

Gastrin-releasing peptide blockade as a broad-spectrum anti-inflammatory therapy for asthma

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Edited* by Susan E. Leeman, Boston University School of Medicine, Boston, MA, and approved December 28, 2010 (received for review October 12, 2010)

Gastrin-releasing peptide (GRP) is synthesized by pulmonary neuroendocrine cells in inflammatory lung diseases, such as bronchopulmonary dysplasia (BPD). Many BPD infants develop asthma, a serious disorder of intermittent airway obstruction. Despite extensive research, early mechanisms of asthma remain controversial. The incidence of asthma is growing, now affecting >300 million people worldwide. To test the hypothesis that GRP mediates asthma, we used two murine models: ozone exposure for air pollution-induced airway hyperreactivity (AHR), and ovalbumin (OVA)-induced allergic airway disease. BALB/c mice were given small molecule GRP blocking agent 77427, or GRP blocking antibody 2A11, before exposure to ozone or OVA challenge. In both models, GRP blockade abrogated AHR and bronchoalveolar lavage (BAL) macrophages and granulocytes, and decreased BAL cytokines implicated in asthma, including those typically derived from Th1 (e.g., IL-2, TNF α), Th2 (e.g., IL-5, IL-13), Th17 (IL-17), macrophages (e.g., MCP-1, IL-1), and neutrophils (KC = IL-8). Dexamethasone generally had smaller effects on all parameters. Macrophages, T cells, and neutrophils express GRP receptor (GRPR). GRP blockade diminished serine phosphorylation of GRPR with ozone or OVA. Thus, GRP mediates AHR and airway inflammation in mice, suggesting that GRP blockade is promising as a broad-spectrum therapeutic approach to treat and/or prevent asthma in humans.

gastrin-releasing peptide receptor phosphorylation | mouse models | bombesin

Inflammation is a universal process of host defense whereby leukocytes react to noxious stimuli including microorganisms, toxins, and mechanical stress. However, the host response itself can be the primary cause of tissue injury, as in viral hepatitis or rheumatic fever (1).

Despite extensive research on mechanisms and treatment of asthma, it remains unclear why asthma is increasing in incidence, afflicting 300 million people worldwide (2), with ~30 million in the United States, where it causes 5,000 deaths annually (3). Despite optimal medical management, many asthmatics are treatment-resistant and/or have progressive disease (4). For example, β -agonists can paradoxically cause clinical decline (5). Disease diversity is attributed to gene–environment interactions, potentially involving hundreds of asthma-associated genes (2), but few are related to known mechanisms of airway disease.

Many acute and chronic inflammatory lung diseases are associated with pulmonary neuroendocrine cell (PNEC) hyperplasia and/or elevated levels of PNEC-derived peptides (6). Approximately 50% of infants with BPD later develop asthma (7, 8), a serious disorder of intermittent airway obstruction, with the greatest resistance arising in small airways. Asthmatic exacerbations can be triggered by allergens, pollution, or infections (2), leading to structural remodeling (9) involving mast cells, eosinophils, and Th2 lymphocytes (CD4⁺ T cells) (10), which produce cytokines and mediators (11). Neuropeptides from sensory nerve fibers, such as substance P, can elicit “neurogenic inflammation” (12), mainly linked to neutrophilic inflammation and vascular leakage, possibly contributing to bronchoconstriction (13, 14). Cigarette smoke elicits nonspecific AHR to substance P in guinea pigs, which is of unclear significance to asthma in humans (15).

PNECs are airway epithelial cells that secrete bioactive neuropeptides including gastrin-releasing peptide (GRP), homologous to amphibian bombesin. In lung, only PNECs produce GRP (16). GRP is present at high levels in human and mouse fetal lung (17, 18), and GRP regulates normal development (18, 19). However, excessive GRP can promote disease (20). Several observations led to our hypothesis that GRP contributes to asthma. Bombesin and GRP are potent, immediate bronchoconstrictors, 10-fold more potent than substance P and 100-fold more than histamine in vitro (21, 22). In guinea pigs, systemic immunization elicits PNEC hyperplasia, and PNEC degranulation follows aerosol challenge (23). Adults with primary idiopathic PNEC hyperplasia have secondary increased AHR (24). Children with primary PNEC hyperplasia, termed neuroendocrine cell hyperplasia of infancy (NEHI), have air trapping and elevated airways reactivity (25). GRP levels double in premature newborns that later develop BPD (6, 20, 26). GRP blockade abrogates acute and chronic lung injury in baboon models of BPD (6, 20). GRP as a mediator of lung injury in BPD is relevant to asthma because BPD patients have ~5- to 10-fold increased risk for developing asthma (7, 8).

We reasoned that asthmatics with PNEC hyperplasia could have symptoms unresponsive to conventional treatment. Classical inflammatory responses in asthma include both the innate and adaptive immune systems (2), triggered by allergens, irritants, smoke, viruses, and other agents. GRP induces host responses typical of asthma, including mast cell chemotaxis, macrophage migration, and proliferation of T cells and fibroblasts (27–31). GRP receptor (GRPR) is expressed by peribronchiolar fibroblasts (32). All these cell types contribute to acute and/or chronic asthma (2).

We tested our hypothesis that GRP mediates asthma by using two distinct mouse models of AHR and airways inflammation: ovalbumin (OVA)-induced AHR and eosinophilic inflammation as a model for allergic asthma, and ozone-induced AHR and neutrophilic inflammation as a model for asthma triggered by air pollution (33). Asthma is a human disease defined functionally (physiologically) as intermittent airway obstruction. We used two different GRP blocking agents in studying responses to air pollution versus allergen.

Results

We tested whether GRP blockade alters AHR and airways inflammation by using BALB/c females, which respond to both O₃ and OVA (34, 35). Females are preferred because they have higher levels of X-linked GRPR (36). A *flexiVent* apparatus was used to carry out PFTs on mice previously exposed to O₃

Author contributions: S.Z., E.N.P., W.M.F., and M.E.S. designed research; S.Z. and E.N.P. performed research; F.C. contributed new reagents/analytic tools; S.Z., E.N.P., W.M.F., and M.E.S. analyzed data; and S.Z. and M.E.S. wrote the paper.

Conflict of interest statement: S.Z., E.N.P., W.M.F., and M.E.S. have submitted a patent application to the Duke Ventures office, but outside contacts have not yet been made.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1014792108/-DCSupplemental.

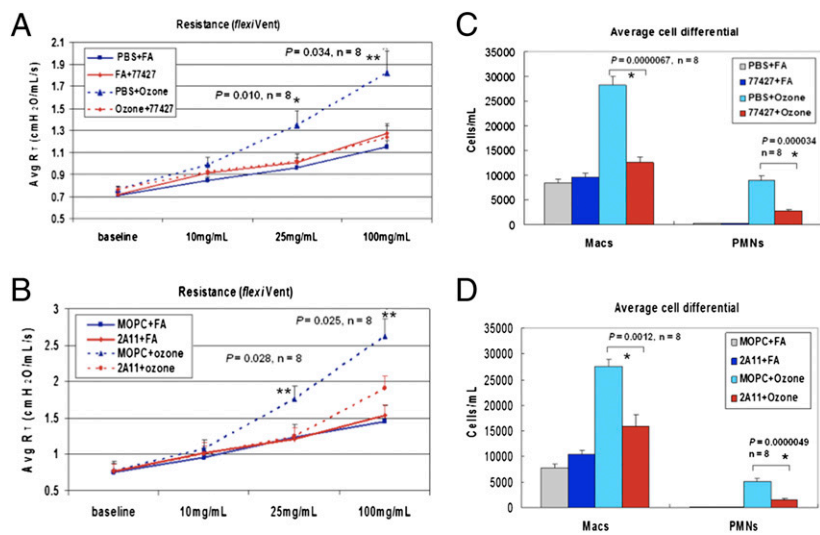


Fig. 1. GRP blockade abrogates O₃-induced AHR and inflammation. (A and B) PFTs on BALB/C mice exposed to O₃ or FA, using the flexiVent system. Mice given 77427 (A) or 2A11 (B) before O₃ had decreased airway resistance at higher MCh doses (25 or 100 mg/mL). **P* < 0.01, ***P* < 0.05, *n* = 8. (C and D) Pretreatment with 77427 (C) or 2A11 (D) reduced numbers of BAL macrophages and PMN induced by O₃. **P* < 0.01, *n* = 8.

(34). These models have been optimized and validated in multiple publications (34). We observed significant O₃-induced AHR, with airway resistance increasing from ~0.7 up to 1.8–2.6 cm H₂O/mL/s (Fig. 1 and Fig. S1), representing 157–271% increased airway resistance over filtered air (FA) controls at baseline. This model has been used successfully in multiple laboratories (34, 37, 38). A recent report (39) showed the kinetics (6–48 h after exposure) of inflammation and robust AHR development to methacholine (MCh) 24 h after O₃.

Dose–response studies showed that 500 nM 77427 is optimal for PFTs and BAL cell analysis (Fig. S1). Subsequently, mice received 77427 (500 nM IP, *n* = 8 per experiment) or vehicle (PBS, *n* = 8). Half the groups were exposed to O₃ (77427+O₃, *n* = 4; PBS+O₃, *n* = 4) or FA (77427+FA, *n* = 4; PBS+FA, *n* = 4). Data from two experiments are pooled in Fig. 1. Mice given PBS+O₃ had increased AHR (*P* = 0.011 at 25 mg/mL MCh and *P* = 0.0035 at 100 mg/mL MCh, compared with PBS+FA). In contrast, 77427 given before O₃ abrogated AHR (*P* = 0.010 at 25 mg/mL MCh and *P* = 0.034 at 100 mg/mL, comparing PBS+O₃ to 77427+O₃). Thus, 77427 normalized O₃-increased airway resistance (Fig. 1A) and associated decreased compliance (Fig. S2).

To validate the GRP specificity of 77427 effects, identical experiments were performed by using monoclonal anti-GRP/

bombesin IgG1 antibody (2A11) or isotype-matched IgG1 negative control, MOPC21 (MOPC). Shown in Fig. 1B, O₃-induced AHR was elevated in mice given MOPC (*P* < 0.05 at 25 mg/mL MCh and *P* < 0.01 at 100 mg/mL MCh), whereas 2A11 reduced AHR (*P* < 0.03 comparing MOPC+O₃ to 2A11+O₃). Decreased compliance in O₃+FA mice was also normalized by 2A11, but this increase was only a trend (*P* = 0.08–0.10) (Fig. S2). We then quantified BAL cells from O₃-exposed mice as a measure of the intensity of airway inflammation. Mice given 77427 (Fig. 1C) or 2A11 (Fig. 1D) had fewer macrophages and neutrophils (PMN) than controls (PBS or MOPC, *P* < 0.002). MOPC is the ideal isotype (IgG1)-matched negative control for 2A11, controlling for nonspecific protein binding and effects from Fc-gamma receptor binding (40, 41).

To determine whether O₃-induced AHR is mediated via GRPR, we compared GRPR-KO mice and WT littermates. Whereas WT mice respond with elevated AHR 24 h after O₃ exposure, GRPR-KO mice do not respond above baseline to O₃ (Fig. S1C).

We then tested whether GRP has a role in the OVA model of allergic airways disease (35). OVA-immunized mice given PBS followed by OVA challenges (OVA/OVA) had the greatest increase in AHR to MCh at 25 and 100 mg/mL (*P* = 0.01 and 0.00004, respectively; Fig. 2A). The 77427 given IP once before

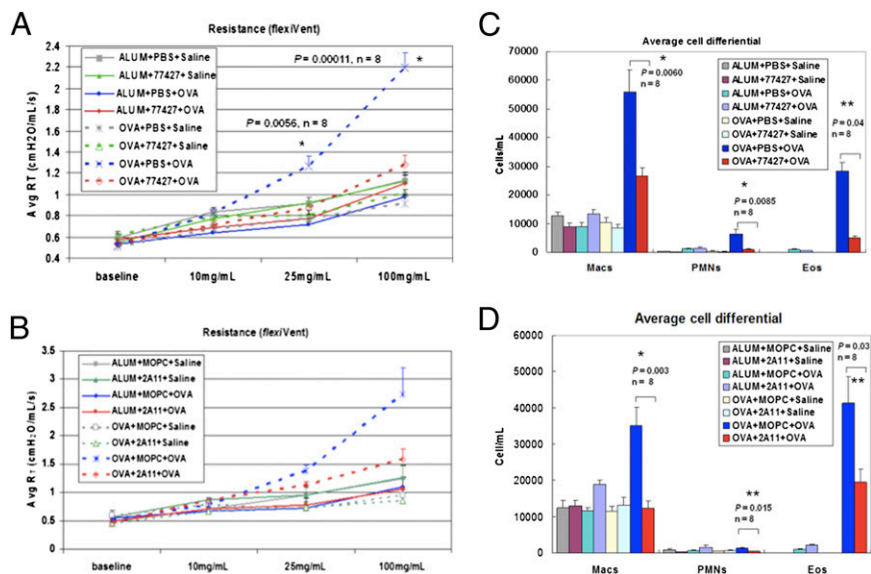


Fig. 2. GRP blockade abrogates OVA-induced AHR and inflammation. (A and B) Pretreatment with 77427 (A) reduced airway resistance to MCh induced by OVA down to baseline. **P* < 0.01, *n* = 8. (B) Treatment with 2A11 showed a similar pattern of responsiveness that was a statistical trend (0.10 > *P* > 0.05). (C and D) OVA/OVA mice pretreated with 77427 (C) or 2A11 (D) had fewer BAL macrophages, PMN, and eosinophils. **P* < 0.01, ***P* < 0.05, *n* = 8.

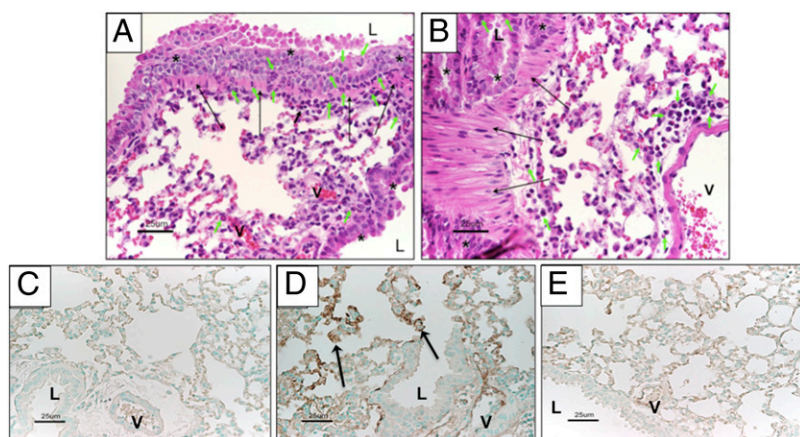


Fig. 3. Lung histopathology and IHC in mouse lungs exposed OVA with or without GRP blockade. (A) Mice treated with OVA/OVA had inflammatory infiltrates with PMN, mononuclear cells, and eosinophils (short green arrows) throughout the airway epithelium (asterisks) and smooth muscle (long arrows) (H&E). (B) In contrast, OVA-immunized mice given 77427 before the first OVA aerosol challenge had rare inflammatory cells (green arrows) in airway epithelium (asterisks) or smooth muscle (long black arrows), except for perivascular infiltrates and a few cells outside airway smooth muscle (H&E). (C) GRP IHC in control mouse lungs treated with Alum/PBS/Saline. (D) GRP IHC of mouse lungs exposed to OVA/PBS/OVA, showing strong GRP signal in cells lining the alveolar ducts (black arrows). The same distribution of staining was observed for the general NE marker, PGP9.5. (E) In parallel to Fig. 3D, GRP IHC was performed by using anti-GRP antibody preabsorbed with excess GRP peptide. (Scale bars: 25 μm .) L, airway lumen; V, vascular lumen.

the OVA challenges reduced OVA/OVA-induced AHR to baseline levels ($P < 0.006$ comparing OVA/PBS/OVA to OVA/77427/OVA; $P > 0.175$ comparing OVA/77427/OVA to other experimental groups). In contrast, 77427 given during the sensitization phase (D1, D7, D14) had no effect on OVA/OVA-induced AHR in BALB/c mice.

Specificity of 77427 for GRP was validated in the OVA model by using 2A11 as an independent GRP-blocking agent. The 2A11 decreased AHR to baseline, whereas MOPC did not (Fig. 2B). This 2A11 effect was a trend compared with MOPC ($0.10 > P > 0.05$). Decreased compliance in OVA/OVA mice was normalized by 77427 or 2A11 (Fig. S3). BAL inflammatory cells were quantified. Mice given 77427 (Fig. 2C) or 2A11 (Fig. 2D) had fewer macrophages, PMN, and eosinophils than OVA/OVA-treated controls ($P < 0.05$ or $P < 0.01$).

Lung histopathology typical of asthma was observed in OVA/OVA mice (Fig. 3A), with eosinophils, mononuclear cells (lym-

phocytes+monocytes), and few PMN infiltrating airway epithelium and smooth muscle. In contrast, OVA/OVA mice given 77427 (Fig. 3B) had infrequent inflammatory cells in airway epithelium or smooth muscle, but did have small perivascular infiltrates. To determine whether GRP might be visible in OVA/OVA lung sections, we carried out GRP immunohistochemistry (IHC). Alum/PBS/Saline negative controls had no detectable GRP (Fig. 3C), whereas OVA/OVA mice had prominent GRP IHC of many cells lining alveolar ducts (Fig. 3D). Another neuroendocrine marker, PGP9.5 (cytoplasmic), was observed in the same staining pattern in all OVA-immunized mice, not appreciably affected by OVA challenge or by 77427. GRP IHC of OVA/OVA lungs using GRP-preabsorbed GRP antibody had reduced IHC (Fig. 3E).

Using a multiplex assay of BAL fluid, we determined the absolute levels of 20 cytokines implicated in asthma. With O₃, 77427 or 2A11 decreased 19/20 cytokines ($P < 0.003$) (Fig. 4,

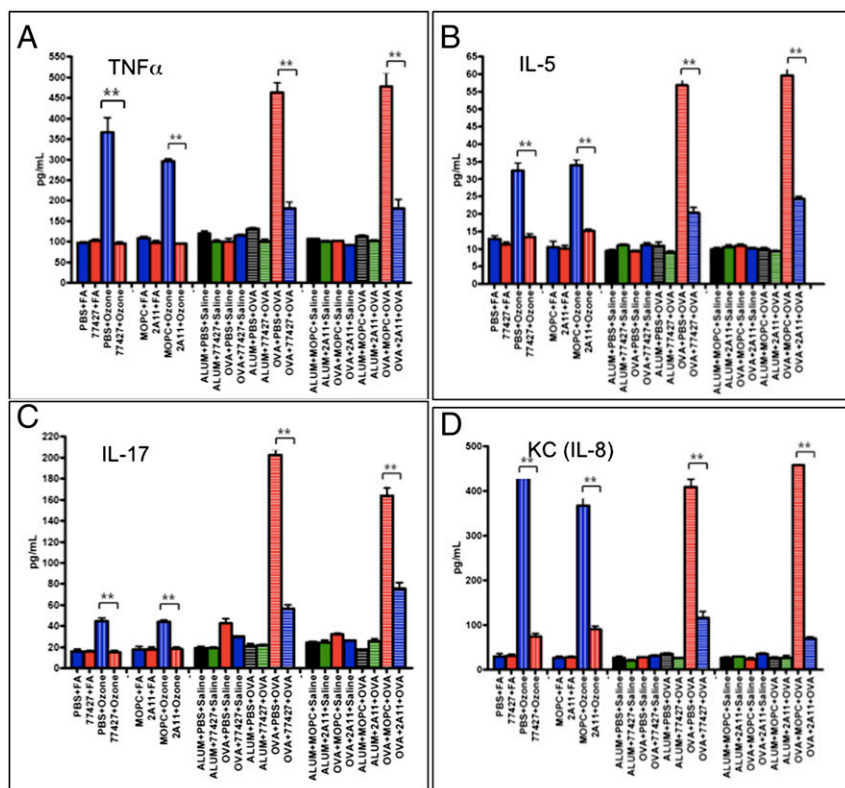


Fig. 4. GRP blockade decreases O₃- or OVA-induced BAL cytokine levels. Quantitative multiplex immunoassays show that GRP blockade by 77427 or 2A11 decreased O₃- or OVA-induced BAL fluid cytokine levels, represented here by a Th1 cytokine, TNF α (A); a Th2 cytokine, IL-5 (B); a Th17 cytokine, IL-17 (C); and PMN-derived cytokine, KC (IL-8) (D). ***P < 0.001. n = 5.

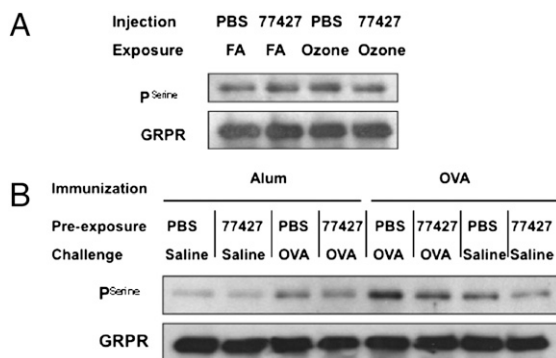


Fig. 6. GRP blockade decreases O₃- or OVA-induced GRPR serine phosphorylation. (A) Immunoprecipitation and Western blot for pGRPR in mouse lungs exposed to O₃ with or without 77427. One representative blot out of four total. (B) Similarly, GRPR serine phosphorylation in the OVA model. One representative blot out of four total. Statistical analysis of densitometry for Fig. 6 A and B is in Tables S3 and S4.

rogated AHR and inflammation after GRP blockade. PNECs are triggered by ROS, such as O₃ or allergens (49), leading to GRP secretion and elevated GRP synthesis. Our observation of GRP-positive PNECs in OVA-immunized mice is unique, because mice are normally devoid of GRP immunostaining (50), suggesting that increased GRP production is linked to systemic immune responses. GRP elicits acute and chronic inflammatory responses with influx of PMN or eosinophils, monocytes/macrophages, and CD4⁺ T cells from peripheral blood. GRP induces cell proliferation, cell differentiation/activation, cell migration, and/or cytokine secretion from macrophages, T cells, and mast cells (27). Consistent with GRPR mRNA in PMN, macrophages, and T cells, GRP blockade suppresses acute and chronic inflammation in both O₃ and OVA models. In baboon models of BPD (6, 20), GRP blockade normalizes thymic maturation and T cell responses and reduces CD4⁺ T cells in the pulmonary interstitium (28). GRP blockade could arrest migration of peribronchiolar CD4⁺ T cells, which contribute to airways inflammation in mice and asthma in humans (51).

In summary, GRP appears to function as a potent proinflammatory mediator by inducing cell differentiation and/or activation of inflammatory cell precursors from multiple hematopoietic cell lineages. Direct effects are also likely in airway smooth muscle constriction (21, 22), endothelial cell activation and angiogenesis (20, 41), and epithelial cell and fibroblast proliferation (6, 31, 52) during remodeling. Indirect effects due to increased cytokine production could occur in parallel, some cytokines being secreted earlier due to direct binding of GRP to GRPR on target cells, amplifying proinflammatory signals via multiple cytokine-specific receptors. It is not known what limits these responses, except perhaps anti-inflammatory cytokines IL-10, IL-12(p40), and IFN- γ (53).

We compared effects of 77427 to Dex because glucocorticoids are the standard-of-care anti-inflammatory treatment for asthma, including acute exacerbations and prevention, despite many adverse side effects (54). Our data indicate that 77427 is more effective than Dex in both O₃ and OVA models. Thus, 77427 may be a promising treatment for asthma in humans. Advantages of 77427 over Dex include its apparently long biological half-life, with one dose effective for at least 3 d during OVA challenge. The 77427 effectively blocks asthmatic responses to common airborne triggers, including both O₃ and OVA. The 77427 has a molecular mass of 139, which should facilitate aerosol delivery to lung, and its synthesis is simple and economical.

We observed similar results with 77427 and 2A11 in all experiments, supporting the GRP specificity of these effects. Two clinical trials with 2A11 (55, 56) included 25 patients total with terminal lung cancer, refractory to conventional treatment, who should be immunosuppressed from chemotherapy and radiation

therapy (57). However, 2A11 did not increase their incidence of infection, suggesting that antimicrobial and homeostatic host defenses may be spared, whereas GRP blockade may reduce harmful overactivation of immunity in asthma. Clinical trials will be necessary to test these hypotheses.

In conclusion, GRP blockade for treating asthma would represent a paradigm shift in the field. However, we cannot rule out similar regulatory functions for other PNEC-derived neuropeptides. PNECs have been implicated as regulators of ventilation-perfusion mismatches in response to hypoxia (16, 58). Bronchoconstrictor and vasoconstrictor responses could be homeostatic in normal lung, maintaining optimal ventilation-perfusion matching despite regional hypoxia. ROS-triggered PNEC activation with GRP secretion may be an example of host defense gone awry, with immediate airflow obstruction, then sustained inflammatory cell activation. GRP blockade by 77427 may provide a promising broad-spectrum approach to asthma therapy, including reversal and/or prevention of asthmatic exacerbations.

Materials and Methods

Detailed methods are in *SI Materials and Methods*.

Animals. Five-week BALB/c mice were housed with low-endotoxin bedding. Experimental protocols were approved by the Institutional Animal Use and Care Committee at Duke University.

O₃ Inhalation Challenge. Mice were exposed to O₃ for 3 h. One hour before exposure, mice were injected IP with one GRP blocking agent [77427 or 2A11], Dex, or appropriate negative control (PBS or MOPC).

OVA Immunization and Challenge. Mice were immunized IP with 10 μ g of chicken OVA in 100 μ L of Alum hydroxide or Alum alone. After 14 d, mice were boosted with OVA or Alum. Seven days later, mice received aerosol challenges (30 min/d for 3 d) with 1% OVA. Mice were given GRP blockade IP [77427 or 2A11] 1 h before the first challenge, and PFTs were performed 24 h after the last challenge.

PFTs. Direct measurements of respiratory mechanics in response to MCh were made by using the *flexiVent* system and reported as total pulmonary resistance (cm of H₂O per mL s⁻¹ at room temperature) or quasi-static compliance (Cst).

BAL Cytokine Assay. Cytokines in BAL were assayed with the Becton Dickinson Milliplex Mouse Immunoassay by following manufacturer's protocols.

Immunoprecipitation and Immunoblotting. Lungs were lysed in modified RIPA buffer with protease/phosphatase inhibitors. Immune complexes were captured with protein A-Sepharose-agarose. Western blots were probed by using anti-phosphoserine or anti-GRPR antibody, followed by HRP-conjugated secondary antibodies.

Histopathology and Immunohistochemistry. Lungs were inflation-fixed in 4% PF then routinely paraffin-embedded. Paraffin sections were stained with H&E. For IHC, sections were stained with anti-GRP or anti-PGP9.5 antibodies, followed by biotinylated secondary antibodies, ABC complex, and diaminobenzidine as substrate.

Cell Isolation. Peritoneal macrophages were elicited by injecting mice with thioglycolate. After 72 h, macrophages were collected. PMN were isolated from mouse bone marrow by using Percoll gradients. Pan T cells and CD4⁺ T cells were isolated from mouse spleen.

QRT-PCR and Statistics. QRT-PCR was conducted using cDNA from 100 ng of total RNA, and SYBR Green Master Mix on ABI-PRISM 7300 detection system with β -actin as control. Student's *t* test was used for statistical analysis of all experiments.

ACKNOWLEDGMENTS. We thank Dr. Robert P. Orange and Prof. Baruj Benacerraf for invaluable discussions. This work was supported by an Established Investigator Award (to M.E.S.) from the American Asthma Foundation. Mouse model development and airway physiology were supported by National Institutes of Health Grants AI081672 and ES016347 (to W.M.F.).

1. Cotran RS, Kumar V, Collins T (1999) *Robbins' Pathologic Basis of Disease* (Saunders, Philadelphia), 6th Ed.
2. Kim HY, DeKruyff RH, Umetsu DT (2010) The many paths to asthma: Phenotype shaped by innate and adaptive immunity. *Nat Immunol* 11:577–584.
3. Beasley R (2002) The burden of asthma with specific reference to the United States. *J Allergy Clin Immunol* 109(5 Suppl):S482–S489.
4. Gaga M, et al., ENFUMOSA Study Group (2005) Risk factors and characteristics associated with severe and difficult to treat asthma phenotype: An analysis of the ENFUMOSA group of patients based on the ECRHS questionnaire. *Clin Exp Allergy* 35: 954–959.
5. Shore SA, Drazen JM (2003) Beta-agonists and asthma: Too much of a good thing? *J Clin Invest* 112:495–497.
6. Sunday ME, Yoder BA, Cuttitta F, Haley KJ, Emanuel RL (1998) Bombesin-like peptide mediates lung injury in a baboon model of bronchopulmonary dysplasia. *J Clin Invest* 102:584–594.
7. Ng DK, Lau WY, Lee SL (2000) Pulmonary sequelae in long-term survivors of bronchopulmonary dysplasia. *Pediatr Int* 42:603–607.
8. Evans M, Palta M, Sadek M, Weinstein MR, Peters ME (1998) Associations between family history of asthma, bronchopulmonary dysplasia, and childhood asthma in very low birth weight children. *Am J Epidemiol* 148:460–466.
9. Bai TR, Knight DA (2005) Structural changes in the airways in asthma: Observations and consequences. *Clin Sci (Lond)* 108:463–477.
10. Hamid Q, Tulic MK, Liu MC, Moqbel R (2003) Inflammatory cells in asthma: Mechanisms and implications for therapy. *J Allergy Clin Immunol*, 111(1 Suppl): S5–S12, discussion S12–S17.
11. Lilly CM (2005) Diversity of asthma: Evolving concepts of pathophysiology and lessons from genetics. *J Allergy Clin Immunol* 115(4 Suppl):S526–S531.
12. Groneberg DA, Quarcoo D, Frossard N, Fischer A (2004) Neurogenic mechanisms in bronchial inflammatory diseases. *Allergy* 59:1139–1152.
13. Joos GF, Germonpre PR, Kips JC, Peleman RA, Pauwels RA (1994) Sensory neuropeptides and the human lower airways: Present state and future directions. *Eur Respir J* 7: 1161–1171.
14. Chanez P, et al. (1998) Bronchial mucosal immunoreactivity of sensory neuropeptides in severe airway diseases. *Am J Respir Crit Care Med* 158:985–990.
15. Dusser DJ, Djokic TD, Borson DB, Nadel JA (1989) Cigarette smoke induces bronchoconstrictor hyperresponsiveness to substance P and inactivates airway neutral endopeptidase in the guinea pig. Possible role of free radicals. *J Clin Invest* 84:900–906.
16. Linnoila RI (2006) Functional facets of the pulmonary neuroendocrine system. *Lab Invest* 86:425–444.
17. Wharton J, et al. (1978) Bombesin-like immunoreactivity in the lung. *Nature* 273: 769–770.
18. Sunday ME, Hua J, Dai HB, Nusrat A, Torday JS (1990) Bombesin increases fetal lung growth and maturation in utero and in organ culture. *Am J Respir Cell Mol Biol* 3:199–205.
19. King KA, Torday JS, Sunday ME (1995) Bombesin and [Leu8]phyllostin promote fetal mouse lung branching morphogenesis via a receptor-mediated mechanism. *Proc Natl Acad Sci USA* 92:4357–4361.
20. Subramaniam M, et al. (2007) Bombesin-like peptides modulate alveolarization and angiogenesis in bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 176:902–912.
21. Impicciatore M, Bertaccini G (1973) The bronchoconstrictor action of the tetradecapeptide bombesin in the guinea-pig. *J Pharm Pharmacol* 25:872–875.
22. Lach E, Haddad EB, Gies JP (1993) Contractile effect of bombesin on guinea pig lung in vitro: Involvement of gastrin-releasing peptide-preferring receptors. *Am J Physiol* 264:L80–L86.
23. Bousbaa H, Fleury-Feith J (1991) Effects of a long-standing challenge on pulmonary neuroendocrine cells of actively sensitized guinea pigs. *Am Rev Respir Dis* 144: 714–717.
24. Aguayo SM, et al. (1992) Brief report: Idiopathic diffuse hyperplasia of pulmonary neuroendocrine cells and airways disease. *N Engl J Med* 327:1285–1288.
25. Deterding RR, Pye C, Fan LL, Langston C (2005) Persistent tachypnea of infancy is associated with neuroendocrine cell hyperplasia. *Pediatr Pulmonol* 40:157–165.
26. Cullen A, et al. (2002) Urine bombesin-like peptide elevation precedes clinical evidence of bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 165:1093–1097.
27. Subramaniam M, et al. (2003) Bombesin-like peptides and mast cell responses: Relevance to bronchopulmonary dysplasia? *Am J Respir Crit Care Med* 168:601–611.
28. Rosen DM, et al. (2006) Accelerated thymic maturation and autoreactive T cells in bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 174:75–83.
29. Del Rio M, De la Fuente M (1994) Chemoattractant capacity of bombesin, gastrin-releasing peptide and neuromedin C is mediated through PKC activation in murine peritoneal leucocytes. *Peptides* 49:185–193.
30. Yule KA, White SR (1999) Migration of 3T3 and lung fibroblasts in response to calcitonin gene-related peptide and bombesin. *Exp Lung Res* 25:261–273.
31. Rozengurt E, Sinnett-Smith J (1990) Bombesin stimulation of fibroblast mitogenesis: Specific receptors, signal transduction and early events. *Philos Trans R Soc Lond B Biol Sci* 327:209–221.
32. Cullen A, et al. (2000) Bombesin-like peptide (BLP) and BLP receptors in two different baboon models of bronchopulmonary dysplasia: Diversity in gene expression yet similarity in function. *Peptides* 21:1627–1638.
33. Dozor AJ (2010) The role of oxidative stress in the pathogenesis and treatment of asthma. *Ann N Y Acad Sci* 1203:133–137.
34. Savov JD, et al. (2004) Ozone-induced acute pulmonary injury in inbred mouse strains. *Am J Respir Cell Mol Biol* 31:69–77.
35. Whitehead GS, Walker JK, Berman KG, Foster WM, Schwartz DA (2003) Allergen-induced airway disease is mouse strain dependent. *Am J Physiol Lung Cell Mol Physiol* 285:L32–L42.
36. Shriver SP, et al. (2000) Sex-specific expression of gastrin-releasing peptide receptor: Relationship to smoking history and risk of lung cancer. *J Natl Cancer Inst* 92:24–33.
37. Hollingsworth JW, et al. (2007) Ambient ozone primes pulmonary innate immunity in mice. *J Immunol* 179:4367–4375.
38. Williams AS, et al. (2008) Role of p38 mitogen-activated protein kinase in ozone-induced airway hyperresponsiveness and inflammation. *Eur J Pharmacol* 600:117–122.
39. Voynow JA, et al. (2009) NAD(P)H quinone oxidoreductase 1 is essential for ozone-induced oxidative stress in mice and humans. *Am J Respir Cell Mol Biol* 41:107–113.
40. Baxter LT, Zhu H, Mackensen DG, Jain RK (1994) Physiologically based pharmacokinetic model for specific and nonspecific monoclonal antibodies and fragments in normal tissues and human tumor xenografts in nude mice. *Cancer Res* 54:1517–1528.
41. Martinez A, Zudaire E, Julián M, Moody TW, Cuttitta F (2005) Gastrin-releasing peptide (GRP) induces angiogenesis and the specific GRP blocker 77427 inhibits tumor growth in vitro and in vivo. *Oncogene* 24:4106–4113.
42. Corjay MH, et al. (1991) Two distinct bombesin receptor subtypes are expressed and functional in human lung carcinoma cells. *J Biol Chem* 266:18771–18779.
43. Williams BY, Wang Y, Schonbrunn A (1996) Agonist binding and protein kinase C activation stimulate phosphorylation of the gastrin-releasing peptide receptor at distinct sites. *Mol Pharmacol* 50:716–727.
44. Chang LY, et al. (2003) A catalytic antioxidant attenuates alveolar structural remodeling in bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 167:57–64.
45. Ally RA, et al. (2003) Agonist- and protein kinase C-induced phosphorylation have similar functional consequences for gastrin-releasing peptide receptor signaling via Gq. *Mol Pharmacol* 64:890–904.
46. De la Fuente M, Del Rio M, Ferrandez MD, Hernanz A (1991) Modulation of phagocytic function in murine peritoneal macrophages by bombesin, gastrin-releasing peptide and neuromedin C. *Immunology* 73:205–211.
47. Lemaire I (1991) Bombesin-related peptides modulate interleukin-1 production by alveolar macrophages. *Neuropeptides* 20:217–223.
48. Meloni F, et al. (1996) Bombesin enhances monocyte and macrophage activities: Possible role in the modulation of local pulmonary defenses in chronic bronchitis. *Respiration* 63:28–34.
49. Caillag A, et al. (2010) Pollen-induced oxidative stress influences both innate and adaptive immune responses via altering dendritic cell functions. *J Immunol* 184: 2377–2385.
50. Polak JM, et al. (1993) Lung endocrine cell markers, peptides, and amines. *Anat Rec* 236:169–171.
51. Levine SJ, Wenzel SE (2010) Narrative review: The role of Th2 immune pathway modulation in the treatment of severe asthma and its phenotypes. *Ann Intern Med* 152:232–237.
52. DeMichele MA, Davis AL, Hunt JD, Landreneau RJ, Siegfried JM (1994) Expression of mRNA for three bombesin receptor subtypes in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 11:66–74.
53. Finkelman FD, Hogan SP, Hershey GK, Rothenberg ME, Wills-Karp M (2010) Importance of cytokines in murine allergic airway disease and human asthma. *J Immunol* 184: 1663–1674.
54. Dahl R (2006) Systemic side effects of inhaled corticosteroids in patients with asthma. *Respir Med* 100:1307–1317.
55. Chaudhry A, et al. (1999) Phase I and imaging trial of a monoclonal antibody directed against gastrin-releasing peptide in patients with lung cancer. *Clin Cancer Res* 5: 3385–3393.
56. Kelley MJ, et al. (1997) Antitumor activity of a monoclonal antibody directed against gastrin-releasing peptide in patients with small cell lung cancer. *Chest* 112:256–261.
57. Fischer JR, et al. (1997) Decrease of interleukin-2 secretion is a new independent prognostic factor associated with poor survival in patients with small-cell lung cancer. *Ann Oncol* 8:457–461.
58. Van Lommel A, Bollé T, Fannes W, Lauweryns JM (1999) The pulmonary neuroendocrine system: The past decade. *Arch Histol Cytol* 62:1–16.

Supporting Information

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SI Materials and Methods

Animals. Five-week-old female BALB/c mice were purchased from Charles River Laboratories and housed in cages with low-endotoxin bedding with supplement of water and chow ad libitum. Experimental protocols were approved by the Institutional Animal Use and Care Committee at Duke University Medical Center. GRPR-null mice were generated and maintained as described (1–3).

O₃ Inhalation Challenge. O₃ exposure was performed in a 55-liter Hinners-style exposure chamber. Chamber air was kept at 20–22 °C and 50–60% relative humidity and renewed at 20 exchanges per hour. O₃ was generated by directing 100% medical grade O₂ through an UV light generator and mixed with the air supply to the chamber. O₃ concentration in the chamber was monitored continuously with an UV light photometer (Model 1003AH; Dasibi). O₃ exposure was at 1 ppm for 3 h. One hour before O₃ exposure, mice were injected IP with GRP blockade [77427 at 500 nM or monoclonal GRP antibody clone 2A11 at 4 mg/kg] versus an equivalent volume of PBS (100 µL) or an equivalent volume and concentration of MOPC. Mice were tested for pulmonary function by using a *flexiVent* apparatus or killed for other studies 24 h after O₃ exposure.

OVA Immunization and Challenge. Mice were immunized IP with 10 µg of chicken OVA (Sigma Chemical) in 100 µL of aluminum hydroxide gel (Alum; Sigma) or with Alum alone. Two weeks after the first immunization, mice were boosted with the same doses of OVA or Alum. Seven days later, mice received three aerosol challenges (30 min/d, 3 d) with 1% OVA (wt/vol) in endotoxin-free PBS or PBS alone. The aerosol was generated by using an ultrasonic nebulizer (UltraNeb 2000; DeVilbiss). One hour before the first OVA challenge, mice were treated with GRP blockade [77427 at 500 nM IP or 2A11 at 4 mg/kg IP as described above], PBS, or MOPC-injected IP. Mice were tested for pulmonary function or killed for other analyses 24 h after last aerosol exposure.

Pulmonary Function Testing. Direct measurements of respiratory mechanics in response to methacholine (MCh) were made by using the *flexiVent* system (SCIREQ) and reported as total pulmonary resistance (cm of H₂O per mL·s^{−1} at room temperature) or quasi-static compliance (Cst). Mice were anesthetized with 60 mg/kg pentobarbital sodium (Sigma) and surgically prepared with a tracheal cannula and then ventilated with a computer-controlled ventilator (*flexiVent*) at a constant tidal volume of 6–8 mL/kg and a peak expiratory end pressure of 3 cm of H₂O. The mice were then given a neuromuscular blockade (0.8 mg/kg pancuronium bromide; Sigma) for 5 min to adjust to the ventilator. Measurements of airway pressure were made at a side port of the tracheal cannula via a differential pressure transducer and airway resistance was monitored from pressure and volume data that were generated by applying a 2-s sine wave volume perturbation to the tracheal cannula with an amplitude of 0.2 mL and a frequency of 2.5 Hz. Responses to MCh aerosol challenges (0, 10, 25, and 100 mg/mL) were determined by resistance measurements every 30 s for 5 min. The resistance (cm of H₂O per mL·s^{−1} at room temperature) or quasi-static compliance (Cst) measurements were then averaged at each dose per sample group and graphed (cm of H₂O per mL·s^{−1} at room temperature) along with the initial baseline measurement pre-MCh challenge.

Bronchoalveolar Lavage (BAL). Whole-lung lavage was performed immediately after pulmonary function testing. The trachea was exposed and intubated with PE-90 tubing (0.86-mm inner diameter, 1.27-mm outer diameter). Sterile saline was instilled 1 mL at a time (total 3 mL) in the tracheal catheter at a pressure of 25 cm of water and retrieved. Return volume was recorded and was consistently >75% of the instilled volume. The cells were then isolated from the whole-lung lavage fluid by centrifugation at 1,400 × g for 15 min. The supernatant was stored at −80 °C for later assessment of cytokine levels. Cells were resuspended in 1 mL of HBSS (Invitrogen) and counted by using 0.5% Trypan blue exclusion (in PBS) and a hemacytometer. An aliquot of each cell suspension was centrifuged on Cytospin 3 slides (Shandon) and stained with Wright-Giemsa stain (Diff Quick Stain Set; Harleco). Differential pulmonary inflammatory cells (macrophages, neutrophils, and eosinophils) were determined by using standard morphological criteria and calculated.

Cytokine Assay. Cytokines in BAL fluid were assayed by using the Beadlyte Milliplex Mouse Immunoassay (Millipore) according to the manufacturer's manual. Briefly, the BAL supernatants were incubated with beads to be conjugated to the cytokines overnight at 4 °C. The matched detection antibodies were then added and incubated for 1 h, followed by the addition of streptavidin-phycoerythrin solution and further incubation of 30 min. Cytokine levels were determined from a standard curve by using a Luminex100 reader (Luminex).

Immunoprecipitation and Immunoblotting. Mouse lung tissues were lysed by sonication in ice-cold modified RIPA buffer containing 50 mM Tris at pH 8.0, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS plus 1 mM PMSF, a protease inhibitor mixture, and a serine/threonine phosphatase inhibitor mixture (Sigma). After incubation for 30 min at 4 °C, lysates were cleared by centrifugation at 20,000 × g for 15 min. The supernatant was precleared with protein A-Sepharose agarose (Sigma) and further incubated with anti-GRP antibody (Abcam) for 60 min at 4 °C with gentle agitation. Immunocomplexes were captured with protein A-Sepharose agarose, washed with lysis buffer, and eluted in Laemmli sample buffer. Samples were then fractionated on 10% SDS/PAGE and transferred to PVDF membrane (BioRad). Western blots were conducted by using anti-phosphoserine antibody (clone 4A4; Millipore) or anti-GRPR antibody (Abcam), the corresponding HRP-conjugated secondary antibodies (R&D Systems), and an enhanced chemiluminescent reagent (Perkin-Elmer).

Histopathology and Immunohistochemistry (IHC). Lung tissues were fixed overnight in 4% PF and embedded in paraffin. Four-micrometer paraffin sections were placed onto Fisher PLUS slides. After deparaffinization and rehydration, some paraffin sections were stained with hematoxylin and eosin (H&E), dehydrated, and mounted. For IHC, additional paraffin sections were permeabilized with 0.3% Triton X-100, blocked with 10% normal serum, and stained with anti-GRP (clone 2A11; Abbott Biotech) or anti-PGP9.5 antibody (Dako), followed by biotinylated anti-mouse or biotinylated anti-rabbit antibody. The paraffin sections were then applied with ABC complex (Vector Laboratories) and DAB (Sigma-Aldrich) for visualization. Antigen-specific absorption of 2A11 was carried out by adding to one of two identical tubes of diluted antibody GRP at a 5-fold molar excess, then placing tubes on a rotator overnight at 4 °C. After centri-

Table S1. Comparison of BAL cytokine levels in mice treated with 77427 or Dex before 3-h exposure to O₃

Cytokine	O ₃ + 77427 vs. O ₃ + vehicle	O ₃ + Dex vs. O ₃ + vehicle
IL-1a	60% decrease, $P < 0.000001$	NS
IL-1b	77% decrease, $P = 0.00001$	NS
IL-2	54% decrease, $P = 0.0001$	NS
IL-3	55% decrease, $P = 0.00001$	NS
IL-4	53% decrease, $P = 0.00003$	NS
IL-5	59% decrease, $P = 0.00005$	NS
IL-6	73% decrease, $P = 0.00003$	NS
IL-9	56% decrease, $P < 0.000001$	20% decrease, $P = 0.0442$
IL-10	75% decrease, $P = 0.00033$	NS
IL-12(p40)	69% decrease, $P = 0.00004$	<u>127% increase, $P = 0.0342$</u>
IL-13	86% decrease, $P < 0.000001$	NS
IL-17	66% decrease, $P = 0.00001$	34% decrease, $P = 0.0232$
GM-CSF	70% decrease, $P < 0.000001$	NS
INF- γ	40% decrease, $P = 0.00002$	NS
KC (IL-8)	83% decrease, $P = 0.000006$	NS
TNF α	74% decrease, $P = 0.00006$	<u>149% increase, $P = 0.0003$</u>
MCP-1	84% decrease, $P < 0.000001$	NS
MIP-1 β	<u>146% increase, $P = 0.0146$</u>	NS
VEGF	68% decrease, $P = 0.00003$	17% decrease, $P = 0.0497$
RANTES	50% decrease, $P = 0.0025$	46% decrease, $P = 0.0214$

Multiplex immunoassays were used to measure the BAL levels of 20 cytokines that have been implicated in the pathogenesis of asthma. GRP blockade with 77427 or 2A11 decreased O₃-induced levels of 19/20 cytokines (P values given here for 77427 data). Comparisons of O₃ + PBS or MOPC to O₃ + 77427 or 2A11 yield $P < 0.001$ ($n = 5$), except $P < 0.003$ for RANTES and $P < 0.02$ for MIP-1 β (increased, underlined). Dex treatment altered levels of six cytokines in the O₃ model: increased levels of IL-12(p40) ($P < 0.05$), and TNF α ($P < 0.0003$) (both underlined). Dex suppressed IL-9, IL-17, VEGF, and RANTES (all with $P < 0.05$).

Table S2. Significance of altered cytokine levels in OVA/OVA mice treated with or without 77427 once before first OVA challenge or with or without Dex before each OVA exposure

Cytokine	OVA/OVA + 77427 vs. OVA/OVA + vehicle	OVA/OVA + Dex vs. OVA/OVA + vehicle
IL-1a	<0.0001	<0.001
IL-1b	<0.00001	< 0.00001
IL-2	<0.00001	< 0.0001
IL-3	<0.001	<0.01
IL-4	<0.001	<0.05
IL-5	<0.00001	<0.001
IL-6	<0.00001	<0.00001
IL-9	<0.00001	<0.01
IL-10	<0.00001	<0.01
IL-12(p40)	<0.00001	<0.00001
IL-13	<0.00001	<0.00001
IL-17	<0.00001	<0.01
GM-CSF	<0.00001	<0.0001
INF γ	<0.00001	<0.00001
KC (IL-8)	<0.00001	<0.00001
TNF α	<0.00001	<0.01
MCP-1	<0.00001	<0.01
MIP-1 β	<0.001	<0.001
VEGF	<0.00001	<0.05
RANTES	<0.001	<0.001

Both 77427 and high-dose Dex significantly decreased most of the tested cytokines. In the OVA model, 77427 suppressed all cytokine levels ($P < 0.001$). Dex suppressed two cytokines (IL-4 and VEGF) with $P < 0.05$ and six cytokines (IL-3, IL-9, IL-10, IL-17, TNF α , and MCP-1) with $P < 0.01$. Reduction in cytokine levels was greater with 77427 than with Dex (Table 1). Corresponding values for percent suppression are given in Table 1.

