TIM-3-Expressing Mast Cells Are Present in Chronic Rhinosinusitis with Nasal Polyps

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objectives. To identify whether TIM-3 expression is present in the mast cell population within nasal polyps and to determine its correlation with clinical severity in patients with chronic rhinosinusitis with nasal polyposis.

Study Design. Basic science, translational study.

Setting. Nasal polyp tissue collected from patients seen at a tertiary care hospital (2015-2016).

Subjects and Methods. Nasal polyp tissue obtained during functional endoscopic sinus surgery (n = 24) was enzymatically digested into epithelial and stromal fractions. Viable mast cells expressing TIM-3 were identified using flow cytometry for the following: CD45, Live/Dead, c-kit, FcεR1, TIM-3. Disease severity was assessed using the Sino-Nasal Outcome Test, Lund-Mackay staging system, Lund-Kennedy staging system, and complete blood counts.

Results. Mast cells were found in both the epithelial and stromal layers of polyps, with a greater %TIM-3+ mast cells in the epithelial layer compared with that of the stromal layer (P = .001). As the percentage of mast cells increased, there was a comparative worsening in endoscopic severity after comparing pre- and postoperative LK scores (ρ = −0.455, P = .029). In a subgroup of patients with concomitant asthma, increased epithelial %TIM-3+ mast cells also correlated with worsening endoscopic appearance postoperatively (ρ = 0.866, P = .001, n = 11). Oral corticosteroid treatment did not change the viability of mast cells nor their influence on the increased postoperative endoscopic disease severity (ρ = −0.544, P = .020, n = 18).

Conclusion. Viable mast cells were found to be present in polyps with increased TIM-3 expression at the epithelial layer. This suggests that TIM-3 may play a role in chronic inflammation in CRSwNP via mast cell activation.

Keywords

chronic rhinosinusitis, nasal polyps, mast cells, TIM-3

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Introduction

Chronic rhinosinusitis (CRS) is a multifactorial inflammatory disorder of yet unknown etiology. CRS is currently subdivided into 2 phenotypic groups, with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP). Possible etiologies of inflammation have been proposed, including genetic susceptibility, environmental triggers, microbial alterations, and barrier and innate immune dysfunction, among others. Specific inflammatory mediators are associated with CRSwNP, including interleukin (IL)–4, IL-5, and IL-13, with corresponding eosinophilia and immunoglobulin E (IgE) production.1

Although there is increased understanding of the role of eosinophils in nasal polyps, mast cells and their activation in CRSwNP is an area that remains poorly understood. Mast cells are found at sites of chronic inflammation and are activated through one of the IgE receptors called FcεRI, meaning it forms a complex with the fragment crystallizable (Fc) region of the epsilon heavy chain of IgE antibodies. It has been reported that mast cells constitutively express cell surface T-cell or transmembrane immunoglobulin and mucin domain protein 3 (TIM-3), a novel immune checkpoint receptor with inhibitory effects on chronically stimulated T cells that has recently been shown to modulate immediate-phase degranulation and late-phase cytokine

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production downstream of FcεRI. In other words, TIM-3 promotes mast cell activation and cytokine production by closely associating with FcεRI and its proximal signaling pathways.

It has been suggested that manipulation of TIM-3 activity on mast cells could be a promising target for the development of novel therapeutic modalities for chronic inflammatory diseases. The primary goals of this study were to identify whether TIM-3 expression was present in the mast cell population within nasal polyps and then determine its correlation with disease severity in patients with CRSwNP.

**Methods**

**Inclusion and Exclusion Criteria**

The study was approved by the University of Pittsburgh institutional review board, and informed consent was obtained from all patients. Inclusion criteria included patients 18 years of age or older with CRSwNP refractory to medical management for at least 6 months. Medical management included at least a 4-week methylprednisolone taper, culture-directed antibiotics, nasal saline irrigations, and a topical nasal steroid spray. Patients were also included with and without comorbid asthma, allergic rhinitis, and aspirin-exacerbated respiratory disease (AERD). This study excluded patients with known mast cell disorders, hematologic disorders, cystic fibrosis, and pregnant patients.

Disease severity was assessed using the following: the 22-item Sino-Nasal Outcome Test (SNOT-22), the Lund-Mackay (LMK) staging system, the Lund-Kennedy (LK) staging system, and complete blood count with differential specifically evaluating eosinophils and basophil levels. Both the preoperative and postoperative SNOT-22 and LK scores were recorded; the change between preoperative and postoperative scores was considered as additional separate variables and denoted as ΔSNOT-22 and ΔLK.

LMK and LK scores were obtained within 90 days before functional endoscopic sinus surgery (FESS) and again within 90 days after surgery, by the same attending otolaryngologist. Use of oral systemic corticosteroid (OCS) treatment prior to surgery was also recorded, with all patients following a standard 4-week course of methylprednisolone (32 mg daily for the first week prior to surgery, then 32 mg for 3 doses per week, 24 mg for 3 doses per week, and 16 mg for 3 doses per week).

**Antibodies and Reagents**

The following reagents were used: collagenase class II (Worthington Biochemical Corp, Lakewood, New Jersey), anti-human CD45 (violetFluor 450 clone H130, Tonbo Biosciences, San Diego, California), Ghost Red 780 viability dye (APC-Cy7, Tonbo Biosciences); anti-human FcεRI (FITC, eBioscience Affymetrix Inc, San Diego, California), anti-human ckit (PerCP-Cy5.5, BD Biosciences, San Jose, California), and anti-human TIM-3 (APC, R&D Systems, Minneapolis, Minnesota).

**Isolation and Quantification of Viable TIM-3+ Mast Cells from Nasal Polyps by Flow Cytometry**

Fresh nasal polyp tissue obtained during FESS (n = 24) was enzymatically digested into epithelial and stromal fractions. Viable mast cells expressing TIM-3 were identified using flow cytometry for the following markers: CD45 (V450), Live/Dead (APC-Cy7), c-kit (PerCP-Cy5.5), FcεRI (FITC), and TIM-3 (APC). Identification of viable mast cells by flow cytometry was determined to be the %APC-Cy7-CD45+c-kit+FceRI+ population (%total mast cells). TIM-3 identification by flow cytometry was determined to be the %APC-Cy7-CD45+c-kit-FcεRI+TIM3+ population (%TIM-3+ mast cells).

The following methods for isolation of viable mast cells from nasal polyps were adapted from Finotto et al. Polyph tissue obtained during FESS was transported on ice in calcium- and magnesium-free Hanks’ balances salt solution (HBSS; Lonza, Walkersville, Maryland) containing 20 mM HEPES (HBSS-CMF). Transport and washing solutions were filtered through a 40-μm nylon mesh cell filtration strainer (Fisherbrand cell strainers, Fisher Scientific, Pittsburgh, Pennsylvania) and saved on ice (fraction 1). The remaining tissue was incubated in HBSS-CMF containing 25 mM HEPES (GE Healthcare Life Sciences, Logan, Utah) and 1.3 mM EDTA (HBSS or EDTA) at 22°C for 60 minutes with stirring, to separate the epithelium from the stroma. The supernatant was decanted and filtered through a 40-μm nylon mesh cell filtration strainer (Fisherbrand cell strainers, Fisher Scientific, Pittsburgh, Pennsylvania) and saved on ice (fraction 1). The remaining tissue was minced (0.3 mm3) and incubated for two 60-minute periods in HBSS-CMF containing 190 U/mL of collagenase (60 mL/g of tissue) at 37°C with stirring. The supernatants were decanted and filtered through the sieve, pooled, and saved on ice (fractions 3+4). The harvested cell suspensions were centrifuged at 100g for 15 minutes and resuspended in fresh HBSS-CMF. Fraction 1+2 was obtained before removal of basement membrane and thus was considered to include the epithelial cells. Fraction 3+4 was obtained after removal of the epithelial layer and thus was considered to include the stromal cells. The total cell number and viability were assessed by a hemocytometer following staining with Trypan blue exclusion dye.

For flow cytometry studies, dead cells were excluded by first staining with viability dye in phosphate-buffered saline (PBS) for 30 minutes on ice. To avoid nonspecific staining, single-cell suspension was incubated with human plasma in a 1:10 dilution with FACS staining buffer (1% bovine serum albumin, 0.1% sodium azide in PBS) on ice for 5 minutes prior to addition of antibodies against human CD45, c-kit, FcεRI, and TIM-3 for 30 minutes on ice. Stained cells were subsequently fixed with 1.5% paraformaldehyde for 10 minutes at room temperature prior to analysis on the BD LSR II or FORTESSA flow cytometer. Unstained and fluorescence minus one controls were included for each analysis.
**Immunohistochemistry**

Slides were deparaffinized and rehydrated. Antigen retrieval was performed using Diva Retrieval (Biocare Medical, Concord, California) and a decloaking chamber at 124°C, 3 minutes, and cooled for 10 minutes. Tryptase was used as a marker for mast cells. Slides were placed on an Autostainer Plus (Dako, Carpinteria, California) using a TBST rinse buffer (Dako) and stained using 3% H₂O₂ (ThermoFisher Scientific, Pittsburgh, Pennsylvania) for 5 minutes and CAS Block (Invitrogen, Grand Island, New York) for 10 minutes, the primary antibody for TIM-3 (polyclonal goat IgG) used per instructions. The secondary antibody consisted of Envision Dual Link + (Dako) polymer for 30 minutes, which was rinsed; a TBST holding rinse was then applied for 5 minutes. The substrate used was 3,3-diaminobenzidine+ (Dako) for 7 minutes and counterstained with hematoxylin.

**Statistical Analysis**

Statistical analysis was performed using IBM SPSS Statistics Version 24.0. Differences were considered to be statistically significant at \( P < .05 \). Log transformation was used to report the data. A Wilcoxon signed rank test and Spearman rho correlation test was used to interpret immunophenotypic data and correlation with disease severity. Disease severity was measured using the SNOT-22 score, LMK staging system, LK staging system, and eosinophil and basophil cell counts. The change between pre- and postoperative SNOT-22 and LK scores was also analyzed in the Spearman rho correlation studies. Stratified analysis was then performed using a Spearman rho correlation test to compare CRSwNP patients with and without concomitant asthma as well as those that did and did not receive OCS treatment, considering concomitant asthma and OCS treatment as possible confounding variables.

**Results**

**Patient Characteristics**

A total of 24 patients were included in the study with the following concomitant diseases: asthma (n = 13, 54.2%), AERD (n = 8, 33.3%), and allergic rhinitis (n = 13, 54.2%; Table 1). Overlap was present among subgroups. Asthma severity was categorized per the National Asthma Education and Prevention Program Expert Panel Report 3 Guidelines for the Diagnosis and Management of Asthma as mild (n = 5, 20.8%), moderate (n = 7, 29.2%), and severe (n = 1, 4.2%). Most patients received OCS treatment 1 week prior to sinus surgery (n = 18, 75%).

**Statistical Analysis**

**Cell yields.** As represented in Figure 1, the %TIM-3⁺ mast cells present in the epithelial and stromal fractions across all specimens was identified as previously described; logarithmic amplification was used to distinguish negative from positive signals. The median for %total mast cells present in the epithelial and stromal fractions across all specimens was 2.99 and 1.56, respectively; the interquartile (IQ) range was 6.46 and 12.38, respectively (Table 2). The median for %TIM-3⁺ mast cells present in the epithelial and stromal fractions across all specimens was 32.8 and 8.68, respectively; the IQ range was 67.30 and 31.78, respectively (Table 2). One patient did not have a TIM-3⁺ mast cell yield from the epithelial cell suspension; thus, statistical analysis involving mast cells in the epithelial layer had fewer samples (n = 23) than that which involved mast cells in the stromal layer (n = 24).

**Correlation between mast cells and clinical severity scales.** The Wilcoxon signed rank test showed a greater %TIM-3⁺ mast cells in the epithelial layer than in the stromal layer of nasal polyps across all specimens (\( P = .001, n = 23 \); immunohistochemistry staining shown in Figure 2a and Figure 2b is representative of this finding.

Spearman’s rho correlation tests displayed a negative correlation between stromal %total mast cells and ΔLK scores (\( \rho = -0.455, P = .029, n = 23 \); Figure 3). The correlations between epithelial and stromal %TIM-3⁺ mast cells and the other clinical severity scales considered in the study, including pre- and postoperative SNOT-22, Lund-Mackay scores, and eosinophil and basophil counts, were not statistically significant (\( P > .05 \)).

Stratified analysis was then performed using a Spearman’s rho correlation test to compare CRSwNP patients with and without concomitant asthma as well as those who did and did not receive OCS treatment. In the absence of asthma, there was a negative correlation between stromal %TIM-3⁺ mast cells and LK postoperative scores (\( \rho = -0.620, P = .042, n = 11 \)) and a negative correlation

<table>
<thead>
<tr>
<th>Table 1. Patient Sample (n = 24) Demographics.*</th>
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<tbody>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Concomitant asthma</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>Concomitant aspirin-exacerbated respiratory disease</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>Concomitant allergic rhinitis</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>Asthma severity</td>
</tr>
<tr>
<td>Mild</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Severe</td>
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<tr>
<td>Oral systemic corticosteroid treatment</td>
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<td>0</td>
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<td>1</td>
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*0 denotes “absence of”; 1 denotes “presence of.”
between stromal %TIM-3\(^+\) mast cells and basophil counts \((\rho = -0.694, P = .038, n = 9; \textbf{Figure 4})\). In the presence of asthma, there was a positive correlation between epithelial %TIM-3\(^+\) mast cells and LK postoperative scores \((\rho = 0.866, P = .001, n = 11)\) and a negative correlation between stromal %total mast cells and LK postoperative scores \((\rho = -0.698, P = .008, n = 13; \textbf{Figure 4})\). In the subgroup of patients who did not receive OCS treatment, there was a negative correlation between stromal %TIM-3\(^+\) mast cells and LK preoperative scores \((\rho = -0.820, P = .046, n = 6; \textbf{Figure 5})\). In the subgroup of patients who received OCS treatment, there was a negative correlation between stromal %total mast cells and the LK postoperative scores \((\rho = -0.673, P = .002, n = 18)\), as well as with the ΔLK scores \((\rho = -0.544, P = .020, n = 18; \textbf{Figure 5})\).

### Discussion

This study demonstrated that viable mast cells are present in nasal polyps and localized preferentially in the epithelial layer, although they were also detected in the stromal layer. Chronic inflammation, which is characteristic of CRSwNP, can be initiated and/or propagated at the epithelial cell layer and perhaps mediated by erroneous initiation of signaling cascades, leading to TIM-3–mediated mast cell activation and degranulation.\(^6\)-\(^8\) A protein called galectin-9 that is strongly expressed in Hodgkin’s disease tissue has been shown to bind to TIM-3 and down-regulate allergic responses by disrupting IgE-antigen complex formation.\(^9\)-\(^11\) Other studies have shown that there is increased TIM-3 expression in the inflammatory response to melanoma with a corresponding up-regulation in mast cells.\(^12\) TIM-3\(^+\) mast cells have also been shown to correlate with the severity of chronic inflammatory periodontitis.\(^13\)

In the current study, mast cells in the stromal layer of nasal polyps were found to correlate with clinical severity outcomes measures in patients with CRSwNP. An increased percentage of mast cells found in the stromal layer across all samples translated to worsening postsurgical course and severe clinical picture. In addition, although TIM-3\(^+\) mast cell expression was found to be higher in the epithelial layer of polyps, preferential activation in the stroma may actually be more important in contributing to disease severity.

### Table 2. Descriptive Study Depicting the Median and Percentiles for %Total Mast Cells and %TIM-3\(^+\) Mast Cells in Epithelial and Stromal Layers of Nasal Polyps across All Specimens (n = 24).

<table>
<thead>
<tr>
<th>%TIM3(^+) mast cells</th>
<th>Median (25th Percentile, 75th Percentile)</th>
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<tbody>
<tr>
<td>Epithelial layer</td>
<td>32.80 (7.00, 74.30)</td>
</tr>
<tr>
<td>Stromal layer</td>
<td>8.68 (1.02, 32.80)</td>
</tr>
<tr>
<td>%Total mast cells</td>
<td></td>
</tr>
<tr>
<td>Epithelial layer</td>
<td>2.99 (1.43, 7.88)</td>
</tr>
<tr>
<td>Stromal layer</td>
<td>1.56 (0.22, 12.60)</td>
</tr>
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</table>
Although a multivariable regression analysis was infeasible because of our sample size, a stratified correlation test was performed to compare CRS patients with and without concomitant asthma as well as CRS patients who did and did not receive OCS. In the subgroup of patients with concomitant asthma, there was a positive correlation between TIM-3\textsuperscript{+} mast cells and LK postoperative scores. This finding indicates that TIM-3 may be up-regulated in this subgroup of patients with concomitant asthma and translate to more recalcitrant disease. In the subgroup who received OCS treatment, administration of steroids prior to surgery changed neither the viability of mast cells nor their influence on the increased postoperative endoscopic disease severity.

Limitations of this pilot study include a small sample size and lack of comparison with healthy sinus control tissue. A baseline level of TIM-3 expression present in nasal polyps mast cells before and after activation has never

Figure 2. (a) Immunohistochemistry tryptase staining of mast cells. (b) Immunohistochemistry staining demonstrates a greater presence of TIM-3\textsuperscript{+} mast cells in the epithelial layer of the nasal polyp.

Figure 3. The percentage of mast cells found in the stromal layer of nasal polyps negatively correlates with the change between pre- and postoperative Lund-Kennedy scores ($p = -0.455$, $P = .029$).

Figure 4. Positive correlation between epithelial %TIM-3\textsuperscript{+} mast cells and Lund-Kennedy postoperative scores in a subgroup of patients with concomitant asthma ($p = 0.866$, $P = .001$, $n = 11$).

Figure 5. Negative correlation between stromal %total mast cells and the change between pre- and postoperative Lund-Kennedy scores in a subgroup of patients who received oral systemic corticosteroid treatment ($p = -0.544$, $P = .020$, $n = 18$).
been quantified; however, studies have shown that TIM-3
down-regulation and activation.2 Future studies are ongoing to
better quantify the level of mast cell activation (ie, by using
markers that identify the presence of granules released after
mast cell degranulation and activation) and comparison with
normal sinus tissue.

Other areas for future study include assessment of how
mast cell activation correlates to concomitant diseases, such
as AERD and allergic rhinitis, the effects of topical and/or
inhaled corticosteroid treatment, environmental factors on
the mast cell milieu, as well as fluctuation with disease
severity, all of which can perhaps further help define our
understanding of differing CRS endotypes and development
of targeted therapy.

Conclusion
Activated mast cells are present in nasal polyps, with
increased TIM-3 positivity found in mast cells located in the
epithelial layer when compared to the stromal layer, suggesting that TIM-3 may play a role in the initiation and/
or propagation of epithelial signaling cascades in CRSwNP.
Increased mast cell percentage in the stromal layer of
polyps, however, was found to correlate with worse endo-
sopic appearance. In the presence of concomitant asthma,
increased epithelial %TIM-3+ mast cells translated to
decreased improvement after FESS by LK score. OCS treat-
ment did not change either the viability of mast cells or
their influence on the increased severity of postoperative
endoscopic disease.

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Erica Corredera, data acquisition, analysis and interpretation,
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John A. Moore, data acquisition, drafting, accountability for all
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