEAS-2B cells was extracted by Trizol reagent (Invitrogen) and the concentration of total RNA was measured with a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was reverse-transcribed from 1 µg of total RNA using random hexamers (Promega, Madison, WI, USA) and reverse transcriptase (Promega). Then, cDNA was amplified by PCR with 0.5 units of *rTaq* DNA polymerase and 10 pmol primers specific to human IL-6 (forward primer: 5'-AGC GCC TTC GGT CCZ GTT GC-3', reverse primer: 5'-TGT GGT TGG GTC AGG GGT GG-3'), human IL-8 (forward primer: 5'-TCT GCA GCT CTG TGR GAA GG-3', reverse primer: 5'-TGA ATT CTC AGC CCT CTT CAA-3'), or GAPDH (forward primer: 5'-GTG GTG GAC CTG ACC TGC-3', reverse primer: 5'-TGA GCT TGA CAA AGT GGT CG-3') with

32 cycles for human IL-6 and IL-8, and 25 cycles for GAPDH. Equal amounts of PCR products were electrophoresed on 1% agarose gels containing Safeview (Abm, Richmond, BC, Canada). The band intensity was quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The mRNA expression of CCL5 (forward primer: 5'-GAA AGA ACC GCC AAG TGT GT-3', reverse primer: 5'-GTA GAA TCT GGG CCC TTC AA-3'), CCL28 (forward primer: 5'-GCT GAT GGG GAT TGT GAC TT-3', reverse primer: 5'-GTT TCG TGT TTC CCC TGA TG-3'), CXCL10 (forward primer: 5'- GAT GTT CTG ACC CTG CTT CA-3', reverse primer: 5'-GAA AGA ATT TGG GCC CCT TG-3'), or GAPDH (forward primer: 5'-AAG GTG AAG GTC GGA GTC AA-3', reverse primer: 5'-ATG ACA AGC TTC CCG TTC TC-3') was determined by using real-time RT-PCR as described previously [32].

2.6. Enzyme-linked immunosorbent assay (ELISA)

BEAS-2B cells were plated in a 48-well culture plate at 1×10^5 cells/ml and cultured overnight. Then, the supernatant of each well was discarded and fresh DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin was added. The cells were stimulated with r-SP, h-SP, or f-SP for 24 h. Then, the cell culture supernatants were collected and the induction of IL-6 and IL-8 was measured with commercial ELISA kits (Biolegend), according to the manufacturer's instructions.

2.7. Cell viability analysis using trypan blue staining and annexin

V/propidium iodide (PI) staining

BEAS-2B cells (1×10^5 cells/ml) were seeded in a 48-well culture plate and incubated overnight. Then, the cells were stimulated with r-SP, h-SP, or f-SP for 24 h. After the culture supernatant of each well was discarded, the cells were detached by trypsin-EDTA at 37°C for 3 min. For trypan blue staining, fresh media were added to inactivate trypsin-EDTA immediately and the suspended cells were mixed with trypan blue solution for enumeration by microscopic analysis. For annexin V/PI staining, the cells were washed twice with PBS and resuspended in 100 μ l of annexin V-binding buffer. After addition of 5 μ l of annexin V and 5 μ l of PI, the cells were incubated at room temperature for 15 min in the dark. Then, 400 μ l of annexin V-binding buffer were added to the cells. The cell viability was analyzed by flow cytometry within 1 h.

2.8. Western blot analysis

BEAS-2B cells (1.5×10^5 cells/ml) were seeded in a 6-well culture plate and incubated until fully confluent. The cells were stimulated with 3×10^7 CFU/ml of r-SP, h-SP, or f-SP for 30 min. After the stimulation, the cells were washed with PBS and lysed in 50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 150 mM NaCl with 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1 mM PMSF, 1 mM Na_3VO_4 , and 1 mM NaF for proteinase inhibition. After bicinchoninic acid (BCA) assay using Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), 20 μ g of protein was separated by 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membrane (Milipore, Bedford, MA, USA). Then, the

membrane was blocked with 5% skim milk at room temperature for 1 h on a shaker and incubated overnight at 4°C with primary antibodies (1:1000) against phosphorylated MAP kinases, MAP kinases, or β -actin. After washing with Tris-buffered saline supplemented with 0.05% Tween 20 (TBST) three times, the membrane was incubated with HRP-conjugated secondary antibodies (1:1000) at room temperature for 1 h. The membrane was washed with TBST and immunoreactive bands were visualized by Chemi Doc MP (Bio-Rad, Hercules, CA, USA) with SUPEX ECL solution (Neuronex, Pohang, Korea). The band intensities were analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD).

2.9. Flow cytometric analysis

To measure NF- κ B activation through TLR2 or TLR4, we used CHO/CD14/TLR2 or TLR4 cells as previously described [30, 33]. Briefly, CHO/CD14/TLR2 or CHO/CD14/TLR4 cells (5×10^5 cells/ml) were seeded in a 96-well plate and incubated overnight. The cells were stimulated for 24 h and stained with APC-conjugated monoclonal antibodies specific to CD25 for 30 min on ice followed by analysis with FACS Verse flow cytometer (BD Biosciences, San Diego, CA, USA) and FlowJo software (TreeStar, San Carlos, CA, USA).

2.10. Visualizing proteins on SDS-PAGE gel by Coomassie blue staining and silver staining

Soluble components of inactivated *S. pneumoniae* were loaded into each well and run at 120 V for 3 h in 10% SDS-PAGE gels. For Coomassie blue staining, the SDS-PAGE gel was stained with Coomassie blue solution containing 0.1% Coomassie blue R-250, 10% acetic acid, and 45% methanol at room temperature for 1 h on a shaker. Then, the gel was treated with de-staining solution containing 10% methanol and 10% acetic acid at room temperature on a shaker overnight. For silver staining, the SDS-PAGE gel was incubated with fixation solution composed of 50% methanol, 12% acetic acid, and 0.0185% CH₂O for 1 h. After washing twice with 50% ethanol for 20 min each, the gel was treated with sensitizing solution containing 0.02% Na₂S₂O₃·5H₂O for 1 min. Then, the gel was washed three times with distilled water for 20 sec each, after which the gel was treated with silver reaction solution composed of 0.2% AgNO₃ and 0.027% CH₂O for 1 h. Subsequently, after washing twice with non-pyrogenic water for 20 sec each, the gel was treated with developing solution containing 6% Na₂CO₃, 0.0004% Na₂S₂O₃·5H₂O, and 0.0185% CH₂O for 5-10 min. When the desired intensity of band was obtained, the gel was washed and incubated with stopping solution composed of 50% methanol and 12% acetic acid to stop the silver reaction.

2.11. Preparation of soluble and insoluble components of inactivated *S. pneumoniae*

Soluble and insoluble components of inactivated *S. pneumoniae* were separated by centrifugation of r-SP, h-SP, and f-SP at 6,090 × g for 10 min. Soluble components were collected as supernatants and the remaining pellets, the insoluble components,

were reconstituted to an equal volume as soluble components in PBS. Soluble and insoluble components of r-SP, h-SP, and f-SP were each aliquoted and stored at -80°C.

2.12. Statistical analysis

All experiments were performed at least three times. Statistical analysis was conducted using Student's *t*-test by comparing stimulated groups with the control group. An asterisk (*) indicates a significant difference between a stimulated group and the control group at $P < 0.05$. Mean values + standard deviation (SD) were determined from triplicate samples for each experimental group.

Chapter III. Results

3.1. r-SP induces the mRNA expression of IL-6 and IL-8 more potently than h-SP or f-SP in BEAS-2B cells

Respiratory epithelial cells encounter intranasal vaccines at the forefront [34]. Notably, IL-6 and IL-8 produced from these cells have a strong influence on vaccine efficacy [35, 36]. To compare IL-6 and IL-8 expression, BEAS-2B cells were stimulated with different doses of r-SP, h-SP, or f-SP at various time points and the mRNA expression of cytokines was analyzed by RT-PCR. As shown in Fig. 2A, r-SP dose-dependently induced the expression of IL-6 and IL-8 mRNA in BEAS-2B cells. Compared with r-SP, h-SP or f-SP hardly induced IL-6 and IL-8 mRNA expression. During the time course, r-SP more potently induced IL-6 and IL-8 mRNA compared with h-SP or f-SP (Fig. 2B). However, there was no difference in the expression of other chemokines, including CCL5 (Fig. 2C), CCL28 (Fig. 2D), and CXCL10 (Fig. 2E), by BEAS-2B cells stimulated with r-SP, h-SP, or f-SP. The IL-6 and IL-8 mRNA expression through dose and time course were triplicated and represented by graph (Fig 3A, B). These results suggest that r-SP efficiently induces IL-6 and IL-8 production in BEAS-2B cells.

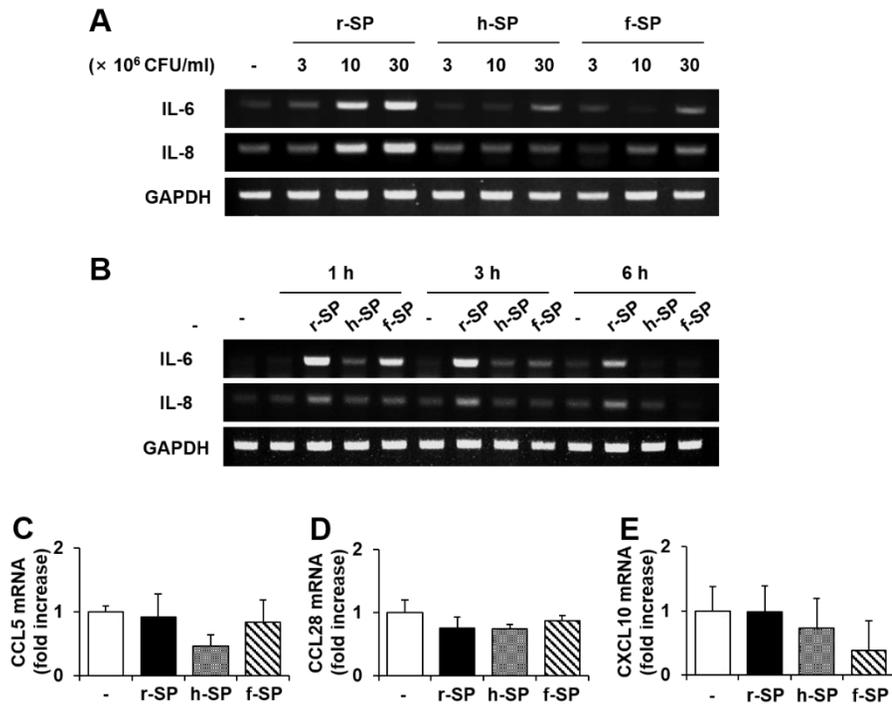


Figure 2. r-SP induces mRNA levels of IL-6 and IL-8, but not CCL5, CCL28, or CXCL10, more potently than h-SP or f-SP in BEAS-2B cells. (A, B) BEAS-2B cells (1.5×10^5 cells/ml) were stimulated with (A) 3, 10, or 30×10^6 CFU/ml of r-SP, h-SP, or f-SP for 3 h or (B) 3×10^7 CFU/ml of r-SP, h-SP, or f-SP for 1, 3, or 6 h. Then, total RNA was extracted and mRNA expression of IL-6 and IL-8 was determined by RT-PCR. (C-E) BEAS-2B cells (1.5×10^5 cells/ml) were treated with 3×10^7 CFU/ml of r-SP, h-SP, or f-SP for 3 h. Then, total RNA was extracted and mRNA expression of (C) CCL5, (D) CCL28, or (E) CXCL10 was determined by real time PCR. Each graph is based on the results of three independent experiments. - denotes non-treatment.

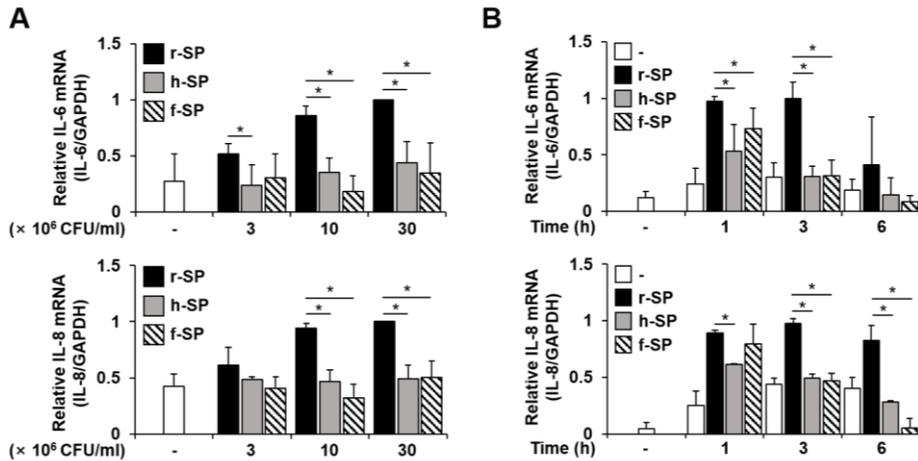


Figure 3. r-SP potently induces IL-6 and IL-8 mRNA expression in BEAS-2B cells. BEAS-2B cells (1.5×10^5 cells/ml) were stimulated with (A) 3, 10, or 30×10^6 CFU/ml of r-SP, h-SP, or f-SP for 3 h or (B) 3×10^7 CFU/ml of r-SP, h-SP, or f-SP for 1, 3, or 6 h. Then, total RNA was extracted and mRNA expression of IL-6 and IL-8 was determined by RT-PCR. Each graph is based on the results of three independent experiments. An asterisk indicates $P < 0.05$. - denotes non-treatment.

3.2. r-SP induces the protein expression of IL-6 and IL-8 more potently than h-SP or f-SP in BEAS-2B cells

To compare the protein expression level of IL-6 and IL-8, BEAS-2B cells were stimulated with r-SP, h-SP, or f-SP and cytokine secretion was measured by ELISA. As shown in Fig. 4A, BEAS-2B cells secreted IL-6 and IL-8 dose-dependently when stimulated with r-SP, h-SP, or f-SP. Particularly, a significant difference between r-SP and h-SP or f-SP to induce IL-6 and IL-8 expression was observed when the cells were stimulated with 1×10^7 to 3×10^7 CFU/ml of inactivated *S. pneumoniae*, which did not affect cell viability (Fig. 5A, B). Moreover, the secretion of IL-6 and IL-8 gradually increased from 3 to 24 hours after r-SP stimulation (Fig. 4B). Taken together, r-SP more potently induces IL-6 and IL-8 both at the mRNA and protein levels than h-SP or f-SP in BEAS-2B cells.

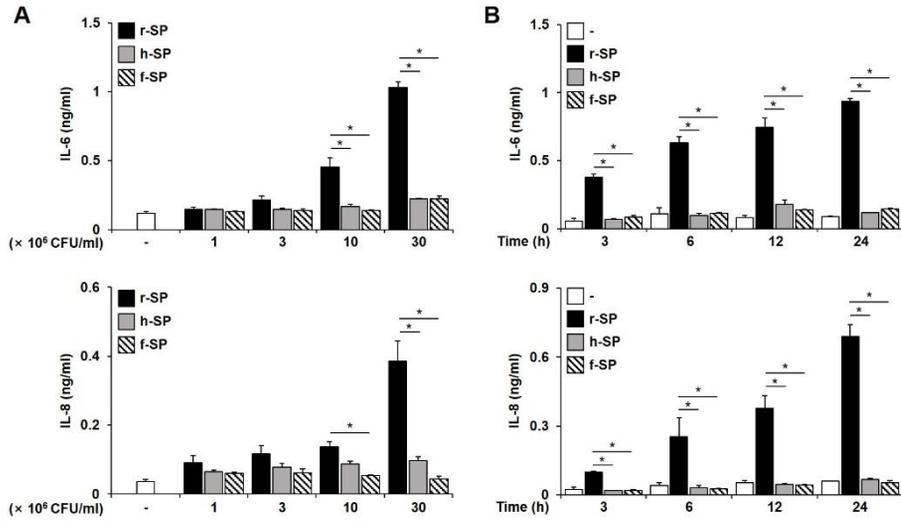


Figure 4. r-SP induces IL-6 and IL-8 secretion more potently than h-SP or f-SP in BEAS-2B cells. BEAS-2B cells (1×10^5 cells/ml) were treated with (A) the indicated concentrations of r-SP, h-SP, or f-SP for 24 h or (B) 3×10^7 CFU/ml of r-SP, h-SP, or f-SP for 0, 3, 6, 12, or 24 h. Then, the levels of IL-6 and IL-8 in the culture supernatants were measured by ELISA. The data are the mean values + S.D. and statistical differences between experimental groups were analyzed by Student's *t*-test. An asterisk indicates $P < 0.05$. Data shown are representative of three independent experiments. - denotes non-treatment.

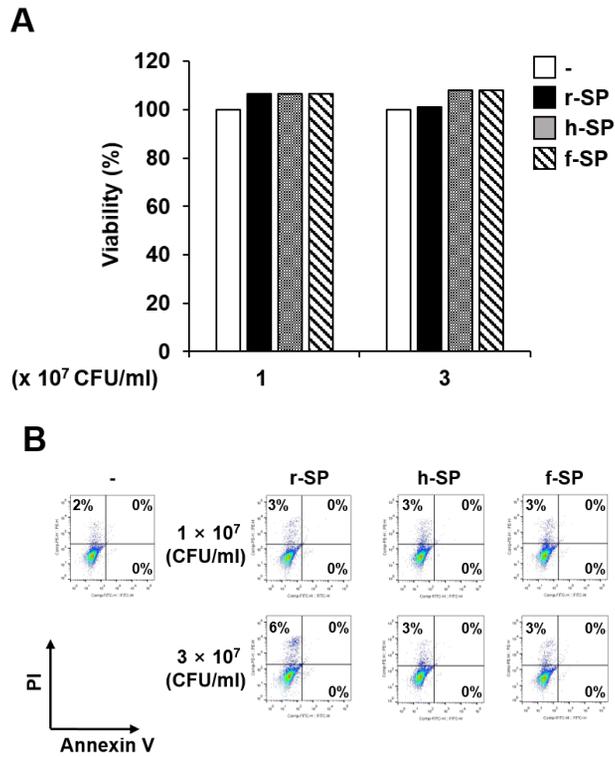


Figure 5. Effect of inactivated *S. pneumoniae* on cell viability in BEAS-2B cells. BEAS-2B cells (1×10^5 cells/ml) were stimulated with 1 or 3×10^7 CFU/ml of r-SP, h-SP, or f-SP for 24 h. After the stimulation, the cell viability was evaluated by (A) trypan blue staining followed by enumerating through microscopic analysis or (B) annexin V/PI staining followed by flow cytometric analysis. - denotes non-treatment.

3.3. r-SP induces the activation of TLR2 more potently than h-SP or f-SP

TLR signaling in respiratory epithelial cells plays important roles in initiating the innate immune response [37]. In host cells, TLR2, rather than TLR4, is an important pattern recognition receptor against Gram-positive bacteria including *S. pneumoniae* [38, 39]. Furthermore, TLR2 is essential for a whole-cell vaccine to effectively induce antibody responses [26]. Thus, to investigate the signaling pathways that the differently-inactivated *S. pneumoniae* activates, the activation of TLR2 or TLR4 was measured using CHO/CD14/TLR2 or CHO/CD14/TLR4 cells, respectively. All of the inactivated *S. pneumoniae* induced activation of CHO/CD14/TLR2 cells to display CD25 expression in a dose-dependent manner (Fig. 6A). Of note, r-SP induced higher TLR2 activation compared with h-SP or f-SP. Interestingly, CHO/CD14/TLR4 cells displayed a slight expression of CD25 by r-SP, but not by h-SP or f-SP (Fig. 6B). However, r-SP could not enhance the amount of intracellularly synthesized TLR2 in BEAS-2B cells (Fig. 7A, B). Therefore, these results suggest that r-SP more potently induces TLR2 and TLR4 activation, not synthesis, than h-SP or f-SP.

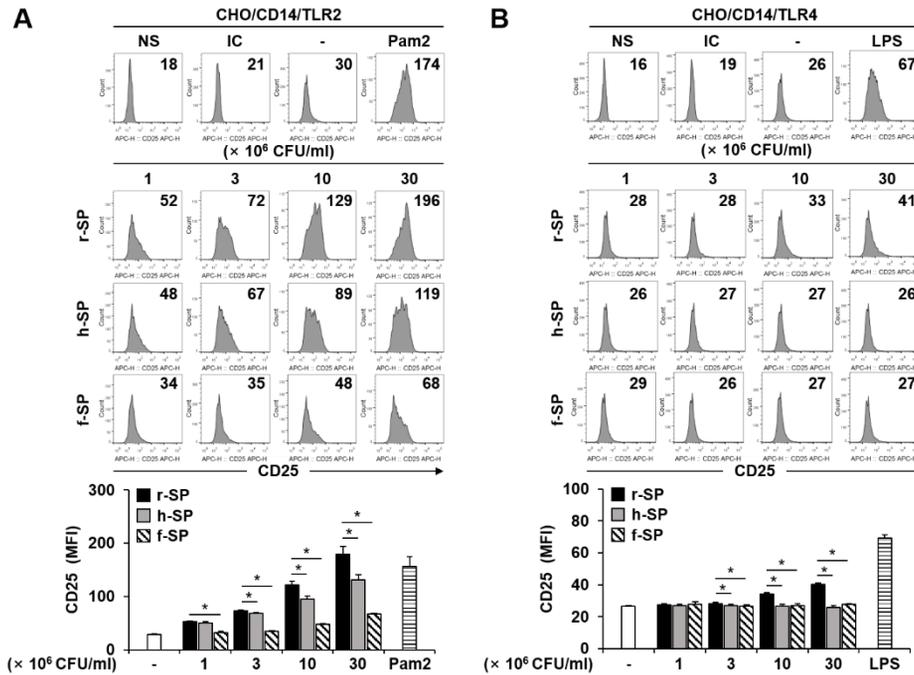


Figure 6. r-SP induces the activation of TLR2 and TLR4 more strongly than h-SP or f-SP. (A) CHO/CD14/TLR2 cells (5×10^5 cells/ml) were treated with 1, 3, 10, or 30×10^6 CFU/ml of r-SP, h-SP, f-SP, or 0.1 μ g/ml of Pam2CSK4 for 24 h. Then, the cells were stained with APC-conjugated anti-CD25 monoclonal antibodies and the levels of CD25 expression were determined by flow cytometry. (B) CHO/CD14/TLR4 cells (5×10^5 cells/ml) were treated with 1, 3, 10, or 30×10^6 CFU/ml of r-SP, h-SP, f-SP, or 0.1 μ g/ml of LPS for 24 h. Then, the cells were stained with APC-conjugated anti-CD25 monoclonal antibodies and the levels of CD25 expression were determined by flow cytometry. The number in each histogram indicates mean fluorescence intensity (MFI) of CD25 positive cells. Each graph on the bottom panels is based on the results of three independent experiments. The data are the mean values + S.D. and statistical differences between experimental groups were analyzed by Student's *t*-test. An asterisk indicates $P < 0.05$. NS denotes no staining. IC denotes isotype control. - denotes non-treatment. Pam2 denotes Pam2CSK4.

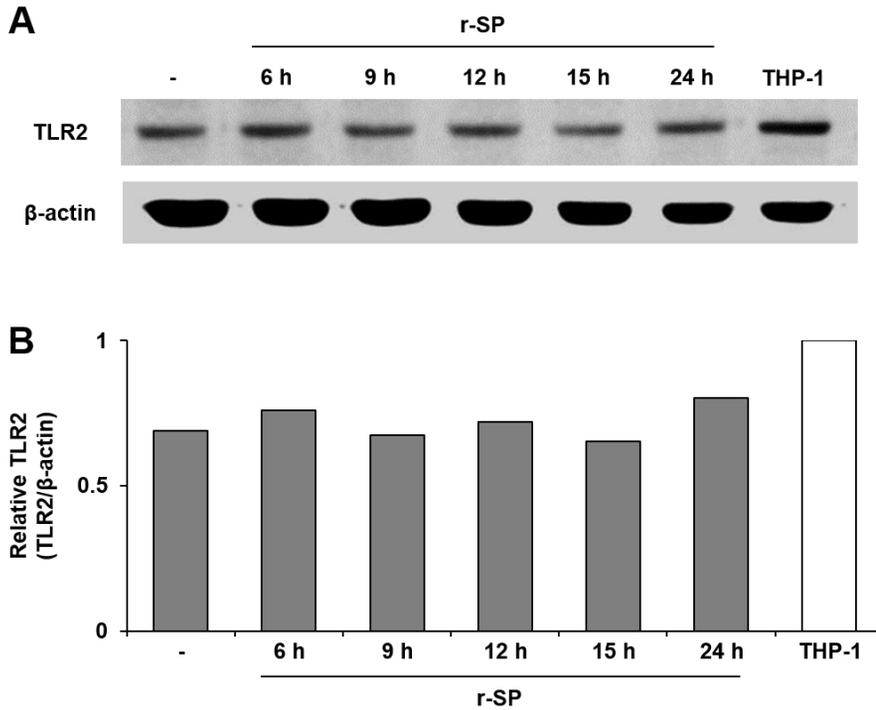


Figure 7. r-SP does not induce TLR2 synthesis in BEAS-2B cells. (A) BEAS-2B cells (1.5×10^5 cells/ml) were treated with 3×10^7 CFU/ml of r-SP for each indicated time and subjected to Western blot analysis using antibodies specific to TLR2. (B) Graph represents the relative band intensities of the results analyzed by ImageJ. - denotes non-treatment. THP-1 cells were used as a positive control of TLR2 synthesis.

3.4. r-SP induces phosphorylation of ERK, JNK, and p38 MAP kinases more potently than h-SP or f-SP in BEAS-2B cells

MAP kinase pathways are the major signaling pathways to express IL-6 and IL-8 in BEAS-2B cells [40, 41]. To determine whether the different levels of IL-6 and IL-8 expression is caused by the difference in MAP kinase activation potency, the cells were stimulated with r-SP, h-SP, or f-SP and phosphorylation of MAP kinase was determined using Western blotting. r-SP more potently phosphorylated all of the MAP kinases, especially JNK and p38, than h-SP or f-SP (Fig. 8A, B). To confirm the involvement of MAP kinase activation in the induction of IL-6 and IL-8 expression, BEAS-2B cells were stimulated with r-SP, h-SP, or f-SP following pretreatment with inhibitors of ERK, JNK, or p38. All three MAP kinase inhibitors, which did not affect cell viability, suppressed the expression of IL-6 and IL-8 (Fig. 9A, B). These results indicate that r-SP phosphorylates MAP kinases more potently than h-SP or f-SP, thereby enhancing IL-6 and IL-8 expression in BEAS-2B cells.

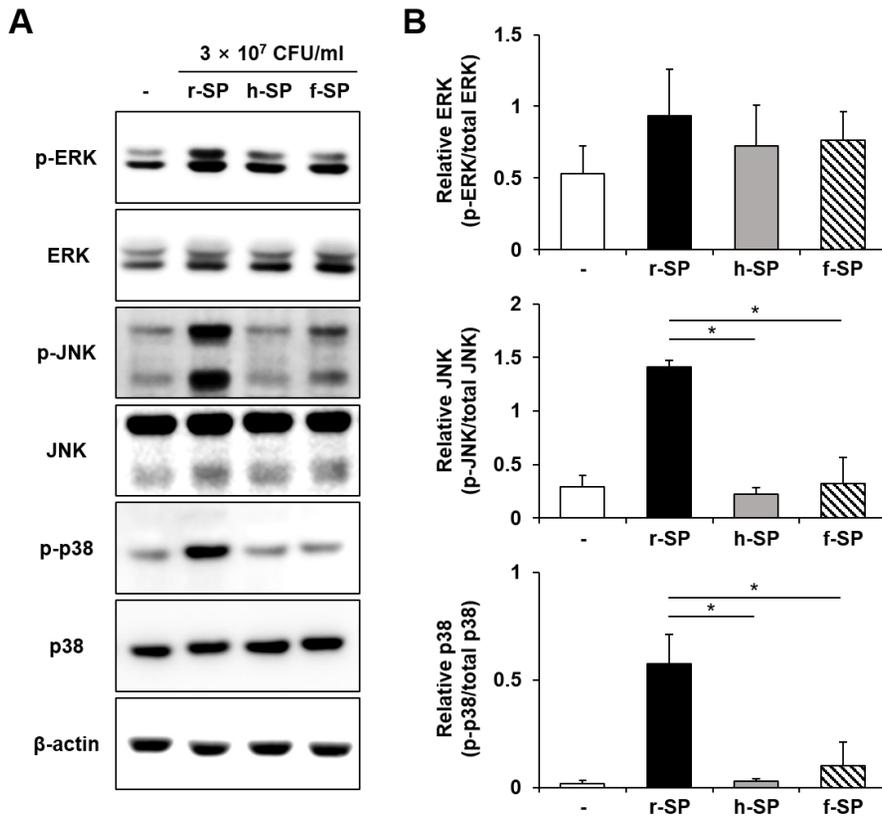


Figure 8. r-SP induces phosphorylation of ERK, JNK, and p38 kinase more strongly than h-SP or f-SP in BEAS-2B cells. (A) BEAS-2B cells (1.5×10^5 cells/ml) were treated with 3×10^7 CFU/ml of r-SP, h-SP, or f-SP for 30 min and subjected to Western blot analysis using antibodies specific to phosphorylated or non-phosphorylated forms of ERK, JNK, or p38 kinase. (B) Each graph is based on the results of three independent iterations. The data are the mean values + S.D. and statistical differences between experimental groups were analyzed by Student's *t*-test. An asterisk indicates $P < 0.05$. Data shown are representative of three independent experiments. - denotes non-treatment.

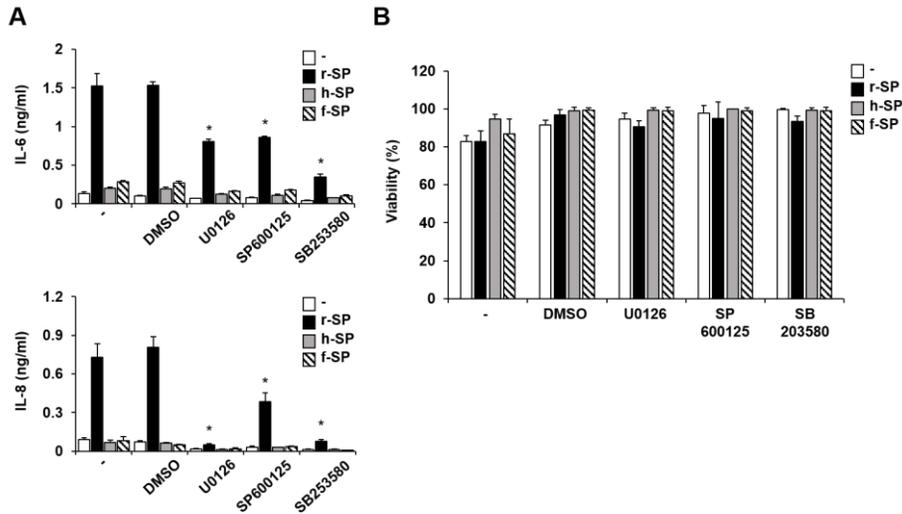


Figure 9. r-SP induces the phosphorylation of ERK, JNK, and p38 kinase more potently than h-SP or f-SP in BEAS-2B cells. (A) BEAS-2B cells (1.5×10^5 cells/ml) were pre-treated with 10 μ M of U0126, SP600125, SB253580, or 0.05% of DMSO (vehicle control) for 30 min. The cells were then stimulated with 3×10^7 CFU/ml of r-SP, h-SP or f-SP for 24 h. After the stimulation, levels of IL-6 and IL-8 in the culture supernatant were measured by ELISA. (B) The effects of inhibitors on cell viability was evaluated by trypan blue staining followed by enumerating through microscopic analysis. The data are the mean values + S.D. and statistical differences between experimental groups were analyzed by Student's *t*-test. An asterisk indicates $P < 0.05$ compared with r-SP group of no treatment group. Data shown are representative of three independent experiments. - denotes non-treatment.

3.5. Re-exposure of r-SP to heat or formalin reduces the inducibility of IL-6 and IL-8

Heat or formalin treatment can damage proteins by breaking hydrogen bonds or crosslinking the proteins [42, 43]. Thus, the inactivation of *S. pneumoniae* by heat or formalin might damage the bacterial immunostimulatory molecules, and lead to decreased induction of IL-6 and IL-8. Therefore, to examine this hypothesis, r-SP was re-exposed to heat (H-r-SP) or formalin (F-r-SP) by the same methods used to make h-SP or f-SP, respectively. When the cells were treated with H-r-SP or F-r-SP, the expression of IL-6 and IL-8 was considerably decreased compared with r-SP (Fig. 10A, B). Thus, re-exposure of r-SP to heat or formalin results in a decrease in the activity of certain components of *S. pneumoniae*, which is responsible for the lower IL-6 and IL-8 secretion. Subsequently, the activation of TLR2 or TLR4 induced by H-r-SP or F-r-SP was measured by flow cytometry. When CHO/CD14/TLR2 or CHO/CD14/TLR4 cells were stimulated with H-r-SP or F-r-SP, CD25 expression was considerably decreased compared with the cells stimulated with r-SP (Fig. 11A, B). These data show that inactivating r-SP with heat or formalin diminishes the ability of r-SP to activate TLR2 and TLR4.

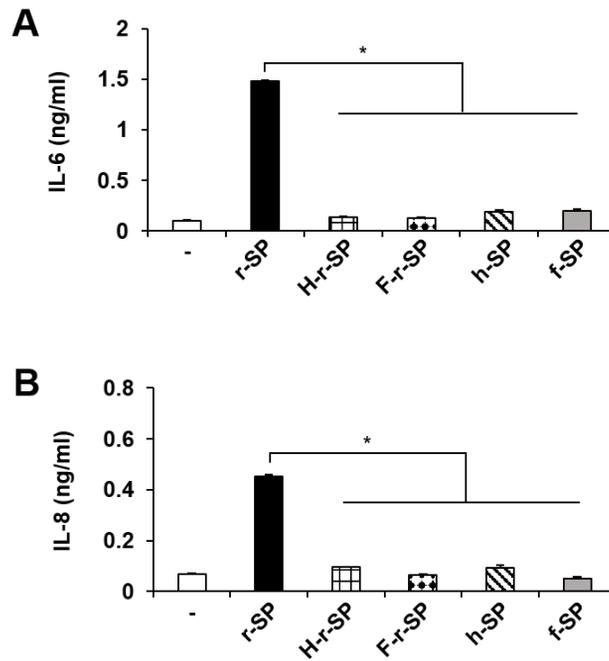


Figure 10. r-SP re-exposed to heat or formalin fails to induce the expression of IL-6 and IL-8. (A-B) BEAS-2B cells (1×10^5 cells/ml) were treated with 3×10^7 CFU/ml of r-SP, H-r-SP, F-r-SP, h-SP, or f-SP for 24 h. Then, (A) IL-6 and (B) IL-8 secretion in the culture supernatants were measured by ELISA. The data are the mean values + S.D. and statistical differences between experimental groups were analyzed by Student's *t*-test. An asterisk indicates $P < 0.05$. - denotes non-treatment.

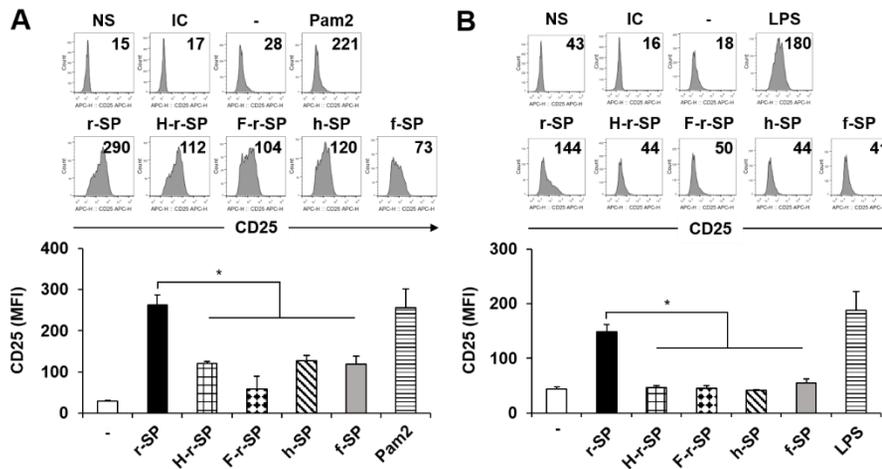


Figure 11. r-SP re-exposed to heat or formalin fails to induce the activation of TLR2 and TLR4 (A) CHO/CD14/TLR2 cells (5×10^5 cells/ml) were treated with 3×10^7 CFU/ml of r-SP, H-r-SP, F-r-SP, h-SP, f-SP, or 0.1 μ g/ml of Pam2CSK4 for 24 h. Then, the levels of CD25 expression were determined by flow cytometry. (B) CHO/CD14/TLR4 cells (5×10^5 cells/ml) were treated with 3×10^7 CFU/ml of r-SP, H-r-SP, F-r-SP, h-SP, f-SP, or 0.1 μ g/ml LPS for 24 h. Then, the levels of CD25 expression were determined by flow cytometry. The number in each histogram indicates mean fluorescence intensity (MFI) of CD25 positive cells. Each graph on the bottom panels is based on the results of three independent experiments. The data are the mean values + S.D. and statistical differences between experimental groups were analyzed by Student's *t*-test. An asterisk indicates $P < 0.05$. NS denotes no staining. IC denotes isotype control. - denotes non-treatment. Pam2 denotes Pam2CSK4.

3.6. Soluble components of r-SP potently induce the expression of IL-6 and IL-8

To investigate which characteristic of r-SP potently induced IL-6 and IL-8 expression, soluble and insoluble components were separated from differently inactivated *S. pneumoniae*. When the cells were stimulated with the whole, soluble, or insoluble components of inactivated *S. pneumoniae*, the overall tendency that r-SP more potently induces IL-6 and IL-8 than h-SP or f-SP was maintained. Remarkably, soluble components of r-SP induced the secretion of both IL-6 and IL-8 as much as whole r-SP (Fig. 12A, B). In case of f-SP, insoluble components induced higher IL-6 secretion compared with soluble components. When the soluble components of each inactivated *S. pneumoniae* were separated by SDS-PAGE followed by staining, a large amount of different proteins from soluble components of r-SP were detected compared with that of h-SP or f-SP (Fig. 13A, B). These results indicate that the major component of r-SP that potently induces IL-6 and IL-8 has a soluble characteristic.

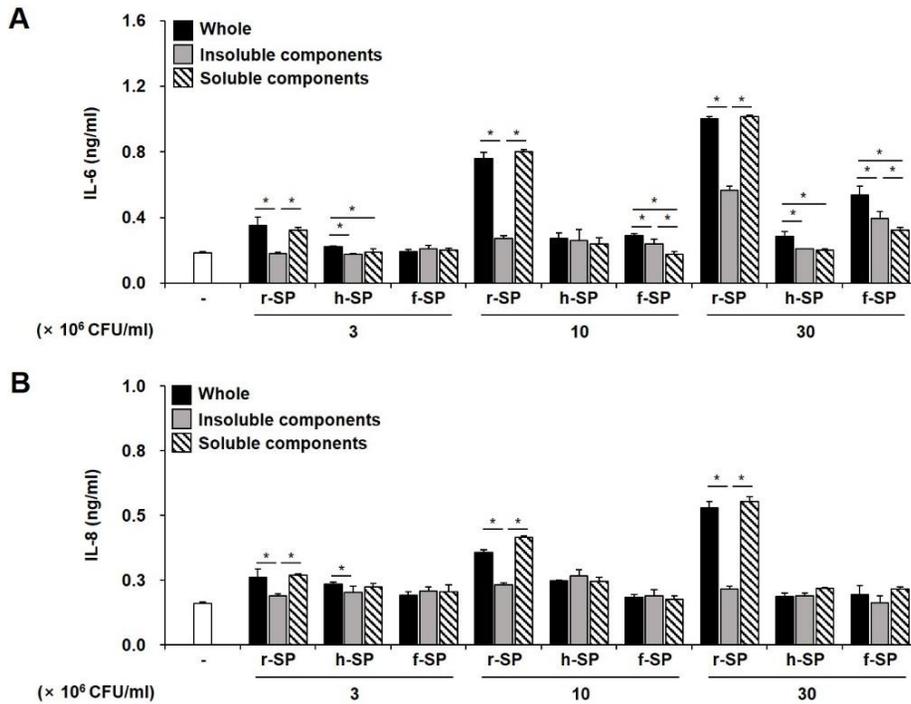


Figure 12. Soluble components of r-SP potently induce the expression of IL-6 and IL-8. BEAS-2B cells (1×10^5 cells/ml) were treated with whole, soluble, or insoluble components of 3, 10, or 30×10^6 CFU/ml of r-SP, h-SP, or f-SP for 24 h. Then, the levels of (A) IL-6 and (B) IL-8 in the culture supernatants were measured by ELISA. The data are the mean values + S.D. and statistical differences between experimental groups were analyzed by Student's *t*-test. An asterisk indicates $P < 0.05$. Data shown are representative of three independent experiments. - denotes non-treatment.

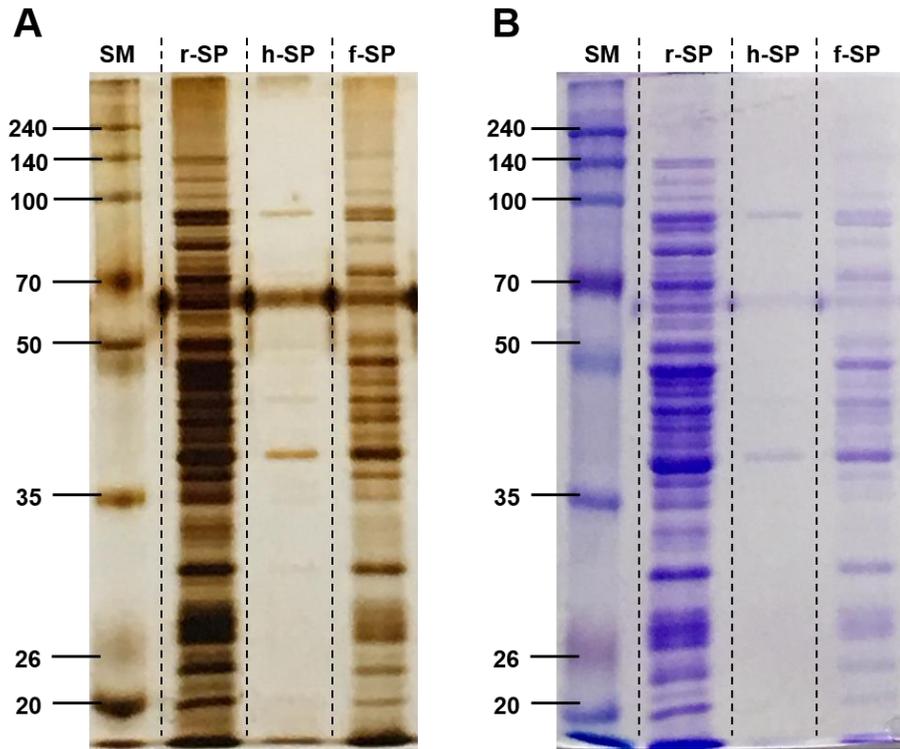


Figure 13. r-SP potently releases soluble components compared with h-SP or f-SP. Proteins from the supernatant of r-Sp, h-Sp, or f-SP were detected by (A) silver staining or (B) Coomassie blue staining. Both of the stainings were conducted after separation by 10% SDS-PAGE. SM denotes size marker.

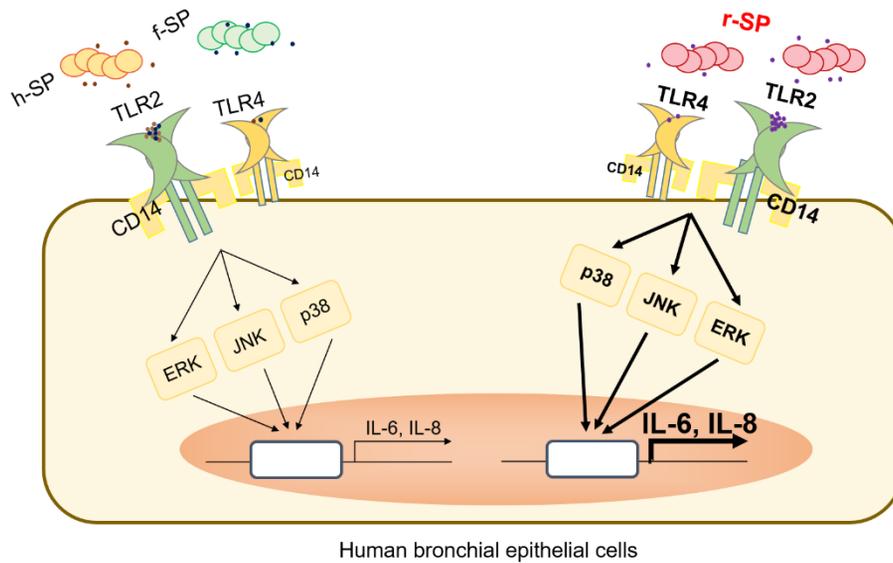


Figure 14. Schematic illustration of the proposed mechanisms of immunostimulatory effect of r-SP in human bronchial epithelial cells. Soluble components of r-SP potentially activate TLR2 and TLR4 and secrete IL-6 and IL-8 through intracellular signaling of MAP kinases, which may influence the adaptive immune response and enhance vaccine efficacy. However, h-SP or f-SP hardly induces the aforementioned activity.

Chapter IV. Discussion

The strength and quality of innate immunity are important to determine vaccine efficacy as innate immunity influences adaptive immune responses that follow [44, 45]. Since the immunostimulatory capacity of an inactivated whole-cell vaccine can vary depending on the inactivation method, the immunostimulatory potential of different inactivation methods needs to be carefully compared. In the present study, we demonstrated that r-SP induces IL-6 and IL-8 expression in BEAS-2B cells more potently than h-SP or f-SP through the MAP kinase pathway, especially JNK and p38. The activation of TLR2 by r-SP was much stronger than that by h-SP or f-SP. In addition, the potent TLR activation and cytokine expression by r-SP were reduced by re-exposure of r-SP with heat or formalin. Furthermore, we found that the major component of r-SP that potently induced inflammatory cytokines had a soluble characteristic. Collectively, these results suggest that r-SP stimulates the human respiratory epithelial cells to produce the cytokines IL-6 and IL-8, which might influence the induction of adaptive immune responses.

We observed that r-SP remarkably induced IL-6 and IL-8 expression while h-SP or f-SP hardly induced them. Similarly, gamma-irradiated *Brucella abortus* induces IL-12 production more potently than heat-inactivated *B. abortus* [46]. Although further studies are needed to clarify the exact molecular mechanism, the differential immunostimulating capacity might be primarily due to structural and/or conformational damage caused by heat or formalin inactivation. Heat or formalin treatment can cause denaturation of key antigens that induce immune responses by

thermal denaturation or chemical modification, respectively [42, 43]. For example, the lipoprotein is a major immunostimulating factor of Gram-positive bacteria including *S. pneumoniae* [47, 48]. Denaturation of the protein moiety in lipoprotein by heat or formalin treatment may decrease its immunostimulatory potency, considering that the amino acid sequence of the lipopeptide can affect its immunostimulatory potency [49].

The different ability of r-SP *versus* h-SP or f-SP to induce IL-6 and IL-8 appears to be due to their different TLR-stimulating potencies as demonstrated by the CHO reporter cell systems. It is important to note that BEAS-2B cells express both extracellular and intracellular TLR2, and that Pam3CSK4, a synthetic bacterial lipopeptide, induces IL-6 production through TLR2 [50]. Concomitant with our results, gamma-irradiated *Listeria monocytogenes* induced maturation and cytokine expression in dendritic cells through TLR2, and its immunostimulatory ability was more potent than that of heat-killed *L. monocytogenes* [51]. Furthermore, we also showed that only r-SP, but not h-SP or f-SP, slightly activates the TLR4 signaling pathway in CHO/CD14/TLR4 cells. TLR4 activation may only have been retained in r-SP, since it was reported that heat-killed *S. pneumoniae* only stimulates TLR2 and not TLR4, due to denaturation of pneumolysin, which normally stimulates TLR4 [52].

We showed that the soluble components of r-SP are responsible for the high expression of IL-6 and IL-8 in BEAS-2B cells. These results are concordant with a previous study that shown that the culture supernatant from nonencapsulated *S.*

pneumoniae CP1200 strain more potently induces IL-8 production compared with heat-inactivated *S. pneumoniae* CP1200 in A549 epithelial cells [53]. Soluble components of *S. pneumoniae* A17 have also been shown to be immunostimulatory, inducing reactive oxygen species production in neutrophils [54]. Although further studies are necessary in order to identify the exact molecules and their mechanisms, some explanations can be suggested. Gamma-irradiation may expose virulence factors in *S. pneumoniae* considering that certain strength of laser irradiation causes toxin release in *Microcystis aeruginosa* [55]. Remarkably, pneumolysin, which potently stimulates human respiratory epithelial cells [56], may be secreted from *S. pneumoniae* in an autolysin- or domain 2-dependent manner [57] when inactivated with gamma-irradiation. Since gamma-irradiated *Brucella melitensis* shows metabolic activity [58], r-SP is likely to retain enzymatic activity, and therefore autolysin activity.

We demonstrated that r-SP potently induces expression of IL-6 and IL-8 through TLR2. Recently, we and Babb *et al.*, reported that immunization with r-SP potently induces *S. pneumoniae*-specific antibodies and leads to protective immunity [25, 59]. However, it is unclear whether the induction of IL-6 and IL-8 by r-SP is responsible for adaptive immune responses eliciting *S. pneumoniae*-specific antibodies *in vivo*. Although further studies are required, since IL-6 promotes the differentiation of B cells into antibody secreting cells [20] and IL-8 activates $\gamma\delta$ T cells which are known to be important in pneumococcal vaccine-mediated protection [24, 25], IL-6 and IL-8 are likely to be important for adaptive immune responses *in vivo*. Besides, we have recently reported that bone marrow-derived dendritic cells stimulated with r-SP

effectively induces Th17 activation, which is important for mucosal immune responses against *S. pneumoniae* infection [21, 60]. However, r-SP was less potent than h-SP or f-SP in the induction of DC maturation and activation including cytokine expression [60]. Since responses by mucosal epithelial cells may vary due to cell tropism, further studies are needed to elucidate the role of cytokines produced by mucosal epithelial cells in vaccine-induced immunity *in vivo*.

In the present study, we demonstrated that r-SP potently induced IL-6 and IL-8 production in epithelial cells, which are important for inducing local B and T cell responses [23, 61]. Furthermore, these responses seem to be closely associated with TLR2. Given the fact that activation of innate immune responses by TLRs can lead to intense adaptive immune responses and persistent antibody responses [62], the potent stimulation of respiratory epithelial cells by r-SP through TLR2 might effectively induce the subsequent adaptive immune responses, leading to long-term immunity.

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감마선 조사 사멸 폐렴구균에 의한 사람 기관지 상피세포의 면역활성

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1. 목 적

*Streptococcus pneumoniae*는 호흡기로 감염되어 높은 사망률을 야기하는 폐렴, 수막염, 폐혈증 등의 질환을 유발하는 대표적인 원인 균이다. 폐렴 백신은 협막다당 기반의 백신이 상용화되고 있으나, 이는 일부 폐렴구균의 혈청형에 대한 예방만 가능하기 때문에 혈청형에 영향을 받지않는 비용 효율이 높은 백신에 대한 연구가 필요하다. 이러한 한계점을 극복하기 위해 감마선 조사로 사멸시킨 비협막의 폐렴구균 전세포를 사용한 경비용 백신의 연구들이 진행되고 있다.

하지만 이의 면역활성에 대해서는 아직 잘 알려져 있지 않다. 따라서 본 연구에서는 열이나 포르말린으로 사멸시킨 폐렴구균 (h-SP or f-SP)과 비교하여 감마선 조사 사멸 폐렴구균 (r-SP)의 사람의 기관지 상피세포에서의 면역활성능력 및 작용기전을 규명하는 연구를 진행하였다.

2. 방 법

혈청형에 영향을 받지 않는 전세포 사백신을 연구하기 위해, 비협막의 *S. pneumoniae* TIGR4 균주를 만들어 이를 감마선 조사, 열, 또는 포르말린으로 사멸시켰다. 사람의 기관지 상피세포주인 BEAS-2B 세포에 r-SP, h-SP, 또는 f-SP를 처리하여 interleukin (IL)-6와 IL-8의 메신저 리보 핵산과 단백질 발현 정도를 각각 역전사효소 중합연쇄반응 (Reverse Transcription-Polymerase Chain Reaction, RT-PCR)과 효소결합 면역분석법 (Enzyme-Linked ImmunoSorbent Assay, ELISA)을 이용하여 확인하였다. CCL5 와 CCL28 및 CXCL10의 메신저 리보 핵산은 실시간 역전사효소 중합연쇄반응 (real time RT-PCR)로 확인하였다. 세포 생존능은 트라이판 블루 염색법과 annexin V/ propidium iodide (PI) 염색법으로 측정하였다. 각각의 방법으로 사멸된 *S. pneumoniae*의 톨유사수용체 (Toll-like receptor, TLR) 활성 정도를 비교하기 위해, 중국 햄스터 난소 (Chinese hamster ovary, CHO) 세포에 CD14와 함께 TLR2 또는 TLR4를 과발현하며 CD25의 리포터 유전자를 가진 CHO/CD14/TLR2 또는 CHO/CD14/TLR4 세포의 NF- κ B 활성을 통해 표면에 발현된 CD25를 유세포분석기로 측정하였다. 세포 내부 신호전달을 알아보기 위해, 미토겐 활성화 단백질 (mitogen-activated protein, MAP) 인산화 효소인 ERK와 JNK 및 p38의 인산화 정도 및 TLR2의 세포 내 합성을 웨스턴 블롯법으로 확인하였다. MAP 인산화효소들과 IL-6 또는 IL-8과의 신호전달체계의 연관성을 확인하기 위해, ERK와 JNK 및 p38 억제제를 전처리한 후 각각의 방법으로 사멸된 *S. pneumoniae*를 처리하여 IL-6와 IL-8의 발현 정도를 ELISA를 이용하여 확인하였다.

각각의 방법으로 사멸된 *S. pneumoniae*는 원심분리기를 이용하여 수용성 성분과 불용성 성분으로 분리하여 실험에 사용하였다. 각각의 방법으로 사멸된 *S. pneumoniae*의 수용성 성분들은 은 염색법과 쿠마시 블루 염색법을 통해 확인하였다.

3. 결 과

r-SP는 h-SP나 f-SP에 비해 BEAS-2B cell를 자극시켜 IL-6와 IL-8의 mRNA 발현을 강하게 유도시켰으며 CCL5 및 CCL28과 CXCL10과 같은 케모카인의 mRNA 발현의 차이는 보이지 않았다. 분비되는 IL-6와 IL-8의 단백질 양에 대한 결과에서도 r-SP가 h-SP나 f-SP에 비해 월등히 높은 양을 나타내었으며, 처리하는 r-SP 농도를 증가시키기에 따라 분비되는 IL-6와 IL-8의 양이 농도의존적으로 증가하였음을 확인할 수 있었다. BEAS-2B 세포에 각각의 방법으로 사멸된 *S. pneumoniae*를 처리시에 세포 생존능에는 영향을 미치지 않았다. CHO/CD14/TLR2 세포를 이용하여 TLR2의 활성화를 확인한 결과에서 r-SP를 처리할 경우 h-SP나 f-SP를 처리할 경우 비해 강한 활성화를 보였다. 또한 CHO/CD14/TLR4 세포를 이용한 TLR4 활성화 실험결과에서는 r-SP에 의해서만 활성화가 유도되었으며, h-SP나 f-SP는 TLR4를 활성화 시키지 못하는 것을 관찰할 수 있었다. 이와 다르게 BEAS-2B 세포에서 r-SP를 처리하였을 때 세포 내에서 합성되는 TLR2의 양은 크게 달라지지 않았다. r-SP는 BEAS-2B 세포에서 세포 내 신호전달 물질인 ERK와 JNK 및 p38과 같은 MAP 인산화 효소의 강한 인산화를 유도하였지만, h-SP나 f-SP는 약한 인산화를 보였다. 또한, MAP 인산화 효소 억제제를 전처리 하였을 경우 IL-6와 IL-8의 생성이 월등히 떨어짐을 통해 유도된 IL-6와 IL-8의 생성에 MAP 인산화 효소의 인산화가 관여됨을 확인하였다. 열이나 포르말린으로 재처리한 r-SP로 BEAS-2B 세포를 자극시켰을 경우 r-SP에 비해 TLR 활성화 및 IL-6와 IL-8의 발현이 감소함을 확인하였다. r-SP에서 분리한 수용성 성분을 처리하였을 경우 분리 전의 r-SP 처리 시 발현되는 IL-6와 IL-8의 양과 비슷한 양의 강한

발현을 확인하였다. r-SP에서는 h-SP와 f-SP보다 다양하고 많은 양의 수용성 성분들이 확인되었다.

4. 결 론

본 연구 결과를 종합해보면 r-SP는 h-SP나 f-SP에 비해 사람의 기관지 세포를 강하게 자극시켜 TLR2 활성화를 통해 세포 내부 신호기작인 MAP kinase 신호전달을 통해 적응면역반응의 유도를 일으켜 백신의 효율을 향상시킬 수 있는 IL-6와 IL-8의 월등한 발현을 일으킨다는 것을 알 수 있다.

주요어 : *Streptococcus pneumoniae*, 감마선 조사, 기관지 상피 세포, 백신, 선천 면역

학 번 : 2016-26545