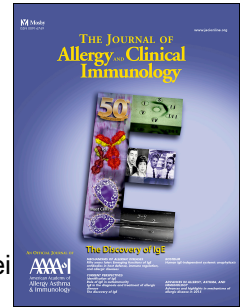


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Title: Sputum autoantibodies in severe eosinophilic asthma

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Abstract:

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

Objectives: To investigate the possibility of a polyclonal autoimmune event in asthmatic airways, and identify associated clinical and molecular characteristics.

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

Results: We report a “polyclonal” autoimmune event occurring in the airways of prednisone-dependent asthmatics with increased eosinophil activity and/or recurrent pulmonary infections, evident by the concomitant presence of sputum anti-EPX and ANAs of the IgG subtype. Extensive cytokine profiling of the sputum revealed a Th2-dominated micro-environment (eotaxin-2, IL-5, IL-18, IL-13), and increased signalling molecules that support the formation of ectopic lymphoid structures (B-cell activating factor, B-cell attracting chemokine-1). Immunoprecipitated sputum immunoglobulins from patients with increased autoantibodies triggered eosinophil degranulation *in vitro*, with release of extensive histone-rich extracellular-

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traps; an event unsuppressed by dexamethasone 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.

Capsule summary:

We report a novel concept of an autoimmune inflammation localised to the airways of severe eosinophilic asthmatics. This is possibly steroid-insensitive, contributing to disease severity, and necessitates identification of these patients for appropriate therapeutic strategies. (35 words)

Keywords: severe asthma, eosinophilia, autoantibodies, sputum, eosinophil degranulation, eosinophil peroxidase, autoimmunity, anti-nuclear antibodies

Abbreviations: FEGs – free eosinophil granules; EPX – eosinophil peroxidase; ANAs – anti-nuclear antibodies; PC₂₀ – provocative concentration causing a 20% drop in FEV₁; IP – immunoprecipitation; Igs – Immunoglobulins; dsDNA – double stranded deoxyribonucleotide acid; LDH – lactose dehydrogenase; TCC – total cell count; Abs₆₀₀ – Absorbance values at 600 nm; EET – eosinophil extra-cellular traps.

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Main Text

Introduction:

A persistence of airway eosinophilia despite daily maintenance oral corticosteroids (OCS) indicates a difficult-to-control population projected to benefit most from anti-eosinophil biologics currently in development¹. However, clinical trials targeting eosinophils in prototype eosinophilic patients, though successful in depleting circulating eosinophils, resulted in only ~50-70% reduction in exacerbations²; 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, leading to frequent exacerbations with characteristic clusters of free eosinophilic granules (FEGs) in sputum^{3, 4}. Increased abundance of FEGs in the airways strongly correlate with sputum eosinophil peroxidase (EPX) content that are released upon luminal eosinophil degranulation, indicative of ‘active’ disease^{5, 6}. Indeed, eosinophil degranulation releasing FEGs indicative of cytolysis or primary lysis is suggested to be pathogenic⁷.

Increased frequency of immune cell degranulation over time leads to peroxidase enzyme release, which has known immunogenic properties by the virtue of its ability to cause tissue damage⁸. Thyroid peroxidase (TPO) and myeloperoxidase (MPO) have established etiologic roles in the pathogenesis of autoimmune disorders like Hashimoto’s thyroiditis⁹ and eosinophilic granulomatosis with polyangiitis¹⁰, respectively. EPX has been reported to cause necrotic lysis of epithelial cells, *in vitro*⁸. We therefore speculated that increased eosinophil degranulation accumulates immunogenic entities like EPX in airways, and subsequent markers of tissue injury, i.e., molecules that activate danger-associated molecular pattern (DAMPs) receptors and trigger a

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localized adaptive immune response leading to breach of immune tolerance as per Matzinger's "danger" model¹¹.

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 without a definite causal relationship. First, circulating ANAs with unknown antigen specificities were seen in 55% of asthmatics with aspirin-sensitivity, although airways were not examined¹². Two recent studies reported naturally occurring circulating anti-IgE IgG in healthy subjects, with raised titers in atopic and non-atopic asthma patients¹³, along with increased circulating immune complexes in the sera of asthmatic subjects with heightened disease severity¹⁴.

To date, an autoimmune mechanism triggered locally in the lungs targeting eosinophil-specific antigens to explain asthma severity has not been investigated. Following our hypothesis of 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 i.e. 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 asthma patients during routine clinical management at the Firestone Clinic, with approval from the local Hospital Research Ethics Board (HiREB), St Joseph's Hospital, Hamilton, ON. Random sputum supernatants used in this study were categorised based on the nature and history of their

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bronchitis, viz.: (i) eosinophilic (EA) patients with current sputum eosinophils > 3%, normal total cell count (TCC) < 9.7×10^6 cells/g, and a history of sputum eosinophilia that requires daily maintenance corticosteroid; (ii) neutrophilic (NA), current infective bronchitis with TCC > 9.7×10^6 cells/g, neutrophils > 65%, eosinophils < 1.5%, and no history of sputum eosinophilia; (iii) Mixed phenotype/ pleiotropic bronchitis (MEA), recurrent infective bronchitis, TCC > 9.7×10^6 cells/g, neutrophils > 65%, eosinophils > 1.5% or history of sputum eosinophils that requires daily maintenance corticosteroids. 15 healthy volunteers with no known respiratory or systemic disorders, no personal/immediate family history of autoimmune disease and no event of an infection within 4 weeks of their sputum induction participated in the study with written consent. All subjects were non-smokers (<10/pack year) or never smokers. Subject characteristics are summarized in Table I, and information collected is with respect to the date of the archived sputum sample used for the study. Asthma diagnosis was based on either post-bronchodilator reversibility >12% and/or PC₂₀ < 8 mg/mL in methacholine provocation challenge, while severity was determined by the maintenance corticosteroid dosage¹⁵.

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

Sputum was induced with hypertonic saline as described previously¹⁶ and the endogenous EPX content was measured by ELISA in the cell-free supernatant.⁵ FEGs in the sputum cytospin were ranked by two independent medical technologists between 0-3 (where, 0 = none, 1 = few, 2 = moderate and 3 = many), for the current sputum sample, and that of two more samples preceding it (based on sputum database maintained for all patients seen at Firestone Clinic). The index was computed as the sum of the ranks over three consecutive sputum samples divided by the maximum possible outcome i.e. $3 \times 3 = 9$, and the mean sum of the two indices thus calculated was

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considered as the FEG index. This allowed us to take into consideration both the degree of current and previous degranulation.

Detection of sputum autoantibodies

In order to detect Ig 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 recently¹⁷. Anti-EPX reactivity was assessed using an indirect ELISA¹⁷, developed in-house, and expressed as absorbance at 600 nm (Abs_{600}) after background correction. An ANA Line Immunoassay strip (IMTEC-ANA-LIA-Maxx, Human Worldwide, Weisbaden, Germany) was adapted for detecting sputum ANAs (using manufacturer's protocol, except for the initial step, where 100 μ L of sputum supernatant was incubated overnight at 4°C, with 700 μ L of diluent). The sputum reactivity against eosinophils was further validated by confocal microscopy (refer to online repository for method)

Sputum cytokines

All reported cytokines and chemokines were analysed by Eve Technologies (Calgary, Canada; HD65 Discovery assay), except for BAFF (Quantikine ELISA, R&D Systems, MN, USA).

Assessing eosinophil degranulation with sputum IP-Igs

Freshly prepared IP-Igs from individual patient samples with high sputum autoantibody titers, low/ no sputum autoantibodies and healthy, were coated onto 48-well tissue-culture plates, blocked with 2.5% human serum albumin for 2 hrs at 37°C. 1×10^5 eosinophils, isolated using MACSxpress® Eosinophil Isolation kit, Miltenyi Biotec GmbH, Germany, as per manufacturer's protocol) in serum-free RPMI1640 media $\pm 10^{-6}$ M dexamethasone (Sigma Aldrich) were added

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to the Ig-coated wells, incubated for 12 hr at 37°C, 5% CO₂. Lactose dehydrogenase activity (LDH assay, Abcam) was measured along with ds-DNA release in the collected supernatants (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes). To visualize the event, 0.5 x 10⁵ eosinophils were added to the IP-Ig coated chamber-slides (Lab-Tek II) for t = 3, 6, 9, 12 hrs. At given time-points, the wells were fixed with 4% PFA, probed with 0.1 µg/mL of anti-histone monoclonal antibody (Millipore, CA, USA), and counterstained with anti-mouse IgG Alexa Fluor 488 (Molecular Probes). Events were visualized with a Nikon Eclipse TE2000-E (Melville, NY, USA) fluorescence microscope and a Q-imaging Retiga 2000R camera (Surrey, British Columbia, Canada). Care was taken not to permeabilize the cells during the protocol.

All other reported protocols and statistical methods are provided in the online data supplement.

Results:

Identification of anti-EPX IgGs in a sub-set of eosinophilic asthmatics “localised” in the airways

We detected anti-EPX IgG in 51 eosinophilic patients diagnosed with asthma (EA) compared to 15 healthy controls (HC) (**Figure 1A**); the former comprising a sub-population of daily OCS users with Abs₆₀₀ values > cut-off. To characterise this sub-population, we segregated the 51 eosinophilic patients based on their daily corticosteroid use. The proportion of anti-EPX IgG was greater in the OCS-dependent group compared to both inhaled (ICS)-dependent and HCs (**Figure 1B**). When we next analyzed the nature of inflammation and bronchitis history, we identified patients within the ICS-group with Abs₆₀₀> cut-off, who had a history of recurrent infections (sputum TCC >14 x 10⁶ cells/g, neutrophils > 65%, eosinophils >1.5% or history of sputum eosinophils requiring daily prednisone). Finally, these 51 EA patient samples were classified into

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three distinct groups based on nature of their bronchitis, with an additional age and sex-matched neutrophilic asthma group (NA) added to constitute the *final cohort* (**Table I**). The data distribution for the final cohort (**Figure 1C**) showed significantly increased anti-EPX IgG signal in the “OCS-EA” sub-group comprising of prednisone-dependent patients with persistent sputum eosinophils and the “MEA” sub-group of patients with airway eosinophilia (despite corticosteroid use)/history of eosinophilia, and recurrent infections with increased sputum TCC. In addition, we chose 15 severe eosinophilic asthmatic patients from within our final study cohort for whom matched serum for the tested sputum sample was available. A direct comparison of sputum IP-IgG and the serum autoantibody titres was conducted, results of which suggested anti-EPX IgGs to be “localised” to the airways (**Figure 1D**).

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 three isotypes in the neat sputum supernatant, the matched eluted IP-Ig and non-Ig wash-through fraction, in parallel (Figure 1E, $n = 10$, severe asthmatic, sputum eosinophils $>3\%$, refer to Table E1 for patient demographics ‘validation cohort’). Anti-EPX reactivity in the sputum supernatant was significantly weaker when probed for IgA and IgM compared to IgG ($P < 0.0001$, Two-way ANOVA). This further validated the use of IP-IgG as the source for detecting anti-EPX reactivity since the signal lost in the wash-through fraction was minimal. We investigated the nature of anti-EPX IgG detected in the sputum IP-IgG of 10 eosinophilic sputa (sputum eosinophils $>3\%$) against non-eosinophilic ($n = 4$, sputum eosinophils $<2\%$) and healthy ($n = 6$) comprising the ‘validation cohort’ ($n = 20$, Table E1). It was confirmed that the anti-EPX antibodies were of IgG subtype (**Figure 1F-H**). Finally, significant binding of sputum IP-IgG to fixed, permeabilized eosinophils allowed a visual confirmation of the phenomenon (**Figure 1I, 1J**, see **Figure E1** for complete panel staining).

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The modest co-localisation 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 towards an autoimmune phenomenon, coupled with the evidence of detectable ANAs in COPD airways²⁰ and anti-double stranded DNA IgG (dsDNA) in the nasal polyp extracts from CRS patients^{21, 22}, we analysed autoantibodies to 17 common autoantigens related to nuclear and extractable nuclear components (ANAs). The ANA scores on the immunoassay strips (refer to **Figure 2A** for an example of the scoring index) were cross-validated with Hep-2 substrate slides (Immco Diagnostics, Buffalo, New York, USA), as described recently¹⁷ (**Figure 2, panel B**). A significant increase in the ANA scores was observed in the OCS-EA and MEA groups (**Figure 2C**). The similar distribution pattern of both ANAs and anti-EPX reactivity in the final cohort (**Figure 2C, 1C**) was corroborated by the significant correlation value (**Figure 2D**), 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 the serum ANA profile between healthy volunteers and 15 severe eosinophilic asthmatics (reported in Figure 1D) with detectable sputum ANAs (**Figure 2E**). 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 autoantibodies in the asthmatic airways contribute to eosinophil “activity”

Statistical analysis revealed significant correlation scores between sputum autoantibody (IgG) levels and both EPX and FEGs, but none with intact eosinophils (**Figure E2**) 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 autoantibodies in the airways, to address whether their presence was a mere epiphenomenon or contributory to the observed airway eosinophil activity. We did not co-IP autoantibodies to determine antigen-specificity (for example, anti-EPX IgG), since the aim of this experiment was to assess the consequence of increased IgG autoantibodies in the airways, and to assess their effects on naïve eosinophils, using an unaltered physiological pool of sputum Igs.

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, indicative of eosinophil extracellular traps (EETs), observed with increasing time-points (**Figure E3**). Indeed, extensive events of EET formation were quantified (LDH activity and dsDNA content in the collected supernatants) and found to be significantly induced by sputum IP-Igs from patients with high autoantibody titres compared to those with low autoantibodies and healthy individuals (**Figure 3**). Addition of dexamethasone at a physiologically relevant concentration (10^{-6} M) failed to suppress sputum Ig-induced degranulation (**Figure 3C, D**), reflecting similar observations by Gleich *et al*²³.

Mechanism of autoimmune susceptibility in severe asthma

The finding that not all eosinophilic asthmatics exhibit detectable sputum autoantibodies suggests the presence of regulatory mechanisms to support local immunogenic tolerance³²⁻³⁵. In autoimmune disorders like systemic lupus erythematosus and rheumatoid arthritis (RA), a strong association with lymphopenia and reduced regulatory cells has been established to be accountable for the loss of peripheral tolerance³⁶. Though the majority of our study subjects have low peripheral lymphocyte counts, we failed to 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 (**Figure 4A-D**). Since 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 i.e. the history of lymphopenia (i.e., evidence of reduced blood lymphocyte counts $<1.5 \times 10^9/L$ in three or more occasions. Though lymphopenia has often been shown to have causality in 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 presence of sputum autoantibodies (**Figure 4A-F**). Since 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 history of lymphopenia (**Figure 4G, 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. Further, we observed that all patients with high sputum TCC with 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 that are associated with localised autoimmune responses we adopted the following strategy considering our sample size. Patients with autoantibody signature were defined as those with anti-EPX IgG or ANA above the cut-off threshold (calculated cut-off based on mean absorbance values of HCs +2 x standard deviation (SD), represented by dotted lines in Figures 1C and 2C). A non-parametric test (independent samples, Mann-Whitney) was conducted for every individual clinical parameters based on the distribution between the two groups (patients with autoantibodies vs patients without autoantibodies). We next performed multivariate regression analysis (method: Stepwise, IBM® SPSS® version 23.0) separately for each autoantibody signature (Anti-EPX IgG/ANA) using the clinical parameters with a significant P value ≤ 0.01 (refer to the online repository Table E 2A for the individual P values, Table E3 for model summary and excluded variables). FEG index and sputum total cell count (TCC) were selected as positive predictors for anti-EPX signature generating a model with $R^2=0.235$, standard error of estimate (SEE) = 0.44, $P = 0.001$, while FEG index was the sole predictor for the presence of ANAs (adjusted $R^2=0.119$, SEE = 0.47, $P = 0.01$) (Table II). The model thus suggested that patients with increased events of eosinophil degranulation (indicated by FEG index) and increased sputum TCC, suggestive of susceptibility to infection, were more likely to have localised auto-inflammatory responses.

Non-parametric analysis of all the 67 inflammatory mediators and correlation studies identified a unique micro-environment in patients with an autoantibody signature i.e. either/or anti-EPX IgG and ANAs (see Table E3, online repository). Eight inflammatory mediators were computed to be significantly (P value ≤ 0.01) increased in these patients (represented in Figure 5) with a significant correlation, viz., IL-13, IL-5, IL-18 (Th2 environment), eotaxin-2 (eosinophil

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recruitment), macrophage-derived chemokine (MDC), IL-16 (Th2 cell recruitment) and finally, 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 value in the non-parametric test was ≤ 0.01 (refer to the online repository Table E 2B for the individual P values, Table E4 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 ($R^2 = 0.458$, $SEE = 0.365$, $P = 0.000$), and eotaxin-2 and BAFF for predicting sputum ANAs ($R^2 = 0.296$, $SEE = 0.428$, $P = 0.000$) (Table II). The molecular signature thus suggested that autoimmune responses were likely to be higher in asthmatics that retained a Th2 environment with increased eosinophil recruitment and B-cell activity.

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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 Th2 inflammation, and B-cell activity.

In our study cohort of 79 subjects, levels of both anti-EPX IgG and ANAs were higher in patients with prednisone-dependent eosinophilic asthma and in those with severe asthma and a pleiotropic bronchitis compared to the patients with mild-to-moderate eosinophilic asthma and patients with neutrophilic asthma. Atopy, smoking status, and body mass index were not determinants of the autoantibody levels (see online supplementary Table E2 and E3). Since prevalence of the detected autoantibodies was significantly less in the mild-to-moderate eosinophilic group (ICS-EA, Figure 1C, 2C), 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 normal subjects were not age-matched to the asthmatic subjects, there was no statistical difference in the ages of the four groups of asthmatic subjects (Table I).

It is unlikely that these observations are secondary to a non-specific binding from increased level of IgGs (HCs vs. 51 EA, Mann Whitney test, $P < 0.001$). When total IgG was analysed in the final cohort (**Figure E4**) a significant increase was charted only for the mixed pleiotropic group (MEA). In addition, staining intensities on Hep-2 slides (**Figure 2B**) were not influenced by total IgG content in sputum. The presence of autoreactive IgE subtypes in these patients is considered unlikely due to the unremarkable total IgE content in the final cohort (**Figure E4, D**), and the non-significant correlation of IgE with sputum autoantibody levels (Anti-EPX: $r = -0.04$, $P = 0.72$; ANAs: $r = 0.136$, $P = 0.33$). It is interesting to note that, similar to our observation of localized autoreactive-IgGs in an eosinophilic micro-environment, there have been recent reports of anti-ds DNA IgGs in nasal polyp extracts from CRS patients²¹, with no evidence of IgE auto-reactivity²². Despite the emerging evidence of a Th17-axis in autoimmunity³⁷, we could not determine any correlation between detected autoantibodies and airway levels of IL-17 or IL-23 (correlation values 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 CRS patients^{21, 22}. Even though a direct causality between chronic eosinophilic inflammation and development of local autoimmunity could not be established, observations reported in CRS patients 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 may simply be the non-specific consequence of the unresolved inflammation.

Chronic unresolved inflammation is considered a critical trigger for iBALT formation^{24, 26}, with a typical chemokine signature comprising of BCA-1, CCL21 and BAFF, as seen in Sjögren's disease³⁸ and RA^{39, 40} presenting with pulmonary complications. Increased sputum BAFF and

BCA-1 levels (**Figure 5**) coupled with BAFF as a positive predictor for the presence of lung autoantibodies (**Table II**), 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; since 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 immunohistological evidence of granulomas in lung biopsies from severe prednisone-dependent patients⁴¹. Again, double transgenic mice *l5/hE2* with severe respiratory inflammation and airway eosinophil degranulation (that resembles our patient population), were seen to have localised anti-EPX IgG in their broncho-alveolar lavage fluid (BALF), in addition to positive immunohistological staining for B-cell clusters (refer to the online methods, results, and Figure E5). The presence of granulomas or ectopic lymphoid structures with higher organisation (iBALT) in our autoantibody-positive patients is possible. Furthermore, localized expression of BAFF, BCA-1, IL-16 and IL-15 (Table E3), would support both T-cell-dependent and -independent B-cell activation and differentiation to antibody-secreting plasma cells *in situ*^{24, 42}, while increased eotaxin-2, IL-13 would enhance eosinophil recruitment into the lungs.

Recently, IgG-mediated eosinophil degranulation with formation of extensive extra-cellular DNA traps, termed as EETosis, was reported *in vitro*⁴³ and *in vivo* in CRS tissue⁴⁴. Gleich and co-workers demonstrated in 1996 that IgG caused eosinophils to degranulate (undergo exocytosis) to release EPX and potentially free granules⁴⁵. Therefore, an increase in IgGs in the asthmatic airways (due to increased autoantibodies) coupled with 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 signalling of autoantibody-target antigen interaction or IgG:Fcγ receptor cross-linking. There is early clinical

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evidence that ragweed-allergen specific IgG from asthmatic sera induced eosinophil degranulation *in vitro*, which was abolished in the presence of anti-IgG, anti-Fc γ RII antibodies but not with anti-IgE or anti-Fc ϵ RII⁴⁶. In addition, increased IgG-autoantigen complexes in the airways as a result of an autoimmune response would lead to ligation of Fc γ RII, which has been shown to inhibit glucocorticoid-induced eosinophil apoptosis⁴⁷. This, coupled with our observation that dexamethasone was unable to suppress the Ig-induced extracellular trap formation (**Figure 3C, 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 the years of prednisone use ($r = 0.25$, $P = 0.08$, Table E2). Further, ‘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 autoimmunity⁴⁸. Finally, *l5/hE2* murine model (refer to the online methods, results, and Figure E5) which resembles our asthmatic population with respect to intense airway eosinophil degranulation were seen 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 the observed autoimmunity cannot definitively be ruled out.

In conclusion, these findings suggest that some patients with severe asthma may have autoantibodies against autologous eosinophilic cellular components that can be detected in sputum. These patients, crudely estimated to be approximately a third of patients with severe eosinophilic asthma, can be identified as those who have luminal eosinophil degranulation,

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lymphopenia, and recurrent infective bronchitis, and a characteristic lung Th2 micro-environment 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.

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Author Contribution: PN conceived the idea, MM designed the experiments, MK, CH recruited the patients, MM, KR, DB, AC, HL, PL performed the human experiments, EJ, SO, JL performed the animal experiments, JM supervised the Ig characterization experiments, MM and PN analyzed the data and prepared the manuscript, PN provides overall guarantee for the paper.

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Table Legends:

Table I: Demographics of Subjects in the final study cohort

Table II: Clinical identification of patients with airway autoimmune endotype

532 **Table I: Demographics of Subjects in the final study cohort**

Patient Groups	ICS-dependent Eos Asthma	OCS- dependent Eos asthma	Mixed Phenotype Eos Asthma	Neutrophilic Infective Asthma	Healthy Controls	<i>p</i> value
Abbreviation used in text	ICS-EA	OCS-EA	MEA	NIA	HC	
Asthma severity	Mild-to-moderate	Severe	Moderate-to-severe	Mild-to-moderate	None	
Bronchitis	Eosinophilic	Eosinophilic	Pleiotropic	Neutrophilic	None	
Subjects, n	13	20	18	13	15	
Female, n (%)	7 (54)	10 (50)	11 (61)	8 (61)	7 (47)	0.8922
Mean Age (range)	56 (23- 80)	53 (28-79)	60 (33-75)	55 (19-73)	34 (20- 56)*	<0.0001
BMI	26.8 ± 4	33.01 ± 5	30.2 ± 7	30.8 ± 4	24.8 ± 4*	0.0023
Asthma Onset (adult, % of n)	11 (84)	16 (80)	16 (88)	6 (46)*	n/a	0.031
<u>Lung function</u>						
FEV₁ %	77.6 ± 18.9*	57.6 ± 15.5	59.2 ± 22	71 ± 28	96.1 ± 9.6*	<0.001
FEV₁/FVC	0.67 ± 0.13	0.6 ± 0.12	0.6 ± 0.14	0.63 ± 0.18	0.85 ± 0.04*	<0.001
<u>Daily Rx</u>						
ICS, median (range)	500 (250-2000)	1200 (500-4000)	1125 (500-2000)	750 (0-3600)	n/a	0.0701
OCS, median (range)	0 (0)*	11.25 (0-40)	6.25 (0-35)	0 (0-20)*	n/a	0.0003
<u>Indices of Airway Inflammation</u>						
Sputum TCC Mean (range)	2.3 (0.3-6.3)	7.2 (0.2-26.6)	48.6 (18.0 - 288)*	45.8 (14.8- 150.9)*	5.19 (1.8- 9.2)	<0.0001
Neutrophil% (range)	48.0 (10.7 - 83.2)	43.3 (5.7 - 88.2)	84.4 * (47 - 99.5)	90.6* (67.3 - 99.7)	32.0 (5.3 - 60.8)	<0.0001

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Eosinophil% (range)	16.5 (3.7-69.3)	27.89 (3.3-87.8)	3.8 (0-14.7)	0.2 (0-0.8)	0.27 (0-1.5)	<0.000 1
<u>Symptoms/Risk factors</u>						
Atopy, n (%)#	9(69)	10 (50)	12 (66)	6 (46)	8 (53)	0.6295
Sinus disease n (%)	9 (69)	13 (65)	13 (72)	6 (46)	n/a	0.4497
CRSwPolyps n (%)	7 (53)	9 (45)	7 (38)	2 (15)	n/a	0.2077
Ex-smoker>10 pack-history, n (%)	4 (31)	2 (10)	5 (27)	8 (62)	0*	0.0016
<u>Indices of Systemic Inflammation</u>						
Total LKCs	8.775 ± 2.5	10.68 ± 4.1	11.46 ± 4.1	9.892 ± 3.7	n/a	0.2782
Absolute eosinophils	0.38± 0.27	0.5 ± 0.5	0.3389 ± 0.3	0.1 ± 0.1*	n/a	0.005
Absolute neutrophils	5.825 ± 2.1	7.767 ± 3.6	8.839 ± 4.2	7.523 ± 3.7	n/a	0.1385
C-reactive protein (CRP)	7.914 ± 15.3	6.97 ± 4.9	16.37 ± 23.1	22.4 ± 36.2	n/a	0.4441
Total serum IgE	260 ±319	650 ±1200	350 ±453	107 ±120	n/a	0.2382
Autoantibodies, n (%)	1 (7)	0	3 (16)	3 (23)	0	0.1154
ANA (n)	1	0	3	2	0	
ANCA (n)	0	0	0	1	0	
Family history of (Autoimmune), n	3	1	0	1	0	
Thyroid complications, n (%)	2 (15)	1 (10)	4 (11)	1 (15)	n/a	0.3982

Data presented as Mean ± SD (standard deviation); Range (min-max); BMI: body mass index; FVC: forced vital capacity; CRS: chronic rhinosinusitis; LKCs: leukocytes; ICS- mcg daily equivalent dose of fluticasone propionate; OCS - prednisolone, mg/daily; ANA - hospital panel includes antibodies against dsDNA, chromatin, Ribosomal P, Centromere B, Sm, smRNP, SSA, SSB; SSB (La); Scl-70; Anti-Topoisomerase; Jo-1; Healthy Reference range for Blood LKCs: 4.0 – 11.0 x 10⁹/L; eosinophils: 0.0-0.3 x 10⁹/L; neutrophils: 2.0 – 7.5 x 10⁹/L; cut-off for CRP (C-reactive protein) 5.0 mg/L, IgE >165 kIU/L; *indicates the group with significant variation; #Atopy was defined as positive reaction to skin prick test; Rh factor, ESR and physician notes from patient charts within 3 months of sputum sample used.

Table II: Clinical identification of patients with airway autoimmune endotype

Regression Model	Predictors	Beta coefficient (standardized)	R square	Adjusted R Square	Std. Error of the Estimate	F (df)	Sig.
1A. Clinical Parameters for Predicting Anti-EPX IgG							
	FEG Index	0.493	0.235	0.206	0.44367	7.992	0.001
	Sputum TCC	0.301					
1B. Clinical Parameters for Predicting ANAs							
	FEG Index	0.345	0.119	0.099	0.47694	6.07	0.018
2A. Molecular Parameters for Predicting Anti-EPX IgG							
	BAFF	0.465	0.458	0.443	0.36504	30.792	0.000
	IL-13	0.431					
2B. Molecular Parameters for Predicting ANAs							
	Eotaxin-2	0.467	0.296	0.274	0.42821	13.048	0.000
	BAFF	0.223					

*Dependent variable: Sputum autoantibody signature (either based on ANA index or anti-EPX IgG). Data summarised from SPSS (version 23) output of model summary, ANOVA table and coefficients. Model summary and list of excluded variables provided in online supplementary (Table E2, E5). Method used: stepwise. Sig.-significance based on ANOVA.

Figure Legends:

Figure 1: Asthmatics with sputum eosinophilia have anti-EPX IgGs in their airways: Anti-EPX IgG reactivity of sputum IP-IgG from patients (A) with eosinophilic asthma (EA, $n = 51$, blue square indicates patients on daily OCS) compared with healthy controls (HCs, $n = 15$), $P = 0.0002$, Mann Whitney U Test. (B) ICS-EA patients, and those on daily maintenance oral corticosteroid (OCS-EA). Purple squares indicate eosinophilic patients with recurrent bronchitis. $P < 0.0001$, Kruskal Wallis, (C) final cohort $n = 79$ grouped into ICS-EA (no infection, $n = 13$), OCS-EA (no infection, $n = 20$), and mixed EA (MEA, recurrent infection, ICS and/or OCS

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dependent, $n = 18$), and an additional neutrophilic, non-eosinophilic asthmatic group (NIA, $n = 13$), *indicates significant difference between groups, Kruskal Wallis, Dunn's Multiple comparison Test. **(D)** Anti-EPX IgG titers of matched sputum vs. sera from 15 severe asthmatics. Data presented as mean $\pm$ SEM for each titer. $P < 0.0001$, Two-way ANOVA with Sidak's multiple comparison test. **(E)** Protocol validation for using Protein A/G eluted immunoprecipitated eluate (IP-Ig) for detection of anti-EPX IgG, IgA, IgM ($n = 10$ severe eosinophilic asthmatics, Two-way ANOVA, Tukey's multiple comparison *indicates $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$). **(F,G,H)** Anti-EPX ELISA conducted on three parallel plates ($n = 20$, Validation cohort, supplementary Table E1) with sputum IP-Igs probed separately for IgG, IgA IgM reactivity. **(I, J)** Representative micrographs of sputum IgG reactivity shown in green, EPX (red) and nuclei visualised using DAPI. Images presented as overlay of the three channels to visualise co-localization (**Figure E1** for individual channels). The fluorescence intensity for sputum IgG binding normalised to the area for all three subjects, $P < 0.0001$, Friedman Test (repeated measures). *N.B. calculated cut-off based on mean absorbance values of HCs $+2 \times$ standard deviation (SD), represented by dotted lines.*

Figure 2: Anti-nuclear/extractable antigen antibodies detectable in asthmatic sputum: (A) A representative scoring pattern is presented for estimating the ANA Index Score. Two blinded observers assigned a score between 0-4 to each band specific for the given autoantigen. A score of 1 is given to a band showing comparable intensity to the internal cut-off control. The sum total score for all 17 autoantigens was divided by the maximum possible score i.e. $17 \times 4 = 68$, and the average of both the scores were used to generate an ANA Index for each patient sample. The ANA indices were validated with Hep-2 substrate slides and representative micrographs are presented in Panel (B). The ANA scores were plotted, and each symbol represents an individual

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subject score represented in (C) final cohort $n = 79$; $P < 0.0001$, Kruskal-Wallis, post Dunn Multiple comparison test. The dotted line represents the cut-off value of 0.08 calculated based on the mean ANA Index of 15 HCs + 2 x SD. (D) Significant positive correlation between sputum ANAs and detected anti-EPX IgG; Spearman's rank test, (E) sera ANA scores between 15 severe asthmatics (with detectable sputum ANAs) and HCs, Mann-Whitney test, at 1:100 as per manufacturer's instructions.

Figure 3: Sputum Igs with increased autoantibody levels cause eosinophil cytolysis with extracellular traps, *in vitro*: Representative images of eosinophil cytolysis induced upon 12 h incubation with immobilised IP-Igs from patient with high autoantibody (aAb) levels ($n = 8$), low aAb level ($n = 7$) and healthy volunteers ($n = 5$). Each symbol represents an sputum from an individual subject. **(A)** Immunostaining with anti-histone monoclonal antibody (green) shows visual confirmation of increased histone rich extra-cellular traps on fixed, non-permeabilized eosinophils with the respective Ip-Igs or uncoated control. Nuclei stained by DAPI. Images captured at 10x and 40x ; **(B)** the respective anti-EPX IgG Abs₆₀₀ values of the sputum IP-Igs used for this experimental set is given, Kruskal-Wallis followed by Dunn's post-hoc multiple comparison test; **(C)** dsDNA release in the collected media was quantified and **(D)** LDH activity, measured as NADH conversion as a function of time, was monitored in the media supernatants from 48 well-plates run in parallel as a quantitative measure of the extent of cytolysis between the samples, uncoated control and assess the suppressive effect of 10^{-6} M dexamethasone. Kruskal-Wallis followed by Dunn's post-hoc multiple comparison test ** Significant difference between the groups, $P < 0.01$, $*P < 0.05$. Data represented as mean $\pm$ SEM; aAb - autoantibody.

Figure 4: Sputum autoantibodies are prevalent in patients with increased airway degranulation and a history of lymphopenia: Association of sputum autoantibodies with **(A)** circulating

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lymphocytes (absolute counts) and **(B)** sputum lymphocytes (absolute counts) and **(C, D)** distribution in the patient sub-sets in the final cohort. The dotted lines represent the reference range for circulating lymphocytes. ANOVA with Holm-Sidak's multiple comparison test **(E, F)** Anti-EPX and ANA scores plotted for all subjects divided into asthmatics with normal lymphocyte counts, and those with a history (Hx) of lymphopenia (assessed as blood absolute lymphocyte count $< 1.5 \times 10^9/L$ on current and past two occasions). Mann-Whitney Test. **(G, H)** Study population divided into four 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. *indicates significant difference between the groups, $P < 0.05$, ** $P < 0.01$, ANOVA with Holm-Sidak's multiple correction. The cut-off thresholds for both autoantibody detections are represented by the dotted line on the respective plots. *Purple indicates patients with sputum TCC and neutrophil% indicative of infection.*

Figure 5: Distribution of mediators included in the molecular signature for autoimmune endotype: Scatter plots for sputum expression levels of the positive predictors - **(A-D)** mediators suggestive of Th2-dominated environment: IL-5, IL-13, IL-18, Eotaxin-2; **(E-F)** mediators indicative of increased B cell activity: BAFF, BCA-1, and **(G, H)** Th2 cell recruitment: IL-16, MDC. Each symbol represents an individual patient; red indicates sputum with positive autoantibody signature (i.e. detectable anti-EPX IgG and/or ANA above respective cut-offs). *indicative of significant difference between groups <0.01 , ** $P < 0.001$, *** $P < 0.0001$, Kruskal-Wallis with Dunn's multiple analysis.

All figures to be printed in colour.

