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Cigarette smoke compromises macrophage innate sensing in response to pneumococcal infection

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ABSTRACT

Background: Cigarette smoking remains a leading cause of mortality worldwide. *Streptococcus pneumoniae*, also known as pneumococcus, is one of the most common pathogens that colonizes the human respiratory tract, causing life-threatening infections. Several studies have reported that cigarette smoke (CS) exposure promotes pneumococcal infectivity; however, the underlying mechanisms remain to be illustrated.

Methods: In this study, we prepared cigarette smoke extract (CSE) from tobacco containing nicotine (0.8 mg/cigarette) and tar (10 mg/cigarette) to investigate the effects of CSE on innate immune response using murine macrophage models.

Results: The results from the cytokine array showed that the production of C-C Motif Chemokine Ligand 2 (CCL2), CCL4, CCL3, C-X-C Motif Chemokine Ligand 2 (CXCL2), and CXCL-10, in pneumococcus-infected cells was reduced upon 5 % CSE treatment. Our results further demonstrated that 5 % CSE exposure, followed by pneumococcal challenge, significantly decreased CCL2 and type I interferon (IFN) production in macrophages by inhibiting nuclear factor (NF)-κB and IFN regulatory factor 3 (IRF3) signaling pathways. Moreover, CSE disrupts macrophage polarization and impedes innate immune signaling to suppress pneumococcal phagocytosis by macrophages.

Conclusion: Our results provide evidence that CS manipulates the signaling molecules to subvert macrophage functions, thereby hindering the innate response against pneumococcal infection.

1. Introduction

Cigarette smoke (CS) is the principal cause of respiratory tract hyperresponsiveness that leads to acute inflammation in the lungs.[1] Bacterial pathogens have been isolated in significant concentrations from distal airways in over 50 % of patients with acute exacerbations (AEs).[2] Although pulmonary diseases are mainly caused by bacterial pathogens, whether CS exposure affects the bacterial burden that

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provokes inflammation or hampers the immune defense remains to be illustrated.

Bacterial colonization is associated with chronic respiratory tract inflammation, which involves an increased frequency of exacerbations and accelerated decrease in lung functions; these factors primarily contribute to pulmonary disease progression.[3] Pneumococcus is a Gram-positive bacterium, exhibiting the form of diplococci and belonging to flora that colonizes the human respiratory tract. Pneumococcal infection causes several severe diseases, including bacteremia, otitis media, meningitis, and most importantly pneumonia, which is a lower respiratory tract disease.[4,5] The nasopharyngeal flora of smokers comprise fewer beneficial microorganisms and more potential pathogens, such as pneumococcus, than that of non-smokers.[6] *In vivo* models have further demonstrated that pneumococcal infection and CS exposure synergistically aggravate lung damage.[7] However, studies exploring the molecular mechanisms behind CS-induced pulmonary inflammation in response to bacterial infection needed to be investigated.

CS in the respiratory tract can recruit immune cells that generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to chronic inflammation.[8] Although research has reported that CS exposure attenuates immune response and enhances pneumococcal colonization *in vivo*,[9] the underlying mechanisms remain to be elucidated. This study investigated whether CS manipulates innate immune signaling against pneumococcal infection. We established cell-based models with CS-exposed murine macrophages and challenged them with pneumococci to assess innate responses and bacterial clearance. Our results reveal that CS modulates signaling molecules to orchestrate inflammation activation, leading to attenuation of macrophage function in response to pneumococcal infection.

2. Materials and methods

2.1. Preparation of cigarette smoke extract (CSE) medium

Tobacco cigarettes (Taiwan Tobacco & Liquor Corporation, Taipei, Taiwan) with nicotine (0.8 mg/cigarette) and tar (10 mg/cigarette) were used to prepare the culture medium as described previously.[10] Briefly, burning cigarette smoke was bubbled through DMEM (Invitrogen), which is considered cigarette smoke extract (CSE) medium. The CSE medium was inflated with 25 cigarettes into 250 mL of medium and set to 100 %. The CSE medium was then diluted to the designed concentrations by DMEM and employed in the following studies.

2.2. Cell culture

RAW264.7 cells (a murine macrophage cell line, TIB-71) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RAW264.7 cells were incubated at 37 °C with 5 % CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % complement-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA). MH-S cells (an alveolar macrophage cell line, CRL-2019, ATCC) were cultured in RPMI 1640 medium supplemented with 10 % FBS and incubated at 37 °C with 5 % CO_2.

2.3. Bacterial culture

Pneumococcal strain TIGR4 (ATCC BAA-334) was grown on blood agar plates (Becton Dickinson, Sparks, MD, USA) and cultured under 5 % CO_2 at 37 °C. In infection experiments, pneumococci were refreshed for 3 h in Todd Hewitt Broth (Becton Dickinson) to reach the logarithmic phase and prepared for the following experiments as described previously.[11]

2.4. Cell viability assay

RAW264.7 cells were treated with CSE (0, 2.5, 5, and 10 %) for 3 h and incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent at 37 °C for 2 h. Formazan crystals were dissolved in isopropanol, and the absorbance at 562 nm was determined by a spectrophotometer (Molecular Devices, San Jose, CA, USA).[12]

2.5. Bactericidal survival assay

Refreshed pneumococci were treated with CSE (0, 2.5, 5, and 10 %) for 3 h, the bacteria were serial diluted and plated onto blood agar plates. After incubation at 37 °C for 24 h, the viable colony-forming units (CFUs) on plates were counted.

2.6. Phagocytosis assay

The phagocytosis activity of macrophages was employed by using a Phagocytosis Assay Kit (IgG FITC) (Cayman Chemical, Ann Arbor, MI, USA). RAW264.7 cells were pretreated with 5 % CSE or 10 μM benzo[a]pyrene (B[a]P, Sigma-Aldrich) for 3 h. FITC-labeled rabbit IgG-coated latex beads were incubated with macrophage cells for an additional 4 h. The cells were washed three times with PBS and fixed with 4 % paraformaldehyde followed by probing with Hoechst 33,342 (AAT Bioquest, Sunnyvale, CA, USA). The signals of phagocyted beads were observed using a laser scanning confocal microscope (LSM780, Carl Zeiss).

2.7. Bacterial internalization assay

The gentamicin protection assay was employed to determine the pneumococcal internalization by macrophages.[13] RAW264.7 cells were treated with 5 % CSE for 3 h followed by infection with pneumococci at an MOI of 10 for 4 h. The infected cells were washed with PBS for three times and then incubated with 100 μg/mL gentamycin (Sigma-Aldrich) for 90 min. The cells were then lysed using sterile water. The cell lysate was serially diluted onto blood agar plates and incubated for 24 h. Viable bacteria were counted and indicated as CFUs.

2.8. Cytokine array assay

RAW264.7 cells were treated with 5 % CSE for 3 h and then infected with pneumococci (MOI = 10) for an additional 4 h. Culture supernatant was prepared to analyze the cytokine production using Proteome Profiler Array (R&D Systems, Minneapolis, MN, USA). Cytokine expression was measured by Azure C400 (Azure Biosystems, Dublin, CA, USA). Data were quantified as fold changes and calculated by Log_2.

2.9. Quantitative real-time reverse transcription-PCR (qRT-PCR)

Total RNA was prepared according to the manufacturer's instructions (Invitrogen) and the mRNA levels of the genes were assessed using qRT-PCR. The oligonucleotide primers used for qRT-PCR were listed in Table S1. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) served as the reference gene for normalization. The mRNA level of each gene was analyzed using SYBR Green I Master Mix with the program of preheated at 95 °C for 10 min and 35 cycles of 95 °C for 10 s and 60 °C for 1 min. The data for each gene was calculated by the $2^{-\Delta\Delta Ct}$ method.[11]

2.10. Immunofluorescence staining

RAW264.7 cells were plated in 6-well plates and cultured for 20 h. The cells were treated with 5 % CSE for 3 h and infected with pneumococci (MOI = 10) for 4 h. The cells were fixed with 4 %

paraformaldehyde for 60 min and blocked with 1 % FBS for 60 min. The cells were incubated with anti–IRF-3 antibody (Cell Signaling Technology, Beverly, MA, USA) followed by staining with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1 antibody (Invitrogen) for 60 min. The nuclei were probed with Hoechst 33,342, and the signals of FITC and Hoechst 33,342 were visualized using a laser scanning confocal microscope (LSM780, Carl Zeiss, Oberkochen, Germany).

2.11. Western blot assay

RAW264.7 cells were treated with 5 % CSE for 3 h, followed by pneumococcal infection (MOI = 10) for an additional 4 h. Cells were washed with PBS and lysed with RIPA buffer containing protease inhibitors and phosphatase inhibitors (Roch, Indianapolis, IN, USA). The protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The samples were resolved by 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5 % skim milk for 1 h and incubated with primary antibodies overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Millipore) were incubated at room temperature and the signals of interest proteins were analyzed by Azure C400 with AzureSpot Analysis Software (Azure Biosystems, Dublin, CA, USA).

2.12. Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells were untreated or treated with 5 % CSE for 3 h, followed by uninfected or infected with pneumococci at an MOI of 10 for 4 h. Supernatants of macrophage culture were collected and subjected to analyze the expression levels of CCL2, IFN-α, IFN-β, soluble high mobility group box 1 (sHMGB1), and TNF-α by ELISA kits (R&D Systems, Minneapolis, MN, USA).

2.13. Analysis of NF-κB-luciferase reporter activity

RAW264.7 cells were transfected with NF-κB-luciferase reporter using jetPEI (Polyplus-transfection, France). Cells were exposed to 5 % CSE for 3 h, followed by pneumococcal infection (MOI = 10) for 4 h. Cells were lysed, NF-κB-luciferase activity was determined by Dual-Luciferase Reporter Assay System (Promega, Madison, MA, USA), and then normalized for transfection efficiency by the co-transfected β-galactosidase expression vector (Promega).[14]

2.14. Statistical analysis

Results are expressed as mean ± SD. The student's *t*-test was conducted to analyze the statistical significance of the experimental results between two groups, with a *P*-value less than 0.05 considered significant. Statistics and figures were performed with the Prism Program (version 9.0.0, GraphPad).

3. Results

3.1. CSE suppresses bacterial phagocytosis by macrophages

To investigate whether CSE influences pneumococcal internalization by macrophages, we first evaluated the effects of CSE on cell viability and bacterial survival. RAW264.7 cells and pneumococci were separately incubated in different concentrations of CSE (0, 2.5, 5, and 10 %) for 3 h to analyze cell viability and bacterial survival, respectively. Our results showed that neither cell viability nor bacterial survival was influenced by CSE treatment (Fig. S1). Therefore, 5 % CSE concentration was selected and subjected to the pneumococcus-infected macrophage model. We then investigated the effect of CSE exposure on macrophage phagocytic activity using FITC-coated latex beads in a phagocytosis assay. Compared to the mock treatment, 5 % CSE exposure suppressed phagocytosis of the fluorescent beads by macrophages (Fig. 1A–B). To validate the effect of CSE exposure on bacterial internalization by macrophages, we replaced fluorescent-beads with pneumococci. Macrophage internalization of pneumococci was significantly reduced upon 5 % CSE treatment (Fig. 1C). These results suggest that phagocytic activity and bacterial internalization by macrophages are hindered by CSE exposure.

3.2. CSE decreases pneumococcus-induced cytokine/chemokine production

To identify differential cytokine production in macrophages, RAW264.7 cells were exposed to CSE prior to pneumococcal infection, and a cytokine array was used to analyze the cytokine/chemokine expressions. As shown in Fig. 2A, co-treatment of CSE and pneumococci elicited the production of several cytokines in macrophages, including G-CSF, CXCL1, CCL1, and TNF-α. Notably, the secretion of CCL2, CCL4, CCL3, CXCL2, and CXCL-10 in pneumococcus-infected cells was reduced owing to CSE treatment. Because pneumococcal infection enhances type I interferon (IFN-α and IFN-β) production that is important for host immune defense against bacterial infection,[15,16] we subsequently assessed the mRNA levels of these cytokines using qRT-PCR. The mRNA levels of *CCL2*, *CXCL-2*, *CXCL-10*, *IFN-α*, and *IFN-β* were increased in the pneumococcal-infected group compared with the mock group; however, the mRNA levels were considerably decreased after CSE exposure (Fig. 2B–F). Similarly, CSE reduced the mRNA level of pneumococcus-induced IFN regulatory factor 3 (*IRF3*) (Fig. 2G). To further validate the effect of CSE exposure on cytokine production in macrophages, the levels of CCL2, IFN-α, and IFN-β were analyzed using ELISA. CCL2, IFN-α, and IFN-β productions were substantially increased in pneumococcus-infected cells compared with the mock group; however, these upregulated expressions were significantly reduced after CSE treatment (Fig. 3). We next investigated whether CSE affects proinflammatory cytokine production using the alveolar macrophage cell line MH-S. Our results showed that CSE treatment inhibited the production of sHMGB1 and TNF-α not only in RAW264.7 cells (Fig. S2) but also in MH-S cells (Fig. S3).

3.3. CSE impedes pneumococcus-induced cytokine production through nuclear factor (NF)-κB and IRF3 signaling pathways

As both Toll-like receptor 2 (TLR2) and nucleotide-binding oligomerization domain 2 (NOD2) signaling are involved in pneumococcus-induced IRF3-mediated cytokine production in innate cells,[17] we next examined the expressions of TLR2 and NOD2 using western blotting. TLR2 and NOD2 levels were reduced in CSE-treated macrophages challenged with pneumococci compared with pneumococcus-infected macrophages (Fig. 4A). These results suggest that CSE hampers the innate ability of macrophages to recognize pneumococci.

Because pneumococcus-induced cytokine production is mediated through NF-κB activation,[18] we investigated whether the NF-κB signaling pathway is involved in CSE-dampened macrophage functions. We demonstrated that pneumococcal infection elevated NF-κB luciferase activity (Fig. 4B). In contrast, CSE exposure significantly suppressed pneumococcus-elevated NF-κB activation. Another transcription factor, IRF3, induces type I IFNs during bacterial infection.[19] To examine whether CSE affects IRF3 activation, followed by the suppression of type I IFN production, the distributions of phospho-IRF3 were determined using immunofluorescence staining. Our results revealed that CSE exposure reduced the upregulation of phospho-IRF3 in pneumococcus-infected macrophages (Fig. 5A and B). To validate the role of IRF3 in pneumococcus-activated signaling pathway for type I IFN production, the levels of phosphorylated IRF3 and TBK1 were analyzed using western blotting. Compared with the mock treatment, CSE alone barely influenced p-IRF3 and p-TBK1 levels (Fig. 5C). However, CSE decreased pneumococcus-induced p-IRF3 and p-TBK-1 expressions. This

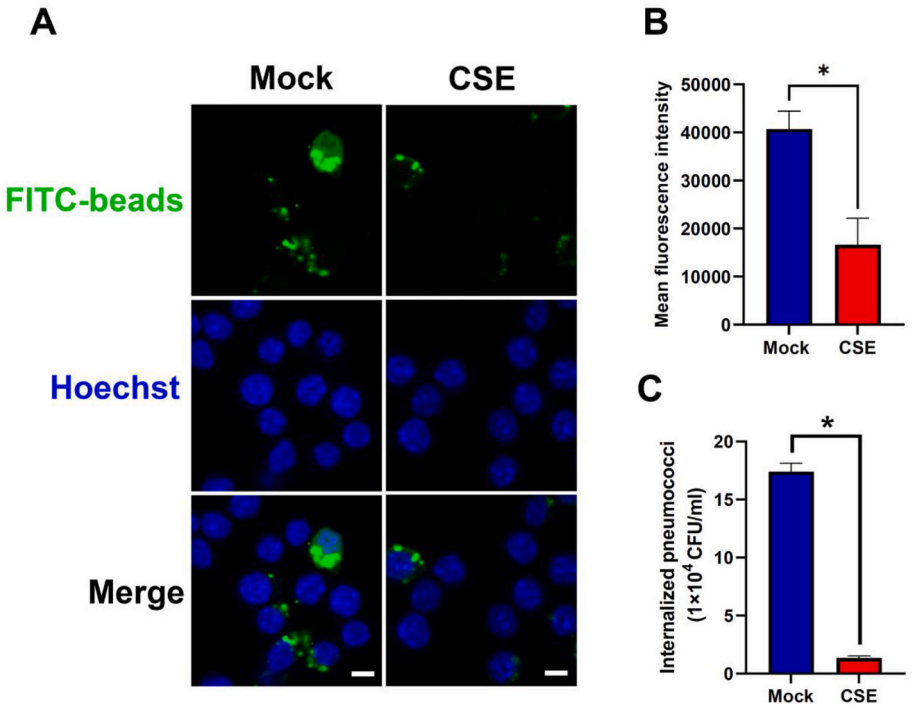

Fig. 1. CSE decreases pneumococcal phagocytosis by macrophages. (A) RAW264.7 cells were exposed to 5 % CSE for 3 h and incubated with latex-fluorescent beads for 4 h to assess phagocytic activity. Nuclei were stained with Hoechst 33,342, and images were analyzed using confocal microscopy. Scale bars, 10 μm. (B) The fluorescent intensity of phagocyted beads was measured. (C) RAW264.7 cells were pretreated with 5 % CSE for 3 h, followed by pneumococcal infection (MOI = 10) for 4 h. Pneumococcal internalization was analyzed by gentamicin-protection assay. Bar represented mean ± SD from triplicate independent experiments. *, $P < 0.05$.

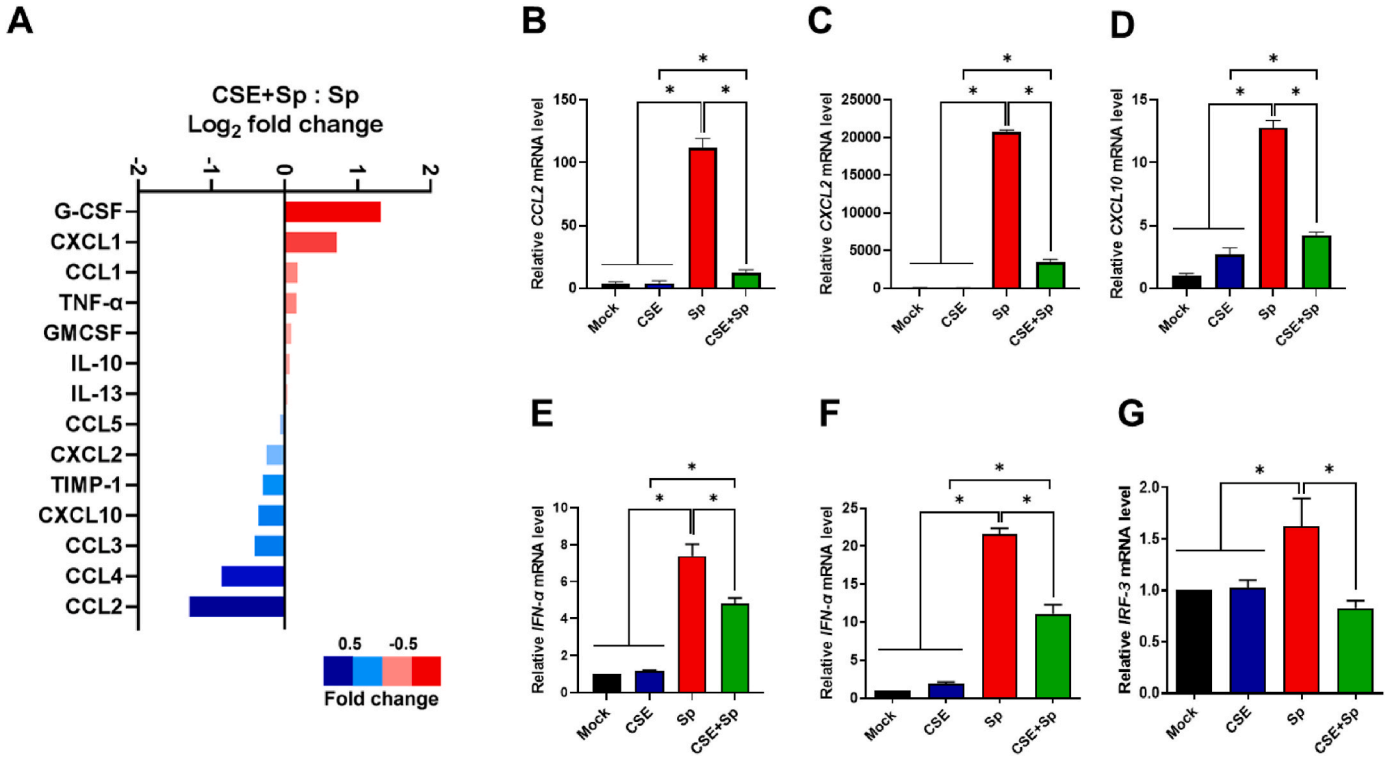

Fig. 2. CSE affects pneumococcus-induced cytokines and chemokines. (A) RAW264.7 cells were exposed to 5 % CSE for 3 h prior to pneumococcal challenge (MOI = 10) for 4 h. The culture supernatant was prepared, and the cytokine production was analyzed using the cytokine array. Cytokine expression was quantified using ImageJ. Log_2 fold changes were calculated for the CSE + pneumococcus cotreated and pneumococcus-infected groups. The relative mRNA levels of (B) *CCL2*, (C) *CXCL-2*, (D) *CXCL-10*, (E) *IFN-α*, (F) *IFN-β*, and (G) *IRF3* were analyzed using qRT-PCR and normalized to the reference gene, GAPDH. Results represented mean ± SD from triplicate independent experiments. *, P < 0.05.

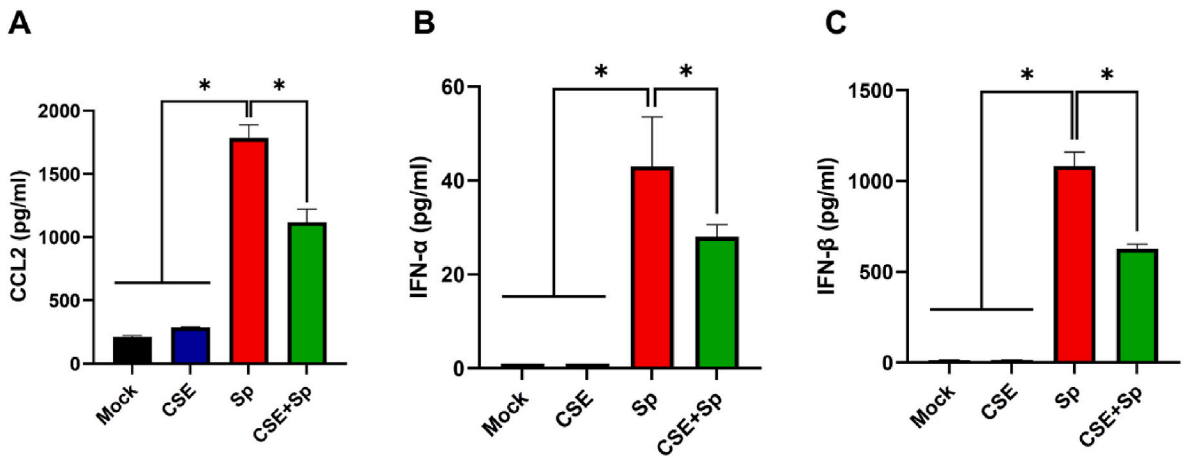

Fig. 3. CSE suppresses pneumococcus-mediated cytokine production. RAW264.7 cells were pretreated with 5 % CSE for 3 h and then infected with pneumococci (MOI = 10) for 4 h. Culture supernatant was prepared and the concentration of (A) CCL2, (B) IFN-α, and (C) IFN-β was assessed by ELISA. Results represented mean ± SD from triplicate independent experiments. *, $P < 0.05$.

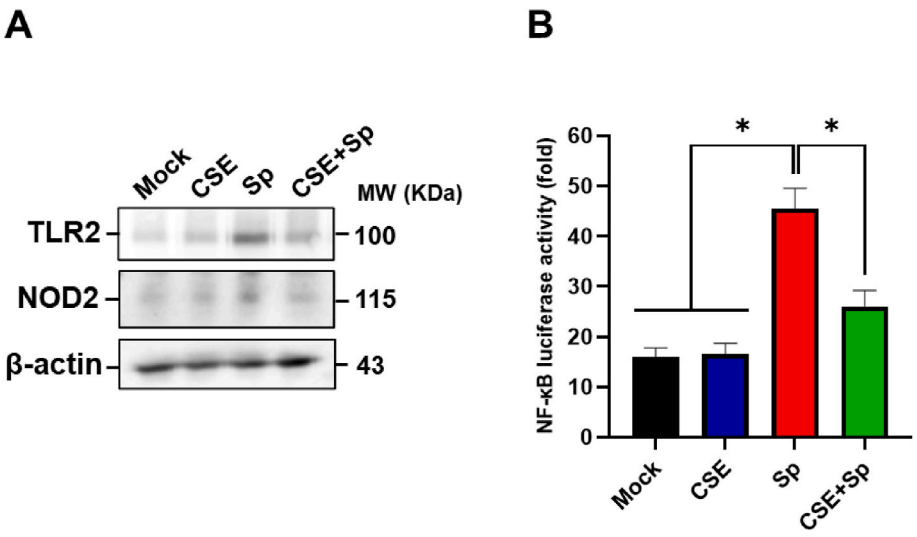

Fig. 4. CSE dampens pneumococcus-induced cytokine production by suppressing NF-κB pathway. (A) RAW264.7 cells were incubated with 5 % CSE followed by pneumococcal infection (MOI = 10) for 4 h. Cell lysates were prepared to analyze the expression levels of TLR2, NOD2, and β-actin using western blotting. β-actin was used as a loading control. (B) NF-κB activation was measured using luciferase assay. *, $P < 0.05$.

trend was also observed in the alveolar macrophage cell model (MH-S cells) (Fig. S4). These results suggest that CSE inhibits pneumococcus-stimulated type I IFN production by suppressing p-IRF3. Together, our results provide evidence that CS dampens pneumococcus-elicited NF-κB activation as well as IRF3 phosphorylation, leading to the suppression of cytokine and chemokine production in macrophages.

3.4. CSE hinders pneumococcus-induced M1 polarization

The phenotypes and functions of macrophages can be divided into M1-type (proinflammatory macrophage) and M2-type (anti-inflammatory macrophage) during pathogen infections.[20] We further assessed the effects of CSE on macrophage phenotypic transition. As shown in Fig. 6, pneumococcal infection activated M1 macrophage markers *CD86* and inducible nitric oxide synthase (*iNOS*); however, this effect was attenuated by CSE treatment. In contrast, CSE elevated the pneumococcus-induced M2 marker, *CD163*. Moreover, CSE exposure increased macrophage marker *F4/80* in pneumococcus-challenged macrophages. These results indicate that CSE suppressed macrophage M1 polarization and influenced M2 macrophage transition in pneumococcus-challenged macrophages.

4. Discussion

Macrophages act as innate immune cells and play a crucial role in defending the body against microbial infections. Despite this powerful immune surveillance, pathogens can often evade host immune attack, particularly by inducing macrophage subversion.[21] Impaired macrophage function reduces the pulmonary immune response, which exacerbates microbial infectivity.[22] Because the breathing process continuously exposes the respiratory tract to the external environment, pathogens can easily infect the host by exploiting this situation. Cigarette smoking is a substantial risk factor for respiratory tract hyperresponsiveness and pathogen infections,[23] particularly invasive pneumococcal disease.[24] However, the detailed mechanism behind how CS affects the recognition ability and phagocytic activity of macrophages that combat pneumococcal infection remains to be explored. In this study, we prepared CSE from tobacco cigarettes containing nicotine and tar, the ingredients and effects of which have been characterized in

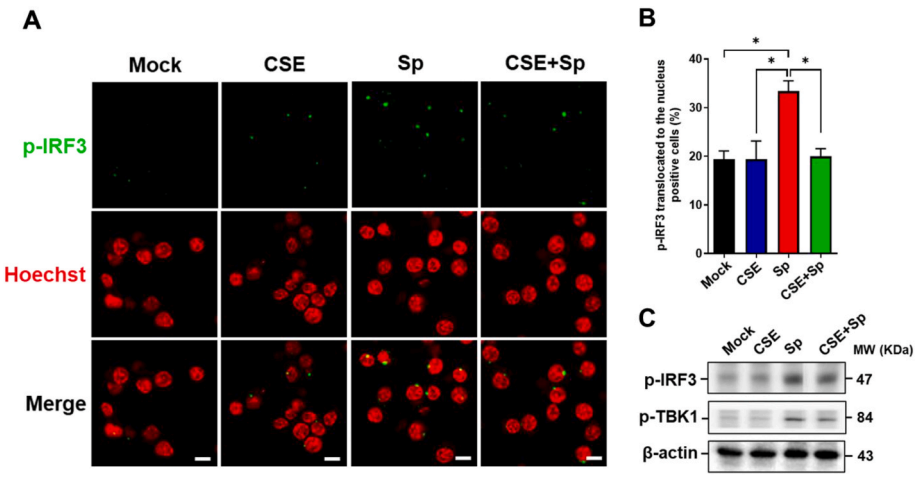

Fig. 5. CSE impedes pneumococcus-mediated type I IFN production by inhibiting IRF3 signaling. (A) RAW264.7 cells were exposed to 5 % CSE and infected with pneumococci (MOI = 10) for 4 h. Cells were fixed and probed with p-IRF3 (green) and stained with Hoechst 33,342 to visualize the nuclei (pseudocolor in red). The image was analyzed using confocal microscopy. Scale bars, 10 μm. (B) The fluorescence intensity of p-IRF3 was quantified. *, $P < 0.05$. (C) The cell lysates from CSE-treatment and/or pneumococcus-challenge were analyzed using western blotting with antibodies against p-IRF-3, p-TBK1, and β-actin, respectively. β-actin was used as a loading control.

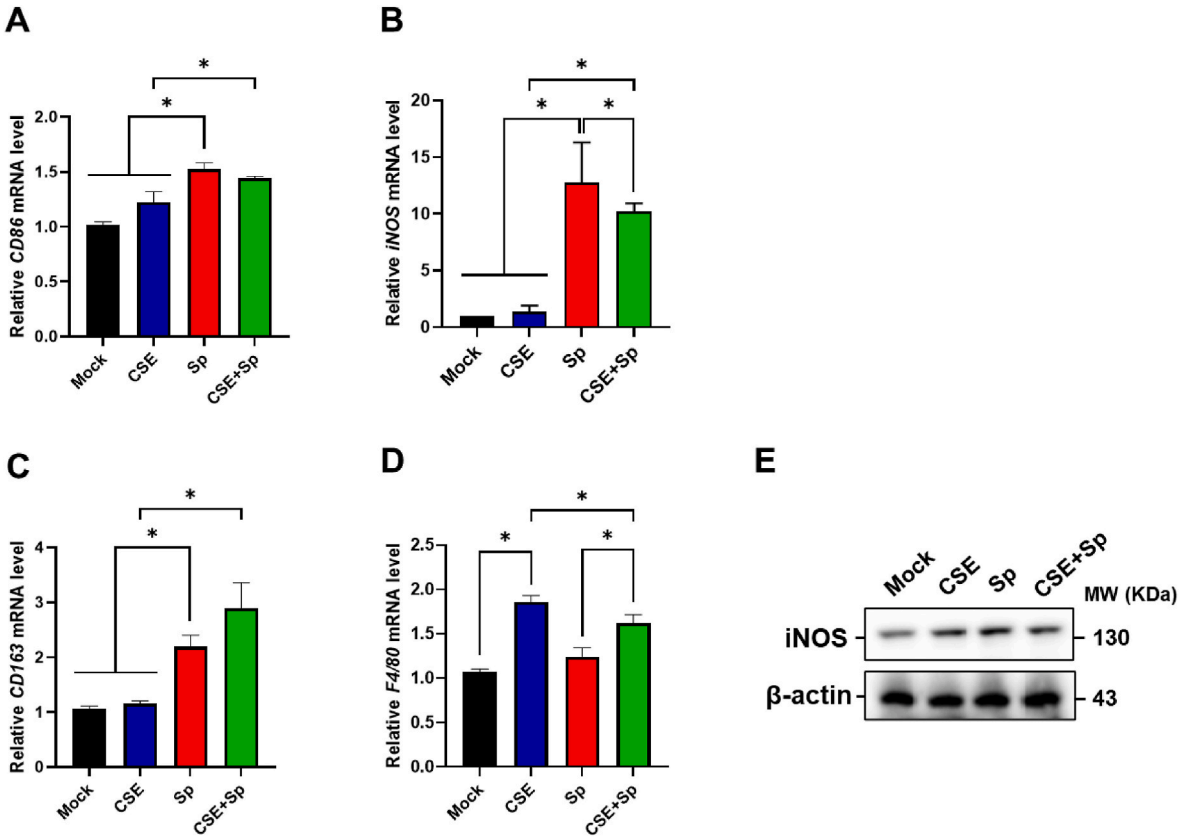

Fig. 6. CSE manipulates macrophage polarization triggered by pneumococci. RAW264.7 cells were exposed to 5 % CSE for 3 h, and infected with pneumococci (MOI = 10) for 4 h. Total RNA was prepared to analyze the relative mRNA levels of (A) *CD86*, (B) *iNOS*, (C) *CD163*, (D) *F4/80* using qRT-PCR, with normalization to the reference gene, *GAPDH*. *, $P < 0.05$. (E) The cell lysates from CSE-treatment and/or pneumococcus-challenge were analyzed using western blotting with antibodies against iNOS and β-actin, respectively. β-actin was used as a loading control.

our recent research.[10,25] In addition, the pathogenic effects of CSE administration in mice are similar to those of CS inhalation.[26,27] The present study established a reliable CSE-exposed macrophage cell model and comprehensively investigated the potential mechanisms of how CSE affects macrophage functions in response to pneumococcal infection.

Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon primarily present in CS, is known to be involved in increasing the susceptibility to bacterial infection.[28] We further examined whether B[a]P has similar activity to CSE in inhibiting macrophage phagocytosis and observed that B[a]P not only diminished the phagocytosis of latex-fluorescent beads by macrophages but also impeded pneumococcal internalization by macrophages (Fig. S5). These results suggest

that B[a]P may be one of the most important components in CSE that disrupt macrophage functions. However, the molecular mechanism underlying this effect by B[a]P needs to be elucidated.

Cigarette smoke exposure has been reported to be harmful to human respiratory tract health. The adverse effects of CS include impairing mucociliary flow, disrupting epithelial barrier integrity, suppressing immune function, and increasing pathogen infectivity in the respiratory tract.[29,30] CS-exposed patients have been clinically associated with invasive pneumococcal diseases, such as pneumonia, meningitis, and bacteremia, which have been documented for decades.[24,31] The present study used the pneumococcal strain TIGR4 (serotype 4) as a model infectious bacterial pathogen, as its genome has been completely sequenced and it is known to exhibit high virulence, causing the most severe invasive pneumococcal disease.[32,33]

Several pneumococcal virulence factors are associated with disease progression. For example, pneumococcal cell wall components, including lipoproteins and lipoteichoic acid, are recognized by TLR2, which elicits innate immune signaling.[34] Pneumococcal peptidoglycan activates NOD2, which then stimulates NF-κB to trigger proinflammatory cytokine production.[35] Macrophage recruitment and pneumococcal clearance have been shown to be impeded in mice lacking TLR2 and NOD2.[36] Additionally, pneumolysin forms pores to release bacterial DNA activating type I IFN signaling, which plays a key role in clearing pneumococcal infection.[37] Both TBK1 and IRF3 are downstream molecules that are involved in interferon induction by pneumococci.[16] As mentioned above, proinflammatory cytokines and chemokines are crucial for providing immune defense against pneumococcal infection.[16,36] Our results showed that cytokine/chemokine production was diminished in CS-exposed macrophages challenged with pneumococci via NF-κB and IRF3 inhibition. Additionally, bacterial phagocytosis by macrophages was reduced upon CS exposure, implicating that pneumococci orchestrate a meticulous strategy to evade the host attack, thereby promoting bacterial survival to exacerbate pathogenesis. These lines of evidence can explain the previous report that CS hampers host defense against pneumococci and predisposes the host to invasive pneumococcal disease.[9]

Macrophages can be differentiated into M1 and M2 phenotypes in different microenvironmental stimuli.[38] Activated M1 macrophages exhibit a proinflammatory state by increasing production of nitric oxide, type I IFNs, and various chemokines including CCL2, whereas M2 macrophages provide an anti-inflammatory response mediated by Th2-secreted cytokines, including IL-4, IL-10, and IL-13.[39] In smokers with chronic obstructive pulmonary disease (COPD), M2 macrophages are predominant[40] and M1 macrophage cytokines are suppressed.[41] A recent study revealed that extracellular vesicles produced by pneumococci polarize macrophages to M2 phenotype and enhance pneumococcal survival in macrophages.[42] Our results indicated that CS exposure skewed M2 macrophage polarization, which plays a crucial role in downregulating antimicrobial activity. This is consistent with previous findings mentioned above.

Although this study reveals a mechanism underlying CS-mediated impediment of macrophage functions after pneumococcal challenge, the *in vitro* model may not fully reflect the host pathophysiology. To validate our findings performed in the cell-based study, we established sets of CS-exposed murine models and investigated whether long-term cigarette smoke exposure impairs the immune response and exacerbates bacteria-induced pulmonary inflammation *in vivo* (Fig. S6A). BALF was prepared and differentiated using the Wright-Giemsa stain, and lung tissues were stained with H&E to evaluate levels of infiltrated inflammatory cells as described previously[43]. Our results showed that the cell counts of macrophages and neutrophils were markedly decreased in the CS + pneumococcus co-treated mice compared to those in the other treatment groups (Figs. S6D and S6E). These results indicate that long-term CS exposure in murine models, followed by bacterial infection, aggravates pulmonary pathogenesis.

Although the present study combined with the cell-based and animal studies, the mechanism of how respiratory tract mucosal cells interact with macrophages and cytokine/chemokine networks in response to pneumococcal infection remains to be elucidated. Moreover, the airway microbiota composition dysregulated by cigarette smoking and pneumococcal infection may potentially cause respiratory disease progression. Further *in vivo* explorations, including human studies and microbiota research, are necessary to fill this gap in translational research.

5. Conclusions

This study exploited murine macrophage models to demonstrate that CSE exposure hampers macrophage functions, including bacterial phagocytosis and cytokine production. Our results indicate that CS suppresses pneumococcus-induced cytokine production by inhibiting NF-κB and IRF3 signaling pathways (Fig. 7). Furthermore, CS alters macrophage polarization and innate immune sensing, leading to macrophage dysfunction that hinders bacterial clearance. Understanding the mechanism of how CS affects macrophage functions may help in the identification of new methods for restoring cellular function in patients with pneumococcal infection.

CRediT authorship contribution statement

Wei-Chih Liao: Conceptualization, Methodology, Writing – original draft. **Chia-Huei Chou:** Conceptualization, Methodology, Writing – original draft. **Mao-Wang Ho:** Validation, Writing – review & editing. **Jo-Tsen Chen:** Data curation, Methodology, Writing – original draft. **Shu-Ling Chou:** Data curation, Methodology. **Yu-Tsen Huang:** Data curation, Methodology. **Ngoc-Niem Bui:** Data curation, Methodology. **Hui-Yu Wu:** Data curation, Methodology. **Chi-Fan Lee:** Data curation, Methodology. **Wei-Chien Huang:** Conceptualization, Supervision, Writing – review & editing. **Chih-Ho Lai:** Conceptualization, Supervision, Writing – review & editing.

Data availability statement

The authors confirm that the data supporting the findings of this

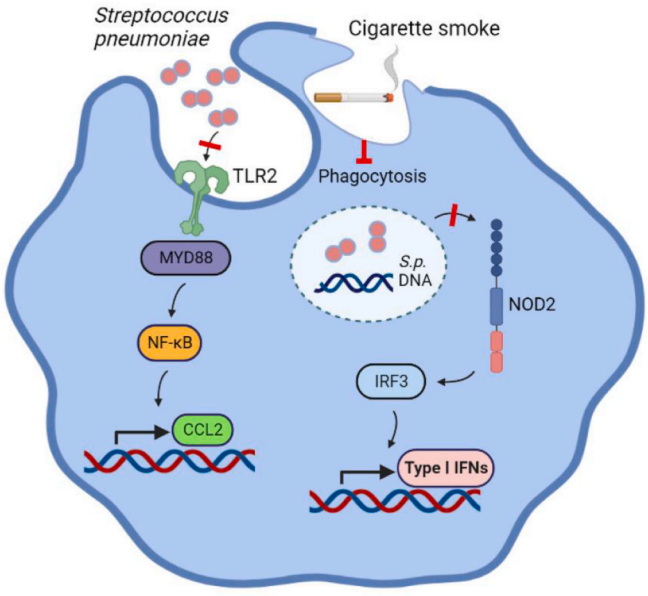

Fig. 7. Model depicting the mechanism of how CSE impairs macrophage functions against pneumococcal infection. CSE exposure impedes phagocytic activity and cytokine/chemokine production by suppressing NF-κB and IRF3 signaling pathways in pneumococcus-infected macrophages.

study are available within the article.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.10.001.

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