

Inhaled p38 α Mitogen-activated Protein Kinase Antisense Oligonucleotide Attenuates Asthma in Mice

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The p38 mitogen-activated protein kinase (MAPK) plays a critical role in the activation of inflammatory cells. Therefore, we investigated the antiinflammatory effects of a respirable p38 α MAPK antisense oligonucleotide (p38 α -ASO) in a mouse asthma model. A potent and selective p38 α -ASO was characterized *in vitro*. Inhalation of aerosolized p38 α -ASO using an aerosol chamber dosing system produced measurable lung deposition of ASO and significant reduction of ovalbumin (OVA)-induced increases in total cells, eosinophils, and interleukin 4 (IL-4), IL-5, and IL-13 levels in bronchoalveolar lavage fluid, and dose-dependent inhibition of airway hyperresponsiveness in allergen-challenged mice. Furthermore, inhaled p38 α -ASO markedly inhibited OVA-induced lung tissue eosinophilia and airway mucus hypersecretion. Quantitative polymerase chain reaction analysis of bronchoalveolar lavage fluid cells and peribronchial lymph node cells showed that p38 α -ASO significantly reduced p38 α MAPK mRNA expression. Nose-only aerosol exposure of mice verified the p38 α -ASO-induced inhibition of OVA-induced pulmonary eosinophilia, mucus hypersecretion, and airway hyperresponsiveness. None of the effects of the p38 α -ASO were produced by a six-base mismatched control oligonucleotide. These findings demonstrate antisense pharmacodynamic activity in the airways after aerosol delivery and suggest that a p38 α MAPK ASO approach may have therapeutic potential for asthma and other inflammatory lung diseases.

Keywords: asthma; bronchoalveolar lavage fluid; eosinophilia; mucus hypersecretion; ovalbumin

Respirable antisense oligonucleotides (ASOs) represent a novel therapeutic approach to the treatment of inflammatory lung diseases, such as asthma. ASOs are usually short strands of nucleic acid (typically 20-mers) that are complementary to the target mRNA. Hybridization of ASO to the intended mRNA can result in specific inhibition of target gene expression by various mechanisms, depending on the chemical makeup of the ASO, resulting in reduced levels of translation of the mRNA species. To circumvent systemic delivery of ASOs, topical application of ASOs has been shown to be highly effective. Furthermore, the lung is considered an excellent tissue for local ASO administration for the following reasons: it can be approached noninvasively using aerosolized ASO; it has a very large absorption surface area; and it is lined with surfactant, a cationic glycolipid at physiologic pH, which serves as a carrier to facilitate ASO uptake into lung cells (1). Accordingly, pharmacologic and

pharmacodynamic effects of an aerosolized adenosine receptor A₁ ASO have been demonstrated in a rabbit asthma model (2).

Allergic asthma is a chronic airway disorder characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) (3). T-helper type 2 (Th2) cells together with mast cells, B cells, and eosinophils are proposed to play a critical role in the initiation and perpetuation of allergic asthma. On activation, Th2 cells produce pleiotropic cytokines, such as interleukin (IL)-4, IL-5, and IL-13, which lead to B-cell maturation, IgE synthesis, airway eosinophilia, mucus secretion, and ultimately AHR (4, 5).

The mitogen-activated protein kinase (MAPK) signaling cascade has been shown to be important in the activation of various immune cells (6). It is activated by a three-tiered sequential phosphorylation of MAPK kinase kinase (MKKK), MAPK kinase (MKK or MEK), and MAPK. There are three major groups of MAPK in mammalian cells, including extracellular signal-regulated protein kinase (ERK), p38 MAPK, and c-Jun NH₂-terminal kinase (JNK). A recent study showed that p38 MAPK activity in the lungs of asthmatic mice was significantly higher as compared with control mice (7). p38 MAPKs exist in four distinct isoforms (α , β , γ , and δ) and are activated by dual phosphorylation on Thr180 and Tyr182 by their direct upstream kinases MKK3 and MKK6. The p38 MAPK signaling pathway is activated on ligation of T cell receptor (TCR) in T cells, B cell receptor (BCR) in B cells, and Fc ϵ RI in mast cells, leading to proliferation, differentiation, cytokine production, and degranulation (8–10). It has also been shown that the p38 MAPK pathway is critically important for the activation of the IL-5 receptor and eotaxin receptors in eosinophils, resulting in survival, activation, degranulation, and chemotaxis of eosinophils (11–13).

A recent study revealed that the p38 MAPK inhibitor SB239063 markedly reduced ovalbumin (OVA)-induced pulmonary eosinophilia in animal models of asthma, suggesting that the p38 MAPK pathway can be a pharmacologic target for the treatment of allergic asthma (14). However, SB239063 is not selective between p38 α and p38 β MAPK isoforms. In addition, in that study, the inhibitor was given systemically, not topically, and airway eosinophilia was the only endpoint reported. In the present study, we have designed and synthesized a potent and selective p38 α -ASO and investigated its antiinflammatory effects in a mouse asthma model. Aerosolized p38 α -ASO was given to conscious free-moving mice by nebulization. The ASO significantly reduced OVA-induced increases in total cell counts, eosinophil counts, and IL-4, IL-5, and IL-13 levels in bronchoalveolar lavage (BAL) fluid. It also inhibited OVA-induced airway mucus production and AHR in asthmatic mice. Our findings support the use of inhaled ASOs as a highly effective pharmacologic approach for pulmonary disorders, and suggest that aerosolized p38 α -ASO may have therapeutic potential for allergic airway inflammation. Some of the results of this study have been previously reported in abstract form (15, 16).

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METHODS

Design and Synthesis of ASOs

The p38 α -ASO was designed to target the coding region of mouse p38 α MAPK. Synthesis and purification of chimeric 2'-*O*-methoxyethyl/deoxy phosphorothioate-modified oligonucleotides were described previously (17, 18). Sequences of the oligonucleotides used in this study are shown in Table 1.

Cell Culture and ASO Transfection

bEND.3 cells, a mouse-brain endothelial cell line, were grown in a complete high-glucose Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (CHyclone, Logan, UT) and 1% penicillin/streptomycin. Cells were transfected for 4 hours in Opti-MEM7 medium (Invitrogen) containing 3 μ g/ml lipofectin (Invitrogen) and indicated amounts of ASO. The cells were allowed to recover for 18 hours in complete high-glucose Dulbecco's modified Eagle medium before mRNA and protein assays were performed.

Mice and Aerosol Dosing Systems

Male Balb/c mice 6 to 8 weeks old (Interfauna, East Yorkshire, UK) were sensitized and challenged with OVA as described previously (19). Aerosolized p38 α -ASO (estimated inhalable doses of 0.3, 1.5, and 3.0 mg/kg), six-base mismatched (MM) control, or saline (vehicle control) was given to mice for 30 minutes in an aerosol chamber from Days 14 to 20. Aerosol was delivered via a DeVilbiss ultrasonic nebulizer (Sunrise Medical, Carlsbad, CA) with a usable nebulizer output at 6 ml/minute and particle sizes of less than 4 μ m. For the nose-only aerosol exposure system, mice were exposed to different doses of ASOs (estimated inhalable doses of 3.3, 33, and 333 μ g/kg) on Days 17, 19, 21, 24, and 26 as described previously (20).

Pharmacokinetic Analysis of ASO in Mouse Lung

For the aerosol chamber dosing system, pulmonary ASO deposition level was quantitated using capillary gel electrophoresis as previously described (21, 22). For nose-only exposure, ASO deposition in lungs was measured using a quantitative hybridization-dependent nuclease ELISA method as described previously (23).

Bronchoalveolar Lavage and ELISA

Mice were anesthetized by an intraperitoneal injection of 100 μ L anesthetic mixture (Hypnorm:Dormicum:H₂O = 1:1:2; Roche, Basel, Switzerland). BAL fluid collection, total cell counts, and differential cell count were performed as described previously (19). Mouse IL-4 and IL-5 ELISA were obtained from BD PharMingen (San Diego, CA). Mouse IL-13 and IFN- γ ELISA were purchased from R&D Systems (Minneapolis, MN).

Histologic Examination

The lungs were fixed in 10% neutral formalin, paraffinized, cut into 6- μ m sections, and stained with hematoxylin and eosin for examining cell infiltration and with periodic acid-Schiff stain for measuring mucus production. Quantitative analysis was performed blinded as previously described (19).

TABLE 1. SEQUENCES OF P38 α -ANTISENSE OLIGONUCLEOTIDE AND CONTROL OLIGONUCLEOTIDES

Oligonucleotides	Sequences
ISIS 101757 (p38 α -ASO)	AGGTGCTCAGGACTCCATTT
ISIS 335400 (one-base MM control)	AGGTGCTCAGGCCTCCATTT
ISIS 335401 (two-base MM control)	AGGTGATCAGGCCTCCATTT
ISIS 335402 (four-base MM control)	AGGTGATCTGGCCTACATTT
ISIS 101758 (six-base MM control)	AGCTGATCTGGCCTACAGTT
ISIS 335403 (uniform MOE control)	AGGTGCTCAGGACTCCATTT
ISIS 118920 (PP2A gapmer control)	GTTCACTTAAAGTGGTCAC

Definition of abbreviations: ASO = antisense oligonucleotide; MM = mismatched; MOE = methoxyethyl.

Measurement of AHR

Mouse airway responsiveness to methacholine was measured using a single-chamber whole-body plethysmograph (Buxco, Sharon, CT), as previously described (19). Temperature equilibration inside the plethysmograph was allowed to occur before measurement of airway responsiveness, as suggested previously by Lundblad and coworkers (24).

mRNA Analysis

Total mRNA from bEND.3 cells was extracted using an RNEASY7 mini kit (Qiagen, Valencia, CA). Total mRNA from BAL fluid cells and peribronchial lymph node cells were extracted using an RNA isolation kit (Ambion, Austin, TX). Primers and probes are shown in Table 2. Quantitative polymerase chain reaction was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).

Statistical Analysis

Data are presented as means $\pm$ SEM. One-way analysis of variance followed by a Dunnett test was used to determine significant differences between treatment groups. The significance level was set at $p \leq 0.05$.

RESULTS

Characterization of p38 α -ASO

An active ASO against mouse p38 α MAPK was identified by screening for p38 α mRNA reduction using Northern blot in mouse b.END.3 cells (data not shown). The selected p38 α -ASO (ISIS 101757) was characterized for potency of target inhibition and specificity for the α isoform. After lipofectin-mediated transfection, the p38 α -ASO reduced the basal mRNA level of p38 α in a dose-dependent manner, as determined by quantitative real-time reverse transcriptase-polymerase chain reaction (Figure 1A). To confirm an antisense mechanism of p38 α reduction, the active sequence was also tested in parallel with oligonucleotides containing one-, two-, four-, and six-base MM controls. Activity was compromised in accordance with increasing number of MM bases in the sequence (Figure 1A), indicating the importance of RNA hybridization for this effect. An irrelevant ASO of similar chemical makeup failed to inhibit p38 α mRNA expression, and an ASO with uniform methoxyethyl modification also was without effect, suggesting that the p38 α -ASO-mediated target reduction depends on RNase H1. Concomitant evaluation of p38 β mRNA levels in p38 α -ASO-treated b.END.3 cells failed to show any change in expression, suggesting specificity of the p38 α -ASO for the α isoform (Figure 1B). p38 α protein levels in b.END cells were also found to be inhibited by the p38 α -ASO, in good agreement with the observed effect on p38 α mRNA (Figures 1C and 1D). These results describe a potent, isoform-specific p38 α -ASO useful for *in vivo* studies in rodent disease models.

TABLE 2. PRIMERS AND PROBES FOR QUANTITATIVE POLYMERASE CHAIN REACTION ANALYSIS

MAPK	Sequences
p38 α	Forward primer: 5'-AAGGGAACGAGAAAAGTCTGTT-3' Reverse primer: 5'-TATTTTAACCAAGTGGTATTATCTGACATCCT-3' Probe: FAM-TTGTAATTTGTGAAGTGGCTGTAATCTGGTATGCC-TAMRA
p38 β	Forward primer: 5'-CGCCAGAGAGTGGCTGTAAA-3' Reverse primer: 5'-TGTCCTCCTCGCTGGAT-3' Probe: FAM-AAFCTGTCTCGCCCTTTCCAATCGC-TAMRA

Definition of abbreviation: MAPK = mitogen-activated protein kinase.

Carboxyfluorescein (FAM) is the fluorescent reporter dye and 6-carboxy-tetramethyl-rhodamine (TAMRA) is the quencher dye.

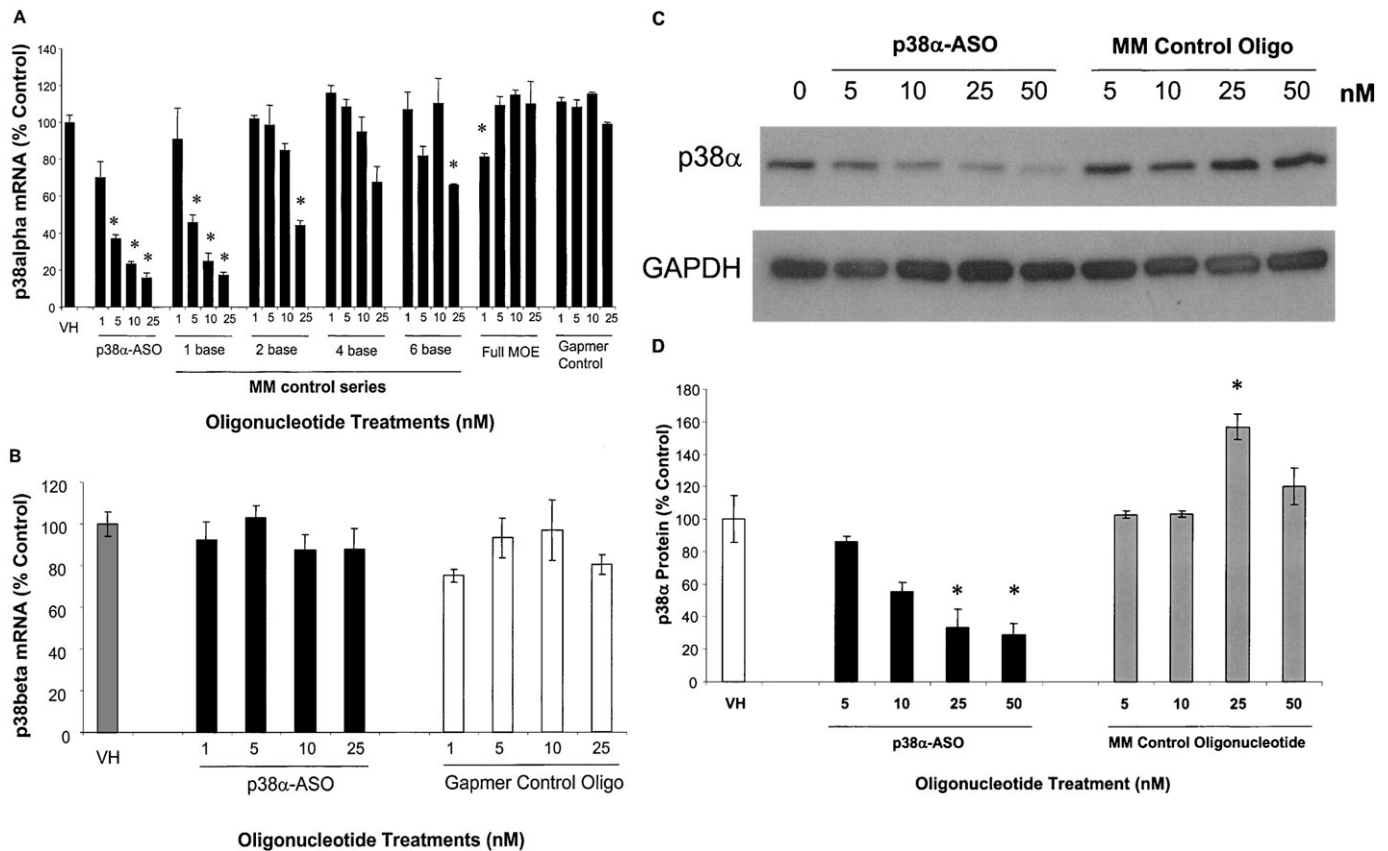


Figure 1. Sequence- and dose-dependent inhibition of p38 α , but not p38 β mRNA, and protein expression by a p38 α -antisense oligonucleotide (ASO). (A) p38 α mRNA levels in b.END.3 cells after transfection with ASO or oligonucleotide control sequences at the indicated concentrations. (B) Effect of p38 α -ASO or an irrelevant control oligonucleotide on p38 β mRNA expression in b.END.3 cells. RNA evaluation performed at 24 hours after transfection. Data is expressed as percentage of vehicle (lipofectin only) treatment. (C) b.END.3 cells were transfected in the presence of lipofectin with p38 α -ASO or control oligonucleotides at the indicated concentrations and whole-cell extracts were prepared 60 hours later and immunoblotted. Five micrograms of protein were separated on 10% Tris-glycine gels (Western blot representative of three separate experiments). (D) Quantitation of Western blot results by Phosphorimager (Bio-Rad, Hercules, CA) analyses. Data are expressed as percentage of vehicle (VH; lipofectin only) treatment. Data shown are representative of three independent experiments (quantitative analysis of triplicate immunoblots from the same representative experiment is shown). GAPDH = glyceraldehyde-3-phosphate dehydrogenase; MM = mismatched; MOE = methoxyethyl. *Significant difference from vehicle, $p < 0.05$.

Deposition of Inhaled p38 α -ASO in Mouse Lung

To characterize the delivery of p38 α -ASO to the lung, naive mice were placed in an aerosol chamber and subjected to a single exposure of the ASO aerosol. The aerosolized dose was derived from nebulization of 12.5, 62.5, and 125 μ g p38 α -ASO/ml solutions for 30 minutes (estimated inhalable doses were calculated to be 0.3, 1.5, and 3.0 mg/kg, respectively). Using capillary gel electrophoresis, p38 α -ASO levels present in lung tissue were determined 24 hours later to be 0.3, 0.8, and 1.1 μ g ASO/g of lung tissue, respectively, indicating dose-dependent accumulation of aerosolized oligonucleotide in lung tissue. ASO aerosol exposure of allergen-sensitized mice after the protocol used to evaluate pharmacologic responses produced lung tissue concentrations 24 hours after the last dose that were similar to the single exposure in naive mice (e.g., between 0.5 and 2 μ g/g lung tissue). These data indicate that relatively little accumulation in whole lung is observed with repeated aerosol exposures in mice at these doses.

p38 α -ASO Inhalation Inhibits OVA-induced Eosinophil Recruitment to Lung

BAL fluid was collected 24 hours after the last OVA aerosol challenge, and total and differential cell counts were performed.

OVA inhalation significantly increased total cell, eosinophil, macrophage, and lymphocyte counts as compared with the saline control (Figure 2). Inhalation of p38 α -ASO (0.3, 1.5, and 3.0 mg/kg) substantially reduced the total cell number recovered in BAL fluid as compared with the MM control oligonucleotide. This effect was mainly caused by a significant reduction in eosinophil count in the p38 α -ASO-treated mice, which occurred in a dose-dependent manner (Figure 2). The numbers of neutrophils, macrophages, and lymphocytes were not affected by the p38 α -ASO. These results suggest that the p38 α MAPK pathway is predominantly involved in eosinophil recruitment during allergic inflammation in this model.

Effects of p38 α -ASO on OVA-induced Eosinophil Infiltration and Mucus Production

Lung tissue was collected 24 hours after the last OVA challenge. OVA aerosol challenge induced marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues as compared with saline challenge (Figures 3A and 3B). As expected, eosinophils constituted the majority of infiltrating inflammatory cells (Figure 3I). Inhalation of p38 α -ASO (1.5 mg/kg) significantly attenuated the eosinophil-rich leukocyte

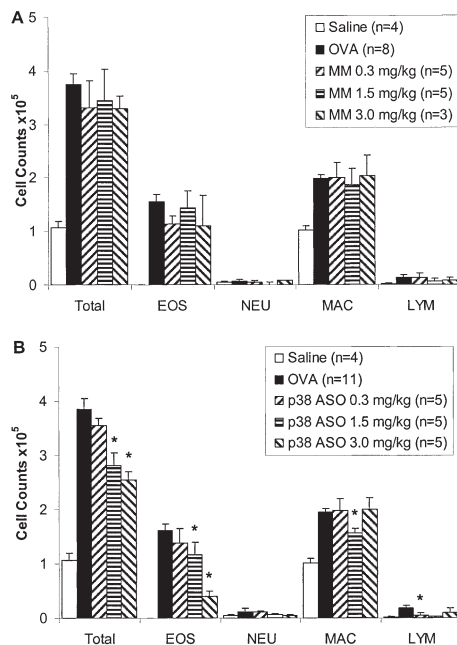


Figure 2. p38 α -ASO-mediated reduction of allergen-induced bronchoalveolar lavage (BAL) fluid total cell and eosinophil infiltration. (A) Inflammatory cell counts in BAL fluid obtained from sensitized mice 24 hours after the last saline aerosol or 10 mg/ml ovalbumin (OVA) aerosol challenge from six-base MM control oligonucleotide-pretreated mice. (B) Effects of p38 α -ASO on OVA-induced inflammatory cell counts in BAL fluid from sensitized mice 24 hours after the last 10 mg/ml OVA challenge. Differential cell count was performed on a minimum of 500 cells to identify eosinophil (EOS), neutrophil (NEU), macrophage (MAC), and lymphocyte (LYM). * Significant difference from OVA control, $p < 0.05$.

infiltration as compared with MM control exposure (Figures 3C, 3D, and 3J). In contrast, OVA-challenged mice, but not saline-challenged mice, developed marked goblet cell hyperplasia and mucus hypersecretion within the bronchi in the lung (Figures 3E and 3F). The OVA-induced mucus secretion was substantially reduced by p38 α -ASO (1.5 mg/kg) as compared with the six-base MM control (Figures 3G, 3H, and 3K).

BAL Levels of Th2 but Not Th1 Cytokines Are Inhibited in Aerosolized p38 α -ASO-treated Mice

To determine the levels of cytokines *in vivo*, BAL fluid samples were collected 2 hours after the last OVA challenge. IL-4, IL-5, IL-13, and IFN- γ levels were measured using ELISA. As shown in Figure 4, OVA inhalation in sensitized mice induced substantial cytokine release into BAL fluid as compared with untreated mice. Treatment of mice with aerosolized p38 α -ASO significantly reduced IL-4, IL-5, and IL-13 levels in BAL fluid as compared with the six-base MM control (Figure 4). In contrast, aerosolized p38 α -ASO exposure did not show any significant effects on IFN- γ levels in BAL fluid.

p38 α -ASO Reduces OVA-induced AHR in Mice

To correlate the antiinflammatory activity of the inhaled p38 α -ASO to airway performance, we investigated the effect of aerosolized p38 α -ASO inhalation on the development of AHR in mice. Sensitized animals challenged with 1% OVA aerosol for 20 minutes daily for 3 consecutive days developed AHR to inhaled methacholine. Airway responsiveness was determined by Penh (19), and was substantially increased in the OVA-challenged group

in response to methacholine provocation, as compared with the saline-challenged group (Figure 5). Inhalation of p38 α -ASO significantly reduced AHR to inhaled methacholine in a dose-dependent manner as shown in Figure 5, suggesting that the decreased immune-mediated pathology resulted in decreased airway smooth muscle constriction as well.

ASO-mediated Inhibition of p38 α Expression in Mouse BAL and Draining Lymph Node Cells

To verify that the palliative effects of the inhaled p38 α -ASO on lung inflammatory and airway responses in the mouse asthma model were mediated by p38 α MAPK knockdown, we examined the effects of the ASO on p38 α MAPK gene expression in BAL fluid cells and peribronchial lymph node cells. Figure 6 shows that the level of p38 α mRNA was significantly reduced in p38 α -ASO-treated mice (3.0 mg/kg) as compared with MM control-treated animals, in both cell populations. Because p38 α is ubiquitously expressed, these data do not allow discrimination of which cell type in BAL fluid or lymph node is impacted by ASO treatment. Because the majority of BAL cells are macrophages, one might speculate that macrophages are likely to be one target cell population of antisense delivered to the lung via aerosol under these conditions.

Nose-Only Aerosol Exposure of Mice to p38 α -ASO Results in Inhibition of OVA-induced BAL and Lung Eosinophilia, Mucus Overproduction, and AHR

To determine whether nose-only exposure of mice would result in similar pharmacology, we reproduced key endpoints using a characterized aerosol delivery system described previously (20). Initial experiments defined median mass aerodynamic diameter of oligonucleotide particles (particle size: range 0.9–1.2 μ m) and demonstrated similar delivery to individual animal ports in the nose-only chamber housing (data not shown). Exposure of mice to aerosolized p38 α -ASO under these conditions resulted in 11.5, 80.3, and 324 ng/g ASO per gram of lung tissue at estimated inhalable doses of 3.3, 33, and 333 μ g/kg. p38 α -ASO delivered at these doses via this apparatus produced pronounced inhibition of BAL eosinophilia and AHR in a dose-related manner and also suppressed mucus overproduction, as determined by periodic acid-Schiff staining (Figure 7). Lung histopathology also showed reduction of tissue eosinophilia and mucus (data not shown).

DISCUSSION

We used a potent and highly selective p38 α MAPK ASO that inhibits the p38 α isoform with a negligible effect against the p38 β isoform to probe the role of p38 α in an allergen-driven mouse model of asthma. Our present findings reveal that inhaled aerosolized p38 α -ASO produces inhibition of the p38 α MAPK expression in BAL and peribronchial lymph node cells and attenuates OVA-induced pulmonary inflammation, release of Th2 cytokines into the airway, airway mucus production, and AHR in sensitized mice.

There is now clear evidence that Th2 cells play an essential role in the pathogenesis of allergic airway inflammation (5, 25). Th2 cytokines can be produced by various resident cells, including bronchial epithelial cells, tissue mast cells, and alveolar macrophages, and by infiltrating inflammatory cells, such as lymphocytes and eosinophils. Our data show that inhaled aerosolized p38 α -ASO significantly reduced the levels of IL-4, IL-5, and IL-13 in BAL fluids. In contrast, the level of IFN- γ , a Th1 cytokine, was not affected. The p38 α MAPK signaling pathway has been shown to be involved in cytokine production from a variety of cell types. TCR engagement and/or CD28 costimulation of CD4⁺

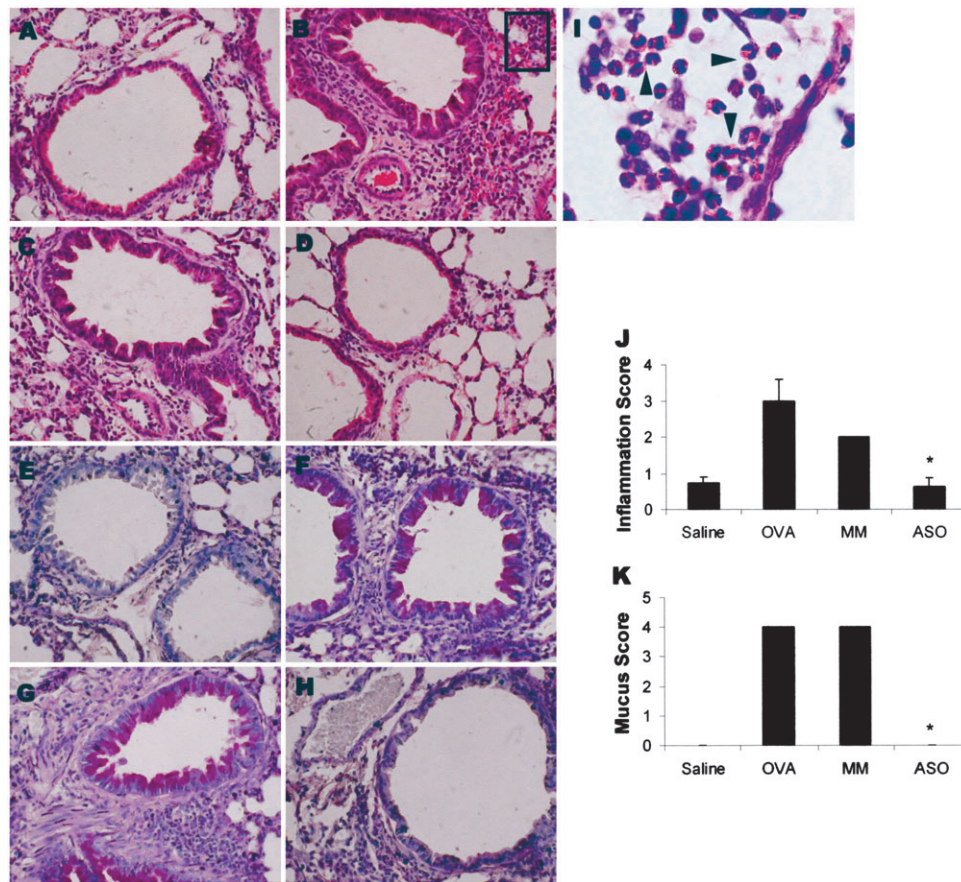


Figure 3. Histologic determination of lung tissue eosinophilia and mucus production after p38 α -ASO inhalation in allergen-challenged mice. Histologic examination of lung tissue eosinophilia (A–D, original magnification $\times 400$) and mucus secretion (E–H, original magnification $\times 400$) 24 hours after the last challenge of saline aerosol (A, E), OVA aerosol (B, F), OVA aerosol plus six-base MM control oligonucleotide (C, G), or OVA aerosol plus 1.5 mg/kg p38 α -ASO (D, H). The rectangular box in B was magnified to $\times 1,000$ to reveal OVA-induced eosinophilia (I). Arrowheads indicate eosinophils, which are characterized by prominent bilobed nuclei and specific granules stained in pinkish red with eosin. Quantitative analyses of inflammatory cell infiltration (J) and mucus production (K) in lung sections were performed blinded based on the methods described previously (19). Lung tissues were fixed, sectioned at 7- μ m thickness, and stained with hematoxylin and eosin for tissue eosinophilia or periodic acid-Schiff (PAS) for mucus production. Values shown are the mean $\pm$ SEM of four separate experiments. *Significant difference from six-base MM control oligonucleotide, $p < 0.05$.

T cells has been shown to induce IL-4 production and Th2 cell differentiation via activation of p38 MAPK (26–28). SB203580, a small-molecule p38 MAPK inhibitor, has been shown to inhibit the synthesis of IL-4, IL-5, and IL-13 from anti-CD3 and anti-CD28 mAb-activated human T cells via p38 MAPK inhibition (29, 30). Airway smooth muscle can also release proinflammatory cytokines during airway inflammation (31, 32). Enhanced p38 MAPK tyrosine phosphorylation has been observed in human airway smooth muscle cells on stimulation with IL-4 and IL-13 (33). In addition, p38 MAPK signaling pathway is critical for eotaxin production from airway smooth muscle cells on stimulation with IL-4 and IL-13 (33, 34). Furthermore, the p38 MAPK signaling pathway has been implicated in cytokine production in bronchial epithelial cells (35) and mast cells (36), and in IL-6 release from human lung fibroblasts (37). As such, the observed reduction of Th2 cytokine levels in BAL fluid from p38 α -ASO-treated mice may be from downregulation of p38 α MAPK signaling in inflammatory and/or airway resident cells.

The eosinophil is believed to be the principal effector cell for the pathogenesis of allergic inflammation. Our present findings showed that p38 α -ASO administration prevented eosinophil infiltration into the airways as shown by a significant drop in total

cell counts and eosinophil counts in BAL fluid. Similarly, tissue eosinophilia was also inhibited as revealed by a substantial reduction of inflammatory cell infiltration in histologic examination. Eosinophil transmigration into the airways is a multistep process that is orchestrated by Th2 cytokines, such as IL-4, IL-5, and IL-13, and coordinated by specific chemokines in combination with adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) (38, 39). IL-5 has been shown to induce p38 MAPK activation in eosinophils, leading to their differentiation and degranulation (12). IL-13 has been shown to be by far the most potent inducer of eotaxin expression by airway epithelial cells (40). Furthermore, the p38 MAPK pathway has been shown to be involved in the intrinsic mechanism of eotaxin-induced eosinophil cytoskeletal rearrangements, chemotaxis, and degranulation (13, 41). Together, the observed reduction in airway eosinophilia by the p38 α -ASO is likely a result of reduction in Th2 cytokine production, leading to downregulation of chemokine production and eosinophil chemotaxis.

Our findings demonstrated a dramatic reduction in mucus production with less goblet cell hyperplasia in p38 α -ASO-treated mice as compared with the MM oligonucleotide control. Studies using undifferentiated tracheobronchial epithelial cells

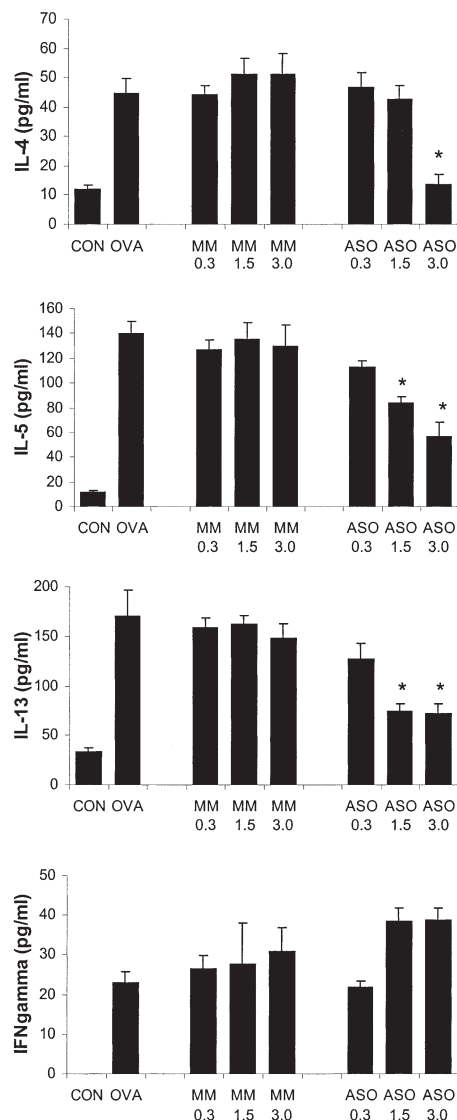


Figure 4. Effects of inhaled aerosolized p38 α -ASO on cytokine levels in BAL fluid. BAL fluids were collected 2 hours after the last OVA challenge. Levels of interleukin 4 (IL-4), IL-5, IL-13, and IFN- γ were analyzed using ELISA (n = 5). Values shown are the mean $\pm$ SEM. CON = control. *Significant difference from six-base MM control oligonucleotide, $p < 0.05$.

or cancer cell lines showed that Th2 cells and cytokines (42, 43) are linked to mucus hypersecretion by their effects on mucin gene expression in the airway epithelium. In addition, a recent study showed that IL-13-induced goblet cell hyperplasia and mucin MUC5AC protein expression in human bronchial epithelial cell cultures were mediated by the p38 MAPK signaling pathway and could be inhibited by SB202190 (44). Therefore, the observed decrease in mucus production in p38 α -ASO-treated lung tissue may be attributed to the substantial drop in Th2 cytokines in asthmatic mice treated with the inhaled ASO and/or downregulation of p38 α MAPK in the IL-13-responsive airway epithelium.

It is believed that inflammatory mediators released during allergic inflammation play a critical role in AHR development (4, 45). Our data showed that aerosolized p38 α -ASO significantly inhibited OVA-induced AHR to inhaled methacholine in a dose-dependent manner. It has been established that IL-5-mediated eosinophilia contributes to AHR by generating cytotoxic prod-

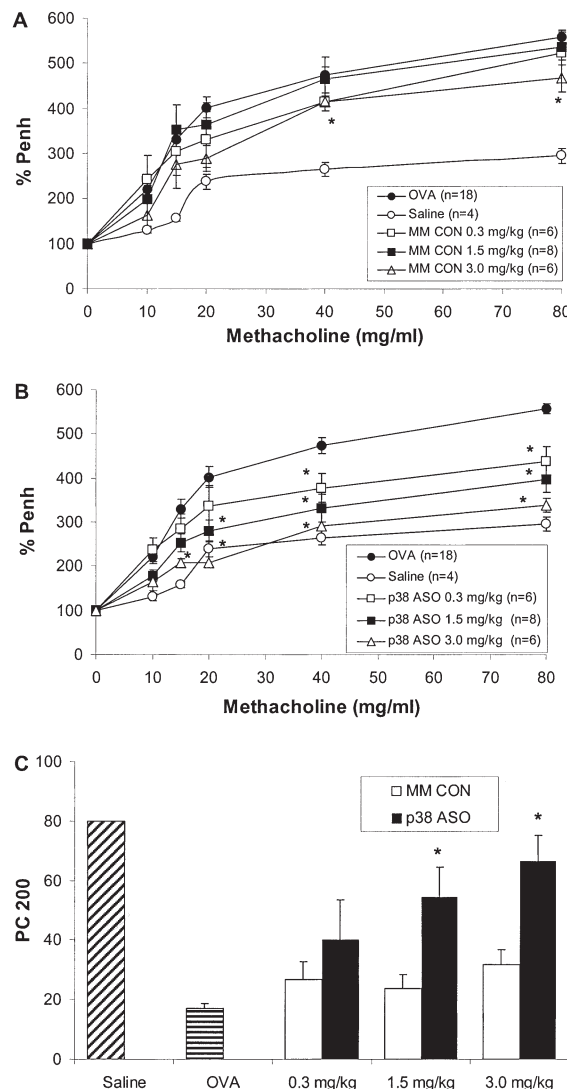


Figure 5. Aerosolized p38 α -ASO treatment dose-dependently reduces airway hyperreactivity in allergen-challenged mice. (A) Airway responsiveness of MM control oligonucleotide-pretreated sensitized mice challenged with aerosolized saline or 10 mg/ml OVA for 3 consecutive days to increasing doses of inhaled methacholine. (B) Effects of p38 α -ASO on inhaled methacholine-induced AHR. (C) AHR to methacholine was expressed as provocation concentrations of methacholine causing 200% increase from basal level of airway responsiveness (PC200). Values shown are the mean $\pm$ SEM. *Significant difference from six-base MM control oligonucleotide, $p < 0.05$.

ucts, such as major basic protein, eosinophilic cationic protein, and other lipid mediators, such as platelet-activated factor and cysteinyl-leukotrienes, which contribute to tissue damage (46). In addition, IL-4 and IL-13 have been shown to induce AHR in mouse asthma models in which cysteinyl-leukotrienes have been implicated to play a major role (47, 48). Moreover, IgE-mediated mast cell activation may contribute to AHR by producing a wide array of inflammatory mediators and cytokines (4, 10). Thus, the observed inhibition of AHR after p38 α -ASO inhalation may be associated with collective reductions in Th2 cytokine production and tissue eosinophilia and allergen-induced mast cell activation.

The differences we observed in effective lung concentrations of ASO after inhalation in the whole-body chamber and nose-

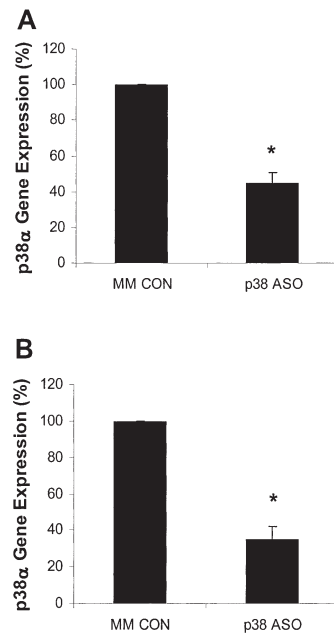


Figure 6. Target mRNA reduction in BAL fluid and peribronchial lymph node cells after aerosolized p38 α -ASO exposure. BAL fluid cells (A) and peribronchial lymph node cells (B) were isolated from mice treated with 3.0 mg/kg MM ASO and p38 α -ASO. Total cellular RNA was extracted using an RNA isolation kit. Primers and probes for quantitative detection of p38 α MAPK were described in METHODS. Values shown are the mean $\pm$ SEM (n = 4). *Significant difference from six-base MM control oligonucleotide, p < 0.05.

only exposure systems may be explained by efficiency of delivery to the deep lung airspace. The estimated particle size of aerosolized ASO in the whole-body exposure chamber is approximately 4.0 μ m, whereas the nose-only system delivered particles in the 0.9- to 1.2- μ m range. These differences would be consistent with greater impaction of ASO particles in the upper respiratory tract in the whole-body chamber compared with the nose-only system. Because our pharmacokinetics measurements do not account for regional differences in lung deposition, we hypothesize that uniform deep lung delivery is more effectively achieved by the use of smaller particle size. In support of this idea, p38 α -ASO solution delivered by intratracheal instillation to mice also required higher lung ASO tissue concentrations to produce similar pharmacology as reported herein (unpublished observations of J.G. Karras and associates).

A previous study documented greater deposition of aerosolized ASO in mouse lung at similar dose levels to those used in our chamber dosing experiments (49). However, the pharmacokinetic data reported therein were obtained after nose-only exposure. Thus, the greater efficiency of delivery, as noted previously, likely contributed to these observed differences. In addition, differences in the respective rate of deposition (higher in the nose-only system) and, potentially, in the humidity of the dosing chambers, which would be expected to affect particle size, may also contribute to these disparate results. Further study of the behavior of aerosolized oligonucleotides *in vivo* will be required to more fully understand these differences.

Allergic airway inflammation and AHR development involve multiple inflammatory cells and a wide array of mediators. We report here for the first time that an inhaled p38 α MAPK ASO molecule effectively reduced the OVA-induced pulmonary inflammatory responses and airway hyperreactivity, which are hallmarks of the asthmatic-like response in mouse models. These findings support a potential role for direct aerosol administration of p38 α MAPK ASO in the treatment of asthma and the suitability of topical ASO administration for inflammatory lung diseases using current metered dose or dry-powder inhaler technologies.

Conflict of Interest Statement: W.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.H.P.C. does not have a financial relationship with a commercial entity that has an interest

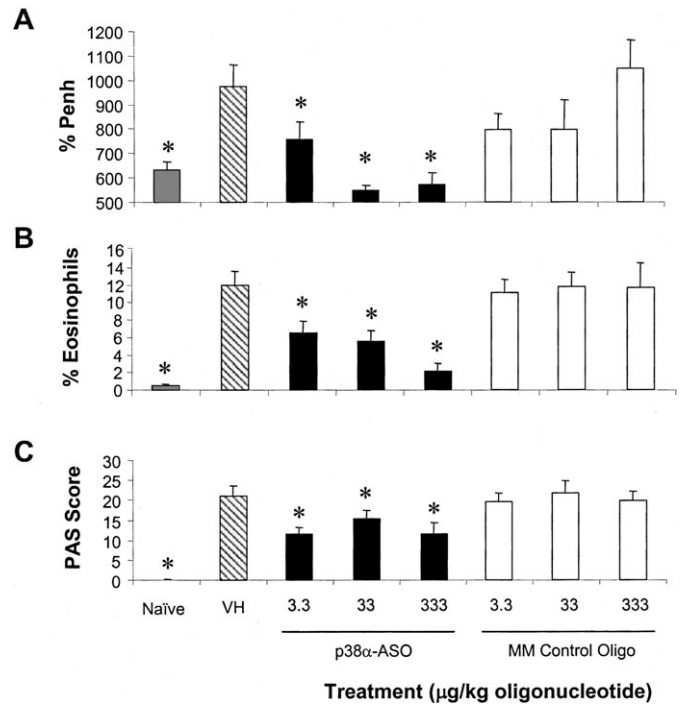


Figure 7. Inhibition of allergen-induced BAL eosinophilia, mucus overproduction, and airway hyperreactivity in mice after nose-only aerosol p38 α -ASO exposure. (A) Response of OVA-sensitized and -challenged mice to 100 mg/ml concentration of methacholine after treatment with p38 α -ASO, MM control oligonucleotide, at the indicated doses, or saline. (B) p38 α -ASO-mediated reduction of allergen-induced BAL fluid eosinophil infiltration. Cell counts were obtained 48 hours after the last saline or OVA aerosol challenge. (C) Mucus expression was visualized by tissue staining of formalin-fixed, paraffin-embedded lung sections with PAS stain. The number of PAS-positive airways are represented (n = 7 mice/group). *Significant difference from VH treatment group, p < 0.05.

in the subject of this manuscript; K.M. was employed by and has stock options through ISIS Pharmaceuticals, Inc., and is named on a pending U.S. patent application on methods of use of p38 α antisense oligonucleotides for the potential therapy of respiratory disease; J.R.C. is an employee of ISIS Pharmaceuticals and has stock options for ISIS Pharmaceuticals; H.H.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; B.P.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.G.K. is an employee of ISIS Pharmaceuticals and owns company stock and options and is named on a pending patent application regarding p38 α antisense molecules for potential therapy of respiratory disease; W.S.F.W. is a coinventor of a pending patent application filed by ISIS Pharmaceuticals on the use of p38 α MAPK antisense oligonucleotide for inflammatory diseases.

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