

ORIGINAL RESEARCH—SINONASAL DISORDERS

Physiologic alterations in the murine model after nasal fungal antigenic exposure

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OBJECTIVE: To determine whether a recently developed murine model of fungus-induced sinonasal inflammation demonstrated alterations in ciliary activity and expression of inflammatory cytokines.

STUDY DESIGN: A prospective randomized controlled study of rhinosinusitis after fungal antigenic sensitization was performed with intraperitoneal aspergillus antigen injection followed by intranasal antigen challenge for 4 weeks. Saline solution was used in a parallel fashion for control animals.

SUBJECTS AND METHODS: Six mice were used to validate the model. Additional 15 mice were used for ciliary beat frequency (CBF) analysis and cytokine expression with multiplex technology. Mean values for degree of inflammation, secretory hyperplasia, CBF, and cytokine expression were compared.

RESULTS: Histologic analyses demonstrated dense chronic inflammation in aspergillus-challenged animals versus sparse inflammatory cells in controls. Significant differences in mean of aspergillus-challenged versus control animals were observed in degree of inflammation ($P < 0.01$), secretory hyperplasia ($P < 0.01$), CBF ($P < 0.00002$), IL-1 α ($P < 0.0002$), IL-1 β ($P < 0.0003$), IL-4 ($P < 0.02$), TNF- α ($P < 0.02$), and RANTES ($P < 0.01$).

CONCLUSION: Alteration in baseline CBF accompanied by increased expression of specific inflammatory cytokines was observed in aspergillus-challenged mice.

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The nose, paranasal sinuses, trachea, and lower airways are lined by a superficial epithelium composed primarily of mucus-producing goblet cells and ciliated cells. It is this very epithelial layer that comprises the mucociliary escalator, the primary mode of defense for the entire respiratory system. Mucus produced by goblet cells traps inhaled particulate and infectious debris, whereas the propulsive force generated by the ciliated cells transports this mucus blanket to the gastrointestinal tract for elimination.¹ Effective mucociliary clearance, therefore, re-

quires both appropriate mucus production and normal ciliary activity.^{1–3}

Normal cilia clear debris-laden mucus by beating in a coordinated and rhythmic manner. Dynamic responses to a variety of mechanical, chemical, hormonal, and thermal stimuli influence this well-established flow pattern toward the sinus ostia.^{1–6} For example, during times of stress (ie, exercise or infection), ciliary beat frequency (CBF) increases to accelerate clearance.^{3–7} As such, compromised mucociliary function results in stasis of sinonasal secretions with subsequent infection and/or persistent inflammation.^{1,2,8–10}

Although chronic rhinosinusitis (CRS) represents a number of clinically indistinguishable yet pathologically distinct entities over the past 25 years, allergic fungal rhinosinusitis (AFRS) has emerged as a particularly challenging form of noninvasive CRS.^{9,10} Although patients with AFRS tend to be younger, atopic, immunocompetent, and often present with unilateral disease, their initial clinical presentation is similar to other forms of CRS with polypoid degeneration of the mucosa.^{1,11,12} Because the disease is highly refractive to conventional therapy, patients also typically report a history of sinus surgery with varying, albeit temporary, degrees of clinical improvement.

The exact pathophysiology of fungus-induced sinonasal inflammation remains a matter of conjecture. Nonetheless, most experts agree that an atopic host is exposed to fungi by means of normal nasal respiration, thus providing an antigenic stimulus.^{10,12} Immune-mediated reactions then trigger an intense eosinophilic response that perpetuates the inflammatory cascade. In fact, species-specific IgE mediated hypersensitivity to the presence of extramucosal fungi within the sinus cavity is observed consistently in AFRS patients.¹² Interestingly, decreased sinonasal clearance of bacterial pathogens has been demonstrated in a mouse model of allergic rhinitis, which suggests that such inflammatory responses may alter mucociliary clearance.¹³

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Much of the challenge in understanding the disease process of fungus-induced rhinosinusitis has been the lack of a reliable animal surrogate. Recently, Lindsay et al¹³ described a model that consistently demonstrated a chronic eosinophilic response in histologic analyses of antigenically challenged murine sinonasal mucosa. As such, our goal was to determine whether this novel animal model could be used to investigate CBF and inflammatory markers associated with fungal antigenic exposure.

METHODS

A prospective randomized controlled trial was performed after approval from the Philadelphia Veterans Medical Affairs Center's (PVAMC) Institutional Animal Care and Use Committee. Evaluation of the outcome parameters, pathologic scoring, inflammatory cytokines, and ciliary beat frequency were performed by three separate individuals uninformed as to the nasal challenge the mice received. Twenty-one BALB/c mice (8 to 10 weeks of age) were obtained (Taconic Farms, Germantown, NY) and housed in pathogen-free conditions in the PVAMC animal research facility for 2 weeks to establish a baseline. A mycelial extract of *Aspergillus fumigatus* (Af) (Hollister-Stier Laboratories, Spokane, WA) was used for intraperitoneal and intranasal usage.¹³

Sensitization and Antigen Presentation

Ten mice were controls and the remaining 11 were designated as study mice. Study mice were subjected to an intraperitoneal injection of Af extract, 200 µg absorbed into 2 mg of alum in 0.5 mL of phosphate-buffered saline solution. The control mice received an intraperitoneal injection of 2 mg alum in 0.5 mL of phosphate-buffered saline solution. The Af extract was diluted to a concentration of 20 mg/mL with normal saline solution. One week after sensitization by intraperitoneal injection, the study mice received nasal antigen challenge of Af extract administered three times per week for four consecutive weeks. The control animals received nasal saline solution at the same time intervals. Mice were given 10 µg (5 µL) of Af antigen or 5 µL of normal saline solution that was applied to each nare by using a micropipette with the mouse held in the supine position. A few seconds after instillation, the mice were held upright for 1 minute to allow inspection of their nasal cavities. All 10 control and 11 study mice were euthanized five weeks after initial sensitization. This time period was chosen as this was a sufficient period of time to induce chronic inflammatory changes in the previously discussed murine model.^{13,14}

Histopathologic Specimen Preparation

Three controls and three study mice were used for histologic analysis. After the removal of the nose and sinonasal com-

plex from the body without separation of the nasal septum, the specimen was placed in formalin. After initial fixation in 10 percent formalin, the rodent skulls were skinned and incubated in decalcifying medium for 1 minute (Decalifier II Solution, Surgipath Inc, Richmond, IL). After decalcification, cross-sections of the sinonasal structures were obtained by sectioning the skulls coronally into four regions for embedding into paraffin: (A) caudal to the incisor teeth, (B) halfway between A and C, (C) anterior margin of bony orbit, (D) posterior margin of the orbit. Hematoxylin and eosin (H and E) stains were performed to characterize the inflammatory infiltrate.

Histopathologic Scoring and Anatomic Method

Nasal diagrams were used to standardize the anatomic locations being examined. In the sections, the vomeronasal organ (VNO), maxilloturbinate, and nasoturbinate were identified (Fig 1). The olfactory mucosa was also identified although this was excluded from examination of the sections. In addition, the nasal-associated lymphoid tissue (NALT) was identified (Fig 1). All sections through the VNO and the NALT were examined histologically for each mouse. The control and aspergillus sinonasal cavities were examined with the examiner blinded to the two groups. All four histologic specimens described earlier were reviewed. The sections were scored for inflammation and secretory hyperplasia.

Inflammation was graded 0 to 4 according to severity with 0 reflecting no changes, 1 minimal changes defined as rare individual inflammatory cells within the mucosa and submucosa, 2 as mild changes defined as a light infiltrate of inflammatory cells, 3 as moderate changes defined as a moderately dense infiltrate of inflammatory cells, and 4 as severe changes defined as a dense infiltrate of inflammatory cells that were dense enough to partially obscure normal architecture of the mucosa or submucosa. The inflammation was also described as focal (F) or diffuse (D).

Secretory hyperplasia was recognized as loss of ciliated epithelium and hyperplasia of nonciliated epithelial cells that exhibited apical cytoplasmic blebbing (apocrine secretion) (Fig 2B). These changes were graded according to severity and extent of involvement of the epithelium with 0 reflecting no changes, 1 minimal changes, 2 mild, 3 moderate, and 4 severe changes that affected most of the respiratory mucosa.

Tissue Harvest

After euthanasia of the remaining 15 mice, the nasal septum was harvested as previously described.¹³ In essence, the animal was placed on a styrofoam dissection table. Pinning of the mouse tail with an 18-gauge needle provided counter-tension during the initial part of the dissection. The skin at the nape of the neck was incised with a fine dissecting scissor and the incision rotated around the entire neck. This skin was dissected anteriorly and completely removed, ex-

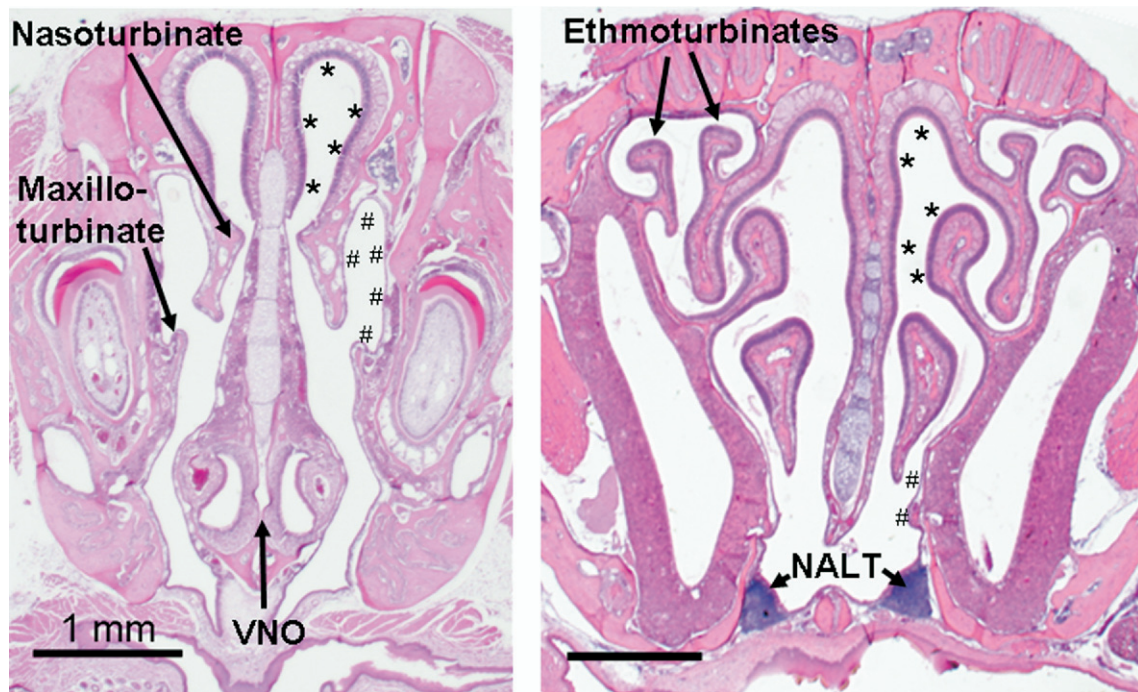


Figure 1 Murine nasal anatomy (hematoxylin-eosin stain). *Left.* Section through the vomeronasal organ (VNO) (original magnification $\times 100$), with nasoturbinate and maxilloturbinate. Respiratory mucosa (#) located on the lateral walls of nasal cavity and on ventral nasal septum; olfactory mucosa (*) is located on the dorsal nasal cavity wall and septum. *Right.* Section through the nasal-associated lymphoid tissue (NALT) (original magnification $\times 100$). Respiratory mucosa is located ventrally (#); expressed olfactory mucosa is dorsally (*).

posing the bone over the entire skull and nose. The skull was sectioned in the coronal plane posterior to the eyes. The remnant of the brain was completely removed, leaving the anterior aspect of the skull base exposed. The most anterior point of the skull base on either side was directly posterior to the mouse nasal cavities. The remaining portion of the skull was removed to the posterior aspect of the nasal cavity. The dorsum of the mouse nose had two lines that represented embryonic fusion planes formed between the two maxillas (laterally) and the ethmoid bone (medially). With this as a guide, a scissor was inserted into the posterior aspect of the nasal cavity and the suture line incised, separating one side of the septum (medially) from the maxilla (laterally). The scissor was rotated inferiorly to section the palate and completely separate the septum from the lateral nasal wall. The procedure was repeated on the other side. The remaining attachment at the nasal tip was severed and the septum completely removed.

Once harvested, the nasal septum's mucosa from each side was gently elevated off the cartilage and bone. One half of the septal mucosa was placed in iced Locke Ringer's and used for CBF analysis as described below; the other half was placed in 200 μ l of 50 mM PBS, 25 mM EDTA, 0.1% Tween 20 and (protease inhibitors and homogenized on ice using a type A 1.5 mL pestle (Eppendorf, Hamburg, Germany). Each sample was centrifuged at 4°C for 10 minutes at $\times 1000$ g. The resulting pellet was resuspended in 200 μ l of homogenization buffer and rehomogenized and centri-

fuged. The supernatants were pooled and aliquoted into 150 μ l samples. One sample was subjected to cytokine profile analysis with luminex technology which has been demonstrated to be a suitable alternative to ELISA for cytokine analysis; the rest of the sample was frozen at 80°C for storage.¹⁵

Nasal Tissue Cytokine Analysis

Luminex technology allows for the evaluation of multiple immune mediators with a smaller volume than conventional ELISA methods. The Beadlyte Mouse 21-Plex Cytokine Detection System (Upstate Lake Placid, NY) was used and the levels of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-12 (p70), IL-13, IL-17, TNF- α , IFN- γ , granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (GCS), monocyte chemoattractant protein-1 (MCP), and macrophage inflammatory protein-18 (MIP) were determined using the Luminex200TM IS instrument (Luminex Corporation, Austin, TX) with data analysis performed with MasterPlex GT (MiriaBio, Alameda, CA).¹⁶

Ciliary Beat Frequency Analysis

Ciliary beat frequency was performed as previously described by our laboratory.¹⁷ The murine nasal septal mucosal explants were placed in Locke Ringer's solution (136 mM NaCl, 5.6 mM KCl, 10 mM HEPES, 14.3 mM NaHCO₃, 1.2 mM MgCl₂, 2.2 mM CaCl₂ and 11.5 mM

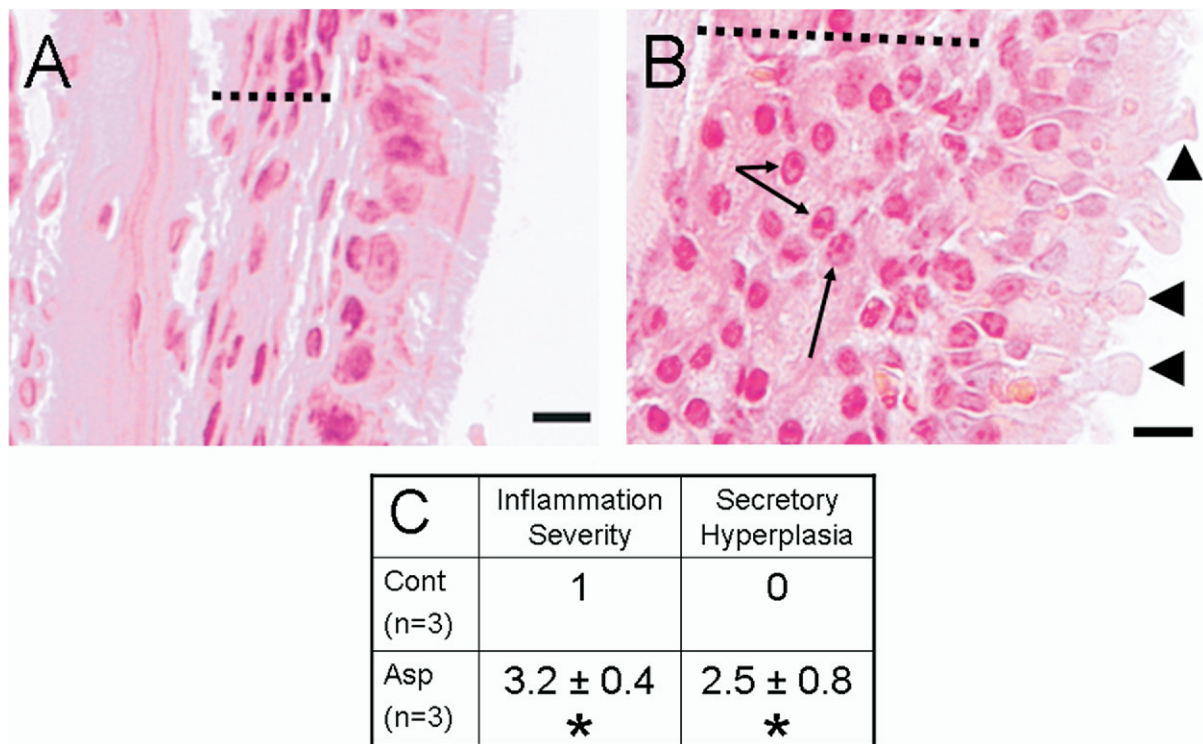


Figure 2 Inflammatory changes of the nasal respiratory epithelium of the aspergillus challenged mice vs the control mice (hematoxylin-eosin stain; original magnification $\times 400$). Sections through inferior nasal septal respiratory epithelium of a control (A) and aspergillus-challenged mouse (B). The lamina propria of the respiratory mucosa of the aspergillus challenged mouse is thickened (*dashed line*) with infiltrating eosinophils (*thin arrow*) compared with the control mice. In addition, the aspergillus challenged respiratory epithelium demonstrates hyperplasia of secretory epithelial cells with apocrine secretion, or blebbing (*thick arrow heads*). (black bar = 10 μ M) (C) Histopathologic scoring of inflammatory severity as well as secretory hyperplasia demonstrate statistically significant differences in the aspergillus challenged mice compared to the control (* $P < 0.005$).

dextrose, pH 7.35). Explants were placed in a glass perfusion chamber on a thermostatically controlled stage and held in place with a nylon grid (1.5 mm) whose outer frame is snapped into the inside of the perfusion chamber. The stage and perfusate were maintained at a temperature between 35.5°C and 37°C with a dual channel heater (Warner Inst, Hamden, CT). The perfusion chamber (Warner Inst) provided superfusion (1 to 2 mL/min) of the tissue with Locke Ringer's solution via a gravity-flow system. Images were visualized with a Leica DMLFSA microscope set on an air table (TMC, Peabody, MA) with a water immersion $\times 63$ objective and differential interference contrast (DIC) optics (Leica Microsystems, Inc, Bannockburn, IL). Images were captured by a Model 500 Redlake MotionPro high-speed monochrome digital video camera (DEL Imaging Systems, LLC, Cheshire, CT). The digital image-sampling rate was set at 250 frames per second (fps) with a resolution of 1280 $\times$ 1024 pixels. A three-second video was recorded at each time point. The video images were written to a PCI 2.2 board and then analyzed using Midas Professional Analyst video image processing software (XCitex, Cambridge, MA). Beating cilia were analyzed with the 1-D line-tracking algorithm included in the motion analysis software. After locating a region of beating cilia, an analysis

grid was created by drawing a line across a segment of beating cilia. The software captured the motion history within the analysis grid for the duration of the video and recorded the gray-scale intensity variation as a function of time. This track history represented the oscillations of the cilia in the form of a time-based waveform graph. The principal frequency was determined with a customized sub-program in MatLab.

Statistical Analysis

A number of assumptions were made in our sample-size calculation. We assumed 100% infectivity of the study animals, a relatively large standard deviation for CBF within groups (approximately 15%), and that changes in cytokine profiles with the multiplexed bead array system are comparable with traditional ELISA. Alpha was set at 0.05. As such, a sample size of 15 (7 controls and 8 experimental animals) was expected to generate a power of 0.8. The data were analyzed with the *t* test to compare differences between ciliary beat frequency in control and study mice as well as their levels of inflammatory markers in the array. The Mann-Whitney *U* test was used to evaluate differences in degree of inflammation and secretory hyperplasia between control and antigenically challenged mice.

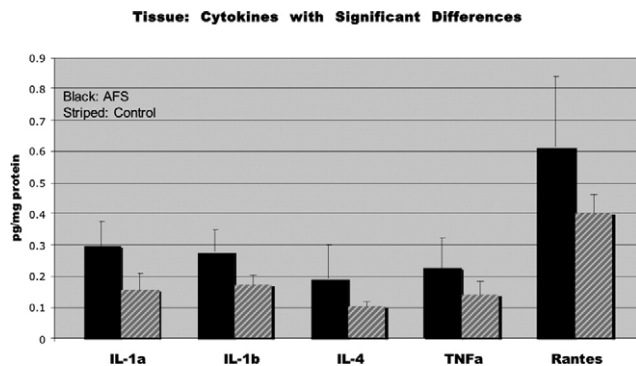


Figure 3 Luminex analysis of cytokine levels. Graph depicts cytokine/chemokine levels in tissue and plasma samples of control and aspergillus-challenged mice. A statistically significant increase in the aspergillus-challenged mice versus the control mice was observed for the following: IL-1 α , IL-1 β , IL-4, TNF- α , and RANTES. The remainder of the cytokines/chemokines analyzed did not demonstrate any statistically significant differences.

RESULTS

Histopathologic Analysis

Significant inflammatory and structural changes were seen under light microscopy between the control and study mice. Histologic analyses of sinonasal complexes demonstrated cells consistent with dense chronic eosinophilia in animals challenged with aspergillus antigen versus sparse inflammatory cell infiltration in saline-challenged controls. Specifically, as shown in Figure 2A, the control mice demonstrated minimal changes with rare inflammatory cells present within the lamina propria of the respiratory mucosa. No hyperplastic changes were evident (Figure 2C, top row). By contrast, the aspergillus-challenged mice demonstrated a moderate to marked inflammatory infiltrate predominately consisting of eosinophils within the lamina propria as well as secretory cell hyperplasia with apocrine secretion (blebbing) (Figure 2B, 2C bottom row). In fact, Mann-Whitney *U* analysis revealed that a significance level of 0.005 was achieved when comparing degree of inflammation as well as sinonasal secretory hyperplasia between controls and antigen-challenged animals.

Cytokine Analysis

To determine whether the histopathologic changes were also associated with altered inflammatory mediator expression, nasal septal tissue was homogenized and evaluated for 21 cytokines, as listed above. Several of the key mediators upregulated in human CRS were also found to be elevated in the murine allergic fungal rhinitis model. Of note, as shown in Figure 3, a significant difference in the study mice versus the control mice for the following cytokines and chemokines was observed: IL-1 α ($P < 0.0002$), IL-1 β ($P < 0.0003$), IL-4 ($P < 0.02$), TNF- α ($P < 0.02$), and RANTES ($P < 0.01$). The remainder of the cytokines and chemokines did not demonstrate any statistically significant difference.

CBF Analysis

Because mucociliary clearance is altered in human CRS, we set out to determine whether cilia function is altered in a murine model. As shown in Figure 4, data are represented as a “Box and Whiskers” plot, where the upper end of the box represents the third quartile, the lower end of the box represents the first quartile, the horizontal bar represents median whereas the vertical lines represent the maximum and minimum values. The mean $\pm$ standard deviation for the control population was 21.1 ± 9.1 while the mean $\pm$ standard deviation for the study population was 25.6 ± 9.7 for study mice ($P < 0.00002$).

DISCUSSION

Currently, the surgical mainstay of management for AFRS involves surgical debulking of diseased mucosa with precise removal of osteal obstructing sinus mucosa as well as the clearance of inspissated fungus containing allergic mucin. Barring any medical contraindications, long-term oral corticosteroids are often added after surgery to improve overall outcomes.¹² In addition, a battery of other therapeutic interventions, including allergen immunotherapy, nasal corticosteroids, antihistamines, oral antileukotrienes, and systemic antifungals, are often combined to varying degrees to meet individual patient requirements.¹ These treatment modalities carry significant individual and collective risks. Thus, a poor understanding of mucociliary dysregulation and sinonasal cytokine expression in fungus-induced sinonasal inflammation creates a void in the array of potential pharmacologic agents that could be used to treat this disease in a safer and/or more specific manner.

The inflammatory responses found in CRS can broadly be divided into T-helper type 1 (Th₁) and T-helper type 2

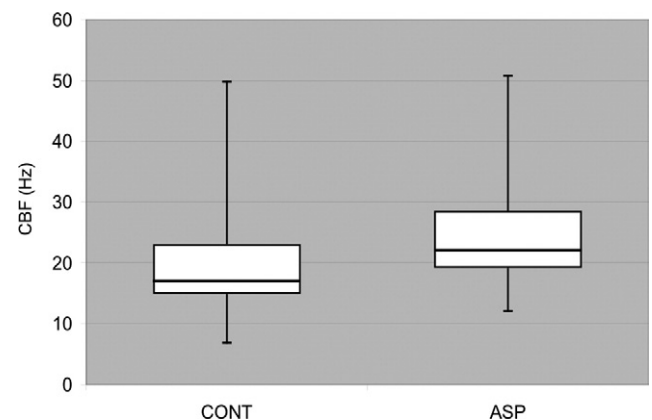


Figure 4 Basal ciliary beat frequency (CBF) in murine model. Baseline CBF is demonstrated in control mice and aspergillus-challenged mice. A statistically significant difference in baseline CBF is shown between control and aspergillus-challenged mice. Data are represented as a “Box and Whiskers” plot, with the median as center, the upper end of the box representing the third quartile, the lower end of the box represents the first quartile.

(Th₂) cascades. Although no general consensus with respect to inflammatory cytokines in CRS has been compiled, several molecules are consistently up-regulated in diseased mucosa by using various techniques including ELISA, reverse transcriptase coupled to quantitative polymerase chain reaction (RT-PCR), immunohistochemistry, and multiplex technology. The Th₁ cytokines and chemokines consistently elevated in CRS include TNF- α , IFN- γ , and IL-8, whereas IL-4, IL-5, eotaxin, and RANTES represent consistently elevated Th₂ cytokines. Furthermore, several of these factors are reduced after treatment with glucocorticoids, a critical component of CRS medical management. Although chemokines and cytokines are primarily responsible for inducing migration, differentiation, activation, and degranulation of subpopulations of leukocytes, several studies have reported cytokine modulation of respiratory cilia function. Recently, IL-8 has been demonstrated to inhibit isoproterenol stimulated CBF in bovine bronchial epithelial cells by negatively modulating adenylyl cyclase, and IL-13 has been shown to decrease basal CBF in a dose- and time-dependent manner.^{4,18,19} Conversely, TNF- α and IL-1 β increase CBF in bovine bronchial epithelial cells.⁴ Consequently, inflammatory cytokines associated with disease processes of the sinonasal cavities may modulate ciliary function for various outcomes.

Interestingly, IL-4 expression, a cytokine responsible for Th₂ lymphocyte proliferation and B-cell class switching to IgE, was significantly induced in our model. In addition, RANTES, a potent chemokine for eosinophils and basophils, is also increased in the mouse model. Both of these chemicals are believed to play critical roles in the development of human allergic fungal rhinosinusitis. Our finding that mean CBF is significantly increased in the aspergillus-challenged mouse correlates with CBF values for human polyposis, a disease state in which CBF has been demonstrated to be accelerated.^{1,2} Both the cytokine analysis as well as the physiologic alterations of CBF provide further confirmation that the murine model used in the current study mimics human polypoid sinus disease in physiologic as well as in inflammatory profiles.

CONCLUSION

The results of our current investigation demonstrate that a novel murine model of fungus-induced sinonasal inflammation can be used reliably to study CBF and changes in local cytokines in the sinonasal mucosa. In particular, our data suggest that at least early in the disease process there is increased expression of TNF- α , RANTES, IL-4, and IL-1 with a concomitant up-regulation in basal CBF. Future investigations will aim to delineate CBF, cytokine expression, and markers of allergic disease (IgE and eosinophils) through the continuum of the disease process to better understand the role of ciliary dysregulation in fungus-induced sinonasal inflammation.

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AUTHOR CONTRIBUTIONS

Ayesha N. Khalid, conducted murine nasal instillations and harvest, wrote grant proposal for AAOA; **Bradford A. Woodworth**, aided in study design for cytokine testing and conducted luminex testing; **Anthony Prince**, conducted murine inoculations; **Sadeq A. Quraishi**, aided in murine study design and researched biomarker profiles, performed statistical analyses of data; **Marcelo B. Antunes**, aided in CBF study design and performed ciliary beat frequency measurements and analysis; **Fenella H.A. Long**, conducted histopathologic examination and staining, analyzed inflammation severity and provided pathologic descriptions; **William E. Bolger**, aided in study design and provided technical support for experiment including obtaining inoculum and analysis of validation study component; **Alexander G. Chiu**, aided in study design with provided bacteriologic expertise in obtaining aspergillus and aided in technique for nasal instillation; **James N. Palmer**, provided equipment and technical support in luminex setup and testing, critical review of manuscript before submission; **Noam A. Cohen**, provided mentorship for entire project including grant writing, experimental design, data analysis, and critical review of manuscript and gave final approval for submission.

FINANCIAL DISCLOSURES

Ayesha N. Khalid, Bradford A. Woodworth, Anthony Prince, Sadeq A. Quraishi, Marcelo B. Antunes, Fenella H.A. Long: none. **William E. Bolger**, Gyrus ENT, consultant, royalty and Acclarent, advisor and shareholder; **Alexander G. Chiu**, Medtronic ENT, consultant, and Brain Lab, consultant; **James N. Palmer**, Medtronic ENT and GE, consultant; **Noam A. Cohen**, Medtronic ENT, consultant.

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