Sputum autoantibodies in patients with severe eosinophilic asthma

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Background: The persistence of eosinophils in sputum despite high doses of corticosteroids indicates disease severity in asthmatic patients. Chronic inflamed airways can lose tolerance over time to immunogenic entities released on frequent eosinophil degranulation, which further contributes to disease severity and necessitates an increase in maintenance corticosteroids.

Objectives: We sought to investigate the possibility of a polyclonal autoimmune event in the airways of asthmatic patients and to identify associated clinical and molecular characteristics.

Methods: The presence of autoantibodies against eosinophil peroxidase (EPX) and anti-nuclear antibodies was investigated in patients with eosinophilic asthma maintained on high-dose corticosteroids, prednisone, or both. The ability of sputum immunoglobulins to induce eosinophil degranulation in vitro was assessed. In addition, the associated inflammatory microenvironment in patients with detectable autoantibodies was examined.

Results: We report a “polyclonal” autoimmune event occurring in the airways of prednisone-dependent asthmatic patients with increased eosinophil activity, recurrent pulmonary infections, or both, as evident by the concomitant presence of sputum anti-EPX and anti-nuclear antibodies of the IgG subtype. Extensive cytokine profiling of sputum revealed a Th2-dominated microenvironment (eotaxin-2, IL-5, IL-18, and IL-13) and increased signalling molecules that support the formation of ectopic lymphoid structures (B-cell activating factor and B cell-attracting chemokine 1). Immunoprecipitated sputum immunoglobulins from patients with increased autoantibody levels triggered eosinophil degranulation in vitro, with release of extensive histone-rich extracellular traps, an event unsuppressed by dexamethasone and possibly contributing to the steroid-unresponsive nature of these eosinophilic patients.

Conclusion: This study identifies an autoimmune endotype of severe asthma that can be identified by the presence of sputum autoantibodies against EPX and autologous cellular components. (J Allergy Clin Immunol 2018;141:1269-79.)

Key words: Severe asthma, eosinophilia, autoantibodies, sputum, eosinophil degranulation, eosinophil peroxidase, autoimmunity, anti-nuclear antibodies

A persistence of airway eosinophilia despite daily maintenance oral corticosteroids (OCSs) indicates a difficult-to-control population projected to benefit most from anti-eosinophil biologics currently in development. However, clinical trials targeting eosinophils in prototype eosinophilic patients, although successful in depleting circulating eosinophils, resulted in only an approximately 50% to 70% reduction in exacerbations, thereby hinting at the presence of alternative mechanisms that sustain local inflammation. Emerging hypotheses suggest the presence of localized mechanisms that are refractory to maintenance corticosteroids, thereby delaying resolution of inflammation and leading to frequent exacerbations with characteristic clusters of free eosinophil granules (FEGs) in sputum. Increased abundance of FEGs in the airways strongly correlates with sputum eosinophil peroxidase (EPX) content released on luminal eosinophil degranulation, which is indicative of “active” disease. Indeed, eosinophil degranulation releasing FEGs indicative of cytolysis or primary lysis is suggested to be pathogenic.

Increased frequency of immune cell degranulation over time leads to peroxidase enzyme release, which has known immunogenic properties by virtue of its ability to cause tissue damage. Thyroid peroxidase and myeloperoxidase have established etiologic roles in the pathogenesis of autoimmune disorders, such as Hashimoto thyroiditis and eosinophilic granulomatosis with polyangiitis, respectively. EPX has been reported to cause necrotic lysis of epithelial cells in vitro. Therefore we speculated that increased eosinophil degranulation accumulates immunogenic entities, such as EPX, in the airways and subsequent markers of tissue injury, such as molecules that activate danger-associated molecular pattern receptors and trigger a localized adaptive immune response, leading to breach of immune tolerance per the Matzinger “danger” model. A breach of mucosal immunity in the lungs is not unprecedented. In the context of asthma, several studies have shown an...
Association with autoimmunity, although these were observational in nature and without a definite causal relationship. First, circulating anti-nuclear antibodies (ANAs) with unknown antigen specificities were seen in 55% of asthmatic patients with aspirin sensitivity, although airways were not examined. Two recent studies reported naturally occurring circulating anti-IgE IgG in healthy subjects, with increased titers in atopic and nonatopic asthmatic patients, along with increased circulating immune complexes in the sera of asthmatic subjects with heightened disease severity.

To date, an autoimmune mechanism triggered locally in the lungs targeting eosinophil-specific antigens to explain asthma severity has not been investigated. According to our hypothesis of a degranulation-mediated increase in immunogenic entities in the airways, we aimed to investigate the presence of autoantibodies targeted against EPX and autologous cellular/nuclear material (ie, ANAs) to further assess the possibility of a localized polyclonal autoimmune event associated with asthma severity.

METHODS

Patient samples

This was a retrospective study conducted with archived sputum samples collected from asthmatic patients during routine clinical management at the Firestone Clinic, with approval from the local Hospital Research Ethics Board, St Joseph’s Hospital, Hamilton, Ontario, Canada. Random sputum supernatants used in this study were categorized based on the nature and history of their bronchitis: (1) patients with eosinophilic asthma (EA), with current sputum eosinophils of greater than 3%, normal total cell count (TCC) of less than 10^6 cells/g, and a history of sputum eosinophils that requires daily maintenance corticosteroids. Fifteen healthy volunteers with no known respiratory or systemic disorders, no personal/immediate family history of autoimmune disease, and no infection within 4 weeks of sputum induction participated in the study with written consent. All subjects were nonsmokers (<10 per pack year) or never smokers.

Subjects’ characteristics are summarized in Table 1, and information collected is with respect to the date of the archived sputum sample used for the study. Asthma diagnosis was based on either postbronchodilator reversibility of greater than 12% and/or PC20 of less than 8 mg/mL on methacholine provocation challenge, and severity was determined based on the maintenance corticosteroid dosage.15

Measurement of airway eosinophil activity (sputum EPX and FEG index)

Sputum was induced with hypertonic saline, as described previously, and endogenous EPX content was measured by means of ELISA in the cell-free supernatant. FEGs in sputum cytosin preparations were ranked by 2 independent medical technologists between 0 and 3 (0 = none, 1 = few, 2 = moderate, and 3 = many) for the current sputum sample and 2 more samples preceding it (based on a sputum database maintained for all patients seen at the Firestone Clinic). The index was computed as the sum of the ranks over 3 consecutive sputum samples divided by the maximum possible outcome (ie, 3 × 3 = 9), and the mean sum of the 2 indices thus calculated was considered the FEG index. This allowed us to take into consideration both the degree of current and previous degranulation.

Detection of sputum autoantibodies

To detect immunoglobulin reactivity in sputum, cell-free soluble fractions of processed sputum supernatants were subjected to immunoprecipitation (IP) with Protein A/G beads to generate IP-Igs, as described recently. Anti-EPX reactivity was assessed by using an indirect ELISA developed in house and expressed as absorbance at 600 nm (Ab₆₀₀) after background correction. An ANA Line Immunosassay strip (IMTEC-ANA-LIA-Maxx; Human Worldwide, Weisbaden, Germany) was adapted for detecting sputum ANAs by using the manufacturer’s protocol (except for the initial step, where 100 µL of sputum supernatant was incubated overnight at 4°C with 700 µL of diluent). The sputum reactivity against eosinophils was further validated by means of confocal microscopy (see the Methods section in this article’s Online Repository at www.jacionline.org).

Sputum cytokines

All reported cytokines and chemokines were analyzed by Eve Technologies (HD965 Discovery Assay; Calgary, Alberta, Canada), except for B cell–activating factor of the TNF family (BAFF; Quantikine ELISA; R&D Systems, Minneapolis, Minn).

Assessing eosinophil degranulation with sputum IP-Igs

Freshly prepared IP-Igs from individual patient samples with high sputum autoantibody titers or low/no sputum autoantibodies and from healthy subjects were coated onto 48-well tissue-culture plates and blocked with 2.5% human serum albumin for 2 hours at 37°C. Eosinophils (1 × 10⁶) isolated by using the MACSxpress Eosinophil Isolation Kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer’s protocol, in serum-free RPMI 1640 medium with or without 10⁻⁴ mol/L dexamethasone (Sigma-Aldrich, St Louis, Mo) were added to the immunoglobulin-coated wells and incubated for 12 hours at 37°C in a 5% CO₂ atmosphere. Lactose dehydrogenase (LDH) activity (LDH Assay; Abcam, Cambridge, United Kingdom) was measured along with double-stranded DNA (dsDNA) release in the collected supernatants (Quant-iT PiCoGreen dsDNA Assay Kit; Molecular Probes, Eugene, Ore). To visualize the event, 0.5 × 10⁷ eosinophils were added to the IP-Ig-coated chamber slides (Lab-Tek II; Thermo Fisher, Waltham, Mass) at 3, 6, 9, and 12 hours. At given time points, the wells were fixed with 4% paraformaldehyde, probed with 0.1 µg/mL anti-histone mAb (Millipore, Temecula, Calif), and counterstained with anti-mouse IgG Alexa Fluor 488 (Molecular Probes). Events were visualized with a Nikon Eclipse TE2000-E (Nikon, Melville, NY) fluorescence microscope and a Q-imaging Retiga 2000R camera (Surrey, British
When we next analyzed the nature of inflammation and bronchitis history, we identified patients within the ICS group with Abs600 values of greater than the cutoff who had a history of recurrent infections (sputum TCC > 14 × 10⁶ cells/g; neutrophils > 65%, eosinophils > 1.5%, or a history of sputum eosinophils requiring daily prednisone). Finally, these 51 samples from patients with EA were classified into 3 distinct groups based on the nature of their bronchitis, with an additional age- and sex-matched neutrophilic asthma (NA) group added to constitute the final cohort (Table I). The data distribution for the final cohort (Fig 1, C) showed significantly increased anti-EPS IgG signal in the subgroup with OCS-dependent eosinophilic asthma (OCS-EA) comprising prednisone-dependent patients with persistent sputum eosinophilia and the MEA subgroup of patients with airway eosinophilia (despite corticosteroid use)/history of eosinophilia and recurrent infections with increased sputum TCCs. In addition,

### TABLE I. Demographics of subjects in the final study cohort

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>ICS-dependent eosinophilic asthma</th>
<th>OCS-dependent eosinophilic asthma</th>
<th>Mixed phenotype eosinophilic asthma</th>
<th>Neutrophilic infectious asthma</th>
<th>Healthy control subjects</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviation used in text</td>
<td>ICS-EA</td>
<td>OCS-EA</td>
<td>MEA</td>
<td>NIA</td>
<td>HC</td>
<td></td>
</tr>
<tr>
<td>Asthma severity</td>
<td>Mild to moderate</td>
<td>Severe</td>
<td>Moderate to severe</td>
<td>Neutrophilic</td>
<td>Mild to moderate</td>
<td>None</td>
</tr>
<tr>
<td>Subjects, no.</td>
<td>13</td>
<td>20</td>
<td>18</td>
<td>13</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Female sex, no. (%)</td>
<td>7 (54)</td>
<td>10 (50)</td>
<td>11 (61)</td>
<td>8 (61)</td>
<td>7 (47)</td>
<td>.8922</td>
</tr>
<tr>
<td>Mean age (y [range])</td>
<td>56 (23-80)</td>
<td>53 (28-79)</td>
<td>60 (33-75)</td>
<td>55 (19-73)</td>
<td>34 (20-56)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8</td>
<td>33.01 ± 5</td>
<td>30.2 ± 7</td>
<td>30.8 ± 4</td>
<td>24.8 ± 4</td>
<td>.0023</td>
</tr>
<tr>
<td>Asthma onset (adult [% of no.])</td>
<td>11 (84)</td>
<td>16 (80)</td>
<td>16 (88)</td>
<td>6 (46)</td>
<td>6 (46)</td>
<td>.031</td>
</tr>
<tr>
<td>Lung function</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁ (%)</td>
<td>77.6 ± 18.9</td>
<td>57.6 ± 15.5</td>
<td>59.2 ± 22</td>
<td>71 ± 28</td>
<td>96.1 ± 9.6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>FEV₁/FVC ratio</td>
<td>0.67 ± 0.13</td>
<td>0.6 ± 0.12</td>
<td>0.6 ± 0.14</td>
<td>0.63 ± 0.18</td>
<td>0.85 ± 0.04</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Daily medication</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICS (mg/d), median (range)</td>
<td>500 (250-2000)</td>
<td>1200 (500-4000)</td>
<td>1125 (500-2000)</td>
<td>750 (0-3600)</td>
<td>NA</td>
<td>.0701</td>
</tr>
<tr>
<td>OCS (mg/d), median (range)</td>
<td>0 (0)</td>
<td>11.25 (0-40)</td>
<td>6.25 (0-35)</td>
<td>0 (0-20)</td>
<td>NA</td>
<td>.0003</td>
</tr>
<tr>
<td>Indices of airway inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum TCC, mean (range)</td>
<td>2.3 (0.3-6.3)</td>
<td>7.2 (0.2-26.6)</td>
<td>48.6 (18.0-288)</td>
<td>84.4 (47.9-95)</td>
<td>90.6 (67.3-99.7)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Neutrophils [%]</td>
<td>48.0 (10.7-83.2)</td>
<td>43.3 (5.7-88.2)</td>
<td>84.4 (47.9-95)</td>
<td>90.6 (67.3-99.7)</td>
<td>32.0 (5.3-60.8)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Eosinophils [%]</td>
<td>16.5 (3.7-69.3)</td>
<td>27.89 (3.3-87.8)</td>
<td>3.8 (0-14.7)</td>
<td>0.2 (0-0.8)</td>
<td>0.27 (0-1.5)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Symptos/factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atopy, no. (%)</td>
<td>9 (69)</td>
<td>10 (50)</td>
<td>12 (66)</td>
<td>6 (46)</td>
<td>8 (53)</td>
<td>.6295</td>
</tr>
<tr>
<td>Sinus disease, no. (%)</td>
<td>9 (69)</td>
<td>13 (65)</td>
<td>13 (72)</td>
<td>6 (46)</td>
<td>NA</td>
<td>.4947</td>
</tr>
<tr>
<td>Ex-smoker &gt;10 pack-year history, no. (%)</td>
<td>7 (53)</td>
<td>9 (45)</td>
<td>7 (38)</td>
<td>2 (15)</td>
<td>NA</td>
<td>.2077</td>
</tr>
<tr>
<td>Indices of systemic inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total LKCs</td>
<td>8.775 ± 2.5</td>
<td>10.68 ± 4.1</td>
<td>11.46 ± 4.1</td>
<td>9.892 ± 3.7</td>
<td>NA</td>
<td>.2782</td>
</tr>
<tr>
<td>Absolute eosinophils</td>
<td>0.38 ± 0.27</td>
<td>0.5 ± 0.5</td>
<td>0.3389 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>NA</td>
<td>.005</td>
</tr>
<tr>
<td>Absolute neutrophils</td>
<td>5.825 ± 2.1</td>
<td>7.767 ± 3.6</td>
<td>8.839 ± 4.2</td>
<td>7.523 ± 3.7</td>
<td>NA</td>
<td>.1385</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>7.914 ± 15.3</td>
<td>6.97 ± 4.9</td>
<td>16.37 ± 23.1</td>
<td>22.4 ± 36.2</td>
<td>NA</td>
<td>.4441</td>
</tr>
<tr>
<td>Total serum IgE</td>
<td>260 ± 319</td>
<td>650 ± 1200</td>
<td>350 ± 453</td>
<td>107 ± 120</td>
<td>NA</td>
<td>.2382</td>
</tr>
<tr>
<td>Autoantibodies, no. (%)</td>
<td>1 (7)</td>
<td>0</td>
<td>3 (16)</td>
<td>3 (23)</td>
<td>0</td>
<td>.1154</td>
</tr>
<tr>
<td>ANA (no.)</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>ANCA (no.)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Thyroid complications, no. (%)</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
| Data are presented as mean ± SDs or ranges (minimums-maximums). Healthy reference range for blood LKCs: 4.0-11.0 × 10⁹/L; eosinophils: 0.0-0.3 × 10⁹/L; neutrophils: 2.0-7.5 × 10⁹/L; cutoff for C-reactive protein: 5.0 mg/L; and IgE: >165 kIU/L. ANCA, Anti-neutrophil cytoplasmic antibody; ANA, Hospital panel includes antibodies against dsDNA, chromatin, ribosomal P, centromere B, Sm, SmRNP, SSA, SSB, SSB (La), Scl-70, anti-topoisomerase, and Jo-1; BMI, body mass index; FVC, forced vital capacity; ICS, equivalent dose of fluticasone propionate; LKCs, leukocytes; NA, not applicable; OCS, prednisolone. 

*CIndicates the group with significant variation. 

†Atopy was defined as positive skin prick test response; Rh factor, erythrocyte sedimentation rate, and physician’s notes from patient charts within 3 months of sputum sample were used.
we chose 15 patients with severe EA from within our final study cohort for whom matched serum for the tested sputum sample was available. A direct comparison of sputum IP-Igs and serum autoantibody titers was conducted, results of which suggested anti-EPX IgGs to be localized to the airways (Fig 1, D).

To address the concern that Protein A/G binds to IgA and IgM with a lower affinity than IgG, we tested the anti-EPX reactivity for all 3 isotypes in the neat sputum supernatant, the matched eluted IP-Ig and non-Ig wash-through fraction, in parallel (Fig 1, E; n = 10, severe asthma; sputum eosinophils > 3%; see Table E1 in this article’s Online Repository at www.jacionline.org for patient demographics, ie, the validation cohort). Anti-EPX reactivity in the sputum supernatant was significantly weaker when probed for IgA and IgM compared with IgG (P < .0001, 2-way ANOVA). This further validated the use of IP-Igs as the source for detecting anti-EPX reactivity because the signal lost in the wash-through fraction was minimal. We investigated the nature of anti-EPX immunoglobulins detected in the sputum IP-Igs of 10 eosinophilic sputa (sputum eosinophils > 3%) against non-eosinophilic (n = 4, sputum eosinophils < 2%) and healthy subjects (n = 6) comprising the “validation cohort” (n = 20, see

![Image](image-url)
It was confirmed that the anti-EPX antibodies were of the IgG subtype (Fig 1, F-H). Finally, significant binding of sputum IP-Igs to fixed permeabilized eosinophils allowed a visual confirmation of the phenomenon (Fig 1, I and J, and see Fig E1 for complete panel staining). The modest colocalization of sputum IgG staining with EPX \((r = 0.23-0.71)\) and extensive cytoplasmic staining suggested the occurrence of autoantibodies of possible multiple reactivities to autologous eosinophilic cellular components.

**Autoantibodies to nuclear and extractable nuclear antigens in the airways**

To investigate the underlying propensity toward an autoimmune phenomenon coupled with the evidence of detectable ANAs in
airways of patients with chronic obstructive pulmonary disease and anti-dsDNA IgG in nasal polyp extracts from patients with chronic rhinosinusitis (CRS), we analyzed autoantibodies to 17 common autoantigens related to nuclear and extractable nuclear components (ANAs). The ANA scores on the immunoassay strips (see Fig 2, A, for an example of the scoring index and the list of 17 autoantigens) were cross-validated with Hep-2 substrate slides (Immco Diagnostics, Buffalo, NY), as described recently (Fig 2, B). A significant increase in ANA scores was observed in the OCS-EA and MEA groups (Fig 2, C). The similar distribution pattern of both ANAs and anti-EPX reactivity in the final cohort (Figs 1, C, and 2, C) was corroborated by the significant correlation value (Fig 2, D), thereby indicating a “polyclonal” autoimmune event. The staining patterns varied from homogenous, speckled, and mitochondria-like between the samples (data not shown).

This further reiterated the presence of autoantibodies of multiple specificities typical of a polyclonal autoimmune response. No significant difference was seen in serum ANA profiles between healthy volunteers and 15 patients with severe EA (reported in Fig 1, D) with detectable sputum ANAs (Fig 2, E). Taken together, the presence of a localized autoimmune event with in situ generation of IgG autoantibodies to EPX and nuclear/cytoplasmic antigens was evident.

Clinical relevance: Increased autoantibody levels in the airways of asthmatic patients contribute to eosinophil activity

Statistical analysis revealed significant correlation scores between sputum autoantibody (IgG) levels and both EPX levels and...
FEGs but none with intact eosinophils (see Fig E2 in this article’s Online Repository at www.jacionline.org), indicating that autoimmune response was abundant in the airways of patients with high eosinophil luminal activity. Therefore we next investigated the clinical relevance of increased autoantibody levels in the airways to address whether their presence was a mere epiphenomenon or contributory to the observed airway eosinophil activity. We did not co-immunoprecipitate autoantibodies to determine antigen specificity (eg, anti-EPX IgG) because the aim of this experiment was to assess the consequence of increased IgG autoantibodies in the airways and to assess their effects on naive eosinophils using an unaltered physiologic pool of sputum immunoglobulins.

We determined that sputum IP-Igs with increased autoantibody titers (both anti-EPX IgG and ANAs) induced eosinophil degranulation in vitro. This was evident from the loss of membrane integrity (LDH release) and extensive extracellular release of histone-coated chromatin traps, which is indicative of eosinophil extracellular traps (EETs), observed with increasing time points (see Fig E3 in this article’s Online Repository at www.jacionline.org). Indeed, extensive events of EET formation were observed online.21

Mechanism of autoimmune susceptibility in patients with severe asthma

The finding that not all patients with EA exhibit detectable sputum autoantibodies suggests the presence of regulatory mechanisms to support local immunologic tolerance.22,23 In patients with autoimmune disorders, such as systemic lupus erythematosus and rheumatoid arthritis, a strong association with lymphopenia and reduced regulatory cell counts has been established to be accountable for the loss of peripheral tolerance.24 Although the majority of our study subjects have low peripheral lymphocyte counts, we did not document any significant correlation of sputum autoantibodies with either circulating or sputum lymphocyte levels using the absolute values documented at the time of sputum induction (Fig 4, A–D). Because the pathomechanism initiated by lymphopenia is time dependent based on the gradual compensatory homeostatic expansion of autoreactive clones, subsequently leading to loss of peripheral tolerance, we also considered using an index that takes into account transient lymphopenic events over time. The history of lymphopenia was based on the evidence of reduced blood lymphocyte counts of <1.5 × 10^9/L on ≥3 occasions. Although lymphopenia has often been shown to have causality in the development of autoimmunity, in our observations neither absolute lymphocyte counts (at the time of sputum induction) nor the history of lymphopenia were seen to be associated with the presence of sputum autoantibodies (Fig 4, A–F).

Because eosinophil degranulation was determined to be associated with the presence of autoantibodies in both clinical and murine studies, we segregated our population into patients with concurrent presence of increased airway activity (degranulation) and a history of lymphopenia (Fig 4, G and H). Indeed, a significant increase in the prevalence of both anti-EPX IgG and ANAs was evident in patients with increased degranulation presenting with a history of lymphopenia. Furthermore, we observed that all patients with high sputum TCCs with a concurrent history of lymphopenia and airway degranulation had autoantibodies in their sputum.

Identifying patients with an autoantibody signature: Clinical and molecular parameters

To identify clinical parameters associated with localized autoimmune responses, we adopted the following strategy, considering our sample size. Patients with autoantibody signatures were defined as those with anti-EPX IgG or ANAs greater than the cutoff threshold (calculated cutoff based on mean absorbance values of HCs +2× SD, as represented by dotted lines in Figs 1, C, and 2, C). A nonparametric test (independent-samples Mann-Whitney test) was conducted for every clinical parameter based on the distribution between the 2 groups (patients with autoantibodies vs patients without autoantibodies). We next performed multivariate regression analysis (method: Stepwise, IBM SPSS version 23.0; IBM, Armonk, NY) separately for each autoantibody signature (anti-EPX IgG/ANA) by using the clinical parameters with a significant \( P \) value of .01 or less (see Table E2, A, in this article’s Online Repository at www.jacionline.org for model summary and excluded variables). FEG index and sputum TCCs were selected as positive predictors for anti-EPX signatures, generating a model with an \( R^2 \) value of .235, a standard error of estimate (SEE) of 0.44, and a \( P \) value of .001, whereas the FEG index was the sole predictor for the presence of ANAs (adjusted \( R^2 = 0.119 \), SEE = 0.47, \( P = .01 \); Table II). Thus the model suggested that patients with increased events of eosinophil degranulation (indicated by FEG index) and increased sputum TCCs, which is suggestive of susceptibility to infection, were more likely to have localized autoimmune responses.

Nonparametric analysis of all 67 inflammatory mediators and correlation studies identified a unique microenvironment in patients with an autoantibody signature (ie, anti-EPX IgG and/or ANAs; see Table E3). Eight inflammatory mediators were computed to be significantly increased in these patients (\( P ≤ .01 \); Fig 5), with a significant correlation among IL-13, IL-5, IL-18 (T H2 environment), eotaxin-2 (eosinophil recruitment), macrophage-derived chemokine, IL-16 (T H2 cell recruitment), and B cell–attracting chemokine 1 (BCA-1) and BAFF (increased B-cell activity). To identify molecular predictors, we used the same strategy as before. Again, only those cytokines/mediators were chosen for the regression model whose \( P \) values in the nonparametric test were .01 or less (refer to Table E2, B, for individual \( P \) values and Table E4 in this article’s Online Repository at www.jacionline.org for model summary and excluded variables). The regression analysis suggested BAFF and IL-13 to be positive predictors for the presence of anti-EPX IgGs (\( R^2 = 0.458 \), \( P = .035 \), \( P = .000 \)) and eotaxin-2 and BAFF to be positive for predicting sputum ANA levels (\( R^2 = 0.296 \), \( P = .0428 \), \( P = .000 \); Table II). Thus the molecular signature suggested that autoimmune responses were likely to be higher in asthmatic patients who retained a T H2 environment with increased eosinophil recruitment and B-cell activity.
FIG 4. Sputum autoantibodies are prevalent in patients with increased airway degranulation and a history of lymphopenia. A-D, Association of sputum autoantibodies with circulating lymphocytes (absolute counts; Fig 4, A) and sputum lymphocytes (absolute counts; Fig 4, B) and distribution in patient subsets (Fig 4, C and D) in the final cohort. Dotted lines represent the reference range for circulating lymphocytes. E and F, ANOVA with the Holm-Sidak multiple comparison test. Anti-EPX and ANA scores plotted for all subjects were divided into asthmatic patients with normal lymphocyte counts and those with a history (Hx) of lymphopenia (assessed as an blood absolute lymphocyte count <1.5×10^9/L currently and on 2 past occasions; Mann-Whitney test). G and H, Study population divided into 4 groups based on history of lymphopenia and degree of degranulation (measured by FEG index scores). Anti-EPX IgG values and ANA index scores were plotted. Significant difference between the groups: *P < .05 and **P < .01, ANOVA with Holm-Sidak multiple correction. The cutoff thresholds for both autoantibody detections are represented by the dotted line on the respective plots. ns, Not significant. Purple symbols indicate patients with sputum TCC and neutrophil percentages indicative of infection.
DISCUSSION

We report a novel observation of pathogenic autoantibodies in the sputum of patients with persistent eosinophilia and severe asthma. Sputum autoantibodies against EPX and several anti-nuclear antigens absent from the circulation suggest that a polyclonal autoimmune event localized to the airways has occurred in these patients, the presence of which directly correlates with asthma severity. Patients with the proposed autoimmune endotype require high doses of corticosteroids to control their asthma, which is characterized by increased airway eosinophilic degranulation coupled with sputum cellularity suggestive of infection and systemic lymphopenia. These patients presented with a unique sputum cytokine signature indicative of increased eosinophil recruitment, chronic T_{H}2 inflammation, and B-cell activity.

In our study cohort of 79 subjects, levels of both anti-EPX IgG and ANAs were greater in patients with prednisone-dependent EA and in those with severe asthma and pleiotropic bronchitis compared with patients with mild-to-moderate EA and those with neutrophilic asthma. Atopy, smoking status, and body mass index were not determinants of autoantibody levels (see Tables E2 and E3). Because the prevalence of the detected autoantibodies was significantly less in the mild-to-moderate eosinophilic group (ICS-EA; Figs 1, C, and 2, C), it is reasonable to assume that the loss of tolerance demonstrated in our findings occurs with disease progression. However, other unknown underlying events cannot be discounted at this stage that could simultaneously be responsible for both the loss of tolerance and disease progression. Finally, as a limitation of our cohort, although the healthy subjects were not age matched to the asthmatic patients, there was no

TABLE II. Clinical identification of patients with the airway autoimmune endotype

<table>
<thead>
<tr>
<th>Regression Model</th>
<th>Predictors</th>
<th>β Coefficient (standardized)</th>
<th>R²</th>
<th>Adjusted R²</th>
<th>SE of the estimate</th>
<th>F (df)</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A. Clinical parameters for predicting Anti-EPX IgG</td>
<td>FEG index</td>
<td>0.493</td>
<td>0.235</td>
<td>0.206</td>
<td>0.44367</td>
<td>7.992</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>Sputum TCC</td>
<td>0.301</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1B. Clinical parameters for predicting ANAs</td>
<td>FEG index</td>
<td>0.345</td>
<td>0.119</td>
<td>0.099</td>
<td>0.47694</td>
<td>6.07</td>
<td>.018</td>
</tr>
<tr>
<td>2A. Molecular parameters for predicting anti-EPX IgG</td>
<td>BAFF</td>
<td>0.465</td>
<td>0.458</td>
<td>0.443</td>
<td>0.36504</td>
<td>30.792</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>IL-13</td>
<td>0.431</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2B. Molecular parameters for predicting ANAs</td>
<td>Eotaxin-2</td>
<td>0.467</td>
<td>0.296</td>
<td>0.274</td>
<td>0.42821</td>
<td>13.048</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>BAFF</td>
<td>0.223</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The dependent variable was the sputum autoantibody signature (either based on ANA index or anti-EPX IgG). Data are summarized from SPSS (version 23) output of model summary, ANOVA table, and coefficients. The model summary and list of excluded variables are provided in Tables E2-E5.

Sig, Significance based on ANOVA.

FIG 5. Distribution of mediators included in the molecular signature for autoimmune endotype. Scatter plots for sputum expression levels of positive predictors are shown. A-D, Mediators suggestive of the T_{H}2-dominated environment: IL-5, IL-13, IL-18, and eotaxin-2. E-F, Mediators indicative of increased B-cell activity: BAFF and BCA-1. G and H, T_{H}2 cell recruitment: IL-16 and macrophage-derived chemokine (MDC). Each symbol represents an individual patient: red indicates sputum with positive autoantibody signature (ie, detectable anti-EPX IgG and/or ANA above respective cutoffs). Significant difference between groups: *P < .01, **P < .001, and ***P < .0001, Kruskal-Wallis test with Dunn multiple analysis.
statistical difference in the ages of the 4 groups of asthmatic patients (Table I).

It is unlikely that these observations are secondary to nonspecific binding from an increased IgG level (HC subjects vs 51 patients with EA; P < .001, Mann-Whitney test). When total IgG levels were analyzed in the final cohort (see Fig E4), a significant increase was charted only for the mixed pleiotropic group (MEA). In addition, staining intensities on Hep-2 slides (Fig 2, B) were not influenced by total IgG content in sputum. The presence of autoreactive IgE subtypes in these patients is considered unlikely because of the unremarkable total IgE content in the final cohort (see Fig E4, D) and the nonsignificant correlation of IgE levels with sputum autoantibody levels (anti-EPX: r = −0.04, P = .72; ANAs: r = 0.136, P = .33).

It is interesting to note that, similar to our observation of localized autoreactive IgGs in an eosinophilic microenvironment, there have been recent reports of anti-dsDNA IgGs in nasal polyp extracts from patients with CRS,19 with no evidence of IgE auto-reactivity.20 Despite the emerging evidence of a TfiR17 axis in auto-immunity,21 we could not determine any correlation between detected autoantibodies and airway levels of IL-17 or IL-23 (correlation values are shown in Table E4). In addition, one of the most commonly observed sputum reactivity bands on the ANA Line Immunoassay was against dsDNA similar to the observations in patients with CRS.19,20,28,34 Even though a direct causality between chronic eosinophilic inflammation and development of local autoimmunity could not be established, observations reported in patients with CRS and our recent findings in severe eosinophilic airways suggest a strong association. It is also important to mention that the potential of a polyclonal autoantibody response, as seen in these patients, might simply be the nonspecific consequence of unresolved inflammation.

Chronic unresolved inflammation is considered a critical trigger for inducible bronchus-associated lymphoid tissue formation,28,29 with a typical chemokine signature comprising BCA-1, CCL21, and BAFF, as seen in patients with Sjögren disease30 and rheumatoid arthritis31,32 presenting with pulmonary complications. Increased sputum BAFF and BCA-1 levels (Fig 5) coupled with BAFF as a positive predictor for the presence of lung autoantibodies (Table II; and see Table E5 in this article’s Online Repository at www.jacionline.org) support the likelihood of local B-cell clusters as the seat of autoantibody generation. We were unable to confirm the presence of B-cell clusters in our patients because we did not have access to resected lung tissue (bronchoscopic mucosal tissue might not suffice). However, a recent description of “asthma granulomatosis” was based on the immunohistologic evidence of granulomas in lung biopsy specimens from prednisone-dependent patients with severe disease.33 Again, double-transgenic l5/hE2 mice with severe respiratory inflammation and airway eosinophil degranulation (resembling our patient population) were seen to have localized anti-EPX IgG in their bronchoalveolar lavage fluid in addition to positive immunohistologic staining for B-cell clusters (see the Methods and Results sections and Fig E5 in this article’s Online Repository at www.jacionline.org). The presence of granulomas or ectopic lymphoid structures with higher organization (inducible bronchus-associated lymphoid tissue) in our autoantibody-positive patients is possible. Furthermore, localized expression of BAFF, BCA-1, IL-16, and IL-15 (see Table E2) would support both T-cell–dependent and independent B-cell activation and differentiation to antibody-secreting plasma cells in situ,8,34 whereas increased eotaxin-2 and IL-13 levels would enhance eosinophil recruitment into the lungs.

Recently, IgG-mediated eosinophil degranulation with formation of extensive extracellular DNA traps, termed EETosis, was reported in vitro35 and in vivo36 in CRS tissue. Weiler et al12 demonstrated in 1996 that IgG caused eosinophils to degranulate (undergo exocytosis) to release EPX and potentially free granules. Therefore an increase in IgG levels in asthmatic airways (caused by increased autoantibody levels) coupled to active eosinophil recruitment is likely to trigger eosinophil degranulation and EETs. However, from our current data set, it cannot be concluded whether EET formation is induced specifically by downstream signaling of autoantibody-target antigen interaction or IgG:Fcγ receptor cross-linking. There is early clinical evidence that ragweed allergen–specific IgG from the sera of asthmatic patients induced eosinophil degranulation in vitro, which was abolished in the presence of anti-IgG and anti-FcγRII antibodies but not with anti-IgE or anti-FcεRI.37 In addition, increased IgG-autoantigen complexes in the airways as a result of an autoimmune response would lead to ligation of FcγRII, which has been shown to inhibit glucocorticoid-induced eosinophil apoptosis.38 This, coupled with our observation that dexamethasone was unable to suppress the immunoglobulin-induced extracellular trap formation (Fig 3, C and D) possibly underlies the steroid insensitivity observed in the putative autoimmune endotype.

In our current set of patients, we failed to demonstrate a significant association between the presence of lymphopenia and years of prednisone use (r = 0.25, P = .08; see Table E2). Furthermore, “prednisone dose” in our multivariate regression model was not computed to be a “predictor” (refer to excluded variable OCS in Table E3). In addition, a recent study shows that variations in the immunome with corticosteroid treatment do not affect regulatory T-cell populations, the suppression of which is implicated in the development of autoimmunity.40 Finally, the l5/hE2 murine model (see the Methods and Results sections and Fig E5), which resembles our asthmatic population with respect to intense airway eosinophil degranulation, was found to develop anti-EPX IgG without being subjected to any steroid treatment. Nevertheless, we acknowledge that without a longitudinal study with a justified sample size, the role of long-term corticosteroid use in the development of observed autoimmunity cannot be ruled out definitively.

In conclusion, these findings suggest that some patients with severe asthma might have autoantibodies against autologous eosinophilic cellular components that can be detected in sputum. These patients, which are crudely estimated to be approximately a third of patients with severe EA, can be identified as those with luminal eosinophil degranulation, lymphopenia, and recurrent infective bronchitis and a characteristic lung TiH2 microenvironment along with increased B-cell activity, features that are often overlooked in routine clinical practice. Comprehensive understanding of the molecular events that lead to the described autoimmune endotype will pave the way for better and novel therapeutic options and improve patient management.

We thank Brenda Helpard and Nicola LaVigne, MLT, Hargreave Sputum Laboratory, St Joseph’s Healthcare, Hamilton, Ontario, Canada, for their expertise with sputum processing. We dedicate this manuscript to our dear colleague Professor Jamie Lee. We sorely miss his wit and wisdom.
Clinical implications: A novel concept of an autoimmune inflammation localized to the airways of severe eosinophilic asthma is reported. This is possibly steroid-insensitive, contributing to disease severity, and necessitates identification of these patients for appropriate therapeutic strategies.

REFERENCES