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Macrophage Infiltrate Is Elevated in CRSwNP Sinonasal Tissue Regardless of Atopic Status

Caroline A. Banks, MD¹, Rodney J. Schlosser, MD¹,2, Eric W. Wang, MD³, Sarah E. Casey⁴, Ryan M. Mulligan⁴, and Jennifer K. Mulligan, PhD¹,5,6

Abstract

Objective. Macrophages are major producers of inflammatory cytokines; however, their role in chronic rhinosinusitis (CRS) has not been clearly defined. The aim of this study was to quantify macrophages in sinus tissue of patients with various subtypes of CRS and determine the impact of atopic status on macrophage infiltrate.

Study Design. Prospective immunohistochemical study of human sinonasal tissue.

Setting. Academic medical center.

Subjects and Methods. Human sinonasal tissue was taken from patients with CRS with nasal polyposis (CRSwNP, n = 8), CRS without nasal polyposis (CRSsNP, n = 8), and controls (n = 8) undergoing surgery for CSF leak repair or endoscopic excision of non-secreting pituitary tumor. Samples were immunohistochemically stained for macrophage/monocyte markers Mac387 and CD68.

Results. CRSwNP patients had significantly increased numbers of Mac387 and CD68 cells compared to control patients (P < .05) or CRSsNP patients (P < .01). CRSsNP had significantly increased number of cells staining for CD68 compared to controls (P < .05). The increased presence of macrophages measured by either marker in CRSwNP was independent of atopic status.

Conclusion. Macrophages are increased in CRSwNP patients regardless of atopic status and may contribute to the immunopathology of CRS.

Keywords
macrophage, chronic rhinosinusitis, nasal polyps, atopy

Introduction

Chronic rhinosinusitis (CRS) is broadly divided into CRS with nasal polypos (CRSwNP) and without nasal polyps (CRSsNP). Histopathologically, tissues from patients with CRSwNP have a higher degree of edema and contain increased eosinophils and plasma cells.¹ The 2 subsets of CRS also differ in biomarker profiles, with higher levels of T helper 2 (Th2) cytokines, such as interleukin (IL)-4, IL-5, and IL-13, in CRSwNP.² Studies investigating cellular profiles suggest that CRSsNP favors a Th1 or a mixed Th1/Th2 response, while CRSwNP displays a Th2 polarization.³ The significance of Th2 cytokine up regulation is that it can promote rhinorrhea, increased mucus production, and hyperplasia.⁴,⁵ Macrophages can both directly and indirectly induce the production of Th2 cytokines, though their role in CRS is poorly understood.

Macrophages are involved in many human diseases, including multiple sclerosis, Crohn’s disease, and lupus.⁶-⁸ Macrophages also play a role in inflammation of the lower airway, and there has been considerable research directed toward the alveolar macrophage and its function in inflammatory lung disease. In the lungs, macrophages are involved in elimination of viral pathogens and recruitment of adaptive immunity.⁹ They also play a critical role in the pathology of COPD, hypersensitivity pneumonitis, and asthma.¹⁰-¹² Macrophages are extensively involved in diseases of the lower airway, and it is likely that macrophages play similar roles in upper airway inflammation.

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Studies have shown increased macrophage mannose receptor (MMR) in patients with CRSwNP when compared to CRSsNP and controls. Van Zele et al showed a trend toward an increased number of macrophages in the tissue of all patients with chronic sinus disease, regardless of subtype. Additionally, a recent study by Krysko et al found that macrophages may contribute to chronic inflammation in CRSwNP; however, atopic status was not addressed. In this study, we quantified macrophages in sinonasal tissue to determine if macrophages were increased in patients with atopic and non-atopic CRSwNP and patients with CRSsNP.

Methods

Clinical Evaluation

This study was conducted prospectively and with the approval of the Institutional Review Board for Human Research at the Medical University of South Carolina. Sinus mucosal biopsies were taken from the middle meatus of patients with CRSsNP and CRSwNP who were undergoing sinus surgery. In patients with CRSwNP, mucosa adjacent to polypoid tissue was examined. Actual polypoid tissue was relatively acellular and was therefore not used in study. Atopic status for CRSwNP patients was determined by skin prick or radioallergosorbent (RAST) testing as per standard of care. Control sinus tissue was taken from sinus tissue free of inflammation in patients undergoing surgery for cerebrospinal fluid leak (CSF) repair or endoscopic excision of non-secreting pituitary tumor. See Table 1 for patient demographics. Patients who were treated with oral steroids within the preceding 30 days, antifungal medications, or immunotherapy, as well as patients with ciliary dysfunction, autoimmune disease, cystic fibrosis, or any known immunodeficiency, were excluded from the study.

Immunohistochemistry

Sinus tissue specimens were placed in Tissue-Tek O.C.T. medium (Sakura Finetec, Torrance, California) and immediately snap-frozen in liquid nitrogen and stored at −80°C until use. A Leica CM 1850 cryostat (Wetzlar, Germany) was used to cut 8 μm sections. Tissue specimens were mounted on Vectabond-coated (Vector Labs, Burlingame, California) glass slides. The slides were frozen and stored at −20°C until processing.

Macrophages were identified by staining tissue sections with commercially available antibodies to 2 macrophage markers, Mac387 (concentration 1:100) and CD68 (concentration 1:200). Prior to primary antibody staining, slides were treated with 10% swine serum for 20 minutes. Primary antibodies or isotype control were incubated for 1 hour. Slides were then counterstained with hematoxylin (Dako, Carpinteria, California), mounted, and coverslipped. Three random photomicrographs of each slide at 40× magnification were taken with a Nikon Eclipse E600 microscope. The photographer was blinded to the stain and to the patient diagnosis. Photomicrographs were presented to a panel of 3 blinded graders who quantified positive staining and determined the mean number of positive cells per high-powered field.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 5.02 software (La Jolla, California). Kruskal-Wallis analysis of variance (ANOVA) with post hoc Mann Whitney was used to calculate statistical significances in the number of macrophages between patient cohorts. Pairwise comparisons were done following significant ANOVA. Data to conduct a power analysis for macrophages from the literature were unavailable. We conducted power analysis based on information from the B-cell literature. B-cells perform a diverse range of functions including antibody and cytokine production, antigen presentation, and inflammatory regulation. Similar to macrophages, B-cells have been examined locally in atopic and non-atopic CRS. Based on that data with plasma B-cell values of 0.718 ± 0.34 in non-atopic and 2.1 ± 1.5 in atopic, a total of 10 patients (5 per group) would be adequate to detect differences in APC infiltrate by atopic status.

Results

Macrophages Are Present in Human Sinonasal Tissue and Are Increased in Patients with CRSwNP

We quantified the number of macrophages in sinonasal tissue in controls, patients with CRSsNP, and patients with CRSwNP (see Table 2, representative photographs shown in Figure 1). CRSwNP patients had statistically significant increased numbers of cells staining positively for Mac387 when compared to controls (P < .05) or CRSsNP (P < .01) (Figure 2). Similarly, patients with CRSwNP had increased numbers of cells staining positively for CD68 when compared to controls (P < .05) or CRSsNP (P < .01). CRSsNP also had slightly elevated numbers of cells staining positively for CD68 when compared to controls, which was statistically significant (P < .05) (Figure 3).

The Increased Presence of Macrophages in CRSwNP Is Independent of Atopic Status

After we determined that there were increased numbers of macrophages in the sinonasal tissue of patients with

<table>
<thead>
<tr>
<th>Race/ethnicity (%)</th>
<th>Control</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>40</td>
<td>80</td>
<td>41</td>
</tr>
<tr>
<td>African American</td>
<td>40</td>
<td>20</td>
<td>59</td>
</tr>
<tr>
<td>Other</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: CRSsNP, chronic rhinosinusitis without nasal polyposis; CRSwNP, chronic rhinosinusitis with nasal polyposis.

Table 1. Patient Characteristics.
CRSwNP, we examined whether there were differences in macrophage infiltrate based on atopic status. For the Mac387 stain, atopic and non-atopic patients with CRSwNP both had elevated numbers of macrophages when compared to controls or CRSsNP (\( P < .05 \)); however, there was no statistical difference observed between the atopic and non-atopic groups (\( P > .05 \)). The CD68 staining demonstrated similar results, with increased numbers of positive-staining cells in both the atopic and non-atopic CRSwNP tissue (\( P < .05 \)) compared to controls, but no significant difference was detected between the 2 groups (Figure 3).

**Discussion**

The inflammatory response of the innate immune system is complex and involves multiple cell types including monocytes, macrophages, and dendritic cells (DC). DC function as major antigen presenting cells and producers of cytokines and have been found to be involved in the inflammatory response of CRS.\(^{16,17}\) Macrophages share similar features with DC including phagocytosis, antigen presentation, and cytokine production.\(^{18}\) but the role of macrophages in CRS remains unclear. In lower airway disease, such as asthma, elevated numbers macrophages along with alterations in their function have been shown to be associated more severe disease.\(^{19}\) In patients with steroid-resistant asthma, alterations macrophage function have been shown to be resistance to glucocorticosteroids, suggesting that macrophage may be driving steroid resistances.\(^{20-22}\) In CRS with or without polyps, the role of macrophages if far less well defined and therefore the subject of our investigation. Macrophages are major producers of inflammatory cytokines and chemokines including TNF-\( \alpha \), IL-1\( \beta \), IL-6, IL-8, regulated on activation, normal T cell expressed and secreted (RANTES), and matrix metalloproteinases (MMPs).\(^{23,24}\) As such, macrophages may contribute to the pathology of CRS. Prior evidence suggests that macrophages are increased in CRS, but a statistically significant increase in macrophages in CRSsNP and CRSwNP has not been proven. A potential pathway of macrophage involvement in CRS is via the production of chemokines. Macrophages have been shown to be a significant cellular source of eotaxin in human nasal mucosa.\(^{25}\) Eotaxin functions as a chemoattractant that is responsible for the recruitment of eosinophils to mucosal inflammation. Immunostaining reveals a dense and diverse network of macrophages and DC in human nasal mucosa,\(^{26}\) but their contribution to the inflammation associated with CRS, especially the role of the macrophage in sinus mucosa, is not clearly defined.

**Table 2. Summary of Macrophage Quantification.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>CRSsNP</th>
<th>Non-atopic CRSwNP</th>
<th>Atopic CRSwNP</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>0.58 ± 0.25</td>
<td>3.21 ± 1.01</td>
<td>8.92 ± 0.21</td>
<td>9.51 ± 2.36</td>
<td>0.0015</td>
</tr>
<tr>
<td>MAC387</td>
<td>2.10 ± 0.77</td>
<td>2.39 ± 0.88</td>
<td>7.75 ± 1.85</td>
<td>9.45 ± 2.57</td>
<td>0.0222</td>
</tr>
</tbody>
</table>

Abbreviations: CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps.

\( ^{a} \)Values shown are mean ± SEM. \( P \)-value shown is Kruskal-Wallis ANOVA.

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**Figure 1.** Immunohistochemical staining of sinonasal mucosa from control, CRSsNP, and CRSwNP patients. Representative staining shown for Mac387 and CD68. Positive staining is identified by arrows. CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps.

**Figure 2.** Quantitative analysis of Mac387 immunohistochemistry staining with atopic status. Data shown are mean numbers of cells per high-powered field. Statistics shown are the result of Kruskal-Wallis ANOVA. CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps. *\( P < .05 \) versus control; \( ^{#} P < .01 \) versus CRSsNP; NS, no significant difference versus non-atopic CRSwNP.
In this study, immunohistochemical staining confirmed that macrophages are present in human sinonasal tissue. We investigated the association between disease state and the presence of macrophages. Our study demonstrated that macrophages are significantly increased in the sinonasal tissue of patients with CRSwNP when compared to controls. A less robust but statistically significant increase in macrophages was seen in patients with CRSsNP when staining for CD68 but not with Mac387. This difference in staining may be accounted for by the difference in specificity between the 2 monoclonal antibodies. CD68 is a universal marker for the macrophage/monocyte and to a lesser extent DCs in the skin, murine spleen, and a non-myeloid DC lineage in circulation in humans.27-29 To ensure that we were staining macrophages and not DCs, Mac387 antibody was chosen, which is highly specific to the macrophage lineage. Mac387 is found on a subset of CD68 positive cells and is specific to a variety of histiocytes including reactive or infiltrating macrophages and monocytes.30

Macrophages can be separated into 2 distinct phenotypes. M1 macrophages, also known as classically activate macrophages, are pro-inflammatory and target intracellular pathogens. Conversely, alternatively activated M2 macrophages are primed by Th2 cytokines, propagate the Th2 response, and are associated with allergic disease.31,32 Recent studies are in agreement with our findings that macrophages are increased in CRSwNP. Krysko et al14 found that M2 macrophages are significantly increased in CRSwNP. Th2 markers were positively correlated with the increased numbers of macrophages.14 Our studies expand upon this work by examining the influence of atopic status on local macrophage infiltrate. CCL23, a chemokine involved in recruitment of macrophages, has also been shown to be significantly upregulated in CRSwNP tissue. Poposki et al13 suggested that CCL23 may recruit macrophages into nasal polyps, and the presence of Th2 cytokines then leads to skewing toward the M2 phenotype.

Our study also investigated the association between systemic atopy and CRS and found that the increase in macrophage numbers in patients with CRSwNP was independent of atopic status. There is discussion in the literature surrounding the involvement of atopy and CRS. Though some research suggests that allergic status plays a key role in Th2 polarization of CRSwNP,34,35 our data found that systemic atopy did not influence the number of macrophages locally. This suggests that the inflammation of CRS is not solely caused by the classic IgE-mediated allergic response. Studies have shown that atopic and non-atopic CRSwNP patients have similar elevations in dendritic cells and mast cells.16,36 Similarly, Psaltis et al37 found that the immune infiltrate of patients with atopic and nonatopic CRSwNP had increased levels of all B-cell subtypes, and this was independent of systemic B-cell response. Additionally, it has been shown that atopy does not determine inflammatory gene expression in CRS.38 The present study supports the view that the Th2 skewing in CRSwNP is driven by other nonallergic mechanisms, and macrophages may be implicated in the pathogenesis of CRS.

Potential limitations of this study include the sample size limitation. B-cell data were used given the lack of available data for macrophage power analysis. There is the possibility the study is underpowered and we were unable to detect differences because of this. An additional potential weakness is that our definition of atopy is based on systemic, not local, clinical measurements. Studies show that local IgE can be elevated in some patients with CRSwNP in the absence of systemic atopy.39

Conclusion

Our study demonstrates that the sinonasal tissue of patients with CRSwNP has increased numbers of monocytes and macrophages when compared to controls. The increased presence of macrophages is independent of atopic status. The role of macrophages in CRS is poorly understood; however, macrophages may contribute to the Th2 skewing found in CRSwNP.

Author Contributions

Caroline A. Banks, substantial contribution to design, data collection and analysis, final approval; Rodney J. Schlosser, substantial contribution to design, interpretation of data, revising article, and final approval; Eric W. Wang, substantial contribution to acquisition of data, article revision, and final approval; Sarah E. Casey, substantial contribution to acquisition of data, article revision, and final approval; Ryan M. Mulligan, substantial contribution to acquisition of data, article revision, and final approval; Jennifer K. Mulligan, substantial contribution to design, data analysis and interpretation, drafting and revising, and final approval.

Disclosures

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