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Cytokine Inductions and Intracellular Signal Profiles by Stimulation of dsRNA and SEB in the Macrophages and Epithelial Cells

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ABSTRACT

Foreign molecules, including viruses and bacteria-derived toxins, can also induce airway inflammation. However, to the best of our knowledge, the roles of these molecules in the development of airway inflammation have not been fully elucidated. Herein, we investigated the precise role and synergistic effect of virus-mimicking double-stranded RNA (dsRNA) and staphylococcal enterotoxin B (SEB) in macrophages and epithelial cells. To identify cytokine expression profiles, both the THP-1-derived macrophages and BEAS-2B epithelial cells were stimulated with dsRNA or SEB. A total of 21 cytokines were evaluated in the culture supernatants. We observed that stimulation with dsRNA induced cytokine production in both cell types. However, cytokine production was not induced in SEB-stimulated epithelial cells, compared to the macrophages. The synergistic effect of dsRNA and SEB was evaluated observing cytokine level and intracellular phospho-signaling. Fifteen different types were detected in high-dose dsRNA-stimulated epithelial cells, and 12 distinct types were detected in macrophages; those found in macrophages lacked interferon production compared to the epithelial cells. Notably, a synergistic effect of cytokine induction by co-stimulation of dsRNA and SEB was observed mainly in epithelial cells, via activation of most intracellular phosphor-signaling. However, macrophages only showed an accumulative effect. This study showed that the type and severity of cytokine productions from the epithelium or macrophages could be affected by different intensities and a combination of dsRNA and SEB. Further studies with this approach may improve our understanding of the development and exacerbation of airway inflammation and asthma.

Keywords: Double-stranded RNA; Staphylococcal enterotoxin B; Macrophages; Epithelial cells

INTRODUCTION

The respiratory system is on the frontline of exposure to foreign molecules. These molecules, which include chemical reagents and biological derivatives, can induce innate immune

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
dsRNA, double-stranded RNA; RSV, respiratory syncytial virus; RV, rhinovirus; SEB, staphylococcal enterotoxin B.

Author Contributions
Conceptualization: Choi JP; Data curation: Choi JP; Formal analysis: Choi JP, Ayoub G, Kim SH, Chang YS; Funding acquisition: Kim SH, Chang YS; Investigation: Choi JP, Ji M; Methodology: Choi JP; Project administration: Choi JP, Kim SH, Chang YS; Resources: Kim SH, Cho SH, Chang YS; Supervision: Kim SH, Cho SH, Chang YS; Validation: Choi JP; Visualization: Choi JP; Writing - original draft: Choi JP, Ji M; Writing - review & editing: Choi JP, Losol P, Kim SH, Cho SH, Chang YS.

response via the activation of the surveillance system of the lung. That system is composed of alveolar macrophages and epithelial cells. Foreign molecules can stimulate immune-related receptors on these cells, such as pattern recognition receptors, including toll-like-, scavenger-receptor or protease activated receptor, and induce airway inflammation, through elevating cytokine production (1,2). These processes may heighten the sensitization to inhaled protein molecules and result in the deterioration of immune tolerance to allergens or the exacerbation of underlying diseases, including asthma or chronic obstructive pulmonary disease (3-5).

Most respiratory viruses, including rhinovirus (RV) and respiratory syncytial virus (RSV), are single-stranded RNA viruses. These viruses make double-stranded RNA (dsRNA) in the host cell as a means to replicate. Although the components and characteristics of each virus are different from one another, the above mechanism is similar across the various viruses (6). The dsRNA is able to activate an innate immune response by stimulating a pattern-recognition receptor, TLR3 and RIG-I, on the macrophage and epithelial cell, and the activated cells produce inflammatory cytokines, including type I Interferons, typically (1). Interestingly, some clinical studies showed that there is a close correlation between viral infection of the airway and development of asthma. Infants who have experienced severe respiratory infection by RV or RSV showed an induction of wheezing and increased risk of eventual asthma development (4,5,7). Nonetheless, although a correlation between asthma development and viral infection has been shown by previous studies, a detailed mechanism explaining the correlation has not been fully understood.

To date, the respiratory tract has been regarded as a sterile environment for a long time. However, with recent progress and advancement in analytical techniques, it has been revealed that there is a microbiome in the airway. The types of microbes in the airway are dependent on the state of the host. For example, in patients with asthma, *Staphylococcus aureus* is more abundant in the upper-airway (8). *S. aureus* has been well-known to colonize the upper-airway, especially in asthma patients. *S. aureus* is able to affect the host via secreting a diverse set of protein molecules, including enterotoxins. Clinical studies have suggested a relationship between *S. aureus* and development of airway inflammation and asthma through colonization or IgE sensitization to staphylococcal enterotoxin B (SEB) (9-11). Moreover, colonization of *S. aureus* could also be related with infection and interaction with respiratory viruses (12). However, to the best of our knowledge, their role and overall mechanism behind how they affect the host innate immunity are poorly understood.

For this reason, we first stimulated the macrophages and epithelial cells with dsRNA and SEB, separately, to study their response to these stimulants in the early phase of innate inflammation. Next, we stimulated the macrophages and epithelial cells with a combination of both dsRNA and SEB to evaluate their potential synergistic effect on the host innate immune system of the airway.

MATERIALS AND METHODS

Cell culture and stimulation

Human bronchial epithelia and monocyte cell lines (BEAS-2B and THP-1) were obtained from America Type Culture Collection (Manassas, VA, USA; CRL-9609 and TIB-202, respectively). CRL-9609 and TIB-202 cells were maintained in DMEM and RPMI 1640 (Dulbecco's modified

eagle medium and Roswell park memorial institute medium; Welgene, Gyeongsan, Korea), respectively, supplemented with 10% FBS (Biowest, Riverside, MO, USA), 1× antibiotics (penicillin and streptomycin; Welgene), and 10 mM HEPES (Welgene). All experiments were approved by the Institutional Review Board (IRB) of Seoul National University Bundang Hospital (IRB number: X-2004/604-903).

For stimulation, BEAS-2B cells were seeded at a density of 1.0×10^6 cells in 750 μl of serum-free medium per well in 6-well culture plates (Corning, Corning, NY, USA). After overnight starvation, cells were stimulated with poly I:C or SEB (Sigma-Aldrich, St. Louis, MO, USA) under serum-free conditions.

For differentiation into macrophages, THP-1 monocytes were seeded similar to epithelial cells with 2 ml of serum-free medium containing 150 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich). After 2 days, the culture media were replaced with fresh serum-free media for one day to stabilize the activated macrophages. The cells were then stimulated following the aforementioned conditions.

The supernatants were harvested 0.5, 3, 6, and 12 h after stimulation and stored at −20°C. To evaluate the synergistic effect of both molecules, cells were stimulated with various concentrations of poly I:C and SEB for 12 h. Then, the cells and supernatants were stored at −20°C for further evaluation.

Evaluation of various cytokines using ELISA

To evaluate the induction of cytokines after stimulation, 21 types of molecules—IL-6, IL-12p70, IL-25, IL-33, TSLP, IFN-λ (IL-29/IL-28B), IFN-β, TNF-α, CCL2 (MCP-1), CCL3 (MIP-1), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (TARC), CCL20 (LARC), CCL22 (MDC), CCL24 (Eotaxin-2), CXCL8 (IL-8), CXCL10 (IP-10), CXCL11 (I-TAC), G-CSF (CSF3), and soluble ICAM-1 (sICAM-1)—were estimated in each sample using the human ELISA Duoset kit (R&D Systems, Minneapolis, MN, USA), per the manufacturer's guidelines.

Evaluation of phosphorylation level using a protein array

The frozen cells were lysed with cell lysis buffer containing protease inhibitor from a Human Phospho-Kinase Array Kit (ARY003C; R&D Systems). All procedures were performed in accordance with the manufacturer's guidelines. In brief, lysates were incubated on the target-specific antibody pre-coated membrane for 2 h at room temperature, and then, the membranes were incubated with the detection antibody mixture. Next, chemiluminescence was detected using an X-ray film in a dark room after HRP-conjugation (**Supplementary Fig. 1**). The film results were measured and digitalized using the ImageJ software (version 1.52a; National Institutes of Health, Bethesda, MD, USA; **Supplementary Tables 1** and **2**). Then, the data were normalized, clustered, and a dendrogram was generated using the heatmap.2 function in the ggplot package of R (R Foundation, Vienna, Austria). Normalization was performed on each row of the map. Heatmaps were generated using GraphPad Prism (version 7.0; GraphPad, La Jolla, CA, USA). Enrichr (developed by the Ma'ayan Lab) and Kyoto encyclopedia of genes and genomes (KEGG, www.genome.jp; developed by Kanehisa Laboratories) were used to investigate the related signaling pathways.

Statistical analysis

GraphPad Prism (version 7.0) was used to construct the graphs. All data are presented as the mean ± SEM. Statistical significance was calculated using one- or 2-way ANOVA with *post hoc*

comparisons (Kruskal-Wallis test or Dunnett test, respectively). Statistical significance was set at $p<0.05$.

RESULTS

dsRNA or SEB stimulation induces different cytokine expression pattern in macrophages and bronchial epithelial cells

To evaluate the cytokine expression by dsRNA or SEB stimulation, we treated macrophages and bronchial epithelial cells with each molecule separately and estimated the production of 21 cytokine types in the culture supernatant at each time point. We detected 15 cytokine types in the culture supernatant derived from activated macrophages with diverse stimulators (**Fig. 1**); IL-12, IL-25, IL-33, TSLP, CCL17 (data not shown), and G-CSF were not detected (**Fig. 1P**). Exposure of macrophages to low-dose dsRNA (1 μg/ml) induced the production of 3 cytokines, namely, CCL24, CXCL10, and CXCL11 at 12 h after stimulation (**Fig. 1G, I, and J**).

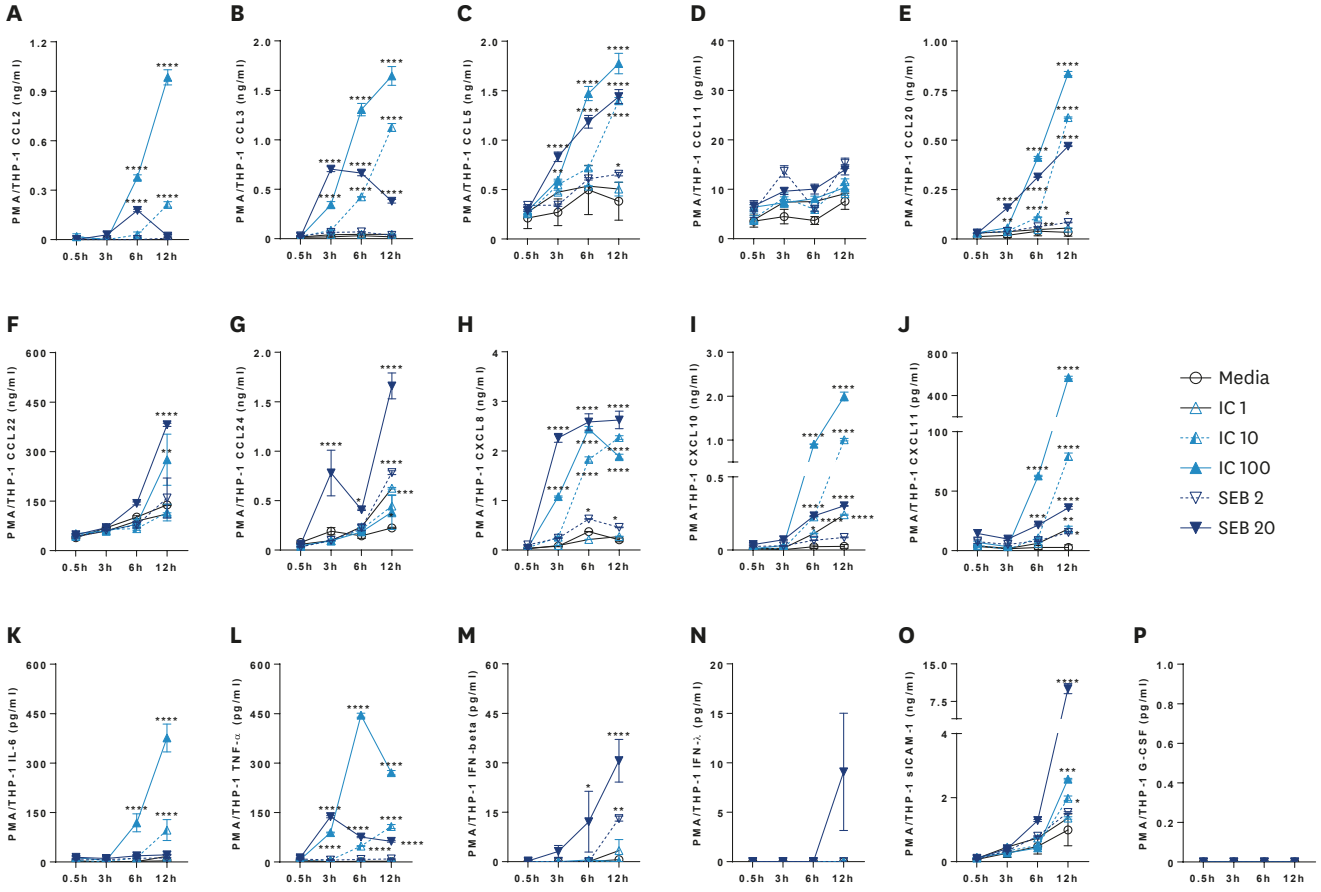

Figure 1. Cytokine induction profile over time after stimulation with dsRNA or SEB in macrophages. Twenty-one types of cytokines were evaluated using ELISA in the culture supernatant derived from THP-1-derived macrophages. After stimulation with each dose of dsRNA or SEB, evaluation was performed at 0.5, 3, 6, and 12 h after stimulation. IL-12p70, IL-25, IL-33, TSLP, and CCL17 (TARC) were not detected in all experiments (data not shown). All experiments were repeated at least twice. In all experiments, comparisons are relative to media control group at each time point. (A) CCL2 (MCP-1). (B) CCL3 (MIP-1α). (C) CCL5 (RANTES). (D) CCL11 (Eotaxin). (E) CCL20 (LARC). (F) CCL22 (MDC). (G) CCL24 (Eotaxin-2). (H) CXCL8 (IL-8). (I) CXCL10 (IP-10). (J) CXCL11 (I-TAC). (K) IL-6. (L) TNF-α. (M) IFN-β. (N) IFN-λ1/3 (IL-29/IL-28B) (O) sICAM-1. (P) G-CSF (CSF-3).
IC, double-stranded RNA.
*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

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Macrophages treated with mid- and high-dose dsRNA (10 and 100 μg/ml) showed diverse and considerable cytokine production (total 15), including CCL2, 3, 5, 20, and 22 (**Fig. 1A-C, E, and F**), CXCL8 (**Fig. 1H**), IL-6, TNF-α (**Fig. 1K and L**), and sICAM-1 (**Fig. 1O**) 3 to 6 h after stimulation. Macrophages exposed to low-dose SEB (2 μg/ml) induced elevated production of 4 cytokines (CCL5, CXCL8, CXCL11, and IFN-β) at 6 h after stimulation (**Fig. 1C, H, J, and M**). High-dose SEB (20 μg/ml) induced the production of 11 other cytokines (including CCL2, 3, 20, 22, 24 [**Fig. 1A, B, and E-G**], CXCL10 [**Fig. 1I**], TNF-α [**Fig. 1L**], and sICAM-1 [**Fig. 1O**]), in addition to the abovementioned cytokines (CCL5 CXCL8, and CXCL11), 3 to 6 h after stimulation.

When the epithelial cells were exposed to low-dose dsRNA, the production of 3 cytokines, CXCL8, 10, and sICAM-1, was induced 6 h after stimulation (**Fig. 2H, I, and O**). Similar to the results of macrophages, mid- and high-dose dsRNA stimulation induced the production of 11 additional cytokines (CCL2, 3, 5, 11, 20, 22 [**Fig. 2A-F**], CXCL11 [**Fig. 2J**], IL-6 [**Fig. 2K**], IFN-β/λ [**Fig. 2M and N**], and G-CSF [**Fig. 2P**]) 3 h after stimulation. In the case of low- and high-dose SEB stimulation, the production of 5 cytokines (CCL2, 22, 24, CXCL8, and 11) was elevated (**Fig. 2A, F-H, and J**). Induction of IL-12p70, IL-25, IL-33, TSLP, CCL17 (data not shown), and TNF-α (**Fig. 2L**) production by dsRNA or SEB stimulation was not detected in epithelial cells.

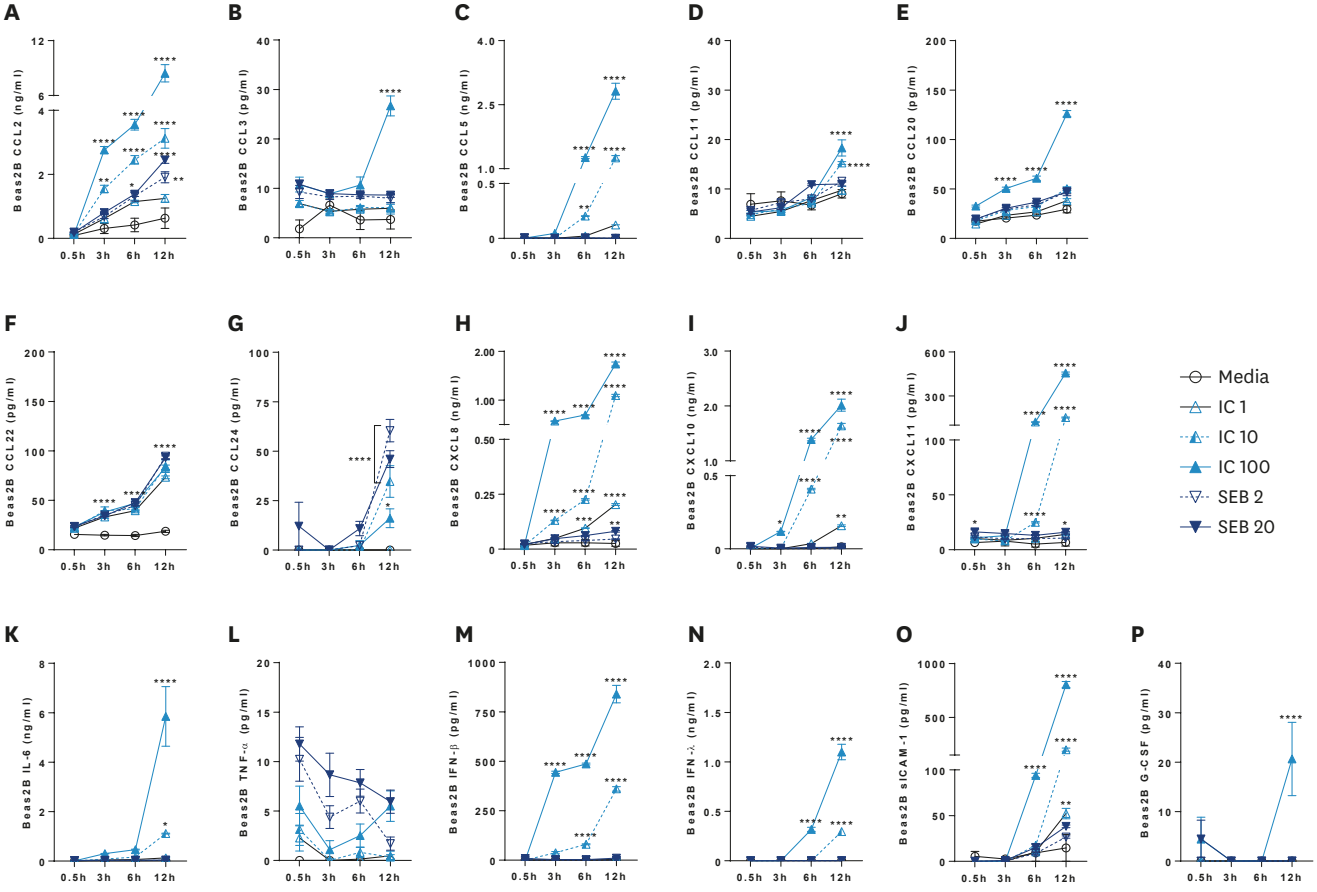

Figure 2. Cytokine production profile over time after stimulation with dsRNA or SEB in epithelial cells. BEAS-2B cells were stimulated with each dose of dsRNA and SEB, and ELISA was performed at each time point to detect 21 types of cytokines in culture supernatants. IL-12p70, IL-25, IL-33, TSLP, and CCL17 (TARC) were not detected (data not shown). All experiments were repeated at least twice. In all experiments, comparisons are relative to media control group at each time point. (A) CCL2 (MCP-1). (B) CCL3 (MIP-1α). (C) CCL5 (RANTES). (D) CCL11 (Eotaxin). (E) CCL20 (LARC). (F) CCL22 (MDC). (G) CCL24 (Eotaxin-2). (H) CXCL8 (IL-8). (I) CXCL10 (IP-10). (J) CXCL11 (I-TAC). (K) IL-6. (L) TNF-α. (M) IFN-β. (N) IFN-λ1/3 (IL-29/IL-28B) (O) sICAM-1. (P) G-CSF (CSF-3).
IC, double-stranded RNA.
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

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Co-stimulation with dsRNA and SEB exerts synergic effects on cytokine expression in macrophages and bronchial epithelial cells

To evaluate the effect of combined exposure to dsRNA and SEB on macrophages and epithelial cells, we treated each cell type with both molecules simultaneously, with varying concentration of each molecule. We then evaluated the production of 21 cytokines 12 h after stimulation, when most of the cytokines were detected.

Similar to previous results, low-dose dsRNA stimulation in macrophages induced CCL24, CXCL10, and CXCL11 (**Fig. 3G, I, and J**) production; however, a synergistic effect of co-stimulation with dsRNA and SEB was not observed. In the case of mid- and high-dose

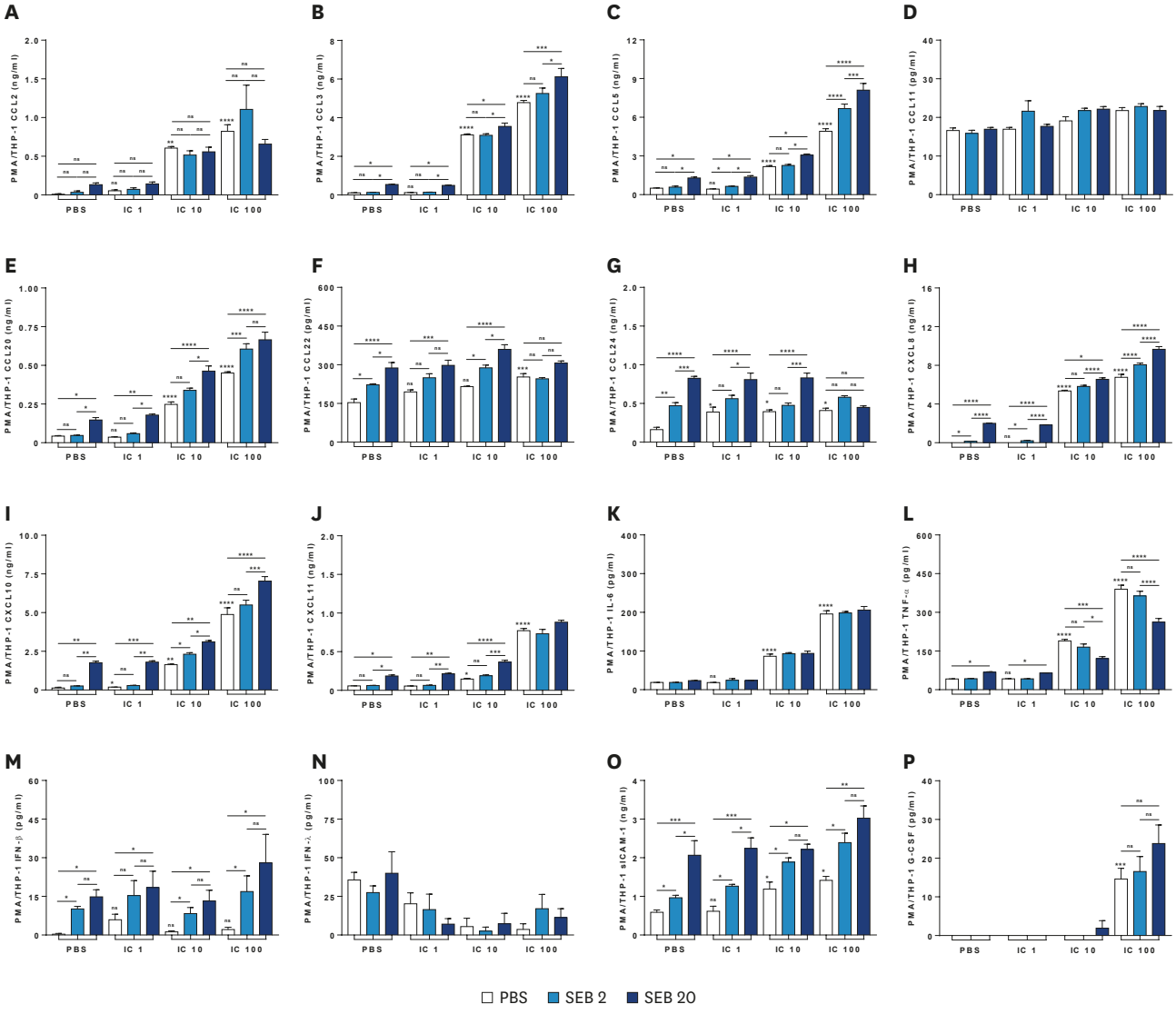

Figure 3. SEB partially assists to synergistically affect cytokine production of macrophages upon dsRNA stimulation. THP-1-derived macrophages were stimulated with different doses of dsRNA and SEB to evaluate their synergistic effect on cytokine production. In the culture supernatant after 12 h stimulation, levels of 21 kinds of cytokines were determined using ELISA. The levels of IL-12p70, IL-25, IL-33, TSLP, and CCL17 (TARC) did not differ between groups or were not detected in all experiments (data not shown). All experiments were performed at least twice. In all experiments, comparisons are relative to their corresponding control group. (A) CCL2 (MCP-1). (B) CCL3 (MIP-1α). (C) CCL5 (RANTES). (D) CCL11 (Eotaxin). (E) CCL20 (LARC). (F) CCL22 (MDC). (G) CCL24 (Eotaxin-2). (H) CXCL8 (IL-8). (I) CXCL10 (IP-10). (J) CXCL11 (I-TAC). (K) IL-6. (L) TNF-α. (M) IFN-β. (N) IFN-λ1/3 (IL-29/IL-28B) (O) sICAM-1. (P) G-CSF (CSF-3).
IC, double-stranded RNA.
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

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dsRNA stimulation, a synergistic effect of SEB was detected in the production of CCL3, CCL5, and IFN-β (**Fig. 3B, C, and M**). G-CSF also showed a synergistic effect, but it was not significant (**Fig. 3P**). Among the others, only CCL20, CXCL8, CXCL10, and sICAM showed the accumulative effect of dsRNA and SEB co-stimulation (**Fig. 3E, H, I, and O**).

Unlike the results for macrophages, the synergistic effect of co-stimulation was observed in the production of 13 cytokines in epithelial cells. Low-dose dsRNA and SEB induced CCL2, CCL5, CXCL8, CXCL10, CXCL11, IL-6, and IFN- IFN-β/λ production (**Fig. 4A, C, H-J, K, M, and N**). In addition, mid- or high-dose dsRNA induced synergistic effects on the production of CCL20 and CCL24 (**Fig. 4E and G**) or CCL3, CCL20, TNF-α, and G-CSF (**Fig. 4B, E, L, and P**), respectively.

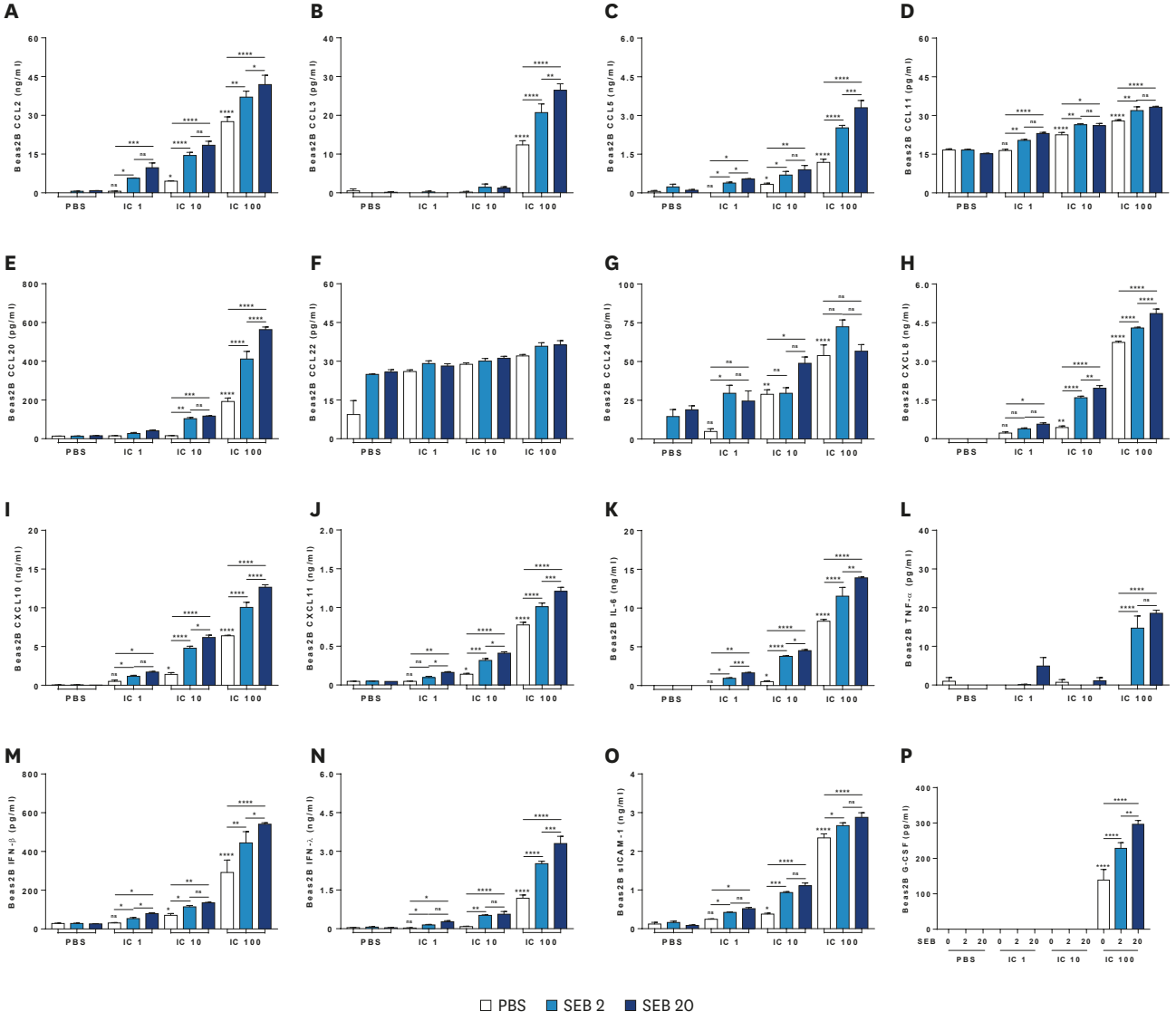

Figure 4. SEB plays an important role synergistic effect in the cytokine production of epithelial cells. Cytokine induction profiles 12 h after stimulation with poly I:C and/or SEB in epithelial cells. Twenty-one kinds of cytokines in the culture supernatants were assessed using ELISA. IL-12, IL-25, IL-33, TSLP, and CCL17 levels did not differ between groups in all experiments (data not shown). The experiments were repeated at least 2 times. In the all experiments, comparisons are relative to control group. (A) CCL2 (MCP-1). (B) CCL3 (MIP-1α). (C) CCL5 (RANTES). (D) CCL11 (Eotaxin). (E) CCL20 (LARC). (F) CCL22 (MDC). (G) CCL24 (Eotaxin-2). (H) CXCL8 (IL-8). (I) CXCL10 (IP-10). (J) CXCL11 (I-TAC). (K) IL-6. (L) TNF-α. (M) IFN-β. (N) IFN-λ1/3 (IL-29/IL-28B) (O) sICAM-1. (P) G-CSF (CSF-3). IC, double-stranded RNA.
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

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Co-stimulation with dsRNA and SEB increases the intra-cellular signaling in macrophages and bronchial epithelial cells

To investigate the mechanism of the synergistic effect of SEB and dsRNA co-stimulation on cytokine production in both cell types, we evaluated the phosphorylation of phospho-kinase and its substrates to reveal the differences in intracellular signaling cascades under the synergistic situation. We found differences in protein expression according to cell type and concentration of stimulators.

In macrophages, an increase in phospho-kinase protein level was observed in the mid-dose dsRNA-stimulated group. Phosphorylation of 18 proteins increased in groups stimulated with mid-dose dsRNA than of the proteins in the groups stimulated with low-dose dsRNA or combined with SEB. Based on the KEGG pathway analysis, most of them were related to chemokine or inflammation (IL) signaling pathways. In addition, co-stimulation with mid-dose dsRNA and low-dose SEB enhanced the expression of these molecules more than co-stimulation with high-dose SEB. Macrophages showed greater effects to strong stimulation and exhibited high activation of the signaling pathway. However, from this group, there was no significant difference in the signaling patterns between each other (**Fig. 5A**).

Compared with that of macrophages, the overall phosphorylation level of epithelial cells was reduced 12 h after stimulation (**Supplementary Fig. 1**). The combination of low-dose dsRNA and SEB had a synergistic effect on epithelial cells. In addition, most of the protein levels increased under these conditions. Interestingly, the highest phosphorylation level was observed in the low-dose dsRNA and SEB co-stimulation group. Phosphorylation levels of some proteins related to the MAPK pathway showed that greater the stimulation intensity, more was the reduction in phosphorylation (**Fig. 5B**).

DISCUSSION

Previous animal studies have shown that differences in stimulation intensity according to the inoculation dose of dsRNA results in the production of type I or II cytokines within 12 h after stimulation (13,14). The purpose of this study was to evaluate whether this phenomenon also occurs in humans. In addition to dsRNA, we examined the stimulatory effects of SEB to evaluate the role of *S. aureus*, a commensal and representative upper-airway bacterium, in the development of asthma and its exacerbation. For this purpose, we used macrophages and epithelial cells, which are on the frontline of the airway immune system. We observed that different doses of both dsRNA and SEB induced different cytokine expression patterns. Furthermore, co-stimulation with both dsRNA and SEB could induce a synergistic effect on macrophages and epithelial cells (**Fig. 6**). This study indicated that type I or II responses can be induced based on the severity of infection in human airways and that SEB can induce a synergistic effect in virus-induced inflammation.

Unlike results of previous *in vivo* animal studies, our results did not show a clear difference in signature cytokine expression based on the dose of dsRNA or SEB (13,14). Instead, we observed different responses depending on the type of stimulator and responder cells. In the case of dsRNA stimulation, both macrophages and epithelial cells showed strong reaction by producing high levels of IL-6, sICAM-1, CC, and CXC chemokines. TNF-α was detected in macrophages, and high levels of IFN-β and λ were detected in epithelial cells specifically. Unlike dsRNA, SEB stimulation showed considerable differences between macrophages

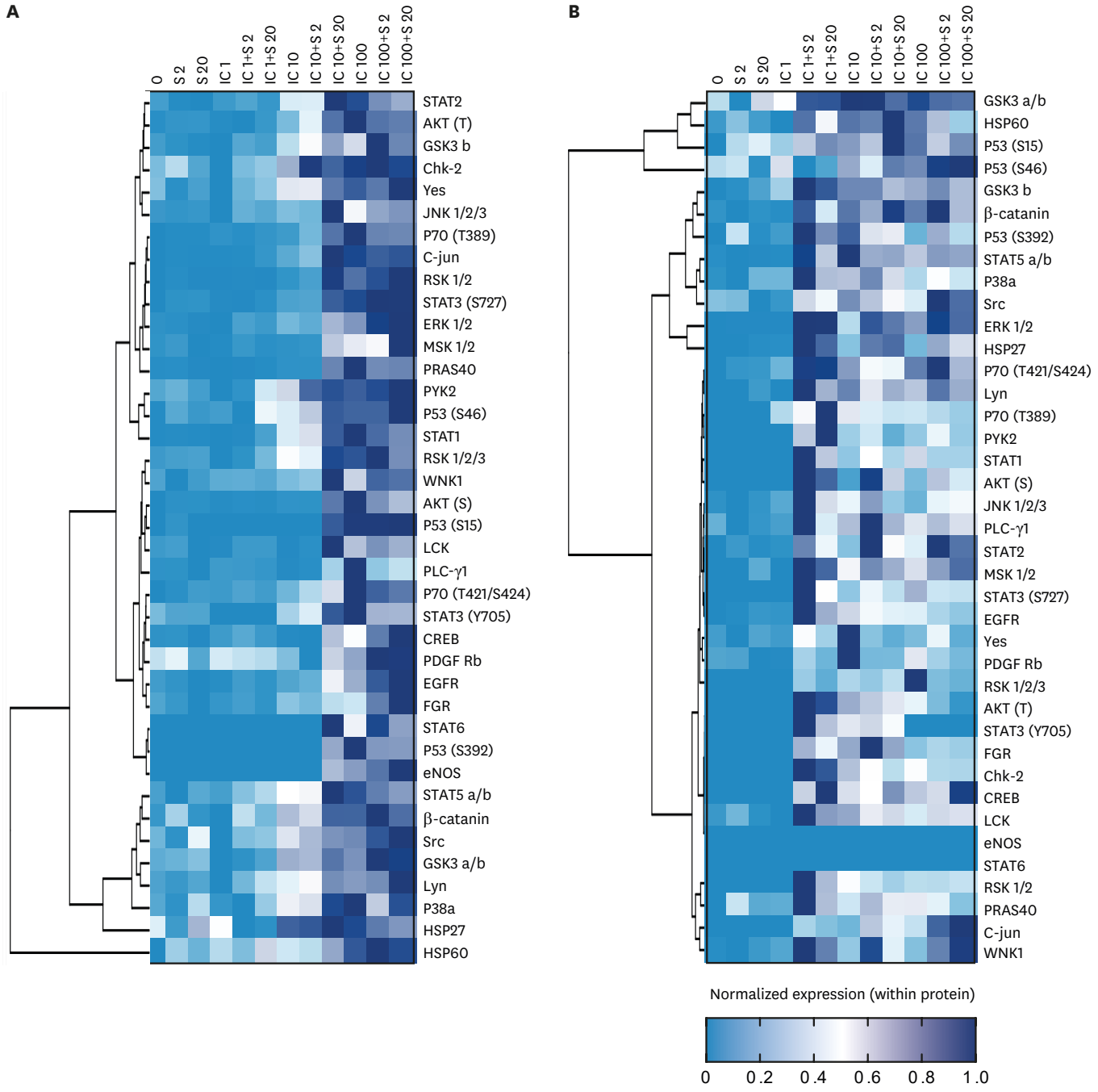

Figure 5. Phospho-kinase proteome profiles 12 h after stimulation with different doses of dsRNA and SEB in both macrophages and epithelial cells. Heatmap and cluster analysis of phosphor-kinase proteome files, including 37 phosphor-kinases and 2 total proteins (β-catenin and HSP60) in macrophages (A) and epithelial cells (B). Heatmap 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 **Figs. 3** and **4**, were used for this analysis.

and epithelial cells. Macrophages stimulated with SEB induced most of the CC and CXC chemokines and high levels of sICAM-1, and IFN-β. However, SEB stimulation did not induce the production of most cytokines in epithelial cells. It is worth noting that SEB induced less chemokine production than dsRNA did in both macrophages and epithelial cells, and epithelial cells showed very low reactivity to SEB stimulation. In the airway, macrophages

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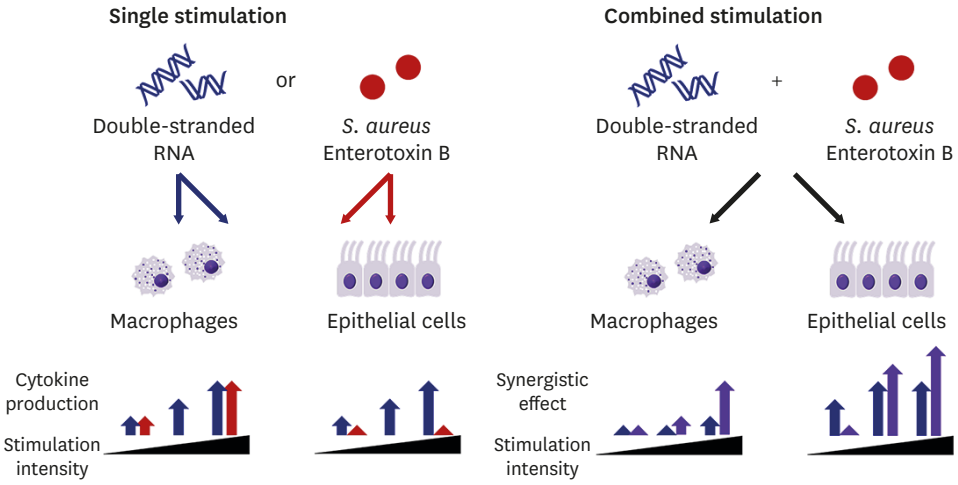

Figure 6. Summary of macrophage and epithelial cell responses to dsRNA and SEB stimulation. Single stimulation of dsRNA induces sufficient cytokine production in both macrophages and epithelial cells (blue arrows in single stimulation). Unlike dsRNA, SEB stimulation is only effective in macrophages but not in epithelial cells (red arrows in single stimulation). Contrary to the results of single stimulation, combined stimulation of dsRNA and SEB in macrophages exerts marginal synergistic effects on the production of few cytokines. However, this co-stimulation induces high synergistic effects on the production of most cytokine types in epithelial cells (blue arrows in combined stimulation). In addition to cytokine production, co-stimulation of both molecules in epithelial cells activates most intracellular signaling pathways compared with when macrophages are co-stimulated (purple arrows in combined stimulation). The height of each arrow indicates the degree of cytokine production or its synergistic effect.

and epithelial cells cooperatively play a surveillance role. In some diseases, however, macrophages play a major role in the immune system (15). Based on these points and our results, we postulate that dsRNA is relevant to the former and SEB to the latter. Another interesting point is that macrophages stimulated with SEB showed 4 to 5 times higher levels of sICAM-1 expression compared with macrophages stimulated with dsRNA. Soluble ICAM-1 inhibits immune cell adhesion (16), and according to a previous study, overexpression of sICAM-1 in gram-negative bacterial infections resulted in exacerbated inflammation and early death (17). Considering this, it appears that the negative effect of SEB on airway inflammation, such as asthma exacerbation, can be induced by macrophages.

Although the intensity of stimulation of dsRNA did not influence cytokine production, it could influence chemokine production. Low-dose dsRNA induced mild and moderate production of CCL24, CXCL10/11, and CXCL8/10 12 h after stimulation in macrophages and epithelial cells, respectively. High-dose dsRNA induced a strong response via the expression of 9 or 11 cytokines as well as CXC chemokines at 3 h after stimulation in each cell. These differences might affect the infiltration of inflammatory cells into airways and may also result in cytokine differences, as shown in previous animal studies. CXCL8, 10, and 11 can induce migration of mast cells, basophils, and NKT cells via CXCR1, 2, or CXCR3 on their surface. In addition, CCL24 recruits eosinophils, basophils, and mast cells via CCR2 (18). In a previous study with a human subject, RSV- or RV-induced bronchiolitis showed a high IL-4/IFN-γ ratio compared with RSV- or RV-induced pneumonia, which are regarded as mild and severe infections, respectively (19). Mild infection with RSV induced an elevated production of type II immune response-related chemokines in the serum compared with severe infection (20). In this context, we speculate that a low intensity of viral infection in the airway has a greater possibility of inducing a type II immune response via CXC8, 10, 11, and CCL24 than a higher intensity do.

Compared to CXC chemokines, other cytokines and CC chemokines were detected in high-dose dsRNA-stimulated macrophages and epithelial cells. Most of these are related to Th1

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immune response. TNF-α, a well-known pro-inflammatory cytokine, can drive immune responses to Th1 development (21,22). Although IL-6 can induce Th17 cell development and inhibit Th1 cell development and activation via SOCS1 and SOCS3 in humans (23-25), it is possible to induce Th1 cells through the plasticity of Th17 cells (26,27). IFN-β and λ, well-known defense cytokines against viral infection, can also induce a Th1 immune response (28,29). CC chemokines can recruit the majority of innate and adaptive immune cells. CCL2 can recruit monocytes/macrophages via CCR2, and CCL3 and CCL5 are able to recruit diverse immune cells as well as neutrophils via CCR 1/5 and 1/2/4/5, respectively. CCL20 is known to be involved in NK cell recruitment (18). Similar to our results, these chemokines are detected in respiratory virus infections, including RSV, influenza, adenovirus, and RV, as well as other viruses related to severe respiratory inflammation accompanied by high levels of neutrophils and monocytes/macrophages (30,31). In addition, the above cytokines might play a complex role with highly elevated CXC chemokines in both cells stimulated with high-dose dsRNA.

In this study, we stimulated both cells with diverse doses of dsRNA and SEB, as well as a combination of both to confirm the synergistic effect. Macrophages showed synergistic effects in the production of CCL3 and 5, and epithelial cells showed synergistic effects in the production of 13 cytokines among the identified 21 cytokines. In addition, co-stimulation with SEB was more effective in high-dose dsRNA stimulation than low-dose dsRNA stimulation. Accordingly, we postulate that airway inflammation caused by severe viral infection can be exacerbated by co-stimulation with SEB via the amplification of cytokine production in macrophages and epithelial cells. Interestingly, a previous study showed that pretreatment with dsRNA induced attenuated response to post-SEB stimulation via IL-10 production in nasal polyp-derived cells (32). However, there are some differences between the previous and present studies. They used nasal polyp-derived mixed cells, not a single cell type, and SEB was exposed for a few hours after dsRNA stimulation. In addition, the evaluations were performed 72 h after the stimulation. In another study, responsiveness was found time-sensitive, indicating that the time interval between stimulation and evaluation may impact the study results (33). These differences may have produced contradictory results between the 2 studies.

This study investigated the mechanism of the synergistic effect of dsRNA and SEB co-stimulation using a phospho-kinase array kit 12 h after stimulation. Based on the heatmap analysis, we found differences in phosphorylation level patterns depending on the intensity of stimulation and cell type. In the mid-dose dsRNA-stimulated group in macrophages, about phosphorylation of 18 proteins related to chemokine and inflammation (IL) signaling pathways had increased, and the phosphorylation levels elevated even further after co-stimulation with SEB. The production of 4 cytokines, namely, CCL3, CCL5, IFN-β, and G-CSF, was also upregulated by co-stimulation. Interestingly, although most of the signaling was activated, strong stimulation by high-dose dsRNA and SEB also showed a synergistic effect in the production of the aforementioned 4 cytokines. Thus, we postulate that these 2 signaling pathways are commonly related with the observed synergistic effects in macrophages. However, in the case of epithelial cells, we could not match specific signaling with cytokine production because signaling pathways were activated simultaneously. In addition, although strong co-stimulation induced a synergistic effect and increased cytokine production, the level of some proteins associated with the signaling pathway were downregulated.

Although, we identified a synergistic effect in response to 2 signaling molecules, there is a possibility that the length of evaluation time confounded our observations. We observed that

the production of CCL3, CCL5, CXCL8, and TNF-α showed a saturating or decreasing tendency 6 h after high-dose dsRNA stimulation. In addition, combining SEB with high-dose dsRNA lowered the level of phosphorylation 12 h after stimulation with some phosphoproteins (**Supplementary Fig. 1**; **Supplementary Tables 1** and **2**). This suggests that a higher degree of stimulation induces a shorter activation time. In this context, combining SEB and dsRNA might enhance the intensity of stimulation. Furthermore, cytokines produced in the early phase of innate inflammation, including CCL2, 5, and CXCL10, can activate these cells via auto- and paracrine signaling (34,35), which might accelerate the intensity of stimulation and shorten the activation and induction times for signaling and cytokines, respectively. Furthermore, 12 h after stimulation, most proteins were phosphorylated in the macrophages but not the epithelial cells. Such a difference in the pattern is possibly attributable to the induction of different cytokines for each cell type treated with stimulators. However, to the best of our knowledge, only a few studies have evaluated the synergistic effects of dsRNA and SEB, and therefore, further studies are warranted.

In this single cell *in vitro* system, evaluations were performed until 12 h after stimulation. Because of the closed system and evaluation time, the effects of interactions with other cells and foreign cytokines were excluded, and some cytokines, such as CCL17, were not detected until the last evaluation time point, compared with previous studies (20,32). However, there is a possibility that our evaluation time was faster than the induction time of those cytokines, including CCL17 (36). For this study, we used poly I:C instead of respiratory viruses, such as RV or RSV, to mimic infection. However, it is important to note that viruses have capsids and envelopes that encloses the genetic material. Among these molecules, the RSV F protein on the envelope can induce immune responses, including asthma exacerbation via TLR4 and CD14 (37). Compared with a previous study, our study did not reflect the effect of viral proteins. However, we could present a cytokine profile at various time points after stimulation. In addition, our results provide a common clue to understand the early immune responses to the respiratory virus without the complex and mixed responses of the whole virus. This is because the production of dsRNA in the host cell is a common step for the replication of single-stranded RNA viruses in respiratory infections.

Since the relationship between respiratory viral infection in early time and the risk of wheezing and allergy have been reported (38,39), some previous cohort studies have shown that RV and RSV infections in the airway may be related to the induction of wheezing disorder and asthma exacerbation, but not related to the development of allergic disorder (40-42). In contrast, other studies have shown that patients who were hospitalized for respiratory viral infections developed allergic diseases (4,5,43). However, these studies could not present precise mechanisms because they primarily focused on finding a clinical correlation. In this context, we showed the aspects of immune responses to dsRNA in macrophages and epithelial cells during the early phase of airway inflammation. We also demonstrated the role of SEB in the induction of cytokines in both cell types to address the findings of previous clinical studies with respect to the relationship between SEB-specific IgE sensitization and asthma, especially in adult or elderly patients with severe, sputum eosinophilia, and chronic sinusitis (9-11). This is the first study to show the synergistic effects of combining dsRNA and SEB, which may provide a better insight into the relationship between SEB sensitization and asthma exacerbation.

Our findings suggest that the types and severity of cytokine production from the epithelium or macrophages can be affected by different intensities and combinations of dsRNA and

SEB. Further studies with this approach are warranted to improve our understanding of the development and exacerbation of airway inflammation and asthma.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Intensity of phospho-kinases in macrophages

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Supplementary Table 1

Intensity of phospho-kinases in epithelial cells

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Supplementary Figure 1

Analysis of phosphor-kinase levels in dsRNA- and SEB-stimulated macrophages and epithelial cells using a phosphor-kinase proteome array. (A) Schematic coordinates of a phosphor-kinase array kit. The expression levels of phosphorylated protein in THP-1-derived macrophages (B) and epithelial cells (C) stimulated with dsRNA and SEB. The levels were evaluated 12 h after stimulation. Black dots indicate the phosphorylation sites of 32 phospho-kinases and 2 total proteins.

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REFERENCES

1. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124:783-801.
PUBMED | CROSSREF
2. Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang MJ, He CH, Takyar S, Elias JA. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu Rev Physiol* 2011;73:479-501.
PUBMED | CROSSREF
3. Martorano LM, Grayson MH. Respiratory viral infections and atopic development: from possible mechanisms to advances in treatment. *Eur J Immunol* 2018;48:407-414.
PUBMED | CROSSREF
4. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F, Kjellman B. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am J Respir Crit Care Med* 2005;171:137-141.
PUBMED | CROSSREF

5. Bacharier LB, Cohen R, Schweiger T, Yin-Declue H, Christie C, Zheng J, Schechtman KB, Strunk RC, Castro M. Determinants of asthma after severe respiratory syncytial virus bronchiolitis. *J Allergy Clin Immunol* 2012;130:91-100.e3.
PUBMED | CROSSREF
6. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 2010;11:373-384.
PUBMED | CROSSREF
7. Saglani S. Viral infections and the development of asthma in children. *Ther Adv Infect Dis* 2013;1:139-150.
PUBMED | CROSSREF
8. Lee JJ, Kim SH, Lee MJ, Kim BK, Song WJ, Park HW, Cho SH, Hong SJ, Chang YS, Kim BS. Different upper airway microbiome and their functional genes associated with asthma in young adults and elderly individuals. *Allergy* 2019;74:709-719.
PUBMED | CROSSREF
9. Song WJ, Sintobin I, Sohn KH, Kang MG, Park HK, Jo EJ, Lee SE, Yang MS, Kim SH, Park HK, et al. Staphylococcal enterotoxin IgE sensitization in late-onset severe eosinophilic asthma in the elderly. *Clin Exp Allergy* 2016;46:411-421.
PUBMED | CROSSREF
10. Kim YC, Won HK, Lee JW, Sohn KH, Kim MH, Kim TB, Chang YS, Lee BJ, Cho SH, Bachert C, et al. *Staphylococcus aureus* nasal colonization and asthma in adults: systematic review and meta-analysis. *J Allergy Clin Immunol Pract* 2019;7:606-615.e9.
PUBMED | CROSSREF
11. Song WJ, Chang YS, Lim MK, Yun EH, Kim SH, Kang HR, Park HW, Tomassen P, Choi MH, Min KU, et al. Staphylococcal enterotoxin sensitization in a community-based population: a potential role in adult-onset asthma. *Clin Exp Allergy* 2014;44:553-562.
PUBMED | CROSSREF
12. Morgene MF, Botelho-Nevers E, Grattard F, Pillet S, Berthelot P, Pozzetto B, Verhoeven PO. Staphylococcus aureus colonization and non-influenza respiratory viruses: Interactions and synergism mechanisms. *Virulence* 2018;9:1354-1363.
PUBMED | CROSSREF
13. Choi JP, Kim YS, Kim OY, Kim YM, Jeon SG, Roh TY, Park JS, Gho YS, Kim YK. TNF-alpha is a key mediator in the development of Th2 cell response to inhaled allergens induced by a viral PAMP double-stranded RNA. *Allergy* 2012;67:1138-1148.
PUBMED | CROSSREF
14. Choi JP, Kim YM, Choi HI, Choi SJ, Park HT, Lee WH, Gho YS, Jee YK, Jeon SG, Kim YK. An important role of tumor necrosis factor receptor-2 on natural killer T cells on the development of dsRNA-enhanced Th2 cell response to inhaled allergens. *Allergy* 2014;69:186-198.
PUBMED | CROSSREF
15. Byrne AJ, Mathie SA, Gregory LG, Lloyd CM. Pulmonary macrophages: key players in the innate defence of the airways. *Thorax* 2015;70:1189-1196.
PUBMED | CROSSREF
16. Rieckmann P, Michel U, Albrecht M, Brück W, Wöckel L, Felgenhauer K. Soluble forms of intercellular adhesion molecule-1 (ICAM-1) block lymphocyte attachment to cerebral endothelial cells. *J Neuroimmunol* 1995;60:9-15.
PUBMED | CROSSREF
17. Mendez MP, Monroy YK, Du M, Preston AM, Tolle L, Lin Y, VanDussen KL, Samuelson LC, Standiford TJ, Curtis JL, et al. Overexpression of sICAM-1 in the alveolar epithelial space results in an exaggerated inflammatory response and early death in Gram negative pneumonia. *Respir Res* 2011;12:12.
PUBMED | CROSSREF
18. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD, et al. International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* 2013;66:1-79.
PUBMED | CROSSREF
19. Yuan XH, Li YM, Shen YY, Yang J, Jin Y. Clinical and Th1/Th2 immune response features of hospitalized children with human rhinovirus infection. *J Med Virol* 2020;92:26-33.
PUBMED | CROSSREF
20. Vojvoda V, Savić Mlakar A, Jergović M, Kukuruzović M, Markovinović L, Aberle N, Rabatić S, Bendelja K. The increased type-1 and type-2 chemokine levels in children with acute RSV infection alter the development of adaptive immune responses. *BioMed Res Int* 2014;2014:750521.
PUBMED | CROSSREF

21. Kanehiro A, Lahn M, Mäkelä MJ, Dakhama A, Fujita M, Joetham A, Mason RJ, Born W, Gelfand EW. Tumor necrosis factor-alpha negatively regulates airway hyperresponsiveness through gamma-delta T cells. *Am J Respir Crit Care Med* 2001;164:2229-2238.
PUBMED | CROSSREF
22. Rudmann DG, Moore MW, Tepper JS, Aldrich MC, Pfeiffer JW, Hogenesch H, Tumas DB. Modulation of allergic inflammation in mice deficient in TNF receptors. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1047-L1057.
PUBMED | CROSSREF
23. Tsukamoto H, Senju S, Matsumura K, Swain SL, Nishimura Y. IL-6-mediated environmental conditioning of defective Th1 differentiation dampens antitumour immune responses in old age. *Nat Commun* 2015;6:6702.
PUBMED | CROSSREF
24. Diehl S, Anguita J, Hoffmeyer A, Zapton T, Ihle JN, Fikrig E, Rincón M. Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1. *Immunity* 2000;13:805-815.
PUBMED | CROSSREF
25. Velazquez-Salinas L, Verdugo-Rodriguez A, Rodriguez LL, Borca MV. The role of interleukin 6 during viral infections. *Front Microbiol* 2019;10:1057.
PUBMED | CROSSREF
26. Lee YK, Turner H, Maynard CL, Oliver JR, Chen D, Elson CO, Weaver CT. Late developmental plasticity in the T helper 17 lineage. *Immunity* 2009;30:92-107.
PUBMED | CROSSREF
27. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity* 2009;30:646-655.
PUBMED | CROSSREF
28. Zhou JH, Wang YN, Chang QY, Ma P, Hu Y, Cao X. Type iii interferons in viral infection and antiviral immunity. *Cell Physiol Biochem* 2018;51:173-185.
PUBMED | CROSSREF
29. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, Salazar AM, Colonna M, Steinman RM. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J Exp Med* 2009;206:1589-1602.
PUBMED | CROSSREF
30. Nuriev R, Johansson C. Chemokine regulation of inflammation during respiratory syncytial virus infection. *F1000 Res* 2019;8:1837.
PUBMED | CROSSREF
31. Schaller M, Hogaboam CM, Lukacs N, Kunkel SL. Respiratory viral infections drive chemokine expression and exacerbate the asthmatic response. *J Allergy Clin Immunol* 2006;118:295-302.
PUBMED | CROSSREF
32. Okano M, Fujiwara T, Kariya S, Higaki T, Makihara S, Haruna T, Noyama Y, Koyama T, Omichi R, Orita Y, et al. Regulatory effect of TLR3 signaling on staphylococcal enterotoxin-induced IL-5, IL-13, IL-17A and IFN-γ production in chronic rhinosinusitis with nasal polyps. *Allergol Int* 2016;65:96-102.
PUBMED | CROSSREF
33. Abdi K, Singh NJ, Matzinger P. Lipopolysaccharide-activated dendritic cells: "exhausted" or alert and waiting? *J Immunol* 2012;188:5981-5989.
PUBMED | CROSSREF
34. Huvenne W, Callebaut I, Reekmans K, Hens G, Bobic S, Jorissen M, Bullens DM, Ceuppens JL, Bachert C, Hellings PW. Staphylococcus aureus enterotoxin B augments granulocyte migration and survival via airway epithelial cell activation. *Allergy* 2010;65:1013-1020.
PUBMED | CROSSREF
35. Robinson SC, Scott KA, Balkwill FR. Chemokine stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-alpha. *Eur J Immunol* 2002;32:404-412.
PUBMED | CROSSREF
36. Sekiya T, Miyamasu M, Imanishi M, Yamada H, Nakajima T, Yamaguchi M, Fujisawa T, Pawankar R, Sano Y, Ohta K, et al. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. *J Immunol* 2000;165:2205-2213.
PUBMED | CROSSREF
37. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 2000;1:398-401.
PUBMED | CROSSREF
38. Martinez FD. Development of wheezing disorders and asthma in preschool children. *Pediatrics* 2002;109:362-367.
PUBMED | CROSSREF

39. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 1999;354:541-545.
PUBMED | CROSSREF

40. Lemanske RF Jr. Inflammation in childhood asthma and other wheezing disorders. *Pediatrics* 2002;109:368-372.
PUBMED | CROSSREF

41. Jackson DJ, Gangnon RE, Evans MD, Roberg KA, Anderson EL, Pappas TE, Printz MC, Lee WM, Shult PA, Reisdorf E, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am J Respir Crit Care Med* 2008;178:667-672.
PUBMED | CROSSREF

42. Jackson DJ, Evans MD, Gangnon RE, Tisler CJ, Pappas TE, Lee WM, Gern JE, Lemanske RF Jr. Evidence for a causal relationship between allergic sensitization and rhinovirus wheezing in early life. *Am J Respir Crit Care Med* 2012;185:281-285.
PUBMED | CROSSREF

43. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, Gustafsson PM. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. *Thorax* 2010;65:1045-1052.
PUBMED | CROSSREF