

Down-regulation of Fine Dusts-induced Airway Inflammation by the PDZ motif peptide of ZO-1

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Abstract

BACKGROUND: Although fine airborne particulate matter (PM) has been known to play a role in many human diseases, there is no direct evidence that therapeutic drugs or proteins can diminish PM-induced diseases. Nevertheless, studies examining the negative control mechanisms of PM-induced diseases are critical to develop novel therapeutic medications.

RESULTS: We found that CLB2.0, a surrogate for PM, induced production of multiple cytokines that alter airway inflammation. Interestingly, deletion of each PDZ domain in the ZO-1 protein dramatically decreased F-actin formation and increased the expression of genes for pro-inflammatory cytokines. We also found that the consensus PDZ peptide in ZO-1 downregulates the expression of pro-inflammatory cytokine genes and F-actin formation; in contrast, the GG24,25AA mutant PDZ peptide upregulates them. Moreover, CLB2.0-induced F-actin formation in 2D and 3D matrix cultures was significantly inhibited by PDZ peptide, but not by the mutant peptide. Induction of IL-8 secretion by CLB2.0 activates CXCR2 signaling, while increased RGS12 controlled by the PDZ peptide inhibits IL-8/CXCR2 signaling. The consensus PDZ peptide also inhibited CLB2.0-induced inflammatory cell infiltration, pro-inflammatory cytokine gene expression, and TEER in bronchoalveolar lavage (BAL) fluid and AM cells.

CONCLUSIONS: Our data indicated that the PDZ domain in ZO-1 is critical for regulation of the CLB2.0-induced inflammatory microenvironment. Therefore, we suggest that the PDZ peptide may be a potential therapeutic candidate during PM-induced respiratory diseases.

Background

Air pollution is a global concern and is becoming increasingly severe in Korea. The annual average airborne 2.5 μm particulate matter (PM_{2.5}) concentration in Korea was 24.0 $\mu\text{g}/\text{m}^3$ in 2018, but was only 12.0 $\mu\text{g}/\text{m}^3$ in Japan, 9.0 $\mu\text{g}/\text{m}^3$ in USA, and 7.9 $\mu\text{g}/\text{m}^3$ in Canada. The PM_{2.5} concentration in Korea is 2.4-fold greater than the 10 $\mu\text{g}/\text{m}^3$ annual guideline for PM_{2.5} recommended by the World Health Organization, and approaches the recommended 25 $\mu\text{g}/\text{m}^3$ 24-hour mean [20]. The reduction in air quality in Korea is due to industrialization, population growth, thermal power plants, and automobile exhaust. Airborne PM comprises particles of many sizes, but fine particulates less than 2.5 μm in diameter are able to enter the alveolar spaces of lungs and infiltrate human skin [32]. Epidemiological and clinical studies have suggested exposure to PM_{2.5} is a risk factor for many conditions, including death due to lung cancer and cardiovascular disease, and brain damage; however, there are currently no specific therapeutic options to address PM_{2.5}-related illness [11, 22]. To date, at least 40 different proteins have been identified or components of tight junctions [17]. Of these, transmembrane proteins, such as claudin and occludin, form intercellular homophilic and heterophilic adhesions, and intracellular plaque proteins, such as Zonula occludens (ZO)-1, -2, and -3, form scaffold proteins between transmembrane proteins and the actin cytoskeleton on the intracellular side of plasma membrane [30]. ZO-1 has been identified as a junctional adaptor protein that binds to many other junctional proteins, including the transmembrane proteins of the claudin and JAM families [39]. Coyne *et al.* reported that co-treatment of cystic fibrosis

(CF) cells or non-CF cells with TNF- α and IFN- γ inhibited tight junction protein production and its own function [8], ZO-1 was likewise affected by pro-inflammatory cytokines and the tight junctional barrier of the upper and lower respiratory tract, as assessed by TEER testing, was reduced. The ZO-1 protein is composed of three PSD95/dlg/ZO-1 (PDZ) domains, an SH3 domain, and a region of homology to guanylate kinase (GUK) [13]; however, there have been few studies of the effects of PDZ of ZO-1 on PM-induced airway inflammation.

Regulator of G protein signaling (RGS) proteins are known to act as critical negative modulators of G-protein-coupled receptor (GPCR) signaling. Many genetic studies suggest RGS proteins are found in many organ systems, and are potential drug targets in many human diseases [34]. The GTPase activity (GAP function) of RGS proteins plays a major role in terminating GPCR signaling. The family of RGS proteins comprises more than 30 members, characterized by the presence of the canonical RGS homology (RH) domain, and has been divided into several sub-families [34]. Of these, RGS12 is highly expressed in lung cancer (non-small cell lung carcinoma) [9]. RGS12 has a PDZ domain that is a putative CXCR2-binding site [35]. Although many studies have suggested that RGS12 acts as a scaffold protein, organizes a signal transduction complex, and promotes cell proliferation and differentiation [41, 46], studies addressing the effects of RGS12 on PM-induced airway inflammation are lacking.

Because controlled ZO-1 expression during CLB2.0-induced airway inflammation plays an important role in the pathogenesis of airway diseases, we examined whether ZO-1 down-regulates F-actin formation and pro-inflammatory cytokine gene expression by activating specific signal transduction pathways. We found the PDS domain of ZO-1 was able to reduce inflammation caused by CLB2.0 more effectively than ZO-1 protein itself. In addition, the consensus PDZ peptide dramatically inhibited CLB-induced airway inflammation *in vitro* and *in vivo*. We also showed that CXCR2 activation secondary to CLB2.0-induced IL-8 secretion was significantly abolished by RGS12 protein.

Methods

Cell Cultures

The human alveolar basal epithelial cell line (A549) was purchased from the American Type Culture Collection (CCL-185; Manassas, VA) and cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin/streptomycin at 37 °C in a humidified chamber with 5% CO₂. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline and re-cultured in DMEM with 0.2% FBS.

Materials

Carboxyl latex beads (CLB; 2 μ m) were purchased from Thermo Fisher Scientific (# C37278). PDZ peptides were synthesized by Peptron (Daejeon, Korea).

Real-time Quantitative Pcr

Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories; Hercules, CA) with iQ SYBR Green Supermix. Reactions were performed in a total volume of 20 μ l, including 10 μ l of 2 \times SYBR Green PCR Master Mix, 300 nM of each primer, and 1 μ l of the previously reverse-transcribed cDNA template.

F-actin Staining

F-actin staining was performed using ActinRed™ 555 ReadyProbes reagent (R37112, Molecular Probes) following the manufacturer's instructions. Briefly, the cells were rinsed with PBS, and the ActinGreen™ 488 ReadyProbe reagent added. The cells were incubated for 30 min, the stain solution removed, and the cells rinsed with PBS. Images were obtained using a Nikon Eclipse 80i microscope (Eclipse 80i) with a 488 nm excitation filter and a 532 nm emission filter.

Actin Polymerization Assay

The effects of PDZ peptides on actin polymerization were examined using an Actin Polymerization Biochem Kit™ (BK003, Cytoskeleton, Denver, CO) following the manufacturer's instructions. Briefly, cells were treated with CLB2.0 for 4 hours, and cell lysate was collected and centrifuged at 150,000 \times g for 1 hour at 4 °C to obtain the supernatant. Cell lysates containing equal amounts of protein were treated with the final reaction mix containing ATP and pyrene-conjugated actin (final concentration = 0.4 mg/mL) in actin polymerization buffer. The kinetics of actin polymerization were visualized according to fluorescence intensity using a microplate reader with a 355 nm excitation filter and a 405 nm emission filter. All analyses were performed using Microsoft Excel.

Transepithelial Electrical Resistance (teer) Testing

Before evaluation, the electrodes were sterilized and corrected according to the manufacturer's instructions (Millicell ERS-2; MERCK). The shorter tip was placed in the culture plate insert and the longer tip was placed in the outer well. The unit area resistance ($\Omega \times \text{cm}^2$) was calculated by multiplying the sample resistance (Ω) by the effective area of the membrane (4.2 cm^2 for 6-well Millicell inserts) [27].

F-actin staining on 2D/3D collagen-coated coverslips, Tracheotomy, Collection of BAL fluid, measurement of cell populations, ELISA, and Alveolar Macrophage (AM) cell culture

The methods used in this study have been described previously [6, 37].

Statistical Analyses

The data are presented as the mean \pm S.D. of at least three independent experiments. Where appropriate, statistical differences were assessed by the Wilcoxon Mann-Whitney test. *P*-values less than 0.05 were considered statistically significant.

Results

CLB2.0 increased F-actin formation and decreased transepithelial electrical resistance in human airway epithelial cells cultured under pro-inflammatory conditions

The A549 cell line has been long utilized in airway research as a suitable substitute for primary cells of the alveolar epithelium [15]. We used the A549 line as it imitates all the major characteristics of primary alveolar type II (ATII) cells, allowing collection of consistent and reproducible data without the technical or ethical issues involved in using primary or stem cells [7]. Although we attempted collection of PM_{2.5} samples from the top of several buildings at Dong-A university (Busan, Korea), the composition of PM_{2.5} is heavily dependent upon weather, sampling technique, and other factors. In addition, the concentrations of heavy metals in PM, which may vary from sample to sample, may influence PM-induced inflammation. Therefore, we used carboxyl charge-stabilized hydrophobic polystyrene microspheres [carboxyl latex beads (CLB), 2 μ m diameter; CLB2.0] as a surrogate for airborne particulate matter [21, 45]. We investigated the concentration dependence of the cytotoxic effects of CLB2.0 in A549 cells for 24 hours using a cell viability assay kit. CLB2.0 at 40 ~ 80 μ g/ml did not have any cytotoxic effects on A549 cells (Fig. 1a). Therefore, a concentration of CLB2.0 within this range was utilized in this study. To determine whether CLB2.0 could induce airway inflammation in the airway cells, we assessed inflammatory cytokines in the cell cultures using qPCR. CLB2.0 induced *IL-6*, *IL-1 α* , *IL-1 β* , and *TNF α* gene expression (Fig. 1b). 160 μ g/ml of CLB2.0 could not affect the expression of inflammatory cytokines, but cytotoxicity did. Chirono *et al.* reported that PM₁₀ induced cytoskeleton remodeling in A549 cells [5]. Thus, we hypothesized that CLB2.0 could also produce similar effects. In our study, CLB2.0 induced enriched F-actin formation in cell-to-cell contacts and in cell membranes, indicating increased cytoskeleton networking in CLB2.0-treated cells (Fig. 1c). In addition, actin formation was increased in a CLB2.0-concentration-dependent manner, but not at 160 μ g/ml of CLB2.0 (Fig. 1d). Next, we assessed transepithelial electrical resistance (TEER), because increased TEER due to increased F-actin formation may be associated with the invasive features of cancer cells. Interestingly, TEER was reduced in a dose-dependent manner (Fig. 1e). These results show that CLB2.0 can increase both the expression of inflammatory cytokines and F-actin formation and decrease the transepithelial resistance in human airway epithelial cells.

The PDZ domain of ZO-1 protein critically reduces F-actin formation and airway inflammation

To examine whether ZO-1 overexpression can affect F-actin formation induced by CLB2.0, F-actin staining was carried out (Fig. 2a). While ZO-1 overexpression increased the density of CLB2.0-induced F-actin formation, siRNA-ZO-1 did not. Because several studies suggest ZO-1 can bind to ZO-2, occludin, and F-actin to form epithelial tight junctions [13, 14, 31], this finding was not unexpected. Interestingly, ZO-1 overexpression increased pro-inflammatory cytokines (*IL-6*, *IL-1 α* , *IL-1 β* , and *TNF α*) gene expression, but si-RNA-ZO-1 significantly reduced their expression (Fig. 2b). Tight junction proteins such as ZO-1 were reported to be disrupted in epithelial cultures from asthmatic subjects [42]. In addition, the same group reported that the allergenic fungus *Alternaria alternate* increased IL-8 and TNF α secretion with an accompanying reduction in TEER [24]. There several possible reasons why overexpression of ZO-1 increased pro-inflammatory cytokine gene expression: First, because CLB2.0 releases/produces inflammatory cytokines to induce airway inflammation, ZO-1 expression increased in proportion to the degree of inflammation in the acute phase, and then ZO-1 negatively regulated pro-inflammatory cytokine gene expression during later stages of inflammation (24 hours; data not shown). Increased ZO-1 expression increased pro-inflammatory cytokine production; however, siRNA-ZO-1 expression reduced these cytokines. We postulated that ZO-1 protein activity/expression might be closely related to CLB2.0-induced inflammation. Although it appeared that ZO-1 played a role as an inducer of cytokine production, cell-cycle arrest was also increased by deletion of ZO-1 (see Discussion, below). Next, to examine which domain of ZO-1 was critical for F-actin polymerization and cytokine production during CLB2.0 exposure, several deletion mutants were generated (Fig. 2c) [13]. After transfection with each deletion mutant construct, F-actin staining was performed. Interestingly, PDZ deletion mutants abolished F-actin formation, but an SH3 deletion mutant construct did not. In addition, *pro-inflammatory cytokines* gene expression was significantly increased in the cells transfected with PDZ deletion mutants (M1-3; Fig. 2d). These results show that PDZ may be critical for PM-induced airway inflammation during acute phase inflammation. Indeed, we synthesized permeable PDZ-containing specific peptide and mutant peptide (Fig. 2e). Interestingly, the consensus PDZ peptide significantly reduced *pro-inflammatory cytokines* gene expression and F-actin polymerization, but the mutant PDZ peptide did not (Fig.s 2e and 2f). Next, we investigated the effects of PDZ peptide on CLB2.0-induced F-actin polymerization in the A549 cells (Fig. 2g). On either 2D (upper panel) or 3D (lower panel) collagen-coated coverslips, the formation of F-actin was enhanced by CLB2.0. While the consensus PDZ peptide significantly reduced F-actin polymerization, the mutant peptide increased polymerization. These results suggest the consensus PDZ peptide reduces CLB2.0-mediated F-actin polymerization, consistent with the observation that the PDZ peptide negatively regulates chemokine-induced movement to control inflammation at the inflamed site; however, the mutant peptide did not affect F-actin polymerization or pro-inflammatory cytokine gene expression. In this context, PDZ peptide has an anti-inflammatory role in the inflamed microenvironment.

CLB2.0 induces intracellular IL-8 secretion and RGS12 inhibits CXCR2-induced signaling activated by CLB2.0

Next, we investigated the physiological mechanism by which CLB2.0 activates pro-inflammatory cytokine gene expression. CLB2.0 may increase *C-X-C motif chemokine receptor (CXCR) 2* and *regulator of G-protein signaling 12 (RGS12)* gene expression in a time-dependent manner (Fig. 3a). CXCR2, a G-protein-coupled receptor, and its ligand IL-8 are the most significantly upregulated chemokine and receptor in cancer and inflammation [10, 18, 23], and ZO-1/CXCL8 (IL-8) signaling also has important functions in tumor angiogenesis [26]. In addition, Longhin *et al.* reported recently that PM2.5 and 10 increased *IL-6* and *IL-8* gene expression and secretion for up to 48 hours [28]. Thus, to firstly determine whether CLB2.0 could induce intracellular secretion of IL-8, the medium from cells incubated with CLB2.0 was assayed using an IL-8-specific ELISA (Fig. 3b). Intracellular IL-8 was secreted from the cells into the medium. Interestingly, the consensus PDZ peptide inhibited IL-8 secretion, but the mutant peptide did not (Fig. 3c). In addition, CXCR2 gene expression was reduced by the consensus PDZ peptide, whereas it was dramatically increased by the mutant PDZ peptide. The PDZ domain was localized at the N-terminal region of RGS12 and RGS12 bound to CXCR2 [35]. The presence of a spliced PDZ domain in RGS12 indicates a physiological mechanism by which RGS12 may target specific CXCR2 receptor systems for receptor desensitization. Interestingly, the consensus PDZ peptide increased *RGS12* gene expression, rather than reducing *CXCR2* (Fig. 3d), suggesting that the consensus PDZ peptide may control RGS12 gene expression in a reciprocal fashion proportional to CXCR2 expression. Next, we assessed cAMP concentrations because CXCR2 is a Gai-coupled receptor, cAMP concentrations were strongly restored by consensus PDZ peptide, but not by the mutant PDZ peptide (Fig. 3e), indicating the consensus PDZ peptide both inhibited IL-8 secretion and abolished signaling from the interaction of CXCR2 and IL-8. We examined whether RGS12 could also regulate CLB2.0-induced cAMP inhibition. RGS12 also restored cAMP concentrations (Fig. 3f). These results indicate that the consensus PDZ peptide acts as a primary negative regulator to maintain homeostasis during CLB2.0-induced airway inflammation and RGS12 acts as a secondary negative regulator protein.

Consensus PDZ peptide diminishes inflammatory cell populations, and decreases inflammatory cytokine production in the mouse lung

Next, to investigate whether the consensus PDZ peptide regulates the inflammatory cell populations in BAL fluid, we measured various inflammatory cell populations after intranasal instillation of the two PDZ peptides but prior to CLB2.0 instillation (Fig. 4a). Mice instilled with consensus PDZ peptide had lower levels of lymphocytes, neutrophils, alveolar macrophages, and total protein in BAL fluid. In contrast, inflammatory cell populations in the BAL fluid of mice administered the mutant PDZ peptide had dramatically increased levels of these markers compared with those in mice administered the CLB2.0/consensus PDZ peptide. To determine whether the consensus PDZ peptide controls the

production of inflammatory cytokines after instillation of CLB2.0, inflammatory cytokines in BAL fluid were measured using specific ELISAs three days after CLB2.0 instillation (Fig. 4b). Treatment with the consensus PDZ peptide dramatically reduced CLB2.0-induced IL-6, IL-1 α , IL-1 β , and TNF α production, whereas mutant PDZ peptide increased the production of these cytokines. We also investigated whether the PDZ peptides altered the production of pro-inflammatory cytokines in the mouse lung. We found the consensus PDZ peptide also reduced IL-6, IL-1 α , IL-1 β , and TNF α gene expression in the lung, whereas the mutant peptide increased their expression (Fig. 4c). Lastly, TEER was also restored by consensus PDZ peptide, but not by the mutant peptide (Fig. 4d). Our results indicate that the consensus PDZ peptide abolishes changes in inflammatory cell populations, and pro-inflammatory cytokine production in both BAL fluid and the lung after CLB2.0 treatment.

Discussion

Air pollution and airborne particulate matter are major public health hazards. Ambient air pollution in both urban and suburban areas was estimated to cause 4.2 million premature deaths worldwide per year in 2016; this mortality is due to exposure to airborne particulates 2.5 μm or less in diameter (PM_{2.5}), which cause respiratory and cardiovascular disease, and cancers [20]. Many epidemiological reports have linked exposure to air pollution and increased morbidity and mortality associated with cardiovascular and respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma [3, 4, 47]. Interestingly, although PM has been known to be a critical risk factor for COPD, evidence supporting the relationship between the incidence of COPD and PM_{2.5} is currently very limited and insufficient. Given PM causes many diseases in the human body, clarification of the physiological mechanisms underlying the negative regulation of PM-induced airway inflammation may provide a greater understanding of airway inflammation.

ZO-1 is a scaffolding protein that connects transmembrane tight junctions with cytoplasmic proteins and the actin cytoskeleton [13, 16]. The ZO-1 protein has several domains, including PDZ, SH3, and GUK. The PDZ domain forms dimers or binds to intracellular proteins and PDZ-containing proteins. The PDZ domain is involved in intracellular signaling, cell adhesion, ion transport, and formation of the paracellular barriers. The roles of the SH3 domain include regulation of enzymes by intramolecular interactions, altering the subcellular localization of components of signaling pathways, and mediating the assembly of large multi-protein complexes [29]. The GUK domain is involved in protein-protein interactions. Unlike the PDZ domain, SH3 and GUK domains did not affect F-actin polymerization and pro-inflammatory cytokine gene expression in our system. The functionality of each PDZ domain did not vary and deletion of each domain abolished F-actin polymerization and pro-inflammatory cytokine gene expression (Fig. 2). Interestingly, the classical PDZ domain at the N-terminus of RGS12 bound selectively to C-terminal (A/S)-T-X-(L/V) motifs as found within both the CXCR2 IL-8 receptor, and the alternative 3' exon form of RGS12, suggesting that this interaction between the PDZ domain in RGS12 and CXCR2 shows a specific mechanism by which RGS12 acts as a desensitization protein that shuts down GPCR signaling (Fig. 3) [2].

Because ZO-1 overexpression increased the expression of pro-inflammatory cytokine genes, whereas siRNA-ZO-1 abolished their expression (Fig. 2b), it was not clear whether ZO-1 acted as an inducer of inflammation. ZO-1 expression increased in proportion to the degree of inflammation in the acute phase, but ZO-1 negatively regulated pro-inflammatory cytokine gene expression at later stages of inflammation (24 hours; data not shown). The reason for the late-stage inhibition of pro-inflammatory cytokines by siRNA-ZO-1 was probably because of induction of cell-cycle arrest and inhibition of both cell proliferation and adhesion *in vitro* [1, 33, 38]. We postulated that the cells were damaged by siRNA-ZO-1. Thus, we were unable to use siRNA-ZO1 to further knock-down ZO-1 expression, and instead used several deletion mutants of each domain of ZO-1 to investigate the function of the ZO-1 protein during CBL2.0-induced airway inflammation.

Recently, it was reported that although PM2.5 did not affect cellular survival or proliferation, PM2.5 activated the stress response mediated by p38 MAPK which, along with RhoA GTPase and HSP27, induced morphological changes, including actin cytoskeletal rearrangements and paracellular gap formation, in BEAS-2B cells [12]. Moreover, PM2.5 activated TGF- β 1/SMAD3 signaling and α -SMA and COL1 upregulation in a human pulmonary fibroblast cell line (HFL-1), and triggered pulmonary fibrosis by targeting pulmonary epithelium, macrophages and fibroblasts [43]; however, no physiological mechanism by which PM affects F-actin polymerization, stress-fiber formation, and cytoskeletal rearrangements in airway epithelial cells has been reported. In addition, PM2.5 and PM10 increased the gene expression of IL-6 and IL-8, and increased their secretion from BEAS-2B human bronchial epithelial cells [28]. We hypothesized that CLB2.0 may interact with CXCR2, the IL-8 receptor of the CXR chemokine receptor family [40]. In addition, administration of a dual CXCR1/2 inhibitor reduced allergen-induced neutrophilic inflammation and allergic airway inflammation in mice [19]. Based on these reports, we investigated the effect of PDZ peptides on CLB2.0-induced F-actin formation in A549 cells. Visualization of F-actin in cells treated with the consensus PDZ peptide or the mutant peptide showed outstanding morphological differences (Fig. 2g). The consensus PDZ peptide significantly reduced F-actin polymerization in 2D and 3D cultures; in contrast, the mutant peptide dramatically increased polymerization. These differences suggest that PDZ peptide-induced downregulation of pro-inflammatory cytokines abolishes airway inflammation in the lung, chemokine-induced migration is not necessary to regulate airway inflammation, and the PDZ peptide controls airway inflammation to maintain homeostasis. Based on our *in vitro* results and our 2D and 3D culture experiments, the consensus PDZ peptide appears to prevent PM-induced airway inflammation. This information provides new knowledge for the development of novel therapeutic drugs in PM-induced respiratory diseases.

As noted previously, incubation of A549 cells with CLB2.0 induced CXCR2 and RGS12 gene expression (Fig. 3a). Secreted IL-8 activated the CXCR4 receptor, reduced intracellular cAMP concentrations, and up-regulated Gai signaling to induce pro-inflammatory cytokine gene expression. However, we suggest that RGS12 acted as a secondary negative regulator in the present study. This report is the first to investigate the CLB2.0-ZO-1-CXCR2-RGS12 axis. In our previous study, we found that RGS4 protein suppressed LPS-induced airway inflammation [36]. This study provides additional insights into the molecular mechanism of negative regulation of LPS-induced mucus hyperproduction and hypersecretion in airway mucosal

inflammation. Taken together, the function of RGS proteins may be critical for GPCR desensitization to down-regulate the signaling. The most interesting finding was that there is a specific PDZ domain in the RGS12 protein. Recently, expression of the PDZ-adaptor protein PDZK1 (NHERF3) was found to be reduced in the inflamed intestine of ulcerative colitis patients and murine models of colitis [25, 44]. As a result, we postulated that PDZ-adaptor protein and PDZ-containing protein suppressed inflammation in several tissue microenvironments. We are investigating the physiological mechanism of RGS12 in PM-induced airway inflammation in a follow-up study. Taken together, our current finding indicate induction of IL-8 secretion by CLB2.0 activated CXCR2 signaling, but PDZ-containing RGS12 protein controlled the physiological functions of IL-8/CXCR2 signaling through GTPase activity during PM exposure.

More interestingly, the consensus PDZ peptide acted as a negative regulator *in vivo* in the BAL fluid from CLB2.0-instilled mice. CLB2.0-induced lymphocytes, neutrophils, alveolar macrophages (AMs), total protein, and pro-inflammatory cytokines production were significantly reduced by the consensus PDZ peptide, whereas all these were increased in response to the mutant PDZ peptide, suggesting that PDZ acts as a negative regulator to maintain homeostasis by shutting down CLB-induced effects *in vivo*. The PDZ peptide produced similar effects in AMs to those noted *in vitro*. Even though such a result provides insight into the negative regulatory mechanism of CLB2.0-induced airway inflammation, it is still unclear how the PDZ peptide can control the inflammatory cell populations in BAL fluid. In addition, if PDZ functions similarly *in vivo* and in BAL fluid, PDZ may mediate apoptotic cell clearance in acute lung injury [27].

Conclusions

We found the PDZ peptide of the ZO-1 protein had an inhibitory effect on CLB2.0-induced airway inflammation and TEER. In addition, IL-8 secretion induced by incubation with CLB2.0 activated CXCR2, while RGS12 negatively regulated CXCR2/IL-8-altered cAMP concentrations by activating GTPase activity. The consensus PDZ peptide increased RGS12 production. PDZ induced reductions of the number of AMs and inflammatory cells in BAL (Fig. 4e). Thus, these results suggest that the PDZ peptide may be a potential therapeutic candidate during PM-induced respiratory diseases.

Declarations

Acknowledgement

Not applicable.

Statement

We have read and have abided by the statement of ethical standards for manuscripts submitted to J Transl Med.

Authors' Contributions

Conceived and designed the experiments: DHK, TJJ, JWK, CHK, and KSS

Performed the experiments: DHK, TJJ, YSS, JDK, and HCL

Analyzed the data: SR, DHK, and KSS

Contributed reagents/materials/analysis tools: YHC, CHK

Wrote the paper: DHK, TJJ, and KSS.

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Available of Supporting Data

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

Ethics Approval

All experimental activities were approved by the committee of Kosin University College of Medicine on Animal Resources

Consent for Publication

Not applicable.

CONFLICT OF INTEREST

We declared we don't have any conflict of interest.

References

1. Balda MS, Matter K. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. *EMBO J.* 2000;19:2024-33.
2. Bauer H, Zweimueller-Mayer J, Steinbacher P, Lametschwandtner A, Bauer HC. The dual role of zonula occludens (ZO) proteins. *J Biomed Biotechnol.* 2010;2010:402593.
3. Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC Jr, and Tager I. Expert Panel on Population and Prevention Science of the American Heart Association: Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation.* 2004;109:2655-71.

4. Brunekreef B, Holgate ST. Air pollution and health. *Lancet*. 2002;360:1233-42.
5. Chirino YI, García-Cuellar CM, García-García C, Soto-Reyes E, Osornio-Vargas ÁR, Herrera LA, López-Saavedra A, Miranda J, Quintana-Belmares R, Pérez IR, Sánchez-Pérez Y. Airborne particulate matter in vitro exposure induces cytoskeleton remodeling through activation of the ROCK-MYPT1-MLC pathway in A549 epithelial lung cells. *Toxicol Lett*. 2017;272:29-37.
6. Choi IW, Ahn do W, Choi JK, Cha HJ, Ock MS, You E, Rhee SM, Kim KC, Choi YH, Song KS. Regulation of Airway Inflammation by G-protein Regulatory Motif Peptides of AGS3 protein. *Sci Rep*. 2016;6:27054.
7. Cooper JR, Abdullatif MB, Burnett EC, Kempshell KE, Conforti F, Tolley H, Collins JE, Davies DE. Long Term Culture of the A549 Cancer Cell Line Promotes Multilamellar Body Formation and Differentiation towards an Alveolar Type II Pneumocyte Phenotype. *PLoS One*. 2016;11:e0164438.
8. Coyne CB, Vanhook MK, Gambling TM, Carson JL, Boucher RC, Johnson LG. Regulation of airway tight junctions by proinflammatory cytokines. *Mol Biol Cell*. 2002;13:3218-34.
9. Dai J, Gu J, Lu C, Lin J, Stewart D, Chang D, Roth JA, Wu X. Genetic variations in the regulator of G-protein signaling genes are associated with survival in late-stage non-small cell lung cancer. *PLoS One* 2011;6:e21120.
10. de Oliveira S, Reyes-Aldasoro CC, Candel S, Renshaw SA, Mulero V, Calado A. Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response. *J Immunol*. 2013;190:4349-59.
11. de Prado Bert P, Mercader EMH, Pujol J, Sunyer J, Mortamais M. The Effects of Air Pollution on the Brain: a Review of Studies Interfacing Environmental Epidemiology and Neuroimaging. *Curr Environ Health Rep*. 2018;5:351-64.
12. Dornhof R, Maschowski C, Osipova A, Gieré R, Seidl M, Merfort I, Humar M. Stress fibers, autophagy and necrosis by persistent exposure to PM2.5 from biomass combustion. *PLoS One*. 2017;12:e0180291.
13. Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* 1998;273:29745-53.
14. Fanning AS, Ma TY, Anderson JM. Isolation and functional characterization of the actin binding region in the tight junction protein ZO-1. *FASEB J*. 2002;16:1835-7.
15. Fernando IPS, Jayawardena TU, Kim HS, Lee WW, Vaas APJP, De Silva HIC, Abayaweera GS, Nanayakkara CM, Abeytunga DTU, Lee DS, Jeon YJ. Beijing urban particulate matter-induced injury and inflammation in human lung epithelial cells and the protective effects of fucosterol from *Sargassum binderi*. *Environ Res*. 2019;172:150-8.
16. Furuse M, Itoh M, Hirase T, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J Cell Biol*. 1994;127:1617-26.

17. González-Mariscal L, Betanzos A, Nava P, Jaramillo BE. Tight junction proteins. *Prog Biophys Mol Biol.* 2003;81:1-44.
18. Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases. *Theranostics.* 2017;7:1543-88.
19. Hosoki K, Rajarathnam K, Sur S. Attenuation of murine allergic airway inflammation with a CXCR1/CXCR2 chemokine receptor inhibitor. *Clin Exp Allergy.* 2019;49:130-2.
20. [https://www.who.int/news-room/fact-sheets/detail/ambient-\(outdoor\)-air-quality-and-health](https://www.who.int/news-room/fact-sheets/detail/ambient-(outdoor)-air-quality-and-health)
21. Kim SS, Kim CH, Kim JW, Kung HC, Park TW, Shin YS, Kim JD, Ryu S, Kim WJ, Choi YH, Song KS. Airborne particulate matter increases MUC5AC expression by downregulating Claudin-1 expression in human airway cells. *BMB Rep.* 2017;50:516-21.
22. Lee BJ, Kim B, Lee K. Air pollution exposure and cardiovascular disease. *Toxicol Res.* 2014;30:71-5.
23. Lee YS, Choi I, Ning Y, Kim NY, Khatchadourian V, Yang D, Chung HK, Choi D, LaBonte MJ, Ladner RD, Nagulapalli Venkata KC, Rosenberg DO, Petasis NA, Lenz HJ, Hong YK. Interleukin-8 and its receptor CXCR2 in the tumour microenvironment promote colon cancer growth, progression and metastasis. *Br J Cancer.* 2012;106:1833-41.
24. Leino MS, Loxham M, Blume C, Swindle EJ, Jayasekera NP, Dennison PW, Shamji BWH, Edwards MJ, Holgate ST, Howarth PH, Davies DE. Barrier disrupting effects of alternaria alternata extract on bronchial epithelium from asthmatic donors. *PLoS One.* 2013;8:e71278.
25. Lenzen H, Lünemann M, Bleich A, Manns MP, Seidler U, Jörns A. Downregulation of the NHE3-binding PDZ-adaptor protein PDZK1 expression during cytokine-induced inflammation in interleukin-10-deficient mice. *PLoS One* 2012;7:e40657.
26. Lesage J, Suarez-Carmona M, Neyrinck-Leglantier D, Grelet S, Blacher S, Hunziker W, Birembaut P, Noël A, Nawrocki-Raby B, Gilles C, Polette M. Zonula occludens-1/NF- κ B/CXCL8: a new regulatory axis for tumor angiogenesis. *FASEB J.* 2017;31:1678-88.
27. London NR Jr., Tharakan A, Rule AM, Lane AP, Biswal S, Ramanathan M. Jr. Air pollutant-mediated disruption of sinonasal epithelial cell barrier function is reversed by activation of the Nrf2 pathway. *J Allergy Clin Immunol.* 2016;138:1736-8.
28. Longhin E, Holme JA, Gualtieri M, Camatini M, Øvrevik J. Milan winter fine particulate matter (wPM_{2.5}) induces IL-6 and IL-8 synthesis in human bronchial BEAS-2B cells, but specifically impairs IL-8 release. *Toxicol In Vitro.* 2018;52:365-73.
29. Mayer BJ. SH3 domains: complexity in moderation. *J Cell Sci.* 2001;114:1253-63.
30. McNeil E, Capaldo CT, Macara IG. Zonula occludens-1 function in the assembly of tight junctions in Madin-Darby canine kidney epithelial cells. *Mol Biol Cell.* 2006;17:1922-32.
31. Odenwald MA, Choi W, Buckley A, Shashikanth N, Joseph NE, Wang Y, Warren MH, Buschmann MM, Pavlyuk R, Hildebrand J, Margolis B, Fanning AS, Turner JR. ZO-1 interactions with F-actin and occludin direct epithelial polarization and single lumen specification in 3D culture. *J Cell Sci.* 2017;130:243-59.

32. Ogino K, Nagaoka K, Okuda T, Oka A, Kubo M, Eguchi E, Fujikura Y. PM2.5-induced airway inflammation and hyperresponsiveness in NC/Nga mice. *Environ Toxicol*. 2017;32:1047-54.
33. Qiao X, Roth I, Féraille E, Hasler U. Different effects of ZO-1, ZO-2 and ZO-3 silencing on kidney collecting duct principal cell proliferation and adhesion. *Cell Cycle*. 2014;13:3059-75.
34. Roman DL, Traynor JR. Regulators of G protein signaling (RGS) proteins as drug targets: modulating G-protein-coupled receptor (GPCR) signal transduction. *J Med Chem*. 2011;54:7433-40.
35. Snow BE, Hall RA, Krumins AM, Brothers GM, Bouchard D, Brothers CA, Chung S, Mangion J, Gilman AG, Lefkowitz RJ, Siderovski DP. GTPase activating specificity of RGS12 and binding specificity of an alternatively spliced PDZ (PSD-95/Dlg/ZO-1) domain. *J Biol Chem*. 1998;273:17749-55.
36. Song KS, Kim HJ, Kim K, Lee JG, Yoon JH. Regulator of G-protein signaling 4 suppresses LPS-induced MUC5AC overproduction in the airway. *Am J Respir Cell Mol Biol*. 2009;41:40-9.
37. Song KS, Yoon JH, Kim KS, Ahn DW. c-Ets1 inhibits the interaction of NF- κ B and CREB, and downregulates IL-1 β -induced MUC5AC overproduction during airway inflammation. *Mucosal Immunol*. 2012;5:207-15.
38. Spadaro D, Tapia R, Jond L, Sudol M, Fanning AS, Citi S. ZO proteins redundantly regulate the transcription factor DbpA/ZONAB. *J Biol Chem*. 2014;289:22500-11.
39. Tornavaca O, Chia M, Dufton N, Almagro LO, Conway DE, Randi AM, Schwartz MA, Matter K, Balda MS. ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation. *J Cell Biol*. 2015;208:821-38.
40. Veenstra M, Ransohoff RM. Chemokine receptor CXCR2: physiology regulator and neuroinflammation controller? *J Neuroimmunol*. 2012;246:1-9.
41. Willard MD, Willard FS, Li X, Cappell SD, Snider WD, Siderovski DP. Selective role for RGS12 as a Ras/Raf/MEK scaffold in nerve growth factor-mediated differentiation. *EMBO J*. 2007;26:2029-40.
42. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, Haitchi HM, Vernon-Wilson E, Sammut D, Bedke N, Cremin C, Sones J, Djukanović R, Howarth PH, Collins JE, Holgate ST, Monk P, Davies DE. Defective epithelial barrier function in asthma. *J Allergy Clin Immunol*. 2011;128:549-56.
43. Xu Z, Li Z, Liao Z, Gao S, Hua L, Ye X, Wang Y, Jiang S, Wang N, Zhou D, Deng X. PM2.5 induced pulmonary fibrosis in vivo and in vitro. *Ecotoxicol Environ Saf*. 2019;171:112-21.
44. Yeruva S, Chodisetti G, Luo M, Chen M, Cinar A, Ludolph L, Lünemann M, Goldstein J, Singh AK, Riederer B, Bachmann O, Bleich A, Gereke M, Bruder D, Hagen S, He P, Yun C, Seidler U. Evidence for a causal link between adaptor protein PDZK1 downregulation and Na⁺/H⁺ exchanger NHE3 dysfunction in human and murine colitis. *Pflugers Arch*. 2015;467:1795-807.
45. Yu H, Li Q, Kolosov VP, Perelman JM, Zhou X. Regulation of particulate matter-induced mucin secretion by transient receptor potential vanilloid 1 receptors. *Inflammation*. 2012;35:1851-9.
46. Yuan X, Cao J, Liu T, Li YP, Scannapieco F, He X, Oursler MJ, Zhang X, Vacher J, Li C, Olson D, Yang S. Regulators of G protein signaling 12 promotes osteoclastogenesis in bone remodeling and pathological bone loss. *Cell Death Differ*. 2015;22:2046-57.

47. Yue W, Tong L, Liu X, Weng X, Chen X, Wang D, Dudley SC, Weir EK, Ding W, Lu Z, Xu Y, Chen Y. Short term PM2.5 exposure caused a robust lung inflammation, vascular remodeling, and exacerbated transition from left ventricular failure to right ventricular hypertrophy. *Redox Biol.* 2019;22:101161.

Figures

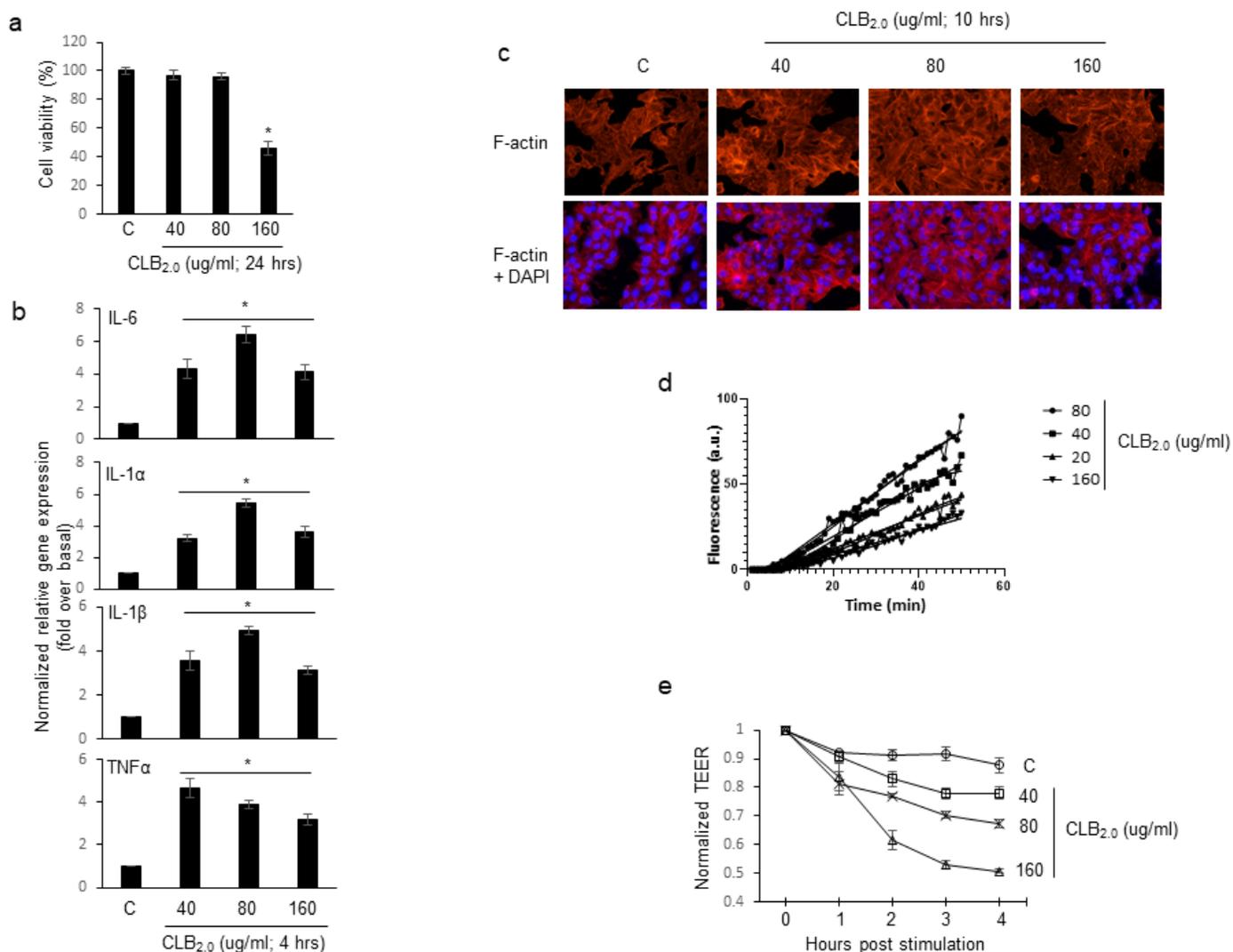


Figure 1

Effect of CLB2.0 on F-actin formation and transepithelial resistance in the A549 human airway epithelial cell line (a) 5,000 transfected cells were incubated for 24 h with CLB2.0. Cell proliferation was measured using a Cell-Counting Kit-8 according to the manufacturer's instructions (Dojindo, Rockville, MD) (n=4). (b) Confluent and quiescent A549 cells were incubated for four hours with various concentrations of CLB2.0, and then lysates were harvested and analyzed by real-time quantitative RT-PCR. * $p < 0.05$ compared to the control. $\beta 2M$, beta-2-microglobulin, was used as a loading control. (c) After treatment with various concentrations of CLB2.0 for 10 hours, the cells were rinsed with PBS, and ActinGreenTM 488 ReadyProbe reagent added. The images were taken using a Nikon Eclipse 80i microscope (Eclipse

80i) with a 488 nm excitation filter and a 532 nm emission filter. (d) The cells were treated with CLB2.0 for four hours, and cell lysates were collected, centrifuged, and then supernatant recovered. Cell lysates with equal amounts of protein were treated with the final reaction mix containing ATP and pyrene-conjugated actin (final concentration = 0.4 mg/mL) in actin polymerization buffer. Actin polymerization was visualized by fluorescence intensity using a microplate reader with a 355 nm excitation filter and a 405 nm emission filter, and the analyses were performed using Microsoft Excel. (e) The cells were treated with CLB2.0 for four hours, and the TEER was measured. Error bars represent the SEM of at least three independent experiments. All f data shown are representative of three independent experiments

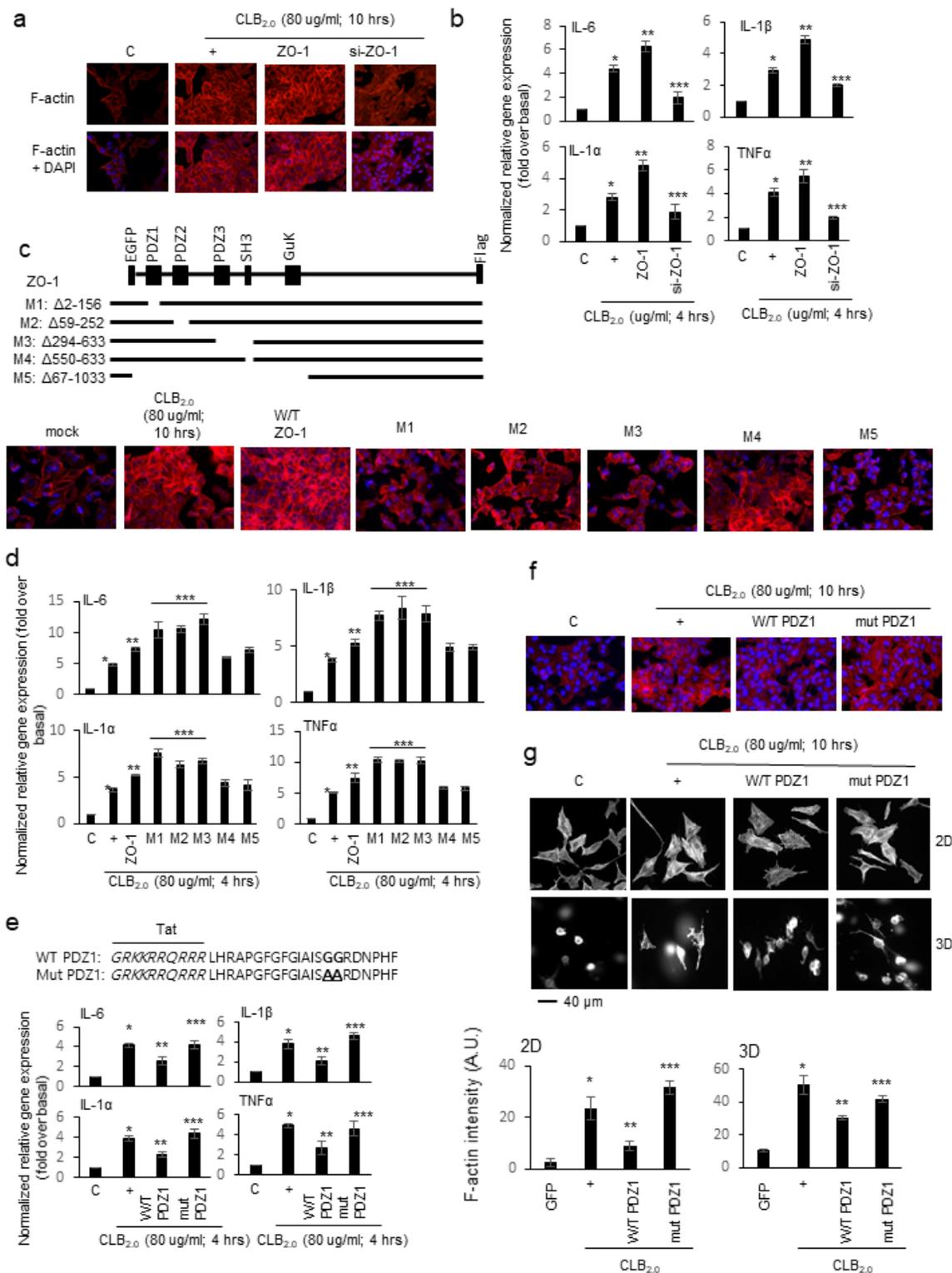


Figure 2

Effect of the ZO-1 protein PDZ domain on CLB2.0-induced airway inflammation. (a) A549 cells were transfected with either a construct driving the expression of wild-type ZO-1 or ZO-1-specific siRNA. Cells were then incubated with CLB2.0 for 10 hours. The cells were stained with ActinGreenTM 488 ReadyProbe reagent. (b) Cells were transfected, and were then incubated with CLB2.0 for four hours prior to the generation of total cell lysates, and then qRT-PCR for pro-inflammatory cytokines transcript was

performed. * $p < 0.05$ compared to the control, ** $p < 0.05$ compared to CLB2.0 only, and *** $p < 0.05$ compared to ZO-1-transfected cells. (c) Constructs were designed according to the amino acids deleted (e.g. M1: 2-156). Black lines represent the magnitude of the sequences encoded by each construct. The cells were transfected with either a ZO-1 overexpression construct or deletion constructs, and were incubated with CLB2.0 for 10 hours. F-actin was stained with a specific reagent. (d) Cells were transfected with deletion constructs, and were then incubated with CLB2.0 for four hours, after which qRT-PCR for pro-inflammatory cytokine transcripts was performed. * $p < 0.05$ compared with the control, ** $p < 0.05$ compared with CLB2.0 only, and *** $p < 0.05$ compared with ZO-1-transfected cells. (e) Peptides were synthesized with Tat region (*italic amino acids*) based on the first PDZ domain sequence (upper panel). Cells were treated with consensus PDZ or GG25,26AA mutant PDZ peptide for 24 hours and then incubated with CLB2.0 for either 4 or 10 hours (f), after which we performed qRT-PCR and F-actin staining. * $p < 0.05$ compared with the control; ** $p < 0.05$ compared with CLB2.0 alone; *** $p < 0.05$ compared with consensus PDZ peptide treatment. (g) Cells were seeded on coverslips and then treated with either consensus PDZ peptide or the mutant PDZ peptide (both at 1.0 $\mu\text{g/ml}$) prior to treatment with CLB2.0. After fixation, rhodamine-conjugated phalloidin was added for 30 min (1:100 dilution). Cells were then stained with DAPI for 2 min (1:10,000 dilution) (2D culture; upper panels). After trypsinizing, collagen was added to the cells (1.5 mg/ml; 2×10^4 /matrix), and subsequently, media containing FBS and CLB2.0 was added and the preparations incubated for 10 hours. After blocking with 2% BSA, the cells were incubated with rhodamine-conjugated phalloidin (3D culture; lower panels). The scale bar is 20 μm . The F-actin intensity was used to assess morphometric differences between cells. * $p < 0.05$ compared with the control (GFP); ** $p < 0.05$ compared with CLB2.0 only; *** $p < 0.05$ compared with CLB2.0 and consensus PDZ peptide treatment. All of data shown are representative of three independent experiments.

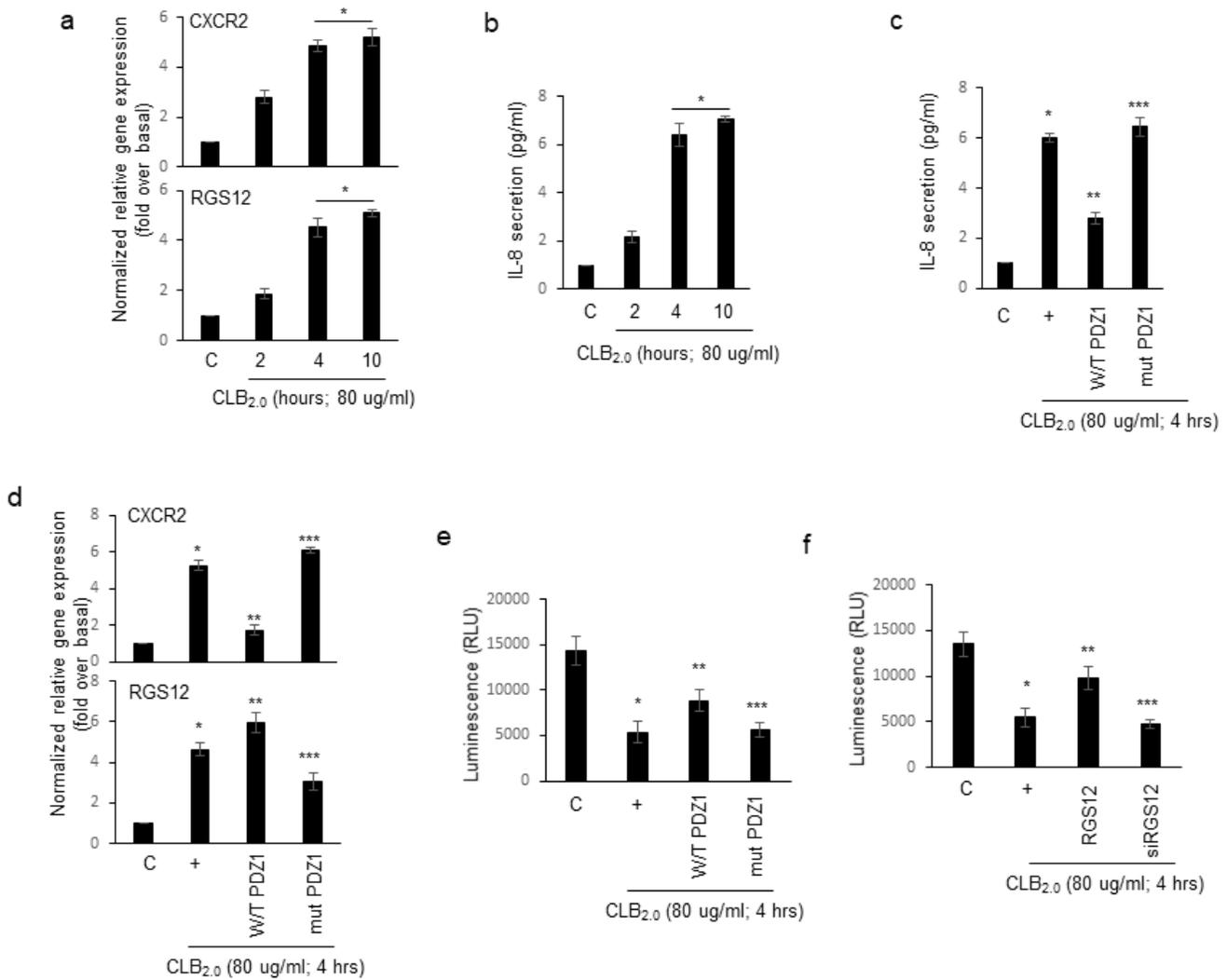


Figure 3

IL-8 Secretion by CLB2.0 activates CXCR2 receptor and RGS12 inhibits CXCR2-induced signaling activated by CLB2.0 in A549 cells. Confluent and quiescent cells were incubated for various durations with CLB2.0, the lysates and medium were harvested, and then analyzed by (a) real-time quantitative RT-PCR and (b) IL-8-specific ELISA. * $p < 0.05$ compared with the control. (c) Cells were treated with consensus PDZ or mutant PDZ peptide for 24 hours and then incubated with CLB2.0 for four hours. An IL-8 was assayed in the medium using ELISA and qRT-PCR was carried out with cell lysates (d). * $p < 0.05$ compared with the control; ** $p < 0.05$ compared with CLB2.0 only; *** $p < 0.05$ compared with CLB2.0 and consensus PDZ peptide treatment. (e) The cells were treated with consensus PDZ or mutant PDZ peptide. After 24 hours, the cells were re-trypsinized, and seeded at 7,000 cells/well into a 96-well plate. The cells were incubated with CLB2.0 for four hours, and a cAMP assay was performed according to the manufacturer's instructions (cAMP-Glo assay; Promega; Madison, WI). * $p < 0.05$ compared with the control; ** $p < 0.05$ compared with CLB2.0 only; *** $p < 0.05$ compared with CLB2.0 and consensus PDZ peptide treatment. (f) The cells were transfected with an RGS12 overexpression construct or siRNA-RGS12, the cells were re-trypsinized, and seeded at 7,000 cells/well into a 96-well plate. The cells were

incubated with CLB2.0 for four hours, and a cAMP assay was performed. * $p < 0.05$ compared with the control; ** $p < 0.05$ compared with CLB2.0 only; *** $p < 0.05$ compared with CLB2.0 and RGS12 treatment. All of data shown are representative of three independent experiments.

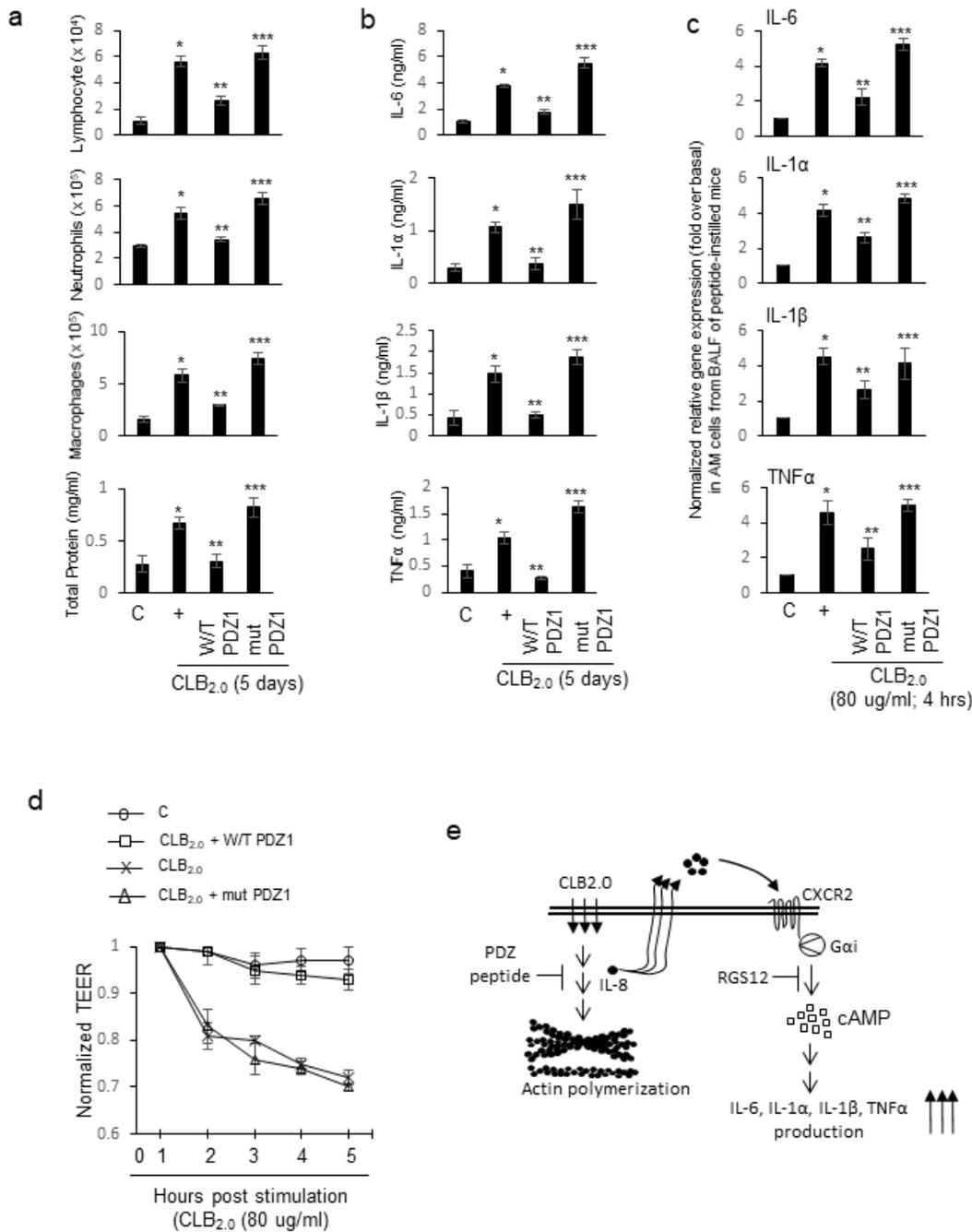


Figure 4

Effect of PDZ peptide on CLB-induced lung inflammatory responses in vivo. (a) Five days after CLB2.0 instillation (30 μ l of 20 mg/kg) into the tracheal lumens of mice that had been injected with either the

consensus PDZ peptide or the mutant peptide (2.0 mg/kg/30 μ l) 24 hours previously. The lymphocytes, neutrophils, alveolar macrophages (AMs), and total protein in the BAL fluid were then assessed. (b) The IL-6, IL-1 α , IL-1 β , and TNF α concentrations in the BAL fluid were measured using specific ELISAs. (c) AMs from the BAL fluid in healthy mice were treated with either the consensus PDZ peptide or the mutant peptide prior to incubation with CLB2.0 for four hours, and then qRT-PCR was performed. (a-c) * $p < 0.05$ compared with saline-treated mice; ** $p < 0.05$ compared with CLB2.0-treated mice; *** $p < 0.05$ compared with CLB2.0- and consensus PDZ peptide-treated mice. (d) The AMs from the BAL fluid in healthy mice were treated with either the consensus PDZ peptide or the mutant peptide prior to incubation with CLB2.0 for various times, and then TEER testing was performed. Error bars represent the SEM of at least three independent experiments. All of data shown are representative of three independent experiments.