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Anti-neutrophilic inflammatory activity of ASP3258, a novel phosphodiesterase type 4 inhibitor

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ABSTRACT

Neutrophil-dominant pulmonary inflammation is an important feature of chronic obstructive pulmonary disease (COPD). Here, we evaluated the *in vitro* and *in vivo* anti-neutrophilic inflammatory activities of ASP3258, a novel, orally active, and selective phosphodiesterase (PDE) 4 inhibitor with anti-inflammatory potency comparable to that of second-generation compound roflumilast but with lower emetic activity *in vivo*. In *in vitro* experiments using human peripheral blood neutrophils, PDE4 inhibitors ASP3258, cilomilast, and roflumilast inhibited fMLP-induced superoxide production in a concentration-dependent manner with IC50 values of 5.0, 96, and 4.7 nM, respectively. ASP3258, cilomilast, and roflumilast also attenuated fMLP-induced neutrophil chemotaxis in a concentration-dependent manner with IC30 values of 18, 270, and 9.7 nM, respectively. In contrast, the glucocorticoid prednisolone inhibited neither superoxide production nor chemotaxis up to 1 μM. In a rat model of lipopolysaccharide (LPS)-induced lung inflammation, orally administered ASP3258, cilomilast, roflumilast, and prednisolone (at 10 or 30 mg/kg) dose-dependently attenuated pulmonary accumulation of neutrophils. The inhibitory effect of ASP3258 was more potent than cilomilast and almost the same as roflumilast and prednisolone. Treatment with ASP3258 inhibited the elevation of TNF-α in the bronchoalveolar lavage fluid following LPS instillation. Histological examination revealed significant inhibition of neutrophil and macrophage infiltration into alveoli by ASP3258. Overall, these findings suggest that ASP3258 has therapeutic potential for treating neutrophilic inflammation such as COPD, partly through direct inhibition of neutrophil activation as well as possibly through inhibition of the TNF-α-mediated pathway.

1. Introduction

Neutrophils, key players in the innate immune defense against infectious diseases, are rapidly recruited to sites of inflammation, killing invading bacteria and certain fungal species through phagocytosis and production of bactericidal factors, including oxygen species and preformed granular enzymes. However, the highly destructive capacity of these cells may also damage healthy tissue in inflammatory diseases including chronic obstructive pulmonary disease (COPD) [1–3]. Glucocorticoids are currently the most effective agent in the treatment of inflammatory diseases such as asthma but are largely ineffective in attenuating inflammation in COPD [4,5]. This observation suggests that neutrophils may be less sensitive to glucocorticoids than other immune cells such as eosinophils and T lymphocytes [6], thus highlighting the importance of developing new drugs that can regulate neutrophil function.

Phosphodiesterase (PDE) 4 inhibitor, an agent with a broad spectrum of anti-inflammatory activity, is one possible candidate. PDE4 is the main cAMP-metabolizing enzyme in inflammatory cells, and its inhibition suppresses the recruitment and activation of these cells, including neutrophils [7,8]. Previous clinical studies have found that second-generation PDE4 inhibitors cilomilast and roflumilast significantly improve lung function and reduce the frequency of exacerbation in patients with COPD [9–12], leading to the recent European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) approval of roflumilast for use in treating COPD [13]. Although roflumilast and cilomilast have improved safety margins over the prototype rolipram with respect to class-specific side effects such as nausea and diarrhea, their therapeutic use remains limited likely due to these adverse effects [9,12].

We recently discovered the novel, orally active PDE4 inhibitor ASP3258 which has a wider therapeutic window than second-generation PDE4 inhibitors cilomilast and roflumilast [14]. Here, we investigated the direct effect of ASP3258 on human peripheral blood neutrophil functions such as superoxide production and chemotaxis compared with that of clinically advanced PDE4 inhibitors roflumilast and cilomilast and the glucocorticoid prednisolone.

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We also investigated the effects of these compounds on lipopolysaccharide (LPS)-induced lung injury in rats, which is a neutrophil-dominant model of inflammation [15–17].

2. Materials and methods

2.1. Animals

Male Wistar rats were purchased from Charles River Japan (Kanagawa, Japan), housed in ordinary animal cages with food and water available *ad libitum*. Five- to seven-week-old animals were used in the studies. All experiments were performed in accordance with the regulations of the corporate Animal Ethical Committee of Astellas Pharma Inc.

2.2. Chemicals

ASP3258, cilomilast, and roflumilast were synthesized at Astellas Pharma Inc. (Tsukuba, Japan), and prednisolone was purchased from Nacalai Tesque (Kyoto, Japan). LPS (*Escherichia coli* serotype 0127:B8; Difco, Detroit, MI, USA), heparin (Shimizu Pharmaceutical Co., Ltd., Shizuoka, Japan) and methylcellulose (Shin-Etsu Chemical Co., Tokyo, Japan) were purchased commercially. Gelatin, bovine serum albumin (BSA), cytochrome c, superoxide dismutase (SOD), and N-formyl-Met-Leu-Phe (fMLP) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Isolation of human neutrophils

Human peripheral blood was drawn from healthy adult donors with a heparinized syringe, after which neutrophils were isolated by a density gradient technique using Mono-Poly Resolving Medium (DS Pharma Biomedical Co., Ltd., Osaka, Japan) according to the manufacturer's directions. Isolated neutrophils were suspended in Hanks' buffered saline solution (HBSS) containing 20 mM HEPES (pH 7.4) and 0.1% gelatin for superoxide production assay or in RPMI 1640 medium containing 1% BSA for chemotaxis assay.

2.4. Superoxide production assay

Neutrophil superoxide production was determined by quantitating SOD-inhibitable reduction of cytochrome c in response to fMLP [18]. Neutrophils (2×10^5 cells/100 μL) suspended in HBSS containing 20 mM HEPES (pH 7.4) and 0.1% gelatin were preincubated with test compounds (20 μL) at 37 °C for 30 min in a 96-well plate. The reaction was initiated by adding 80 μL of 750 nM fMLP and 0.375 mM cytochrome c. The absorbance at 550 nm was then measured at intervals of 1 min for 60 min using a microplate autoreader with the plate maintained at 37 °C. The area under the absorbance–time curve was used as an indication of the superoxide production. Negative control samples were given 20 μg/mL SOD in the presence of fMLP.

2.5. Chemotaxis assay

Neutrophil chemotaxis assays were performed using 96-well chemotaxis chambers with 3-μm pore polyvinylpyrrolidone-free polycarbonate filters (Neuro Probe, Gaithersburg, MD, USA). Cells suspended at 1×10^6 cells/mL in RPMI 1640 medium containing 1% BSA were preincubated at 37 °C for 30 min with various concentrations of test compounds and then added to the upper wells (200 μL) without removing the compounds from the medium. The chemoattractant fMLP (100 nM, 29 μL) was added to the lower wells. After 1 h incubation at 37 °C, the filter was stained with Diff-Quik and observed via light microscopy to count the number of cells that migrated through the filter in three randomly selected fields per well.

2.6. LPS-induced pulmonary inflammation in rats

Rats were anesthetized with a ketamine–xylazine mixture (39 mg/kg ketamine and 4.3 mg/kg xylazine, i.p.) and then intratracheally instilled with 200 μL of LPS solution (10 μg/mL). Saline-instilled rats were used in the saline group. At 2 (for BAL cell analysis) or 24 h (for BAL cytokine/chemokine analysis) after LPS instillation, animals were sacrificed under pentobarbital anesthesia (50 mg/kg, i.p.), after which their tracheas were cannulated. The lungs were lavaged with 2 mL of ice-cold saline containing 1 U/mL heparin five times (for BAL cell analysis) or twice (for BAL cytokine/chemokine analysis) via the cannula. The BAL fluid was centrifuged at $400\times g$ for 10 min at 4 °C. The resultant cell pellet was resuspended in 0.5 mL of ice-cold heparinized saline for measurement of the total cell count, and the supernatant was stored at −80 °C until use. The total number of leukocytes in the BAL fluid was counted using an automated cell counter (Celltac-α; Nihon Kohden, Tokyo, Japan), and differential cell count was performed using a cytospin preparation stained with Diff-Quik (Sysmex International Reagent Corp., Kobe, Japan). A minimum of 300 cells were identified and differentiated as mononuclear cells, neutrophils, or eosinophils using the standard morphological criteria. TNF-α and cytokine-induced neutrophil chemoattractant (CINC)-1 concentrations in the BAL fluid were measured by ELISA (Rat TNF-α ELISA System; GE Healthcare UK Ltd., Buckinghamshire, UK, and rat GRO/CINC-1 assay kit; IBL Co., Ltd., Takasaki, Japan). All tested compounds were suspended in 0.5% (w/v) methylcellulose solution and orally administered at 3 mL/kg 1 h prior to LPS instillation. The saline and control groups were treated with vehicle (0.5% methylcellulose).

2.7. Histological evaluation

To avoid inflicting possible traumatic damage on BAL, histological assessment of the lung tissue was performed in separate animals. Animals were sacrificed 24 h after LPS instillation, after which the lungs were removed and fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 2-μm sections, and stained with hematoxylin and eosin. The lung sections were analyzed in a blinded fashion, and the extent of pathological changes, including infiltration of neutrophils and macrophages into the bronchioles or the alveoli, was scored as follows: 0, none; 1, slight; 2, mild; 3, moderate; and 4, severe.

2.8. Statistical analysis

All statistical analyses were conducted using the SAS system (SAS Institute Inc., Cary, NC, USA). Data were expressed as means ± S.E. The statistical significance of differences between groups was determined using Student's t-test, Dunnett's multiple range test, or Wilcoxon rank sum test. Values of $P<0.05$ were considered significant.

3. Results

3.1. Effects of PDE4 inhibitors ASP3258, cilomilast, and roflumilast, and the glucocorticoid prednisolone on superoxide production and chemotaxis in human peripheral blood neutrophils

We performed superoxide production and chemotaxis assays using human peripheral blood neutrophils to evaluate the ability of PDE4 inhibitors ASP3258, cilomilast, and roflumilast, and the glucocorticoid prednisolone to directly downregulate neutrophil function. fMLP (300 nM) stimulated significant superoxide production from human neutrophils but this was significantly suppressed by PDE4 inhibitors ASP3258, cilomilast, and roflumilast in a concentration-dependent manner with respective IC50 values of 5.0, 96, and

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4.7 nM. In contrast, prednisolone did not inhibit fMLP-induced superoxide production even at concentrations up to 1000 nM (Fig. 1). In the chemotaxis assay, pretreatment with ASP3258, cilomilast, or roflumilast significantly and concentration-dependently inhibited human neutrophil chemotaxis toward fMLP (100 nM) with maximum inhibition at approximately 60% and respective IC30 values of 18, 270, and 9.7 nM. As in the case of superoxide production, prednisolone did not inhibit chemotaxis at concentrations up to 1000 nM (Fig. 2).

3.2. Effects of ASP3258 and reference compounds on neutrophilic pulmonary inflammation induced by LPS instillation in rats

Intratracheally instilled LPS (10 μg/mL, 200 μL/animal) caused a significant increase in leukocyte counts in the BAL fluid at 24 h after instillation. Differential cell count analysis revealed that the increased cells were comprised almost entirely of neutrophils (data not shown). Orally administered ASP3258, cilomilast, and roflumilast, as well as prednisolone (at 10 or 30 mg/kg) dose-dependently inhibited the increase in neutrophils (Fig. 3). The inhibitory effect of ASP3258 was more potent than that of cilomilast but similar to those of roflumilast and prednisolone.

3.3. Effects of ASP3258 and prednisolone on elevated TNF-α and CINC-1 levels in BAL fluid induced by LPS instillation in rats

We next examined the BAL fluid levels of TNF-α, a representative proinflammatory cytokine in COPD [19], and CINC-1, a rat homologue of human neutrophil chemoattractant GRO-α [20,21]. Intratracheal injection of LPS induced significant increases in TNF-α and CINC-1 levels in the BAL fluid 2 h post injection. Orally administered ASP3258 (10 and 30 mg/kg) dose-dependently inhibited these increases in levels of TNF-α, but not CINC-1 (Fig. 4), an effect similar to that observed on prednisolone administration (10 and 30 mg/kg).

3.4. Effect of ASP3258 on intratracheally instilled LPS-induced histological changes in the lung

To further characterize the inhibitory effect of ASP3258 in the LPS-induced lung injury model, we examined the lung tissues histologically. In the lung sections, marked neutrophilic infiltration of the small airways and alveoli was observed in the LPS-exposed control group compared with the saline group. In contrast to the results obtained on BAL cell analysis, an accumulation of macrophages in the alveoli was also observed in the LPS-instilled control group. Treatment with ASP3258 (30 mg/kg, p.o.) significantly reduced the histological scores with respect to the accumulation of neutrophils and macrophages in the alveoli (Figs. 5 and 6).

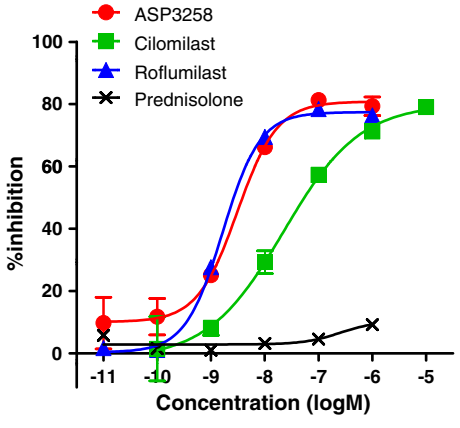

Fig. 1. Effects of ASP3258, cilomilast, roflumilast, and prednisolone on fMLP-induced superoxide production in human neutrophils. Neutrophils were preincubated with test compounds at 37 °C for 30 min and then stimulated with 300 nM fMLP at 37 °C for 60 min. Superoxide production was measured by cytochrome c reduction assay. The area under the absorbance (OD 550 nm)–time curve was used as an indication of superoxide production. Data are expressed as the mean ± S.E. of experiments performed in triplicate. Representative data from three separate experiments with similar results are shown.

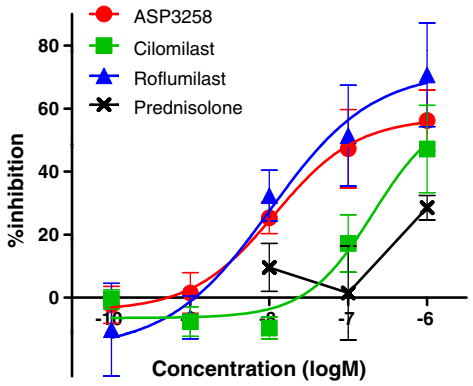

Fig. 2. Effects of ASP3258, cilomilast, roflumilast, and prednisolone on fMLP-induced human neutrophil chemotaxis. Neutrophils were pretreated with test compounds at 37 °C for 30 min. The chemotactic activity of the pretreated neutrophils toward fMLP (100 nM) was then determined using the Boyden chamber method. Data are expressed as the mean ± S.E. of three independent experiments.

4. Discussion

Asthma and COPD are both characterized by pulmonary inflammation and airflow limitation; however, while inhaled glucocorticoids are highly effective in treating asthma, they are largely ineffective against COPD [4,5]. This difference in efficacy is believed to be partly due to the difference in glucocorticoid responsiveness of inflammatory cells; neutrophils that are dominant in COPD may respond less to glucocorticoids than asthma-related cell types such as eosinophils and T lymphocytes [6]. Therefore, the development of drugs that regulate neutrophil functions may be beneficial for the treatment of COPD. In the present study, we demonstrated that the novel, orally active PDE4 inhibitor ASP3258 directly suppresses the function of human neutrophils as well as limits lung injury in a LPS-induced, neutrophil-dominant, pulmonary inflammation model in rats.

In vitro, PDE4 inhibitors ASP3258, cilomilast, or roflumilast all significantly and concentration-dependently suppressed fMLP-induced superoxide production and chemotaxis of human peripheral blood neutrophils, in contrast to prednisolone. These results suggest that

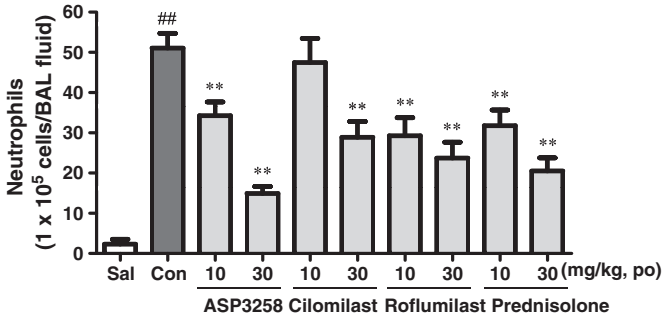

Fig. 3. Effects of ASP3258, cilomilast, roflumilast, and prednisolone on LPS-induced pulmonary neutrophil accumulation in rats. Rats were anesthetized and then instilled intratracheally with LPS (10 μg/mL, 200 μL/animal) or saline. BAL was performed 24 h after instillation. Test compounds (10 or 30 mg/kg) or vehicle were orally administered 1 h prior to the instillation. Data are expressed as the mean ± S.E. of 9 or 10 animals. $^{\#\#}P<0.01$, significantly different from the saline group (Student's t-test). $^{**}P<0.01$, significantly different from the control group (Dunnett's multiple range test).

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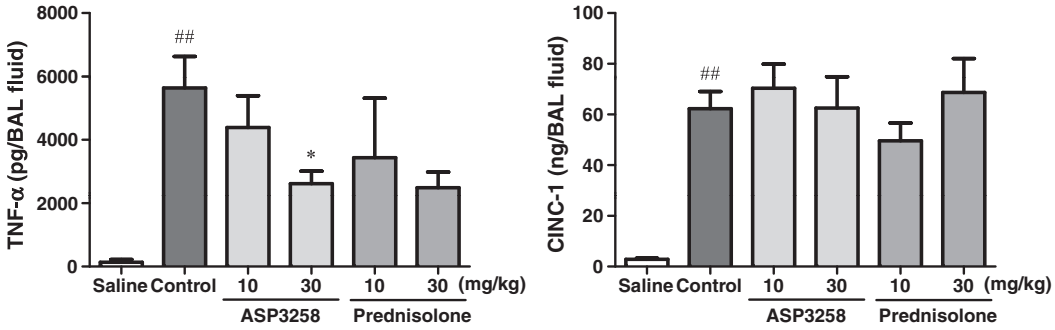

Fig. 4. Effects of ASP3258 and prednisolone on TNF-α and CINC-1 levels in BAL fluids of rats exposed to LPS. Rats were anesthetized and then instilled intratracheally with LPS (10 μg/mL, 200 μL/animal) or saline. BAL was performed 2 h after instillation. Concentrations of TNF-α and CINC-1 in the supernatant of BAL fluid were detected by ELISA. Test compounds (10 or 30 mg/kg) or vehicle were orally administered 1 h prior to the instillation. Data are expressed as the mean ± S.E. of 6 or 7 animals. $^{\#\#}P<0.01$, significantly different from the saline group (Student's t-test). $^{*}P<0.05$, significantly different from the control group (Dunnett's multiple range test).

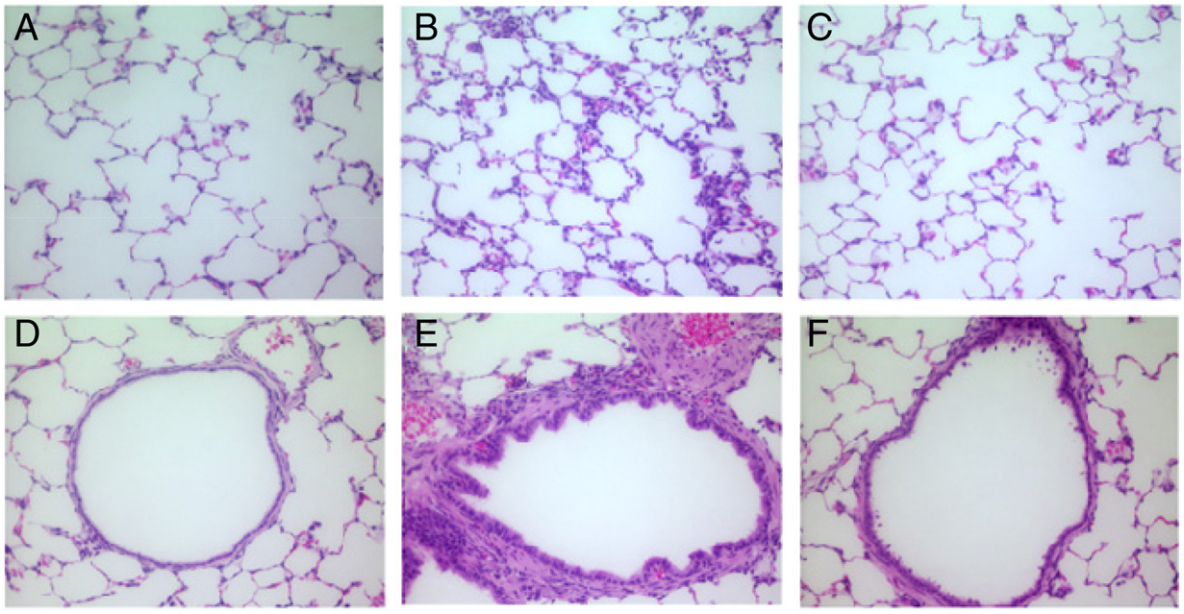

Fig. 5. Representative micrographs of lung tissue stained with hematoxylin and eosin. Lung sections from saline-instilled (A and D), LPS-instilled (B and E), and LPS-instilled and ASP3258 (30 mg/kg)-treated (C and F) animals are shown.

human neutrophils are sensitive to PDE4 inhibitors but not to glucocorticoids, at least in part, possibly explaining the lack of efficacy of inhaled glucocorticoids in COPD.

In contrast to the *in vitro* findings, both the PDE4 inhibitors and prednisolone inhibited pulmonary neutrophil accumulation *in vivo* in the LPS-induced lung injury model. To further examine the *in vivo* inhibitory effects of the PDE4 inhibitors and glucocorticoid in this model, we analyzed the cytokine/chemokine levels in the BAL fluid and found that both ASP3258 and prednisolone attenuated increases in levels of TNF-α but not CINC-1 following LPS instillation.

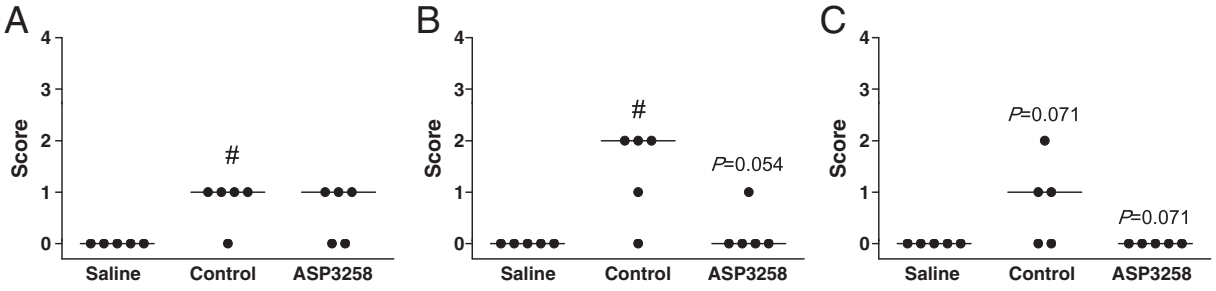

Fig. 6. Quantitative assessment of histological scores of the lungs from saline-, control-, and ASP3258 (30 mg/kg)-treated rats. We scored histological changes observed in the lung, namely, A) infiltration of neutrophils into the bronchioles, B) infiltration of neutrophils into the alveoli, and C) infiltration of macrophages into the alveoli. Each circle represents the data point of one animal. Horizontal lines indicate the medians. $^{\#}P<0.05$, significantly different from the saline group (Wilcoxon rank sum test). $^{*}P<0.05$, significantly different from the control group (Wilcoxon rank sum test).

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TNF-α mediates the trafficking of neutrophils to airway epithelial cells and vascular endothelial cells through the upregulation of adhesion molecules, such as intracellular adhesion molecule (ICAM)-1 [22,23], and exerts a direct chemotactic effect on neutrophils [24]. Further, TNF type 1 receptor (TNFR1)-deficient mice exhibited diminished pulmonary neutrophilia in response to inhaled LPS [25]. From these observations, we speculate that TNF-α plays a central role in neutrophil accumulation in the rat model of lung injury, such that prednisolone or ASP3258 inhibition of TNF-α production subsequently suppresses neutrophil infiltration into the lung, although ASP3258 may also directly inhibit neutrophil migration. The present model may not be capable of distinguishing between the anti-inflammatory effects of PDE4 inhibitors and glucocorticoids. Given its relative insensitivity to glucocorticoids, cigarette smoke-induced lung injury has been recently recognized as a well-defined model for COPD [26–28]. Using a wide variety of models may therefore aid in predicting the clinical efficacy of potential therapeutic agents for COPD.

With respect to their pharmacological properties, the inhibitory effect of ASP3258 in the present study was more potent than that of cilomilast but similar to that of roflumilast. We previously reported that ASP3258 as well as cilomilast and roflumilast dose-dependently attenuated ovalbumin (OVA)-induced asthmatic lung inflammation in sensitized Brown Norway rats [14]. Although the effective doses of ASP3258, cilomilast, and roflumilast in the current LPS-induced neutrophilic inflammation model were higher than those in the OVA-induced model, the rank order potency of the tested compounds was identical between these two models. Therefore, ASP3258 is considered to have broad spectrum anti-inflammatory activity with potency similar to roflumilast, the first PDE4 inhibitor approved for the treatment of COPD [13].

Histological examination of the lung sections was conducted to further investigate the effect of ASP3258 in the LPS-induced pulmonary inflammation model. Notably, either or both peribronchiolar or alveolar accumulation of neutrophils and macrophages was observed in the LPS-instilled control animals at 24 h after the instillation (Figs. 5 and 6), whereas no significant increase in macrophage numbers was noted on BAL cell analysis at the same time point (data not shown). However, a significant increase in macrophage numbers in the BAL fluid was seen at 48 h after LPS exposure, when neutrophil numbers had already returned to near normal levels (data not shown), raising the possibility of a time delay in macrophage accumulation in areas covered by the BAL. ASP3258 treatment significantly reduced the histological scores for neutrophil and macrophage infiltration into alveoli. Given that numerous studies suggest a crucial role of macrophages in the inflammation of COPD in addition to neutrophils, the effect of ASP3258 on both neutrophils and macrophages may be beneficial for the treatment of COPD.

In summary, we demonstrated the inhibitory effect of the PDE4 inhibitor ASP3258 in a rat model of LPS-induced pulmonary inflammation. In addition, ASP3258 directly suppressed human neutrophil functions which were insensitive to prednisolone. Given its wider therapeutic window over roflumilast and cilomilast [14], ASP3258 is a promising candidate for the treatment of COPD.

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