INTRODUCTION

Eosinophilic esophagitis (EoE) is a relatively recently described chronic immune and antigen mediated disease. The histopathological characteristics include increased eosinophilic inflammation within the esophagus which is not driven by gastroesophageal reflux disease (GERD), eosinophilic predominant inflammation within esophageal biopsies (typically \( \geq 15 \) eosinophils/hpf). Additional histologic abnormalities including peak eosinophil concentration, eosinophilic microabscesses, surface predominance of eosinophilic infiltration, eosinophilic degranulation, basal cell hyperplasia, dilation of squamous intracellular spaces and lamina propria fibrosis may also be reported\(^8\).

The inflammation should be isolated to the esophagus, elimination of other causes of eosinophilia (particularly PPI responsive eosinophilia) should be performed, and patients should demonstrate improvement with therapies including dietary modification and/or topical corticosteroids\(^9\). Clinically, patients may present from any age group and frequently suffer from dysphagia and food impaction, failure to thrive, vomiting, epigastric or chest pain\(^10,11\). Endoscopic findings may include esophageal rings, strictures, furrowing, and white plaques, although the esophagus may often appear normal endoscopically as well. The disorder is common, affecting 15% of patients being evaluated for dysphagia and up to 6.5% of patients undergoing upper endoscopy in recent studies\(^12\).
The precise cause of the aberrant inflammatory response that characterizes this disorder has not yet been identified, although there has been a proposed role for several Th2 cytokines including IL-5, IL-13 and IL-15[6,7]. A genome wide association study reported an association in pediatric patients with EoE with variants at chromosome 5q22, a locus that includes genes related to the Th2 immune response[1].

Interleukin (IL)-33 is a recently identified member of the IL-1 family of immunoregulatory proteins which had been found to exert function via Toll-like receptor (TLR)/IL-1R superfamily, and has been the subject of extensive recent investigation into the regulation and functioning of the cytokine in differing cell lines and disease states[8,9]. The IL-33 receptor ST2 had been previously cloned and the ligand subsequently identified 12 years later. The membrane bound ST2 receptor (ST2L) has been identified predominantly in cells of myeloid origin including mast cells, basophils, eosinophils, T cells, NK cells and NK cells, and activation of the receptor complex leads to a predominantly Th2 cytokine profile[11].

Expression of IL-33 has been identified in tissues and organs throughout the body including endothelial cells, smooth muscle, fibroblasts, adipocytes, epithelial cells, and hematopoietic cells[12-14]. The IL-33 protein localizes to the nucleus, an unusual although not unique pattern in cytokines. Although the role on intranuclear IL-33 continues to be the subject of investigation, studies showed that IL-33 functions to modify transcription of NF-κB induced gene expression, leading to dampened pro-inflammatory signaling resulting in cellular necrosis and tissue damage leading to functional cytokine[15]. Clinically, increased expression of the IL-33 activity has been identified in several pro-inflammatory conditions including asthma, autoimmune disease, arthritis, and anaphylaxis[16]. The Th2 profile induced by IL-33 leads to increased IgE levels, eosinophilic inflammation in the lung and gastrointestinal tract, and endothelial injury.

There is a remarkable association of EoE with Connective Tissue Disease (CTD) and it has been demonstrated that IL-33 plays an important role in the development and progression of rheumatic disease[17-21]. Thus far there have been no studies evaluating the role of IL-33 in the esophagus in patients with EoE. We hypothesized that excessive levels of IL-33 may be associated with EoE, particularly as compared with an alternative promoter of esophageal eosinophilia such as GERD.

METHODS

A retrospective designed study where distal and mid esophagus biopsy samples which were previously obtained from patients diagnosed with GERD and EoE were retrieved from archive storage. The samples were randomly selected from our clinical data base of GERD and EoE diagnosed patients between the years 2009-2013.

All patients were diagnosed, treated, and followed-up at the University of Iowa Hospital and Clinics outpatient clinic. The study was approved by the University of Iowa Hospitals and Clinics institutional review board.

A total of 60 samples were retrieved, 20 from patients diagnosed with EoE, 20 from patients with GERD and 20 normal esophageal biopsies. In patients with symptoms related to esophageal dysfunction EoE was diagnosed according to recent guidelines[1]:

1. Eosinophil- predominant inflammation on esophageal biopsy with a peak value of ≥15 eos/hpf after 8 weeks of Proton Pump inhibitors treatment

2. Eosinophilia limited to the esophagus.

3. Exclusion of GERD based on documentation of normal esophageal acid exposure.

All biopsies had been previously formalin-fixed and paraffin-embedded. All biopsy samples were randomly selected from a database of patients with diagnosis of EoE and GERD. The GERD biopsy specimens were obtained from symptomatic patients who were found to have erosions on endoscopy, reflux esophagitis on microscopic analysis, and documentation of increased esophageal acid exposure based on 24 hour pH monitoring. Diagnosis of EoE was established based on the maximum esophageal eosinophil count per high-power field (hpf) (400×) and lack of improvement with acid suppression therapy. GERD patients typically had 0-14 eosinophils/hpf and no basal layer expansion, whereas EoE samples had ≥15 eosinophils/hpf.

The slides were numbered and patient identifiers removed. Staining had been previously performed with standard H&E preparation. These biopsy specimens were then stained with an Anti-IL33 antibody [Nessy-1] monoclonal antibody (mAb) directed against IL-33 (Biotin). Concentration and control staining were performed. Two experienced pathologists (LG, AB) rated 60 subjects in three different groups (EoE, GERD, Normal; 20 subjects each) in ordinal scale (1 to 4 in severity) by Nuclear staining and Nuclear distribution methods in two different blocks (proximal and distal). The slides were reviewed independently and the raters were unaware of the patient diagnosis or others rating. The biopsies were graded with respect to the intensity of IL-33 staining in a nuclear or cytoplasmic predominance, and with regards to where in the depth of the biopsy the staining was distributed (basal to surface). The scoring system utilized (Table 1) is based on similar scales developed for evaluation of staining in other tissues previously described[11].

Data were analyzed by Mann-Whitney analysis given the non-parametric nature of the scoring used and non-paired samples obtained. P values less than 0.05 were considered statistically significant with a two-tailed analysis.

<table>
<thead>
<tr>
<th>Scoring</th>
<th>Nuclear</th>
<th>Cytoplasmic</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Surface</td>
<td>Mild</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Surface</td>
<td>Mild</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Surface</td>
<td>Moderate</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Surface</td>
<td>Strong</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Basal</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Full thickness</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Scoring based on anti-IL33 immunoreactivity.
IL-33 staining was identified in cytoplasmic and nuclear aspects of esophageal squamous cells (Figure 1 and 2). The mean intensity of nuclear IL-33 staining in EoE subjects was significantly higher compared to GERD and healthy subjects (3.4±1.2, 2.4 ±1.2, p=0.02). The cytoplasmic intensity of IL-33 staining in EoE patients did not significantly differ compared to GERD and healthy subjects (P=0.7) as shown in Figure 1 and demonstrable histologic image as seen in Figure 3 (A and B). The comparison of nuclear and cytoplasmic distribution in the thickness of the biopsy specimens of IL-33 staining was not statistically different between the three groups (p=0.07 and p=0.35 respectively) (Table 4).

### Table 2 Patient Demographics.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Normal (n = 20)</th>
<th>EoE (n = 20)</th>
<th>GERD (n = 20)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (sd)</td>
<td>43.3 (16.2)</td>
<td>28.6 (13.7)*</td>
<td>46.1 (12.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (sd)</td>
<td>31.0 (7.2)</td>
<td>28.1 (5.9)</td>
<td>31.4 (7.2)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex, male (%)</td>
<td>3 (15)</td>
<td>14 (70)</td>
<td>8 (40)</td>
<td>* &lt; 0.01</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>19 (95)</td>
<td>19 (100)</td>
<td>17 (89)</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

### Table 3 Rater Agreement on Nuclear Staining Method.

<table>
<thead>
<tr>
<th>Agreement Between Raters</th>
<th>Nuclear Staining (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal, Overall</td>
<td>0.71 (0.61; 0.82)</td>
</tr>
<tr>
<td>Proximal in EoE</td>
<td>0.73 (0.54; 0.91)</td>
</tr>
<tr>
<td>Proximal in GERD</td>
<td>0.70 (0.53; 0.87)</td>
</tr>
<tr>
<td>Proximal in Normal</td>
<td>0.69 (0.48; 0.88)</td>
</tr>
</tbody>
</table>

### Table 4 Results from Wilcoxon Rank-Sum analyses of Raters’ scores in Nuclear Staining and Nuclear Distribution methods in proximal esophagus.

| Rater | N  | Nuclear Staining | | | Nuclear Staining | | | |
|-------|----|------------------|---|---|------------------|---|---|
|       |    | Rank sum         | Expected | p-value | Rank sum         | Expected | p-value |
| Rater 1 | 20 | 822.5           | 6100.0 | <0.001* | 822.5           | 6100.0 | <0.001* |
|        | 20 | 572.5           | 6100.0 |           | 572.5           | 6100.0 |           |
|        | 20 | 435.0           | 6100.0 |           | 435.0           | 6100.0 |           |
| Rater 2 | 20 | 730.0           | 6100.0 | 0.025*   | 730.0           | 6100.0 | 0.025*   |
|        | 20 | 642.5           | 6100.0 |           | 642.5           | 6100.0 |           |
|        | 20 | 457.5           | 6100.0 |           | 457.5           | 6100.0 |           |

*1: EOE vs GERD <0.05 (Bonferroni adjusted p=0.009); EOE vs Normal <0.05 (Bonferroni adjusted p<0.001); EOE vs Normal >0.05.
*2: EOE vs Normal <0.05 (Bonferroni adjusted p=0.013); Normal vs GERD <0.05; EOE vs GERD <0.05.

DISCUSSION

Eosinophils are major effector cells in type 2 inflammatory responses and become activated in response to IL-4 and IL-33, yet the molecular mechanisms and cooperative interaction between these cytokines remain unclear. Recently, Bouffi et al have identified a novel activation pathway in murine eosinophils that is induced by IL-33 and differentially dependent upon an IL-4 auto-amplification loop. Although IL-33 has been investigated in patients with asthma and generalized inflammatory disorders, there have been no prior evaluations of its role in patients with EoE.

We compared esophageal biopsies of patients with EoE patients with GERD, a disorder that has also been shown to result in increased esophageal intraepithelial eosinophils although via
differing mechanisms, and healthy subjects. We utilized a sensitive and specific method of identifying IL-33 in biopsy specimens. IL-33 immunoreactivity was demonstrated in both cytoplasmic and nuclear compartments, however there was significantly increased staining within the nuclei in patients with EoE as compared with the GERD specimens. The nuclear localization is consistent with the currently understood behavior of IL-33, although the role of the increased levels in patients with EoE remains uncertain. The potential functions of IL-33 in this population include induction of IL-6, IL-5 and IL-13, degranulation of mast cells, with subsequent attraction of eosinophils and additional pro-inflammatory cascade. Although we did not evaluate the levels of IL-4, IL-5, and IL-13 in our study, these have been investigated previously[24].

While IL-33 is cleaved and inactivated by proteases during apoptosis, once bound to the heterodimer receptor complex of ST2 and IL-1R accessory protein, intracellular signaling is initiated. It has been recently shown that Anti-S T2 monoclonal antibody inhibits eosinophil infiltration[19]. The specific signaling pathways appear variable depending on the cell lines involved, but include association of myeloid differentiation primary response protein 88 (MYD88) and subsequent activation of nuclear factor κB (NF-κB) inhibitor of NF-κBβ-α (IkBa), extracellular signal-related kinase 1 (ERK1), ERK2, phosphor JUN-terminal kinase-1 (JNK-1) and (TRAF6) which have been described elsewhere[20,21,22]. The resulting actions include predominantly Th2 immunity and inflammatory reaction with mast cell degranulation, production of IL-1, IL-3, IL-4, IL-5, IL-6, IL-13 and TNFα[21]. Ho et al recently demonstrated that genetic variation of IL1RL1 can result in increased levels of sST2 and alter immune and inflammatory signaling through ST2/IL-33 pathway[22].

IL-33 is localized simultaneously to nuclear euchromatin and membrane-bound cytoplasmic vesicles, and is secreted by living cells to carry out its extracellular functions without the need for cellular necrosis[23]. Although it is constitutively produced and sequestered in the nucleus, it does not induce there Th2 immune deviation and necessitates appropriate Damage-Associated Molecular Pattern molecules (DAMPs) to initiate the inflammatory process[24].

IL-33 appears to promote broad pro-inflammatory changes by initiating a cascade of downstream effects leading to recruitment and degranulation of eosinophils, activation of dendritic cells, and TNFα release. Thus far it is not known what is IL-33’s exact role in the in the nucleus. Our hypothesis is that it does not induce any genes that support allergic inflammatory disorders, and, more specifically genes that EC actually make like eotaxins, IL-25, and TSLP, or alternatively, induces homeostasis or repair genes. In order for it to play a role in the generation of Th2 cytokines, it must be secreted. Therefore, we assume that the intra-nuclear IL-33 in esophageal squamous mucosa actually serves as a holding area for eventual secretion in EoE patients.

Our findings provide information which could have future implications in terms of diagnostic utility particularly in differentiating patients with EoE from those with other disorders that lead to esophageal eosinophilia such as GERD. This could be useful clinically as these differing disorders are managed by different treatment strategies.

The other important consideration from the findings reported here are the potential utility of IL-33 as a therapeutic target which could allow inhibition of pro-inflammatory changes characteristic of EoE at an early common pathway of inflammation rather than treating the downstream effects as currently being performed with topical and systemic steroids.

Although initial treatments in animal models directed at depletion of IL-5 and IL-13 showed promise, subsequent human studies of IL-5 inhibition have been relatively unsuccessful in treatment of patients with EoE[17,20]. The pro-inflammatory effects of IL-33 bring into question the potential role of this cytokine in the disease. Recently Ravi et al proposed the possible existence of a subgroup of EoE patients who have low-grade esophageal eosinophilia (<15 Eos/HPF) and, at minimum, defy the guidelines used to diagnose this disorder[20].

Other modifiers of IL-33 expression and function may also be useful for future investigation. In particular, TGFβ has been shown to mediate improvement in other models of intestinal inflammation and reduce IL-33 production[21].

This intra nuclear localization may pose a challenge for directed therapeutic modalities such as antibodies currently used to decrease circulating levels of functional cytokines. Future studies designed to evaluate the mechanisms which lead to IL-33 promotion in EoE, the diagnostic utility of IL-33 in patients with other esophageal hypereosinophilia syndromes, and treatment with Anti IL-33 inhibition are warranted.

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CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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