

ORIGINAL RESEARCH

Immune modulatory oligonucleotides in prevention of nasal allergen–induced Eustachian tube dysfunction in rats

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OBJECTIVES: Develop a model of nasal allergen–induced Eustachian tube dysfunction (ETD) in a rat and investigate the role of immune modulatory oligonucleotides (IMOs) in the prevention of nasal allergen–induced ETD.

STUDY DESIGN AND SETTING: Prospective, randomized study. Brown Norway rats were sensitized to ova albumin (OVA) and randomized to receive pretreatment with IMOs or phosphate-buffered saline. All animals were challenged intranasally with aerosolized OVA. Dynamic measures of Eustachian tube (ET) function were analyzed.

RESULTS: Animals that were OVA-sensitized and IMO-pretreated had significantly lower mean passive opening (95% confidence interval [95% CI] 15.0,19.4) and closing (95% CI 4.8,7.8) ET pressures compared with those of (95% CI 24.1,32.7) and (95% CI 12.1,18.8) OVA-sensitized untreated rats, respectively. In addition, the IMO-pretreated animals demonstrated the ability to actively clear a significantly higher proportion of negative pressure (95% CI 0.64,0.96) compared with the untreated animals (95% CI 0.09,0.39). IMO-pretreated animals also demonstrated significantly improved mean mucociliary clearance times in seconds (95% CI 115,195) than those in untreated animals (95% CI 308,668).

CONCLUSIONS: Pretreatment with IMOs prevented allergen-induced allergic inflammation around the Eustachian tube (ET) and resulted in improved ventilatory function of the ET compared with sensitized untreated animals. IMOs offer considerable promise in the management of nasal allergic disease as well as otitis media with effusion.

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The Eustachian tube (ET) plays a critical role in middle ear function by providing a pathway for drainage of secretions into the nasopharynx, regulating air pressure, and protecting the middle ear from nasopharyngeal secretions and sound pressure.^{1,2} Obstruction of the ET alters the partial pressure of middle ear gases, resulting in negative middle ear

pressure, improper ventilation, and a supportive milieu for bacteria reproduction.^{3,4} Thus, inflammation within or around the nasopharyngeal portion of the ET leads to obstruction and subsequent increased risk for the development of otitis media (OM) and otitis media with effusion (OME).⁴

The etiology of Eustachian tube dysfunction (ETD) is thought to be multifactorial. However, recent evidence demonstrates that inflammation within the nose resulting from allergic disease may play an important role in the development of significant ET obstruction and OME.³⁻⁸ In a study of 100 patients with recurrent OME, nearly one third of the cases were attributed directly to the nasal allergic response through the action of inflammatory mediators including cytokines released by mucosal mast cells, other inflammatory cells, and epithelial cells in the nose and nasopharynx.^{8,9} Other studies have shown that as many as 50 percent of all patients with otitis media have allergic rhinitis and over one fifth of patients with nasal allergy have otitis media.¹ Nguyen et al⁵ demonstrated that the allergic inflammation seen in atopics with OME occurred in both the middle ear and the nasopharynx.

There is a clear interrelated relationship between the ME, ET, and the nasopharynx. It is now commonly thought that a parallel relationship exists between the upper and lower airways in allergic disease.^{5,10,11} This united airway concept holds that the middle ear, Eustachian tube, and the nasopharynx participate in the allergic T_H 2 immune-mediated hypersensitivity response in a manner analogous to that of the remainder of the upper and lower airways.^{10,11} Therefore, allergic inflammation is not confined to a specific site or target organ but is considered to range throughout the common airway.^{7,10,11}

An innovative strategy for modifying the chronic inflammatory response associated with allergic disease employs immune modulatory oligodeoxynucleotides (IMOs) containing synthetic immunostimulatory motifs.¹² These oligo-

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nucleotides stimulate cellular-signaling pathways through the Toll-like receptor 9 (TLR9). The binding to the TLR9 ultimately creates an immunomodulatory cascade, resulting in the activation of B and T lymphocytes, natural killer cells, monocytes, macrophages, and dendritic cells.^{12,13} IMOs have been shown to bias the antigen-induced T_H2 immune response toward a T_H1-type response in antigen-sensitized and challenged mice.^{13,14} Through enhancement of the T_H1-biased immunity, IMOs have been shown to successfully abate both the early- and late-phase allergic airway responses by attenuating the levels of eosinophils, interleukin (IL)-10, IL-4, IL-5, and IL-6.¹³

Since IMOs alter the immune response by preventing naïve T-lymphocyte differentiation and reducing IgE levels, these substances have been applied to the treatment of allergic-like airway inflammation and airway hyperresponsiveness. Previous studies have investigated the use of IMOs in the prevention and treatment of allergen-induced OME and ETD in sensitized rats.^{15,16} These studies used a transtympanic allergen challenge protocol. In contrast, the present study examined a more practical method of allergen challenge, an intranasal approach. To our knowledge, there has been no research investigating the feasibility of these compounds to inhibit nasal allergen-induced ETD. The aim of the present study was to investigate the role of IMOs in the prevention of nasal allergen-induced ETD in a rat model.

SUBJECTS AND METHODS

All animals were handled and housed according to the standards and protocols of the Institutional Animal Care and Use Committee in accordance with the requirements described by the National Institutes of Health Committee on Care and Use of Laboratory Animals. Eighteen, nonallergic, male Brown Norway (BN) rats weighing between 200 to 300 g were randomly assigned into one of three groups: 1) untreated allergen-sensitized; 2) untreated, nonsensitized control; and 3) IMO-pretreated allergen-sensitized.

IMO Synthesis and Purification

IMO (5'-TCTGACRTTCT-X-TCTTRCAGTCT-5', wherein R and X stand for 2'-deoxy-7-deazaguanosine and glycerol linker, respectively) was synthesized, purified, and analyzed as previously described.¹⁴ The purity of full-length IMO was greater than 93 percent, with the rest being shorter by one or two nucleotides (n-1 and n-2), as determined by capillary gel electrophoresis and/or denaturing polyacrylamide gel electrophoresis. IMO contained less than 0.2 EU/mL of endotoxin as determined by the Limulus assay (Bio-Whittaker, Walkersville, MD, USA).

OVA Albumin Sensitization of Rats

BN rats in the sensitized and the sensitized IMO-pretreated groups were sensitized to ova albumin (OVA: A5503; Ovalbumin, grade V, Sigma Chemical Co, St Louis, MO)

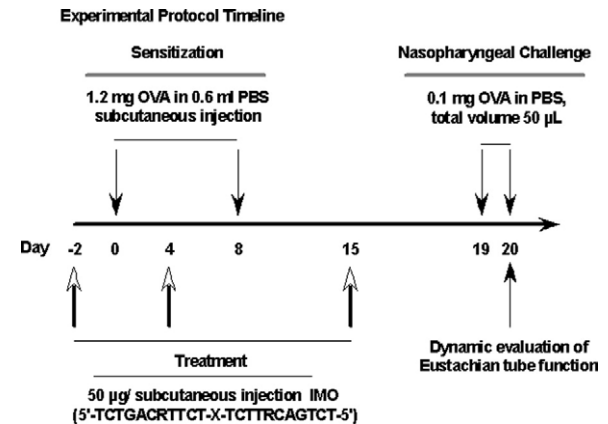


Figure 1 Experimental protocol. Nonallergic, male Brown Norway (BN) rats were randomly assigned into two of three groups: I, untreated OVA-sensitized; II, untreated, nonsensitized control; III, IMO-pretreated OVA-sensitized. Animals in the pretreatment group received three subcutaneous injections of IMO (50 µg/injection) on days -2, 4, and 15, and were OVA-sensitized on days 0 and 8. On days 19 and 20, these animals received intranasal OVA challenge. On day 20 the ET function was assessed. The untreated OVA-sensitized animals received PBS injections in place of IMO treatment on days -2, 4, and 15. The nonsensitized control group received only PBS injections. All animals were challenged with OVA intranasally.

via a subcutaneous injection of 1.2 mg OVA in 0.6 mL phosphate-buffered saline (PBS; 1X PBS-Cell Culture Grade, Lineberger Tissue Culture Facility, Chapel Hill, NC) mixed with 5.14 mg aluminum hydroxide (AX0696-1 Aluminum Hydroxide Gel Dried Powder USP, EM Science, Gibbstown, NJ) in 0.6 mL PBS as a local adjuvant on days 0 and 8 (Fig 1). The nonsensitized control group received a sham sensitization with sterile PBS (1X PBS-Cell Culture Grade).

IMO Pretreatment Protocol

Animals in the pretreatment group received three subcutaneous injections of IMO (50 µg/injection) dissolved in PBS, as well as OVA sensitization as described above (Fig 1). The first dose of IMO was administered two days prior to initial OVA sensitization, the second on day four, and the third on day 15 (Fig 1). (Both the untreated sensitized and nonsensitized control groups at these time intervals received sham treatments with PBS.) The OVA-sensitized IMO-pretreated group ultimately received an intranasal OVA challenge followed by an assessment of ET function. All animals were challenged with OVA via an intranasal spray.

Intranasal Challenge

On day 19, animals were anesthetized with 0.1 mL/100 g body weight of a 1:1 mixture of ketamine (20 mg/mL): xylazine (100 mg/mL). The depth of anesthesia was tested by applying joint pressure. All animals were given 80 µL/min/kg of 300 mOsm Krebs Ringer solution (140 mmol/L Na⁺, 120 mmol/L Cl⁻, 5.2 mmol/L K⁺, 25

mmol/L HCO_3^- , 1.1 mmol/L Ca^{2+} , 1.2 mmol/L Mg^{2+} , 0.4 mmol/L HPO_4^{2-} , and 5.6 mmol/L glucose) subcutaneously to maintain blood pressure and renal perfusion during the experiment. To maintain body temperature at 37°C, a thermistor-controlled heating pad was used each time anesthesia was administered.

Once anesthetized, all animals were placed in the left lateral recumbent position and the tympanic membranes (TMs) were assessed with the use of an operating microscope to exclude obvious middle ear pathology. All subjects then were placed in the prone position and received an intranasal challenge with a solution of 0.1 mg OVA in PBS, total volume 50 μL sprayed into the left nostril, with a Paasche airbrush (Paasche Airbrush VL231135, Chicago, IL) via a filtered pipette tube. Animals were given 24 hours of repose and were subsequently reanesthetized to evaluate the TM under the operating microscope and subjectively grade them for the presence or absence of middle ear effusion (day 20). If effusion was present, the effusate was aspirated prior to a second intranasal OVA challenge. One hour after the second intranasal injection, the TM and middle ear were again examined, and any residual fluid was aspirated before assessment of the ET's ventilatory and clearance capacity was performed.

Assessment of ET Function

This procedure has been described previously.¹⁵ Briefly, while under the described anesthetic protocol, subjects were placed in the lateral recumbent position. The tympanic membrane was visualized with the use of an operating microscope, and a myringotomy was made in the antero-inferior and postero-inferior quadrants. The myringotomies functioned as ports for the application of positive and negative pressure to the middle ear.

Passive opening and closing pressures of the ET were measured with a pressure measurement apparatus that was created by inserting the end of a 6F latex pediatric foley catheter with a 3-mL balloon (Willy Rusch, AG, Duluth, GA) under direct visualization into the external auditory canal and inflating the balloon to create an airtight seal.¹⁵ The catheter was connected to a water manometer, creating a closed system, which allowed for the application of positive pressure via a Harvard infusion pump. Slow uniform pressure (20 mL/min) was infused until the ET opened, marked by the meniscus of the water column changing from convex to concave. This "breaking point" was designated the *passive opening pressure* (POP). At this time, the infusion was discontinued and pressure escaped through the opened ET, causing the meniscus of the water column to descend. The point at which the column stopped descending and became stabilized was considered the closure of the ET. This point was designated as *passive closing pressure* (PCP).

To assess the capability of the ET to actively clear middle ear pressure, subjects were then placed in the recumbent position. Once the catheter balloon was inserted and inflated in the external auditory canal, 14 cm H_2O of

pressure was withdrawn from the middle ear by the Harvard infusion pump. This process created negative pressure within the middle ear. Rats were then stimulated to swallow with polyethylene tubing, which caused the tensor veli palatini to contract and actively open the ET. Each swallow allowed for ventilation of the middle ear and caused the water column to rise (effectively decreasing the negative pressure). Rats were stimulated to swallow until negative pressure could no longer be cleared (the point at which the water column no longer changed after five stimulated swallows). The difference in the pressures between these two points was divided by 14 cm H_2O to compute the percent capability to actively clear negative pressure (CACNP).

To assess the mucociliary clearance function of the ET, subjects were deeply anesthetized to prevent swallowing and active opening of the ET. The rats were placed in the supine position, and a standard nasal speculum was used to aid in the visualization of the soft palate. A small midline incision (10 mm) was made in the soft palate posterior to the posterior border of the hard palate. Hemostasis and anesthesia were maintained by swabbing the incision site with Xylocaine (lidocaine HCl 1% and epinephrine 1:100,000; AstraZeneca LP, Wilmington, DE). A miniature hand mirror was inserted through the incision to visualize the ET orifice under the operating microscope. Rats were then placed in the right lateral recumbent position. With the use of a micropipette, 35 μL of blue dye (Coomassie Brilliant Blue, B-0149; Sigma Chemical Co, St Louis, MO) was injected through incisions in the tympanic membrane into the middle ear. At this time a stopwatch was started and the rats were returned to the supine position. The nasopharyngeal orifice of the ET was again visualized through the palate incision by using the miniature hand mirror. The time at which dye appeared at the orifice signaled the *mucociliary transit time* (MCTT) of the tubotympanum. A cutoff was established at 15 minutes on the basis of numerous previous experiments in our laboratory if no dye was seen.

Statistical Analysis

All statistical analyses were performed with SigmaStat Statistical software, version 2.03 (Systat Software Inc, Point Richmond, CA). Statistical significance and sample size were determined a priori for *P* values of < 0.05 and a power of 0.80, with an expected standard deviation of 0.50 and an anticipated effect size of 40 percent reduction in ET pressures. Pressures were analyzed by 1-way analysis of variance with pair-wise comparisons of means evaluated by paired *t* tests. Active clearance of negative pressure was converted to a percentage of total possible pressure for clearance and then analyzed. Graphs were constructed with the use of means and the SEM. Confidence intervals were calculated and are reported in the figures above each column measure.

RESULTS

Subjects that were OVA-sensitized and pretreated with IMO required significantly less pressure (cm H₂O) to both open ($P < 0.001$) and close ($P < 0.001$) the ET compared with OVA-sensitized untreated animals (Fig 2). There were no statistically significant differences between the OVA-sensitized IMO-pretreated and the nonsensitized control groups for either POP ($P = 0.62$) or PCP ($P = 0.68$). According to the analysis of variance, the differences in the means among and within the IMO group for both POP and PCP are greater than would have been expected by chance ($P < 0.001$). None of the animals had middle ear effusions 24 hours postintranasal OVA challenge (95% CIs are noted above each group, Fig 2).

Pretreatment with IMO also considerably enhanced the capability to actively clear negative pressure from the middle ear compared with allergic untreated animals ($P < 0.05$, Fig 3). Figure 3 shows the mean percent change of CACNP (in cm H₂O) for the 3 experimental groups, and significant differences are marked by asterisks (95% CIs are noted above each group). There was no significant difference in the CACNP for the allergic IMO-pretreated and nonallergic animals ($P > 0.05$).

The mucociliary clearance function of the ET for the three experimental groups is shown in Figure 4. Pretreatment with IMO significantly reduced mean mucociliary transit times compared with OVA-sensitized untreated animals ($P < 0.001$). The average time for dye to be transmitted through the ET was 488 (95% CI 308, 668) seconds for the OVA-sensitized untreated group compared with 154

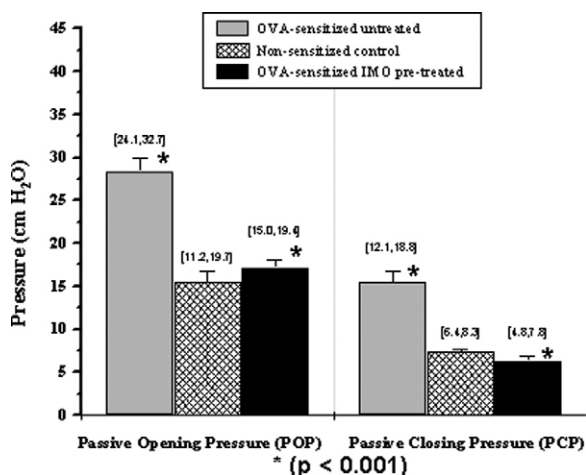


Figure 2 Mean passive opening and closing pressures (in cm H₂O) for the OVA-sensitized untreated (gray), the nonsensitized control (cross-hatch), and the OVA-sensitized IMO-pretreated (black) groups. Pretreatment with IMO resulted in significantly decreased passive opening and closing pressures compared with the untreated OVA-sensitized animals. There were no significant differences in the mean passive opening or closing pressures between the nonsensitized and the OVA-sensitized IMO-pretreated groups. Error bars represent SEM and confidence intervals are indicated above each column.

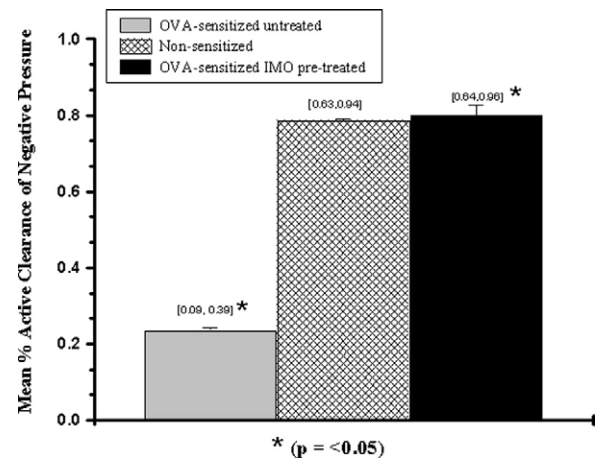


Figure 3 Mean percent capability of active clearance of negative pressure (in cm H₂O) for the OVA-sensitized untreated (gray), the nonsensitized control (cross-hatch), and the OVA-sensitized IMO-pretreated (black) groups. The percent clearance was significantly greater for the IMO-pretreated OVA-sensitized group compared with the untreated OVA-sensitized animals. The maximum amount of possible percent clearance was 100 percent. There were no significant differences between the means of the nonsensitized and the OVA-sensitized IMO-pretreated groups. Error bars represent SEM and confidence intervals are indicated above and/or beside each column.

(95% CI 115, 195) seconds for the OVA-sensitized IMO-pretreated animals. Thus, mucociliary transit time was on average 3.2 times less for the pretreated IMO group than the untreated group. There was no significant difference in the mean MCTT of the nonsensitized and the OVA-sensitized IMO-pretreated groups ($P = 0.67$).

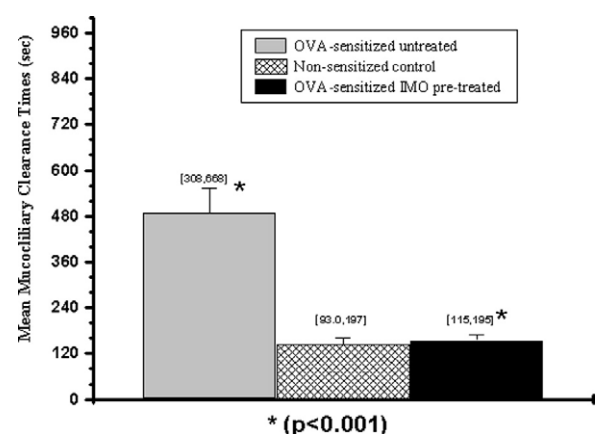


Figure 4 Mean mucociliary transit times (MCTTs) (in seconds) for the OVA-sensitized untreated (gray), the nonsensitized control (cross-hatch), and the OVA-sensitized IMO-pretreated (black) groups. MCTT was significantly longer for the untreated OVA-sensitized group compared with the IMO-pretreated OVA-sensitized group. There were no significant differences between the means of the nonsensitized and the IMO-pretreated groups. Error bars represent SEM and confidence intervals are indicated above each column.

DISCUSSION

These data suggest that the pretreatment of rats with an immune modulatory oligodeoxynucleotide (IMO) containing synthetic immunostimulatory motifs results in enhanced ventilatory and ciliary functions of the ET after intranasal OVA challenge compared with OVA-sensitized controls that were not pretreated with IMO. Significantly lower pressures were needed to passively open and close the ET in the OVA-sensitized IMO-pretreated group compared with the untreated OVA-sensitized group. Because it is commonly thought that the most accurate and significant measure of ET function is the equilibration of negative pressure within the middle ear by deglutition, the clearance of significantly more negative pressure by the subjects in the IMO-pretreated OVA-sensitized group than in the untreated OVA-sensitized group is of unique importance.² The ciliary function of the ET in the IMO-pretreated animals was also significantly better when compared with OVA-sensitized untreated animals as demonstrated by the reduction of the MCTT of the tubotympanum. These data demonstrate that IMOs can prevent allergic-like inflammation of ET postintranasal allergen exposure.

Although the exact manner in which nasal allergic disease (allergic rhinitis) contributes to ETD is not fully understood, it is felt that allergic inflammation within or around the nasopharyngeal portion of the ET may lead to the loss of its protective functions, resulting in increased risk for the development of OM and OME.^{8,9,17,18} Several theories have been proposed to explain ET obstruction secondary to an inflammatory process. ETD may result from a retrograde spread of edema attributable to congestion of the nasal mucosa. Poor mucociliary function resulting from an inflammatory response may also contribute to impaired clearance of secretions and obstruction of the lumen of the ET.^{9,17} In addition, direct allergic inflammation within the ET may cause obstruction from intrinsic venous engorgement and hypersecretion of mucus, thus isolating the middle ear space and thereby affecting gas exchange.¹⁹ The subsequent development of negative pressure in the middle ear then disrupts cellular tight junctions and allows for a transudation of fluids into the middle ear space.^{8,19} Thus, the persistent obstruction of the ET results in chronic ME inflammation, mucosal metaplasia, increased glandular activities of goblet cells, and an inability to clear middle ear fluid.^{8,19}

Repeated allergic exposures and development of ETD may lead to repeated episodes or persistence of OME. In children, this can be of particular importance and may produce impairments in receptive language skills, balance, coordination, maintaining attention, and school readiness.^{9,20} The health care–related expenditures associated with OME have been estimated to be anywhere between \$3 and \$5 billion dollars annually,³ which most likely underestimates the true impact on society. The burden of disease may also affect caregivers of those with OME. Quality of life surveys have found that more than 50 percent of the

caregivers displayed physical problems: poor sleep, poor eating, or irritability due to recurrent AOM and chronic OME in children.²¹

While the untreated OVA-sensitized subjects in this study developed significant ETD compared with the IMO-pretreated and nonsensitized groups, none of the animals developed middle ear effusions. It is theorized that the nasal inflammation from the two intranasal OVA challenges did cause ETD, either through edematous changes in the mucosa or the overproduction of secretions. However, assessment of the ventilatory function of the ET within 24 hours was not sufficient time to result in the development of MEE. Future study will be directed to investigating the long-term effects of IMOs on nasal allergen challenge.

Because there are few effective medical therapeutic regimens for allergy-induced OME, it is important to investigate innovative treatment strategies. The use of novel bimolecular instruments in the treatment of allergic disease has great potential. Immune modulators have the ability to modify and redirect the allergic inflammatory response.¹²⁻¹⁴ To our knowledge this is the first time a TLR9 agonist has been used for the prevention of nasal allergen–induced ETD. These data are consistent with previous work showing that IMOs successfully treat allergic-like airway inflammation and airway hyperresponsiveness.^{13,14} Previous studies in our laboratory have demonstrated that pretreatment and postsensitization treatment of IMO result in improved ventilatory and ciliary functions of the ET after a transtympanic OVA challenge.^{15,16}

While the injection of an allergen into the middle ear, as was used in previous studies, is not a practical *in vivo* simulation, nasal exposure to allergen is a common mode of exposure and a potential significant contributor to ETD and OME.^{18,19} Multiple studies have supported this link between poorly controlled allergic rhinitis and the development of OME.^{6,7,17} Lazo-Saenz et al¹⁷ recently demonstrated that patients with allergic rhinitis were at an increased risk to developing ETD.

The prevention of nasal OVA–induced ETD in rats is likely due to the ability of IMO to abate the overproduction of inflammatory mediators such as eosinophils, IL-4, IL-5, IL-6, and IL-13, and to stimulate the production of T_H1 cytokines, IL-12, and interferon- γ .^{13,14,22} This study clearly builds on our previous work examining the role of allergy in ETD and represents the development of a nasal allergen model to continue investigation in this area.

CONCLUSIONS

There are few proven therapeutic regimens for allergy-induced OME. This study utilizes novel bimolecular instruments in the treatment of allergic disease, which has great potential. Immune modulators have the ability to modify and redirect the allergic inflammatory response.¹²⁻¹⁴ These data show that IMOs offer considerable promise in the management of nasal allergic disease as well as OME.

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Drs Agrawal and Kandimalla are both employees of and have employee stock options in Idera Pharmaceuticals.

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