

Spleen tyrosine kinase inhibition attenuates airway hyperresponsiveness and pollution-induced enhanced airway response in a chronic mouse model of asthma

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Background: Asthma is a chronic inflammatory disease characterized by airways hyperresponsiveness (AHR), reversible airflow obstruction, airway remodeling, and episodic exacerbations caused by air pollutants, such as particulate matter (PM; PM <2.5 µm in diameter [PM_{2.5}]) and ozone (O₃). Spleen tyrosine kinase (Syk), an immunoregulatory kinase, has been implicated in the pathogenesis of asthma.

Objective: We sought to evaluate the effect of Syk inhibition on AHR in a chronic mouse model of allergic airways inflammation and pollutant exposure.

Methods: We used a 12-week chronic ovalbumin (OVA) sensitization and challenge mouse model of airways inflammation followed by exposure to PM_{2.5} plus O₃.

Respiratory mechanics and methacholine (MCh) responsiveness were assessed by using the flexiVent system. The Syk inhibitor NVP-QAB-205 was nebulized intratracheally by using a treatment-based protocol 15 minutes before assessment of MCh responsiveness.

Results: Syk expression increased significantly in the airway epithelia of OVA-sensitized and OVA-challenged (OVA/OVA) mice compared with OVA-sensitized but PBS-challenged (OVA/PBS) control mice. OVA/OVA mice exhibited AHR to MCh, which was attenuated by a single administration of NVP-QAB-205 (0.3 and 3 mg/kg). PM_{2.5} plus O₃ significantly augmented AHR to MCh in the OVA/OVA mice, which was abrogated by NVP-QAB-205. Total inflammatory cell counts were significantly higher in the bronchoalveolar lavage fluid from OVA/OVA than OVA/PBS mice and were unaffected by PM_{2.5} plus O₃ or NVP-QAB-205.

Conclusion: NVP-QAB-205 reduced AHR and the enhanced response to PM_{2.5} plus O₃ to normal levels in an established model of chronic allergic airways inflammation, suggesting that Syk inhibitors have promise as a therapy for asthma. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Airways hyperresponsiveness, Syk, asthma, pollution, mouse

Asthma is a chronic disorder of the airways characterized by airways hyperresponsiveness (AHR), inflammation, reversible airflow obstruction, and airway remodeling.¹⁻³ Despite the availability of effective anti-inflammatory and bronchodilator therapies, asthma remains a major health issue, particularly in developed countries, where the incidence of asthma has continued to grow over the past several decades.⁴ This has been attributed to a combination of genetic and environmental factors, such as allergens, respiratory tract viruses, and pollutants.^{2,3} Indeed, large-scale epidemiologic studies have shown that exposure to air pollutants, such as particulate matter (PM) and ozone (O₃), increases both the risk of asthma *de novo*⁵⁻⁸ and exacerbations of established asthma.⁹⁻¹² The underlying cellular mechanisms are not fully elucidated but appear to involve ongoing dysregulated inflammation that leads to altered airway structure and function (ie, subepithelial fibrosis, increased smooth muscle mass, and goblet cell hyperplasia).¹⁻³ Identification of specific mechanisms that contribute to the disease pathogenesis might provide new targets for therapeutic intervention.

Recent studies have identified spleen tyrosine kinase (Syk) as a novel therapeutic target in the management of chronic inflammatory diseases, such as rheumatoid arthritis¹³ and allergic rhinitis.^{14,15} Syk is a potent inflammatory and immune regulator that is well characterized in leukocytes.¹⁶ Our laboratory has established a role for Syk in regulating viral cell entry and the airway epithelial inflammatory response to human rhinovirus, a common cause of asthma exacerbations.^{17,18} We have also shown that human rhinovirus can induce Syk activation and Syk-mediated cell signaling, leading to expression of inflammatory mediators, such as IL-8 and vascular endothelial growth factor (VEGF).¹⁹⁻²¹ Thus epithelial expression of Syk likely contributes to the inflammatory response of the airways independently of inflammatory cells.

Studies in animal models of asthma have suggested that Syk plays a role in mediating allergic airways inflammation.²²⁻²⁵ Using Brown Norway rats sensitized with ovalbumin (OVA), Stenton et al^{22,23} demonstrated that aerosolized Syk antisense oligodeoxynucleotides (ASOs) administered before OVA challenge significantly attenuated airways inflammation and acetylcholine-induced contractility of isolated tracheas *ex vivo*. These effects were attributed to ASO-induced attenuation of Syk expression

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Abbreviations used

AHR:	Airways hyperresponsiveness
ASO:	Antisense oligonucleotide
BAL:	Bronchoalveolar lavage
CC-10:	Clara cell protein 10
DMSO:	Dimethyl sulfoxide
FA:	High-efficiency particulate air–filtered air
KC:	Keratinocyte-derived chemokine
LCM:	Laser-capture microdissection
MCh:	Methacholine
NF- κ B:	Nuclear factor κ B
O ₃ :	Ozone
OVA:	Ovalbumin
OVA/OVA:	OVA sensitized and OVA challenged
OVA/PBS:	OVA sensitized but PBS challenged
PAS:	Periodic acid–Schiff
PI3:	Phosphatidylinositol 3
PM:	Particulate matter
PM _{2.5} :	PM less than 2.5 μ m/L in diameter
R _{rs} :	Total respiratory resistance
Syk:	Spleen tyrosine kinase
VEGF:	Vascular endothelial growth factor

and activity in alveolar macrophages and other leukocyte populations. In a second model of OVA-induced allergic airways disease using BALB/c mice, administration of R406, a Syk-selective inhibitor, concomitant with OVA challenge prevented the development of airways inflammation.^{24,25} Furthermore, *ex vivo* airway contractility was also attenuated by R406 treatment, a response attributed to decreased dendritic cell and mast cell activation.²⁵ Together, these early studies in which Syk was inhibited during induction of the disease phenotype suggest that Syk plays a role in the development of asthma.

We hypothesize that Syk contributes to airways inflammation and the augmented responsiveness to bronchoconstrictors and that inhibition of Syk activity can attenuate AHR and airways inflammation in established disease. We tested this hypothesis using a chronic OVA sensitization and challenge mouse model, which exhibits less airways inflammation and more pronounced airway remodeling compared with the models used previously,²⁶ and determined airways responsiveness to methacholine (MCh) *in vivo*. After this sensitization-challenge period, mice were exposed to particulate matter of less than 2.5 μ m in diameter (PM_{2.5}) plus O₃, and the effect of Syk inhibition was evaluated by using a protocol that better mimics the clinical setting of established asthma and management of symptoms triggered by exposure to common air pollutants.

METHODS

Additional experimental details are available in the **Methods** section in this article's Online Repository at www.jacionline.org.

Murine OVA sensitization and challenge model of allergic airways inflammation, PM_{2.5} plus O₃ exposure, and Syk inhibition

Animal protocols were approved by the University of Toronto Faculty Advisory Committee on Animal Services and conducted in accordance with the guidelines of the Canadian Council on Animal Care. The subacute, acute, and chronic models of OVA sensitization and challenge manifest primary

phenotypes of airways inflammation, hyperresponsiveness, and remodeling, respectively (see Fig E1, A, in this article's Online Repository at www.jacionline.org).^{27–29} In all models female BALB/c mice (aged 6–8 weeks; mean body weight, 21.4 g; Charles River Laboratory, Saint-Constant, Quebec, Canada) were sensitized to chicken OVA (25 μ g administered intraperitoneally, with 1 mg of aluminum hydroxide gel in 0.2 mL of PBS, pH 7.4) on days 0 and 7 followed by repeated challenge with nebulized OVA or PBS (25 min/d; see Fig E1, A). Twenty-four hours after the last OVA (or PBS) challenge, mice were either anesthetized for pulmonary function testing with the flexiVent (Scireq, Montreal, Quebec, Canada) or randomized to exposure with high-efficiency particulate air–filtered air (FA) or PM_{2.5} plus O₃ for 4 hours before pulmonary function testing (see Fig E1, B). After determination of baseline respiratory mechanics, 0.3 or 3 mg/kg NVP-QAB-205 (or dimethyl sulfoxide [DMSO] vehicle) was nebulized directly into the ventilatory circuit 15 minutes before reassessment of pulmonary mechanics and determination of MCh responsiveness. We used dosages of NVP-QAB-205 that have been validated in a rat model of OVA-induced airways hyperreactivity (personal communication, GlaxoSmithKline, Research Triangle Park, NC).³⁰ Bronchoalveolar lavage (BAL) was performed 15 minutes after MCh challenge. Mice were then killed, and tissues were harvested for subsequent analysis.

Mice were exposed, as previously described,²⁹ by using a modified inExpose nose-only inhalation system (Scireq). PM_{2.5}, drawn from real-world particles outside the Gage Occupational and Environmental Health Unit in downtown Toronto, is concentrated to 350 to 400 μ g/m³ and exposed at 2 L/min. O₃ was produced by an arc generator and introduced into the concentrator at 300 ppb. These concentrations were used based on previous inhalational exposures of rodents.²⁹

Lung homogenization and Western blotting

Mouse lungs were homogenized for Western blot analysis of Syk protein expression and quantification, as described previously.^{28,29}

Immunohistochemical staining of mouse lung sections

Lung tissue sections were prepared, as described previously.²⁸ Sections were double-immunostained with 1:100 rabbit polyclonal anti-Syk antibody (clone N-19; Santa Cruz Biotechnology, Santa Cruz, Calif) and either 1:400 mouse monoclonal anti- α -actin antibody (Sigma Chemical, Mississauga, Ontario, Canada) or 1:400 goat polyclonal anti-Clara cell 10 protein antibody (Santa Cruz Biotechnology) to determine the tissue localization of Syk. Images were captured with a $\times 63$ objective on a Zeiss LSM510 Laser Scanning Confocal Microscope (Carl Zeiss, Oberkochen, Germany).

Hematoxylin and eosin and periodic acid–Schiff (PAS) staining were performed at the Pathology Core Facility of the Centre for Modeling Human Disease (Toronto Centre for Phenogenomics, Toronto, Ontario, Canada). Slides were then visualized on an inverted Leica DMIL microscope (Leica, Wetzlar, Germany) with an attached Olympus DP71 camera (Olympus, Center Valley, Pa) for image acquisition with $\times 4$ and $\times 20$ objectives.

Laser-capture microdissection of airway epithelial cells

For laser-capture microdissection (LCM), frozen tissue sections were prepared by embedding lungs in OCT Compound (Tissue-Tek; Sakura Finetek, Torrance, Calif)–filled cryomolds. The molds were then placed in liquid nitrogen and processed at the Pathology Core Facility (Toronto Centre for Phenogenomics). Frozen tissue specimens were sectioned (8- μ m thickness) with a cryostat (H/I Bright, OTF5000; Hacker Instruments, Fairfield, NJ) before staining and fixation on slides. Airway epithelial cells were isolated by means of LCM (PixCell II System; Arcturus, Mountain View, Calif). RNA was isolated with the PicoPure RNA isolation kit (Arcturus), followed by reverse transcription to cDNA with Riboamp HS PLUS cDNA kit (Arcturus), according to the manufacturer's instructions.

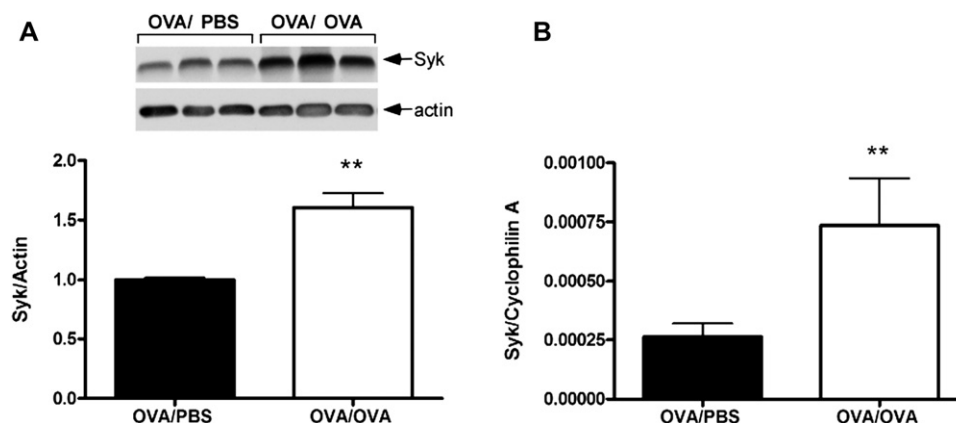


FIG 1. Syk expression is upregulated in the chronic OVA exposure model of allergic airways inflammation. **A**, Western blot analysis of lung homogenates revealed Syk protein expression to be upregulated 1.6-fold in lungs from the chronic OVA exposure model of allergic airways inflammation. *Top panel*, Representative blot; *bottom panel*, densitometry normalized to actin ($n = 6$ per group). **B**, Syk mRNA expression is upregulated 2.8-fold in the whole-lung homogenates from OVA/OVA mice ($n = 5$ per group). ** $P < .01$ compared with OVA/PBS control lungs.

Real-time PCR

Total RNA was extracted from the lungs and purified with the RNeasy Mini Kit. Aliquots (2 μ L) of first-strand cDNA, Power SYBR Green PCR Master Mix, and Syk or cyclophilin A primers (see the [Methods](#) section in this article's Online Repository) were mixed in a total reaction volume of 10 μ L. Quantitative RT-PCR was performed in triplicate by using the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, Calif).^{31,32} Expression of Syk and cyclophilin A was determined at the copy-number level by using standard curves generated from the respective plasmid cDNAs,^{31,32} and Syk expression was calculated relative to the cyclophilin A level in each sample.

BAL

Total and differential cell counts in the BAL fluid were assessed by 2 blinded examiners, as described previously.²⁹

Statistical analysis

All data are expressed as means $\pm$ SEMs. Binary comparisons were made by using the Student t test. Comparisons between multiple groups were made by using 1-way ANOVA, with *post hoc* comparison with the Dunn multiple test. Individual dose differences of the MCh dose-response relationship were compared by using 2-way ANOVA. All analyses were conducted with GraphPad Prism software (version 4.0c; GraphPad Software, La Jolla, Calif).

RESULTS

Syk expression in the OVA model of allergic airways inflammation

We initially investigated Syk expression in the acute, subacute, and chronic models of OVA-induced allergic airways inflammation, which exhibit primary phenotypes of airways inflammation, hyperresponsiveness, and remodeling, respectively.^{28,29,33} In the chronic model Syk protein expression was increased significantly 1.6-fold in the whole-lung homogenates from OVA-sensitized and OVA-challenged (OVA/OVA) mice compared with OVA-sensitized but PBS-challenged (OVA/PBS) control mice ([Fig 1, A](#)). Syk mRNA expression was similarly augmented 2.8-fold in the chronic OVA/OVA mice compared with that seen in the OVA/PBS mice ([Fig 1, B](#)). We did not observe any significant

changes in Syk protein expression in either the acute or subacute OVA models, in which airways inflammation based on BAL fluid cell counts was more prominent (see [Fig E2](#)). These observations suggest that the primary source of increased Syk expression in the chronic model likely arises from cell populations other than leukocytes. Subsequent studies were performed only in the chronic OVA model, in which increased Syk expression was observed.

To investigate which cell populations express Syk in the chronic OVA model, we assessed Syk expression using immunohistochemistry and fluorescence microscopy. A notable increase in Syk staining was observed throughout the lungs in OVA/OVA mice compared with that seen in the OVA/PBS control mice ([Fig 2](#)); staining was primarily localized to the airway epithelia in both the OVA/PBS and OVA/OVA mice, as well the peribronchial inflammatory cells in the OVA/OVA mice. To confirm the identity of the Syk-expressing cells, we performed costaining experiments with markers of airway epithelial cells using Clara cell protein 10 (CC-10). We observed costaining of Syk with CC-10 ([Fig 2, A](#)), confirming Syk expression in airway epithelia in the OVA/PBS and OVA/OVA mice. Quantitative PCR of RNA from airway epithelial cells isolated by using LCM (see [Fig E3](#) in this article's Online Repository at www.jacionline.org) demonstrated that Syk RNA expression was upregulated 11-fold in the chronic OVA model compared with that seen in the OVA/PBS control animals ([Fig 2, B](#)). In contrast to the 2.8-fold increase in Syk mRNA expression in whole lungs, this substantial enrichment of Syk mRNA expression in airway epithelial cells suggests the airway epithelia to be a major source of Syk in the allergically inflamed lung.

Acute administration of NVP-QAB-205 attenuates airways responsiveness to MCh in patients with established disease

Consistent with our previous reports,^{28,29} chronic OVA/OVA mice exhibited moderate increased MCh responsiveness of the total respiratory system (total respiratory resistance [R_{rs}]) compared with OVA/PBS control animals ([Fig 3](#)). Intratracheal administration of a single dose of the Syk inhibitor

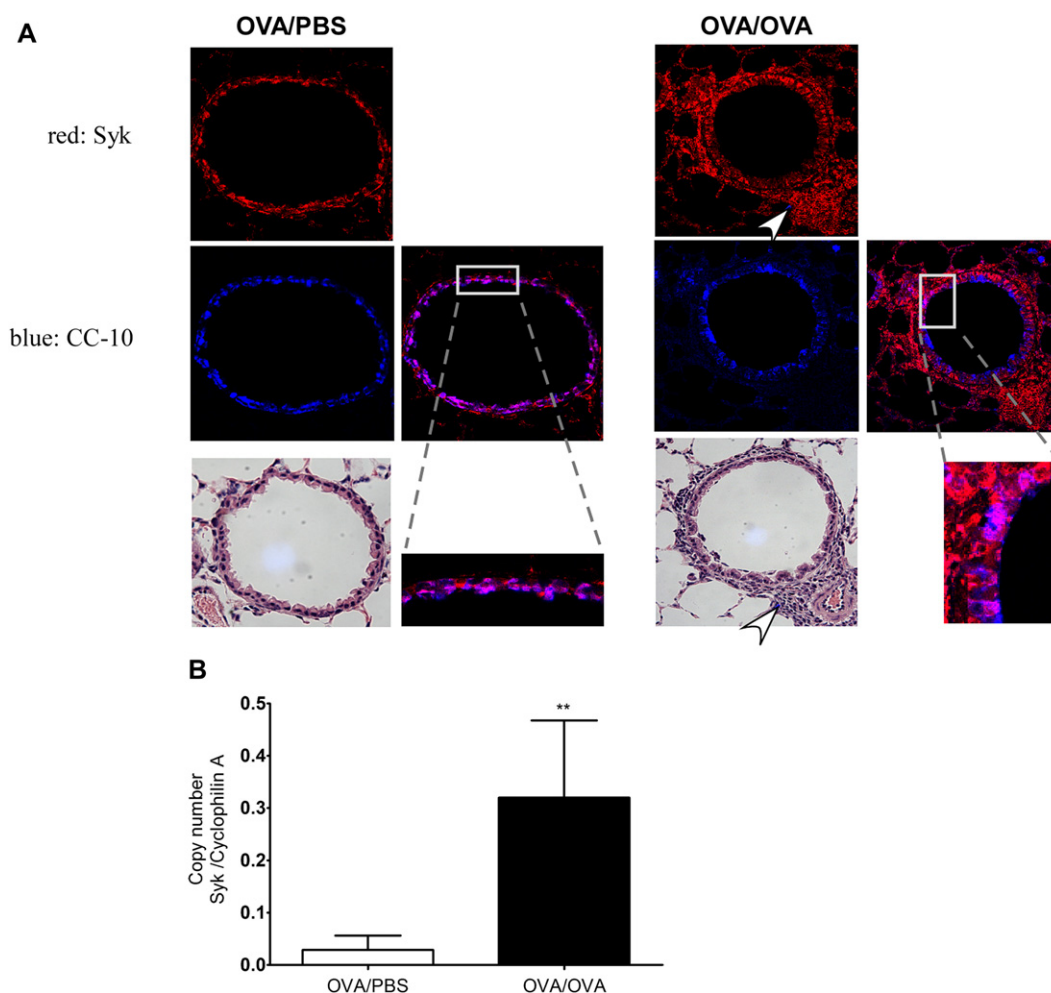


FIG 2. Syk expression is upregulated in the airway epithelia in the chronic OVA exposure model. **A**, Immunohistochemical staining showed a visible increase of Syk staining throughout the lungs in the chronic OVA model (OVA/OVA) compared with that seen in control OVA/PBS mice. Syk-positive (red) staining was observed in inflammatory infiltrates (arrowheads) in the OVA/OVA samples, as well as in the airway epithelium. Costaining with an antibody to CC-10 (blue), an airway epithelial cell marker, demonstrated colocalization of Syk in airway epithelial cells (purple, right lower panel inset). Images are representative of 3 independent experiments. **B**, Syk expression was significantly upregulated 11-fold in airway epithelial cells from OVA/OVA mice that were isolated by using LCM. ** $P < .01$ to OVA/PBS ($n = 6$ per group).

NVP-QAB-205 (0.3 or 3 mg/kg body weight) significantly reduced the increased R_{rs} , particularly at the maximum concentration of MCh evaluated (Fig 3, B). NVP-QAB-205 also reduced the central airways resistance (Newtonian resistance) to MCh in the OVA/OVA mice to levels observed in the OVA/PBS control animals (see Fig E4, A, in this article's Online Repository at www.jacionline.org). Although the tissue damping was significantly greater in the OVA/OVA mice compared with that seen in OVA/PBS mice, NVP-QAB-205 did not reduce the response to MCh (see Fig E4, B). Thus NVP-QAB-205 appeared to affect the airways alone. NVP-QAB-205 had no effect on respiratory mechanics parameters (ie, quasistatic compliance and elastance, total lung capacity, and the Salazar-Knowles parameters) or respiratory parameters in the OVA/PBS mice (data not shown). No differences were observed between the higher and lower doses of NVP-QAB-205. Therefore only the lower dose was used in subsequent studies to avoid off-target effects.

PM_{2.5} plus O₃ augments airways responsiveness to MCh in OVA/OVA mice, a response that is abrogated by NVP-QAB-205

After the OVA sensitization and challenge protocol, mice were exposed to FA or PM_{2.5} plus O₃ for 4 hours, as described in the Methods section. As expected, PM_{2.5} plus O₃ exposure enhanced the R_{rs} to MCh when compared with FA in the OVA/OVA mice, whereas no changes were observed in the OVA/PBS mice (Fig 4, A). Treatment of the OVA/OVA mice with 1 dose of NVP-QAB-205 after PM_{2.5} plus O₃ exposure reduced R_{rs} to levels less than those seen in OVA/OVA mice exposed to FA, approximating those observed in the OVA/PBS mice (Fig 4).

Effect of NVP-QAB-205 on airways inflammation

We found a significant increase in BAL fluid total cell counts in the OVA/OVA mice compared with those seen in the OVA/PBS control animals; no effect was observed after either PM_{2.5} plus O₃

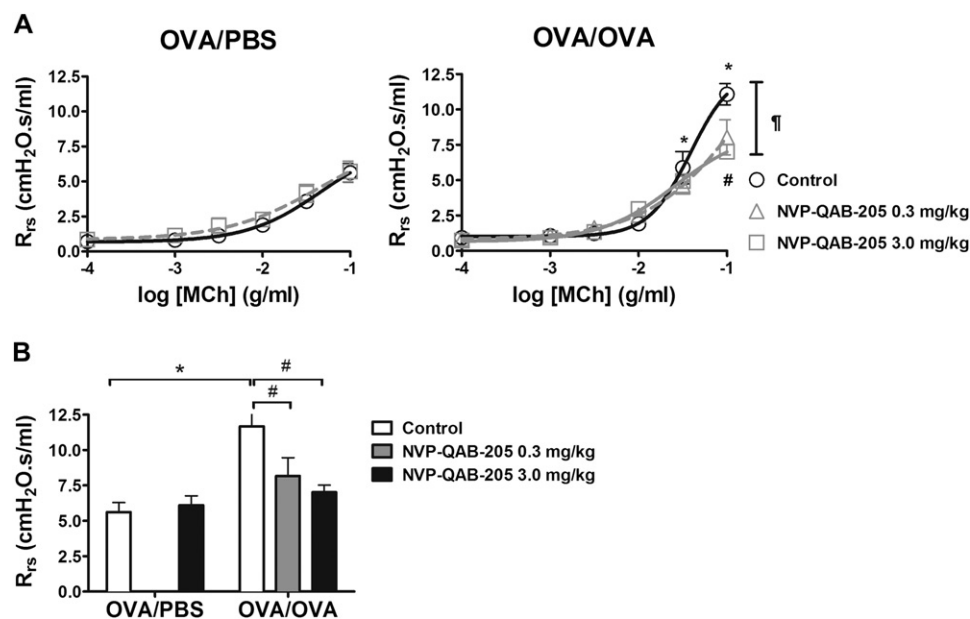


FIG 3. A single administration of the Syk inhibitor NVP-QAB-205 (0.3–3.0 mg/kg) significantly decreased R_{rs} in response to increasing doses of MCh (**A**) and the maximal response to MCh response (**B**) in the OVA/OVA mice compared with the OVA/PBS mice and vehicle control animals. * $P < .05$ to OVA/PBS control animals. # $P < .05$ to vehicle-treated animals by means of 1-way ANOVA. ¶ $P < .05$ to control animals by using the F-test ($n = 10$ –12 per group). Open bars, DMSO; solid bars, 3 μ g/g NVP-QAB-205; gray bar, 0.3 μ g/g NVP-QAB-205.

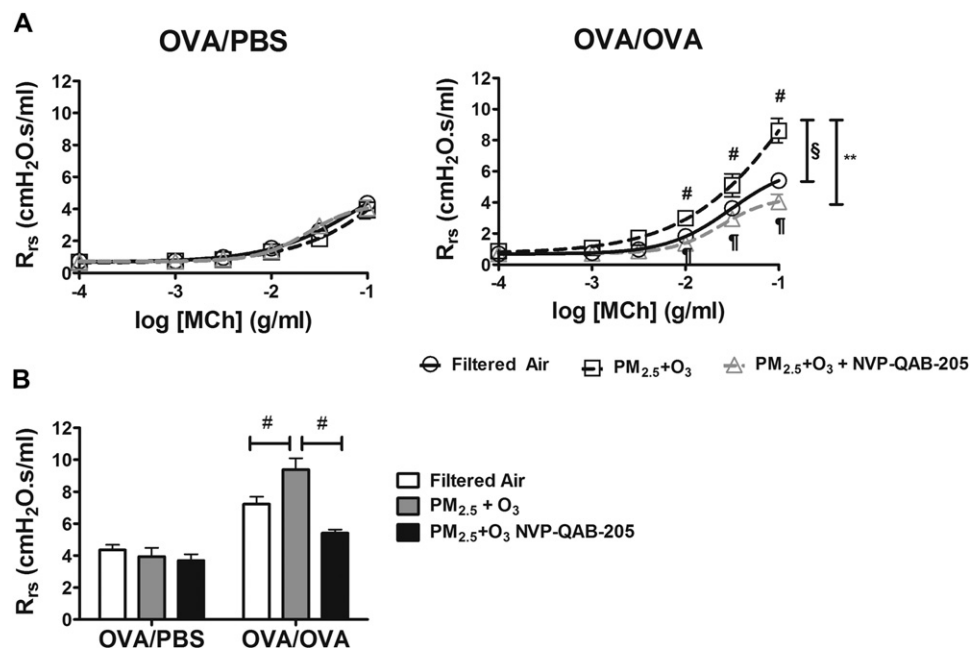


FIG 4. PM_{2.5} plus O₃ augments AHR to MCh in OVA/OVA mice, a response that is abrogated by NVP-QAB-205. The increased R_{rs} in response to increasing doses of MCh (**A**) and the maximal response to MCh challenge (**B**) were augmented by exposure to PM_{2.5} plus O₃ only in the OVA/OVA mice compared with values seen in the OVA/PBS control animals. The augmentation was restored to normal by treatment with NVP-QAB-205. # and ¶ $P < .05$ to filtered air and PM_{2.5} plus O₃, respectively, by means of either 1-way or 2-way ANOVA, where appropriate. § and ** $P < .05$ to filtered air and PM_{2.5} plus O₃, respectively, by using the F-test ($n = 10$ –12 per group).

exposure or NVP-QAB-205 administration (Fig 5, A). BAL differential cell counts revealed significant neutrophilia and eosinophilia in OVA/OVA mice, with no differences between

nonexposed, FA-exposed, and PM_{2.5} plus O₃-exposed mice (see Fig E5, A, in this article's Online Repository at www.jacionline.org).

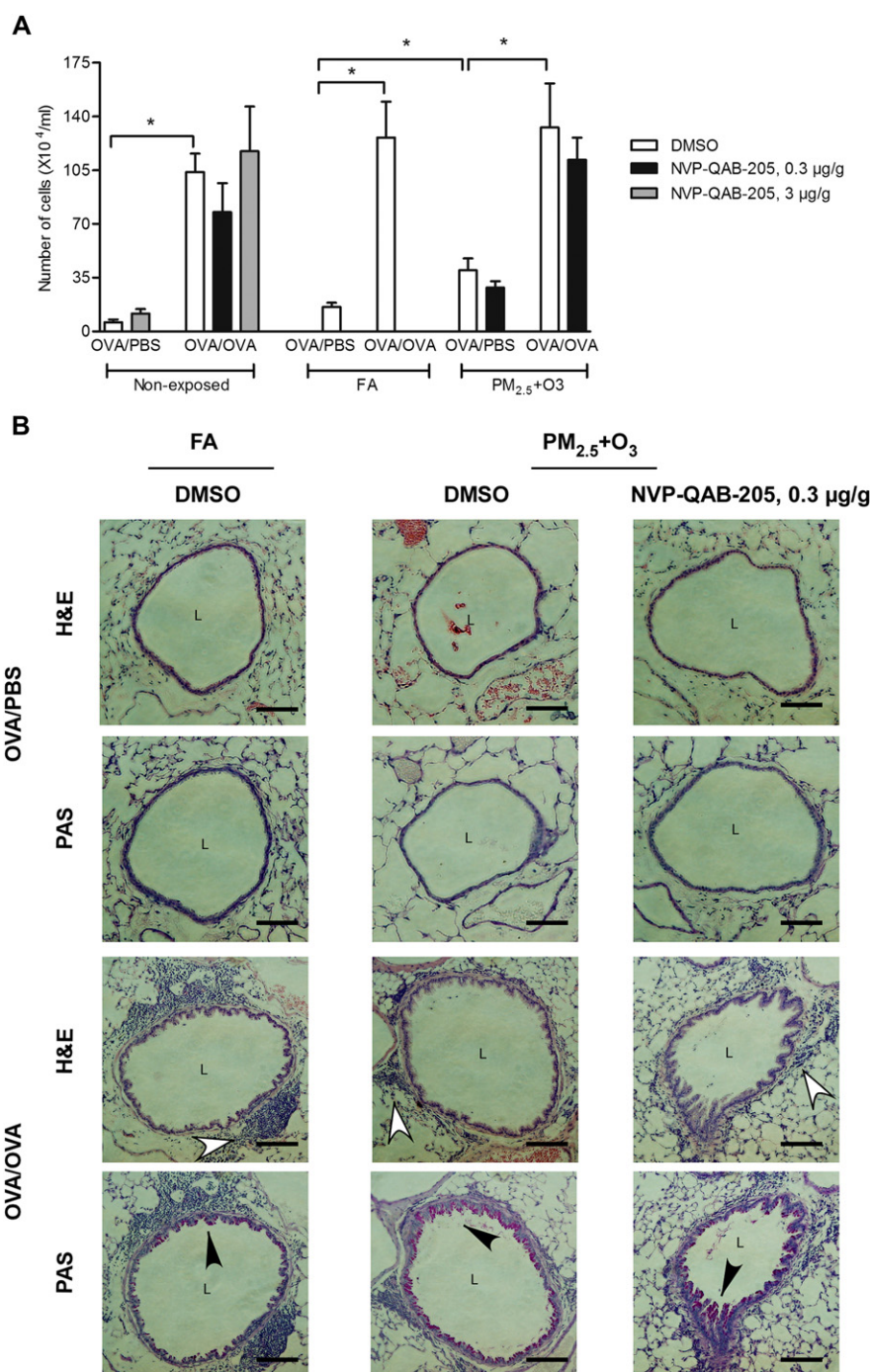


FIG 5. Syk inhibition and PM_{2.5} plus O₃ do not alter the BAL fluid total cell counts in the chronic OVA exposure model. **A**, Total BAL fluid cell counts were increased in the OVA/OVA mice when compared with those seen in the OVA/PBS mice. No significant changes were observed after PM_{2.5} plus O₃ exposure or treatment with NVP-QAB-205 in the OVA/OVA mice. In the OVA/PBS mice PM_{2.5} plus O₃ increased total cells counts, but this effect was not affected by NVP-QAB-205. **P* < .05 (*n* = 4-6 per group). **B**, Hematoxylin and eosin (H&E) and PAS images of FA-exposed versus PM_{2.5} plus O₃-exposed mice revealed increases in peribronchial inflammatory infiltrates (open arrowheads) and PAS-positive cells along the airway epithelial layer (solid arrowheads) in the OVA/OVA mice compared with the OVA/PBS mice, with no difference between the FA- and PM_{2.5} plus O₃-exposed mice or after treatment with NVP-QAB-205. Size bar = 100 µm. L, Airway lumen.

Histologic examination of lung sections revealed significant inflammatory cell peribronchial infiltrates along with increased PAS-positive cell numbers in the airways of OVA/OVA compared

with OVA/PBS mice (Fig 5, B) and are congruent with our previous findings in the chronic OVA-induced model of allergic airways inflammation.³³ Neither PM_{2.5} plus O₃ exposure nor

treatment with NVP-QAB-205 had any effect on the presence of inflammatory infiltrates or the presence of PAS-positive cells (Fig 5, B).

PM_{2.5} plus O₃ induced keratinocyte-derived chemokine and VEGF expression in the BAL fluid of mice

We specifically assessed production of inflammatory mediators that have previously been shown to be regulated by Syk^{20,21,34} and observed increased keratinocyte-derived chemokine (KC), an IL-8 mouse homolog, and VEGF concentrations in the BAL fluid from the PM_{2.5} plus O₃-exposed mice when compared with concentrations seen in the FA-exposed control animals (Fig 6). PM_{2.5} plus O₃ also increased IL-6 production; however, this increase was not statistically significant (data not shown). OVA/OVA mice exhibited high KC and VEGF expression in the FA exposure group, with a trend toward further enhanced expression after PM_{2.5} plus O₃ exposure. However, these changes were not statistically significant.

DISCUSSION

We report, for the first time, that inhibition of Syk *in vivo* with a treatment-based administration protocol in the setting of established disease abrogates AHR in this mouse model of chronic allergic airways inflammation. Syk inhibitors also attenuated the enhanced AHR induced by PM_{2.5} plus O₃, a common clinical trigger of asthma exacerbations. Furthermore, the increased Syk expression is localized primarily to the airway epithelial cells, and recruitment of inflammatory cells into the lungs was unaffected by drug treatment. These findings suggest that airways contractility and reactivity in asthmatic patients are modulated by communication between epithelial and smooth muscle cells through Syk.

Studies in human subjects have shown that airway remodeling occurs early in life and can precede clinical manifestation of asthma.³⁵⁻³⁷ To dissect the role for Syk in the asthma phenotype, we initially assessed Syk expression in 3 different mouse models of allergic airways inflammation by using OVA as the offending allergen: the subacute model, which is characterized by significant inflammatory cell influx but no AHR; the acute model, which exhibits significant airways inflammation, increased inflammatory cell infiltration in the airways, and robust AHR; and the chronic model, which is characterized by prominent airway remodeling and moderate AHR but less marked airways inflammation.^{28,29,33,38} Of these 3 models, only the chronic model exhibited significantly increased Syk expression in whole-lung homogenates. Although Syk was also detected in inflammatory cells, infiltration of inflammatory cells was less marked than in the subacute and acute models and, as such, would be less likely to be the primary source of the augmented Syk protein expression. Not unexpectedly, the single dose of NVP-QAB-205 did not affect the BAL fluid total or differential cell counts in the OVA/OVA mice. Subsequent histologic examination of the lungs and quantitative PCR on LCM specimens confirmed a significant increase in Syk expression in the airway epithelia of chronic OVA/OVA mice.

BAL fluid analysis revealed increases in KC and VEGF expression after PM_{2.5} plus O₃ exposure. Our studies with primary airway epithelial cells and PM_{2.5} collected at the time of

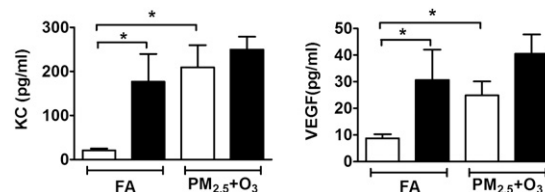


FIG 6. Production of KC and VEGF was increased in BAL fluid of OVA/OVA mice (solid bars) compared with that seen in OVA/PBS mice (open bars). PM_{2.5} plus O₃ only increased KC and VEGF production in OVA/PBS mice. **P* < .05 (*n* = 5-6 per group).

mouse exposures revealed that PM_{2.5} induced activation of Syk and phosphatidylinositol 3 (PI3) kinase (see Fig E6 in this article's Online Repository at www.jacionline.org) and that Syk mediated PM-induced activation of the transcriptional factors nuclear factor of activated T cells and nuclear factor κ B (NF- κ B; see Fig E7 in this article's Online Repository at www.jacionline.org), as well as production of the inflammatory mediators IL-8 and IL-6 (see Fig E8 in this article's Online Repository at www.jacionline.org). These *in vitro* studies offer some insights regarding the cellular mechanisms that are activated by PM exposure. Together, they suggest that the augmented epithelial cell expression of Syk is exquisitely poised to contribute to the communication between epithelial and smooth muscle cells in airways in the chronic model by modulating the airway responsiveness to MCh and regulation of inflammatory mediator production.

In the OVA model the use of the nose-only restraint for the exposure studies resulted in altered AHR under basal conditions (ie, when MCh responsiveness is compared between the OVA/OVA-DMSO control mice [Fig 3] and the OVA/OVA-FA control mice [Fig 4]). Stress resulting from restraint has previously been reported and found to induce a number of stress-response genes, including *GILZ*, a mediator of glucocorticoid-induced immunosuppression that acts in part through activation of NF- κ B.³⁹ It is notable that in the PM_{2.5} plus O₃-exposed OVA/OVA mice, treatment with the Syk inhibitor NVP-QAB-205 reduced the maximal R_s to levels less than those of the OVA/OVA FA-exposed control mice (Fig 4), levels comparable with those seen in the nonrestrained Syk inhibitor-treated OVA/OVA mice that were observed in Fig 3. Taken together, our observations would suggest that Syk plays a role in mediating AHR in this model of chronic airways inflammation and in the exacerbation of symptoms induced by air pollution.

Although previous investigations evaluated the role of Syk by using administration of R406^{24,25} and Syk ASO^{22,23} concomitant with acute and subacute allergen challenges and suggested a role for Syk inhibition in preventing the development of airways inflammation and AHR, we investigated the effects of NVP-QAB-205 after having established airways inflammation and AHR (ie, a treatment-based protocol that is more clinically relevant in the management of asthma and asthma exacerbations after pollution exposure). We also investigated AHR *in vivo* in contrast to previous studies in which AHR was assessed by using either isolated trachea^{22,24} or by characterizing the total resistance of the respiratory system.²⁵ Interestingly, our studies showed that a single dose of NVP-QAB-205 completely abrogated AHR in the setting of established disease, whereas the previous studies achieved reductions of approximately 70% despite prophylactic therapy. Differences in restoration of normal AHR might be

related to differences in potency/uptake between the inhibitors (ie, R406 and NVP-QAB-205 exhibit inhibitory concentration of 50% values of 46 and 10 nmol/L, respectively, against Syk activation *in vitro*) and accessibility/delivery of ASOs to the airway epithelial cells *in vivo*.

Several novel Syk inhibitors have shown promise in the treatment of inflammatory disorders in which tissue remodeling is prominent.⁴⁰ A phase 2 study with the Syk inhibitor R788 in 457 patients with rheumatoid arthritis demonstrated clinical efficacy and acceptable safety profiles when administered orally over 6 months.¹³ It is noteworthy that opportunistic infections were not observed as an adverse event, despite the importance of Syk as a regulator of innate immunity; common adverse events (nausea, diarrhea, hypertension, and neutropenia) were tolerable.¹³ Similar clinical efficacy and adverse events have also been reported in a phase 1/2 study of oral R788 in 82 patients with non-Hodgkin lymphoma and chronic lymphocytic leukemia.⁴¹

Topical instillation by means of inhalation is an established mode of drug delivery in the treatment of respiratory diseases, such as asthma and chronic obstructive lung disease. Adverse reactions are anticipated to be fewer with inhaled drugs because systemic absorption is expected to be lower. In a double-blind placebo-control trial of 317 patients with allergic rhinitis, nasal instillation of the Syk inhibitor R112 (3 μ g administered by means of a metered-dose pump every 4 hours $\times$ 2) was associated with few adverse events: only 1% of patients reported local irritation to the site of instillation, whereas symptoms were significantly reduced compared with those seen in control subjects.¹⁴ Other Syk inhibitors have recently been patented for topical delivery.⁴²

The current findings support the efficacy of Syk inhibitors in controlling AHR in this mouse model of chronic airways inflammation and suggest that topical Syk inhibitors, such as NVP-QAB-205, have potential as a new therapy for the management of asthma. We further posit that epithelial Syk expression and not inflammatory cell expression of Syk regulates the increased reactivity of the allergen-induced inflamed airway. Further investigations are needed to determine the specific role of Syk in the development of other characteristics of the asthma disease phenotype.

Key message

- Syk inhibitors have proved beneficial in clinical trials for rheumatoid arthritis. Our studies indicate that Syk inhibitors are potential therapeutic options in the management of asthma.

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METHODS

Mouse OVA sensitization and challenge model of allergic airway inflammation

Preliminary investigations used our subacute, acute, and chronic models of OVA sensitization and challenge, which manifest the primary phenotypes of airways inflammation, hyperresponsiveness, and remodeling, respectively,^{E1-E3} to determine changes in Syk expression. The subacute and acute models use sensitization to chicken OVA (25 µg of OVA administered intraperitoneally, endotoxin free, [grade V; Sigma Chemical] with 1 mg of aluminum hydroxide gel in 0.2 mL of PBS, pH 7.4 [Invitrogen, Grand Island, NY]) on days 0 and 7, followed by repeated daily exposures to nebulized 6% OVA or PBS (25 min/d) on days 14 to 16 and 14 to 20, respectively.

In the main study testing the role of Syk in the AHR of the chronic OVA exposure model, 60 female BALB/c mice aged 6 to 8 weeks (mean body weight, 21.4 g; Charles River Laboratory) underwent the chronic 12-week OVA sensitization and challenge protocol, as previously described.^{E2,E3} In brief, mice were sensitized to chicken OVA on days 0 and 7, as with the other 2 models. On day 14, mice were randomized to repeated exposure to nebulized 2.5% OVA in PBS or PBS alone for 2 consecutive days (25 min/d) every 2 weeks up to week 12 (ie, 2 consecutive days of exposure followed by 12 days of rest between exposures). Twenty-four hours after the last challenge with OVA or PBS, pulmonary function testing and MCh challenge were performed, just before death and tissue harvesting. These protocols were approved by the University of Toronto Faculty Advisory Committee on Animal Services and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Pulmonary function testing and MCh challenge

Twenty-four hours after the final OVA or PBS challenge, mice were anesthetized with ketamine (50 mg/kg administered intraperitoneally; Bioniche, Belleville, Ontario, Canada)–xylazine (10 mg/kg administered intraperitoneally; Bayer, Toronto, Ontario, Canada) and then intubated with an 18-gauge stainless steel cannula (BD Biosciences Canada, Mississauga, Ontario, Canada) for *in vivo* ventilator-based assessment of MCh responsiveness with use of the flexiVent system (Scireq). After baseline determination of airway resistance, mice were challenged with MCh (0–100 mg/mL in sterile PBS; Sigma) nebulized directly into the ventilatory circuit (AeroNebLab nebulizer). Two models of respiratory mechanics were used to assess lung resistance: the linear first-order single-compartment model, which provides resistance of the total respiratory system, and the constant-phase model, which uses forced oscillation to differentiate between central airway Newtonian resistance and peripheral tissue damping.^{E4,E5} Anesthesia was maintained throughout the experiment by means of repeated administration of 25% of the initial dose of ketamine–xylazine every 25 minutes until completion. All data points were collected by using the FlexiVent software and analyzed offline in Excel (Microsoft, Redmond, Wash).

Syk inhibition *in vivo*

NVP-QAB-205, a purine derivative, is a competitive inhibitor of ATP and was gift from GlaxoSmithKline. It is a Syk-selective inhibitor with an inhibitory concentration of 50% of 10 nmol/L,^{E6,E7} which is highly bioavailable when administered topically. For our studies, after determination of baseline respiratory mechanics in the OVA-sensitized and OVA- or PBS-challenged mice, 0.3 or 3 mg/kg NVP-QAB-205 (or DMSO vehicle) was delivered by means of nebulization directly into the ventilatory circuit 15 minutes before reassessment of pulmonary mechanics and initiation of the MCh dose-response curve.

Lung homogenization and Western blotting

Mouse lungs were homogenized 1:1 (wt/vol) in 0.1% Triton X-100 containing 0.7 µg/mL pepstatin A (Bioshop, Burlington, Ontario, Canada), 0.5 µg/mL antipain (Bioshop), and 2 mmol/L EDTA (pH 7.3). Insoluble debris was cleared by means of centrifugation at 20,000g for 15 minutes at 4°C. Protein concentrations were determined by using the bicinchoninic acid method (Pierce, Rockford, Ill). For Western blot analysis, 40 µg of tissue homogenate

was mixed directly with boiling Laemmli sample buffer. After electrophoresis on 10% polyacrylamide gels, proteins were transferred to nitrocellulose membranes, blocked with 5% BSA in PBS with 0.05% Tween-20, and incubated overnight at 4°C with primary antibody Syk (clone 01, Santa Cruz Biotechnologies). After washing, blots were incubated with horseradish peroxidase-conjugated anti-mouse at 1:10,000 dilution for 1 hour at room temperature. Proteins were visualized with the Western Lightning reagent (PerkinElmer Life Sciences, Boston, Mass), and membranes were exposed to X-Omat Blue XB-1 film (Kodak, Rochester, NY) in a Fluor-S Max MultiImager carrying a CCD camera operating at –35°C (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Blots were stripped (10% SDS, 1 mol/L Tris [pH 6.8], and 0.8% β-mercaptoethanol at 50°C for 25 minutes) and reprobed with mouse anti-mouse β-actin antibody (Sigma Chemical) to normalize protein loading. Densitometry was performed with GelEval 1.22 software (Frogdance Software, University of Dundee, Dundee, Scotland).

Immunohistochemical staining of mouse lung sections

Mouse lung tissue paraffin sections were prepared as described previously (Pathology Core Facility of the Centre for Modeling Human Disease).^{E2} Slides were visualized on an inverted Leica microscope (Leitz DMRB), and images were captured digitally with a micropublisher RTV 5.0 camera with QCapture image capture software (Quorum Technologies, Guelph, Ontario, Canada).

For heat-induced epitope retrieval, paraffin-embedded lung sections were treated with DakoCytomation Target Retrieval Solution Citrate pH 6 (DakoCytomation, Glostrup, Denmark) and immersed in xylene for 15 minutes to remove wax before staining. The dewaxed sections were rehydrated through a graded series of ethanol and blocked with BSA/milk in PBS buffer containing normal donkey serum (Jackson ImmunoResearch Laboratories, Bar Harbor, Me) for 30 minutes. Tissue sections were incubated overnight with rabbit polyclonal anti-Syk (clone N-19) antibody (1:100 dilution, Santa Cruz Biotechnology) and goat polyclonal anti-CC-10 antibody (1:400 dilution, Santa Cruz Biotechnology) at 4°C. After three 5-minute PBS washes, tissues were incubated with the donkey anti-rabbit IgG CY3-conjugated secondary antibody or donkey anti-goat IgG CY5-conjugated secondary antibodies, respectively, for 1 hour at room temperature. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories and used at 1:500 dilutions in blocking buffer. Controls were stained with secondary antibody alone. Fluorescence images were captured with a Zeiss LSM510 Laser Scanning Confocal Microscope driven by the LSM510 software package, and imported as .tiff images into Canvas X (ACD Systems International, Victoria, British Columbia, Canada).

Hematoxylin and eosin and PAS staining of lung sections were performed at the Pathology Core Facility of the Centre for Modeling Human Disease. In brief, paraffin-embedded lung tissue sections were deparaffinized with xylene and rehydrated by passing through a graded series of alcohol. For hematoxylin and eosin staining, sections were stained with Mayer hemalum solution, rinsed with 0.1% HCl solution, and differentiated in running water. Slides were stained with 0.5% aqueous eosin Y solution for 3 minutes, rinsed with water, and mounted. For PAS, hydrated lung sections were oxidized in 0.5% periodic acid solution. Slides were placed in Schiff reagent for 15 minutes, washed with lukewarm water, and counterstained with Mayer hematoxylin before mounting. Slides were then visualized on an inverted Leica DMIL microscope with an attached Olympus DP71 camera for image acquisition with ×4 and ×20 objectives.

LCM of airway epithelial cells

Frozen tissue sections were prepared by embedding lungs in Optimal Cutting Temperature Compound (Tissue-Tek, Sakura Finetek)–filled cryomolds. The molds were then placed in liquid nitrogen and processed at the Pathology Core Facility Pathology, Centre for Modeling Human Disease. Frozen tissue specimens were sectioned (8-µm thickness) with a cryostat (H/I Bright, OTF5000, Hacker Instruments) and collected onto charged slides before fixation and staining. Airway epithelial cells were then isolated by using LCM on the PixCell II System (Arcturus). mRNA was isolated from the epithelial cells by using the PicoPure RNA isolation kit (Arcturus), followed

by 1 round of linear amplification (Riboamp HS PLUS cDNA kit, Arcturus) and reverse transcription (Super-Script First-Strand Synthesis System; Invitrogen, Burlington, Ontario, Canada) with random hexamers, according to the manufacturer's instructions.

Real-time PCR

Total RNA from the lung tissue was extracted and purified with the RNeasy Mini Kit (Qiagen, Germantown, Md). By using random hexamers, 1 μ g of the total RNA was then reverse transcribed into the first-strand cDNA by using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). An aliquot (2 μ L) of the first-strand cDNA, Power SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, Calif), and primers were mixed in a total reaction volume of 10 μ L. The sequences for the Syk (accession no. NM 011518) primers used were as follows: forward primer 5'-AAATGCCCATCCCGTG-3' and reverse primer 5'-GTCAGCATAA GGGCTCTCG-3', as previously described.^{E8} Cyclophilin A (forward primer 5'-TGCTGGACCAACACAAACG-3' and reverse primer 5'-GCCTTCT TTCACCTTCCCAA-3') was used as a housekeeping gene.^{E9}

The quantitative RT-PCR was performed on samples in triplicate by using the ABI-Prism 7900 sequence detection system (Applied Biosystems). Expression levels of Syk and cyclophilin A were determined at the copy-number level by using standard curves generated from the respective plasmid cDNAs (obtained from Open Biosystems [Huntsville, Ala] and OriGene [Rockville, Md], respectively).

BAL

BAL was performed by using 2 instillations of 1 mL of PBS. BAL fluid recovery was recorded for each mouse and was generally greater than 90%. Total cell counts in the BAL fluid were determined with a hemacytometer using Trypan blue (Wisent, St Bruno, Quebec, Canada) exclusion. For differential cell counts, the cells were spun onto a slide by using the Cytospin and stained with Diff-Quik (Invitrogen Life Technologies). The results shown are the average of the differential cell counts obtained under light microscopy by 2 blinded examiners.

Cell culture and PM stimulation

Primary airway epithelial cells isolated from donor airways at the time of lung transplantation were obtained from donors with no history of smoking and underlying lung disease (gift of Dr S. Keshavjee, University Health Network). Cells were passaged in BEGM medium supplemented with BulletKit (Lonza, Mississauga, Ontario, Canada) to 80% confluence in T75 flasks and harvested with ReagentPack (Lonza), according to the manufacturer's instructions. Cells were then plated onto Transwell inserts (0.4- μ m pore size, Corning, Lowell, Mass) and cultured at the air-liquid interface in 1:1 BEBM/GIBCO DMEM-H-Glucose (Invitrogen) supplemented with the BEGM SingleQuots kit (Lonza) for 28 days before experimentation.

PM_{2.5} from Teflon filters collected during the mouse exposure studies was reconstituted by means of sonication (Ultrasonic, model no. 08849-00; Cole-Parmer, Vernon Hills, Ill) for 25 minutes in normal cell culture medium and exposed at 100 μ L/mL for 4 hours, a concentration and period that have previously been validated to be physiologically relevant.^{E10,E11} Supernatants, cell lysates, and nuclear extracts were collected for subsequent analyses.

Western blot analysis of whole-cell lysates has previously been described.^{E12,E13} Antibodies for Syk (clone 4D10) and phospho-Syk-Y^{525,526} were purchased from Santa Cruz Biotechnology, and those for phospho-PI3 kinase p85 (Tyr⁴⁵⁸)/p55 (Tyr¹⁹⁹) and PI3 kinase p85 were from Cell Signaling (Boston, Mass).

ELISA and Luminex analysis

Determination of IL-8, IL-6, and VEGF levels in BAL fluid was performed by using an ELISA, according to the manufacturer's instructions. The kits for IL-8 (Hu IL-8/NAP-1), IL-6, and VEGF (Hu VEGF) were from Mediorp

(Montreal, Quebec, Canada). Luminex assays were performed according to manufacturer's instructions by using a 3-plex Milliplex MAP Mouse Cytokine/Chemokine kit (Millipore, Billerica, Mass). Data were acquired on a Luminex 200 with the Luminex XY plate handling platform and the Luminex SD sheath fluid delivery system and analyzed with 3.1 xPonent and MILLIPLEX Analyst software.

Electrophoretic mobility shift assay

Nuclear fractions were harvested from cell lysates by using the EpiQuik Nuclear Extraction Kit I (Epigentek; Cedarlane Laboratories, Mississauga, Ontario, Canada), and electrophoretic mobility shift assays for NF- κ B and nuclear factor of activated T cells were performed by using kits from Signosis (Sunnyvale, Calif), according to the manufacturer's instructions.

Statistical analysis

All data are expressed as means $\pm$ SEMs. Binary comparisons were made with the Student *t* test, and multiple comparisons were made with 1-way or 2-way ANOVA, as appropriate. Dose-response relationships to MCh were fitted to a sigmoidal variable-slope equation. Comparisons of baseline resistance measures, maximum responses, median effective concentration values, and Hillslopes were used to compare the fit of the dose-response curves by using the F-test, and effects at each dose were compared by using 2-way ANOVA. All analyses were conducted with GraphPad Prism software (version 4.0c, GraphPad Software).

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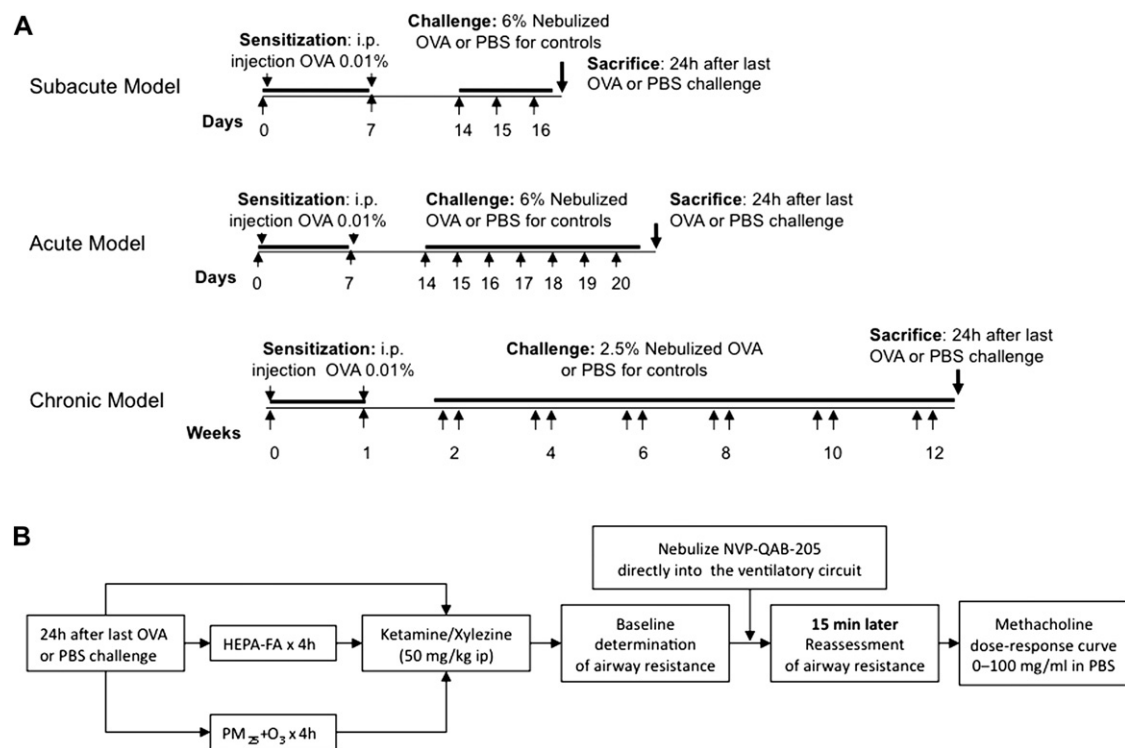


FIG E1. Induction of the 3 OVA exposure models of allergic airways inflammation and timeline of exposure and interventions.

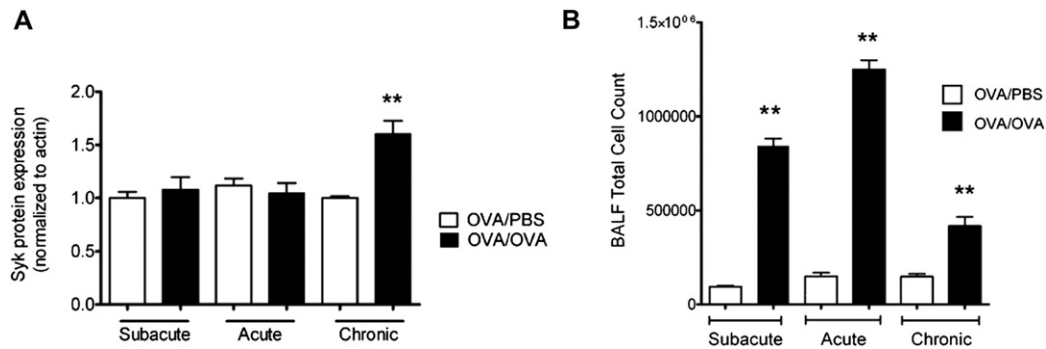


FIG E2. Syk expression and BAL fluid cell counts in the 3 OVA exposure models of allergic airways inflammation. **A**, Syk protein expression in the acute, subacute, and chronic models of OVA-induced allergic airways inflammation was assessed by using Western blotting.^{E2,E3} Syk protein expression was only increased in the chronic OVA model lungs (1.6-fold compared with the lungs from the OVA-sensitized but PBS-challenged control mice). ** $P < .05$ ($n = 6$ per group). **B**, In the acute and subacute models, in which airways inflammation, as manifested by increases in BAL fluid (BALF) total cells counts, is more prominent than in the chronic model, we did not observe any significant changes in Syk protein expression in the OVA/OVA mice compared with that seen in the OVA/PBS control mice. ** $P < .01$ compared with OVA/PBS control mice in the same model ($n = 6$ per group). These observations suggest that the increased Syk expression in the chronic model likely arises from cell populations other than leukocytes.

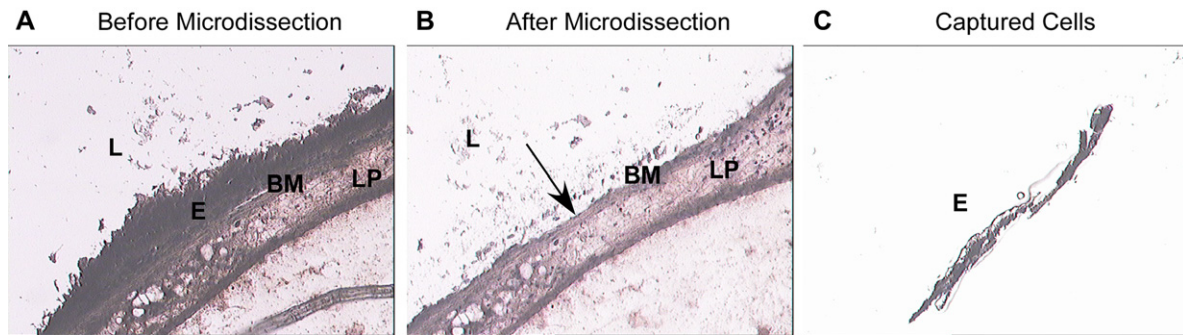


FIG E3. LCM of lung sections. Images of a mouse lung section before (**A**) and after (**B** and **C**) LCM show efficient capture of airway epithelial cells (*E*) and differentiation from lung parenchyma (*LP*) and basement membrane (*BM*). *L*, Airway lumen. Results are representative of lung sections from 12 mice.

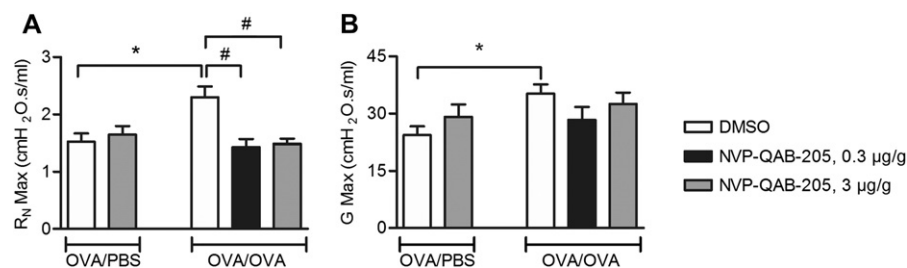


FIG E4. Syk inhibition abrogates AHR to MCh in the chronic OVA-challenge model. **A**, The central airways resistance (R_N) to MCh was increased in the OVA/OVA mice compared with that seen in OVA/PBS control mice. A single dose of NVP-QAB-205 was sufficient to decrease R_N to levels observed in the OVA/PBS control mice. **B**, Although the tissue damping (G) was significantly higher in the OVA/OVA mice compared with that seen in the OVA/PBS mice, NVP-QAB-205 did not reduce the response to MCh. * $P < .05$ to OVA/PBS control mice. # $P < .05$ to vehicle-treated animals by means of 1-way ANOVA ($n = 10$ -12 per group).

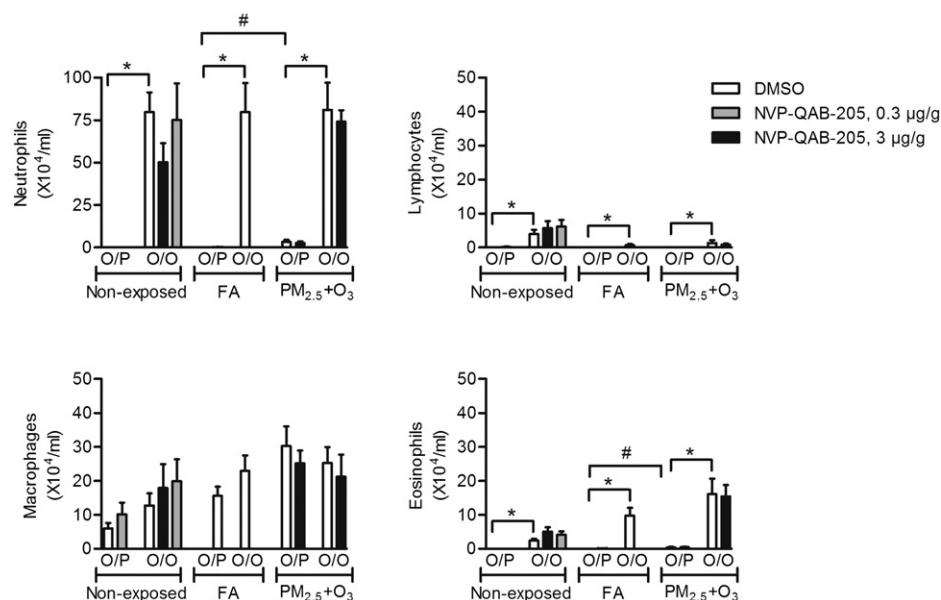


FIG E5. NVP-QAB-205 and $\text{PM}_{2.5}$ plus O_3 had no effect on the BAL fluid differential cell counts of OVA/OVA mice. The increase in total BAL fluid cell counts observed in the OVA/OVA mice was primarily due to increases in neutrophil and lymphocyte counts. Administration of NVP-QAB-205 (both 0.3 mg/kg and 3 mg/kg) did not alter the total cell counts or differential cell populations when compared with those seen in OVA/PBS mice. Similarly, no significant changes in total or differential cell counts were observed after $\text{PM}_{2.5}$ plus O_3 exposure in OVA/OVA mice. However, $\text{PM}_{2.5}$ plus O_3 induced a significant increase in total cell counts, as well as in neutrophil and eosinophil counts, in OVA/PBS mice. * $P < .05$ to OVA/PBS mice. # $P < .05$ to FA control mice, same treatment ($n = 4$ -6 per group). O/O, OVA/OVA; O/P, OVA/PBS.

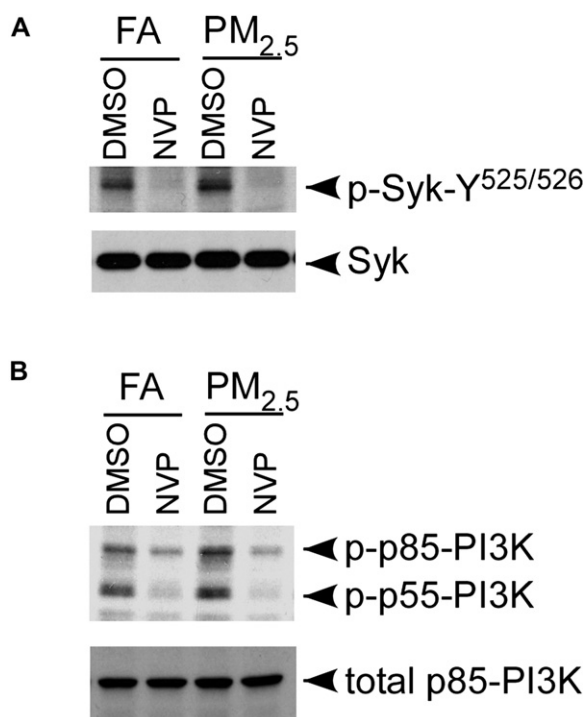


FIG E6. PM_{2.5} induces Syk activation and Syk-mediated PI3 kinase phosphorylation in human airway epithelial cells. To gain a better insight on the potential underlying regulatory mechanisms involved, we used an *in vitro* cell culture model with PM_{2.5} collected during the *in vivo* mouse exposures and primary human airway epithelial cells. PM_{2.5} (100 μ g/mL) was applied for 4 hours to the apical surface of cells grown at an air-liquid interface. Cell lysates were harvested at 24 hours and subjected to Western blot analysis with specific antibodies, as indicated. Phospho-Syk-Y^{525/526} is the activated form of Syk. **A**, PM_{2.5} induces phosphorylation of Syk and is abrogated by treatment with the Syk inhibitor NVP-QAB-205. **B**, PM_{2.5} induced phosphorylation of the p85 subunit of PI3 kinase, a response that was abrogated by NVP-QAB-205. Densitometry revealed a 1.3-fold in both the phospho-Syk-Y^{525/526}/Syk and phospho-p85/PI3 kinase ratios in the PM_{2.5}-exposed cells when compared with those seen in FA controls in the DMSO-treated cells. $P < .05$ ($n = 3$). NVP, NVP-QAB-205 (2 μ mol/L).

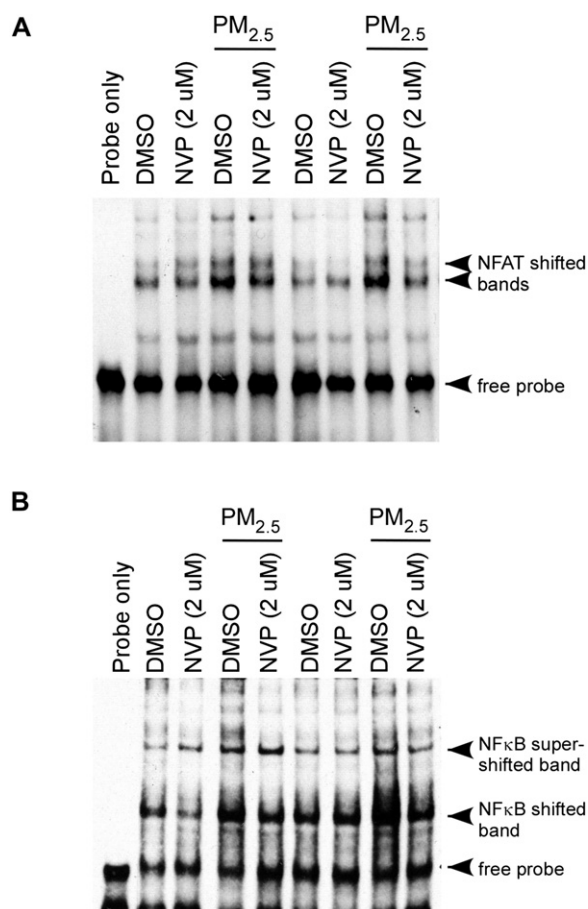


FIG E7. PM_{2.5} induces Syk-dependent transcriptional factor activation in primary airway epithelial cells. Primary airway epithelial cells were exposed to PM_{2.5}, as described above. Nuclear extracts were harvested and subjected to electrophoretic mobility shift assay. PM_{2.5} induced a significant shift in the nuclear factor of activated T cells (*NFAT*) and nuclear factor κ light chain enhancer of activated B cells (*NFκB*) bands, indicating activation of the transcriptional factors. Treatment with 2 μ Mol/L NVP-QAB-205 attenuated the PM_{2.5}-induced shift of both *NFAT* and *NFκB* when compared with DMSO. DMSO had no effect in either the nonexposed nor PM_{2.5}-exposed cells. Results are representative of 3 independent experiments.

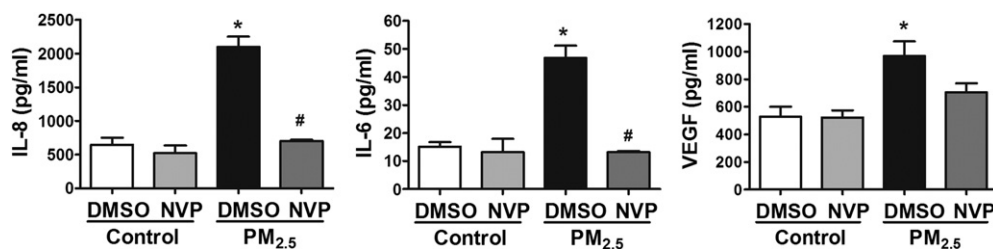


FIG E8. PM_{2.5} activates airway epithelial cells and induces Syk-dependent expression of IL-6 and IL-8. Primary human airway epithelial cells grown at the air-liquid interface were exposed at the apical surface to 100 μ g/mL PM_{2.5} (collected at time of the *in vivo* exposures) for 4 hours. Basal media were collected 24 hours after exposure and analyzed by using ELISAs, Luminex assays, or both. PM_{2.5} induced significant expression of IL-6, IL-8, and VEGF at 24 hours compared with that seen in nonexposed control cells. * $P < .05$ ($n = 3$). NVP-QAB-205 (2 μ mol/L) had no effect on FA-exposed control cells but reduced PM_{2.5}-induced IL-6 and IL-8 expression compared with that seen in cells treated with the vehicle DMSO. # $P < .05$ ($n = 3$). There was a trend toward reduced VEGF expression after treatment with NVP-QAB-205, but this was not statistically significant.