

Peripheral blood mononuclear cells from allergic fungal rhinosinusitis adults express a Th2 cytokine response to fungal antigens

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ABSTRACT

Background: The etiology of allergic fungal rhinosinusitis (AFRS) remains controversial. Initially thought to represent an immunoglobulin E (IgE)-mediated hypersensitivity to fungal antigens, additional data have implicated other non-IgE and cellular-mediated pathways. The aim of this study was to characterize T-helper type 1 (Th1) and Th2 immune responses of blood lymphocytes from AFRS patients by fungal antigen stimulation to help differentiate these possible pathways.

Methods: Peripheral blood mononuclear cells (PBMCs) isolated from AFRS patients ($n = 10$) and healthy controls (HCs; $n = 11$) were exposed to four different fungal extracts (*Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*) in duplicate. After a 72-hour incubation, the supernatants were analyzed for cytokine levels of three Th1 (interferon [IFN] γ , interleukin [IL]-2, and tumor necrosis factor α) and three Th2 (IL-10, IL-5, and IL-4) cytokines by cytometric bead array flow cytometry. Serum fungal-specific IgE levels were measured by ImmunoCAP (Pharmacia Diagnostics, Kalamazoo, MI).

Results: Fungal extracts of *Alternaria* and *Cladosporium* stimulated higher levels of IL-5 from PBMCs in AFRS when compared with HCs ($p < 0.05$). IL-4 was also elevated for *Alternaria* in AFRS versus HCs ($p < 0.05$). A skewed Th2 response to fungal antigen exposure was confirmed by an elevated IL-5/IFN- γ ratio in AFRS subjects ($p < 0.05$). Initial studies suggest a correlation between percent T-cell activation and IL-5 expression to IgE levels. Fungal antigens stimulated a notable but not statistically significant increase in IL-10 response in HCs.

Conclusion: In AFRS patients, fungal antigens stimulated T-cell activation, inducing a predominately Th2 immune response. Healthy controls expressed an inhibitory cytokine IL-10 when exposed to these fungal antigens, possibly serving as a protective response.

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Key words: Allergic fungal rhinosinusitis, chronic rhinosinusitis, cytokine, fungal antigens, IL-10, Th1, Th2

Allergic fungal rhinosinusitis (AFRS) represents a clinically defined subtype of chronic rhinosinusitis (CRS) in which its inciting agent has been linked to fungal antigen exposure. A series of studies conducted at the University of Texas Southwestern (UTSW) Medical Center suggested that an immunoglobulin E (IgE)-mediated pathway triggered by fungal antigens may be important in the pathophysiology of AFRS.^{1,2} Manning and Holmes indicated that AFRS patients with culture-positive *Bipolaris* eosinophilic mucin also had elevated *Bipolaris* serum IgE levels. In addition, these patients had a positive skin test to the *Bipolaris* fungal antigen.³

In 1999, a broader role of fungi in the pathophysiology of rhinosinusitis was proposed. Using more sensitive detection methodologies, fungal antigens were identified within the

nasal cavity of 100% of healthy controls and 96% of patients with CRS.⁴ *In vitro* challenges of peripheral blood mononuclear cells (PBMCs) with fungal antigens only activated mononuclear cells from CRS patients and not from healthy controls. These tissue culture studies suggested that fungal antigens played a direct role in initiating an immune response that was not limited to only a subset of CRS sufferers. Interestingly, both T-helper type 1 (Th1) and Th2 cytokines were elicited on fungal antigen stimulation.

Antigen-activated T-helper lymphocytes, cells central to the adaptive immune response, differentiate classically into one of two major subtypes of effector cells, Th1 or Th2 cells. These subtypes release unique cytokine profiles orchestrating different immune responses. The Th1 cells secrete interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and macrophage activating factor, whereas Th2 cells secrete a different profile of cytokines including interleukin (IL)-4, IL-5, IL-10, and IL-13. Th1 cells incite a strong cell-mediated immune response, targeting intracellular pathogens. Th2 cells, *via* specific cytokines, function to orchestrate a humoral immune response stimulating B-cell antibody production. IL-4 stimulates primarily IgE. IL-5 activates the differentiation and maturation of eosinophils, leukocytes central in this immune response. It is activated *via* the Th2 pathway that results in the allergic inflammatory response. The aim of this study was to characterize and compare the Th1 and Th2 cytokine profile of PBMCs challenged by fungal antigens from AFRS individuals and healthy controls (HCs).

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Table 1 Culture and histology of fungi from eosinophilic mucin of AFRS patients

Study ID	Culture	Histology
002	No growth	Septated fungal hyphae
003	<i>Bipolaris</i>	Positive GMS
009	<i>Exserohilum</i>	Negative
010	No growth	Positive GMS
013	No growth	Septated fungal hyphae
014	No growth	Positive GMS
015	No growth	Positive GMS
016	<i>Aspergillus</i> <i>Curvularia</i>	Negative
017	No growth	Pseudohyphae
019	No growth	Positive GMS
020	<i>Bipolaris</i>	Negative

Fungi identified within eosinophilic mucin of AFRS either on histology or positive culture results.

AFRS = allergic fungal rhinosinusitis; GMS = Gomori methenamine silver.

MATERIALS AND METHODS

Patients

A total of 10 AFRS patients and 11 healthy nonatopic (HCs) donors were recruited for this study as approved by UTSW Institutional Review Board. All participants provided informed consent. Patients with the diagnosis of AFRS who were coming for their routine clinic appointment were recruited. All had undergone previous sinus surgery. Three of the 11 patients complained of worsened symptoms at the time of enrollment, and the remaining 8 patients were symptomatically and endoscopically stable. All patients were under the care of one of our authors (B.F.M). AFRS was defined by historical, endoscopic, radiographic, histological, and laboratory finding as defined by the Bent and Kuhn criteria.⁵ Specifically, patients had rhinosinusitis symptoms for >12 weeks. Computer tomography showed evidence of heterogeneity within opacified sinuses and/or expansion of the affected sinuses. Finally, eosinophilic mucin obtained during operative procedures showed evidence of noninvasive fungal elements or cultures positive for fungi (Table 1).

Determination of Fungal Antigen-Specific IgE Levels

Serum levels of IgE kilowatts per liter of blood (kUA/L) to the four fungal antigens (*Penicillium notatum*, *Aspergillus fumigatus*, *Alternaria alternata*, and *Cladosporium herbarium*) were determined by the Pharmacia ImmunoCAP fluorescence enzyme immunoassay system (Pharmacia Diagnostics, Kalamazoo, MI). All HCs had fungal antigen-specific IgE levels and four other common antigens (Bermuda grass, cat dander, common ragweed, and *Dermatophagoides pteronyssinus*) measured to meet criteria as an HC. Serum was available for only 5 of the 10 AFRS subjects used for determination of fungal-specific IgE levels.

Cell Preparation

Heparinized whole venous blood was obtained from AFRS and HCs as approved by UTSW Institutional Review Board guidelines. PBMCs were purified from blood by density gradient separation using Ficoll-Paque (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) at $130 \times g$ (1250 rpm) for 15 minutes. The platelets were removed by aspiration from the pelleted PBMCs with a $70 \times g$ (900 rpm) centrifugation for 10 minutes. The cells were resuspended at 2×10^6 cells/mL in RPMI (MediaTech, Inc., Manassas, VA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 0.3 mg/mL of glutamine (Invitrogen, Carlsbad, CA), and 100 μ g/mL of streptomycin sulfate (Invitrogen). Cell viability and absence of platelets were confirmed by Trypan Blue exclusion (Sigma Chemical Co., St. Louis, MO).

Culture

The cells (0.2 mL) were then plated with extracts of four airborne fungi, *A. alternata*, *A. fumigatus*, *C. herbarium*, and *P. notatum* (Greer Laboratories, Lenoir, NC) separately. The fungal antigens were reconstituted with RPMI in aliquots at a concentration of 1 mg/mL and stored at -20°C . Purified phytohemagglutinin (PHA; Wellcome Diagnostics, Greenville, NC) at 1 μ g/mL final concentration or tetanus toxoid (MCDC Biologics, Jamaica Plain, MA) at a 1:2000 final dilution were used as positive controls. Cells were cultured at 37°C and 5% CO_2 in 96-well plates (Costar, Cambridge, MA) for 72 hours. The supernatants were collected and stored at -20°C . Preliminary studies were performed optimizing the fungal antigen concentration for cytokine expression and minimizing cell toxicity. A range of 5–50 μ g/mL of fungal antigens were tested in both AFRS and HCs. For both groups, a concentration of 5–10 μ g/mL was determined to be optimal based on minimal cell toxicity as noted by microscopic examination of cells after the 72-hour incubation and maximal cytokine expression levels. All cytokines had greater expression levels at the 5 μ g/mL when compared with the 50- μ g/mL fungal antigen concentration for both groups.

Analysis of Cytokine Production

The levels of three Th1 representative cytokines (IL-2, IFN- γ , and TNF- α) and three Th2 representative cytokines (IL-10, IL-5, and IL-4) were assayed in duplicate using the Human Th1/Th2 Cytokine Bead Array kit (BD Biosciences, San Diego, CA). Samples were collected with a FACSCalibur four-color cytometer according to the manufacturer's instructions and results were analyzed using the BD CBA 6 Bead Analysis software (BD Biosciences). The detection range for the kit was from 20 to 5000 pg/mL for each cytokine.

Characterization of T-Cell Populations

Immunofluorescence labeling for flow cytometry was performed by incubating PBMCs, after culture with or without allergens, with anti-CD4-FITC and anti-CD69-PE-Cy5 antibodies (BD Biosciences Pharmingen, San Diego, CA). Flow cytometry was performed using a FACSCalibur with CellQuest software (BD Biosciences, San Jose, CA). Analysis was performed using FloJo software (Tree Star, Inc., Ashland, OR). After Boolean gating for live cells and lymphocytes by

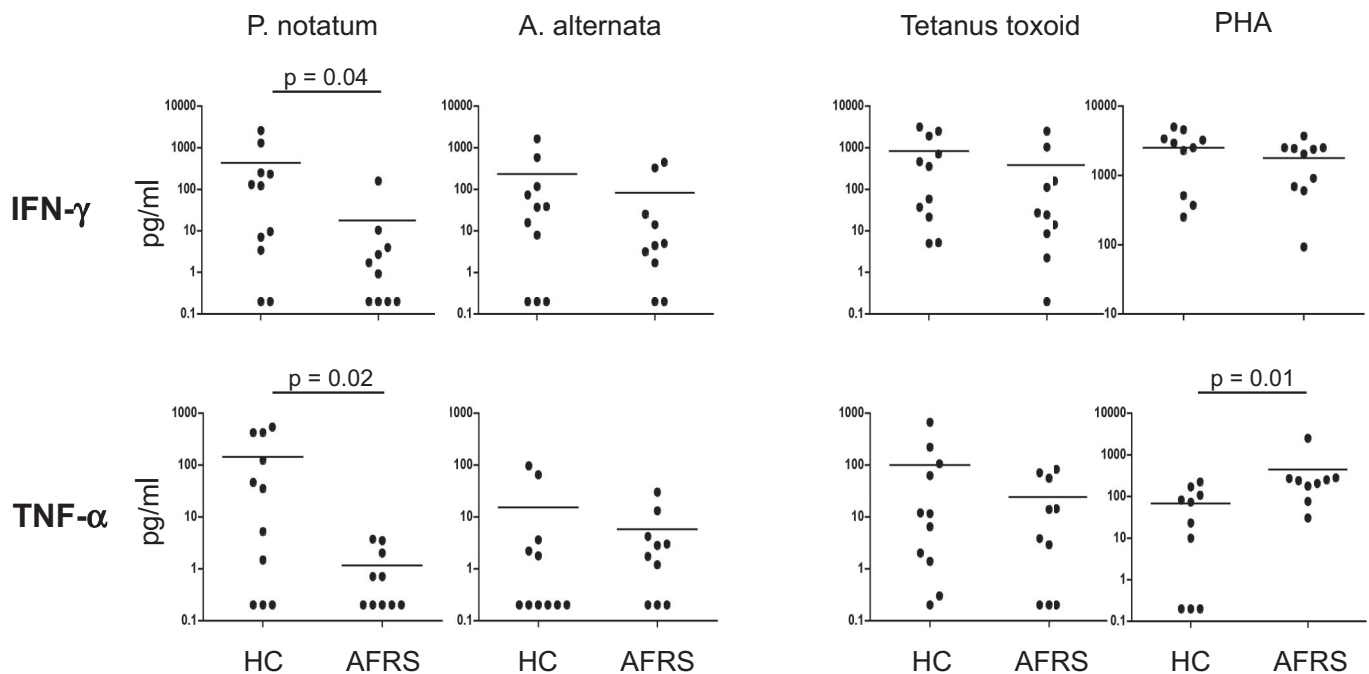


Figure 1. Th1 cytokines interferon (IFN) γ (top) and tumor necrosis factor (TNF) α (bottom) responses to fungal antigens recall antigen tetanus toxoid and mitogen phytohemagglutinin (PHA) in peripheral blood mononuclear cells (PBMCs) from allergic fungal rhinosinusitis (AFRS; $n = 10$) and healthy controls (HCs; $n = 11$). PBMCs were stimulated as detailed in the Methods section, and the culture supernatants were harvested at 72 hours. Data shown are individual values with group mean noted by horizontal bar. A value of 0.2 was substituted for 0 values only for visualization on graph.

forward versus side scatter, we determined the frequency of CD69⁺ cells in the CD4⁺ lymphocyte subset for each sample.

Statistical Analysis

T-cell cytokines are reported as the mean of duplicate determinations for each sample. Computations of significance between HCs and AFRS patients were calculated using the Mann-Whitney U test using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) for each cytokine. The Pearson correlation coefficient was applied to assess bivariate relationships between CD69 expression and allergen-specific IgE levels. A best-fit curve and 95% confidence interval was applied only to better visualize the relationships.

RESULTS

Th1 and Th2 Responses to Fungal Allergens

PBMCs were isolated from AFRS subjects and HCs and cultured with fungal allergens to determine whether allergen-specific cytokine responses could be detected in mononuclear cells from the peripheral blood. Four different fungal antigens were chosen because they are frequently isolated from the nasal cavity.^{6,7} In addition, a recent study had compared cytokine expression to these four fungal antigens from PBMCs of HCs and subjects with chronic rhinosinusitis.⁴ Figure 1 shows the results obtained for Th1 cytokines from 10 AFRS subjects and 11 healthy donors. For these experiments, PBMCs were activated with each of the four fungal allergens, a recall antigen, tetanus toxoid, or the T-cell mitogen, PHA. Increased levels of IFN- γ and TNF- α were produced by healthy donors when

compared with AFRS ($p < 0.05$) in response to *Penicillium* and this trend was also observed for *Alternaria*. No detectable differences in Th1 cytokines were observed between HCs and AFRS patients to *Aspergillus* and *Cladosporium*. Similarly, no statistically significant difference in IFN- γ production was observed between HCs and AFRS subjects in response to the recall antigen or PHA. PBMCs from AFRS patients did produce increased levels of the proinflammatory cytokine, TNF- α , when compared with HCs ($p < 0.05$) in response to mitogen. No differences in HCs versus AFRS Th1 responses were observed to recall antigen.

The Th2 cytokine, IL-5, was preferentially produced by PBMCs from AFRS subjects when compared with HCs ($p < 0.05$) in response to the fungal allergens *Alternaria* and *Cladosporium* (Fig. 2). A statistically significant difference ($p < 0.05$) in IL-4 production was also noted in response to *Alternaria*. PHA also elicited increased IL-5 and IL-4 production from AFRS subjects' PBMCs when compared with healthy controls ($p < 0.05$). No other significant differences in IL-5 or IL-4 responses were noted to fungal allergens or recall antigen. No statistically significant difference was noted in the expression of IL-2 to any of the four fungal antigens, PHA mitogen, or tetanus toxoid control between AFRS and HC groups (data not shown).

Elevated IL-5/IFN- γ Ratios by PBMCs from AFRS Patients to Fungal Allergens

To illustrate the expression of Th1 and Th2 cytokines to fungal antigens, we determined the IL-5/IFN- γ ratios detected for each sample assessed in the bead array assay (Fig.

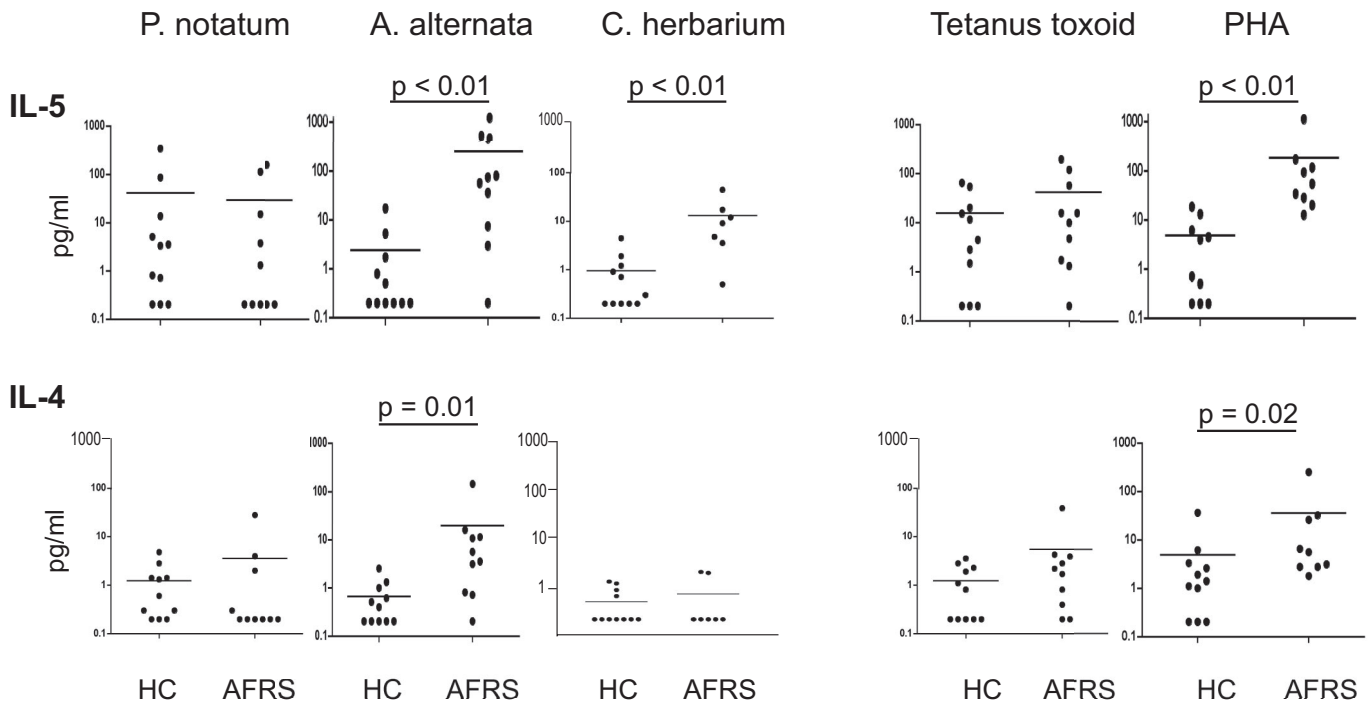


Figure 2. Th2 cytokines interleukin (IL)-5 (top) and IL-4 (bottom) responses to fungal antigens, recall antigen tetanus toxoid, and mitogen phytohemagglutinin (PHA) in peripheral blood mononuclear cells (PBMCs) from allergic fungal rhinosinusitis (AFRS; $n = 10$) and healthy controls (HCs; $n = 11$). PBMCs were stimulated as detailed in the Methods section, and the culture supernatants were harvested at 72 hours. Data shown are individual values with group mean noted by horizontal bar. A value of 0.2 was substituted for 0 values only for visualization on graph.

3). A statistically significant increase in the IL-5/IFN- γ ratio was observed in supernatants from AFRS patients' PBMCs when compared with HCs ($p < 0.005$) in response to the fungal allergens *Alternaria* and *Cladosporium*.

Correlation of Allergen-Specific IgE Levels with T-Cell Activation and IL-5 Secretion

Preliminary experiments examined whether stimulation with fungal allergens could induce CD4⁺ T-cell activation as measured by the expression of the activation marker, CD69. After culturing with allergens for at least 24 hours, PBMCs were collected and stained for flow cytometry. As shown in Fig. 4 A, allergen-specific CD4⁺CD69⁺ T lymphocytes were detected in cultured AFRS PBMCs and the frequency of CD4⁺CD69⁺ cells appeared to correlate with allergen-specific serum IgE levels ($r_p = 0.896$; $p < 0.05$). No activation of CD4⁺CD69⁺ T lymphocytes to fungal antigens was noted in HCs. IL-5 is secreted by memory CD4⁺ T cells after exposure of naive CD4⁺ T cells to exogenous antigen. As seen in Fig. 4 B, allergen-specific IL-5 levels correlated with allergen-specific serum IgE levels from AFRS patient samples ($r_p = 0.996$; $p < 0.05$). These studies support the contention that allergen-specific CD4⁺ T lymphocytes can be detected in the periphery of AFRS patients and that IL-5 production in response to allergen stimulation of PBMCs strongly correlates with allergen-specific serum IgE levels.

IL-10 Responses to Fungal Allergens

Analysis of IL-10 production by allergen-stimulated PBMCs revealed no statistically significant differences be-

tween AFRS subjects and HCs (Fig. 5). However, there was a trend toward increased IL-10 production by HCs to all four fungal allergens examined. Within the AFRS group, the ranges of IL-10 expression to fungal antigens *Penicillium*, *Aspergillus*, *Alternaria*, and *Cladosporium* were 0.0–6.4 pg/mL, 3.6–147.1 pg/mL, 0–37.3 pg/mL, and 0–1.8 pg/mL, respectively. However, the ranges of IL-10 amount for HCs in response to exposure to the same four fungal antigens were 0–46.6 pg/mL, 0–378.9 pg/mL, 0–91.6 pg/mL, and 0–5.7 pg/mL, respectively. The corresponding p values comparing AFRS to HCs were 0.08, 0.19, 0.31, and 0.25, respectively. Of importance, PBMCs from both HCs and AFRS patients responded comparably with optimal stimulation with recall antigen or the T-cell mitogen.

DISCUSSION

From our studies, we found that fungal antigens, specifically *Alternaria* and *Cladosporium*, stimulate predominately Th2 cytokines, IL-4 and IL-5, from PBMCs in AFRS patients. This phenomenon is not observed in healthy controls. IL-4 is well known for its role in the differentiation of CD4⁺ T cells to Th2 lymphocytes. In addition, IL-4 also activates human respiratory epithelial cells to make eosinophil chemoattractant cytokines.^{8,9} Together with IL-5, a key mediator for eosinophil activation, the stimulation of IL-4 and IL-5 production in AFRS to specific fungal antigens may be responsible for the characteristic eosinophilic mucin within the paranasal sinuses, similar to the pathophysiology of allergic bronchopulmonary aspergillosis. Using the major allergen of *Aspergillus* to challenge T-cell clones from allergic bronchopulmo-

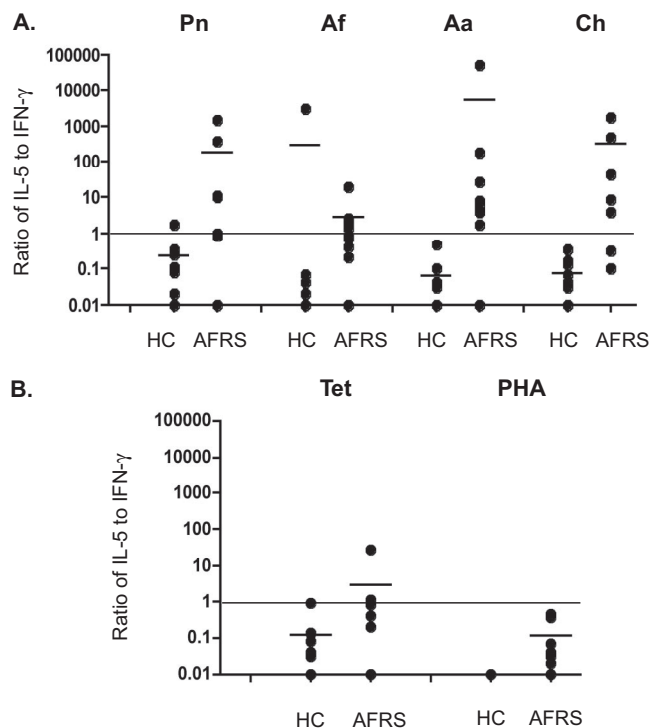


Figure 3. Ratio of interleukin (IL)-5 to interferon (IFN) γ expression to fungal antigens (A) *Penicillium notatum* (Pn), *Aspergillus fumigatus* (Af), *Alternaria alternata* (Aa), and *Cladosporium herbarium* (Ch) or (B) recall antigen *Tetanus toxoid* (Tet) or mitogen *phytohemagglutinin* (PHA) (B) in peripheral blood mononuclear cells (PBMCs) of allergic fungal rhinosinusitis (AFRS; $n = 10$) and healthy controls (HCs; $n = 11$). PBMCs were stimulated as detailed in the Methods section, and the culture supernatants were harvested at 72 hours. The horizontal bar marks the value one. Above the bar, IL-5 expression predominates and below the bar IFN- γ predominates.

nary aspergillosis, IL-5 and IL-4 were the primary cytokines expressed and levels were significantly higher than IFN- γ levels.¹⁰ Highlighting this skewed Th2 response to fungal antigens in AFRS, Fig. 3 illustrates that a majority of the subjects within the AFRS group had a predominate IL-5 to IFN- γ response (as noted by a ratio of greater than one).

Further supporting the predominance of the Th2 lymphocyte subset in AFRS, PHA stimulated elevated levels of IL-4 and IL-5. PHA is a mitogen that stimulates T lymphocytes nonspecifically. Hence, the reflected IL-4 and IL-5 response to PHA suggests a greater prevalence of Th2 lymphocytes within AFRS subjects. A similar enhanced expression of IL-5 and IL-13 was reported from PBMCs challenged with PHA from atopic children.¹¹

Unlike the predominate Th2 expression found in AFRS, Shin *et al.* found that PBMCs from CRS patients expressed both Th1 (IFN- γ) and Th2 (IL-5 and IL-13) cytokines when challenged with *Alternaria*, regardless of atopy status.⁴ CRS represents a spectrum of clinical diseases with several proposed etiologies grouped together clinically by common sinonasal symptoms lasting at least 12 weeks. As such, multiple pathophysiological pathways have been identified in different subtypes of CRS.¹² Cytokine and histological studies compar-

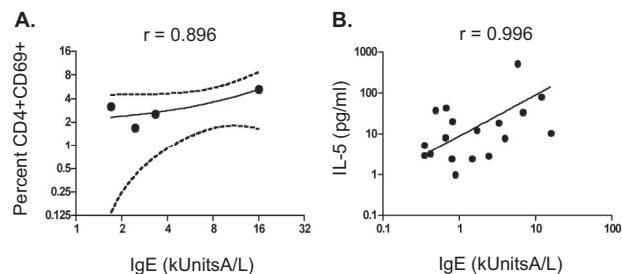


Figure 4. Correlation between immunoglobulin E (IgE) and CD4⁺CD69⁺ T-cell stimulation to fungal antigens after exposure for 48 hours from peripheral blood mononuclear cells (PBMCs) of one AFRS subject was assessed by flow cytometry as detailed in the Methods section. (A) Fungal-specific IgE levels were plotted versus percent frequency of CD4⁺CD69⁺ cells. The solid line shows the linear regression line, and the dashed lines show the 95% CIs. (B) Fungal-specific IgE levels from five AFRS subjects were plotted versus the amount of interleukin (IL)-5 cytokine. The solid line shows the linear regression line.

ing these different clinical subgroups such as CRS with and without nasal polyps have identified varied cytokine and lymphocyte profiles.^{13,14} Although Shin *et al.* showed that *Alternaria* stimulated both Th1 and Th2 expression from PBMCs of an unselected group of CRS subjects, the significant cellular and cytokine differences known to exist within subsets of CRS makes interpreting this study difficult.

We found optimized cytokine expression was noted at the low fungal antigen concentration of 5 $\mu\text{g/mL}$. In fact, the higher concentration of fungal antigens ($>50 \mu\text{g/mL}$) resulted in significant reduction of overall cytokine expression. Flow cytometric analysis of the PBMC population from both HCs and AFRS groups showed a high percentage of cell death when incubated for 72 hours at the higher fungal antigen concentration of 50 $\mu\text{g/mL}$ and greater. This lower fungal antigen concentration used in our study when compared with the 50- $\mu\text{g/mL}$ concentration used by Shin *et al.* might be another reason for the difference in results.

A skewed Th2 response to fungal antigens in AFRS supports a cellular response by PBMCs, but it does not reflect the role of an IgE-mediated pathway in its pathogenesis. In a preliminary study, we found a strong correlation of fungal-specific IgE levels to T-cell activation by the same fungal antigen as noted by an increase in CD4⁺CD69⁺ cells. In addition, IgE levels appear to correlate with IL-5 levels as illustrated in Fig. 4 B. Additional studies are needed to further investigate this finding but would support a role for an IgE-mediated pathway in the pathogenesis of AFRS. Although it appears that both allergic rhinitis and AFRS are activated by an IgE-mediated pathway, the significant differences in clinical presentation suggest that other pathways must be important in the pathogenesis of AFRS.

IL-10 is a suppressive cytokine, secreted by a number of cells including mast cells, dendritic cells, and antigen-induced T regulatory cells, that appears to regulate Th2 response in allergic asthma.¹⁵ Specifically, it has been shown to modulate B-cell production of IgE switch to IgG4, suppress mast activation of cytokine production, and inhibit eosinophil survival and cytokine production.¹⁶ It has been hypothesized that deficient levels of IL-10 may play a role in the pathophysiology

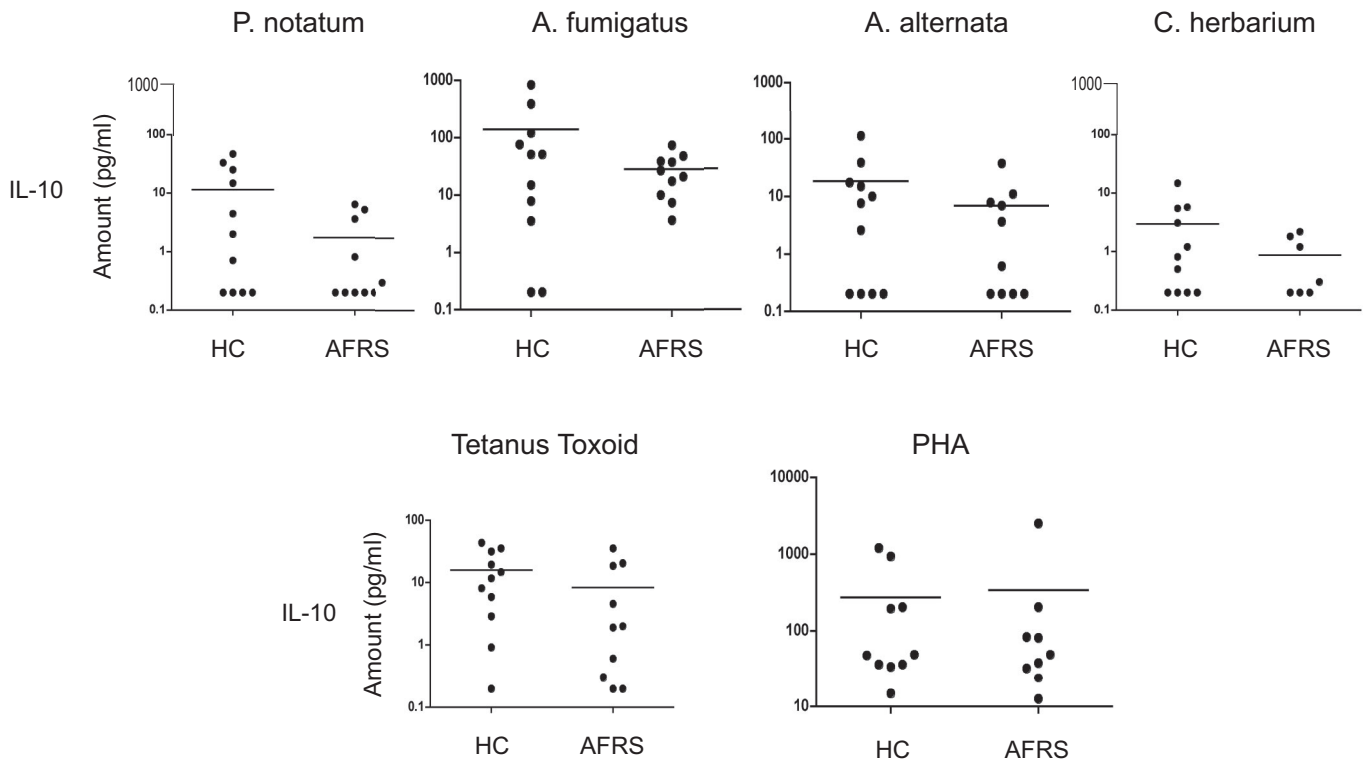


Figure 5. (A) Interleukin (IL)-10 responses to fungal antigens, (B) recall antigen tetanus toxoid, and mitogen phytohemagglutinin (PHA) in peripheral blood mononuclear cells (PBMCs) from allergic fungal rhinosinusitis (AFRS; $n = 10$) and healthy controls (HCs; $n = 11$). PBMCs were stimulated as detailed in the Methods section, and the culture supernatants were harvested at 72 hours. Data shown are individual values with group mean noted by horizontal bar. A value of 0.2 was substituted for 0 values only for visualization on graph.

of allergic patients.¹⁷ In our study, PBMCs, when challenged with fungal antigens, showed a notable elevation of IL-10 expression in HCs. Although the mean IL-10 levels were not statistically significant between the HCs and AFRS groups, every HC subject except one stimulated a pronounced IL-10 response to at least one of the four fungal antigens tested (data not shown). IL-10 production to fungal antigen was not as pronounced in AFRS subjects and unlike the HCs where IL-10 was stimulated by several different fungal antigens, the IL-10 response was found primarily with *Aspergillus*. Although additional studies are needed to further investigate this observation, it could suggest that in HCs, exposure to nonpathogenic antigens results in IL-10 secretion, which functions to suppress the initiation of an immune response. Other defensive molecules such as LL-37, an innate defense peptide, are expressed in nasal tissue from CRS but not eosinophilic mucin CRS patients when exposed to fungal antigens.¹⁸

In summary, PBMCs from AFRS individuals elicit a Th2 cytokine response to fungal antigen exposure. Meanwhile, challenge of PBMCs to fungal antigens in healthy individuals appears to incite secretion of IL-10. Future studies will be aimed at identifying the cells within AFRS individuals responding to the fungal antigens. In addition, cytokine characterization of the local response within the paranasal sinuses to the fungal antigen exposure is necessary to differentiate a systemic atopic versus a local paranasal response. Finally, the role of IL-10 will be investigated to determine if decreased levels of IL-10 on fungal antigen exposure contributes to the vulnerability of the paranasal sinus from AFRS patients.

CONCLUSION

In AFRS patients, fungal antigens stimulate T-cell activation, inducing a predominately Th2 immune response. Pilot studies suggest a strong correlation of Th2 responses to IgE levels, suggesting a possible role of an IgE-mediated pathway in the pathogenesis of AFRS. Finally, healthy controls appear to respond with elevated IL-10 production when exposed to fungal antigens, which may serve as a protective response.

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