

Nasal polyp cell populations and fungal-specific peripheral blood lymphocyte proliferation in allergic fungal sinusitis

Harshita Pant, M.B.B.S., Ph.D.,^{1,2} Dimitra Beroukas, B.Sc.,² Frank E. Kette, M.B.B.S., Ph.D.,³ William B. Smith, M.B.B.S., Ph.D.,³ Peter J. Wormald, M.B.B.S., M.D.,¹ and Peter J. Macardle, Ph.D.²

ABSTRACT

Background: Allergic fungal sinusitis (AFS) is considered a different disease from other polypoid chronic rhinosinusitis diseases (CRS) with eosinophilic mucus (EM) termed eosinophilic mucus chronic rhinosinusitis (EMCRS). To substantiate this, studies on cellular responses to fungi and sinus mucosal inflammatory cell populations in AFS and other EMCRS diseases are required. This study was designed to examine polyp inflammatory cell populations and peripheral blood fungal-specific T-cell responses in AFS, other EMCRS subgroups (defined later), and polypoid CRS without EM.

Methods: A prospective study was performed. Clinical characteristics, including CRS symptoms, sinus computed tomography (CT) scans, allergy status, intraoperative endoscopy, presence of EM, and fungal culture results were used to define patient groups. Polyps and peripheral blood were examined for populations of eosinophils, lymphocytes (CD4⁺, CD8⁺ T cells, natural killer cells, and B cells), and neutrophils using immunohistochemistry, cytospin preparations and flow cytometry. Fungal-specific peripheral blood lymphocyte proliferation was examined in AFS patients, other EMCRS patients, CRS patients, and controls.

Results: There was no significant difference in the percentage of cell populations and fungal-specific lymphocyte proliferation between AFS and other EMCRS diseases. However, AFS and other EMCRS polyps had a higher percentage of eosinophils and CD8⁺ T cells whereas CRS polyps had higher CD4⁺ T cells. Fungal-specific lymphocyte proliferation was significantly greater in AFS and other EMCRS patients regardless of fungal allergy, whereas in CRS and controls, higher proliferation was observed in fungal-allergic individuals.

Conclusion: These findings question the basis for differentiating AFS from other EMCRS diseases based on fungal allergy and fungi in EM. Fungal-specific cellular response was present in AFS and other EMCRS diseases, different from that associated with fungal allergy, suggesting a nonallergic fungal immune response. Increased CD8⁺ T cells in EMCRS polyps signify a different type of inflammation to CRS that may be driven by CD8⁺ T cells.

(Am J Rhinol Allergy 23, 453–460, 2009; doi: 10.2500/ajra.2009.23.3356)

Key words: Allergic fungal sinusitis, CD4⁺ T cells, CD8⁺ T cells, chronic rhinosinusitis, eosinophilic mucus chronic rhinosinusitis, fungal allergy, mucosal inflammation, nasal polyp, sinus mucosal cell populations, T cells

Immunoglobulin E (IgE)-mediated fungal allergy and fungi in sinus eosinophilic mucus (EM) are considered pathologically important in EM chronic rhinosinusitis (EMCRS) and have been used to subgroup these patients into the following proposed groups: allergic fungal sinusitis (AFS), AFS-like, nonallergic fungal eosinophilic sinusitis (NAFES), and nonallergic nonfungal eosinophilic sinusitis (NANFES).^{1,2} Historically, AFS is considered to be a separate disease from other subgroups of EMCRS diseases³ and is argued to be an IgE-mediated allergic reaction to fungi present in sinus EM. However, eosinophilic mucosal inflammation, nasal polyps, EM, and fungi in EM are also present in nonallergic CRS patients. Also, previous studies show that the clinical features, fungal-specific IgE responses, and other humoral responses are not significantly different between AFS and other EMCRS subgroups.^{2,4} Furthermore, there is controversy and limited evidence for an important role for IgE-mediated fungal allergy and for a unique role of fungi in the pathogenesis of AFS.

Several studies have described the clinical characteristics and possible etiologic agents in CRS, including allergy and fungi, but those

examining pathogenic mechanisms are few. An immune response, innate and adaptive, is involved in allergy and fungal immunity where lymphocytes form an integral component of such a response. A study of lymphocyte populations and antigen-specific responses may assist in defining the type of inflammation and in determining the pathological significance of fungal allergy and fungi in CRS, including AFS.

We hypothesized that EMCRS is a distinct type of mucosal inflammation that is associated with fungi and that AFS may not be significantly different from the other subgroups of EMCRS patients in terms of mucosal inflammatory cells and fungal-specific responses. Direct identification and analysis of antigen-specific T cells requires knowledge of the antigen epitopes in the context of major histocompatibility multimers.⁵ Other methods independent of defined peptides include enzyme-linked immunospot assays, cytometric cytokine secretion assays, and cytotoxic assays. These methods are strictly dependent on the specific effector functions of T cells after provocation with antigen *in vitro*. In CRS, the nature of the immunodominant peptides of fungal antigens, fungal-specific T-cell phenotype, and specific effector functions are unknown. Hence, studies were designed to examine fungal-specific T cells using cell proliferation-based methods. Clonal expansion is characteristic of T cells on stimulation by cognate antigen and measurement of T-cell proliferation is an estimate of the presence of antigen-specific T lymphocytes.

The aims of this study were to (1) obtain a more comprehensive understanding of the cell infiltrate in the sinonasal mucosa of AFS patients by determining the major inflammatory cell populations and (2) determine the cellular response to fungi, measured by the magnitude of fungal-specific peripheral blood (PB) cell proliferation. Results were compared with those from other subgroups of EMCRS patients, CRS patients with polyps and no EM, allergic rhinitis with fungal allergy (ARFA) patients, and healthy volunteers (HV).

From the Departments of ¹Otorhinolaryngology, Head and Neck Surgery, Queen Elizabeth Hospital and the University of Adelaide; ²Immunology, Arthritis, and Allergy, Flinders Medical Centre and the Flinders University of South Australia; and ³Clinical Immunology, Royal Adelaide Hospital, Adelaide, South Australia, Australia. This project was funded by a grant from the Garnett Passe and Rodney Williams Foundation, Suite 3, Level 8, 372-376 Albert Street, Victoria 3002, Australia. The authors had no conflicts of interest.

Ethics Board: Approval from the ethics committees of the following hospitals were obtained before the commencement of the study: Queen Elizabeth Hospital, Flinders Medical Centre, and Royal Adelaide Hospital, Adelaide, South Australia. Address correspondence and reprint requests to Harshita Pant, M.B.B.S., Ph.D., University of Pittsburgh Physicians, University of Pittsburgh Medical Centre, Suite 500, Eye and Ear Hospital, 200 Lothrop Street, Pittsburgh, PA 15213. Copyright © 2009, OceanSide Publications, Inc., U.S.A.

Table 1 Clinical characteristics of the study groups for mucosal studies

	CRS (n = 16)	AFS (n = 10)	AFS-like (n = 5)	NAFES (n = 12)	NANFES (n = 11)
Age (yr) (median IQR)	53 (31–59)	36 (28–47)	41 (34–50)	50 (30–64)	40 (31–58)
Male/female	9/7	6/4	3/2	8/4	6/5
Fungi in EM*	NA	7	0	8	0
Fungal culture positive	NA	5	0	9	0
Fungal allergy#	5	10	5	0	0
Nonfungal allergy§	7	10	5	5	4
Aspirin sensitivity	1	1	0	0	1
Sinus CT scan findings					
LM score (median IQR)	11 (8–18)	17 (14–21)	14 (7–20)	18 (11–22)	13 (9–19)
Double densities¶	0	7	1	8	4
Bone remodeling	2	9	3	9	7
Extra sinus involvement**	0	2	0	1	1

*By histology.

#By positive fungal-specific IgE or skin prick test.

§Mixes of tree pollen, grass pollen, house-dust mite, and animal dander.

¶Double densities, heterogeneous signal intensity within affected sinuses.

||Bone erosion or sclerosis.

**Extra sinus involvement, orbital in all cases.

IQR = interquartile range (25th–75th percentiles); EM = eosinophilic mucus; CT = computed tomography; LM = Lund and Mackay score; NA = not applicable; CRS = chronic rhinosinusitis; AFS = allergic fungal sinusitis; NAFES = nonallergic fungal eosinophilic sinusitis; NANFES = nonallergic nonfungal eosinophilic sinusitis.

MATERIALS AND METHODS

Patients and Controls

Patients were recruited prospectively from rhinology and allergy clinics and at the time of surgery. Human ethics approval was obtained from the relevant institutions and written informed consent from every individual. Results of nasoendoscopy, sinus computed tomography (CT) scans, sinus mucus histopathology, skin-prick tests, serology, relevant laboratory findings, and fungal culture were available for stratification into the study groups as previously described.² EMCRS was defined as CRS patients with nasal polyps and thick, tenacious-colored mucus in the sinuses at surgery, confirmed to be EM by histology. EMCRS patients were divided into subgroups depending on the presence of (a) fungi in EM by histology or culture and (b) allergy to any fungi, determined by elevated levels of mold mix-specific serum IgE or by positive skin-prick tests. AFS: EMCRS with fungi in EM and fungal allergy; AFS-like: EMCRS with fungal allergy and no fungi in EM; NAFES: EMCRS with fungi in EM and no fungal allergy; NANFES: EMCRS with no fungi in EM and no fungal allergy. CRS patients with nasal polyps and no EM on intraoperative findings are referred to as CRS. Rhinitis and fungal allergy patients with no evidence of sinusitis on history, endoscopic exam, and CT scan are referred to as allergic rhinitis with fungal allergy (AFRA). Healthy volunteers (HV) had no evidence of rhinitis and sinusitis determined by sinonasal outcome test 22 score of ≤ 1 , endoscopic exam, and an absent mold-mix-specific serum IgE.

Allergen-specific IgE was tested using the ImmunoCAP system (UniCAP-100; Pharmacia Diagnostics AB, Uppsala, Sweden) and by skin-prick testing using antigen extracts from Hollister-Stier Laboratories, LLC (Spokane, WA) for the following allergens: (1) molds—*Aspergillus*, *Alternaria*, *Helminthosporium*, *Penicillium*, *Candida*, *Epicoccum*, *Fusarium*, *Mucor*, *Rhizopus*, *Cladosporium*, *Pullularia*, and *Trichophyton* species; (2) nonfungal allergy, mixes of tree pollen, grass pollen, weed pollen, house-dust group, animal dander, and protein.

Individuals with coexisting medical problems, including allergic bronchopulmonary aspergillosis, Churg-Strauss syndrome, and those treated with systemic corticosteroids or other immunosuppressive therapy were excluded. Subjects had not smoked; had an upper respiratory tract infection; or used topical corticosteroids, antihista-

mines, anticholinergic, or homeopathic preparations in the preceding 4 weeks before collection of tissue and blood samples.

Tissue and PB Samples

Polyp and nonpolyp sinonasal tissue from the same individual was obtained from every EMCRS and CRS patient during sinus surgery. Tissue were placed in 10% formalin and embedded in paraffin blocks for histology. For flow cytometry, tissue was passed through 80G sieves and single cell suspensions were washed twice with RPMI 1640 before suspending in 5% newborn bovine serum in PBS (Gibco BRL, Grand Island, NY). Matched PB samples were also obtained immediately before surgery for cell proliferation studies and to serve as controls for tissue immunophenotyping.

Immunohistochemistry

Indirect immunoperoxidase staining was used to examine the distribution of cells in tissue sections. The first section of tissue series from every individual was also stained with hematoxylin and eosin (H&E) for histological comparison. Serial tissue sections of 4- μ m thickness were cut and dried onto 2% HistoGrip-coated slides (Zymed Laboratories, Inc., South San Francisco, CA). The sections were deparaffinized in xylene, rehydrated through descending grades of ethyl alcohol through to Tris-buffered saline. Sections were incubated overnight with optimized dilutions of primary antibody (eosinophilic cationic protein [EG2; Pharmacia]), CD3 (Cell Marque, Austin, TX), CD4 (Novocastra Laboratories, Ltd., Newcastle upon Tyne, U.K.), CD8 (Cell Marque), and negative isotype control antibodies. After washing, sections were incubated with biotinylated goat anti-mouse IgG (Dako, Glostrup, Denmark), washed and incubated further with avidin-biotin-horseradish peroxidase (Dako), and washed and counterstained with hematoxylin.

Immunophenotyping of Tissue and PB Samples

CD4 and CD8 T-cell, B-cell and NK-cell populations were determined using Tritest antibodies: CD3 FITC/CD4 R-PE/CD45 PerCP, CD3 FITC/CD8 R-PE/CD45 PerCP, CD3 FITC/CD19 R-PE/CD45 PerCP, and CD3 FITC/CD16 CD56 PE/CD45 PerCP (BD Biosciences, San Jose, CA). Cell surface expression of other molecules was deter-

mined using antibodies to major histocompatibility complex class II (MHC II) and CD25. In experiments with PB samples, red blood cell lysis was performed after immunofluorescence staining by adding 2 mL of fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences). Samples were analyzed on FACScan (BD Biosciences) and data were analyzed using CellQuest (BD Biosciences) software. Ten thousand gated events were acquired from each sample of PB and 2500–5000 gated events were acquired from tissue samples. Lymphoid cells were selected using side scatter properties and CD45^{high} expression. Appropriate isotype controls were used to set quadrant markers.

Cytospin Preparations

Triplicate samples of tissue single cell suspension (50 μ L at 5×10^5 /mL) were loaded onto cytospin cuvettes (Shandon, Inc., Pittsburgh, PA), mounted onto histogrip-coated slides (Zymed Laboratories, Inc.) and paper cards, and centrifuged at $800 \times g$ for 2 minutes to allow a monolayer of cells to deposit on the slides. Slides were air-dried and counterstained with H&E and the cell types were identified by their nuclear morphology and cytoplasmic staining. All cell counts were performed using light microscopy and a hemocytometer. Specimens with cell viability of $>95\%$ were analyzed.

Fungal-Specific Cell Proliferation

Fungal Antigens. *Alternaria tenuis* (alternata) (Cat. No. 5009JF10) and *Aspergillus fumigatus* (Cat. No. 5021JF10), were obtained from Hollister-Stier Laboratories, LLC. The reported value for the fungal-specific proliferative response in an individual corresponds to the highest value obtained for either fungus-specific proliferation.

Proliferation Assay. PB mononuclear cells (PBMCs) were prepared by Lymphoprep (Axis-Sheild PoC AS, Oslo, Norway). Cells were washed twice before suspension at 10^7 cells/mL in RF5 (RPMI 1640 supplemented with 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, 0.3 mg/mL of glutamine, and 5% fetal calf serum). PBMCs at 10^5 cells in a final volume of 200 μ L/well were added in triplicate to flat-bottom, 96-well tissue culture plates (Cell Star, Frickenhausen, Germany) and stimulated with fungal antigens at a final concentration of 7.5 μ g/mL. The same patients' unstimulated PBMCs were kept in a 37°C 5% CO₂ incubator for 36 hours. At 36 hours, tissue culture wells were washed and unstimulated PBMCs were added to antigen-primed cells. Cells were harvested after 96 hours for cellular tritiated thymidine incorporation, where the amount incorporated by PBMCs in response to stimulant was compared with that from control wells to yield a stimulation index (SI). Internal PBMC controls included (a) a negative control with tissue culture medium alone and (b) a positive control with phytohemagglutinin at a final concentration of 12.5 μ g/mL. Numbers of CD4⁺ and CD8⁺ T cells, B cells, NK cells, monocyte, and macrophage populations were determined in PBMC before setting up the proliferation assays.

Statistics

Analysis for independent groups of data was performed with the Mann-Whitney *U* test for comparison between two groups and with the Kruskal-Wallis test with Dunn's *post hoc* test for comparison between multiple groups using GraphPad Prism software, Version 4.0a for Macintosh (GraphPad, San Diego, CA). Data are presented as a median and the 25th–75th percentiles, the limits of the interquartile range (IQR). A value of $p < 0.05$ was considered significant.

RESULTS

Mucosal Studies

The mucosal studies included histology, immunohistochemistry, cytospin preparations, and flow cytometry. The clinical characteristics of study groups are shown in Table 1. There were no significant

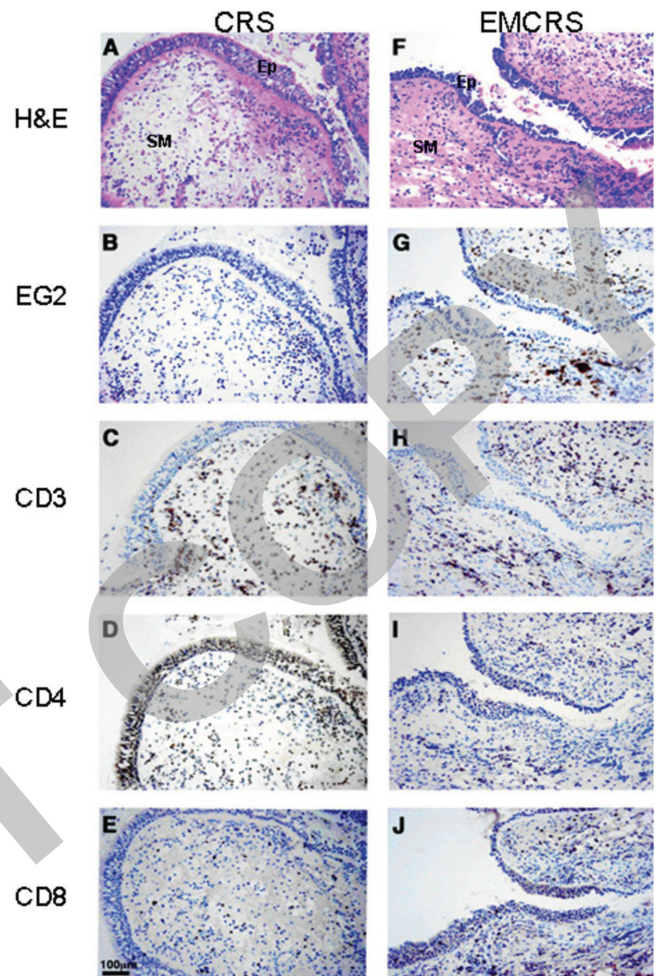


Figure 1. Immunohistochemical localization of eosinophils, CD3⁺, CD4⁺, and CD8⁺ cells in nasal polyps. (A and F) These images show the typical tissue architecture in sections stained with hematoxylin and eosin and the characteristic distribution of the inflammatory cells in sections stained with immunoperoxidase in a CRS and an EMCRS (AFS) patient. Cells that stained brown were positive for one of the following: (B and G) eosinophil cationic protein (EG2), (C and H) CD3, (D and I) CD4, and (E and J) CD8. AFS, allergic fungal sinusitis; CRS, chronic rhinosinusitis; EMCRS, eosinophilic mucus chronic rhinosinusitis; SM, submucosa; Ep, epithelium.

differences in the results of mucosal studies between polyp and nonpolyp tissue from the same individual; hence, data reported here are from polyp analysis.

Histology and Immunohistochemistry

Paraffin-embedded, H&E-stained serial sections of nasal polyps from 10 AFS, 5 AFS-like, 12 NAFES, 11 NANFES, and 16 CRS patients were examined for tissue architecture and distribution of inflammatory cell populations. The tissue architecture between AFS, AFS-like, NAFES, and NANFES were similar. The epithelial layer and submucosal glandular structures were better preserved in CRS polyps than in the EMCRS subgroups (AFS, AFS-like, NAFES, and NANFES). A predominant eosinophil population was present in the polyps from all patient groups. Eosinophils were found throughout the lamina propria, in the intraepithelial layer, and in the mucus. The tissue distribution of the lymphocyte populations was not significantly different between the study groups, where CD3⁺ cells and CD8⁺ cells were abundant in the intraepithelial region. In the lamina propria and

Table 2 Percentages of eosinophil, neutrophil, lymphocyte, mast cell, and monocyte populations in nasal polyps

	CRS (n = 16)	AFS (n = 10)	AFS-like (n = 5)	NAFES (n = 12)	NANFES (n = 11)	p Value*
Eosinophils	41.4 (38.5–46.1)	53.1 (48.6–61.4)	58 (49.9–64.6)	57.7 (50.6–63.9)	54 (49.1–58.3)	0.0005#
Neutrophils	8 (2.6–15.6)	3.5 (1.4–14)	5 (1–15.5)	5.8 (2–11.5)	6 (2.2–12)	0.91
Lymphocytes	26.5 (21.9–30.4)	25.1 (21.2–30.8)	22 (18.4–30.2)	25.7 (20.6–30.3)	22.2 (21.6–30.8)	0.89
Mast cells	2.6 (0.6–4.1)	0.75 (0–2.9)	1 (0–2.7)	1.5 (0–2.8)	1 (0–2)	0.52
Monocyte	17.3 (15.7–21.1)	15.7 (8.6–23.9)	11 (7.7–22.8)	13.5 (14.9–26.6)	16 (16–26.5)	0.36

Results are expressed as a median value and the interquartile range in parenthesis.

*Kruskal-Wallis test with post hoc Dunn test if $p < 0.05$.

#CRS vs AFS, CRS vs AFS-like, CRS vs NANFES, $p < 0.05$; CRS vs NAFES, $p < 0.01$.

CRS = chronic rhinosinusitis; AFS = allergic fungal sinusitis; NAFES = nonallergic fungal eosinophilic sinusitis; NANFES = nonallergic nonfungal eosinophilic sinusitis.

periglandular areas both CD4⁺ and CD8⁺ cells were present. Images from a representative experiment from a CRS and an EMCRS patient (AFS subgroup) are shown in Fig. 1.

Cytospin Preparations

The percentages of polyp inflammatory cells including eosinophils, neutrophils, lymphocytes, mast cells, and monocytes were determined (Table 2). There was a significant difference in the percentage of eosinophils between the study groups. *Post hoc* testing showed no significant difference between the EMCRS subgroups and a greater percentage of eosinophils in all the EMCRS patients compared with CRS patients ($p < 0.0001$; Fig. 2). There was no significant difference in the percentages of neutrophils, total lymphocytes, mast cells, and monocytes between all of the study groups.

Flow Cytometry Studies

Lymphocyte Populations (Total T Cells, CD4⁺ T Cells, CD8⁺ T Cells, B Cells, and NK Cells). Matched polyp and PB samples from CRS, AFS, AFS-like, NAFES, and NANFES patient groups were examined for the percentage of total T-cell (CD45⁺CD3⁺), CD4⁺ T-cell (CD45⁺CD3⁺CD4⁺), CD8⁺ T-cell (CD45⁺CD3⁺CD8⁺), B-cell (CD45⁺CD19⁺), and NK-cell (CD45⁺CD56⁺/CD16⁺) populations. In blood, there was no significant difference in the percentage of these cell populations between all of the study groups (data not shown). However, in polyps, there was a significant difference in the percentage of cell populations between the groups (Table 3). The *post hoc* statistical difference was present between CRS and each of the EMCRS subgroups. Importantly, there was no significant difference in the percentages of the cell populations between AFS and other EMCRS patient subgroups. Accordingly, further analysis and results from the EMCRS subgroups were combined into a single EMCRS group ($n = 38$) and compared with those from CRS patients ($n = 16$). Mann-Whitney *U* test was used to examine statistically significant differences between the two groups.

The percentage of T cells in polyps was significantly greater in EMCRS (median = 86.6; IQR = 83.3–92.3) compared with CRS (median = 76.0; IQR = 66.4–81.0; $p = 0.002$; Fig. 3 B). The percentage of T cells in PB was not significantly different between CRS (median = 79.0; IQR = 69.0–81.3) and EMCRS patients (median = 77.7; IQR = 69.2–80.8; $p = 0.98$).

CD4⁺ and CD8⁺ T Lymphocyte Populations. As shown in Fig. 4, the percentage of CD8⁺ T cells was strikingly higher in EMCRS polyps (median = 62.9; IQR = 54.2–68.3) compared with CRS (median = 31.5; IQR = 26.1–43.3; $p < 0.0001$) and the percentage of CD4⁺ T cells was significantly lower in EMCRS polyps (median = 23.6; IQR = 19.7–26.7) compared with CRS (median = 38.6; IQR = 30.0–49.4; $p < 0.0001$). There was no statistically significant difference in the percentage of PB CD4⁺ (CRS, median = 46.0 and IQR = 43.9–56.8; EMCRS, median = 44.6 and IQR = 41.2–53.9; $p = 0.42$) and CD8⁺ (CRS, median = 23.7 and IQR = 17.2–28.8; EMCRS, median = 29.3

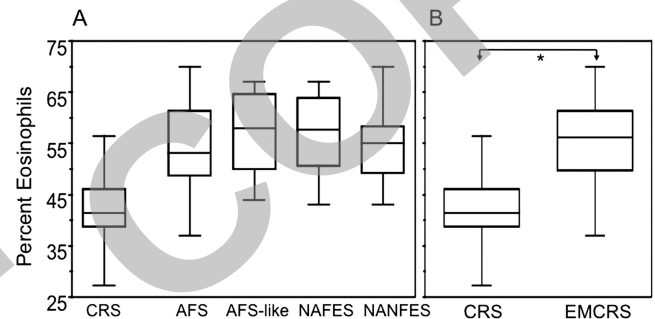


Figure 2. Percentage of eosinophils in the inflammatory cell infiltrate of nasal polyps in CRS and EMCRS patients. Lower and upper limits of the boxes indicate the 25th and 75th percentiles (interquartile range), respectively. The vertical bars show the ranges and horizontal bars the medians. A comparison between (A) all the study groups and (B) between CRS and EMCRS is shown. * $p < 0.0001$, Mann-Whitney *U* test. CRS, chronic rhinosinusitis; EMCRS, eosinophilic mucus chronic rhinosinusitis.

and IQR = 21.6–32.8; $p = 0.24$) T-cell subsets between CRS and EMCRS patients.

MHC II and CD25 Expression on T Cells. The proportion of CD4⁺ and CD8⁺ T cells expressing MHC II in polyps and PB was not significantly different between CRS and EMCRS patients. In both CRS and EMCRS, the percentage of CD4⁺ T cells expressing MHC II was significantly higher in polyps (CRS, median = 41.5 and IQR = 35–50; EMCRS, median = 38.4 and IQR = 26–57.1) than in PB (CRS, median = 7 and IQR = 4.25–13.11; EMCRS, median = 4.8 and IQR = 3.9–9.3; $p = 0.007$). Similarly, the percentage of CD8⁺ T cells expressing MHC II was significantly higher in polyps (CRS, median = 41.7 and IQR = 37.6–56.9; EMCRS, median = 63.3 and IQR = 26.5–75.7) than in PB (CRS, median = 15.8 and IQR = 11.9–18; EMCRS, median = 4.7 and IQR = 4–14.3; $p = 0.007$).

Similarly, the fraction of T cells expressing CD25 was not significantly different between CRS and EMCRS patients. The percentage of CD4⁺ T cells expressing CD25 was higher in polyps (CRS, median = 33.7 and IQR = 23.5–47.3; EMCRS, median = 24.8 and IQR = 20.9–37.8) than in PB (CRS, median = 4.4 and IQR = 2.7–8.7; EMCRS, median = 4 and IQR = 2.8–7.3) of CRS ($p = 0.002$) and EMCRS ($p = 0.008$) patients. Likewise, the percentage of CD8⁺ T cells expressing CD25 was significantly higher in polyps (CRS, median = 20.6 and IQR = 11.3–31.6; EMCRS, median = 12.9 and IQR = 6.9–22) than in PB (CRS, median = 0.8 and IQR = 0.3–2.2; EMCRS, median = 0.6 and IQR = 0.4–1.6) of CRS ($p = 0.002$) and EMCRS ($p = 0.02$) patients.

Fungal-Specific Proliferation Studies

Magnitude of Fungal-Specific Mononuclear Cell Proliferation. Tritiated thymidine incorporation into PBMCs in response to fungal antigens was measured in 12 HVs, 15 ARFA patients, 15 CRS patients, and 34

Table 3 Percentage of lymphocyte populations in nasal polyps

	CRS (n = 16)	AFS (n = 10)	AFS-like (n = 5)	NAFES (n = 12)	NANFES (n = 11)	p Value*
CD3 ⁺ T cells	68.8 (60–75.5)	88.2 (83.7–94.7)	88 (83.2–90)	86.9 (80.7–89.9)	86.3 (81–90.3)	0.0003#
CD4 ⁺ T cells	38.6 (31.2–49.4)	20.7 (18.1–24.9)	21.3 (16.5–24.8)	25.2 (19.1–32.4)	25.1 (20.7–26.7)	<0.0001§
CD8 ⁺ T cells	31.5 (26.2–43.3)	65.95 (60–73.9)	67 (61–69)	61.1 (43.8–65.9)	57 (48.1–72.4)	<0.0001¶
B cells	9.3 (4.9–17.5)	5.75 (2.6–8.7)	6.3 (4.5–17.2)	7 (5.4–9.1)	3.4 (2.3–11.0)	0.38
NK cells	10.1 (6.2–14.9)	4.3 (2.1–7.1)	6 (3.2–10.4)	5.2 (4.2–9.9)	5.15 (2.4–8.5)	0.06

Results are expressed as a median value and the interquartile range in parenthesis.

*Kruskal-Wallis test with post hoc Dunn test if $p < 0.05$.

#CRS vs AFS, $p < 0.001$; CRS vs AFS-like, CRS vs NAFES, and CRS vs NANFES, $p < 0.05$.

§CRS vs AFS, $p < 0.001$; CRS vs AFS-like, $p < 0.01$; CRS vs NAFES and CRS vs NANFES, $p < 0.05$.

¶CRS vs AFS, $p < 0.001$; CRS vs AFS-like, $p < 0.01$; CRS vs NAFES and CRS vs NANFES, $p < 0.05$.

CRS = chronic rhinosinusitis; AFS = allergic fungal sinusitis; NAFES = nonallergic fungal eosinophilic sinusitis; NANFES = nonallergic nonfungal eosinophilic sinusitis.

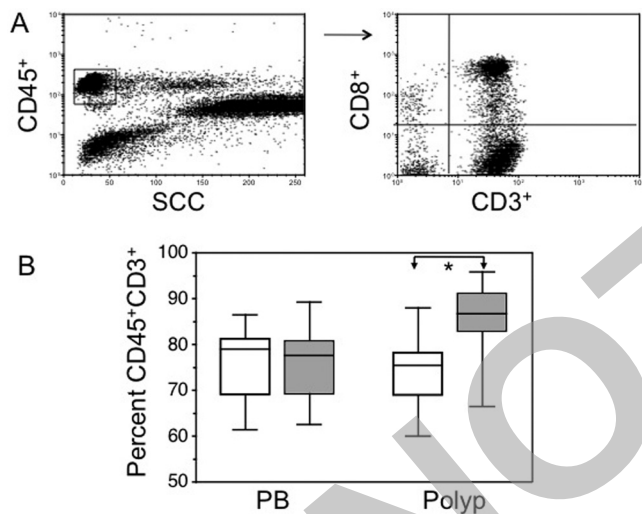


Figure 3. Lymphocyte subsets in the PB and nasal polyps of CRS and EMCRS patients. Dot plots are gated on (A) CD45^{high} and the (B) the percentage of CD3⁺ cells were determined in 16 CRS (clear boxes) and 38 EMCRS (shaded boxes) patients. Lower and upper limits of the boxes indicate the 25th and 75th percentiles (interquartile range), respectively. The vertical bars show the ranges and horizontal bars the medians. * $p = 0.002$, Mann-Whitney U test. CRS, chronic rhinosinusitis; EMCRS, eosinophilic mucus chronic rhinosinusitis; PB, peripheral blood.

EMCRS patients (AFS, $n = 12$; AFS-like, $n = 5$; NAFES, $n = 9$; NANFES, $n = 8$). The clinical characteristics of the study groups are summarized in Table 4. The reported value for the fungal-specific proliferative response in an individual corresponds to the highest value obtained for either *Alternaria alternata*- or *Aspergillus fumigatus*-specific proliferation. The proliferation results for each study group, including the negative and positive control values expressed as the mean $\pm$ SEM counts per minute is provided in Table 5 and results expressed as the median SI is shown in Fig. 5.

The extent of fungal-specific PBMC proliferation was not significantly different between the EMCRS subgroups ($p = 0.46$; Fig. 5 A), regardless of fungal allergy or of the detection of fungi in EM. As a single group, EMCRS patients had a greater proliferative response compared with HVs ($p < 0.0001$) but not when compared with ARFA ($p = 0.33$) or CRS ($p = 0.29$) patients. In CRS patients, fungal-specific PBMC proliferation was higher in fungal-allergic compared with nonfungal-allergic patients ($p = 0.01$; Fig. 5 B). Further analysis showed that there was no significant difference in the SI between

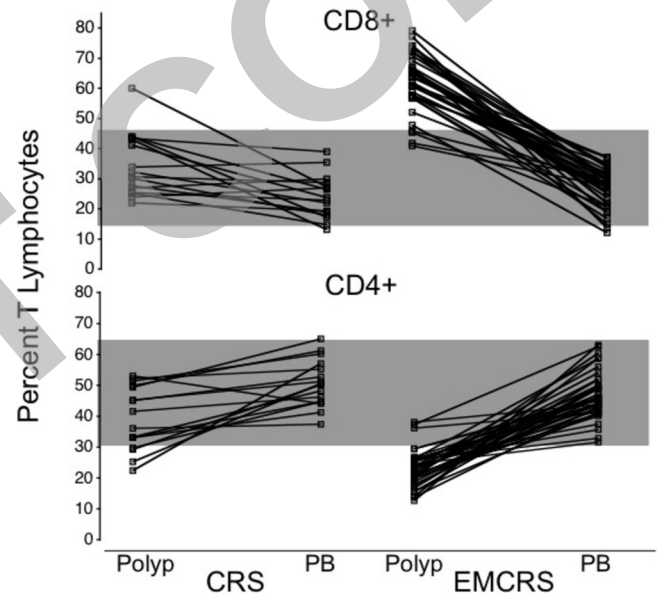


Figure 4. Comparison of percentages of CD8⁺ and CD4⁺ T lymphocytes between nasal polyp and PB in 16 CRS and 38 EMCRS patients. The lines connect the percentage values of lymphocytes between matched samples of polyp and PB for each individual. The shaded areas indicate the normal reference ranges for CD8⁺ and CD4⁺ T lymphocytes in PB. CRS, chronic rhinosinusitis; EMCRS, eosinophilic mucus chronic rhinosinusitis; PB, peripheral blood.

allergic CRS patients, allergic EMCRS patients, and nonallergic EMCRS patients ($p = 0.34$).

DISCUSSION

This study showed that mucosal inflammatory cell populations and fungal-specific T-cell responses were not significantly different between AFS and other EMCRS study groups. As a single group, EMCRS polyps had significantly greater percentages of eosinophil and T-cell populations compared with CRS polyps. Further analysis of polyp T cells showed a significantly higher percentage of CD8⁺ T cells in EMCRS compared with a higher percentage of CD4⁺ T cells in CRS. These differences were found in the mucosal tissue and were not a reflection of PB CD4⁺ and CD8⁺ T-cell ratios because these cell numbers in blood were within normal limits. This important difference in the proportions of mucosal inflammatory cells supported our hypothesis that EMCRS patients had a different type of mucosal inflammation compared with CRS patients. This may, in part, explain

Table 4 Clinical characteristics of the study groups for cell proliferation studies

	HV (n = 12)	ARFA (n = 15)	CRS (n = 15)	AFS (n = 12)	AFS-like (n = 5)	NAFES (n = 9)	NANFES (n = 8)
Age (yr) (median IQR)	37 (24–33)	41 (35–52)	50 (32–56)	42 (27–46)	51 (32–49)	42 (24–51)	55 (32–53)
Male/Female	7/5	8/7	8/7	7/5	3/2	5/4	5/3
Fungus in EM*	NA	NA	NA	100%	0%	100%	0%
<i>Alternaria alternata</i>				6		3	
<i>Aspergillus fumigatus</i>				1		2	
Both				5		4	
Other fungi#				6		4	
Fungal allergy§	0%	100%	47%	100%	100%	0%	0%
<i>Alternaria alternata</i>		5	1	2	2		
<i>Aspergillus fumigatus</i>		1	1	1	2		
Both		8	2	4	1		
Other fungi¶		11	3	5	3		
Nonfungal allergy (%)	42	80	60	83	100	71	40
Aspirin sensitivity	0	1	1	1	0	0	1
Sinus CT scan findings	NA						
LM score (median IQR)		0 (0–0)	11 (8–18)	17 (13–21)	14 (7–20)	19 (11–22)	14 (9–18)
Double densities**		0	0	7	1	7	3
Bone remodeling##		0	2	9	3	8	5
Extrasinus involvement§§		0	0	2	0	1	1

*By histology or fungal culture.

#*Bipolaris*, *Drechslera*, *Trichothecium*, *Candida*, *Penicillium*, *Scedosporium*, *Acremonium*, *Devriessii*, *Phialophora*, and *Cladosporium* species.

§By positive fungal-specific serum IgE or skin prick test.

¶*Helminthosporium*, *Penicillium*, *Candida*, *Epicoccum*, and *Cladosporium* species.

||Mixes of tree pollen, grass pollen, house-dust mites, and animal dander.

**Double densities, heterogeneous signal intensity within affected sinuses.

##Bone erosion or sclerosis.

§§Extra sinus involvement, orbital in all cases.

QR = interquartile range (25th–75th percentiles); EM = eosinophilic mucus; CT = computed tomography; LM = Lund and Mackay score; NA = not applicable; CRS = chronic rhinosinusitis; AFS = allergic fungal sinusitis; NAFES = nonallergic fungal eosinophilic sinusitis; NANFES = nonallergic nonfungal eosinophilic sinusitis.

Table 5 Fungal-specific peripheral blood mononuclear cell proliferation expressed as the mean ± SEM counts per minute

Study Groups	Peripheral Blood Mononuclear Cell Treatment		
	Unstimulated (Control)	Fungal Antigen	Phytohemagglutinin
HV (n = 12)	905 ± 231	2001 ± 319	20,533 ± 2322
ARFA (n = 15)	798 ± 122	11641 ± 266	42,667 ± 1661
CRS (n = 15)	963 ± 36	7028 ± 87	22,444 ± 1242
AFS (n = 12)	1109 ± 115	9816 ± 233	55,521 ± 1099
AFS-like (n = 5)	893 ± 209	8291 ± 180	51,339 ± 988
NAFES (n = 9)	572 ± 138	9022 ± 221	58,666 ± 1022
NANFES (n = 8)	1021 ± 96	9163 ± 253	60,887 ± 1886

CRS = chronic rhinosinusitis; AFS = allergic fungal sinusitis; NAFES = nonallergic fungal eosinophilic sinusitis; NANFES = nonallergic nonfungal eosinophilic sinusitis.

the spectrum of clinical phenotypes of chronic sinonasal inflammation, with EMCRS being a more destructive form of inflammation than that seen in CRS, suggested by a greater percentage of eosinophils and T cells and less-preserved tissue architecture. Also important, the cell populations were not significantly different between polyp and nonpolyp tissue in the same individual, indicating a generalized mucosal inflammation.

T-cell responses to fungal antigens were investigated because fungi are implicated in the pathogenesis of CRS and in AFS, where fungal allergy is proposed to be a major pathogenic mechanism.^{6,7} In this study, all EMCRS subgroups had an elevated T-cell proliferation response to fungi compared with HVs, irrespective of fungal allergy

or detection of fungus in EM. Although fungal-specific T-cell proliferation was significantly higher in EMCRS patients compared with HVs, it was not significantly greater compared with ARFA and CRS patients. In ARFA patients, increased stimulation is likely to be attributed to fungal allergy. Similarly, given that increased cell proliferation was limited to fungal-allergic CRS, allergy is the likely explanation for elevated proliferation responses in these patients. In contrast, both fungal-allergic (AFS and AFS-like) and nonallergic (NAFES, NANFES) EMCRS patients had an elevated fungal-specific proliferation. Also, we have previously shown that fungal-specific humoral response was elevated in all EMCRS subgroups, irrespective of fungal allergy.² Furthermore, CD4⁺ T cells, commonly present in

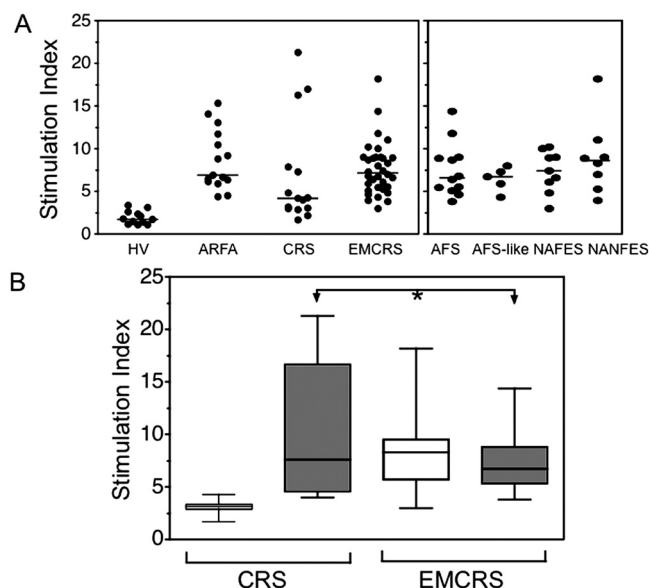


Figure 5. Fungal-specific peripheral blood mononuclear cell proliferation expressed as SI in HVs, ARFA, CRS, and the EMCRS patients. The maximum proliferative response to either *Alternaria alternata* or *Aspergillus fumigatus* is plotted for every individual. The horizontal bars illustrate the median SI values. (A) Comparison of SI between HVs ($n = 12$), ARFA ($n = 15$), CRS ($n = 15$), and a single group of EMCRS ($n = 34$) patients and between the EMCRS subgroups (AFS, $n = 12$; AFS-like, $n = 5$; NAFES, $n = 9$; and NANFES, $n = 8$) is shown. (B) Comparison of SI between nonfungal-allergic (□) and fungal-allergic (■) CRS and EMCRS patients is depicted. * $p = 0.34$, Kruskal-Wallis test. A summary of the proliferation results expressed as the mean $\pm$ SEM counts per minute is provided in Table 5. AFS, allergic fungal sinusitis; ARFA, allergic rhinitis with fungal allergy; NAFES, nonallergic nonfungal eosinophilic sinusitis; NANFES, nonallergic nonfungal eosinophilic sinusitis; CRS, chronic rhinosinusitis; EMCRS, eosinophilic mucus chronic rhinosinusitis; HVs, healthy volunteers; SI, stimulation index.

allergic conditions, were relatively low in EMCRS mucosa.^{8,9} These findings show a separation of cellular and humoral immune responses from fungal allergy, supporting the hypothesis that mechanisms other than IgE-mediated allergy may account for the elevated fungal-specific immune response in EMCRS patients.

The elevated immune response to fungi in EMCRS may be due to chronic stimulation caused by fungi trapped in the EM or by an altered host immune response to fungal antigens. Chronic local exposure to fungal antigens in EMCRS as a cause may be advocated because fungal hyphae are often found within EM, as in AFS and NAFES subgroups.⁴ It could also be argued that fungi can potentially be detected in a greater proportion of EM specimens, hence, AFS-like and NANFES, by using more sensitive fungal detection methods.¹⁰ On the other hand, such elevated and distinct immune responses in the absence of allergy to these common environmental fungi in EMCRS may signify a dysregulated immune mechanism associated with an altered response to fungi.

Although an elevated fungal-specific immune response was present in EMCRS, a direct causal link between fungi and the inflammation in EMCRS still needs to be established. Elevated fungal-specific IgG and IgA from the humoral studies suggests that a mucosa-derived antigen was involved in EMCRS patients.² In EMCRS, the presence of fungi may contribute to inflammation by several mechanisms. Eosinophils are believed to be the major effector cell mediating mucosal damage.^{11,12} Fungi may recruit eosinophils directly *via* Toll-like receptors, indirectly *via* Ig Fc receptors and by

inducing proeosinophilic cytokine secretion by other cells or by as yet undefined mechanisms. Fungal proteins are also directly toxic to cilia and epithelial cells, affect mucus production, and alter innate responses.¹³ Furthermore, T cells can influence eosinophil responses to fungi.

Both CD4⁺ and CD8⁺ T cells are essential in providing primary immunity to fungi, mediating pathogen clearance, damage to surrounding microenvironment and in immune-regulatory function.^{12,14–16} We found that EMCRS mucosa had an abundant CD8⁺ T-cell population with a relative absence of CD4⁺ T cells. Fungi have been reported in inflammation associated with CD8⁺ T cells.¹⁷ Fungi can recruit mucosal CD8⁺ T cells in a nonspecific manner either directly because of their toxic effects or indirectly by the generation and maintenance of cytolytic CD8⁺ T cells *via* fungal-specific CD4⁺ T cells.^{15,18,19} Hence, it is plausible that in genetically susceptible individuals, a dysfunctional population of CD8⁺ T cells and a relative lack of CD4⁺ T cells may be responsible for the ineffective clearance of fungi and an exacerbation of the sinonasal inflammation.

CONCLUSIONS

Except for the defining criteria of fungal allergy and fungi in sinus EM, in these prospective studies, there appear to be no significant differences between the EMCRS subgroups with regard to their clinical parameters, fungal-specific cellular and humoral responses, and mucosal inflammatory cell populations. Taken together, this suggests that the fundamental process driving the inflammatory response in EMCRS may not be significantly different between the subgroups. There is increasing evidence that factors other than fungal allergy, including an altered mucosal immune system, may contribute to sinonasal inflammation in AFS and other EMCRS subgroups. Also, all EMCRS subgroups have a greater fungal-specific immune response regardless of the presence of fungi by histology or culture. Hence, these findings further question the basis for defining “AFS” on the presence of fungal allergy and of fungi in EM and for placing “AFS” in a separate category from other EMCRS subgroups. Accordingly, categorizing EMCRS patients into subgroups based on fungal allergy and detection of fungi in EM may not be of central pathological relevance. It is becoming apparent that AFS and other EMCRS patients have worse clinical outcomes and perhaps a different form of inflammation from other polypoid CRS patients. Thus, an understanding of the underlying inflammatory mechanisms, including and beyond allergy and fungi, remains an important goal. Future clinical research and mucosal studies of the inflammatory cells and mediators, including functions of CD8⁺ and CD4⁺ T cells in well-defined patient groups and controls, may lend further insight into susceptibility for chronic inflammation, pathogenic mechanisms, and potential therapeutic targets.

REFERENCES

1. Bent JP III, and Kuhn FA. Diagnosis of allergic fungal sinusitis. *Otolaryngol Head Neck Surg* 111:580–588, 1994.
2. Pant H, Kette FE, Smith WB, et al. Fungal-specific humoral response in eosinophilic mucus chronic rhinosinusitis. *Laryngoscope* 115:601–601, 2005.
3. Ferguson BJ. Eosinophilic mucin rhinosinusitis: A distinct clinicopathological entity. *Laryngoscope* 110:799–813, 2000.
4. Pant H, Kette FE, Smith WB, et al. Eosinophilic mucus chronic rhinosinusitis: Clinical subgroups or a homogeneous pathogenic entity? *Laryngoscope* 116:1241–1247, 2006.
5. Altman JD, Moss PAH, Goulder PJR, et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94, 1996.
6. Ponikau JU, Sherris DA, Kern EB, et al. The diagnosis and incidence of allergic fungal sinusitis. *Mayo Clin Proc* 74:877–884, 1999.
7. Manning SC, and Holman M. Further evidence for allergic pathophysiology in allergic fungal sinusitis. *Laryngoscope* 108:1485–1496, 1998.
8. Cohn L, Elias JA, and Chupp GL. Asthma: Mechanisms of disease persistence and progression. *Annu Rev Immunol* 22:789–815, 2004.

9. Gould HJ, Sutton BJ, Beavil AJ, et al. The biology of IgE and the basis of allergic disease. *Annu Rev Immunol* 21:579–628, 2003.
10. Taylor MJ, Ponikau JU, Sherris DA, et al. Detection of fungal organisms in eosinophilic mucin using a fluorescein-labeled chitin-specific binding protein. *Otolaryngol Head Neck Surg* 127: 377–383, 2002.
11. Ponikau JU, Sherris DA, Kephart GM, et al. Striking deposition of toxic eosinophil major basic protein in mucus: Implications for chronic rhinosinusitis. *J Allergy Clin Immunol* 116:362–369, 2005.
12. Inoue Y, Matsuwaki Y, Shin SH, et al. Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. *J Immunol* 175:5439–5447, 2005.
13. Ooi EH, Wormald PJ, Carney AS, et al. Fungal allergens induce cathelicidin LL-37 expression in chronic rhinosinusitis patients in a nasal explant model. *Am J Rhinol* 21:367–372, 2007.
14. Mody CH, Paine R III, Jackson C, et al. CD8 cells play a critical role in delayed type hypersensitivity to intact *Cryptococcus neoformans*. *J Immunol* 152:3970–3979, 1994.
15. Santoni G, Boccanera M, Adriani D, et al. Immune cell-mediated protection against vaginal candidiasis: Evidence for a major role of vaginal CD4(+) T cells and possible participation of other local lymphocyte effectors. *Infect Immun* 70:4791–4797, 2002.
16. Wuthrich M, Filutowicz HI, Warner T, et al. Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous antigen presentation to CD8+ T cells: Implications for vaccine development in immune-deficient hosts. *J Exp Med* 197:1405–1416, 2003.
17. Badauy CM, Barbachan JJ, Rados PV, et al. Relationship between *Candida* infection and immune cellular response in inflammatory hyperplasia. *Oral Microbiol Immunol* 20:89–92, 2005.
18. Yekeler H, Bitmis K, Ozcelik N, et al. Analysis of toxic effects of *Alternaria* toxins on esophagus of mice by light and electron microscopy. *Toxicol Pathol* 29:492–497, 2001.
19. Ma LL, Spurrell JC, Wang JF, et al. CD8 T cell-mediated killing of *Cryptococcus neoformans* requires granulysin and is dependent on CD4 T cells and IL-15. *J Immunol* 169:5787, 2002. □

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.