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ARTICLE

Increased intracellular Cl^- concentration promotes ongoing inflammation in airway epithelium

Yi-Lin Zhang[1], Peng-Xiao Chen[1], Wei-Jie Guan[2,3], Hong-Mei Guo[1,4], Zhuo-Er Qiu[1], Jia-Wen Xu[1], Yu-Li Luo[1], Chong-Feng Lan[1], Jian-Bang Xu[1], Yuan Hao[5], Ya-Xia Tan[2], Ke-Nan Ye[1], Zhao-Rong Lun[1], Lei Zhao[6], Yun-Xin Zhu[1], Jiehong Huang[1], Wing-Hung Ko[5], Wei-De Zhong[7], Wen-Liang Zhou[1] and Nan-Shan Zhong[2]

Airway epithelial cells harbor the capacity of active Cl^- transepithelial transport and play critical roles in modulating innate immunity. However, whether intracellular Cl^- accumulation contributes to relentless airway inflammation remains largely unclear. This study showed that, in airway epithelial cells, intracellular Cl^- concentration ($[Cl^-]_i$) was increased after *Pseudomonas aeruginosa* lipopolysaccharide (LPS) stimulation via nuclear factor-κB (NF-κB)–phosphodiesterase 4D (PDE4D)–cAMP signaling pathways. Clamping $[Cl^-]_i$ at high levels or prolonged treatment with LPS augmented serum- and glucocorticoid-inducible protein kinase 1 (SGK1) phosphorylation and subsequently triggered NF-κB activation in airway epithelial cells, whereas inhibition of SGK1 abrogated airway inflammation in vitro and in vivo. Furthermore, Cl^-–SGK1 signaling pathway was pronouncedly activated in patients with bronchiectasis, a chronic airway inflammatory disease. Conversely, hydrogen sulfide (H_2S), a sulfhydryl-containing gasotransmitter, confers anti-inflammatory effects through decreasing $[Cl^-]_i$ via activation of cystic fibrosis transmembrane conductance regulator (CFTR). Our study confirms that intracellular Cl^- is a crucial mediator of sustained airway inflammation. Medications that abrogate excessively increased intracellular Cl^- may offer novel targets for the management of airway inflammatory diseases.

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INTRODUCTION

Airway epithelium plays crucial roles in host defense; the dysfunction of which has been implicated in airway inflammatory diseases including cystic fibrosis (CF) and bronchiectasis.[1–3] Airway surface liquid (ASL) is essential for maintaining mucociliary clearance which effectively eliminates pathogen invasion.[4] To maintain the fluid homeostasis in ASL, airway epithelium actively secrets Cl^- and dynamically modulates intracellular Cl^- concentration ($[Cl^-]_i$) via a constellation of Cl^- transport proteins including CF transmembrane conductance regulator (CFTR), a cAMP-dependent Cl^- channel.[5] Additionally, defective Cl^- transport was causally linked to clinical diseases that are characterized by impaired host defense, indicating crucial roles of Cl^- in maintaining innate host defense.[6] Although cations (e.g. Ca^{2+}, Na^+) have been proven to contribute to inflammation,[7,8] the association between intracellular Cl^- and airway innate immunity is still largely unclear. Previous evidence showed that CFTR dysfunction and extracellular administration of sodium chloride (NaCl) elicited nuclear factor-κB (NF-κB) activation in airway epithelial cells.[9–13] Furthermore, changes in $[Cl^-]_i$ modulated the expression of different genes and the secretion of the pro-inflammatory cytokine interleukin-1β (IL-1β) in IB3-1 CF bronchial epithelial cells.[14,15] In light that multiple Cl^--sensing proteins have been documented,[16] we interrogated whether intracellular Cl^- may regulate downstream immunity signaling pathways by activating some Cl^--sensing proteins. In this study, therefore, we sought to investigate whether intracellular Cl^- is implicated in *Pseudomonas aeruginosa* (*P. aeruginosa*) lipopolysaccharide (LPS)-induced inflammation in airway epithelium and identify the underlying mechanism.

RESULTS

P. aeruginosa LPS increased $[Cl^-]_i$ via up-regulation of PDE4D in airway epithelial cells

To verify the association between $[Cl^-]_i$ and airway inflammation, we initially determined the temporal variation of LPS-induced NF-κB activation and changes in $[Cl^-]_i$. Intriguingly, we observed a dual-phase phosphorylation of the inhibitor of κB (IκB) in human airway epithelial BEAS-2B cells at 2–4 and 12–24 h after LPS stimulation (Fig. 1a). $[Cl^-]_i$ was markedly increased from 38.27 ±

[1]School of Life Sciences, Sun Yat-sen University, Guangzhou, China; [2]State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangzhou Institute for Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou, China; [3]Sino-French Hoffmann Institute, Guangzhou Medical University, Guangzhou, China; [4]Biology and Food Engineering Institute, Guangdong University of Education, Guangzhou, China; [5]School of Biomedical Sciences, The Chinese University of Hong Kong, N. T., China, China; [6]Key Laboratory of Protein Modification and Degradation, School of Basic Medical Sciences, Affiliated Cancer Hospital and Institute of Guangzhou Medical University, Guangzhou, China and [7]Department of Urology, Guangdong Key Laboratory of Clinical Molecular Medicine and Diagnostics, Guangzhou First People's Hospital, Guangzhou Medical University, Guangzhou, China
Correspondence: W-L. Zhou (lsszwl@mail.sysu.edu.cn) or N-S. Zhong (nanshan@vip.163.com)
These authors contributed equally: Yi-Lin Zhang, Peng-Xiao Chen, Wei-Jie Guan and Hong-Mei Guo.

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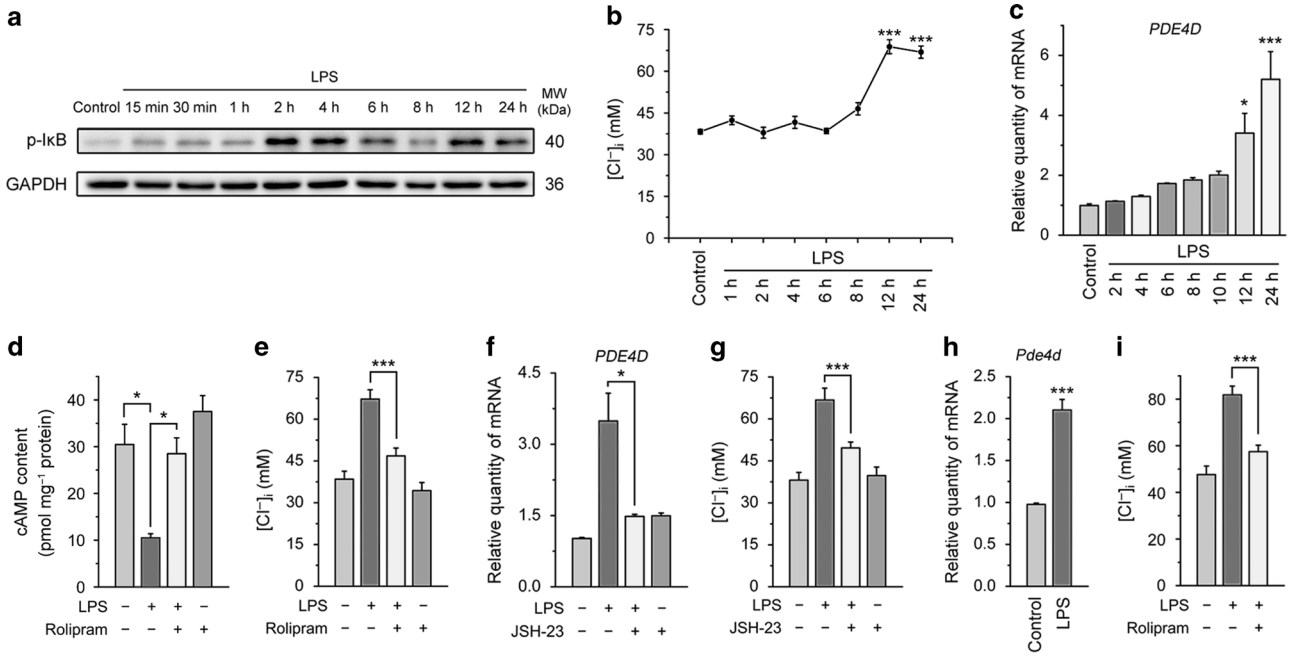

Fig. 1 Lipopolysaccharide (LPS) increased intracellular Cl^- concentration ($[Cl^-]_i$) via up-regulation of phosphodiesterase 4D (PDE4D). **a** Western blot images showing inhibitor of κB (IκB) phosphorylation after LPS (10 μg ml^{-1}) stimulation in BEAS-2B cells. **b** Alteration in $[Cl^-]_i$ after LPS (10 μg ml^{-1}) stimulation in BEAS-2B cells ($n = 26$–40 cells at each time point, ***$P < 0.001$ versus control). **c** PDE4D mRNA expression was measured in LPS-stimulated BEAS-2B cells ($n = 3$ independent experiments, *$P < 0.05$, ***$P < 0.001$ versus control). **d** Effects of rolipram (10 μM) on intracellular cAMP concentration after LPS (10 μg ml^{-1}) stimulation for 12 h in BEAS-2B cells ($n = 3$ independent experiments, *$P < 0.05$). **e** Effect of rolipram (10 μM) on $[Cl^-]_i$ after LPS (10 μg ml^{-1}) stimulation for 12 h in BEAS-2B cells ($n = 9$–13 cells per group, ***$P < 0.001$). **f** Effect of JSH-23 (20 μM) on PDE4D mRNA expression ($n = 3$ independent experiments, *$P < 0.01$) and **g** $[Cl^-]_i$ ($n = 9$–15 cells for each group, ***$P < 0.001$) after LPS (10 μg ml^{-1}) stimulation for 12 h in BEAS-2B cells. **h** PDE4D mRNA expression was measured in lung tissues from mice after intratracheal instillation with LPS (1 μg per gram of body weight) for 24 h ($n = 3$ independent experiments, ***$P < 0.001$ versus control). **i** $[Cl^-]_i$ was measured in airway epithelial cells freshly isolated from LPS-challenged mice, with or without incubation with rolipram (10 μg per gram of body weight) ($n = 8$–10 cells isolated from three mice per group, ***$P < 0.001$). Data are presented as mean ± s.e.m.

0.83 mM at baseline to 68.80 ± 2.52 mM at 12 h (Fig. 1b). To exclude a possible nonspecific effect of LPS due to contamination, effects of the pathogen *P. aeruginosa* were again tested. A similar biphasic phosphorylation of IκB and an increased $[Cl^-]_i$ in BEAS-2B cells were observed (Supplementary Figure S1), indicating that NF-κB activation due to either LPS or *P. aeruginosa* stimulation at the latter phase might be associated with elevated $[Cl^-]_i$.

Because cAMP-activated CFTR plays critical roles in modulating $[Cl^-]_i$, and cyclic nucleotide phosphodiesterase 4 (PDE4), the enzyme that degrades cAMP, reportedly mediated inflammatory responses,[17–19] we interrogated whether PDE4 mediated the LPS-induced increase in $[Cl^-]_i$. First, mRNA expression of PDE4 variants A–D was detected in BEAS-2B cells. PDE4D (Fig. 1c), but not other PDE4 variants (Supplementary Figure S2), was significantly upregulated at 12 h after LPS stimulation, which was parallel to the decrease in intracellular cAMP (Fig. 1d), a finding consistent with increased $[Cl^-]_i$. Similarly, an in vivo study showed that PDE4D mRNA was also upregulated in lung tissues derived from mouse models of LPS-induced airway inflammation (Fig. 1h). Next, we tested whether PDE4D was implicated in LPS-induced elevation in $[Cl^-]_i$. Pretreatment with rolipram, a selective PDE4 inhibitor,[20] reversed the effect of LPS on $[Cl^-]_i$ both in vitro and in vivo (Fig. 1e, i). In light of the temporal variations in $[Cl^-]_i$, we questioned whether LPS induces the initial phase of NF-κB activation which subsequently led to PDE4D up-regulation and the changes in $[Cl^-]_i$. Further studies showed that JSH-23, an inhibitor of NF-κB p65 nuclear translocation,[21] suppressed LPS-induced PDE4D up-regulation (Fig. 1f) and the increase in $[Cl^-]_i$ (Fig. 1g), indicating a positive feedback loop in LPS-induced airway epithelial inflammation, which were similar to the IL-1β-positive feedback loop as described previously.[13,14,22] Collectively, LPS induced up-regulation of PDE4D resulted in increased $[Cl^-]_i$ via activating NF-κB pathways.

Higher $[Cl^-]_i$ elicited LPS-induced airway inflammation via SGK1 activation

Because LPS-induced increase in $[Cl^-]_i$ coincided with the latter phase of NF-κB activation, we hypothesized that higher $[Cl^-]_i$ activated NF-κB via some Cl^--sensitive kinases. Higher extracellular concentrations of NaCl reportedly activated SGK1, a serine–threonine kinase upregulated in airway inflammatory diseases,[23–25] suggesting that SGK1 might be regulated by $[Cl^-]_i$ and could be implicated in airway inflammation. Our in vitro study demonstrated that, compared with Na^+ and K^+, Cl^- selectively augmented SGK1 activity in a concentration-dependent fashion (Fig. 2a). At cellular levels, phosphorylation at threonine 256 locus was mandatory for maximal activation of SGK1.[24] This prompted us to detect phosphorylation at threonine 256 locus of SGK1 at different $[Cl^-]_i$. Airway epithelial cell model with different $[Cl^-]_i$ concentrations was established, showing more pronounced SGK1 and IκB phosphorylation when clamping $[Cl^-]_i$ at a higher level (Fig. 2b, c). Moreover, higher $[Cl^-]_i$ triggered airway epithelial inflammation by augmenting SGK1 activity. GSK-650394, the SGK1 inhibitor,[26] potently inhibited high $[Cl^-]_i$-induced IκB phosphorylation and up-regulation of pro-inflammatory cytokines/chemokines including IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) (Fig. 2c, d).

While higher $[Cl^-]_i$ activated NF-κB in experimental settings, we sought to validate that SGK1 was implicated in LPS-induced airway epithelial inflammation in physiological conditions. As shown in Fig. 3a, Fig. 4a, and Supplementary Figure S3, SGK1 phosphorylation was significantly augmented, whereas SGK1

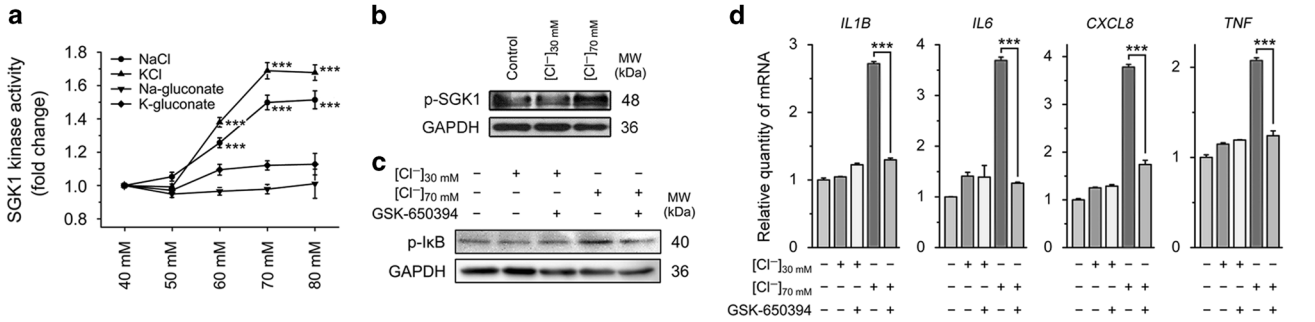

Fig. 2 High intracellular Cl^- concentration ($[Cl^-]_i$) elicited airway epithelial inflammation via serum- and glucocorticoid-inducible protein kinase 1 (SGK1) activation in vitro. **a** Recombinant human SGK1 was incubated with different Cl^- or gluconate solution for 1 h, and SGK1 kinase activity was assessed ($n = 3$ independent experiments, $^{***}P < 0.001$ versus 40 mM group). **b** Western blot images showing SGK1 phosphorylation in airway epithelial cell model with different gradients of $[Cl^-]_i$. **c** Effect of GSK-650394 (1 μM) on inhibitor of κB (IκB) phosphorylation and **d** mRNA expression of pro-inflammatory cytokines ($n = 3$ independent experiments, $^{***}P < 0.001$) in ex vivo cultures of airway epithelial cell model with different gradients of $[Cl^-]_i$. Data are presented as mean ± s.e.m.

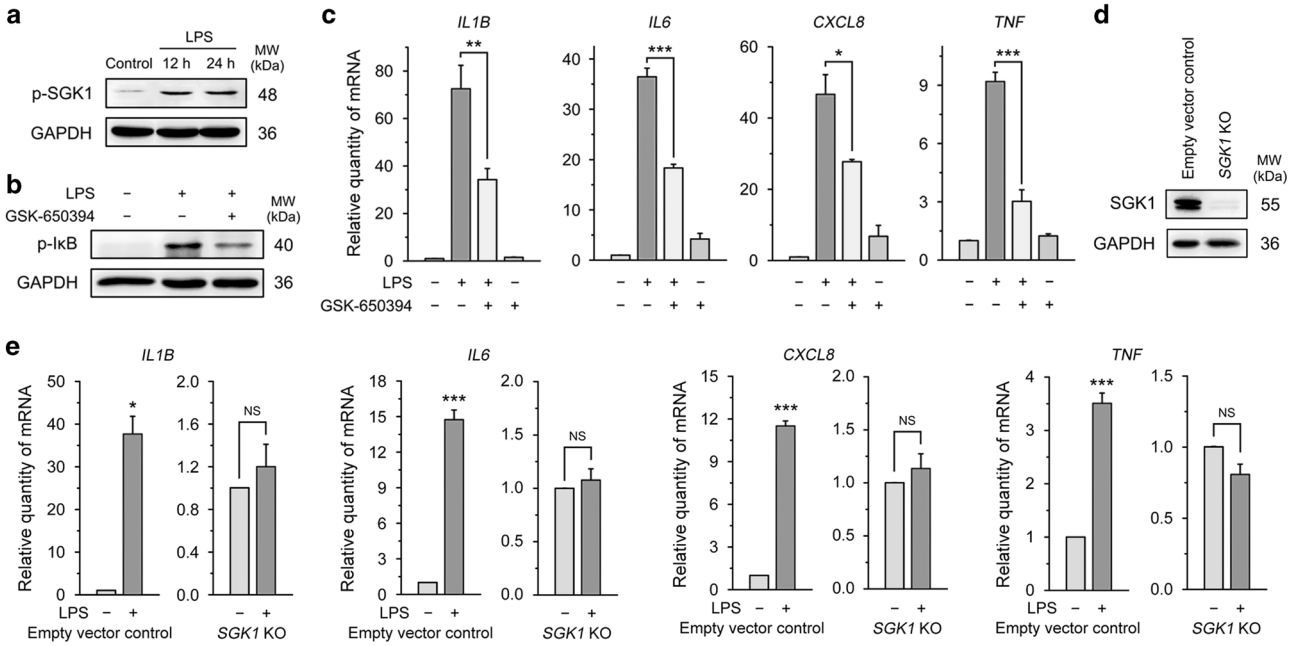

Fig. 3 Prolonged treatment with lipopolysaccharide (LPS) triggered sustained airway epithelial inflammation via serum- and glucocorticoid-inducible protein kinase 1 (SGK1) activation in vitro. **a** Western blot images showing SGK1 phosphorylation after LPS (10 μg ml^{-1}) stimulation in BEAS-2B cells. **b** Effects of GSK-650394 (1 μM) on inhibitor of κB (IκB) phosphorylation and **c** mRNA expression of pro-inflammatory cytokines ($n = 3$ independent experiments, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) in LPS-stimulated BEAS-2B cells. **d** Western blot images showing SGK1 expression after *SGK1* knockout (KO) in BEAS-2B cells. **e** Effects of *SGK1* KO on mRNA expression of pro-inflammatory cytokines in LPS-stimulated BEAS-2B cells ($n = 3$ independent experiments, $^*P < 0.05$, $^{***}P < 0.001$ versus LPS-untreated group. NS, no significant difference was observed). Data are presented as mean ± s.e.m.

mRNA expression was slightly upregulated after LPS stimulation in vitro and in vivo, indicating a regulatory role of SGK1 in NF-κB activation. Reassuringly, pretreatment with GSK-650394 significantly attenuated LPS-induced NF-κB activation and up-regulation of pro-inflammatory cytokines (Fig. 3b, c). Moreover, intraperitoneal pretreatment with GSK-650394 in mice potently suppressed LPS-induced airway inflammation and neutrophil infiltration in bronchoalveolar lavage fluids (Fig. 4b–e). The involvement of SGK1 in LPS-induced NF-κB reactivation was further confirmed in an experimental model of *SGK1* knockout (KO) BEAS-2B cells using CRISPR-Cas9 technology (Fig. 3d). Expression of the pro-inflammatory cytokines/chemokines was significantly tempered compared with the empty vector control, demonstrating that SGK1 was a prerequisite for LPS-induced NF-κB reactivation in airway epithelial cells (Fig. 3e). Taken together, LPS stimulation led to heightened $[Cl^-]_i$ which subsequently activated SGK1, resulting in the ongoing airway inflammatory response.

Heightened $[Cl^-]_i$ and SGK1 phosphorylation levels in bronchiectasis patients

Because in vitro and in vivo studies have verified that high $[Cl^-]_i$ plays crucial roles in eliciting airway inflammation, we sought to validate our hypothesis in patients with chronic airway inflammatory diseases. Bronchiectasis is a chronic debilitating inflammatory disease characterized by chronic cough, sputum production, and/or sometimes hemoptysis.[27] Persistent airway infection (*P. aeruginosa* as the most common pathogen in Guangzhou, China[28]), inflammation and structural damage collectively contribute to the vicious cycle which has rendered bronchiectasis an optimal model for our

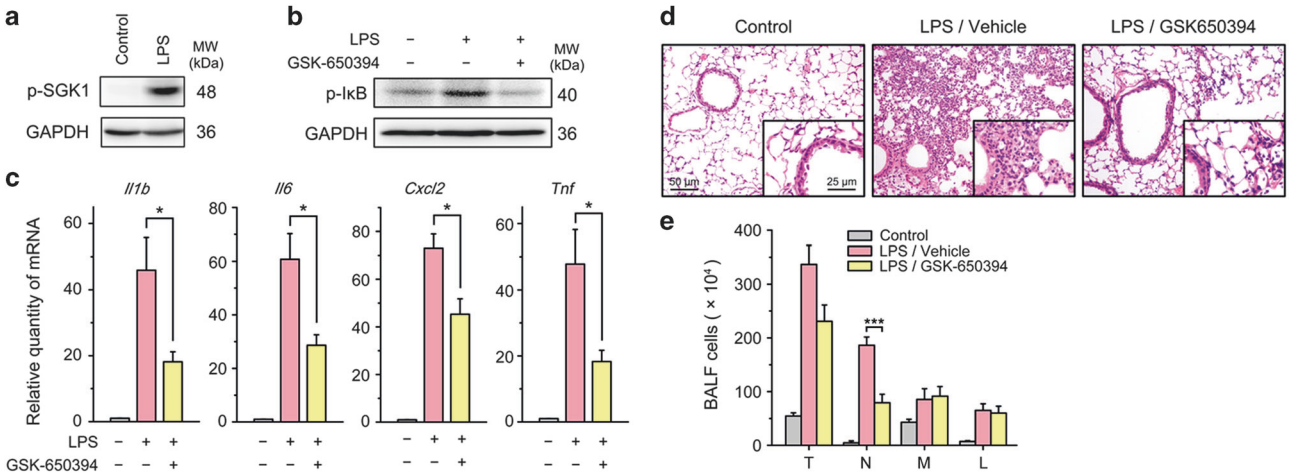

Fig. 4 Serum- and glucocorticoid-inducible protein kinase 1 (SGK1) mediated lipopolysaccharide (LPS) induced airway inflammation in vivo. **a** Western blot images showing SGK1 phosphorylation in lung tissues derived from mice after intratracheal instillation with LPS (1 μg per gram of body weight) for 24 h, with or without incubation with GSK-650394 (1 μg per gram of body weight). **b** Phosphorylation of inhibitor of κB (IκB) and **c** mRNA expression of pro-inflammatory cytokines ($n = 3$ independent experiments, $^*P < 0.05$) was measured in lung tissues from the different group of mice after bronchoalveolar lavage. **d** Hematoxylin and eosin (HE) staining of lung tissues from mice. **e** Cells were collected from lung tissues of mice by performing bronchoalveolar lavage. Bronchoalveolar lavage fluid total and differential cell counts were quantified ($n = 4$ mice per group, $^{***}P < 0.001$). T total cells, N neutrophils, M mononuclears, L lymphocytes. Data are presented as mean ± s.e.m.

investigation.[3,29] We thus sampled bronchial mucosa via bronchoscopy from control subjects and bronchiectasis patients (Supplementary Tables S1–S3). Compared with bronchial mucosae in the least significant bronchiectatic lobes and those from control subjects, significantly augmented IκB phosphorylation was observed in the most significant bronchiectatic lobes (Fig. 5a). Next, we observed substantially increased $[Cl^-]_i$ in epithelial cells isolated from the most, but not the least, significant bronchiectatic lobes (Fig. 5b), where SGK1 phosphorylation was consistently augmented (Fig. 6c). Therefore, elevated $[Cl^-]_i$ and SGK1 activity might have, at least partially, accounted for chronic airway inflammation in bronchiectasis.

H_2S protected against LPS-induced airway inflammation via down-regulation of $[Cl^-]_i$

Next, we abrogated airway epithelial inflammation via down-regulation of $[Cl^-]_i$. Hydrogen sulfide (H_2S), the sulfhydryl-containing gasotransmitter, reportedly mediated Cl^- transportation in various cells.[30–32] Therefore, we determined whether H_2S could influence on $[Cl^-]_i$, thereby verifying its anti-inflammatory effects in airway epithelial cells. Sodium hydrosulfide (NaHS), the H_2S donor, elicited a sustained increase in short-circuit current (I_{sc}) response as a consequence of Cl^- secretion by activating CFTR via adenylate cyclase (AC)–cAMP pathway. By using the patch-clamp technique, CFTRinh-172-sensitive CFTR whole-cell currents were consistently observed in response to NaHS treatment (Supplementary Figure S4).

We further determined whether H_2S could abrogate the increase in $[Cl^-]_i$ and the subsequent NF-κB activation elicited by LPS. Notably, H_2S abolished the increase of $[Cl^-]_i$, decreased phosphorylated IκB expression, and inhibited the up-regulation of pro-inflammatory cytokines in airway epithelial cells upon LPS stimulation in a CFTR-dependent manner (Fig. 6a–d), which was similar to the effects observed after disruption of the IL-1β loop by using IL-1β receptor antagonist in CF bronchial epithelial cells.[15] Consistent with these in vitro findings, intraperitoneal pretreatment with NaHS mitigated the elevation of $[Cl^-]_i$ in freshly isolated airway epithelial cells, suppressed airway inflammation (including neutrophil infiltration), and inhibited NF-κB activation by restoring the function of CFTR in LPS-treated mouse models (Fig. 6e–h).

DISCUSSION

Cl^- is the major anion responsible for transepithelial transportation in airway epithelium, whereas studies on Cl^- mainly focused on its cellular–biological roles such as fluid secretion and cell volume regulation.[16] Numerous pro-inflammatory pathogens and particles, including *P. aeruginosa*, *Toxoplasma gondii*, H5N1 influenza virus hemagglutinin and cigarette smoke extracts, significantly suppressed Cl^- secretion in airway epithelium,[33–36] indicating an intimate link between defective Cl^- transport and airway infection. Unfortunately, whether disequilibrium in $[Cl^-]_i$ occurs after infection in airway epithelial cells has not been fully revealed. In our study, the resting $[Cl^-]_i$ of airway epithelial cells, including BEAS-2B cell line and cells isolated from human or mouse, was around 30–40 mM, which was consistent with previous reports.[37,38] However, after LPS stimulation or *P. aeruginosa* infection, $[Cl^-]_i$ was significantly increased after a certain period. This prompted us to speculate that elevated $[Cl^-]_i$ may be common, but frequently neglected, responses to pathogen infection.

Similar with other ions, elevated $[Cl^-]_i$ serves as an intracellular signal.[14] Our study has, for the first time, shown that kinase activity of SGK1 was augmented in a Cl^--dependent manner. In view that kinases including with-no-lysine kinase 1 (WNK1) and cathepsin C displayed their Cl^--sensing property via mechanisms involving the Cl^- binding-dependent conformational change,[39,40] we postulated that Cl^- may interact with some specific domains of SGK1, thus transforming it into a structurally activated state, although further investigations are needed.

Inflammatory responses are beneficial when delicately balanced, but may cause persistent disease symptoms. The robust infection-independent inflammation was reportedly manifested as an aberrant cytokine network, leading to disproportionate and exaggerated immune responses to pathogen insults.[41] Our results showed that elevated $[Cl^-]_i$ triggered the up-regulation of pro-inflammatory cytokines, suggesting that alteration in $[Cl^-]_i$ may be a cause of both infection-dependent and -independent inflammatory response. A recent study demonstrated that $[Cl^-]_i$ (biphasic response to Cl^- with maximal response at 75 mM) modulated the IL-1β expression and secretion in airway epithelial cells, supporting the regulatory role of intracellular Cl^- in airway inflammation and cytokine network equilibrium.[15] It should be noted that a decrease of $[Cl^-]_i$ reportedly potentiated TNF-α-evoked NF-κB activation in

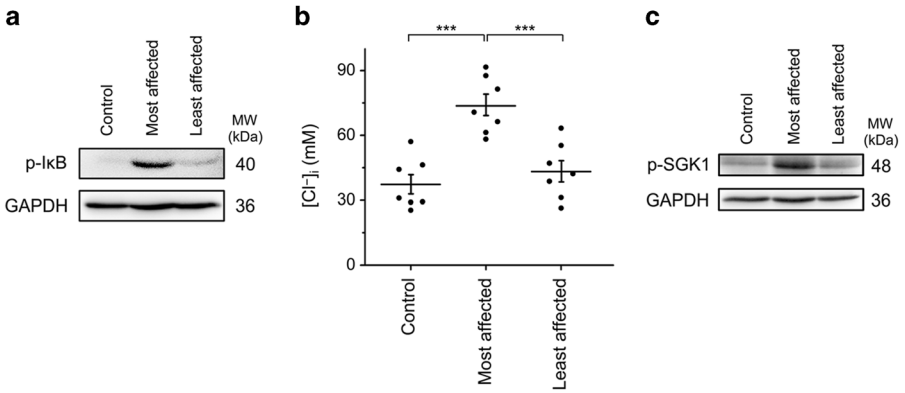

Fig. 5 Elevated intracellular Cl^- concentration ($[Cl^-]_i$) and augmented serum- and glucocorticoid-inducible protein kinase 1 (SGK1) phosphorylation in bronchiectasis patients. **a** Western blot images showing inhibitor of κB (IκB) phosphorylation in bronchial mucosae collected from three control subjects (for etiologic diagnosis of chronic cough and pulmonary nodules) and three bronchiectasis patients (including the least and most significantly affected lobes from the same patient). **b** $[Cl^-]_i$ was measured in cells isolated from bronchial mucosae collected from different subject ($n = 7$ individuals for control subjects or bronchiectasis patients, $^{***}P < 0.001$). Data are presented as mean ± s.e.m. **c** Western blot images showing SGK1 phosphorylation in bronchial mucosa collected from three control subjects and three bronchiectasis patients

endothelial cells.[42] In our study, however, NF-κB activity was enhanced by an increase of $[Cl^-]_i$ in a SGK1-dependent manner. The findings that both high and low $[Cl^-]_i$ could augment NF-κB activation seems contradictory. Nevertheless, previous studies have demonstrated that WNK1 could be activated by low $[Cl^-]_i$.[39] Moreover, WNK1 could act as a positive-regulator of SGK1.[43] Thus, we have postulated that NF-κB activation could be triggered through an indirect activation of SGK1 via WNK1 signaling at low $[Cl^-]_i$, and through direct activation of SGK1 via Cl^--binding mechanism at high $[Cl^-]_i$. The dynamic regulatory role of intracellular Cl^- in inflammatory responses suggested that intracellular Cl^- acts as a second messenger for eliciting chronic airway inflammation.[14,15]

The scientific interests in exploring the mechanisms responsible for chronic inflammation in CF have blossomed for decades. The recent studies have demonstrated that CFTR disorders upregulated $[Cl^-]_i$ and triggered an IL-1β autocrine and positive feedback loop in CF bronchial epithelial cells.[13,15,38] In our in vitro and in vivo study, higher $[Cl^-]_i$ evoked NF-κB signaling via SGK1 activation. The evidence indicated that abnormal Cl^- signaling might contribute to the sustained airway inflammation in CF. The prevalence of CF was reportedly very low in China, whereas bronchiectasis shares similar pathophysiologic manifestations in terms of relentless airway inflammation. Our study has for the first time demonstrated that airway epithelial $[Cl^-]_i$ and SGK1 phosphorylation were consistently more pronounced in bronchiectasis patients compared with control subjects, suggesting that the chronic inflammation in CF and bronchiectasis airways might be associated with elevated $[Cl^-]_i$. Our findings may provide possible research directions for pathological mechanisms of dysregulated inflammation in chronic airway inflammatory diseases such as bronchiectasis.

H_2S reportedly conferred dual regulatory effects on inflammation, depending on the concentrations and duration of treatment.[44] Based on in vitro and in vivo studies, we found that at micromolar concentration, H_2S protected against LPS-induced airway inflammation via activation of CFTR, resulting in lower $[Cl^-]_i$ which approached to resting levels. This may help elucidate the dual regulatory effect of H_2S. On the one hand, reduction of $[Cl^-]_i$ by H_2S to resting level resulted in anti-inflammatory responses. On the other hand, NF-κB activity would be reactivated in case $[Cl^-]_i$ becomes sufficiently low.[42] Importantly, our study has for the first time offered novel therapeutic targets for intervention with PDE4 inhibitors (e.g. rolipram, roflumilast) and thiol-containing compounds (e.g. *N*-acetylcysteine, carbocisteine), which reportedly reduced the risks of exacerbations of chronic obstructive pulmonary disease.[45–48] Our findings have significantly extended the previously recognized pharmacological actions of PDE4 inhibitors which are not solely associated with increased intracellular cAMP levels but also contribute to the regulation of $[Cl^-]_i$ and subsequent changes in airway inflammation. Thiol-containing compounds have been conventionally prescribed in light of their expectorant and anti-oxidative properties. However, our findings on H_2S implied that sulfhydryl-containing mucolytics (e.g. carbocisteine) might mitigate airway inflammation via the $[Cl^-]_i$-regulatory capacity for the treatment of chronic airway diseases such as bronchiectasis.

In summary, *P. aeruginosa* LPS elicited increased $[Cl^-]_i$ via NF-κB-dependent up-regulation of PDE4D in airway epithelial cells. Higher $[Cl^-]_i$ induced NF-κB reactivation by augmenting SGK1 phosphorylation. Conversely, H_2S confers anti-inflammatory property via increasing intracellular cAMP and reducing $[Cl^-]_i$, confirming that Cl^- functions as a critical regulator of NF-κB activity. Disequilibrium of $[Cl^-]_i$ may elicit chronic airway inflammation, offering critical novel insights into the pathophysiological function of Cl^-.

METHODS

Mice and animal experiments

Kunming (KM) mice were purchased from the Experimental Animal Center of Guangdong Province. Mice were anesthetized with chloral hydrate (5 μl 4% (w/v) water solution per gram of the body weight) intraperitoneally. Animal model of LPS-induced airway inflammation was established with a modified procedure as described previously.[49] For the LPS group, anesthetized mice received intratracheal instillation of 1 μg g^{-1} LPS (Sigma Aldrich, St Louis, MO, USA) dissolved in 50 μl phosphate-buffered saline (PBS). Control mice were instilled with an aliquot of PBS. Before LPS administration, mice were intraperitoneally injected with 10 μg g^{-1} rolipram[50] (Sigma Aldrich, St Louis, MO, USA), or 1 μg g^{-1} GSK-650394 (Sigma Aldrich, St Louis, MO, USA), or 56 nM g^{-1} NaHS[51] (Sigma Aldrich, St Louis, MO, USA), or 2 μg g^{-1} CFTRinh-172[52] (Sigma Aldrich, St Louis, MO, USA) dissolved in 56 nM g^{-1} NaHS (volume: 150 μl). All mice were sacrificed at 24 h and the subsequent experiments were performed. All mouse experiments were performed according to the guidelines of the Sun Yat-sen University Animal Use Committee (Guangzhou, China). All procedures were approved by the Sun Yat-sen University Animal Use Committee.

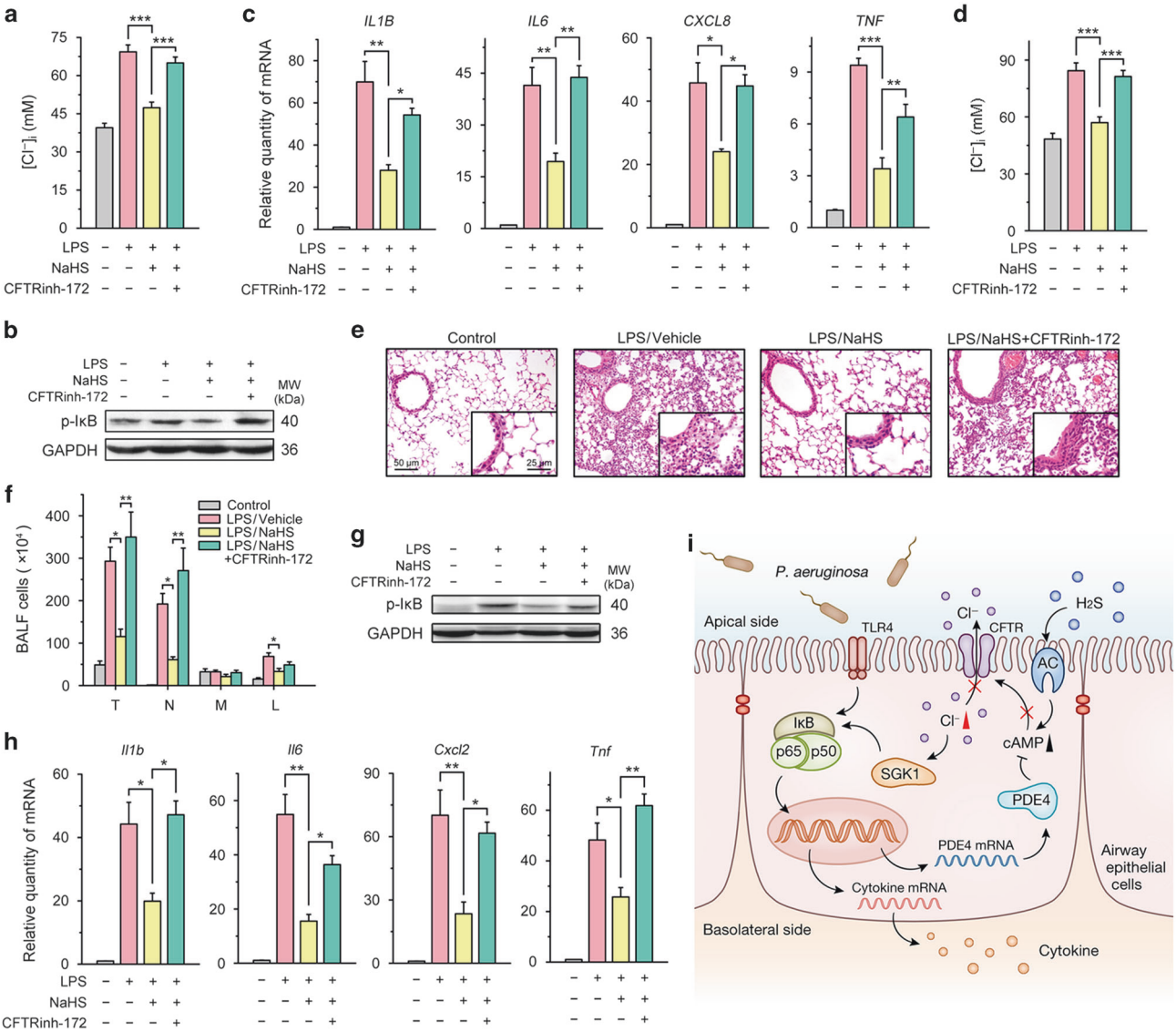

Fig. 6 Hydrogen sulfide (H_2S) suppressed the lipopolysaccharide (LPS)-induced airway epithelial inflammation via cystic fibrosis transmembrane conductance regulator (CFTR) and the schematic diagram of intracellular Cl^- accumulation in eliciting ongoing airway epithelial inflammation. **a** Intracellular Cl^- concentration ($[Cl^-]_i$) was measured in BEAS-2B cells after LPS (10 μg ml^{-1}) stimulation, with or without pretreatment with NaHS (200 μM) and CFTRinh-172 (10 μM) ($n = 9$–17 cells per group, ***$P < 0.001$). **b** Phosphorylation of inhibitor of κB (IκB) and **c** mRNA expression of pro-inflammatory cytokines ($n = 3$ independent experiments, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) was measured in BEAS-2B cells. **d** $[Cl^-]_i$ was measured in airway epithelial cells freshly isolated from mice after intratracheal instillation with LPS (1 μg per gram of body weight) for 24 h, with or without incubation of NaHS (56 nM per gram of body weight) and CFTRinh-172 (2 μg per gram of body weight) ($n = 7$–10 cells isolated from three mice per group, ***$P < 0.001$). **e** Hematoxylin and eosin (HE) staining of lung tissues isolated from different groups of mice. **f** Bronchoalveolar lavage fluid cells were collected from lung tissues of mice and quantified ($n = 4$ mice per group, *$P < 0.05$, **$P < 0.01$). T total cells, N neutrophils, M mononuclears, L lymphocytes. **g** Phosphorylation of IκB and **h** mRNA expression of pro-inflammatory cytokines ($n = 3$ independent experiments, *$P < 0.05$, **$P < 0.01$) was measured in lung tissues from mice after bronchoalveolar lavage. Data are presented as mean ± s.e.m. **i** In airway epithelial cells, *Pseudomonas aeruginosa* LPS stimulation upregulates the expression of phosphodiesterase 4D (PDE4D) via the early-phase nuclear factor-κB (NF-κB) activation and results in a decrease of intracellular cAMP, leading to CFTR dysfunction. Subsequently, intracellular Cl^- is accumulated and reactivates the NF-κB pathway via serum- and glucocorticoid-inducible protein kinase 1 (SGK1) phosphorylation. Contrarily, H_2S harbors anti-inflammatory property via activating adenylate cyclase (AC), thus ameliorating intracellular Cl^- overload

Patients and bronchial mucosal sample collection

Ten control subjects (who underwent bronchoscopy for diagnostic tests of pulmonary nodules or chronic cough) and ten bronchiectasis patients were recruited from the outpatient clinics, The First Affiliated Hospital of Guangzhou Medical University. Bronchial mucosal samples were collected via bronchoscopy, based on the localization assessed with chest high-resolution computed tomography. The extent of bronchiectasis was determined according to modified Reiff score. Biopsy samples were collected from the least (or even an absence of) and most significant bronchiectatic lobes within the same patient for pairwise comparisons. For control subjects, bronchial mucosae in the left or right lower lobes were biopsied. Western blot analysis was performed on samples collected from three randomly selected individuals per group. $[Cl^-]_i$ measurement was performed on seven randomly selected replicate samples. The study was

approved by Ethics Committee of The First Affiliated Hospital of Guangzhou Medical University. All participants gave written informed consent. See Supplementary Tables S1–S3 for details.

Cell culture, treatment with drugs and establishment of airway epithelial cell model with different gradients of $[Cl^-]_i$

BEAS-2B cells were purchased from JENNIO biological technology (Guangzhou, China). BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium, high glucose (Hyclone, Logan, UT, USA) medium with 10% (v/v) fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% (v/v) penicillin−streptomycin (Hyclone, Logan, UT, USA). Before LPS stimulation, BEAS-2B cells were starved of serum for 12 h. Inhibitors including rolipram (10 μM[50]), JSH-23 (20 μM[53]), GSK-650394 (1 μM[54]), and CFTRinh-172 (10 μM[34]) were added to the cells 1 h before LPS stimulation. The inhibition curves of the main inhibitors are provided in Supplementary Figure S5. Cell model with different gradients of $[Cl^-]_i$ was established with a modified procedure as described previously.[14,55] Briefly, human airway epithelial Calu-3 cells (by courtesy from Dr. Wing-Hung Ko at The Chinese University of Hong Kong, HK, China) were incubated with normal HEPES solution containing (in mM) HEPES (20), NaCl (128), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1) and glucose (15) (pH = 7.4) as control, or HEPES buffer containing the K^+–H^+ exchanger nigericin (7 μM) and Cl^-–OH^- exchanger tributyltin (10 μM) with an ionic composition of (in mM) Na^+ (22.6), K^+ (133), Mg^{2+} (1), HCO_3^- (22.6), and glucose (15) (pH 7.4) to equilibrate the $[Cl^-]_i$.[55] Gluconate was used to replace Cl^- at different concentrations (30 or 70 mM) and the osmolarity was adjusted to 340 mmol kg^{-1}.

Construction of SGK1 gene knockout (KO) cells using CRISPR-Cas9

SGK1 KO BEAS-2B cells were constructed by HYY Medical Science (Guangzhou, China) with a modified procedure, as previously described.[56] Briefly, BEAS-2B cells were transduced with the plasmid PXC9-puro, a lenti-CRISPR V2 vector[57] (by courtesy from Feng Zhang at Massachusetts Institute of Technology, Cambridge, Massachusetts, USA, Addgene plasmid # 52961), with either a single guide RNA (sgRNA) targeting SGK1 gene at 5′-GCACATTG-CAGGTACGAAGG-3′ site (*SGK1* KO) or, as a control, a scrambled gRNA 5′-GTAGAGAGCGGCGCGCCTAC-3′ (Empty vector control) with no target by using a lentivirus-based system (HYY Medical Science, Guangzhou, China). Production and transduction of the lentivirus was performed according to the manufacturer's protocol. The positively transfected cells were selected with puromycin (0.5 μg ml^{-1}). Single cells were isolated by serial dilution of positively transfected cells and observed for colony forming. From each clone, half of the cells were used for the subsequent experiments and the other half were used to verify the efficiency and specificity of KO using western blot analysis (Fig. 3d).

Western blot analysis

Western blot was performed with a modified procedure as previously described.[58] Briefly, total protein was extracted from cells or lung tissues by using radio immunoprecipitation assay buffer with phenylmethylsulfonyl fluoride (1 mM) and protease and phosphatase inhibitor cocktail (1:100, BestBio Science, Shanghai, China) according to the manufacturer's instructions. The membrane was blocked in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) albumin from bovine serum for 2 h at room temperature, and then incubated with the following antibodies at 4 °C overnight: anti-phospho-IκB-α (^{32}Ser/^{36}Ser, 1:1000; Cell Signaling Technology, 2859, Beverly, MA, USA), anti-phospho-SGK1 (^{256}Thr, 1:1000; Upstate, 36-002, NY, USA), anti-SGK1 (1:1000; Abcam, ab59337, Cambridge, UK), anti-GAPDH (1:1000; Cell Signaling Technology, 5174, Beverly, MA, USA). The membrane was incubated with an HRP-conjugated secondary antibody (Earthox, E030120-01, Millbrae, CA, USA) for 1 h at room temperature. Respective proteins were visualized by using Immobilon Western Chemiluminescence HRP substrate (Merck Millipore, Darmstadt, Germany) and the bands were digitized using a chemiluminescent imaging system (Tanon, 5200, Shanghai, China).

Intracellular Cl^- measurement

$[Cl^-]_i$ was measured with a modified procedure as previously described.[55,59] Briefly, BEAS-2B cells were cultured on glass coverslips. Mouse airway epithelial cells were scraped from the trachea, and human bronchial mucosal cells were dissociated from bronchial mucosa samples using 0.25% (w/v) trypsin (Gibco, Grand Island, NY, USA) at 37 °C for 5–10 min before attachment onto the coverslips. Cells were subsequently loaded with 5 mM *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE; Invitrogen, E3101, Carlsbad, CA, USA) for 30 min at 37°C. Fluorescence excited at 350 nm was recorded using an imaging system (Olympus, IX83, Tokyo, Japan). To calibrate the change of fluorescence intensity with the change of intracellular $[Cl^-]_i$ that was set to a reference value, the cells were permeabilized by using nigericin (7 μM) and tributyltin (10 μM) in high-K^+ solution.[55] Then the calibration curve could be obtained by fitting fluorescence intensity corresponding to Cl^- concentration and $[Cl^-]_i$ of cells was calculated.

Real-time quantitative reverse transcriptase-polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was performed following the manufacturer's instructions. The total RNA was extracted by RNAprep Pure Kit (Tiangen, Beijing, China). The reverse transcriptase (RT) reaction was performed using Prime-Script RT reagent Kit with gDNA Eraser Kit (Takara, RR047A, Tokyo, Japan). The PCR reaction was performed using SYBR Green (TOYOBO, QPK-201, Tokyo, Japan) on a LightCycler 480 instrument (Roche, Basel, Switzerland). The $2^{-\Delta\Delta Ct}$ method was applied for calibrations and normalization. The relative quantities of mRNAs were normalized using human or mouse hypoxanthine phosphoribosyltransferase 1 (HPRT1) as the endogenous control. The primers used in this study are shown in Supplementary Table S4.

cAMP assay

cAMP in the lysates was quantified with direct cAMP enzyme immunoassay kit (R&D Systems, KGE002B, Minneapolis, MN, USA) following the manufacturer's instructions. Protein concentrations of the lysates were determined using BCA Protein Assay Kit (KWBIO, kw0014, Beijing, China). The concentration of cAMP was normalized to that of protein in the same lysates.

Kinase activity assay

SGK1 kinase activity was measured by an SGK1 kinase assay (Promega, V9671, Madison, WI, USA) according to the manufacturer's instructions. Recombinant human SGK1 enzyme, substrate, and ATP were diluted in kinase buffer containing different concentrations of Cl^- and incubated at room temperature for 1 h. Luminescence was recorded using GloMax 96 Microplate Luminometer Accessory (Promega, Madison, WI, USA).

Histologic assay

Histological examination was performed with a modified procedure as previously described.[60] Briefly, excised lung tissues were fixed in 4% (w/v) buffered paraformaldehyde for 24 h, embedded in paraffin, and sectioned at 4 μm. Sections were stained with hematoxylin and eosin (HE). The HE staining figures were captured by using an upright microscope (Nikon ECLIPSE 50i, Tokyo, Japan).

Bronchoalveolar lavage fluid collection and cell counting

Bronchoalveolar lavage fluid (BALF) and collection and cell counting was performed with a modified procedure as previously described.[49] Mice were euthanized, the lungs were instilled for three times with 0.8 ml ice-cold PBS to collect BALF cells, and smear of BALF cells was prepared and stained with HE. Differential counts were determined for 400 cells.

Statistical analysis

Statistical analysis was conducted using Origin Pro 8.0 (OriginLab Corporation, MA, USA). Data were presented as mean ± s.e.m. The Student's *t*-test (two-tailed) was used to compare the differences between two groups. For three or more groups, data were analyzed with one-way analysis-of-variance followed by Bonferroni for multiple comparisons. $P < 0.05$ was considered statistically significant.

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AUTHOR CONTRIBUTIONS

Y.L.Z., P.X.C., W.J.G., and H.M.G. designed the experiments, performed the experimental work, analyzed the results, and wrote the manuscript. Z.E.Q., J.W.X., Y. L.L., C.F.L., J.B.X., and Y.H. performed part of the experimental work and/or analyzed the results. W.H.K., K.N.Y., Z.R.L., Y.X.T., L.Z., Y.X.Z, and J.H. were involved in manuscript preparation. N.S.Z. and W.D.Z. facilitated the manuscript preparation. W.L.Z. conceptualized the study, designed the experiments, and revised the manuscript.

ADDITIONAL INFORMATION

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REFERENCES

1. Hallstrand, T. S. et al. Airway epithelial regulation of pulmonary immune homeostasis and inflammation. *Clin. Immunol.* **151**, 1–15 (2014).
2. Cohen-Cymberknoh, M., Kerem, E., Ferkol, T. & Elizur, A. Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications. *Thorax* **68**, 1157–1162 (2013).
3. Fuschillo, S., De Felice, A. & Balzano, G. Mucosal inflammation in idiopathic bronchiectasis: cellular and molecular mechanisms. *Eur. Respir. J.* **31**, 396–406 (2008).
4. Whitsett, J. A. & Alenghat, T. Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat. Immunol.* **16**, 27–35 (2015).
5. Hollenhorst, M. I., Richter, K. & Fronius, M. Ion transport by pulmonary epithelia. *J. Biomed. Biotechnol.* **2011**, 174306 (2011).
6. Wang, G. & Nauseef, W. M. Salt, chloride, bleach, and innate host defense. *J. Leukoc. Biol.* **98**, 163–172 (2015).
7. Tauseef, M. et al. TLR4 activation of TRPC6-dependent calcium signaling mediates endotoxin-induced lung vascular permeability and inflammation. *J. Exp. Med.* **209**, 1953–1968 (2012).
8. Roson, M. I. et al. Acute sodium overload produces renal tubulointerstitial inflammation in normal rats. *Kidney Int.* **70**, 1439–1446 (2006).
9. Chan, M. M., Chmura, K. & Chan, E. D. Increased NaCl-induced interleukin-8 production by human bronchial epithelial cells is enhanced by the DeltaF508/W1282X mutation of the cystic fibrosis transmembrane conductance regulator gene. *Cytokine* **33**, 309–316 (2006).
10. Tabary, O. et al. High susceptibility for cystic fibrosis human airway gland cells to produce IL-8 through the I kappa B kinase alpha pathway in response to extracellular NaCl content. *J. Immunol.* **164**, 3377–3384 (2000).
11. Weber, A. J., Soong, G., Bryan, R., Saba, S. & Prince, A. Activation of NF-kappaB in airway epithelial cells is dependent on CFTR trafficking and Cl- channel function. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **281**, L71–L78 (2001).
12. Perez, A. et al. CFTR inhibition mimics the cystic fibrosis inflammatory profile. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **292**, L383–L395 (2007).
13. Clauzure, M. et al. Disruption of interleukin-1beta autocrine signaling rescues complex I activity and improves ROS levels in immortalized epithelial cells with impaired cystic fibrosis transmembrane conductance regulator (CFTR) function. *PLoS ONE* **9**, e99257 (2014).
14. Valdivieso, A. G., Clauzure, M., Massip-Copiz, M. & Santa-Coloma, T. A. The chloride anion acts as a second messenger in mammalian cells—modifying the expression of specific genes. *Cell. Physiol. Biochem.* **38**, 49–64 (2016).
15. Clauzure, M. et al. Intracellular chloride concentration changes modulate IL-1beta expression and secretion in human bronchial epithelial cultured cells. *J. Cell. Biochem.* **118**, 2131–2140 (2017).
16. Duran, C., Thompson, C. H., Xiao, Q. & Hartzell, H. C. Chloride channels: often enigmatic, rarely predictable. *Annu. Rev. Physiol.* **72**, 95–121 (2010).
17. Tarran, R. Regulation of airway surface liquid volume and mucus transport by active ion transport. *Proc. Am. Thorac. Soc.* **1**, 42–46 (2004).
18. Banner, K. H. & Trevethick, M. A. PDE4 inhibition: a novel approach for the treatment of inflammatory bowel disease. *Trends Pharmacol. Sci.* **25**, 430–436 (2004).
19. Michalski, J. M., Golden, G., Ikari, J. & Rennard, S. I. PDE4: a novel target in the treatment of chronic obstructive pulmonary disease. *Clin. Pharmacol. Ther.* **91**, 134–142 (2012).
20. Muller, T., Engels, P. & Fozard, J. R. Subtypes of the type 4 cAMP phosphodiesterases: structure, regulation and selective inhibition. *Trends Pharmacol. Sci.* **17**, 294–298 (1996).
21. Shin, H. M. et al. Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. *FEBS Lett.* **571**, 50–54 (2004).
22. Massip-Copiz, M. M., Clauzure, M., Valdivieso, A. G. & Santa-Coloma, T. A. CFTR impairment upregulates c-Src activity through IL-1beta autocrine signaling. *Arch. Biochem. Biophys.* **616**, 1–12 (2017).
23. Lee, S. M., Lee, Y. J., Yoon, J. J., Kang, D. G. & Lee, H. S. Effect of Poria cocos on hypertonic stress-induced water channel expression and apoptosis in renal collecting duct cells. *J. Ethnopharmacol.* **141**, 368–376 (2012).
24. Lang, F. et al. Pathophysiological significance of the serum- and glucocorticoid-inducible kinase isoforms. *Physiol. Rev.* **86**, 1151–1178 (2006).
25. Waerntges, S. et al. Excessive transcription of the human serum and glucocorticoid dependent kinase hSGK1 in lung fibrosis. *Cell. Physiol. Biochem.* **12**, 135–142 (2002).
26. Sherk, A. B. et al. Development of a small-molecule serum- and glucocorticoid-regulated kinase-1 antagonist and its evaluation as a prostate cancer therapeutic. *Cancer Res.* **68**, 7475–7483 (2008).
27. O'Donnell, A. E. Bronchiectasis. *Chest* **134**, 815–823 (2008).
28. Guan, W. J. et al. Sputum bacteriology in steady-state bronchiectasis in Guangzhou, China. *Int. J. Tuberc. Lung Dis.* **19**, 610–619 (2015).
29. Flight, W. G. & Jones, A. M. Cystic fibrosis, primary ciliary dyskinesia and non-cystic fibrosis bronchiectasis: update 2008-11. *Thorax* **67**, 645–649 (2012).
30. Njie-Mbye, Y. F., Opere, C. A., Chitnis, M. & Ohia, S. E. Hydrogen sulfide: role in ion channel and transporter modulation in the eye. *Front Physiol.* **3**, 295 (2012).
31. Tan, B. H., Wong, P. T. & Bian, J. S. Hydrogen sulfide: a novel signaling molecule in the central nervous system. *Neurochem. Int.* **56**, 3–10 (2010).
32. Hennig, B. & Diener, M. Actions of hydrogen sulphide on ion transport across rat distal colon. *Br. J. Pharmacol.* **158**, 1263–1275 (2009).
33. Rubino, R. et al. Pseudomonas aeruginosa reduces the expression of CFTR via post-translational modification of NHERF1. *Pflug. Arch.* **466**, 2269–2278 (2014).

34. Guo, H. M. et al. Infection by Toxoplasma gondii, a severe parasite in neonates and AIDS patients, causes impaired anion secretion in airway epithelia. *Proc. Natl. Acad. Sci. USA* **112**, 4435–4440 (2015).
35. Cao, K. et al. H5N1 virus hemagglutinin inhibition of cAMP-dependent CFTR via TLR4-mediated Janus tyrosine kinase 3 activation exacerbates lung inflammation. *Mol. Med.* **21**, 134–142 (2015).
36. Clunes, L. A. et al. Cigarette smoke exposure induces CFTR internalization and insolubility, leading to airway surface liquid dehydration. *FASEB J.* **26**, 533–545 (2012).
37. Treharne, K. J., Crawford, R. M. & Mehta, A. CFTR, chloride concentration and cell volume: could mammalian protein histidine phosphorylation play a latent role? *Exp. Physiol.* **91**, 131–139 (2006).
38. Valdivieso, A. G., Mori, C., Clauzure, M., Massip-Copiz, M. & Santa-Coloma, T. A. CFTR modulates RPS27 gene expression using chloride anion as signaling effector. *Arch. Biochem. Biophys.* **633**, 103–109 (2017).
39. Piala, A. T. et al. Chloride sensing by WNK1 involves inhibition of autophosphorylation. *Sci. Signal.* **7**, ra41 (2014).
40. Cigic, B. & Pain, R. H. Location of the binding site for chloride ion activation of cathepsin C. *Eur. J. Biochem.* **264**, 944–951 (1999).
41. Aldallal, N. et al. Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. *Am. J. Respir. Crit. Care. Med.* **166**, 1248–1256 (2002).
42. Yang, H. et al. Decrease of intracellular chloride concentration promotes endothelial cell inflammation by activating nuclear factor-kappaB pathway. *Hypertension* **60**, 1287–1293 (2012).
43. Heise, C. J. et al. Serum and glucocorticoid-induced kinase (SGK) 1 and the epithelial sodium channel are regulated by multiple with no lysine (WNK) family members. *J. Biol. Chem.* **285**, 25161–25167 (2010).
44. Szabo, C. Hydrogen sulphide and its therapeutic potential. *Nat. Rev. Drug Discov.* **6**, 917–935 (2007).
45. Fabbri, L. M. et al. Roflumilast in moderate-to-severe chronic obstructive pulmonary disease treated with longacting bronchodilators: two randomised clinical trials. *Lancet* **374**, 695–703 (2009).
46. Varelogianni, G., Oliynyk, I., Roomans, G. M. & Johannesson, M. The effect of N-acetylcysteine on chloride efflux from airway epithelial cells. *Cell Biol. Int.* **34**, 245–252 (2010).
47. Zheng, J. P. et al. Effect of carbocisteine on acute exacerbation of chronic obstructive pulmonary disease (PEACE Study): a randomised placebo-controlled study. *Lancet* **371**, 2013–2018 (2008).
48. Zheng, J. P. et al Twice daily N-acetylcysteine 600 mg for exacerbations of chronic obstructive pulmonary disease (PANTHEON): a randomised, double-blind placebo-controlled trial. *Lancet Respir. Med.* **2**, 187–194 (2014).
49. Allen I. C. The utilization of oropharyngeal intratracheal PAMP administration and bronchoalveolar lavage to evaluate the host immune response in mice. *J. Vis. Exp.* **86**, e51391 (2014).
50. Komatsu, K. et al. Inhibition of PDE4B suppresses inflammation by increasing expression of the deubiquitinase CYLD. *Nat. Commun.* **4**, 1684 (2013).
51. Chen, Z. F. et al. Hydrogen sulfide regulates vascular endoplasmic reticulum stress in apolipoprotein E knockout mice. *Chin. Med. J. (Engl.)* **124**, 3460–3467 (2011).
52. Salinas, D. B. et al. CFTR involvement in nasal potential differences in mice and pigs studied using a thiazolidinone CFTR inhibitor. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L936–L943 (2004).
53. Tamminen, J. A., Myllarniemi, M., Hyytiainen, M., Keski-Oja, J. & Koli, K. Asbestos exposure induces alveolar epithelial cell plasticity through MAPK/Erk signaling. *J. Cell. Biochem.* **113**, 2234–2247 (2012).
54. Isikbay, M. et al. Glucocorticoid receptor activity contributes to resistance to androgen-targeted therapy in prostate cancer. *Horm. Cancer* **5**, 72–89 (2014).
55. Zhang, L. L., Pathak, H. R., Coulter, D. A., Freed, M. A. & Vardi, N. Shift of intracellular chloride concentration in ganglion and amacrine cells of developing mouse retina. *J. Neurophysiol.* **95**, 2404–2416 (2006).
56. Zheng, Y. et al. ARRDC3 inhibits the progression of human prostate cancer through ARRDC3-ITGbeta4 pathway. *Curr. Mol. Med.* **17**, 221–229 (2017).
57. Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat. Methods* **11**, 783–784 (2014).
58. Zhang, L. et al. Paeonol inhibits B16F10 melanoma metastasis in vitro and in vivo via disrupting proinflammatory cytokines-mediated NF-kappaB and STAT3 pathways. *IUBMB Life* **67**, 778–788 (2015).
59. Guo, J. H. et al. Glucose-induced electrical activities and insulin secretion in pancreatic islet beta-cells are modulated by CFTR. *Nat. Commun.* **5**, 4420 (2014).
60. Dong, J. et al. Dynamically regulated CFTR expression and its functional role in cutaneous wound healing. *J. Cell. Physiol.* **230**, 2049–2058 (2015).