Contrasting the Microbiomes From Healthy Volunteers and Patients With Chronic Rhinosinusitis

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Chronic rhinosinusitis (CRS) is the persistent inflammation of the sinus and nasal passages lasting over 3 months. The etiology of CRS is not well understood.

OBJECTIVE To obtain insights into the disease process, we contrasted the microbiome and immune response from patients with CRS and healthy controls.

DESIGN, SETTING, AND PARTICIPANTS A case vs control design was used. Samples were collected in the operating room in an institutional hospital or clinic. Thirty patients with CRS and 12 healthy controls undergoing surgery were recruited.

MAIN OUTCOMES AND MEASURES The microbiome was analyzed by deep sequencing of the bacterial 16S and fungal 18S ribosomal RNA genes. Immune response was measured by quantification of 30 different cytokines by multiplexed enzyme-linked immunosorbent assay, and immune cells in the lavage were identified by flow cytometry. The immune response of peripheral blood leukocytes to the lavage microbiota was assessed by interleukin (IL)-5 enzyme-linked immunospot assay.

RESULTS While quantitative increase in most bacterial and fungal species was observed in patients with CRS relative to controls, the microbiomes of patients with CRS were qualitatively similar to the controls. Because these results indicated that bacteria and fungi are not triggering CRS, we undertook a more detailed characterization of the immune response. Patients with CRS had increased levels of the following cytokines: IL-4, IL-5, IL-8, and IL-13, along with increased levels of eosinophils and basophils in the lavage. Importantly, peripheral blood leukocytes isolated from patients with CRS responded to control lavage samples (i.e., commensals) to produce IL-5. In contrast, the same lavage sample evoked no IL-5 production in leukocytes from healthy controls.

CONCLUSIONS AND RELEVANCE These findings support the theory that in some cases CRS results from an immune hyperresponsiveness to commensal organisms.
More recently, attention has turned to the role of fungi in this disease process. Ponikau et al\textsuperscript{14} used a novel culturing method to demonstrate the presence of fungi in 96% of the 210 patients with CRS examined. Several fungal species were identified: on average 2.2 fungal species per patient with CRS. In their patient population a few fungal species including \textit{Alternaria}, \textit{Aspergillus}, \textit{Cladosporium}, and \textit{Penicillium} showed statistical differences between patients with CRS and controls. The survey of fungi has been repeated in patients with CRS in a number of countries using the polymerase chain reaction against the fungi with varying results.\textsuperscript{15-18} For these analyses, organism-specific primers for number of fungi identified by Ponikau et al\textsuperscript{14} were used, but consistently no statistical differences in the presence of fungi in patients with CRS and control samples were found.

Most of the studies evaluating host immune response in patients with CRS have identified an elevation of cytokines associated with a T helper (Th) 2 response. In some patient subsets, predominantly in Asian populations, a Th1 and Th17 response has also been observed.\textsuperscript{19} Current opinion holds that CRS is likely a multifactorial disorder that is generally characterized by Th1,2 response in the host. Additional studies have shown that the immune response can be Th1- skewed in white patients without polyps.\textsuperscript{20} Although some authors have suggested that CRS is a response to fungi, the exact role of microbes in the pathophysiologic mechanisms of CRS is unknown and remains the source of ongoing debate.\textsuperscript{21}

It has long been known that the majority of microorganisms in most environments do not readily grow in culture. Although the idea of missing organisms goes back to the 1960s,\textsuperscript{22} the earliest demonstrable evidence for this came from studies by Woese and colleagues\textsuperscript{23-25} in mid 1980s, who showed by amplification of the highly conserved 16S ribosomal RNA gene (rRNA) that less than 1% of the organisms isolated from deep sea vents could be cultured in the laboratory. Organisms may be unculturable in the laboratory because they grow in a specific microenvironment (eg, nutrient requirement or within a narrow range of carbon dioxide and oxygen concentrations) that cannot be easily reproduced in the laboratory. Importantly, because the search space of conditions is very large, only a small number of conditions can reasonably be tested. Under any given condition, the growth of only a subset of organisms would be observed. For many years, little could be learned about these organisms without culturing. More recently, as next-generation methods have become available and become inexpensive, deep sequencing has been used to identify the organisms in many environments.\textsuperscript{26-27}

On the basis of these experiments, we reasoned that the culturing methods used in the reported studies of CRS might also have detected only a subset of the organisms actually present. We therefore used an “unbiased” or culture-independent method of amplifying and sequencing the bacterial 16S and fungal 18S rRNA genes from the lavage of patients with CRS and controls. To explore the potential interaction between the microbiome and host, we also measured the host immune response by measuring the cytokines and the identification of immune cells present by fluorescence-aided flow cytometry.

**Methods**

**Patient Recruitment**

Thirty patients with medically refractory CRS undergoing endoscopic sinus surgery were consecutively recruited to this study by 2 of the authors (R.S. and T.S.) between October and December 2007. All patients met the American Academy of Otolaryngology–Head and Neck Surgery criteria for CRS and had notable disease on posttherapy computed tomographic imaging. As controls, 12 patients undergoing endonasal surgery for conditions other than CRS (pituitary tumor resection, endoscopic orbital procedures, and septoplasty) were also recruited. Patients were required to give written consent to participate in the study. The institutional review board at Saint Louis University School of Medicine approved this study.

**Sample Collection**

Following induction of general anesthesia, lavage of the middle meatus was performed prior to any surgical intervention. With endoscopic guidance, 10 mL of sterile normal saline was instilled into the superior middle meatus and collected by gentle suction using a Fraser-tip suction cannula and a sterile suction trap. Samples were collected from both nasal passages. The lavage samples were labeled with a patient identifier in the operating room to comply with Health Insurance Portability and Accountability Act (HIPAA) regulations and were stored on ice until they were transported to the laboratory (within 2 to 8 hours). All samples were processed in a laminar flow hood using disposable plasticware to prevent cross-sample contamination and contamination by airborne microbes. A 500-μL aliquot of the sample was withdrawn and processed immediately for flow cytometry analysis, and the remainder was frozen. Only the samples from the left side were thawed, aliquoted (approximately 250 μL), and frozen at −80°C. Lavage samples from the right side were labeled and archived unopened at −80°C.

**Preparation of Genomic DNA**

An aliquot of the lavage was rapidly thawed and spun at 16,000 g for 15 minutes at 4°C. The supernatants were used for cytokine assay. Zirconia/silica beads (125 μg of 0.1-mm beads; Biospec Products) were added to pellet, which was then frozen in liquid nitrogen. The samples were lyophilized to dryness under vacuum (Savant SpeedVac; Thermo Scientific) for 1.5 hours and then pulverized (Mini-beadbeater-16; Biospec Products) for 3 minutes. The lysate was diluted with 2 volumes of 10 mM Tris (pH 8.0) and 25 mM EDTA and transferred to new tubes after a brief spin and treated with RNase (Promega) for 20 minutes at 37°C. The samples were extracted with phenol/chloroform and then ethanol precipitated in the presence of ammonium acetate. DNA was quantitated using a Nanodrop 3300 fluorospectrometer using PicoGreen (Molecular Probes) dye.

**Polymerase Chain Reaction Amplification**

Polymerase chain reaction amplification of the bacterial 16S rRNA was performed on 20 ng (quantified by PicoGreen [Invitrogen] using NanoDrop fluorometer [Thermo]) of DNA using
27F(5′-AGGGTTCTGAGCTGACGAG-3′)and518R(5′-CCATATTACGCGCTGCTG-3′)primers. Polymerase chain reaction amplification of the fungal 18S rRNA was performed using 40 ng (quantified by PicoGreen) of DNA using FFi(5′-GT'TTTGATCMTGGCTGCG-3′)andFRi(5′-CTTCTAGTCGTAATCTTTATG-3′)primer pairs. The primer designs chosen were based on published literature evaluations,28-31 and the primers in the SILVA ribosomal RNA database.32 The forward primers also included at the 5′ end the multiplex identifier sequences (MIDS) and a 4-base pair (bp) key. A different MIDS (total of 43: 12 nondiseased individuals + 30 patients with CRS and 1 normal saline control) was used for each lavage sample. The reverse primers had in addition the Roche capture primer sequences at the 5′ end. All reactions were set up in laminar hood to prevent contamination. Normal saline processed in parallel was included as a negative control in all cases. The amplified products (35 cycles) were resolved on a 1% agarose gel and visualized by ethidium bromide staining.

Roche 454 Sequencing

The MIDS-coded 16S (or 18S) amplicons were pooled (50 ng per patient) and subject to emulsion polymerase chain reaction. The amplicons were sequenced at MOgene, LC, on a 454 GS-FLX Titanium sequencer (Roche). The mean read length was 382.6 bp for 16S and 422.4 bp for 18S rRNA. The sequence data for this study have been submitted to NCBI Sequence Read Archive and can be accessed through BioProject ID PRJNA192977.

Data Analysis

The sequence data were analyzed using scripts in the QIIME (Quantitative Insights into Microbial Ecology)33 software suite for chimera elimination and other functions. Additional custom scripts were written for data analysis. The August 5, 2012, version 111 of SILVASSU (small subunit) database was used for annotation (http://www.arb-silva.de). Similarity was measured using the Yue-Clayton Theta index34 as follows:

\[
\theta_{YC} = \frac{\sum_{i=1}^{S_{F}} a_{i} b_{i}}{\sum_{i=1}^{S_{F}} a_{i} - \sum_{i=1}^{S_{F}} a_{i} b_{i}}
\]

where \(S_{F}\) is total number of species, and \(a_{i}\) and \(b_{i}\) are the relative abundance of each species (by 16S or 18S rRNA sequence) in the sample. The data of Grice et al35 sampling 20 different topological areas of human skin were downloaded from NCBI GenBank (BioProject ID PRJNA30123; accession numbers EU534411-EU540623).

Flow Cytometry

Sterile EDTA was added to final concentration of 25mM to an aliquot of the lavage to minimize cell aggregation, which was then centrifuged at 400g for 5 minutes. Cells were stained using a commercial kit (catalogue No. 337166, BD TBNK 6-color multitest; BD Biosciences) per manufacturer’s instructions. The kit contains fluorescein isothiocyanate (FITC)-labeled CD3, clone SK7; phycoerythrin (PE)-labeled CD16, clone B7.3, and CD56, clone NCAM 16.2; peridinin-chlorophyll protein complex (PerCP)-Cy5.5-labeled CD45, clone 2D1 (HLe-1); PE-Cy7-labeled CD4, clone SK3; allophycocyanin (APC)-labeled CD19, clone SJ25C1; and APC-Cy7-labeled CD8, clone SK1. Fluorescent beads were used to normalize counts between samples. For determining eosinophil and basophil counts, PerCP-Cy5.5-labeled CD45, clone 2D1 (HLe-1); PE-labeled CD, clone Y1/82A; APC-labeled CD49D, clone 9F10; FITC-labeled CD203, clone 97A6; and Pac-Blue conjugated CD123 (eBiosciences) were used. Flow cytometry was performed on an LSR II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc).

Cytokine Assay

Supernatants from the 16 000g spin were used for cytokine assay. Cytokines were quantified using the Lumienx multiplexed enzyme-linked immunosorbent assay (ELISA) system (Millipore) according to the manufacturer’s instructions.

Isolation of Leukocytes From Blood

Peripheral blood (7 mL) was collected in EDTA-coated BD Vacutainer tubes (BD Biosciences) and used within 2 hours of collection. Five milliliters of the each patient’s blood was spun (15 minutes at 8000g) through Histopaque 1077 (Sigma), and the leukocyte layer (peripheral blood mononuclear cells) was collected with a sterile Pasteur pipette. The cells were washed, counted, and used in the enzyme-linked immunospot (ELISpot) assay directly.

IL-5 ELISpot Assay

The capture antihuman interleukin (IL)-5 antibody (BD Biosciences) was coated overnight at 4°C, in a 96-well tissue culture-treated plate following the supplier’s protocol. All assays were carried out in triplicate wells using 3 dilutions: 20 000, 100 000, and 500 000 leukocytes. One hundred microliters of frozen lavage samples were pelleted at 16 000g, then resuspended in Roswell Park Memorial Institute (RPMI) media (RPMI + 10% heat-inactivated fetal bovine serum [ΔFBS] + penicillin, streptomycin, and glutamine + 50 μM β-mercaptoethanol) and added per well to leukocytes and incubated for 72 hours. Bound IL-5 was detected by horseradish peroxidase (HRP)-conjugated antibody using AEC (3-aminobenzidine; Sigma) as a substrate. The spots were counted using an automated ImmunoSpot micro Analyzer (CTL Inc).

Results

Patients

We enrolled 42 individuals (30 patients with CRS and 12 controls) between October 1 and December 23, 2007, who were scheduled to undergo surgery. Samples were collected during this short period to avoid potential confounding seasonal effects on the microbiome. The demographics of the patients and controls are provided in the Table. As previously noted, many of the patients with CRS also had asthma. All patients resided within 50 miles of the metropolitan St Louis, Missouri, area. One patient had visible fungal infection (acute or
allergic fungal sinusitis) at the time of surgery, in addition to the chronic inflammation. Samples were collected by lavage of the middle meatus using endoscopic guidance after induction of anesthesia as described in the Methods section.

Deep Sequencing of Microbiome of the Middle Meatus

Fungal cells have rigid cell walls and are very resistant to lysis using chemical methods. We therefore developed a method for efficient method of lysis for both bacteria and fungi. Our method is to freeze cells in liquid nitrogen and then use beads in a bead beater to pulverize the frozen pellet (see Methods section). The DNA was amplified using “universal” 16S bacterial28,36,37 or 18S fungal38 primers in separate reactions. The 5’ rRNA primer also contained a sequence tag (“barcode” or Roche MIDS) that is unique to each lavage sample. The bacterial 16S amplicons were pooled (22 ng per patient) and sequenced on half of the plate, and the 18S amplicons, on the other half of the plate, on a Roche 454 GS-FLX Titanium sequencer. A total of 800 827 reads were obtained from the 16S sequence, originating in the Gulf of Mexico. These second most abundant bacteria in both CRS and control samples.

Bacterial Microbiome in the Lavage of the Middle Meatus

The approximate 800 000 sequences were annotated by using the BLAST program (National Center for Biotechnology Information) against a library of rRNA (SILVA) database. The rRNA sequences were analyzed at the operational taxonomic unit (OTU) level. The OTU definition considers rRNA sequences with 95% sequence identity at the nucleotide level as a single species. A total of 2333 species of bacteria belonging primarily to 5 different phyla were identified in the normal control individuals. In contrast, 3780 species were identified in patients with CRS, indicating that the diversity of bacteria increased in patients with chronic inflammation (Figure 1A). To contrast the microbiomes, we used the Yue-Clayton dissimilarity parameter θ because it considers both diversity and abundance (see Methods section). Assessment of interpatient differences were performed by pairwise comparison of each patient against all others. The Yue-Clayton θ for all patients with CRS was, on average, 0.1 at the OTU level. A θ value close to zero indicates that the microbiomes among the cohort of patients with CRS was nearly the same. Similarly, the mean θ for all pairwise comparisons for the healthy controls was also 0.1, indicating high similarity of the microbiomes as well. The mean θ for patients with CRS vs healthy controls was 0.14 (at the OTU level), also indicating high similarity and reflecting the high degree of overlap shown in Figure 1A.

As expected from other environmental and physiological microbiome studies, there was a dominant organism followed by other species present at lower abundance (Figure 2B). This finding (Figure 1B) further documents the increased diversity of organisms in patients with CRS relative to control cohorts. The number of species of bacteria increased in patients with CRS, relative to controls, across all phyla (Figure 1C). Proteobacteria were also well represented, with nearly 1200 species that are second most abundant bacteria in both CRS and control samples.

Next, we examined the abundance of individual species that are found in all patients (Figure 1D). The most abundant species, representing on average nearly 36% of the reads, was an uncultured Cyanobacteria, present in both healthy and chronically inflamed sinus samples. The 16S rRNA sequences had 100% identity (BLAST E-value = 0.0) to the reference sequence, originally isolated in the Gulf of Mexico. The second most abundant species present in all 29 patients, and present at very low abundance in the 13 controls, was Curtobacterium. Curtobacterium is found in soil and on plants and has been used for fermentation and cheese production for hundreds of years. Clinical isolates of Curtobacterium from sputum, wounds, and eye discharge have been described. Funke et al39 noted that the Curtobacterium clinical isolates grew optimally at 32°C, an observation that may indicate why this species is found in the airway passages.

The most abundant species of bacteria with a statistically significant increase in patients with CRS relative to controls was Corynebacterium accolens (Figure 1D). Also noteworthy is that the presence of 2 species, Alicyclobacillus and Cloacibacterium, was decreased in patients with CRS relative to the control set.

We next compared the population of bacteria found in the sinus of healthy patients with the skin microbiome. Grice et al40 have performed deep sequencing of microbes from 20 different topographical areas of the skin. We were particularly interested in assessing if the sinus microbiome was similar to the nares because both of these areas filter the air, although they are different types of surfaces (mucosal vs skin). Therefore, we performed pairwise comparison using each of the 20 topological areas of the skin35 to the aggregate healthy sinus.

Table. Characteristics of Patients With Chronic Rhinosinusitis and Control Cohorts

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients With CRS (n = 30)</th>
<th>Control Volunteers (n = 12)</th>
<th>P Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD), y</td>
<td>48.0 (18.9)</td>
<td>51.4 (10.3)</td>
<td>.63</td>
</tr>
<tr>
<td>Male/Female, No.</td>
<td>11/19</td>
<td>5/7</td>
<td>.80</td>
</tr>
<tr>
<td>Smokers, No.</td>
<td>4</td>
<td>1</td>
<td>.90</td>
</tr>
<tr>
<td>Polyp, No.</td>
<td>20</td>
<td>0</td>
<td>2 × 10−16</td>
</tr>
<tr>
<td>Asthma, No.</td>
<td>16</td>
<td>2</td>
<td>.04</td>
</tr>
<tr>
<td>AFS, No.</td>
<td>2</td>
<td>0</td>
<td>&gt; .99</td>
</tr>
<tr>
<td>Allergy test positive, No.</td>
<td>2</td>
<td>2</td>
<td>&gt; .99</td>
</tr>
</tbody>
</table>

Abbreviation: AFS, allergic fungal sinusitis.
a Values in boldface indicate statistical significance.
A. The overlap of all species identified by Roche 454 sequencing of the 16S ribosomal RNA (rRNA) amplicons is shown (values represent numbers of species). The patients with CRS had a greater diversity of organisms. Although 37% of the species overlap, the CRS- and control-specific sets are species present at very low abundance (<0.1%). Furthermore, these species were not present in all 30 CRS and 12 control lavage samples. B. The distribution of species (rank order vs number of raw reads for each of the species) is shown. The plot shows that 1 species was dominant and most species were present at less than 0.1% (<800 reads for a species/800,000 total reads). C. The number of species within each phylum and corresponding fraction of reads are shown for CRS and control samples. All species identified, regardless of distribution across patient samples, are given in this table. D. The species with the highest abundance in patients with CRS is shown, along with their fractional abundance in the control samples. Only those species that were found in all 29 patients with CRS are listed. P values were calculated using a 2-tailed Mann-Whitney test. NS indicates nonsignificance (P > .05). E. A pairwise comparison of the overlap of the (healthy) control sinuses microbiome to the 20 topological skin areas sampled by Grice et al\(^3\) using the Yue-Clayton θ dissimilarity measure. The external auditory (Ext aud) canal had the highest similarity (lowest θ value) due in part to the abundance of Cyanobacteria and number of Actinobacteria species. Axill vault indicates axillary vault; RA crease, retroauricular crease. *Increased abundance in patients with CRS relative to controls (panel D). **Increased abundance in controls relative to patients with CRS (panel D).
The approximate 800,000 18S fungal rRNA were assigned to 106 species from the lavage of control subjects and 132 species in patients with CRS. The overlap between patients with CRS and controls was 17.5% (Figure 2A) in the fungal species compared with 35% in the bacterial species (Figure 1A). Again, more diversity is observed in patients with chronic inflammation relative to the control population. There is an increased abundance of all species in patients with CRS as well as evidenced by the distribution plot (Figure 2B). Figure 2B also documents that a single species was dominant in the sinus of both the control and CRS populations. Three fungal phyla were represented in the data (Figure 2C), although Chytridiomycota was only observed in the control population at very low levels (accounting for only 0.5% of the reads). Surprisingly, the most abundant fungus in both control and CRS microbiomes was *Cryptococcus neoformans* variety *neoformans* (strain D) was the dominant species in both CRS and control samples.

**The Fungal Microbiome of the Middle Meatus**

The approximate 800,000 18S fungal rRNA were assigned to 106 species from the lavage of control subjects and 132 species in patients with CRS. The overlap between patients with CRS and controls was 17.5% (Figure 2A) in the fungal species compared with 35% in the bacterial species (Figure 1A). Again, more diversity is observed in patients with chronic inflammation relative to the control population. There is an increased abundance of all species in patients with CRS as well as evidenced by the distribution plot (Figure 2B). Figure 2B also documents that a single species was dominant in the sinus of both the control and CRS populations. Three fungal phyla were represented in the data (Figure 2C), although Chytridiomycota was only observed in the control population at very low levels (accounting for only 0.5% of the reads). Surprisingly, the most abundant fungus in both control and CRS microbiomes was *Cryptococcus neoformans* variety *neoformans* (strain D). In patients with CRS, *C neoformans* represented 90% of the reads, relative to 61% of reads in controls. *Cryptococcus neoformans* is a relatively common fungus found nearly ubiquitously, but its abundance in the sinus has not been previously documented. While *C neoformans* was present in the range of 55% to 63% across all control samples, the second most abundant
Fungal species was *Rhodosporidium diabovatum*. *Rhodosporidium diabovatum* was found in the range of 1.8 to 2.5% in CRS samples, but it was not detected in any of the control samples (Figure 2D). Two species (by 18S sequence) of *Malessezia* were abundant in the control samples but were found in decreased abundance in CRS samples.

Comparison of the interpatient intercontrol microbiomes yielded a mean (SD) Yue-Clayton θ of 0.10 (0.008), indicating high similarity. Cross-comparison of all 30 CRS against all 12 control microbiomes yielded a modestly higher θ of 0.15 (0.01), also indicating high similarity.

A detailed examination of the list of both bacterial and fungal species provided little evidence for presence of an organism that could trigger a chronic inflammatory response.

**Cytokine Response**

To determine the type of immune response and to measure interpatient variance in the chronic immune response, we quantified the cytokines in the lavage samples. We measured 30 different cytokines and chemokines by multiplexed ELISA. As shown in Figure 3, of these 30 cytokines, we only found IL-4, IL-5, IL-8, and IL-13 to be significantly elevated (*P* < .0001) in patients with CRS relative to controls. As expected from previous studies, levels of these potent inflammation mediators, especially IL-4 and IL-5, and IL-13 were significantly higher in the patients with CRS. No interferon γ, typically associated with a T_{h1} response, nor IL-17 was observed in any of patients. Given the lack of precision in the recovery of instilled saline solution, the results of interpatient variation for these cytokines was surprisingly small. One reason for quantifying cytokines was to assess cytokines levels as a measure of disease severity and to correlate the disease severity to levels of specific microbes present in the lavage. However, our results indicate that all patients have similar levels of cytokines, and no correlation was observed.

**Immune Cells in Middle Meatus Lavage**

To determine which cells produce the cytokines and to provide an independent measure of interpatient variance, we quantified the numbers of immune cells using flow cytometry. Again, surprisingly the intrapatient variance was surprisingly small (Figure 4B and see Methods section for details). The increased levels of eosinophils in CRS have been previously documented. The lack of appreciable levels of B or T cells in healthy controls and patients with CRS was surprising. Interestingly, we also detected significant levels of basophils in all patients with CRS, which were not detected in healthy controls. To our knowledge, the presence of high levels of baso-

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**Figure 3. Assessment of Cytokines From the Lavage of Patients With Chronic Rhinosinusitis (CRS) and Healthy Controls**

![Figure 3](image_url)
Leukocyte Hyperreactivity in Patients With CRS

Original Investigation  Research

Figure 4. Characterization of the Immune Response Using Fluorescent-Activated Cell Sorter

A. The gating scheme for identification of the immune cells. B. The quantification of the immune cells. The numbers given in the “Controls” and “Patients With CRS” columns are the mean (SD) percentage of the parent cell population. For instance, 1.6% (0.8%) of CD3+ (T cells) were CD4+ T cells. P values in bold indicate statistical significance. CRS indicates, chronic rhinosinusitis; ND, not detected; and NK, natural killer.

Hyperresponsiveness to Commensal Microbiome in Patients With CRS

Although there are subtle differences in the abundance of the CRS and control microbiomes as assessed by lavage of the middle meatus, there is also significant overlap, as indicated by the mean (SD) cross-comparison θ of 0.15 (0.01). This result suggested that overall, chronic inflammation distorts the commensals, allowing some of the organisms to thrive and others to be eliminated, but that these organisms were not in themselves causing disease. Indeed, we hypothesize that the immune system in the patients was responding inappropriately to commensal organisms. To test this notion, we exposed 10,000 peripheral blood leukocytes to the microbiome (lavage samples) from the healthy individuals. Lavage samples (250 μL) were pelleted at 10,000 × g and resuspended in 100 μL of RPMI media, and 25 μL was added to leukocytes and cocultured for 72 hours. To measure immune response we used an ELISpot assay for IL-5 secretion (see Methods for details).

If the patients were hyperresponsive, we would expect a stronger response from leukocytes from the patients with CRS cocultured with a normal (commensal) microbiome, relative to leukocytes from healthy individuals cocultured with the same microbiome. However, there could be factors present in the lavage that were evoking IL-5 secretion (ie, a Th2 response). To test this possibility, we cocultured leukocytes from healthy individuals with lavage from patients with CRS. Finally, we also cocultured CRS lavage with leukocytes from the patients with CRS to complete the “all vs all” comparison.

As shown in Figure 5, we observed 6- to 7-fold more IL-5-positive ELISpot spots with leukocytes from patients with CRS co-cultured with lavage from healthy individuals relative to leukocytes from healthy individuals with the identical lavage. This result indicates that there were more peripheral circulating leukocytes responding to commensal organisms.

Our experiment design also allows us to test for the presence of factors present in the lavage of patients with CRS that can trigger IL-5 production in leukocytes from healthy individuals. The results (Figure 5) show that more leukocytes from healthy individuals responded to CRS lavage, relative to normal lavage. The leukocytes from patients with CRS also responded with increased IL-5 production when cocultured with CRS lavage. These results indicate that there are factors present in CRS lavage that evoke a strong IL-5 response (ie, Th2 immune response) even in healthy individuals. We interpret these results to indicate that patients

Ten thousand or 50 thousand peripheral blood leukocytes isolated from either 5 patients with CRS or 5 healthy control volunteers (NOR) were cocultured with lavage from the same control (nondiseased) individuals. The immune response was measured using an interleukin (IL)-5 enzyme-linked immunospot (ELISpot). Each data point is an average of 6 to 8 wells. Leukocytes from 1 patient and 1 control were cocultured with 2 different lavage pellets: 1 from self and 1 from a nonself lavage. The leukocytes from the patients with CRS produced 278.3 [50.3] mean (SD) IL-5-positive spots compared with leukocytes from healthy volunteers that produced 12.2 [5.8] spots (P = .005). Coculturing leukocytes from patient with CRS with lavage from patients with patients produced 350 [47.3] positive ELISpot spots, compared with leukocytes from healthy volunteers cocultured with the same CRS lavage that produced 75.0 [34.8] IL-5-positive ELISpot spots. To test for IL-5 carryover from the CRS lavage samples, we also performed ELISpot assay from lavage alone, and we observed 3.3 [(1.2) spots. P values were calculated using the 2-tailed Mann-Whitney test in Prism 5.0d statistical package (GraphPad). The dashes in the y-axis labels indicate that the component (leukocytes or microbiota) was omitted in those set of wells.
Cyanobacteria found in moist soil and marine environments are phototrophes, and therefore their abundance in the sinus (a relatively dark environment) was surprising. Although Cyanobacteria (Figure 1E) has been observed in skin and gut microbiomes, the high abundance of Cyanobacteria in the sinus has not been previously documented. Cyanobacteria are abundant in marine (ocean, brackish, and fresh water) and in terrestrial soil samples.42 They are abundant, especially in the first meter of a water column, so they can collect light and absorb carbon dioxide for photosynthesis. It is relevant for a carbohydrate-rich environment like the sinus that many Cyanobacteria can grow as heterotrophs and survive for extended periods of time in complete darkness.43,44 Furthermore, some species have been shown to be allergens.45 Consistent with the bacteria species that are often found by culturing samples from patient with CRS, we observed 2 Pseudomonas species, including P aeruginosa.

16S rRNA sequencing also identified Corynebacterium in accord with a recent study by Abreu et al.46 Recently, Abreu et al46 identified Corynebacteria tuberculosis as significantly overrepresented in patients with CRS.46 Corynebact-

eria accolens and C tuberculosis are highly related at the 16S rRNA sequence, and since Abreu et al46 used microarrays, the difference of species assignment may lie in fine details that would be resolved by additional studies. In contrast to Abreu et al, however, we observed an overall increase in the diversity of bacterial species in lavage of patients with CRS relative to the control population.

Our study is the first to date to identify Cryptococcus neoformans variety neoformans as a major constituent of the sinus fungal microbiome (Figure 2). This and other fungi detected by 18S rRNA sequencing have been previously detected ubiquitously and are present in both CRS and control samples. Indeed, a detailed examination of all of the bacterial and fungal species identified by deep sequencing produced no obvious candidate as an allergen or pathogen that could trigger an inflammatory response (but see discussion below). Therefore, we undertook a more detailed characterization of the immune response.

Flow cytometry analysis of the lavage showed significant increase in eosinophils and basophils (Figure 4). The eosinophilia has been previously noted by several studies.47-50 The presence of basophils has only been previously documented in a single publication.53 We observed a statistically significant increase in the Th2 cytokines: IL-4, IL-5, and IL-13 (Figure 3). Interleukin 8 was also significantly increased in patients with CRS (Figure 3). Other chemokines such as RANTES (regulated upon activation normal t cell expressed and secreted) and IP-10 (interferon γ inducible protein 10) were present at significant levels in control and CRS lavage but were not statistically different. While Th2 cytokines are often associated with T-cell responses, we find few CD4 T cells in our la-

vage samples (Figure 4); it is possible that these T cells are present in the mucosa and not released by the gentle lavage procedure used here.

Finally, based on our observation that nearly all the fun-
gal and bacterial species we identified are commensals, we hypothesized that the patients were hyperresponsive to these

Discussion

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versity analysis was performed to identify organisms that could potentially trigger an inflammatory response. We used deep sequencing of bacterial 16S and fungal 18S rRNA in the lavage samples from the normal and chronically inflamed individuals. Our goal was to both identify how chronic inflammation alters the microbiome and also to identify if there potential pathogens present that may be triggering an immune re-

sponse. The sequencing identified a number of species that have not been previously shown to colonize the sinus cavity, as well as those that have been identified by culturing. Among the phyla, Cyanobacteria were the most abundant (Figure 1C), with 104 species present in the lavage of both the normal and chronically inflamed individuals with nearly 47% of the reads.

Figure 6. Microbiome, Host Response, and Disease

The microbiome is a spectrum of symbionts (mutually beneficial to microbe and host), commensals (neutral for microbe and host), and pathogens (detrimental to host) that has been described as mutualism.54 In addition to the mutualism axis shown on the x-axis, the immune response (y-axis) and disease severity (z-axis) are also drawn. The illustration is intended to show that the immune response keeps the levels of commensals in check as an appropriate immune response. In contrast, too strong an immune response to commensals can lead to inflammation and damage to host.

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commensals. To test this notion, we used enriched blood leukocytes to measure immune response by an IL-5 ELISpot assay. We found that blood leukocytes from patients with CRS produce on average 6- to 7-fold more spots per 10,000 cells compared with the control group when cocultured with a commensal microbiome, a clear indication of hyperresponse. The ELISpot assay does not distinguish between a 6-fold increase in numbers of leukocytes and a 6-fold increase in the IL-5 production by a constant number of leukocytes (Figure 5). Additional studies are needed to resolve this question.

An important insight from these experiments is that patients with CRS are hyperresponsive to normal microbiota. The inflammatory response and the resulting damage from the prolonged inflammation are due to aberrant immune response by the host (Figure 6).

Our experiment design of all vs all allowed us to test a counter hypothesis: are there factors present in the CRS lavage that could trigger an inflammatory response? The data indicate that (pellets from) CRS lavage could evoke IL-5 production in leukocytes from healthy donors and produced a higher response in leukocytes from patients with CRS (relative to microbiome from a control lavage; Figure 5). The latter data need to be interpreted with caution: while it is possible that there are microorganisms present in the lavage of patients with CRS that can evoke an IL-5 response, it is also possible that host factors present in the lavage may be triggers for an immune response. For instance, an immune response could be evoked because the microorganisms are bound with complement or antibodies or form immune complexes. In addition, the diversity analysis informs us about “who is there” but not “what are they doing there?” In this vein, it is also possible that the microorganisms in the microbiome could produce allergens that can evoke a Th2 response when they find themselves in an inflammatory environment but not when they are in the normal sinus environment. Additional studies are needed to resolve the contribution of host factors from the microbial products in triggering inflammation.

In conclusion, cytokine levels, immune cells, antibodies, and bacterial and fungal microbiomes were measured from lavage of the middle meatus from 30 patients with CRS and 12 control individuals. This study has generated a detailed description of the environment (“ecosystem”) in the normal and chronically inflamed sinus. On the basis of the description that showed that none of the organisms identified were pathogenic and that qualitatively the microbiomes of the patients with CRS were very similar to the controls, we tested the hypothesis that the immune response was aberrantly hyperresponsive in the patient with CRS. Our result, using peripheral blood leukocytes, indicates that some patients with CRS mount a substantial response to the normal microbiome (Figure 6). This result also indicates that the hyperresponsiveness to a normal microbiome is not simply localized to the sinus mucosa. These results may explain why systemic steroid treatment provides relief for some patients with CRS, as well as other comorbidities observed in patients with CRS, including asthma and skin disorders such as urticaria and atopic dermatitis.