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# Repeated and alternate stimulations with dsRNA and SEB alter responses in macrophages and epithelial cells

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## ABSTRACT

**Introduction:** The innate immune system is activated by foreign molecules via pattern recognition receptors and other surveillance systems, producing diverse cytokines that recruit and activate other immune cells. Recent studies have shown that once activated by foreign molecules, the innate immune system exhibits altered responses upon subsequent exposure to the same or different infectious agents, such as lipopolysaccharides (LPS) or bacteria. However, as these alterations in response to viral infection and staphylococcal enterotoxin B (SEB) in the airways have not been fully elucidated, we focused on the changes in immune responses induced by repeated stimulation of macrophages and epithelial cells with foreign molecules.

**Methods:** THP-1-derived macrophages and BEAS-2B epithelial cells were stimulated with dsRNA (double-stranded RNA) or SEB and cultured in fresh complete medium for 4 days. Subsequently, the cells were re-stimulated with different doses of dsRNA or SEB, and the cytokine and signal phosphorylation levels were evaluated.

**Results:** Repeated stimulation with high dose of dsRNA or SEB, induced an increase in IL-10, CCL2, CCL22, CCL24, CXCL10, and CXCL11 in macrophages, while only repeated stimulation with dsRNA stimulation resulted in an increase in IL-6, CCL2, CCL5, CCL24, CXCL11, and TGF-β in epithelial cells. Cross-stimulation with SEB-dsRNA induced an increase in CCL5, CCL20, CCL22, CCL24, CXCL10, and CXCL11 levels in macrophages. However, in epithelial cells, SEB-dsRNA stimulation increased the levels of CCL5, CXCL11, and TGF-β, while dsRNA-SEB stimulation elevated CCL1, CCL20, CXCL10, and CXCL11. These cytokine changes were driven by distinct phosphorylation patterns in macrophages and epithelial cells, depending on the type and intensity of stimuli.

**Conclusion:** Repeated stimulation with the same or cross-over stimuli induced alterations in the immune response of macrophages and epithelial cells. These observations indicated that persistent airway stimulation can lead to changes in airway inflammation, potentially leading to asthma.

**Keywords:** dsRNA, SEB, Macrophage, Epithelial cell

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## INTRODUCTION

The innate immune system recognizes pathogens and tissue damage using pattern recognition receptors that sense various pathogens or damage-associated molecular patterns.[1] Immunological memory has long been assumed an exclusive characteristic of adaptive immune responses. However, a growing body of evidence indicates that innate immune cells may have a memory function called, trained immunity.[2,3] After exposure to certain stimuli, innate immune cells adjust their responses to subsequent insults from the same or other stimulating agents. *Bacillus Calmette-Guérin* (BCG) vaccination against *Mycobacterium tuberculosis* induces cross-protection and reduced mortality in severely combined immunodeficient mice that lack T and B lymphocytes upon inoculation with a lethal *Candida albicans* dose.[4] In other experimental models, β-glucan induced protection against *Staphylococcus aureus* (*S. aureus*) and the peptidoglycan component against *Toxoplasma* infection.[5,6] Evidence of trained immunity has also been reported in human studies. BCG vaccination induces non-specific activation of innate immune cells and protects against diseases, such as yellow fever and malaria. Furthermore, BCG vaccination can induce antitumor effects and can be used in the treatment of cancers, such as bladder cancer and melanoma.[3] Some evidence of adaptation has also been demonstrated, such as LPS tolerance, which is associated with the silencing of genes encoding inflammatory and antimicrobial molecules.[7] Moreover, characteristics of trained immunity are reported to occur in stromal and epidermal stem cells, as well as in innate immune cells.[8] Mechanism of the induction of innate immune memory is not yet clearly defined and epigenetic reprogramming and metabolic rewriting are suggested to be involved.[9]

The innate immune response is important as the first line of defense against pathogens; however it is also crucial in the pathogenesis of chronic inflammatory respiratory diseases, such as asthma.[1] Before the development of adaptive responses to allergens, innate and structural immune cells sense and respond to environmental stimuli and activate adaptive immune cells. Moreover, innate immunity plays a role in differentiating types of inflammation and inducing or protecting against inflammation.[10] RNA viruses, such as rhinoviruses, and bacteria-derived toxins, such as staphylococcal enterotoxin B (SEB), are well-known environmental factors associated with the development and exacerbation of asthma. In our previous study, we demonstrated that virus-mimicking double-stranded RNA (dsRNA) and SEB stimulated macrophages and bronchial epithelial cells, and induced cytokines were related to airway inflammation in asthma.[11] However, it is unclear how repeated or cross-subsequent exposure to dsRNA and SEB influences the innate and airway epithelial cells in terms of trained immunity. In the present study, we investigated the changes in cytokine production and intracellular phosphorylation caused by repeated and alternating stimulations with ds-RNA and SEB in macrophages and bronchial epithelial cells.

## MATERIALS AND METHODS

### Cell culture and stimulation

As in our previous study,[11] human bronchial epithelial and monocyte cells (BEAS-2B and THP-1, respectively) were purchased from the American Type Culture Collection (Manassas, VA, USA; CRL-9609 and TIB-202, respectively). BEAS-2B and THP-1 cells were cultured in complete Dulbecco's modified Eagle medium (DMEM; Welgene, Gyeongsan, Korea) and Roswell Park Memorial Institute medium 1640 (RPMI 1640; Welgene) each containing 10% FBS (Welgene) and 1x antibiotics (Welgene, 100 units/ml of penicillin, 100 μg/ml of streptomycin).

BEAS-2B cells were seeded at a density of $1.0 \times 10^{-}6$ cells in 1 ml of serum-free medium per 60 mm cell culture dish (Corning, NY, USA). After serum starvation, the cells were stimulated with different concentrations of polyinos-inic:polycytidylic acid (poly I:C (IC), Calbiochem, San Diego, CA, USA) at 1 μg/ml (IC1), 10 μg/ml (IC10), and 100 μg/ml (IC100) or SEB (Sigma-Aldrich, St. Louis, MO, USA) at 1 μg/ml (SEB1) and 10 μg/ml (SEB10) in 1 ml of serum-free medium. Culture supernatants were harvested 24 h after stimulation and complete culture medium was added after washing the cells with sterile 1x phosphate-buffered saline (PBS, Welgene). The complete medium was changed every second day and

serum starvation was repeated on day 5. After serum starvation, the cells were stimulated with poly I:C or SEB in 1 ml of serum-free medium. Supernatant and cells were harvested 24 h after second stimulation and stored at −20 °C for further analysis. The PBS control group received an equal volume of PBS as the dsRNA or SEB in serum-free medium.

THP-1 cells (1 × 10-6) were seeded in 6-well culture plates (Corning) with 2 ml of complete RPMI medium containing 20, 50, 100, and 200 nM phorbol 12-Myristate 13-Acetate (PMA, Sigma-Aldrich) to induce monocyte-macrophage differentiation. After 2 days of culture, serum starvation was performed in serum-free medium. The first stimulation and subsequent procedures were performed in the same manner as in the experiment with BEAS-2B cells.

### RNA extraction, reverse-transcriptase polymerase chain reaction (PCR), and quantitative real-time PCR

To isolate the RNA from differentiated THP-1 and BEAS-2B cells, all procedures followed manufacturer's guideline for RNeasy mini-prep kit (Qiagen, Hilden, Germany). Frozen cells were lysed and precipitated with 70% ethanol. The solution was then inserted into the column, and RNA was eluted using RNase-free water after washing.

Isolated RNA level was evaluated with NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was synthesized with isolated RNA, oligo dT (BIONEER, Daejeon, Korea), RT-premix (BIONEER), and RNase-free water (Invitrogen, Waltham, MA, USA) under the T100 Thermal Cycler (BIO-RAD, Hercules, CA, USA).

Finally, the cDNA template was reacted with target forward (5′)/reverse (3′) primers (Table 1), SYBRGreen PCR master mix (Thermo Fisher Scientific), and RNase-free water in a 384-well MicroAmp plate (Thermo Fisher Scientific) using the QuantStudio 7 Flex real-time PCR system (Thermo Fisher).

### Cytokine evaluation using ELISA

To compare cytokine production after repeated stimulation, 20 human cytokines, including IL-1β, IL-6, IL-10, IL-25, IL-33, TSLP, TGF-β, TNF-α, CCL1 (I-309), CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (TARC), CCL18 (PARC), CCL20 (LARC), CCL22 (MDC), CCL24 (Eotaxin-2), CXCL10 (IP-10), and CXCL11 (I-TAG), were evaluated in each culture supernatant after the second stimulation using human ELISA Duoset kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's guidelines.

### Phosphorylation array and evaluation

All procedures followed the manufacturer's guidelines for the Proteome Profiler and were performed using components from the kit (R&D Systems, ARY003C). Briefly, frozen macrophages and epithelial cells were dissolved in a cell lysis buffer containing protease and phosphatase inhibitors. After lysis, equal amounts of proteins were incubated with reconstituted antibody-bound membranes. Spots detected by conjugated HRP and the substrate were analyzed using

| Name | Forward (5′) | Reverse (3′) | Size | Reference ID |
|---|---|---|---|---|
| CD11b | GAACCAGCCCAGAGGTGACTG | GGATGACAAACGACTGCTCCTG | 107 | AH004143.2 |
| CD14 | ACTTGCACTTTCCAGCTTGC | GCCCAGTCCAGGATTGTCAG | 200 | BC010507.2 |
| CD68 | ACCTCGACCTGCTCTCCCTG | CGAGGAGGCCAAGAAGGATC | 138 | BC015557.2 |
| TLR3 | ACAACTTAGCACGGCTCTGGA | ACCTCAACTGGGATCTCGTCA | 124 | NM_003265.3 |
| RIG-I | GCCTTCAGACATGGGACGAA | ACTGCTTTGGCTTGGGATGT | 258 | AF038963.1 |
| MDA5 | TGTGCTGGACTACCTGACCT | ACGAATTCCCGAGTCCAACC | 150 | AF095844.1 |
| β-actin | ATTGCCGACAGGATGCAGAA | GCTGATCCACATCTGCTGGAA | 150 | NM_001101.5 |

**Table 1.** Information of primer sequences.

the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA) and Quick Spots (Ideal Eyes, Bountiful, UT, USA). After loading the images, all calculated values were subtracted from the background and normalized using a loading control. Z-score heat maps were derived by secondary normalization using GraphPad Prism (ver. 8.0) and Excel. A dendrogram was constructed using Hierarchical Clustering Explorer (HCE, Ver. 3.5).

### Graph and statistics

To draw the graphs, GraphPad Prism (Ver. 8.0) was used, and all data are presented as the mean ± SD. Statistical significance was calculated using a two-way ANOVA with *post hoc* comparisons (Tukey's test) and significance set at $p < 0.05$.

## RESULTS

### Low concentration of PMA induced sufficient differentiation of macrophages

Before stimulating macrophages and epithelial cells with foreign molecules, such as dsRNA and SEB, we evaluated the characteristics of these cells, including differentiation markers and pattern recognition receptors, to confirm their ability to respond adequately to the stimuli (Fig. 1A).

THP-1 cells treated with different concentrations of PMA induced an increase in cell size compared to the PBS control. However, there were no significant differences in size or morphology between different PMA concentrations (Fig. 1B). All macrophage differentiation markers, such as CD11b, CD14, and CD68, were upregulated at all PMA concentrations compared to the PBS control. Interestingly, higher PMA concentrations significantly reduced the expression of CD14 and CD68 (Fig. 1C). Pattern recognition receptors, including Toll-like receptor 3 (TLR3), Retinoic acid-inducible gene-I (RIG-I), Melanoma differentiation-associated protein 5 (MDA-5), were also induced by PMA stimulation, but significant differences between each PMA concentration were not observed. Additionally, low concentration of PMA (20 nM) did not show big differences compared to BEAS-2B cells (Fig. 1D). When these macrophages were stimulated with different concentration of dsRNA or SEB, all PMA concentrations induced sufficient production of IL-1β, IL-6, TNF-α, CCL22, and CCL24. Interestingly, cytokines exhibited 2 opposing patterns: IL-1β, IL-6, TNF-α increased with higher concentration, while CCL22 and CCL24 decreased (Fig. 1E).

### Repeated stimulations with dsRNA or SEB enhance cytokine production in macrophages

To evaluate the effect of the first stimulation with each foreign molecule on the second stimulation with the same molecule on the innate immune responses of the airways, macrophages and epithelial cells were initially stimulated with different doses of dsRNA or SEB. The cells were then exposed to various doses of the same molecule after 4 days culture, and cytokines were evaluated in the culture supernatant (Fig. 2A). Among these 20 cytokines, IL-25, IL-33, TSLP, CCL11, and CCL17 were not detected (data not shown).

When macrophages were stimulated with dsRNA or SEB and subsequently exposed to the second stimulation with the same molecule, several cytokines were induced including interleukins (IL-1β, IL-6, and IL-10), TNF-α, CC (CCL1, CCL2, CCL3, CCL5, CCL18, CCL20, CCL22, and CCL24), and CXC (CXCL10 and CXCL11) chemokines, but not TGF-β (Fig. 2 and Supplementary Fig. 1). Higher cytokine production was measured in groups which were stimulated with higher concentrations of dsRNA or SEB during the first stimulation compared to their single dsRNA or SEB stimulation during second stimulation. In addition, the cytokine levels induced by repeated stimulation with dsRNA were significantly higher than those induced by SEB (Fig. 2B). In particular, we observed 2 opposing, dose-dependent cytokine production pattern in response to the second stimulation. The levels of IL-10, CCL2, CCL22, CCL24, CXCL10, and CXCL11 secreted by the cells increased with increasing doses of dsRNA during second stimulation (Fig. 2C–H). In contrast, the concentration of IL-1β, IL-6, TNF-α, and CCL20 decreased with increasing doses of dsRNA during second stimulation (Supplementary Fig. 1). Similar to the results obtained for dsRNA, repeated stimulation with SEB also enhanced the levels of CCL2, CCL24, CXCL10, and CXCL11 (Fig. 2D, F–H).

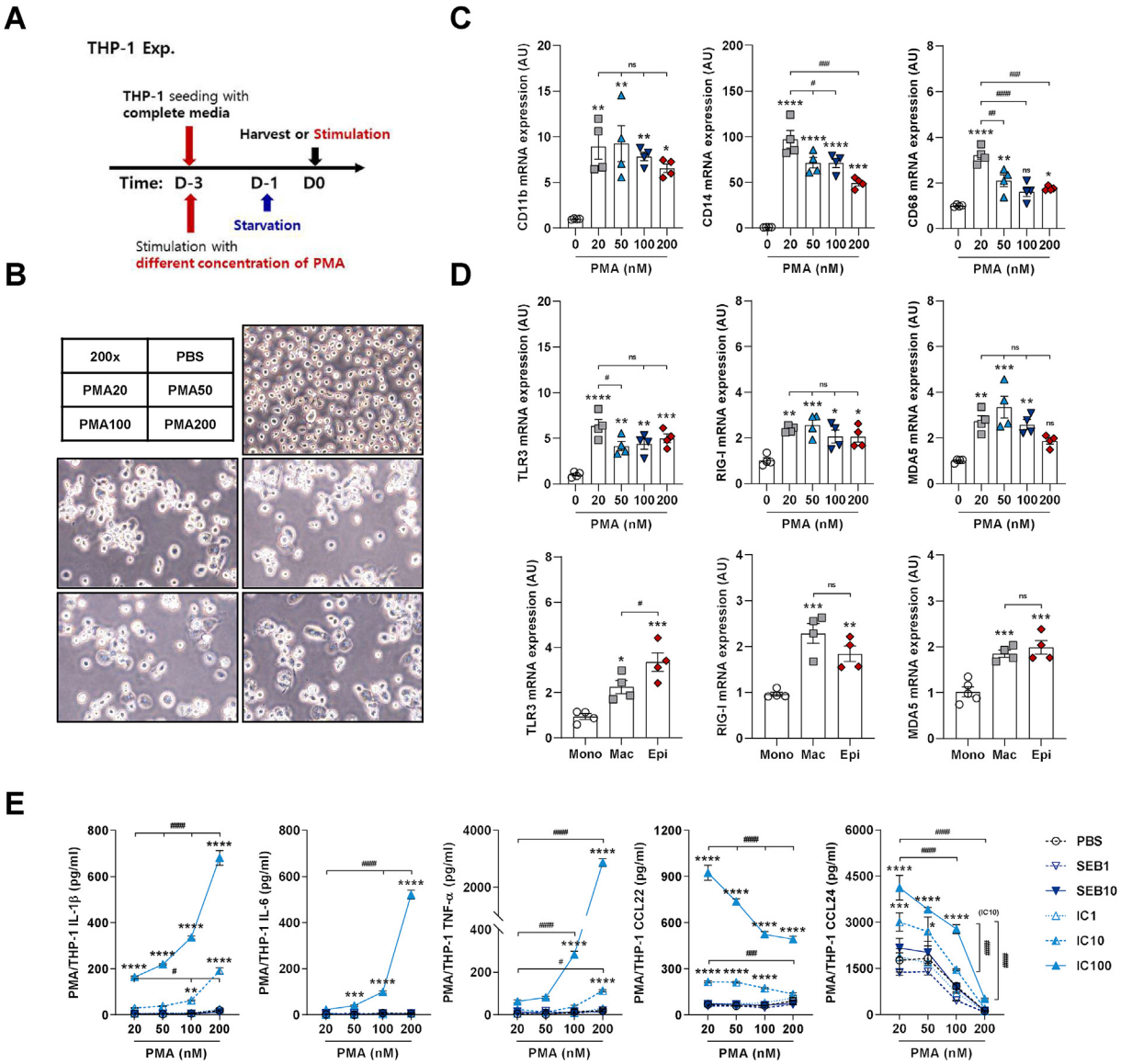


**Fig. 1 The characteristics of PMA stimulated THP-1 and BEAS-2B cells.** Macrophage differentiation was induced with different concentration of PMA. Cell surface receptors and cytokine production in response to foreign molecules were evaluated to confirm adequate differentiation. (A) Schematic diagram illustrating cell differentiation, culture, and stimulation. (B) Cell size and morphology observed under a light microscope on day 0. (C) mRNA expression of macrophage differentiation markers, including CD11b, CD14, and CD68, on day 0. (D) mRNA expression of pattern recognition receptors including, TLR3, RIG-I, and MDA5, at day 0 in PMA stimulated TPH-1 and BEAS-2B cells. (E) Cytokine production after stimulation with different concentration of dsRNA or SEB in 20 nM PMA stimulated THP-1 cells. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 for comparison with its PBS control group; #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001, ####p ≤ 0.0001 for comparison between each group.

## Repeated stimulations with dsRNA or SEB elevate cytokine production in epithelial cells

Repeated stimulation with dsRNA or SEB in epithelial cells (Fig. 3A) resulted in the induction of 14 different cytokines including interleukins (IL-1β, IL-6, and IL-10), TNF-α, TGF-β, CC (CCL1, CCL2, CCL3, CCL5, CCL20, CCL22, and CCL24), and CXC (CXCL10 and CXCL11) chemokines but not CCL18 (Fig. 3 and Supplementary Fig. 2) compared to the response after single dsRNA or SEB stimulation during second stimulation period. Similar to the results obtained from the experiments with the macrophages, we observed bigger differences in the in the cytokine production when the epithelial cells repeatedly stimulated dsRNA, compared to SEB (Fig. 2B). Among the groups stimulated with foreign

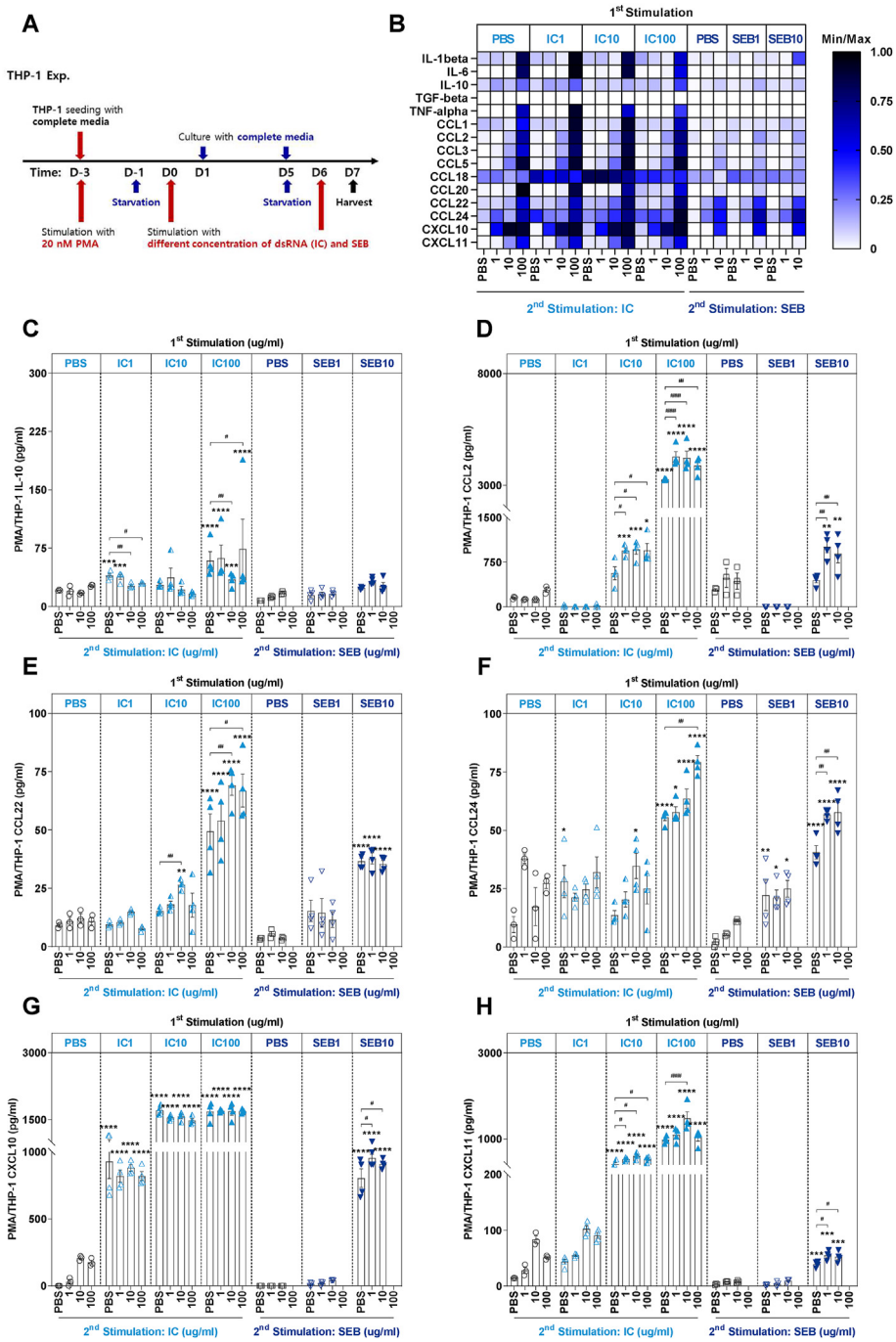


**Fig. 2 Cytokine profile after repeated stimulation with dsRNA or SEB in macrophages.** THP-1 derived macrophages were exposed to repeated-stimulation with varying doses of dsRNA or SEB. The cell culture supernatants were analyzed for the expression of 20 cytokines. (A) Schematic diagram illustrating cell differentiation, culture, and stimulation. (B) Heat-map analysis results of cytokine expression (excluding IL-25, IL-33, TSLP, CCL11, and CCL17). (C–H) The detailed results of cytokine expression demonstrating significant changes, IL-10 (C), CCL2 (D), CCL22 (E), CCL24 (F), CXCL10 (G), and CXCL11 (H). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ for comparison with its PBS control group; #$p \leq 0.05$, ##$p \leq 0.01$, ###$p \leq 0.001$, ####$p \leq 0.0001$ for comparison between each group.

molecules, a few cytokines (CCL5 and CCL24, Fig. 3C-D) increased with increasing doses of the foreign molecules during second stimulation, while others (TNF-α, CCL1, CCL3, CCL20, and CXCL10, Supplementary Fig. 2) decreased in the group stimulated with the highest concentration (IC100). In the group stimulated with middle concentration (IC10) stimulated group, CCL2 and CCL24 were upregulated, and IL-6, CCL5, CCL20, and CXCL10 were downregulated depending on

the concentration of second stimulation (Fig. 3 and Supplementary Fig. 2). The first low concentration (IC1) stimulation of epithelial cells also induced changes in the levels of CCL5, CCL24, CCL2, IL-6, and CXCL11 (Fig. 3C–D, F-H). Additionally, TGF-β was induced in this group in a dose-dependent manner by the second stimulation (Fig. 3E). Unlike with the macrophages, the cytokines TGF-β and CCL2 were significantly induced by a strong initial SEB-stimulation in

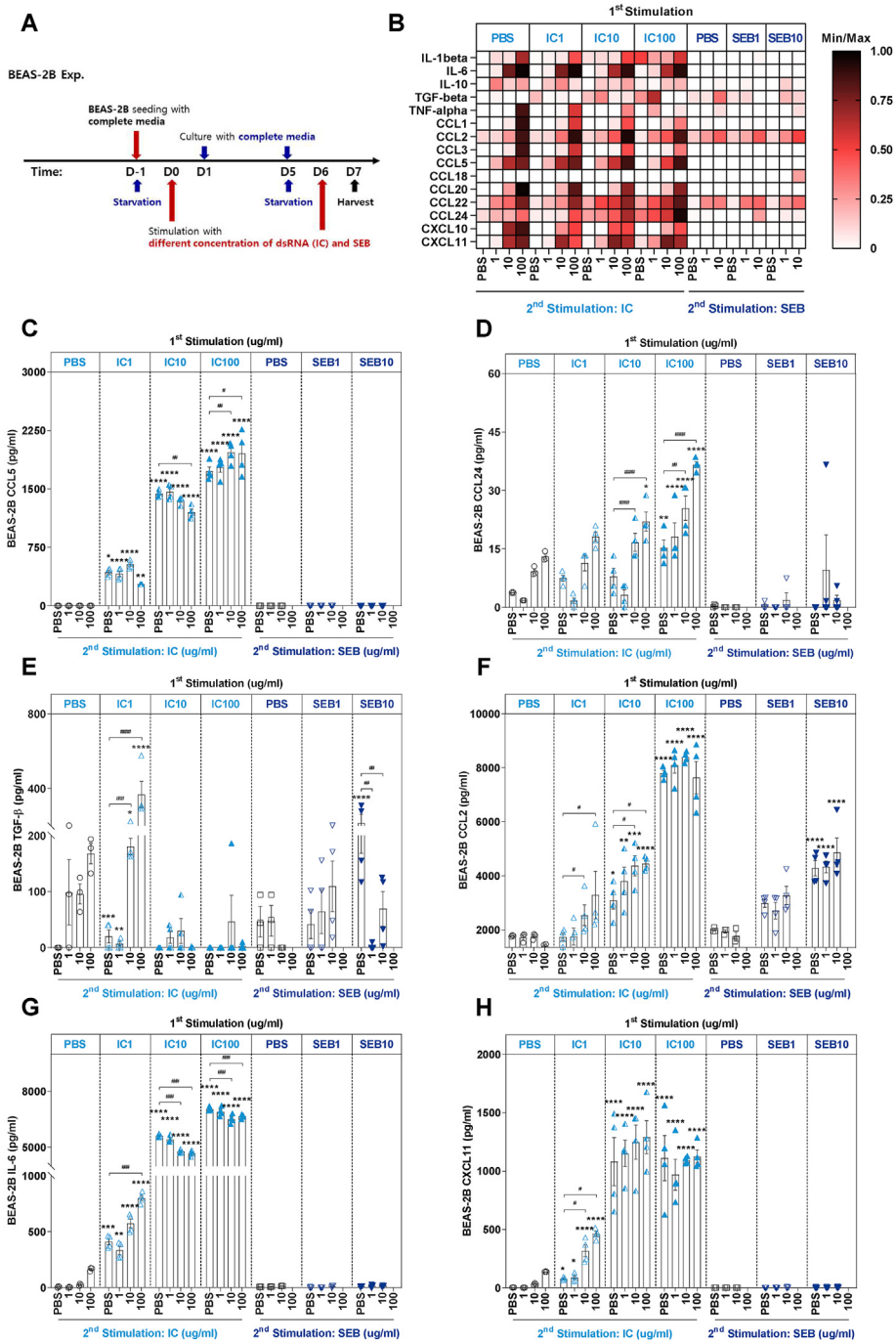


**Fig. 3 Cytokine profile after repeated stimulation with dsRNA or SEB in epithelial cells.** Epithelial cells **repeatedly underwent** stimulated with different doses of dsRNA or SEB, and the cell culture supernatants were analyzed for the expression of 20 cytokines. (A) Schematic diagram illustrating cell culture and stimulation. (B) Heat-map analysis results of cytokine expression (excluding IL-25, IL-33, TSLP, CCL11, and CCL17). (C–H) The detailed results of cytokine expression highlighting significant changes, CCL5 (C), CCL24 (D), TGF-β (E), CCL2 (F), IL-6 (G), and CXCL11 (H). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 for comparison with its PBS control group; #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001, ####p ≤ 0.0001 for comparison between each group.

contrast to dsRNA-stimulation. Moreover, TGF-β was suppressed when higher doses were used during the second stimulation (Fig. 3E and F). Other cytokines including CCL24, IL-1β, IL-10, TNF-α, CCL18, and CCL22, and were not significantly induced compared to each control group (Fig. 3D and Supplementary Fig. 2).

### Alternate-stimulation with dsRNA and SEB or vice versa alters cytokine production compared to repeated-stimulation with the same molecules in macrophages

Next, when macrophages were initially stimulated with dsRNA and subsequently cross-exposed to SEB (Fig. 4A), 14 cytokines were induced (IL-1β, IL-6, IL-10, TNF-α, CCL1, CCL2, CCL3, CCL5, CCL18, CCL20, CCL22, CCL24, CXCL10, and CXCL11, Fig. 3B), similar to the results obtained with respect to the macrophages stimulated twice with dsRNA. Except for these cytokines, there was no significant increase in the production of the other cytokines. Compared to a single stimulation with SEB, groups pre-stimulated with IC10 and IC100 exhibited a significant increase in cytokine induction. However, 2 opposing, dose-dependent cytokine production responses to the second SEB stimulation were observed in alternate-stimulation with IC100-SEB: CCL24 and IL-1β increased, while IL-6, TNF-α, CCL1, and CCL2 decreased (Fig. 4F and Supplementary Fig. 3). In the case of SEB-dsRNA alternate-stimulation, most significant differences were observed in the group stimulated with a high concentration of SEB (SEB10) compared to their respective control groups, i.e., single dsRNA stimulations or PBS control group: CCL2, CCL5, CCL20, CCL22, CCL24, CXCL10, and CXCL11 increased, while TNF-α decreased (Fig. 4C–H, and Supplementary Fig. 3).

### Alternate-stimulation with dsRNA and SEB or vice versa modified cytokine production compared to repeated-stimulation with the same molecules in epithelial cells

Alternate-stimulation of dsRNA-SEB and vice versa in epithelial cells (Fig. 5A) induced the production of 10 cytokines, including IL-6, TNF-α, TGF-β, CCL1, CCL2, CCL3, CCL5, CCL20, CXCL10, and CXCL11, and there was no statistically significant difference in the other cytokines (Fig. 5B and Supplementary Fig. 4). When epithelial cells were subsequently stimulated with dsRNA and SEB, 10 cytokines were primarily induced in the groups initially stimulated with dsRNA10 or 100, compared to the control group with single SEB stimulation. However, an increase was observed in only 4 cytokines (CCL1, CCL20, CXCL10, and CXCL11), with higher concentration observed with increasing doses during the second SEB stimulation compare to the PBS controls (Fig. 5C and E-G). However, in the SEB-dsRNA stimulation groups, significant alterations were observed in 4 cytokines (CCL5, TGF-β, TNF-α, and CCL2) when stimulated with a subsequent high concentration of SEB (SEB10) and dsRNA (IC100), compared to single dsRNA stimulation during the second stimulation. (Fig. 5D, H, and Supplementary Fig. 4). Among them, 2 opposing changes were observed: CCL5 and TGF-β increased, while TNF-α decreased.

### Macrophages and epithelial cells repeatedly stimulated with dsRNA or SEB exhibit different phosphorylation patterns

To investigate changes at the intracellular signaling level, we focused on both macrophages and epithelial cells repeatedly stimulated with a high dose of dsRNA or SEB, which showed significant differences in cytokine production.

In macrophages, phosphorylation changes were identified, and the phosphor-proteins were divided into 7 groups based on their hierarchical dendrogram and phosphorylation patterns. In groups 1, 2, 4, 6, and 7, repeated stimulation with dsRNA induced an increase in the phosphorylation of each protein compared to that in the control PBS group. However, groups 1, 2, and some part of group 6 exhibited an increase in phosphorylation, regardless of the repetition of stimulation. With respect to SEB stimulation, groups 1, 5, 6, and 7 showed a significant increase in protein phosphorylation compared to the control PBS- and single-SEB-stimulated groups. Group 3 exhibited increased phosphorylation, however it was lower than that in the single-stimulation group. Some proteins of group 4 showed a tendency of decreasing phosphorylation (Fig. 6A).

In epithelial cells, repeated stimulation with dsRNA induced an increase in phosphorylation in

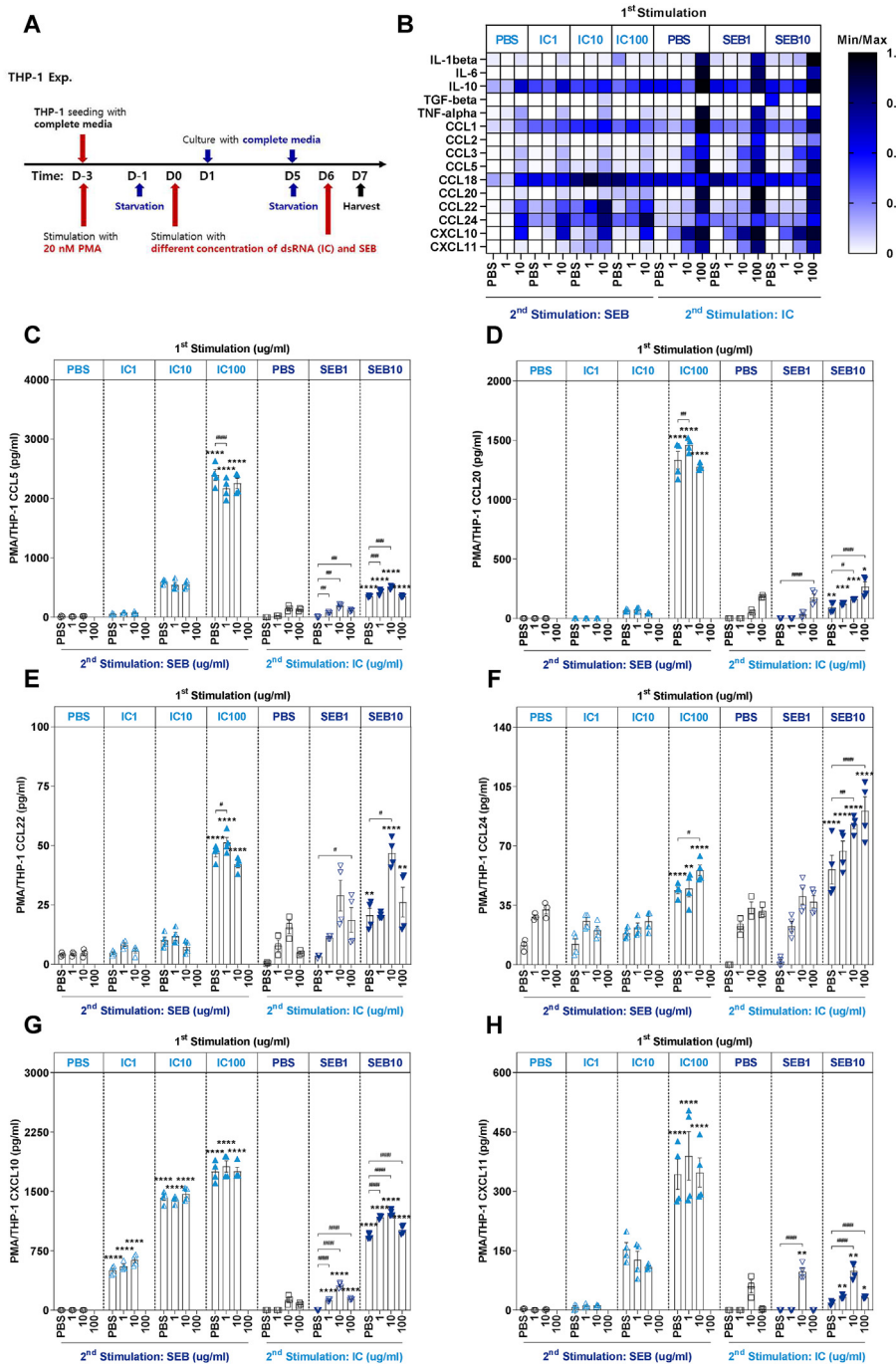


**Fig. 4 Cytokine profile after alternate-stimulation with dsRNA and SEB in macrophages.** THP-1 derived macrophages were alternately stimulated with various doses of dsRNA and SEB. The cell culture supernatants were analyzed for the expression of 20 cytokines. (A) Schematic diagram illustrating cell differentiation, culture, and stimulation. (B) Heat-map analysis results of cytokine expression (excluding IL-25, IL-33, TSLP, CCL11, and CCL17). (C–H) The detailed results of cytokine expression demonstrating significant changes, CCL5 (C), CCL20 (D), CCL22 (E), CCL24 (F), CXCL10 (G), and CXCL11 (H). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 for comparison with its PBS control group; #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001, ####p ≤ 0.0001 for comparison between each group.

groups 1 and 3. However, compared with the single-stimulated group, group 3 exhibited a lower degree of phosphorylation. This tendency was also observed in groups 4, 5, 6, and 7. In these groups, the phosphorylation levels were lower than those in the single-stimulated groups. The same was observed for the groups 1, 2, 3, 5, and 6, when stimulated with SEB. Repeated stimulation with

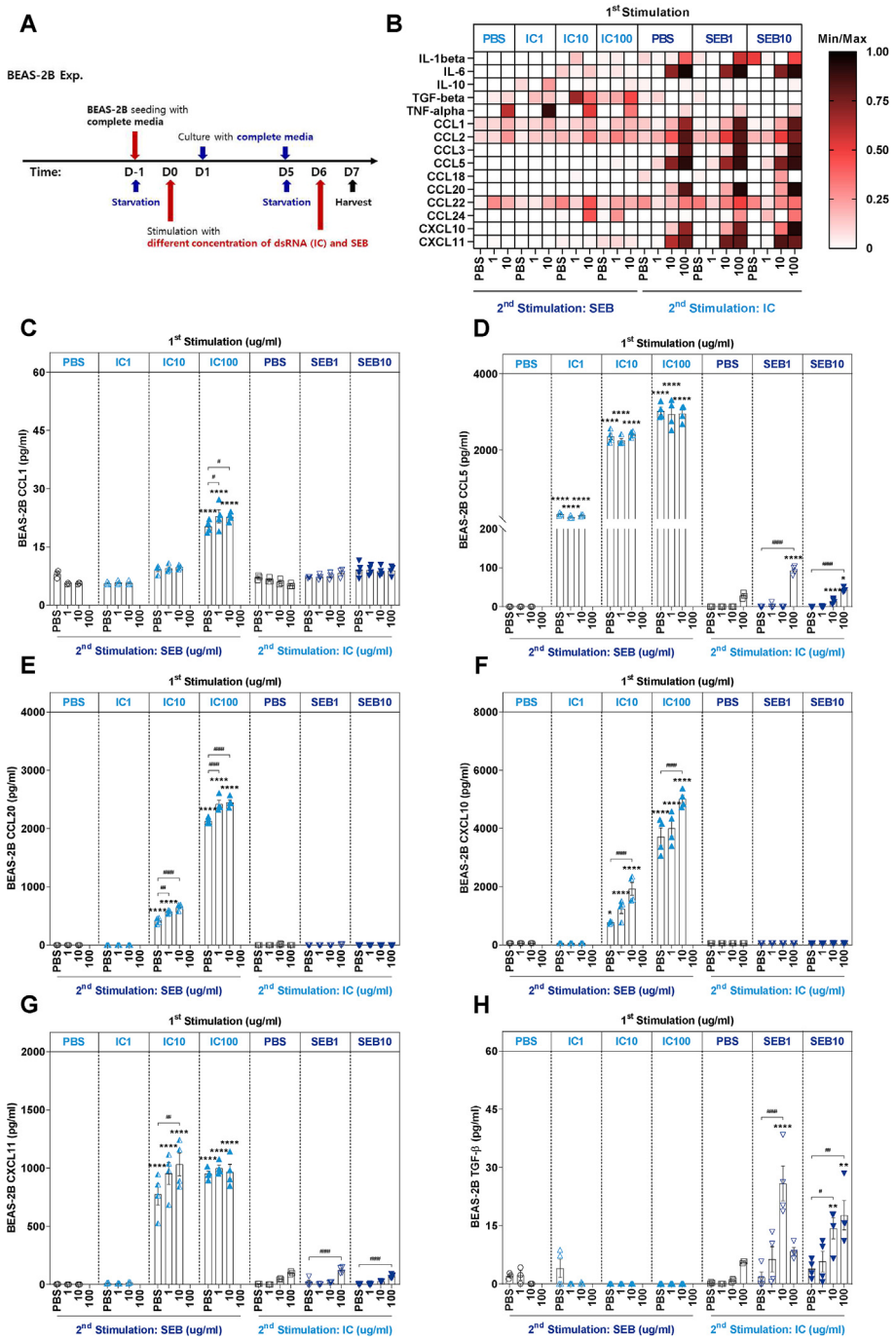


**Fig. 5 Cytokine profile after alternate-stimulation with dsRNA and SEB in epithelial cells.** Epithelial cells were subjected to alternate stimulation with various doses of dsRNA and SEB, and the cell culture supernatants were analyzed for the expression of 20 cytokines. (A) Schematic diagram illustrating cell differentiation and stimulation. (B) Heat-map analysis results of cytokine expression (excluding IL-25, IL-33, TSLP, CCL11, and CCL17). (C–H) The detailed results of cytokine expression demonstrating significant changes, CCL5 (C), CCL24 (D), TGF-β (E), CCL2 (F), IL-6 (G), and CXCL11(H). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 for comparison with its PBS control group; #p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001, ####p ≤ 0.0001 for comparison between each group.

SEB induces an increase in phosphorylation in groups 4 and 7. However, the results in group 4 were independent of repetition, and those in group 7 were similar to the results of dsRNA in group 3 (Fig. 6B).

## DISCUSSION

In the respiratory system, *S. aureus* can be found in the upper airways of the respiratory system and it affects the upper and lower airway immune

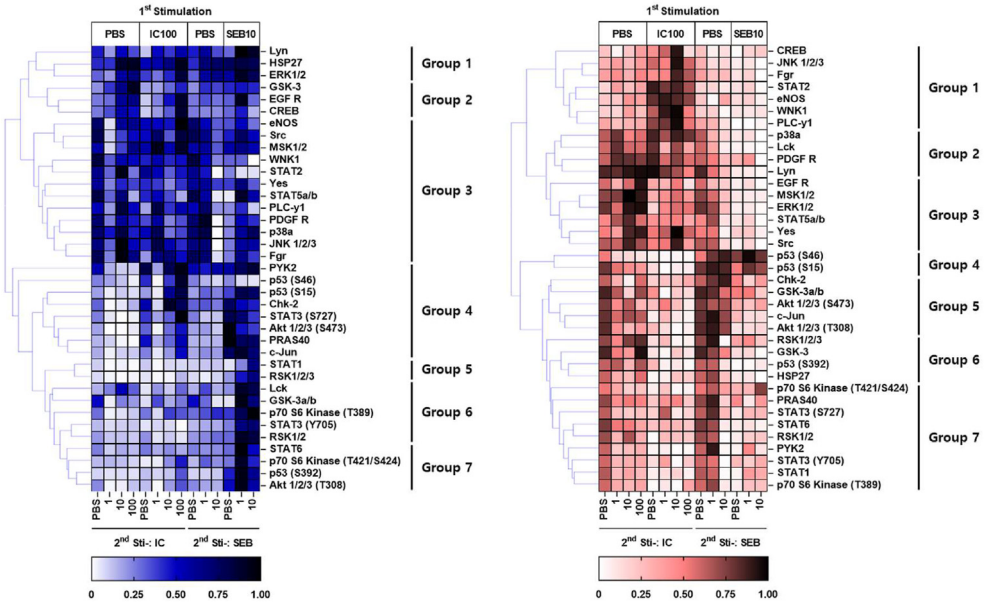


**Fig. 6 Phosphor-kinase proteome arrays after repeated-stimulation with dsRNA or SEB in macrophages and epithelial cells.** Heat-map and cluster analysis of phosphor-kinase proteome arrays, including 37 phosphor-kinases normalized with total protein (β-catenin) in macrophages (A) and epithelial cells (B). Heat-map colors show scaled, normalized protein levels in each sample. THP-1-derived macrophages and BEAS-2B cells stimulated with different doses of dsRNA and SEB, as specified in some part of Figs. 1 and 2, were used for this analysis.

systems by secreting extracellular molecules including toxins. SEB stimulation may amplify immune responses induced by a combination of lower airway infections with respiratory viruses. In a previous study, we demonstrated this synergistic effect using a combination of dsRNA and SEB in epithelial cells and macrophages, the first responders in the respiratory system.[11] This study focused on the immune alterations upon simultaneous exposure to both molecules. However, infections caused by various factors can occur multiple times in a host. Thus, in this study, we focused on the immune responses induced by repeated exposure to or cross-stimulated by dsRNA or SEB with an interval of several days between 2 subsequent stimulations.

We observed that repeated stimulation using dsRNA or SEB in macrophages and epithelial cells could modify immune responses of both cell types compared to a single stimulation. Moreover, the effect of the first stimulation persisted until the second stimulation, even though the culture medium changed during the interval period to remove the effect of the first stimulation. With respect to dsRNA primary stimulation in both macrophages and epithelial cells, among the 20 evaluated cytokines, 13 cytokines were still produced even 7 days later without any additional stimulation. Repeated stimulation with SEB showed a similar but weaker tendency. With respect to cross-stimulation with dsRNA and SEB, the persistence of the primary stimulation effect was observed to varying degrees in both macrophages and epithelial cells.

Previous studies have investigated repeated stimulation of both macrophages and epithelial cells with innate immune stimulators, including bacteria and their components.[12–14] However, compared to our study, there were some differences, such as a shorter interval between stimulations, higher concentration of stimulation, and less cytokines were measured to evaluate changes in inflammation. Despite these differences, we found that a weak initial stimulation can enhance the response to a second stimulation, whereas a strong initial stimulation tends to suppress the response in previous studies and in some parts of the present study. This indicates that the characteristics of both macrophages and epithelial cells were altered by primary stimulation, and these changes were maintained until the end of secondary stimulation. According to the concept of priming and trained immunity described in a

previous study, primary stimulation can induce epigenetic changes such as histone modifications in cytokine genes, which can affect secondary stimulation by persisting.[9] Although we did not present direct evidence of histone modification in cytokine gene expression in this study, we can speculate that epigenetic changes occurred following dsRNA or SEB stimulation and persisted for at least 1 week based on the results of cytokine profiles.

In the present study, most cytokines were induced by strong stimulation with dsRNA or SEB. Moreover, among the cytokines induced by macrophages, the production of IL-1β, IL-6, TNF-α, and CCL20 decreased, while the levels of CCL22 and CCL24 increased by repeated exposure to strong dsRNA stimulation (IC100 group). As for the repetition of strong stimulation with SEB (SEB10 group), CCL3 and CCL5 levels decreased and CCL24 levels increased, similar to the results obtained with dsRNA stimulation. The decrease in cytokines caused by the repetition of strong dsRNA or SEB stimulation are related to the host defense system, including the recruitment of innate and adaptive immune cells, such as macrophages, dendritic cells, natural killer (NK) cells, and Th1 and Th17 cells, to defend against respiratory viral infections. Increased levels of CCL22 and CCL24 are well-known inducers of eosinophils, basophils, mast cells, and Th2 cell recruitment for type II cytokine production via their receptors CCR4 and CCR3, respectively.[15] Previous studies have shown that ILC2 and T cells isolated from the human lung show high expression of CCR2/CCR5/CCR6 and CCR3/CCR4/CCR6, respectively[16] and that respiratory viral infections induce type II cytokines in the nasal lining fluid of normal and asthmatic patients.[17] Moreover, repetition of strong dsRNA stimulation leads to an increase in CCL1/CCL2/CCL3, which can recruit diverse immune cells, including eosinophils, basophils, mast cells, and Th2 cells, through recognition by CCR8, CCR2, and CCR1/CCR5.[15]

Similar to the results obtained from the experiments with the macrophages, most significant changes in cytokine production of the epithelial cells were induced by strong repeated stimulation with dsRNA or SEB. However, little difference was observed in the degree and pattern of these changes. In addition to decrease in IL-6, TNF-α, and CCL20 in macrophages, CCL1, CCL3, and CXCL10 were also down-regulated, while only CCL24 increased in response to dsRNA. With respect to SEB, stimulation did not induce efficient cytokine production, as observed in our previous study,[11] and did not induce significant changes during repeated stimulation. CCL1 is recognized by CCR8, CCL3 by CCR1/CCR5, and CXCL10 by CXCR3. They play a role in recruiting immune cells related to type I immune responses or viral defense, including NK cells, NKT cells, Th1 cells, CD8 T cells, monocytes, macrophages, and dendritic cells.[15,18] Repeated stimulation, especially with dsRNA, can induce the down regulation of type I immune responses against viral invasion, as observed in macrophages. In addition, IL-10 and TGF-β, known for their immunosuppressive effect, were respectively induced in macrophages and epithelial cells. These results show the possibility that repeated stimulation of macrophages and epithelial cells with dsRNA and SEB leads to unfavorable alterations in the host defense system.

Similarly, alternate stimulation with dsRNA-SEB and SEB-dsRNA altered both macrophage and epithelial cell responses compared to their respective control single stimulations. However, when compared to the results of repeated stimulation with the same molecules in both macrophages and epithelial cells, these changes were found to be lesser. Low- and medium-concentration stimulations were less effective in both cell types. If SEB stimulation was followed by a string dsRNA stimulation, IL-6 and TNF-α production was notably reduced, along with an increase in CCL24. Additionally, dsRNA stimulation followed by SEB stimulation upregulated CCL22 and CCL24 production in macrophages. Meanwhile, in epithelial cells, dsRNA-SEB stimulation induced a decrease in TNF-α and increase in CCL20, CXCL10, and CXCL11. Moreover, SEB-dsRNA stimulation induced TGF-β production, in addition to reduction in TNF-α. Although, the degree of cytokine production and amplification was lower than repeated stimulation with the same dsRNA or SEB, alternate-stimulation also induced down-regulation of TNF-α related with invasion of foreign molecules. Additionally, it led to the upregulation of type 2 immune response-related

chemokines CCL20, CCL22, and CCL24. Even, TGF-β might induce the development of Th2/17 immune responses or fibrosis.[19]

In both macrophages and epithelial cells, dsRNA and SEB are recognized by TLR3/RIG-I/MDA-5 and CD40 molecules, respectively.[20,21] These receptors can activate well-known signaling molecules such as p38, JNK, ERK, and AKT.[20,22–24] In this study, we detected alterations in these and related phosphoproteins by stimulation with dsRNA and SEB. Moreover, when comparing the results of macrophages with those of epithelial cells, an increase in phosphorylation was induced by repeated stimulation with SEB in macrophages but not in epithelial cells. This may be because macrophages have the aforementioned SEB-binding proteins. However, epithelial cells lack MHC II and B7 molecules and express lower levels of CD40 than macrophages.[25] In macrophages, the protein of groups 6 and 7 in our study (Fig. 5) showed an increase in phosphorylation upon repeated stimulation with dsRNA or SEB. However, phosphorylation of group 4 was induced by dsRNA, whereas phosphorylation of group 1 and 5 were induced by SEB. In epithelial cells, repeated stimulation with dsRNA or SEB induced phosphorylation in group 1 or 4, respectively. Based on these results, we postulate that stimulation-specific phosphorylation plays an important role in cellular responses, including cytokine production. However, it is important to note that the total phosphorylation pattern and stimulation-specific phosphorylation may vary depending on cytokine expression levels and array evaluation time. In addition, this study was performed in the "closed system," where products, such as cytokines, from primary immune responses by dsRNA or SEB can affect to themselves. This may stimulate both macrophages and epithelial cells continuously and eventually induce superactivation.[26] Similar to previous reports, our previous study also demonstrated a rapid increase in TNF-α and other pro-inflammatory cytokines by strong stimulation with dsRNA in macrophages. Thus, this phenomenon can reflect auto- and paracrine state in the innate immune system.

Cohort studies have revealed a correlation between early viral infection in the respiratory system and persistent/recurrent wheezing disorders and asthma.[27,28] Some studies supported this possibility by studying patients hospitalized for respiratory virus infections who developed allergic disease.[29–31] However, other studies have suggested that RV and RSV infections in the respiratory system may be related to wheezing disorders and asthma exacerbations and not to the development of allergic disorders.[32–34] This remains a controversial issue, primarily focusing on clinical findings that lack the precise mechanisms. *S. aureus* colonization of the upper airway is also associated with the development of wheezing and asthma in young individuals and adults.[35,36] The product of *S. aureus*, SEB, can cause asthma-like airway inflammation due to inhaled proteins.[37] Our previous study demonstrated cytokine induction by dsRNA and SEB and the synergistic effects of both molecules.[11] Additionally, in this study, repeated and alternating stimulation with dsRNA and SEB induced an unfavorable alteration in the host innate immune system response to viral infection. Our previous and current data may be able to enhance our understanding of the precise mechanisms and correlations between viral infections and the development of asthma. In addition, our data indicated that repeated and alternate stimulation of foreign immunostimulators, such as respiratory viruses and secreted molecules from the upper-respiratory bacteria, should be avoided to prevent these immune alterations.

## CONCLUSION

Our study showed various cytokine changes following repeated and alternate stimulation with dsRNA and SEB, compared to single stimulation. While further precise mechanistic studies are needed, this study may present insights into how frequent respiratory virus infections and the synergistic effect of viral infection and SEB in the airway affect the development and exacerbation of asthma.

## ABBREVIATIONS

SEB, *S. aureus* enterotoxin B; dsRNA, double-stranded RNA; CCL, C–C chemokine ligand; CXCL, C-X-C chemokine ligand; IL, Interleukin; TGF-β, Transforming growth factor beta; BCG, *Bacillus Calmette-Guérin*; LPS, Lipopolysaccharide;

TSLP, Thymic stromal lympopoietin; TNF-α, Tumor necrosis factor alpha; Th, T helper; NK, Natural killer; NKT, Natural killer T; TLR, Toll-like receptor; RIG-I, Retinoic acid-inducible gene-I; MDA-5, Melanoma differentiation-associated protein 5; CD, Cluster of differentiation; MHC, Major histocompatibility complex; RV, Rhinovirus; RSV, Respiratory Syncytial virus.

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### Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Ethics approval
All experiments were approved by the Institutional Review Board (IRB) of Seoul National University Bundang Hospital (IRB number: X-2004/604-903).

### Consent for publication
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### Authors' contributions
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IRB approval: YSC.
Writing the manuscript: JPC, KSH.
Revision: JPC, KSH, YSC, MKK.

### Declaration of competing interest
The authors declare no potential conflict of interest.

### Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2025.101026.

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