REGULATION OF ALLERGEN-INDUCED UPPER AIRWAY INFLAMMATION BY TOPICAL CORTICOSTEROID AND RELATIONSHIP TO ALARMIN EXPRESSION IN ALLERGIC RHINITIS

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LAY ABSTRACT

Allergic rhinitis (AR) is a comorbidity in asthmatic patients and characterized by type 2 airway inflammation and alarmin cytokines. We proposed that alarmin cytokines will increase in nasal airways after nasal allergen challenge (NAC), and this increase will be inhibited by intranasal corticosteroids (INCS). We compared the effects 2 weeks INCS treatment versus placebo on nasal samples collected from mild, asthmatic participants with AR after NAC. There was an allergen-induced decrease in IL-33 post-NAC in nasal tissue ($p<0.05$). INCS treatment inhibited the allergen-induced changes nasal symptoms, eosinophils and T2 cytokines (IL-4, IL-5, and IL-13). There was a numerical but not statistically significant reduction in IL-25 and TSLP in nasal lavage at 7h post-allergen with INCS versus placebo, but no effect of INCS on alarmin levels in tissue 24h post-allergen ($p>0.05$). A larger sample size will be required to effectively determine the effect INCS on alarmin expression in upper airways.

ABSTRACT

Allergic rhinitis (AR) is a comorbidity in asthmatic patients and characterized by type 2 (T2) airway inflammation and expression of epithelial alarmins (interleukin (IL) 25, 33 and thymic stromal lymphopoietin (TSLP). In 10 mild allergic asthmatics with AR, we examined the effect of 2 weeks intranasal corticosteroid treatment with triamcinolone 220 μg BID on the regulation of T2 allergic responses to nasal allergen challenge (NAC), with a focus on alarmin cytokines in the upper airways. NAC increased total nasal symptom scores (TNSS) and reduced peak nasal inspiratory flow rate (PNIF) over the 7-hour measurement period. Eosinophil numbers increased at 24 hours post allergen in nasal biopsies. In nasal lavage, eosinophils and T2 cytokines IL-4, IL-5 and IL-13 increased at 7 and 24 hours post-allergen ($p<0.05$), and alarmin cytokines IL-25 and TSLP numerically increased above baseline at 7 hours post-allergen. IL-33-positive cells in nasal tissue decreased 24 hours post-NAC ($p<0.05$), but there was no change in the number of cells immunopositive for IL-25 or TSLP. INCS treatment for 2 weeks significantly attenuated allergen-induced changes in TNSS and PNIF, and inhibited allergen-induced eosinophils and T2 cytokines IL-4, IL-5, and IL-13 in the nasal lavage ($p<0.05$). INCS treatment had no effect on the number of alarmin immune-positive cells in nasal tissue. A consistent numerical reduction of IL-25 and TSLP levels in nasal lavage measured post-allergen with INCS versus placebo provides some evidence that these alarmin cytokines may be regulated by corticosteroids. Power calculations indicated that a doubling of the sample size would be needed to adequately test the effect of intranasal corticosteroid on alarmin levels in nasal lavage, thus larger studies will be needed to further examine the effects of corticosteroids on alarmin production in upper airways.

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DECLARATION OF ACADEMIC ACHIEVEMENT

While I was the major contributor for the immunofluorescence microcopy work presented in this thesis, the clinical trial and sample collection required the collaborative efforts of many people.

Dr. Gail Gauvreau designed the study with input from Dr. Rebecca Bauer from Genentech Inc. Dr. Roma Sehmi, Dr. Kieran Killian, Dr. Paul Keith, Dr. Jonathan MacLean, Dr. Judah Denburg, and Dr. Doron Sommer were co-investigators of the clinical trial and provided expertise for methodology. Patient visits were conducted by Sue Beaudin, Yanqing Xie, Caroline Munoz, Jennifer Wattie, and John Paul Oliveria. Medical procedures were supervised by Dr. Ruth Cusack.

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I performed all immunofluorescent staining of nasal biopsies, performed the cellular counts for IL-33+2D7+Tryptase, IL-25+2D7, and IL-25+Tryptase-stained sections, analyzed all data, and prepared the figures and tables for this dissertation.

CHAPTER 1: INTRODUCTION

1.1 Overview of Allergic Rhinitis

Allergic rhinitis affects up to 500 million people worldwide, and up to 100 million people in North America and Europe alone (1). The 2016 ARIA Guidelines and 2021 GINA report have characterized allergic rhinitis by the presence of rhinorrhea, sneezing, nasal itching, and ocular symptoms such as conjunctivitis, redness and eye irritation (2,3). Asthma is present in up to 40% of AR patients and impacts quality of life in both social and professional settings, posing difficulties in overall quality of life. Nasal, ocular, and pruritic symptoms accompany the onset of allergic rhinitis and when patients are unable to manage their symptoms, this results in disease progression (4).

Allergic rhinitis presents as either seasonal (SAR) or perennial (PAR). Pollen seasons presents patients with the most common environmental triggers, causing AR flares. Particularly for patients who suffer from concomitant asthma and seasonal allergic rhinitis (AS+SAR), quality of life deteriorates and physical functioning is impaired (5). The degree to which quality of life is affected by AR and asthma is more pronounced in women compared to men, due to their susceptibility to allergic sensitivity (6).

The 2016 ARIA guidelines advise that seasonal allergic rhinitis (SAR) be treated with a combination of intranasal corticosteroid (INCS) and H1-antihistamine (INAH) rather than an INCS alone (3). Additional choices of treatment include leukotriene receptor antagonists (LTRA) or an oral H1-antihistamine (OAH) (3).

1.2 Overview of Allergic Inflammation

Allergens are substances that trigger an allergic immune response and subsequent sensitization. Allergens produce a reaction by inducing IgE-mediated inflammation via type 2 cytokines such as IL-4, IL-5 and IL-13, and rely on various antigen-presenting cells (APCs) to initiate an immune response. An increased number of mast cells in nasal tissue is a hallmark of allergic inflammation, similar to their infiltration in the lower airways in increased numbers in sputum post-allergen challenge (7). The cross-linking of IgE with the FcεRI receptor expressed on mast cells and basophils propagates the reaction, thereby orchestrating the release of bronchoconstrictor mediators that facilitates allergic inflammation (8–10). Similarly, basophils infiltrate the lung and are present in sputum after antigen challenge, supporting their role in the pathophysiology of allergic asthma (7,11). The CD34+ progenitor cells have also been shown to secrete IL-4, IL-5, and IL-13 and assist in the class switching of B cells for the production of IgE, while facilitating the differentiation and maturation of eosinophils (12–15). Dendritic cells assist in presenting antigens to naïve T cells, thus polarizing and priming T cells to help elicit immune responses in a Th1 or Th2-dependent manner (16,17).

Studies in murine models demonstrate that innate lymphoid type-2 (ILC2) cells contribute to local tissue sensitization in a Th2 manner in response to antigen exposure (18). In humans, augmented levels of ILC2 cells found in the peripheral blood of asthmatic patients have been shown to correspond with the increase of type 2 cytokines (19). Specifically, the heightened level of ILC2s was specific to patients with allergic asthma, when compared to patients with allergic rhinitis or healthy controls. These allergic asthmatic patients were also found to have higher levels of type 2 cytokines produced by PBMCs (19).

1.3 Eosinophils

Eosinophils are multifunctional leukocytes that are recruited from circulation to a source of inflammation during an immune response (20). They are characterized by their bilobed structure and large granules that contain eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase, and major basic protein (MBP) (21). Under the influence of growth factors such as IL-3, IL-5, and GM-CSF, eosinophils differentiate in the bone marrow from progenitor cells. These growth factors cause proliferation and differentiation of progenitors, with IL-5 acting as a key cytokine for terminal differentiation of eosinophils. GM-CSF and stem cell factor (SCF) found in human umbilical vein endothelial cells (HUVEC) act as another source for hematopoietic cytokines that enhance IL-3 and IL-5 expression. GM-CSF and stem cell factor (SCF) promote IL-3 and IL-5-induced eosinophil differentiation from CD34+ cells (22). Allergen inhalation challenge in mild asthmatics demonstrate increased lung homing of bone-marrow derived CD34+ hematopoietic cells. It has been proposed that TSLP and IL-33 increase lung homing of bone-marrow derived CD34+ hematopoietic cells through an IL-13 axis (23). *In vitro* experimentation demonstrates how the IL-33 and TSLP alarmins prime the migration response of HPCs via IL-13, thus contributing to the onset of airway eosinophilia in allergic asthma (23).

Adhesion molecules such as selectins and integrins are located on the surface of the eosinophilic membrane and facilitate the tethering, rolling, and eventual extravasation of eosinophils onto the endothelium from peripheral blood circulation. Among these adhesion molecules is P-selectin, a receptor on the endothelial surface that binds to the P-selectin glycoprotein ligand-1 (PSGL-1) located on the eosinophil (24–26). Once bonded together, the eosinophil is tethered to the endothelium. The VLA-4 integrin on the eosinophil binds to the vascular cell adhesion molecule-

1 (VCAM-1), and eotaxin will mediate eosinophil activation and subsequent extravasation into the endothelium (27–32). Following extravasation, the eotaxin chemoattractant gradient assists in the migration and accumulation of eosinophils from the endothelium to specific sites of allergic inflammation (32,33).

Eosinophil infiltration is mediated via eotaxin and CCR3-regulated activity in epithelial and endothelial cells. Eotaxin expression is primarily expressed in epithelial cells, macrophages, T cells and mast cells. Eotaxin expression in the submucosa occurred in endothelial cells with less expression in macrophages, T cells, mast cells and neutrophils. The resulting eosinophil infiltration in the bronchial mucosa of asthmatics is associated with airway hyperresponsiveness (34). Tissue homogenates from the sinus mucosa of nasal polyposis patients express the chemotactic cytokine RANTES in eosinophils and epithelial cells, with a 40-fold increase in comparison to normal subjects. This suggests that RANTES contributes to eosinophil recruitment in the nasal polyp microenvironment (35). Eotaxin and RANTES are produced at sites of inflammation by both epithelial and endothelial cells, facilitating the onset of allergic inflammation as an allergic immune response.

Allergen inhalation induces T lymphocyte trafficking and IL-5 expression in human bone marrow in asthmatics. In atopic asthmatics allergen inhalation challenge results in T lymphocyte trafficking to the bone marrow, and in dual responder asthmatics the T lymphocytes from bone marrow cells produce IL-5 (36). This suggests that allergen exposure in the lungs increases EoP proliferation via upregulated IL-5 production. Other studies of human bone marrow demonstrate an increase in sputum CD34+ cells 7 hr post-allergen challenge. In contrast, increases in sputum CD34+IL-5Ra+ cells, eosinophils and IL-5 expression are observed 24 hr post-allergen challenge in dual responding asthmatics. These findings suggest a mechanism for CD34+ migration to the airways after inhalation challenge with concurrent IL-5 upregulation, indicating how allergen challenge can promote eosinophil differentiation (37). These results emphasize the role of bone marrow-derived progenitors and eosinophil precursors in EoP proliferation and differentiation. Further studies, involving cellular , have demonstrated the presence of IL-5 mRNA in the bronchial mucosa of asthmatics (38,39).Taken together, these results implicate a correlation between IL-5 levels inducing eosinophil recruitment in asthma, and subsequent allergic inflammatory response (39).

Mouse models of allergic airway inflammation have shown that eosinophils contribute to airway remodelling (40). In addition, in a patient population of moderate to severe allergic rhinitis patients, higher disease severity was associated with the presence of CD44+ and CD69+ eosinophils in the nose, whereas this increase in activated eosinophils was seen in patients with both mild as well as moderate to severe disease severity when measured in the peripheral blood (41). Additionally, patients with moderate to severe allergic rhinitis have higher levels of granule proteins eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX) measured in serum, as well as increased CCL11-induced ECP and EPX release from blood eosinophils, when compared to mild allergic rhinitis patients or healthy controls (41). Significant correlations exist between serum ECP and methacholine PD_{20} in asthmatic patients exposed to seasonal allergen while on placebo treatment, suggesting that inflammatory markers contribute to pathogenesis via in airway hyperresponsiveness (42). Other eosinophil-derived products such as serum EPX are associated with asthma pathogenesis due to its associated increase with asthma severity, and subsequent decrease with asthma exacerbation resolution (43). Additionally, eosinophils from bronchoalveolar lavage fluid and sputum collected after antigen and inhalation challenges show higher expression of CD44 and CD69 (44,45). Taken together, these findings suggest that activated eosinophils and their granule products may contribute to the pathogenesis and mechanisms of allergic rhinitis in patients with varying levels of disease severity.

Eosinophils and eosinophil cationic protein, and can be measured in the sputum, blood, and serum (46,47). However, samples from induced sputum could more accurately measure the proportion of eosinophils in the asthmatic airway. Sputum eosinophil measurements demonstrate a higher sensitivity and specificity in receiver-operating characteristic (ROC) curves when compared to eosinophils derived from the blood (47). These findings indicate that sputum eosinophil measurements accurately indicate asthmatic airway inflammation, and can be used to differentiate between healthy and asthmatic populations, when assessing clinically relevant parameters used in asthma diagnosis (47). While serving as an effective and non-invasive approach to measuring eosinophilic airway inflammation, sputum analysis is often met with hesitation. A common critique is the quantitative variability and challenges with reproducibility/repeatability in sputum biomarker measurements, thus presenting with inconsistencies when determining asthma phenotypes. Additionally, sputum induction can not be easily performed by young children, and clinicians must rely on other methods to form a differential diagnosis for pediatric asthma patients.

Blood eosinophil counts are easier to collect and are much more often used to measure the clinical importance of eosinophils in asthmatic patients. Several studies have shown its efficacy in identifying patients with severe eosinophilic asthma, predicting asthma-related outcomes, and determining overall disease burden (48,49). Adults with persistent asthma who have a high blood eosinophil count have an increased risk of suffering from asthma exacerbations, thereby contributing to disease burden (50).

1.4 Mast Cells

Mast cells are derived from nonmyeloid progenitors (51), and previous studies have established how mast cells and basophils both originate from CD34+ human progenitor cells (52). These multipotent hematopoietic progenitors differentiate in the bone marrow and rely on stem cell factor (SCF) to initiate granulation and drive subsequent proliferation and differentiation forward (53). Recombinant human SCF (rhSCF) enhances mononuclear cells, derived from peripheral blood and bone marrow, to induce mast cell differentiation (54). Additional studies have emphasized how SCF augments the differentiation of CD34+ bone marrow progenitor cells and when stimulated with thrombopoietin (TPO), mast cell differentiation is significantly enhanced (53).

Upon Th2 stimulation *in vitro*, asthmatic human ASM cultures release mediators that are chemotactic for mast cells, whereas mediators released from non-asthmatic ASM has been shown to inhibit mast cell chemotaxis (55). Human lung mast cells have been shown to accumulate in airway smooth muscle, thereby contributing to the asthma phenotype. This mast cell migration is facilitated through the CXCL10/CXCR3 axis, suggesting that CXCL10 (IP-10) may serve as a possible therapeutic target for mediating mast cell-induced inflammation (56). CXCR3 is an integral chemokine receptor expressed on human lung mast cells, but not in bone-marrow mast cells (57). Additional evidence suggests that both ASM and mast-cell derived CCL19 help regulate ASM migration and subsequent hyperplasia in patients with asthma via the CCL19/CCR7 axis (58). CCR7 is expressed on ASM and fibroblasts, and this chemotactic axis assists in mast-cell mediated ASM migration (58).

Human airway epithelial cells have been shown to release TSLP in response to allergic stimuli, thereby inducing T2 cytokine production from mast cells. When combined with IL-1/TNF, TSLP induces the release of IL-5, IL-13, IL-6, GM-CSF, CXCL8 and CCL1 in stimulated mast cells (59). This suggests that TSLP can propagate allergic inflammation by acting on the innate immune system via stimulation of mast cells (59). Of particular interest is the shift in mast cell infiltration that takes place from the submucosa to the epithelium that is regulated by IL-33 signalling in asthmatics (60). When primed with IL-33, mast cells express type 2 cytokines to regulate epithelial IL-33 expression through a feed-forward loop (60). Additionally, type 2 cytokines induced by these mast cells contribute to indirect airway hyperresponsiveness in response to inhaled mannitol (60). Taken together, these findings indicate an effect of type 2 inflammation and indirect AHR on airway epithelium mast cell infiltration due to IL-33 signalling. Finally, immunoreactivity staining, of late-phase allergen-induced responses in atopic human bronchial and skin biopsies show IL-25+ and IL-25R+ colocalization with mast cells (61,62), amongst other inflammatory cells such as eosinophils, T cells and endothelial cells (61).

Mast cells can be enumerated from lung biopsies when stained appropriately. Using SCF receptor, c-kit, antibodies, mast cells have been identified in the airway mucosa from bronchial biopsies to help determine their association with asthma exacerbations and eosinophilic asthma (63). Biopsies can also be stained using immunohistology methods to identify tryptase-only mast cells, as well as tryptase and chymase-positive mast cells (64). Recent studies suggest higher sensitivity assays are more effective in analyzing mast cells, and that flow cytometry can identify viable mast cells following a gating strategy of: FcεR1α-positive, CD45-positive, CD14 low, CD16 low to intermediate, HLA-DR low, CD117-positive, CD123 low, CD206 low and 7-AAD-negative) can be conducted on sputum samples to assess for the mast cell levels present in atopic airways of asthmatic subjects (65).

1.5 Basophils

Increased numbers of basophils are recruited into the airways during a late asthmatic response in dual responders as compared to early responders, suggesting an important role of these cells in causing allergen-induced airway responses. Basophil granulocytes originate from hematopoietic progenitor cells and differentiate in the bone marrow before entering circulation (66–68). Growth-factor cytokines such as IL-3, IL-5, and GM-CSF drive this differentiation of eosinophil/basophil colony forming unitsEo/B differentiation.

IgE and its high-affinity receptor, FcεRI, assist in inducing basophil migration in asthmatic airways (69). Basophils have been shown to infiltrate the bronchial mucosa post-allergen challenge via IL-4 (70). PGD2 also acts as a basophil chemoattractant via the DP2 receptor expressed on basophils (71–73). Additionally, several monocyte chemotactic protein ligands such as CCL11 and CCL2 have been suggested to help to facilitate the transendothelial migration of basophils (74), thereby emphasizing the importance of CCL11 in chemotaxis as it is released from airway epithelium in asthma and allergic rhinitis.

Basophil activation can occur in a type 2 inflammatory response after allergic stimulation. Studies investigating the effect of alarmins on the role of basophils in allergic asthma have demonstrated a heighted level of basophil activation post-allergen challenge when in the presence of IL-25 and IL-33 (75). *In vitro* stimulation has shown that these alarmins induce an upregulation in the number of basophils expressing IL-17RB and ST2, as well as an increased migratory potential towards eotaxin (75). This increase in the percentage of basophils expressing surface activation markers and intracellular cytokines may be partially due to an IL-3 dependent mechanism, suggesting a possible effect of IL-25 and IL-33 on IL-3 and pro-inflammatory mediators in the airways (75).

Previous studies examining the role of TSLP in basophil activation in allergic asthmatics have shown allergen-induced increases in intracellular Th2 cytokine expression, and upregulation in TSLPR and IL-3α in basophils found in peripheral blood, sputum, and bone marrow samples, thus demonstrating an IL-3 dependent mechanism in basophil migration and activation (76). Basophils incubated *in vitro* with TSLP resulted in increased intracellular Th2 cytokine expression, as well as an upregulation for TSLPR and IL-3α receptor, further suggesting an IL-3 dependent autocrine loop for regulating TSLP-activated basophils (76), similar to that in IL-25 and IL-33 induced responses (75). Taken together, these findings corroborate the role of alarmin-induced basophil activity in propagating pro-inflammatory effects during early and late asthmatic responses (EAR and LAR) in allergic asthmatics.

Differential blood cell counts can help clarify the role of circulating basophils in the inflammatory asthmatic response; studies have shown a decrease in basophil numbers when an asthma exacerbation is resolved (77). Immunohistochemistry methods are another means of basophil enumeration and is used when measuring basophils in biopsy samples. Immunohistochemistry has demonstrated a difference in basophil numbers between late phase asthmatic in bronchial biopsies compared to late phase skin biopsies, in which bronchial biopsies have markedly fewer basophils compared to skin biopsies (78). Additionally, sputum samples collected post-allergen inhalation challenge from dual responder asthmatics can be analysed for basophils through cytospin preparations (7). Basophil enumeration performed using flow cytometry analysis has been suggested as superior compared to other hematology analyzers, with markers consisting of CCR3, CRTH2, CD123, FcεRI/IgE, and CD203c (79,80).

1.6 Alarmins in Allergic Inflammation

Allergen-induced inflammation of asthmatic airways is driven by multiple pro-inflammatory cytokines, including the alarmin cytokines. IL-33, IL-25, and TSLP that operate to initiate type 2 immune responses to mediate allergic disease. Release of alarmin proteins from airway epithelial cells depends on stimulation of transmembrane or intracellular pattern recognition receptors (PRRs) by foreign triggers (81–83), hence the alarmins are highly expressed in epithelium as a protective response to the frequent exposure to environmental triggers.

IL-25 is a member of the IL-17 cytokine family, and is secreted by inflammatory cells such as basophils and eosinophils, as well as alveolar macrophages and mast cells (61,84–86). Stored in the cytoplasm of cells, IL-25 depends on a heterodimeric receptor complex consisting of IL-17Rα and IL-17Rβ to regulate T2 allergic responses (87,88). Similar to IL-25, the IL-25 receptor is expressed on human basophils, eosinophils, mast cells, CD4+ T memory cells, epithelial cells, human vascular endothelial cells (HUVEC), and human airway smooth muscle cells (HASMC) (61,84,89–96). IL-33 is a member of the IL-1 cytokine family, and is located in the nuclei and the cytoplasm of the epithelium (97). While commonly expressed in keratinocytes, fibroblasts, endothelial cells, epithelial cells, and eosinophils, this alarmin may contribute to asthma pathogenesis as well as to airway remodeling, as suggested by *in vitro* studies of human lung fibroblasts showing IL-33 induced the production of fibronectin and type 1 collagen (97–101). In human and murine models, the IL-33 receptor, ST2, is widely expressed on mast cells, eosinophils, CD4+ T cells, and dendritic cells (101–104).

Thymic stromal lymphopoietin (TSLP) and its receptor, TSLPR, are members of the IL-7 and IL-7Rα cytokine family (105,106), and are upregulated in the airways of allergic asthmatics (107). TSLP induces its effects on a variety of cells, including dendritic cells, mast cells, basophils, and macrophages (59,76,108,109). TSLPR has been shown to be expressed on basophils and dendritic cells in patients with atopic dermatitis (110). In allergic rhinitis, overexpression of TSLP is associated with disease severity, and is related with IL-4 expression in the nasal mucosa (111). Children with atopic dermatitis have been shown to have significantly greater levels of serum TSLP than compared to patients who were normal controls (112).

IL-33 and its respective ST2 receptor are a part of the IL-1 cytokine family, and facilitate allergic inflammation through mediators such as mast cells, basophils, and eosinophils (75,113,114). Studies have shown that IL-33 mediated cross-talk between mast cells and basophils propagates an inflammatory response in murine skin anaphylaxis models (115). In patients with allergic rhinitis, the IL-33/ST2 axis is dependent on several signalling pathways to orchestrate an immune response in the nasal epithelium. *In vitro* human nasal epithelial cell experiments in the same study have demonstrated how GM-CSF and IL-8 production controlled by IL-33 are regulated by signalling inhibitors such as ERK, p38 MAPK, JNK, NF-κB, and epidermal growth factor (EGFR) (116).

Alarmins act upstream of eosinophils in asthma and contribute to the pathogenesis of allergic disease and in regulating type 2 immune responses. Allergen exposure enhances alarmin expression as measured by immunoreactivity within the epithelium and submucosa (62). TSLP, IL-33 and IL-25 in the bronchial mucosa has been associated with asthma pathogenesis and airway inflammation. Allergen-induced late-phase airway airway obstruction is associated with alarmin activity as determined by negative correlation with reduced FEV_1 values measured 4 hr and 24 hr post-allergen (62). As mediators of asthma, alarmins drive airway inflammation through the activation of T2 cytokines (IL-4, IL-5, and IL-13) which subsequently stimulate dendritic cells and induce eosinophilia (117,118). Alarmins regulate type 2 inflammation in allergic disease by being released from the epithelium, thereafter polarizing Th2 cells and activating ILC2 cells (59,119). Epithelial-derived TSLP stimulates the activation of human mast cells to induce the production of Th2 (IL-5, IL-13, GM-CSF) and pro-inflammatory cytokines and chemokines (IL-6, CCL1, CXCL8) (59). In TSLP-activated dendritic cells, the OX40 ligand assists in inducing the differentiation of Th2 follicular cells (120). Additionally, TSLP-activated dendritic cells contribute to the polarization and priming of Th2 memory T cells, thus facilitating the propagation of allergic disease (121). Other immune cells such as mast cells and macrophages promote ILC2 activation via IL-33 expression, thereby elucidating the role of alarmins and the onset of helminth infection in propagating allergic disease (122,123). Additionally, in asthmatic lung tissue, IL-25 augments Th2 polarization and proliferation while enhancing cytokine production of Th2 TSLP-DC-activated memory cells, thereby suggesting its role in facilitating type 2 immune responses. IL-25 induces the polarization of Th2 cells. IL-25 induces augmented Th2 memory cell function due to the increased expression of the c-MAF, GATA-3, and JunB transcription factors (84,124).

Studies with IL-25 transgenic mice have shown that IL-25 regulates proallergic responses and eosinophil recruitment by inducing the expression of chemokines such as MDC and eotaxin (125). Normal human bronchial epithelial cells constitutively produce cytoplasmic IL-25, which undergoes allergen-induced alarmin release when challenged with house dust mite (HDM) (126). Additionally, allergen exposure induces protease-dependent IL-25 mRNA transcription in bronchial epithelial cells of allergic patients as well as in nasal epithelial cells of healthy patients (126). Bronchial expression of IL-25 and the IL-25R is higher in asthmatic patients than in healthy controls. Molecular phenotyping has shown that IL-25 is present in airway epithelial cells, and these endotyping results can help distinguish between asthma phenotypes in corticosteroid-responsive patients (92). Additional studies identify the significance of alarmin-induced eosinophilic inflammation via the role of IL-25 and IL-25R on the surface of eosinophils in dual responder asthmatics. Increased IL-25 in eosinophils persist at 24 h post-allergen challenge both intracellularly and in peripheral blood (127). Studies targeting the bronchial mucosa and dermis of atopic patients have identified upregulated expression of IL-25 and its receptor in asthmatic airways, suggesting that this alarmin expression is a result of possible eosinophil proliferation and differentiation, alongside other epithelial cell activity (61). These findings indicate how IL-25 and the IL-25 receptor can act as a target for local allergic inflammation. IL-25 activated eosinophils from human blood induce IL-6, IL-8, MCP-1, MIP-1α and ICAM-1 release, which is mediated by the JNK, p38 MAPK, and NF-κB intracellular signalling mechanisms (124,128,129). In contrast, the same signalling pathways are responsible for the suppression of the ICAM-3 and L-selectin adhesion molecules in eosinophils when enhanced with IL-25 (124,129).

Studies examining primary human cells have shown how IL-33 targets basophils and eosinophils to induce activation and drive allergic inflammation forward (114). For example, IL-33 promotes eosinophil adhesion, migration, and survival by augmenting CD11b expression in human eosinophils (124,130). By blocking the IL-33 receptor, ST2, this prevents IL-33 from boosting CD11b expression *in vitro*. These findings suggest that the IL-33/ST2 axis plays a role in regulating eosinophil-induced tissue inflammation (124,130). Additional studies investigating the role of alarmins reveal how blood and sputum samples collected from asthmatic patients post-allergen challenge show an increase in eosinophil ST2 expression, implicating that the IL-33 alarmin plays an inherent role in asthmatic inflammation (131). Studies using murine models of allergic asthma have elucidated the role of IL-33 as a contributor to a type 2 allergic response when the immune system is exposed to a relevant allergen. Mice treated with anti-IL-33 and soluble ST2 (sST2) had significantly reduced levels of classical Th2 cytokines in bronchoalveolar lavage fluid after OVA sensitization, thus reinforcing the possible therapeutic benefits of anti-IL-33 therapy in managing airway inflammation (132). Similar to IL-25, IL-33 relies on the p38 MAPK, and NF-κB intracellular signalling mechanisms to induce functional effects on eosinophils and basophils (114,124). and IL-33 activates eosinophils in murine allergic asthma models in an IL-4 auto-activation-dependent manner (124,133).

Several factors can induce the expression of TSLP in asthmatic airways, such as IL-1β and TNF-α, which is mediated by the NF-κB signalling pathway (134). TSLP hinders eosinophil apoptosis and concurrently boosts eosinophil survival via chemotactic factors. TSLP also augments the expression of adhesion molecules CD18 and ICAM-1 in human eosinophils, stimulates the intracellular release of IL-6, CXCL8, CXCL1, and CCL2, while simultaneously suppressing the expression of L-selectin (135). This chemotactic activity is reliant on the p38 mitogen-activated kinase and NF-κB signalling pathway to propagate an allergic response forward (135).

Compared to healthy individuals, allergen challenge induces an increase of alarmin expression in the airways of asthmatic patients, reflecting how allergic airways respond to an environmental trigger or allergen exposure, as well as their role in the pathophysiology of asthma. Several clinical trials have assessed the impact of anti-alarmin monoclonal antibodies in mediating allergic disease and have proven the importance of alarmins in allergic inflammation of the lower airways. A phase 2a clinical trial investigating the anti-TSLP biologic (tezepelumab) has demonstrated the importance of TSLP for eosinophilic asthma by showing that tezepelumab ameliorates allergen-induced eosinophilic inflammation and bronchoconstriction in the early and late phase response in patients with mild allergic asthma (136). Participants receiving tezepelumab treatment experienced a decrease in fraction of exhaled nitric oxide, and eosinophils present in the blood and airways, respectively (136). These findings connote that anti-TSLP therapy is effective in improving lung inflammation, thereby highlighting the contribution of TSLP in allergic airway inflammation. A 1-year treatment course of combined tezepelumab/ subcutaneous immunotherapy (SCIT) treatment in patients with moderate to severe allergic rhinitis showed a significant reduction in the total nasal symptom score (TNSS) calculated as area under the curve (AUC) and peak score during the first hour when compared to patients who were receiving SCIT alone (137). Recent reports of tezepelumab use amongst patients with physician-diagnosed severe, uncontrolled asthma convey a significant decrease in annualized asthma exacerbation rate (AAER) (138). Additional mechanistic phase 2 studies further substantiate the efficacy of tezepelumab when assessing for airway submucosal inflammation. A reduction in airway eosinophils supports previous findings in which anti-TSLP therapy has the ability to attenuate type 2 inflammation while also potentially contributing to improved airway hyperresponsiveness when participants are challenged with mannitol (139).

Other clinical trials have been conducted by targeting IL-33 as another form of anti-alarmin therapy. A recent study has shown how patients treated with an anti-IL-33 biologic, itepekimab were able to gain better control of their asthma by minimizing the incidence of events that indicate loss of asthma control, while simultaneously improving lung function (140). Similarly, anti-ST2 clinical trials using astegolimab indicate a reduced AAER in severe asthma patients at week 54 of use (141). Anti-alarmin clinical trial studies employ the use of biologics in addition to corticosteroids that were already prescribed to asthmatic patients. This suggests that anti-alarmins may act as a beneficial add-on therapy to inhaled corticosteroids (ICS) to help target alarmin-specific immune mechanisms that drive allergic disease in the asthmatic airways.

1.7 Corticosteroids Treatment for Asthma and Allergic Rhinitis

1.7.1 Molecular Mechanisms of Action Glucocorticoids

First-line therapies for asthma include the use of ICS either alone, or together with inhaled long-acting inhaled β2-agonists, and prescribed with care due to the onset of strong adverse side effects following long-term use (142). Patients with persistent asthma symptoms benefit from the addition of long-acting inhaled β2-agonists, like formoterol, to budesonide therapy mainly due to the observed improvement in lung function without worsening of asthma control (143). Moreover, the addition of formoterol exerts further anti-inflammatory effects in the allergic airways by attenuating eosinophilic inflammation when compared to asthmatic subjects receiving budesonide alone (144). Intranasal corticosteroids (INCS) and ICS remain the gold standard therapy in treating allergic rhinitis and asthma respectively. INCS are liposoluble molecules known for their anti-inflammatory properties, ability to suppress eosinophilic inflammation, and overall inhibitory pharmacodynamic effects in suppressing T2 cytokines during a type 2 inflammatory response.

Glucocorticoids function by binding to the cytoplasmic glucocorticoid receptor (GR) and inducing a conformational change in the GR, thereby forcing its dissociation from the heat shock protein (hsp) (145–147). In the absence of a glucocorticoid, the otherwise inactive GR-hsp complex prevents the GR from nuclear infiltration (146). Upon entry into the nucleus, the GR dimer complex binds to a glucocorticoid recognition sequence (GRE) and alters gene transcription, thus inducing effects on gene transcription by compromising messenger RNA (mRNA) stability and inhibiting protein synthesis of inflammatory cytokines, chemokines, or adhesion molecules (145–147).

1.8 Regulation of T2 Inflammation by Corticosteroids

1.8.1 Corticosteroid Regulation of T2 Cytokines and Inflammation in Patients with Allergic Airway Diseases

Multiple studies have measured the efficacy of INCS and ICS in managing nasal and ocular symptoms in AR and asthma, respectively. Budesonide is one of the most commonly used inhaled corticosteroids used in the treatment of asthma. Budesonide interferes with the activation and proliferation of airway-immune inflammatory cells, including eosinophils, mast cells, dendritic cells and T lymphocytes. Budesonide has been studied for its potency and dose-dependent response in which seasonal allergic rhinitis (SAR) patients exhibited anti-inflammatory effects and clinical efficacy against nasal symptoms when following a once-daily treatment for two weeks (148). GM-CSF and IL-5 levels in mucosal fluids of seasonal allergic rhinitis patients are suppressed when compared with cytokine levels of patients during a placebo treatment (149). Corticosteroid use has been shown to inhibit expression of T2 cytokines and inflammatory cell infiltration in airways of asthmatics. Pretreatment with topical corticosteroids such as fluticasone propionate can reduce the level of classical Th2 cytokines (IL-4, IL-5, and IL-13) in nasal secretions post-nasal allergen challenge (150), whereas previous a report using data from the current study to evaluate the efficacy of corticosteroids post-nasal allergen challenge has demonstrated its effects on airway function and eosinophilia during both the early and late phase airway responses (151).

1.8.2 Corticosteroid Regulation of Allergen-Induced Outcomes

Another ICS, mometasone furoate delivered by dry powder inhaler in dual responding, mild asthmatic subjects effectively attenuated early- and late-phase responses, airway hyperresponsiveness and sputum eosinophilia. In this study, a dose-dependent relationship existed during the late-phase response and the change in eosinophils 24 hours after patients were induced with allergen (152). These findings indicate how the use of mometasone furoate can inhibit allergen-induced outcomes.

Similarly, topical budesonide alleviates allergic rhinitis symptomology and inflammation induced by 7 days of nasal allergen challenges, and the addition of formoterol was not shown to increase the clinical benefit l (153). Ocular manifestations of SAR (including itching, redness of the eyes, swelling and watering) may be relieved through administering fluticasone proprionate nasal spray for 14 days when compared to placebo, proving to be efficacious when assessed using a patient-rated reflective total ocular symptom score (rTOSS) (154). The development and implementation of ICS and INCS treatment in recent decades has allowed for clinical evaluation of allergic symptoms in addition to its use in exploring mechanisms of AR.

Ahlstrom and colleagues (2007) used an upper airway nasal allergen challenge as a model to replicate peak pollen seasons in which allergic disease is most pervasive (153). A corresponding lowering of tryptase levels/mast cell activity and α2-macroglobulin levels/plasma exudation was observed in nasal lavage obtained post allergen challenge while receiving budesonide treatment (153). Other studies have reported that intranasal triamcinolone pre-treatment partially relieves type 2 inflammation driven by local ILC2 and CD4+ T cells in the nasal mucosa in response to an allergen challenge (151). A reduction of terminal eosinophil progenitor differentiation leading to a reduction in mature eosinophils in response to INCS demonstrates a mechanism for attenuated eosinophilia, however eosinophil progenitors themselves levels are not reduced. Additionally, triamcinolone pre-treatment significantly improves the peak nasal inspiratory flow (PNIF) % change from baseline and TNSS results in both early (2-7 hr) and late (24 hr post-nasal allergen challenge) phase responses following nasal allergen challenge, hence relieving some of the symptom burden involved in allergic rhinitis (151). Whole lung allergen challenge has been shown to increase lower airway submucosal myofibroblasts, and when treated with budesonide/formoterol, decreases the number of these myofibroblasts more effectively than budesonide monotherapy alone (144). Additionally, allergen-induced sputum eosinophilia decreases more significantly in magnitude in the lower airways than compared to pretreatment with budesonide alone. Together, these findings suggest that corticosteroid treatment attenuates bronchial eosinophilia as well as key cytokines and inflammatory pathways involved in asthma immunopathology of the lower airways. Repeatedly, corticosteroids have been demonstrated to be an effective therapy in reducing allergen-induced inflammation, and investigational studies support the idea that immunomodulatory mechanisms of type 2 inflammation can be regulated when patients are treated with corticosteroids.

1.8.3 Corticosteroid Regulation of Alarmins

There have been a few *in vitro* studies that have examined an effect of corticosteroids on alarmin expression in respiratory disease, and conflicting evidence exists for the effect of glucocorticoids on IL-33 and TSLP expression. TSLP has been reported amongst those inflammatory cytokines that undergo trans-repression and decreased transcription in response to glucocorticoids (155).

Some studies have reported a suppression in post-allergen IL-33 levels in murine models when pre-treated with dexamethasone (156). In this study, IL-33 was found to be expressed in M2 macrophages and dendritic cells found in the bronchial epithelium (156). In asthma patients categorized into either an IL-25-high or IL-25-low subset according to the level of IL-25 transcripts present in bronchial brushings, ICS treatment resulted in improved pulmonary function after 4 or 8 weeks of daily budesonide treatment when compared to patients with IL-25-low asthma. (92). In the same study, patients with elevated plasma IL-25 observed greater improvements in pulmonary function at 4 and 8 weeks as measured through FEV_1 measurements and delta PD20 (provocation dose of methacholine causing a 20% decline in FEV_1) from baseline when receiving ICS treatment. This suggests that ICS responsiveness is associated with plasma IL-25 levels in asthmatic subjects (92). Other studies have reported IL-33 and TSLP being insensitive to corticosteroid therapy, specifically in participants with higher disease severity who were taking systemic corticosteroids (157). Furthermore, neonatal mice and endobronchial specimens from pediatric patients with severe therapy-resistant asthma show that IL-33 was steroid-resistant against budesonide treatment (158). Therefore, there is evidence to suggest that alarmins demonstrate insensitivity to systemic steroid therapy, and that alternative or additional anti-alarmin therapies may be an effective treatment approach for some patients.

1.9 Allergen Challenge Tests

1.9.1 Inhaled Allergen Challenge Model

The inhaled allergen challenge is a highly reproducible asthma exacerbation model that allows researchers to investigate the mechanisms of early and late airway bronchoconstriction responses, airway hyperreactivity, and drug efficacy (159–163). Asthmatic patients with atopic sensitization inhale increasing incremental doses of a relevant aeroallergen to induce bronchoconstriction in the airways (162). The early- and late-phase asthmatic responses were first described in the 1950s, and have categorized asthmatic patients into two groups depending on their bronchoconstrictor responses to inhaled allergen (164). The early asthmatic response (EAR) is defined as bronchoconstriction occurring within the first 10-15 minutes after allergen inhalation, reaches maximum intensity at ~30 minutes post-inhalation, and resolves within one to three hours (165–167). Bronchoconstriction in the late asthmatic response (LAR) begins at 3-4 hours post-allergen inhalation and may peak at 6-12h post inhalation (165–167).

The immune response to allergic exposure is initiated allergens binding to and subsequently crosslinking allergen-specific IgE that is bound to high-affinity receptors (FcεRI) found on the surface of mast cells, basophils, and dendritic cells (168–170). Subsequent mast cell degranulation results in the release of mediators such as histamine, cysteinyl leukotrienes and prostaglandins that cause smooth muscle constriction (167,171). The cellular influx of mast cells, eosinophils, and basophils is a result of increased chemokine levels present in the tissue. Allergens also activate epithelial pattern recognition receptors (PRRs) on epithelial cells, such as protease-activated receptor (PAR2), that detect pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), thereby stimulating alarmin release (172–174).

1.9.2 Nasal Allergen Challenge

Nasal allergen challenge (NAC) is a safe and reproducible model that allows for the study of allergic rhinitis mechanisms in both pediatric and adult patients. The goal of the NAC is to imitate the natural exposure to aeroallergens that are responsible for producing an immune response characteristic to allergic rhinitis. One method to deliver allergens to study participants is via environmental exposure chambers, in which participants receive controlled levels of environmental allergen in a custom room with a controlled ventilation system (175). Additionally, allergens can be delivered via micropipette or metered nasal spray, with both methods being equally safe and reliable in administering doses of relevant aeroallergens to patients (176). Nasal sprays using allergen extracts in a liquid-based form help to spread the allergen in the nasal mucosa to help replicate a natural exposure disease model more efficiently than allergens that are in a dry, powder form (177,178). Patients currently taking medications when performing a NAC are required to undergo a washout period to reduce the interference of any drug with the onset of exacerbated allergic symptoms that follow post-NAC (179).

Allergen exposure models employing the use of house dust mite (HDM) provocation successfully demonstrate how pre-treatment with the INCS, fluticasone propionate reduced symptoms of perennial allergic rhinitis when assessed using the visual analogue scale (VAS) score (180). The increased reactivity to repeated antigen challenge (otherwise known as "priming") that increases the percentage of eosinophils in mucosal samples, as well as aggravated ocular symptoms were all reduced when treated with the INCS (181,182). The alleviation of these symptoms suggest the nasal-ocular reflex may contribute to the mechanism(s) involved in facilitating allergic rhinitis pathophysiology (182).

While exact outdoor environmental conditions that trigger an allergic response cannot be replicated in a test setting, it is important to account for the room conditions (humidity, temperature, time of day test is performed) in which the NAC is being performed in to reduce any variability between patient outcomes (179,183,184). The acute nasal allergen provocation model induces symptoms such as sneezing, itching, congestion, as well as nasal obstruction and rhinorrhea in an early-phase reaction, which may last upwards of one hour post-provocation (178,185). It is recommended that studies following a repeated-exposure approach should have the patient repeat nasal provocation at 7 days apart, so that they are able to return to a baseline condition in a time-sensitive manner that does not carry over any priming effect (178,186).

Given the similar increase in cellular inflammation and T2 cytokine responses of upper and lower airways after nasal and inhaled allergen challenges, respectively, one might anticipate allergen-induced effects on alarmin cytokines to also be similar between upper and lower airways. Alarmin cytokines act upstream of eosinophils to drive an immune reaction in response to allergen exposure. Thus far, previous reports have highlighted the effects of INCS treatment in attenuating cellular and T2 cytokine inflammation in the lower airways of asthmatic patients. In particular, hallmark indications of a Th2 response such as allergen-induced eosinophilic and T2 cytokine infiltration are inhibited post-allergen challenge when patients have received INCS pre-treatment. The current study aims to extend existing literature by examining the effects of nasal allergen challenge on alarmin levels in the upper airways, and the effects of intranasal triamcinolone pre-treatment for two weeks on alarmin expression, cellular inflammation, and cytokine expression in the nasal lavage and nasal mucosa post-allergen challenge.

Hypothesis and Aims

Hypothesis: It is hypothesized that treatment with topical corticosteroids reduces allergen-induced inflammation through a reduction in expression of alarmin cytokines

Aim 1: To investigate the effects of nasal allergen challenge on cellular inflammation and protein expression of alarmins IL-25, IL-33 and TSLP in the nasal mucosa and nasal lavage of subjects with mild allergic asthma and allergic rhinitis.

Aim 2: To study the effects of intranasal corticosteroid treatment on alarmin expression in nasal tissue post-allergen challenge in subjects with mild allergic asthma and allergic rhinitis.

Aim 3: To evaluate the relationship between eosinophil levels and alarmin levels in nasal samples from subjects with mild allergic asthma and allergic rhinitis.

CHAPTER 2: METHODS

2.1 Study Population / Participants

Eligible participants (n=10) were required to have allergic asthma and a history of allergic rhinitis. Inclusion criteria included participants who were between 18 to 65 years of age, a positive skin-prick test to common aeroallergens (including cat, dust mite, grass, pollen) and had asthma as confirmed by a methacholine PC_{20} ≤16 mg/ml (provocation concentration of methacholine causing a 20% fall in FEV_1). Participants had mild to moderate asthma as determined by baseline forced expiratory volume (per cent) in one second (FEV_1) ≥ 70% of the predicted value. To be eligible for the study participants were also required to have mild allergic rhinitis with baseline total nasal symptom score (TNSS) <3 and <2 for each individual symptom; and confirmed by demonstrating a total nasal symptom score ≥10 and reduced peak nasal inspiratory flow rate of ≥ 60% during the screening nasal allergen challenge.

Exclusion criteria were subjects who were current or former smokers with >10-pack-year history and participants with a current or previous history of other significant respiratory disease; participants with significant systemic disease, including history of current malignancy or autoimmune disease; those who had an asthma exacerbation or upper/lower respiratory tract infection within the previous 8 weeks; pregnancy; history of clinically significant hypotensive episodes or symptoms of fainting, dizziness, or lightheadedness; history of clinically significant hematologic abnormality, including coagulopathy; treatment with omalizumab in the previous 3 months; chronic use of medication for treatment of allergic lung disease other than short-acting β2-agonists; chronic use of other medication for treatment of allergic rhinitis; lung disease other than mild to moderate allergic asthma; and unwillingness or inability to comply with the study protocol for any other reason. Medication restrictions included use of corticosteroids within 28 days prior to the first study visit, use of nonsteroidal anti-inflammatory drugs (NSAIDs) within 48 hours of study visits or aspirin with 7 days of study visits; use of antihistamines including those in cold and allergy medications within 72 hours of study visits; use of caffeine-containing products within 4 hours of study visits.

2.2 Study Design

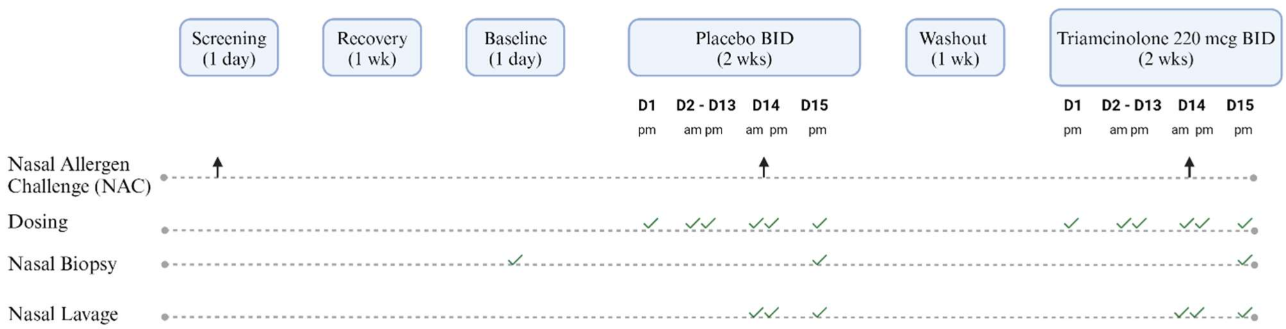


Figure 1: Study Schematic

Participants that met eligibility criteria during the screening period were enrolled into the study, and samples of the inferior nasal turbinate were collected at study baseline. Participants received a placebo (0.9% saline nasal spray) treatment BID for 15 days and after a 1-week washout participants received intranasal corticosteroid (INCS) (220 mcg BID triamcinolone nasal spray) for 15 days. Treatments began on the evening of day 1 and ended in the morning of day 15, 1 hour prior to the 24 hours post-NAC sampling. Nasal allergen challenge was conducted in the morning of day 14 of each treatment period using a bolus aeroallergen dose that was determined during the screening period. Samples of the inferior nasal turbinate were collected on the morning of day 15 24 hrs post-NAC. Nasal lavage was collected at baseline, 7 hours post-NAC, and 24 hours post-NAC. Placebo treatment was followed by INCS treatment to eliminate any potential carry-over effects from intranasal triamcinolone into the placebo period and was therefore the treatment periods were not randomized.

2.3 Study Procedures:

2.3.1 Methacholine inhalation test

Technical standards have been developed and consistently revised in order to use methacholine in the use of bronchial challenges (187). Methacholine chloride (Methapharm Inc., Brantford, Canada) was prepared at 128 mg/mL in physiological saline and was further diluted to use concentrations ranging from 0.031 mg/mL to 128 mg/mL. During the inhalation test, methacholine was inhaled for 2 minutes using a Hans Rudolf valve connected to a Wright nebulizer (Roxon Medi-Tech, Montreal, PQ, Canada). Participants inhaled normal saline, followed by doubling concentrations of methacholine for 2 minutes. FEV_1 was measured using a Cosmed spirometer (Cosmed, Rome, Italy) at 30, 90, 180, and 300 seconds after each inhalation. Once FEV_1 dropped below 20%, inhalation test was stopped and methacholine PC_{20} was calculated using linear interpolation.

2.3.2 Nasal Allergen Challenge - Screening Period

Allergen sensitivity of each participant was determined from the results of a skin prick test to a panel of aeroallergens. Each allergen extract (ALK, Hørsholm, Denmark) was applied to the skin and the skin was scratched to allow exposure. After 10 minutes, the local reaction to the allergen extract was evaluated by measuring the size of wheal horizontally and vertically. A wheal greater than 2 x 2 mm in area defined a positive reaction to the allergen assuming a positive (histamine) and negative (diluent) control were also reacting appropriately. The allergen extract causing the largest skin wheal was selected for the nasal allergen challenge. TNSS questionnaire administered with permission from Mapi Research Trust, and PNIF using the In-Check PNIF meter (G M Instruments, Irvine, United Kingdom) was measured followed by a diluent challenge of 100 μL of 0.9% normal saline (Baxter International Inc., Illinois, United States) sprayed into each nostril using Aptar VP7 nasal spray pump (Aptar, France) to rule out nasal hyper-responsiveness. PNIF and TNSS were measured in triplicate 5 minutes after diluent challenge and the highest PNIF value was used as the baseline to calculate the allergen-induced % change. Using an escalating dose protocol, allergen extracts were prepared into serial dilutions of 1:2048, 1: 512, 1: 128, 1: 32, 1:8, and 1:2. 100 μL of the 1:2048 diluted allergen was sprayed into each nostril, followed by PNIF and TNSS measurements at 5 and 15 minutes later. Successive doses of allergen were administered in 4-fold increments until PNIF had fallen ≥50% and TNSS of ≥10/12 had been achieved. PNIF and TNSS were measured regularly until 24 hours post-NAC. The cumulative allergen dose was calculated and recorded for use during the treatment period NAC.

2.3.3 Nasal Allergen Challenge - Treatment Period

TNSS and PNIF were measured pre-challenge followed bya diluent challenge of 100 μL of 0.9% normal saline sprayed into each nostril using Aptar VP7 nasal spray pump (Aptar, France) to rule out nasal hyper-responsiveness. PNIF and TNSS were measured in triplicate 5 minutes after diluent challenge and the highest PNIF value was used as the baseline to calculate allergen-induced % change.

Allergen: A single bolus with cumulative allergen dose determined from screening challenge was administered in a volume of 100 µL per nostril. PNIF and TNSS were measured at 5, 15 and 30 minutes, 1 hour, and then hourly for 7 hours post-NAC.

2.4 Nasal sampling

2.4.1 Nasal Biopsies

One biopsy was collected from the inferior nasal turbinate of each nostril.

Using forceps, gauze dampened with nasal decongestant and lidocaine spray was inserted into the nasal cavity to soak tissue for 5-10 minutes for local freezing. Lidocaine hydrochloride 2% and epinephrine 1:100,000 was injected at site of nasal curettage to take effect for 5 minutes before biopsies were acquired from participants. Participants were seated in an upright position with head tilted back slightly when biopsies were obtained. The surgeon achieved optimal visualization of the nasal turbinate by using a nasal speculum. Using cutting nasal forceps, a small biopsy was collected from the inferior nasal turbinate of each nostril. Pressure was applied with gauze to prevent any bleeding. Biopsies were transferred into petri dishes by gently flushing forceps with chilled PBS using transfer pipettes. Scissors were used to remove the end of a disposable transfer pipette. The biopsy was gently drawn up into PBS-filled tip and released in cassette. After the biopsy was placed in its' own individual cassette and placed in cold 10% NBF for 4 hours, they were transferred to local histology lab to be processed into paraffin blocks within 24 hours of the start of fixation. Biopsies were embedded into individual paraffin block using local standard embedding protocol and stored at room temperature.

2.4.2 Nasal Lavage

A 10 mL syringe was filled with 10 mL of room temperature 0.9% saline with the correct size of foam plug attached to the end of the syringe. In a sitting position, participants were asked to gently blow their nose prior to procedure. Participants would lean their head forward and look down at the ground at a position 45° from horizontal while holding a sterile specimen collection container under their nose. Participants were asked to breathe through their mouth instead of their nose and to place their tongue at the back of the palate to close the velopharyngeal aperture and block saline from entering the posterior nasopharynx.

A foam plug was placed into the right nostril while 10 mL of saline from the syringe was slowly instilled until all saline had entered the nasal cavity, or until saline began to leak from the opposite nostril. Leaking saline was collected in the sterile container and any injection of saline was stopped. Using the same syringe, this volume of instilled saline was withdrawn from the nostril. This process (instill/withdraw) was repeated 5 times with the volume of saline that was collected/remaining in the syringe but was stopped if the volume in the syringe reached below 7 mL. Once completed, the participant expelled all fluid from the nasal cavity into the sterile container.

Saline from the sterile container was drawn in the syringe containing nasal washing fluid from the right nostril and was used to wash the left nostril. The foam plug was inserted into the left nostril and 10 mL of saline was instilled from the syringe into the nostril until all saline had entered the nasal cavity, or until saline began to leak from the opposite nostril. Leaking saline was collected in the sterile container and any injection of saline was stopped. Saline was withdrawn from the nostril into the syringe. This process (instill/withdraw) was repeated 5 times with the volume of saline that was collected/remaining in the syringe but had to be stopped if the volume in the syringe reached below 7 mL. Once completed, the subject expelled all fluid from the nasal cavity into the sterile container. Volume of collection was recorded, and contents were pipetted into a 15 mL centrifuge tube to be processed. 7-10 mL of fluid should have been collected.

2.4.3 Processing and storage of Nasal Lavage

Protease inhibitor was prepared prior to nasal lavage processing. Storage instructions for the 7X stock solution of protease inhibitor consisted of being stored at 2°C to 8°C for short term (1-2 weeks) and at -15°C to -25°C for long term (at least 12 weeks). Protease inhibitor solution was prepared by adding a Mini tablet (Sigma-Alrdich, St. Louis, Missouri, USA) to a solvent of either 1.5 mL of distilled water, or to 100 mM phosphate buffer at pH 7.0. 170 µL of 7X stock solution was added to 2 mL Corning cryovials (430488) and kept frozen at -20°C. 1 tube was used for each 1 mL of nasal lavage fluid and kept on ice until used.

After sample was centrifuged at 500g for 10 minutes at 4°C. After transferring supernatant into intermediate 15 mL tube, sample underwent hard spin at 1500g to pellet debris. Supernatant was aliquoted + protease inhibitors and stored at -80 degrees C. 0.1% DTT was added to cell pellet. The sample was vortexed, rocked for 15 minutes, then vortexed again. The cell suspension was filtered through 48 µm nylon mesh into a 15 mL conical tube. Cell counts were performed using trypan blue and hemocytometer. Cells were washed by centrifuging at 500g for 10 minutes at 4°C and resuspended in a cold volume of DPBS to achieve a cell concentration of 0.7-1 x 10^6 cells/mL.

2.3.4 Cytospins:

Four slides were assembled in a Shandon cytospin. Cytospin filter cards were pre-wet with 50 µL of DPBS before adding cell suspension and were spun at 300rpm for 5 minutes. 50 µL of cell suspension was added to each slide, before spinning at 300rpm for 5 minutes. Two slides were stained with Diff-quik for differential counts of macrophages, eosinophils, epithelial cells, lymphocytes, and neutrophils. 400 cells counted per cytospin.

2.4 Clinical Data

2.4.1 PNIF

PNIF is a commonly used method to assess for nasal airflow obstruction and that reflects nasal congestion, therefore verifying the subjective TNSS. For this study, the In-Check PNIF meter (G M Instruments, Irvine, United Kingdom) was used to assess PNIF.

The participant was asked to fully exhale prior to measuring PNIF, with the device held horizontally and forming a tight seal around the nose area, then was asked to complete the peak nasal inspiratory manoeuvre by inhaling forcibly through the nose for one second. Peak inspiratory flow was recorded, and this process was repeated thrice.

2.4.2 Total Nasal Symptom Score (TNSS)

TNSS is a subjective measure of onset inflammation and action of medications that is measured at intervals. It is a valid score used for measuring changes in symptomology amongst participants. The reliability of TNSS has been confirmed using internal consistency methods amongst participants with chronic allergic and non-allergic rhinitis (188). Symptoms such as sneezing, congestion, itching and rhinorrhea are assessed on a scale from 0-3 as reported by the study participants. The sum of the score are added together to determine the total TNSS score. Grading is as follows:

0=None, no symptoms evident

1=Mild, symptom present but easily tolerated

2=Moderate, definite awareness of symptom; bothersome but tolerable

3=Severe, symptom hard to tolerate; would interfere with daily activity

2.5 Immunofluorescent Staining

Refer to **Figure 2** for a summary of the immunofluorescence staining protocol.

2.5.1 IL-33+2D7+Tryptase+ Stain

Formalin-fixed paraffin-embedded tissue sections were deparaffinized using xylene for 9 minutes, followed by decreasing concentrations of ethanol and distilled water (100% EtOH for 6 minutes, 95% EtOH for 3 minutes, 70% EtOH for 3 minutes, 50% EtOH for 3 minutes, dH_2O for 5 minutes). Antigen retrieval was performed in heated citrate buffer (pH=6) for 5 minutes. 0.2% Triton was applied for 30 minutes at room temperature. Sections were incubated with 3% normal donkey serum for 30 minutes at room temperature and then incubated with anti-IL-33 antibody (Invitrogen PA5-47006, Waltham, Massachusetts, USA) at 1:40 dilution, with isotype negative slides incubated with Goat IgG antibody (Invitrogen 31245, Waltham, Massachusetts, USA) at 1:250 dilution at 4°C overnight. Following this, TRITC (594) 2^0 secondary antibody (Invitrogen A11058, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250

dilution and used for detection. Slides were incubated with 3% normal donkey serum for 30 minutes at room temperature and then incubated with 2D7 antibody (Abcam ab155577, Cambridge, United Kingdom) at 1:250 dilution, with isotype negative slides incubated with Mouse IgG1 antibody (Abcam ab170190, Cambridge, United Kingdom) at 1:250 dilution factor at 4°C overnight. Following this, FITC (488) 2^0 secondary antibody (Invitrogen A11001, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution and used for detection. Slides were incubated with 3% normal donkey serum for 30 minutes at room temperature and then incubated with Tryptase antibody (Abcam ab134931, Cambridge, United Kingdom) at 1:200 dilution, with isotype negative slides incubated with Rabbit IgG antibody Abcam ab171870, Cambridge, United Kingdom) at 1:200 dilution factor at 4°C overnight. Then, Cy5 (647) 2^0 secondary antibody (Invitrogen A21244, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution factor and used for detection. DAPI stain was applied for 3 minutes, and slides were cover-slipped with Fluoromount.

2.5.2 TSLP+2D7+ Stain

Formalin-fixed paraffin-embedded tissue sections were deparaffinized using xylene for 9 minutes, followed by decreasing concentrations of ethanol and distilled water (100% EtOH for 6 minutes, 95% EtOH for 3 minutes, 70% EtOH for 3 minutes, 50% EtOH for 3 minutes, dH_2O for 5 minutes). Antigen retrieval was performed in heated citrate buffer (pH=6) for 5 minutes. 0.2% Triton was applied for 30 minutes at room temperature. Sections were incubated with 1.5% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with 2D7 antibody (Abcam ab155577, Cambridge, United Kingdom) at 1:250 dilution, with isotype negative slides incubated with Mouse IgG1 antibody (Abcam ab170190, Cambridge, United Kingdom) at 1:250 dilution factor at 4°C overnight. Following this, FITC

(488) 2^0 secondary antibody (Invitrogen A11001, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution and used for detection. Slides were incubated with 1.5% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with anti-TSLP antibody (Abcam ab47943, Cambridge, United Kingdom) at 1:200 dilution, with isotype negative slides incubated with Rabbit IgG antibody Abcam ab171870, Cambridge, United Kingdom) at 1:200 dilution factor at 4°C overnight. Then, TRITC (594) 2^0 secondary antibody (Invitrogen A11012, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution and used for detection. DAPI stain was applied for 3 minutes, and slides were cover-slipped with Fluoromount.

2.5.3 TSLP+Tryptase+ Stain

Formalin-fixed paraffin-embedded tissue sections were deparaffinized using xylene for 9 minutes, followed by decreasing concentrations of ethanol and distilled water (100% EtOH for 6 minutes, 95% EtOH for 3 minutes, 70% EtOH for 3 minutes, 50% EtOH for 3 minutes, dH_2O for 5 minutes). Antigen retrieval was performed in heated citrate buffer (pH=6) for 5 minutes. 0.2% Triton was applied for 30 minutes at room temperature. Sections were incubated with 3% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with Tryptase antibody (Abcam ab2378, Cambridge, United Kingdom) at 1:500 dilution, with isotype negative slides incubated with Mouse IgG1 antibody (Abcam ab170190, Cambridge, United Kingdom) at 1:500 dilution factor at 4°C overnight. Following this, Cy5 (647) 2^0 secondary antibody (Invitrogen A21235, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution factor and used for detection. Slides were incubated with 3% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with anti-TSLP antibody (Abcam ab47943, Cambridge, United Kingdom) at

1:200 dilution, with isotype negative slides incubated with Rabbit IgG antibody (Abcam ab171870, Cambridge, United Kingdom) at 1:200 dilution factor at 4°C overnight. Then, TRITC (594) 2^0 secondary antibody (Invitrogen A11012, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution and used for detection. DAPI stain was applied for 3 minutes, and slides were cover-slipped with Fluoromount.

2.5.4 IL-25+2D7 Stain

Formalin-fixed paraffin-embedded tissue sections were deparaffinized using xylene for 9 minutes, followed by decreasing concentrations of ethanol and distilled water (100% EtOH for 6 minutes, 95% EtOH for 3 minutes, 70% EtOH for 3 minutes, 50% EtOH for 3 minutes, dH_2O for 5 minutes). Antigen retrieval was performed in heated citrate buffer (pH=6) for 5 minutes. 0.2% Triton was applied for 30 minutes at room temperature. Sections were incubated with 1.5% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with 2D7 antibody (Abcam ab155577, Cambridge, United Kingdom) at 1:250 dilution, with isotype negative slides incubated with Mouse IgG1 antibody (Abcam ab170190, Cambridge, United Kingdom) at 1:250 dilution factor at 4°C overnight. Following this, FITC (488) 2^0 secondary antibody (Invitrogen A11001, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution and used for detection. Slides were incubated with 1.5% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with anti-IL-25 antibody (Abcam ab108530, Cambridge, United Kingdom) at 1:200 dilution, with isotype negative slides incubated with Rabbit IgG antibody Abcam ab171870, Cambridge, United Kingdom) at 1:200 dilution factor at 4°C overnight. Then, TRITC (594) 2^0 secondary antibody (Invitrogen A11012, Waltham, Massachusetts, USA) was applied for

1 hour at room temperature at 1:250 dilution and used for detection. DAPI stain was applied for 3 minutes, and slides were cover-slipped with Fluoromount.

2.5.5 IL-25+Tryptase Stain

Formalin-fixed paraffin-embedded tissue sections were deparaffinized using xylene for 9 minutes, followed by decreasing concentrations of ethanol and distilled water (100% EtOH for 6 minutes, 95% EtOH for 3 minutes, 70% EtOH for 3 minutes, 50% EtOH for 3 minutes, dH_2O for 5 minutes). Antigen retrieval was performed in heated citrate buffer (pH=6) for 5 minutes. 0.2% Triton was applied for 30 minutes at room temperature. Sections were incubated with 3% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with Tryptase antibody (Abcam ab2378, Cambridge, United Kingdom) at 1:500 dilution, with isotype negative slides incubated with Mouse IgG1 antibody (Abcam ab170190, Cambridge, United Kingdom) at 1:500 dilution factor at 4°C overnight. Following this, Cy5 (647) 2^0 secondary antibody (Invitrogen A21235, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution factor and used for detection. Slides were incubated with 3% normal goat serum and 1.5% normal horse serum for 30 minutes at room temperature and then incubated with anti-IL-25 antibody (Abcam ab108530, Cambridge, United Kingdom) at 1:200 dilution, with isotype negative slides incubated with Rabbit IgG antibody Abcam ab171870, Cambridge, United Kingdom) at 1:200 dilution factor at 4°C overnight. Then, TRITC (594) 2^0 secondary antibody (Invitrogen A11012, Waltham, Massachusetts, USA) was applied for 1 hour at room temperature at 1:250 dilution and used for detection. DAPI stain was applied for 3 minutes, and slides were cover-slipped with Fluoromount.

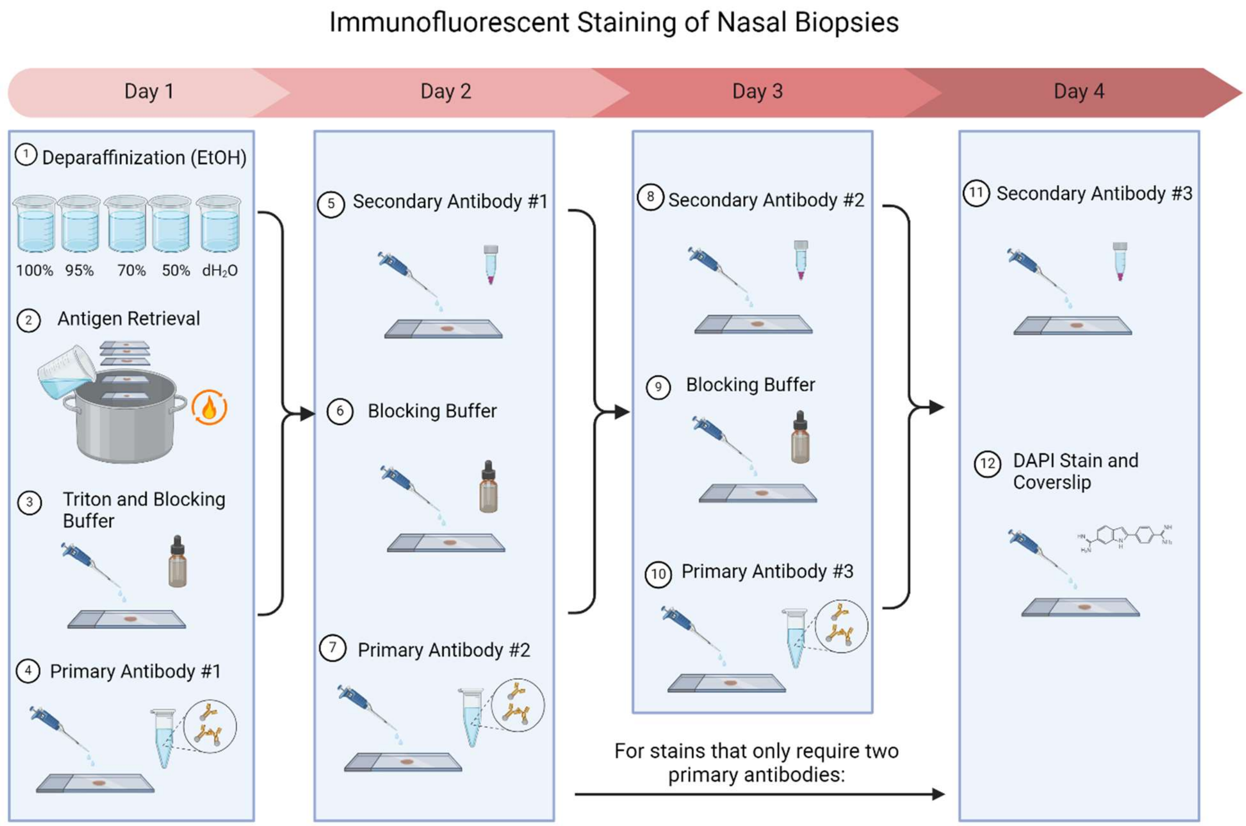


Figure 2: Summary of Immunofluorescent Staining Protocol (Created with BioRender.com)

2.6 Immunofluorescence Microscopy

Biopsy images were scanned with an ECLIPSE Ni-U Nikon fluorescence microscope and the NIS-Elements Imaging System in the Fluorescence Mode. Refer to **Table 1** for acquisition times for the separate staining panels.

Table 1: Acquisition times for immunofluorescence microscopy across multiple IF stains

	IL- 33+ 2D7+ Tryptase+	TSLP+ 2D7+	TSLP+ Tryptase+	IL-25+ 2D7+	IL-25+ Tryptase+

DAPI	80-100 ms	80-100 ms	80-100 ms	80-100 ms	80-100 ms
FITC	100 ms	150 ms	-	150 ms	-
TRITC	200 ms	250 ms	250 ms	250 ms	250 ms
Cy5	350 ms	-	500 ms	-	500 ms

2.7 Cell Quantification

Regions of Interest (ROI) for cell enumeration in the epithelium were identified by outlining the distinct structure of the epithelium along the perimeter of each biopsy. Areas of biopsy that were separated from the overall biopsy were not included when identifying epithelium ROIs. Submucosa ROIs were identified as the structurally different tissue that was more central yet consistently adjacent to the epithelium. Areas containing any vessels, cartilage, or excessive smooth muscle were excluded when identifying submucosa ROIs. All ROIs were measured in mm^2. Cells positive for alarmin proteins alone were enumerated as those staining positive for one of IL-33, TSLP, or IL-25, per mm^2 of the nasal epithelium and submucosa. Isotype negative control slides were used to help identify non-specific staining amongst the tissue. Mast cells were enumerated as tryptase positive and 2D7 negative, per mm^2 of the nasal epithelium and submucosa. Basophils were enumerated as 2D7 positive and tryptase negative, per mm^2 of the nasal epithelium and submucosa. Mast cells expressing IL-33 were enumerated as tryptase positive, IL-33 positive, and 2D7 negative per mm^2 of the nasal epithelium and submucosa. Mast cells expressing IL-25 were enumerated as tryptase positive and IL-25 positive per mm^2 of the nasal epithelium and submucosa. Mast cells expressing TSLP were enumerated as tryptase positive and TSLP positive per mm^2 of the nasal epithelium and submucosa. Basophils expressing IL-33 were enumerated as 2D7 positive, IL-33 positive, per mm^2 of the nasal epithelium and submucosa. Basophils expressing IL-25 were enumerated as 2D7 positive and IL-25 positive per mm^2 of the nasal epithelium and submucosa. Basophils expressing TSLP were expressed as 2D7 positive and TSLP positive per mm^2 of the nasal epithelium and submucosa.

2.8 Mesoplex Assays

Meso-Scale Discovery (MSD) and QuickPlex SQ120 Imager (Meso Scale Diagnostics, Rockville, Maryland, USA) were used to analyze nasal lavage samples. Specifically, the Human V-PLEX 36-cytokine panel was used to measure biomarkers and analytes consisting of chemokines, growth factors, T1 cytokines, T2 cytokines, and pro-inflammatory cytokines. The Uplex kit was used to measure levels of alarmins (IL-33, IL-25 and TSLP).

Compared to a traditional ELISA assay, the QuickPlex SQ120 Imager (Meso Scale Diagnostics, Rockville, Maryland, USA) is an instrument with greater sensitivity when measuring for analytes. An advantage in using the QuickPlex SQ120 Imager (Meso Scale Diagnostics, Rockville, Maryland, USA) machine is its ability to detect analyte levels from small volumes of samples, which means fewer samples fall below the minimum detection range. The QuickPlex SQ120 Imager (Meso Scale Diagnostics, Rockville, Maryland, USA) uses updated immunoassay technology to over a traditional or even high-detection ELISA to identify specific molecules from biological samples more effectively using electrochemiluminescence.

The following cytokines were measured by MSD:

TL1A, IL-17A/F, IL-17E/IL-25, IL-33, TSLP, Eotaxin, Eotaxin-3, IL-8, IP-10, MCP-1, MCP-4, MDC, MIP-1a, MIP-1b, TARC, GM-CSF, IL-12/IL-23P40, IL-15, IL-16, IL-17, IL-1a, IL-5, IL-7, TNF-b, VEGF, IFN-gamma, IL-10, IL-12p70, IL-13, IL-1B, IL-2, IL-4, IL-6, and TNF-a.

Cytokines with volumes lower than the limit of detection were substituted with halved values from the lowest limit of quantitation (LLOQ), derived from standard curves generated from the measured analytes.

2.9 Statistical Analysis

2.9.1 Physiology and Measure of Symptoms Data

Statistical analysis of PNIF and TNSS values at each different time point for all ten participants was done using the non-parametric Wilcoxon matched pairs signed rank test. Missing values in the analyses (TNSS/PNIF were not measured by or for a patient) were replaced with the respective timepoint mean TNSS/PNIF values calculated with data from all patients prior to analysis. Statistical analyses were conducted using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, CA, USA).

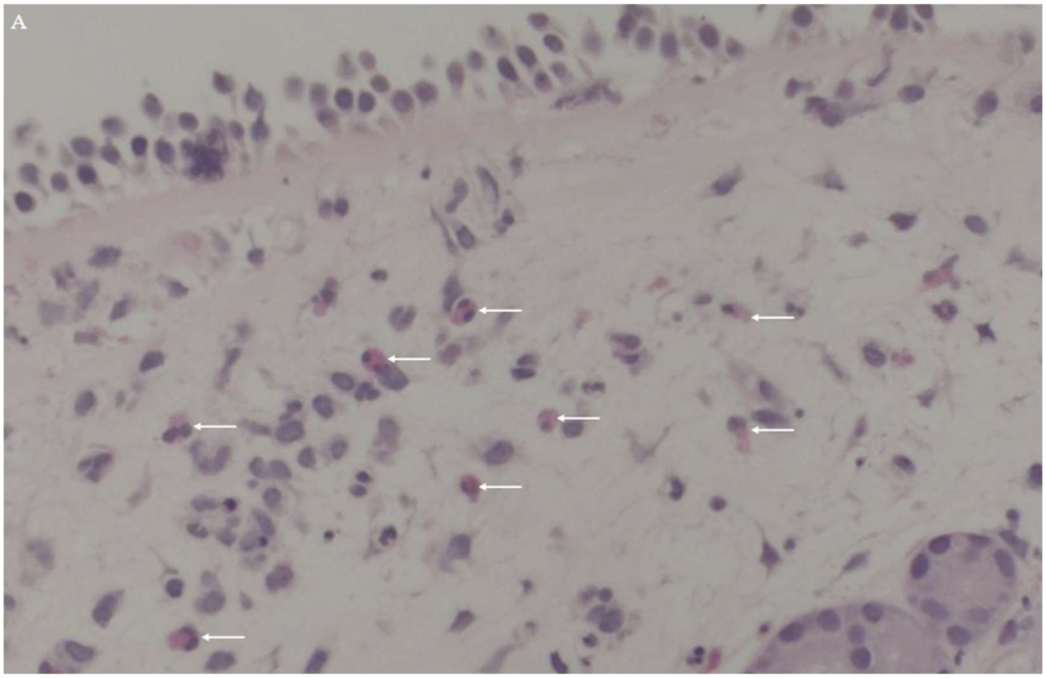
2.9.2 Nasal Lavage Data

Statistical analysis for cytokines and percent eosinophils in nasal lavage was performed using two-way repeated measures ANOVA with Bonferroni post-hoc test, and between group analysis of percent eosinophils at 24h was conducted using a paired t-test. Values were log10-transformed prior to analysis if data sets were non-parametric. Values below lowest limit of detection (LLOD) was replaced with LLOD/2 and missing values were replaced with the timepoint mean value of the analytes calculated with data from all patients. Statistical analyses were conducted using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, CA, USA). Multivariate analyses were conducted using IBM SPSS Advanced Statistics version 25 (IBM Corp., Armonk, NY, USA).

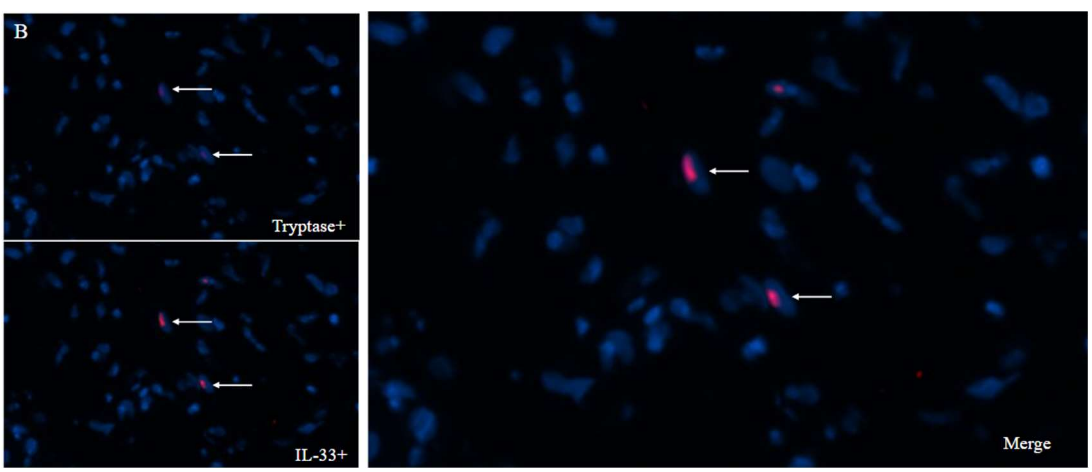
2.9.3 Nasal Biopsy Epithelial-Derived Cytokines, Mast cells, Basophils, and Eosinophils

Statistical analysis for epithelial-derived cytokines in IF-stained biopsies and eosinophils in H&E-stained biopsies was performed using one-way repeated measures ANOVA with Tukey's post-hoc test. Values were log10-transformed prior to analysis if data sets were non-parametric. Number of eosinophils or cells expressing alarmin (IL-33+, TSLP+, or IL-25+) or mast cell and basophil biomarker (tryptase+, or 2D7+) per unit area (mm^2) of tissue per patient visit was measured by taking the sum of the cells positive for eosinophil/mast cell/basophil/alarmin and divided by the sum of the ROIs for both left and right nostril. Missing data for one nostril was accounted for by using the remaining single nostril data alone. Missing data for a complete study visit was replaced with the median number of cells expressing alarmin/ mm^2 across the patient population for the respective visit. Values of 0 were replaced with LLOQ/2 per unit area (mm^2) of tissue of the respective visit. Statistical analyses were conducted using GraphPad Prism 9.4.1 (GraphPad Software, San Diego, CA, USA).

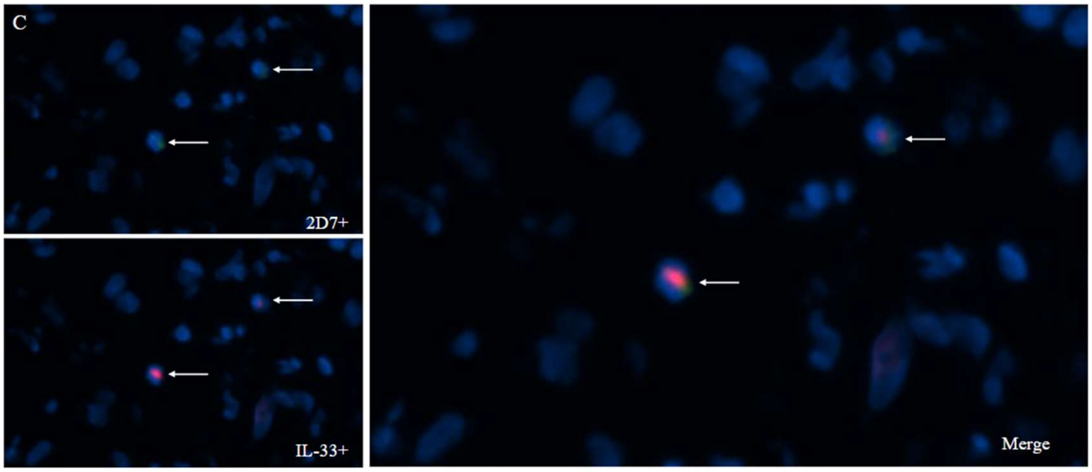
Representative Images



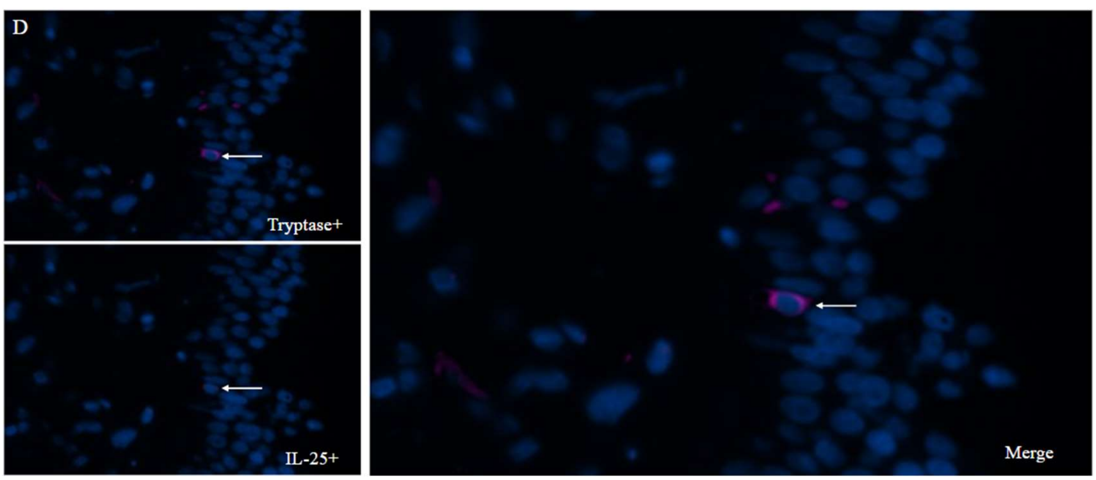
Eosinophils are identified in the haematoxylin and eosin (H&E) stain as cells with a purple-stained bilobed nucleus and pink granules.



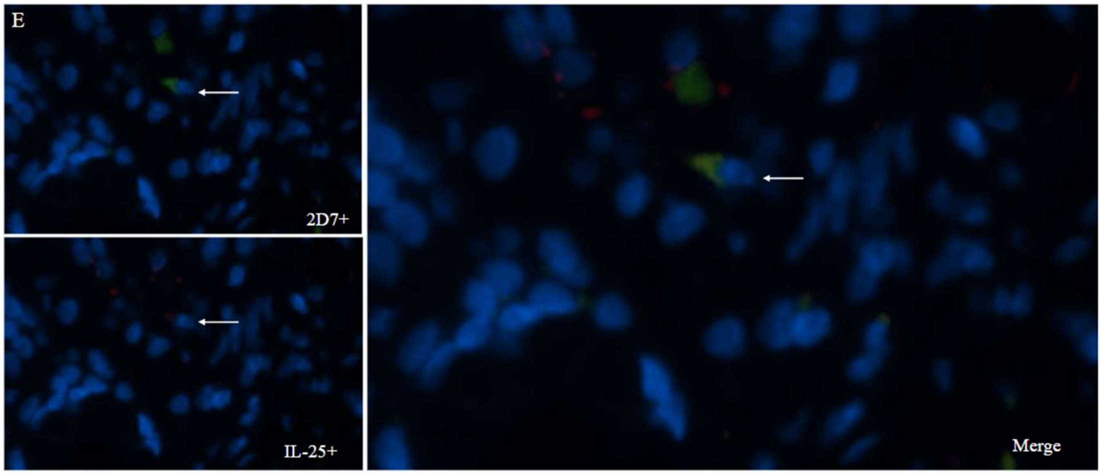
Mast cells colocalized for IL-33 are identified as tryptase (Cy5 – violet) and IL-33 (TRITC – red) positive.



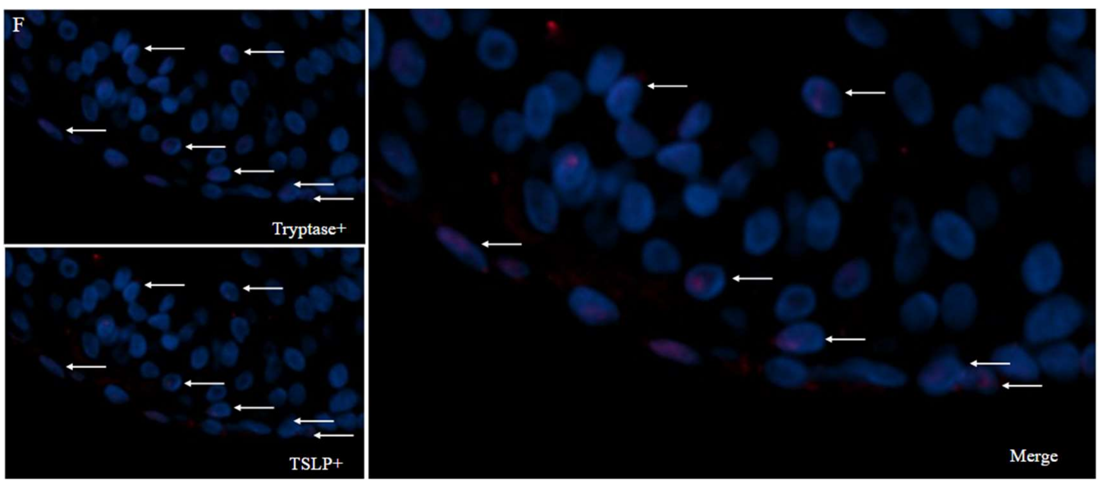
Basophils colocalized for IL-33 are identified as 2D7 (FITC – green) and IL-33 (TRITC – red) positive.



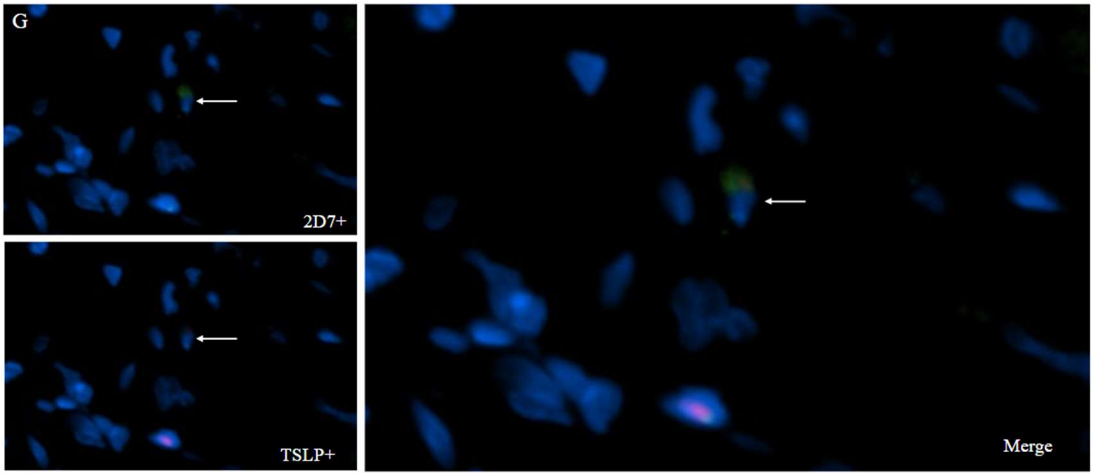
Mast cells colocalized for IL-25 are identified as tryptase (Cy5 – violet) and IL-25 (TRITC – red) positive.



Basophils colocalized for IL-25 are identified as 2D7 (FITC – green) and IL-25 (TRITC – red) positive.



Mast cells colocalized for TSLP are identified as tryptase (Cy5 – violet) and TSLP (TRITC – red) positive.



Basophils colocalized for TSLP are identified as 2D7 (FITC – green) and TSLP (TRITC – red) positive.

Figure 3: Representative images of H&E (A) and immunofluorescent stained biopsies of nasal inferior turbinate (B-G). White arrows indicate positively stained cells of interest.

CHAPTER 3: RESULTS

Participants enrolled in the study

Ten participants with mild allergic asthma and allergic rhinitis met the eligibility criteria and completed the study. The median (STD) age was 26 years (11.46) (Table 2).

Table 2: Participant demographics.

ID#	Age	Sex	Challenge Allergen	Allergen Dose (Units)	FEV_1 (% Predicted)	TNSS	PNIF (L/min)	MCh PC_{20} (mg/mL)*	Blood Eosinophil (10^9/L)
1	37	M	HDM	30.8	67	0	55	0.7	0.8
2	30	F	Cat	166.5	85.8	2	90	8.7	0.4
3	28	M	Kentucky Bluegrass	1665.0	93.8	2	250	8.7	0.1
4	24	F	HDM	499.5	112	3	150	3.2	0.3
5	57	M	HDM	1999.5	95	0	260	5.1	0.4
6	23	M	Horse	415.0	99.7	2	140	1.2	0.5
7	22	F	HDM	499.5	114.1	2	150	6.2	0.2
8	37	M	Ragweed	666.0	109.7	0	150	6.7	0.1
9	19	F	HDM	124.5	85.3	0	135	0.1	0.4
10	21	F	HDM	499.5	109.5	0	210	13.8	0.2
Median (STD)	26 (11.5)	5M 5F		499.5 (655.6)	97.4 (15.0)	1.0 (1.2)	150 (64.8)	3.0 0.1-13.70	0.35 (0.21)

*Geometric mean and range; HDM: house dust mite, FEV_1: forced expiratory volume in one second, TNSS: total nasal symptom score, PNIF: peak nasal inspiratory flow rate, MCh PC_{20}: provocative concentration of methacholine for 20% fall in FEV_1.

All participants had mild allergic asthma with median FEV_1 of 97.35% predicted (14.98), and methacholine PC_{20} ranging from 0.1 to 13.8 mg/mL. All participants had documented allergic rhinitis. The median TNSS at baseline was 1 (1.20), with PNIF of 150 L/min (64.84). The median blood eosinophil count was 0.35 x 10^9/L (0.21) , ranging from 0.1 to 0.8 x 10^9/L.

Effect of nasal allergen challenge on upper airway physiology and symptoms

During the placebo treatment period, allergen challenge reduced PNIF and increased TNSS during early (0-2h) and late (3-7h) responses. Compared to pre-allergen baseline, PNIF dropped a maximum of 56.4% at 30 minutes post-NAC, followed by a second fall by 43.1% at 6h post-NAC. TNSS increased from a score of 0.6 to 6.0 at 30 minutes post-NAC, and gradually improved to 3.6 during the 7h post-NAC. At 24h post-NAC the PNIF was 8.6% lower than pre-challenge, and the TNSS of 3.3 was still not back to baseline (**Figure 4**).

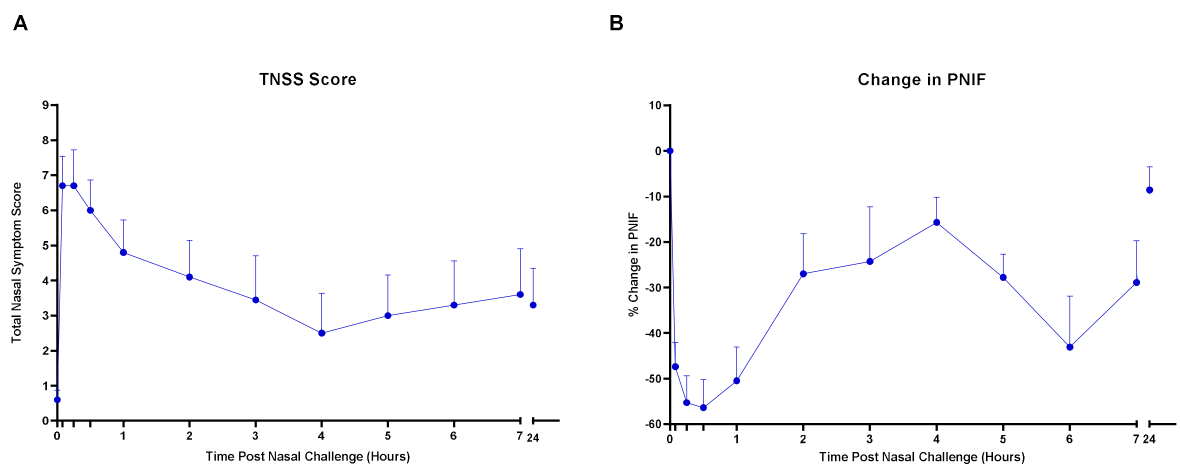


Figure 4: Upper airway physiology and symptoms before and after NAC (nasal allergen challenge) measured by change in peak nasal inspiratory flow (PNIF) and Total Nasal Symptom Score (TNSS).

Effect of nasal allergen challenge on eosinophil levels in upper airways

There was a significant increase in eosinophils in nasal lavage and nasal tissue 24 hours post-NAC (**Figure 5**). In nasal lavage, the baseline percent of eosinophils increased significantly at 7 hr (p= 0.0186) and 24 hr post-NAC (p= 0.0013). Similar results were seen in nasal biopsies, where allergen challenge induced a significant increase in the number of eosinophils/mm^2 at 24 hr post-NAC in the inferior turbinate tissue in patients during the placebo treatment period when compared to baseline (p=0.0273).

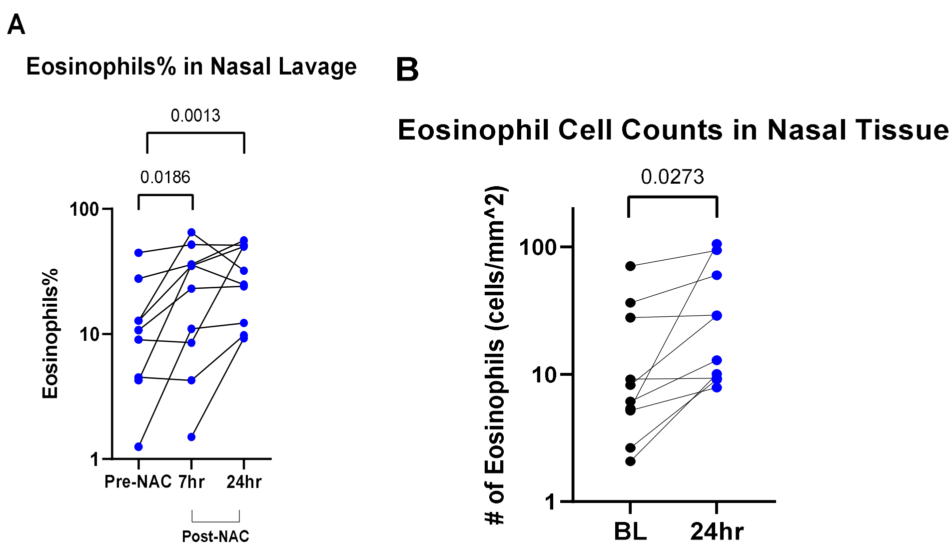


Figure 5: Eosinophils in nasal lavage (A) at 7 hours (7hr) and 24 hours (24hr) after nasal allergen challenge (NAC) compared to pre-challenge; number of eosinophils in nasal biopsies (B), per mm^2 in the inferior turbinate at 24 hours post-NAC compared to pre-treatment baseline (BL). Eosinophils in nasal lavage were measured from cytospins stained with Diff Quick, and raw data values are shown on a log10 scale. Data was analyzed using 2-way ANOVA and Bonferroni's multiple comparisons test. Eosinophils from tissue biopsies were measured with H&E staining. Eosinophils were identified as cells that were eosin-positive and had a bi-lobed nucleus. Raw data values are shown on a log10 scale. Data was non-parametric and log-transformed prior to analysis and analyzed using one-way ANOVA and Tukey's multiple comparisons test.

Effect of nasal allergen challenge on basophils and mast cells in nasal tissue

The number of mast cells (identified as cells immunopositive for tryptase) and basophils (identified as cells immunopositive for 2D7) were measured in biopsies before 24 hours post-NAC are shown in **Figure 6**. There was no significant change in mast cells or basophils in nasal tissue at 24 hours post-NAC compared to pre-challenge baseline (p>0.05).

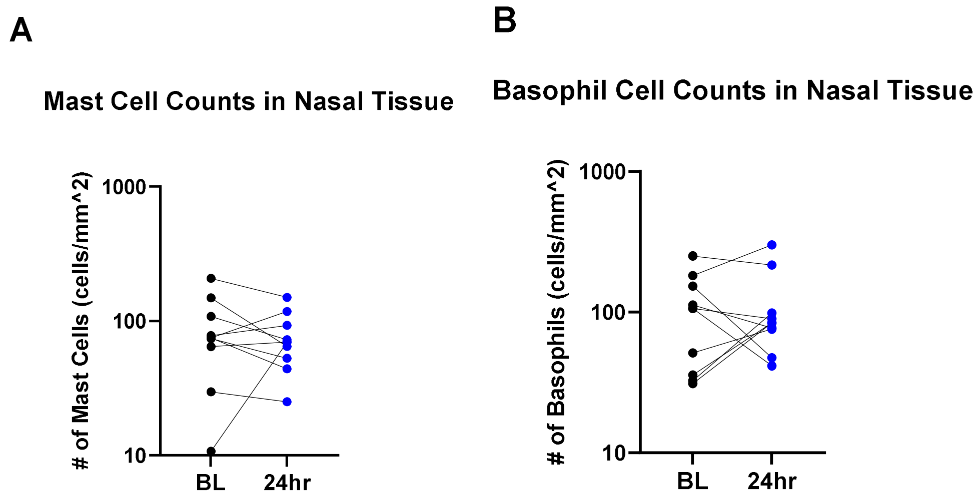


Figure 6: Total number of mast cells and basophils in nasal biopsies in the inferior turbinate 24 hours (24hr) post nasal allergen challenge compared to pre-challenge baseline (BL). Mast cells were measured through IF staining as tryptase-positive cells and basophils as 2D7-positive cells. Raw data values are shown on log10 scale. Mast cell data was non-parametric prior to analysis and was log10 transformed. Data analyzed with repeated measures one-way ANOVA and Tukey's multiple comparisons test.

Effect of nasal allergen challenge on alarmin cytokine levels in nasal lavage

The effect of nasal allergen challenge on IL-25 and TSLP levels in nasal lavage is shown in **Figure 7**. In the nasal lavage fluid of patients with allergic rhinitis and mild asthma, although median TSLP and IL-25 levels were numerically elevated at 7 hr post-challenge, there was no statistically significant change in TSLP levels or IL-25 levels from baseline to 7hr – or 24 hr-post NAC. IL-33 was below the level of detection in many nasal lavage samples and could not be analyzed.

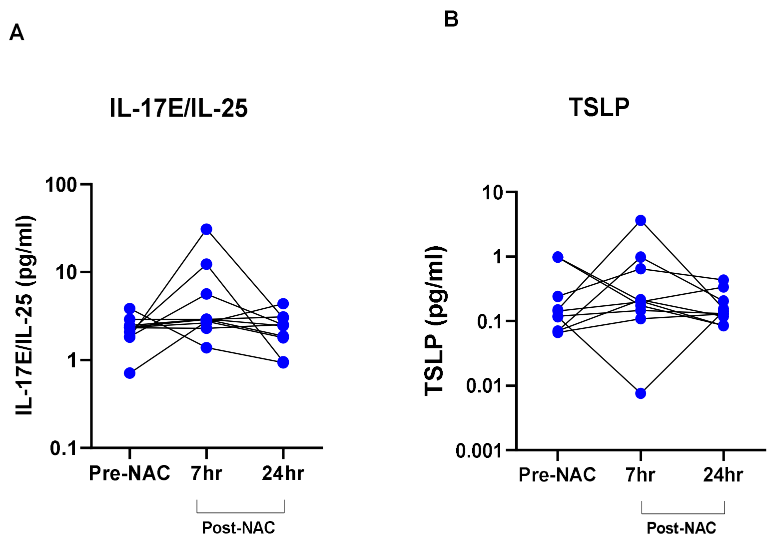


Figure 7: Level of alarmins IL-25 (A) and TSLP (B) in nasal lavage at 7 hours (7hr), and 24 hours (24hr) post nasal allergen challenge (NAC) compared to pre-challenge. Raw data values are shown on log10 scale. Data was non-parametric prior to analysis and was log10 transformed. Data analyzed using 2-way ANOVA with Bonferroni's multiple comparisons test.

Effect of nasal allergen challenge on IL-33 expression in nasal tissue

In nasal biopsies, there was a significant decrease in the number of cells expressing IL-33 (identified as cells immunopositive for IL-33) 24h post-NAC compared to pre-challenge baseline ($p=0.0288$). However, there was no significant change in the levels of mast cells expressing IL-33 (identified as cells immunopositive for tryptase and IL-33) at 24h post-NAC compared to pre-challenge baseline (p>0.05). The levels of basophils co-expressing IL-33 were too low within biopsies and could therefore not be statistically analyzed (**Figure 8).**

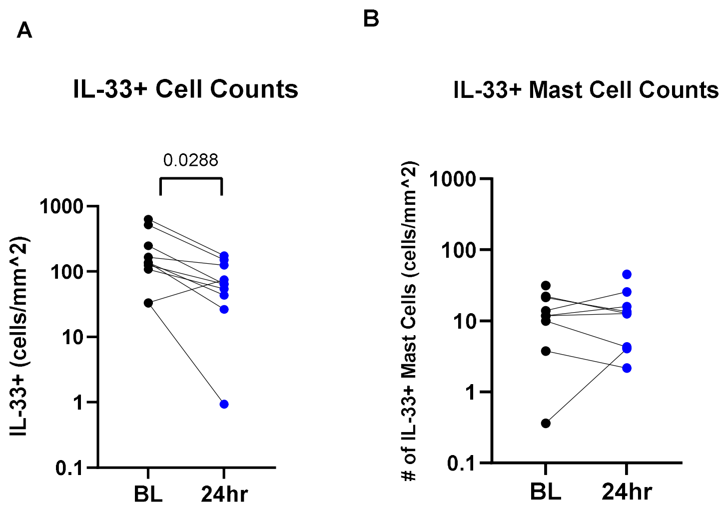


Figure 8: Total number of cells (A) and mast cells (tryptase-positive cells) (B) immunopositive for IL-33 per mm^2 of inferior turbinate, measured 24 hours (24hr) post nasal allergen challenge compared to pre-challenge baseline (BL). Raw data values are shown on log10 scale. Data was non-parametric prior to analysis and log10 transformed. Data analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test. Biopsies collected 24 hrs post-NAC.

<u>Effect of nasal allergen challenge on IL-25 expression in nasal tissue</u>

In nasal biopsies, there was no significant change in the total number of cells expressing IL-25 (identified as cells immunopositive for IL-25) at 24 hours post-NAC compared to baseline (p>0.05). Similarly, there was no significant change in the number of mast cells expressing IL-25 (identified as cells immunopositive for tryptase and IL-25) (p>0.05), or in the number of basophils expressing IL-25 (identified as cells immunopositive for 2D7 and IL-25) 24h post-

NAC (p>0.05). The effect of nasal allergen challenge on cellular expression of IL-25 expression is shown in **Figure 9.**

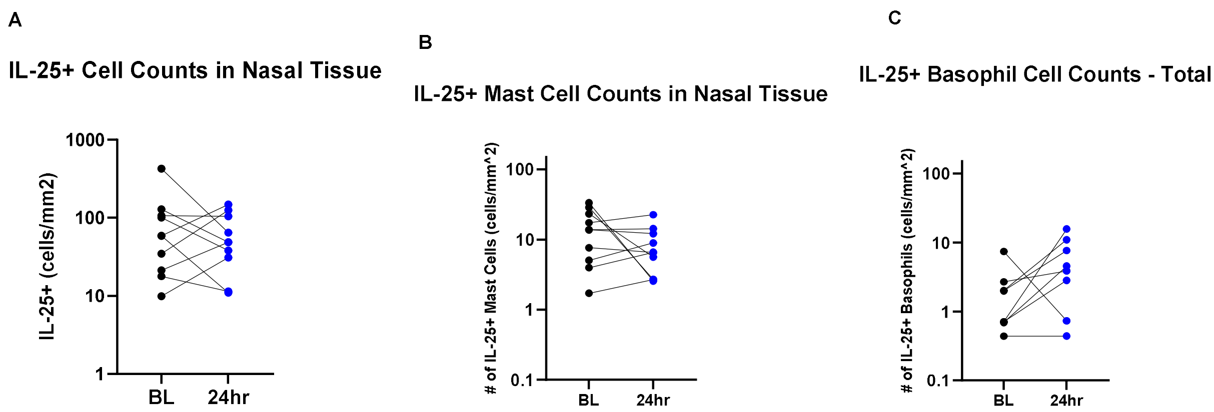


Figure 9: Total number of cells (A), mast cells (tryptase-positive cells) (B) and basophils (basogranulin-positive cells) (C) immunopositive for IL-25 per mm^2 in the inferior turbinate measured 24 hours (24hr) post nasal allergen challenge compared to pre-challenge baseline (BL). Raw data values on log10 scale are shown. Biopsies collected 24 hrs post-NAC. Data for IL-25+2D7+ stain was non-parametric prior to analysis and log-transformed. All data analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test.

<u>Effect of nasal allergen challenge on TSLP expression in nasal tissue</u>

In nasal biopsies, there was no significant change in the number of cells expressing TSLP (identified as cells immunopositive for TSLP) post-NAC compared to pre-challenge baseline (p>0.05). Similarly, there was no significant change in the number of mast cells expressing TSLP, (identified as cells immunopositive for tryptase and TSLP) or in the number of basophils expressing TSLP (identified as cells immunopositive for 2D7 and TSLP) at 24h post-NAC (p>0.05). The effect of nasal allergen challenge on cell expression of TSLP is shown in **Figure 10.**

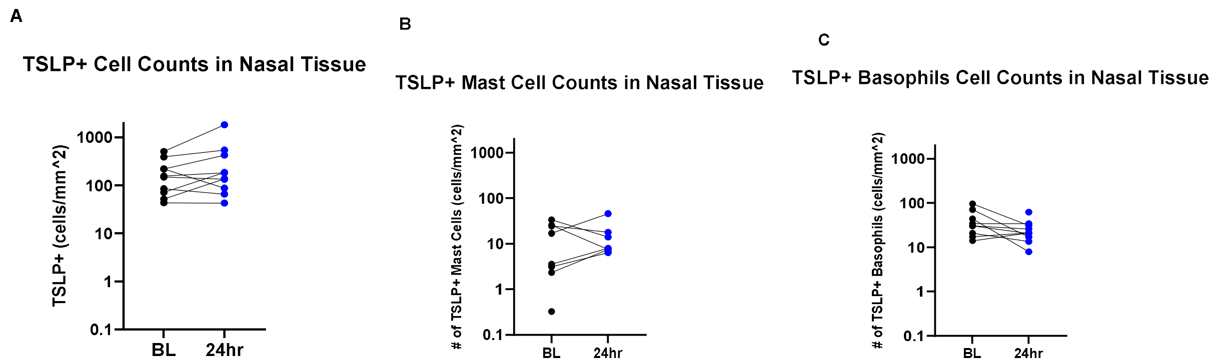


Figure 10: Total number of cells (A), mast cells (tryptase-positive cells) (B) and basophils (basogranulin-positive cells) (C) immunopositive for TSLP per mm^2 in the inferior turbinate measured 24 hours (24hr) post nasal allergen challenge compared to pre-challenge baseline (BL). Raw data values on log10 scale are shown. Biopsies collected 24 hrs post-NAC. Data was non-parametric prior to analysis and log-transformed. All data analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test.

Effect of nasal allergen challenge on cytokine levels in nasal lavage

In nasal lavage, there was an NAC-induced increase from baseline in eotaxin, MCP-1, MDC, MIP-1α, TARC, IL-1α, IL-5, IL-10, IL-13, IL-4, and TNF-α. In addition, eotaxin, MCP-1, MDC, and IL-1α observed trends for increase ($0.05<p<0.1$). In contrast, there was a NAC-induced decrease from baseline in IL-7 ($p=0.0264$). No significant change in IL-25, TSLP, GM-CSF, IL-15, Eotaxin-3, IL-8, IP-10, MCP-4, MIP-1β, IL-12/IL-23P40, IL-16, IL-17, VEGF, IL-2 or IL-6 was observed from baseline levels of expression. NAC-induced effects on cytokines are summarized in **Table 3.**

Table 3: Effect of nasal allergen challenge (NAC) cytokines measured in nasal lavage samples. Values are shown as mean (SD).

Cytokine	Pre-NAC	7 h post-NAC	24 h post-NAC
Eotaxin	36.6 (23.3)	64.2 (52.2) **	61.5 (32.0)
Eotaxin-3	359.5 (811.1)	649.2 (1477.1)	269.2 (254.3)
IL-8	513.0 (368.1)	267.8 (112.0)	693.6 (330.9)
IP-10	993.3 (1136.1)	449.5 (227.4)	769.0 (513.4)
MCP-1	24.2 (8.7)	41.9 (28.2)	35.2 (15.3)**
MCP-4	5.1 (8.1)	3.5 (3.0)	5.1 (4.4)
MDC	28.7 (18.6)	42.2 (34.8)	65.4 (58.0)**
MIP-1α	10.9 (6.1)	15.5 (14.8)	15.0 (9.5)*
MIP-1β	12.9 (17.3)	32.4 (42.2)	46.6 (66.9)
TARC	15.2 (11.9)	57.0 (66.9)	92.6 (115.6)*
GM-CSF	0.12 (0.1)	1.64 (2.5)	0.18 (0.1)
IL-12/IL-23P40	0.53 (0.3)	0.84 (0.9)	0.93 (0.6)
IL-15	0.43 (0.2)	0.32 (0.1)	0.41 (0.1)
IL-16	62.7 (79.8)	71.9 (84.8)	79.1 (71.8)
IL-17	0.38 (0.5)	0.90 (1.7)	0.71 (1.4)
IL-1α	15.8 (10.9)	11.0 (7.3) **	15.2 (10.8)
IL-5	1.5 (1.5)	51.3 (84.1)*	27.8 (57.2)
IL-7	14.0 (7.4)	8.3 (3.2) *	10.3 (2.9)
VEGF	288.0 (124.1)	232.9 (88.8)	236.9 (66.1)
IL-10	0.11 (0.07)	0.11 (0.09)	0.21 (0.10) *
IL-13	1.8 (0.7)	4.2 (4.3) *	3.8 (1.4) *
IL-1β	3.2 (3.3)	1.4 (1.1)	3.2 (2.2)
IL-2	0.26 (0.2)	1.1 (1.3)	0.40 (0.2)
IL-4	0.07 (0.0)	0.58 (0.6) *	0.13 (0.1)
IL-6	1.3 (0.9)	2.8 (2.5)	3.4 (3.0)
TNF-α	0.29 (0.2)	0.47 (0.5)	0.82 (0.4) *

*= $p<0.05$ significant change observed vs pre-allergen baseline

**= $p<0.10$ trends for increase vs pre-allergen baseline

Multivariate Analysis of Chemokines and relation to %Eosinophils

To further determine the effect of chemokines on the percent of eosinophils present in the nasal lavage, multivariate analysis was performed between eotaxin, IL-5, GM-CSF, and the percent of eosinophil data. Eotaxin had a weak to moderately strong association with the percent of eosinophils and demonstrated a trend towards statistical significance (p=0.081). IL-5 and GM-CSF, however, did not have a statistically significant association with eosinophils **(Table 4).**

Table 4: Multivariate analysis between chemokines in nasal lavage and percent of eosinophils

	Coefficient of Determination (r^2)	p-value
Eotaxin	0.218	0.081
IL-5	0.181	0.155
GM-CSF	0.087	0.261

When examining the individual effect of eotaxin on the percent of eosinophils, eotaxin was shown to have a statistically significant and moderately strong association (r^2=0.3799, p=0.0003) with the percent of eosinophils present in the nasal lavage of patients throughout all timepoints, as seen in **Figure 11**.

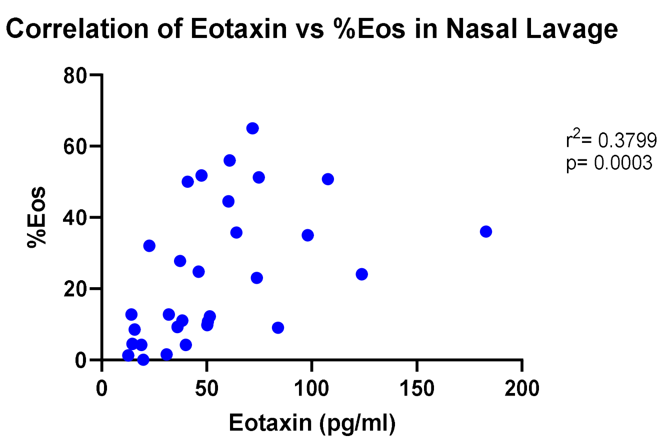


Figure 11: Correlation between eosinophil levels and eotaxin in nasal lavage samples collected during the placebo period (blue) at pre-nasal allergen challenge and 7h and 24h post-NAC timepoints from subjects with mild allergic asthma and allergic rhinitis. Data was non-parametric and analyzed using Spearman's test (r^2=0.3799, p=0.0003). R-values were squared to determine coefficient of determination.

Effect of INCS on allergen-induced airway physiology

When compared to placebo, INCS treatment significantly attenuated allergen-induced changes in PNIF during the late response at 5h and 7h post-NAC ($p<0.05$). TNSS scores from the INCS treatment period were significantly reduced in both the early (2h and 3h, $p<0.05$) and late phase response (5h, 6h, and 7h, $p<0.05$) (**Figure 12**). The levels of PNIF and TNSS after challenge were not completely inhibited by steroid.

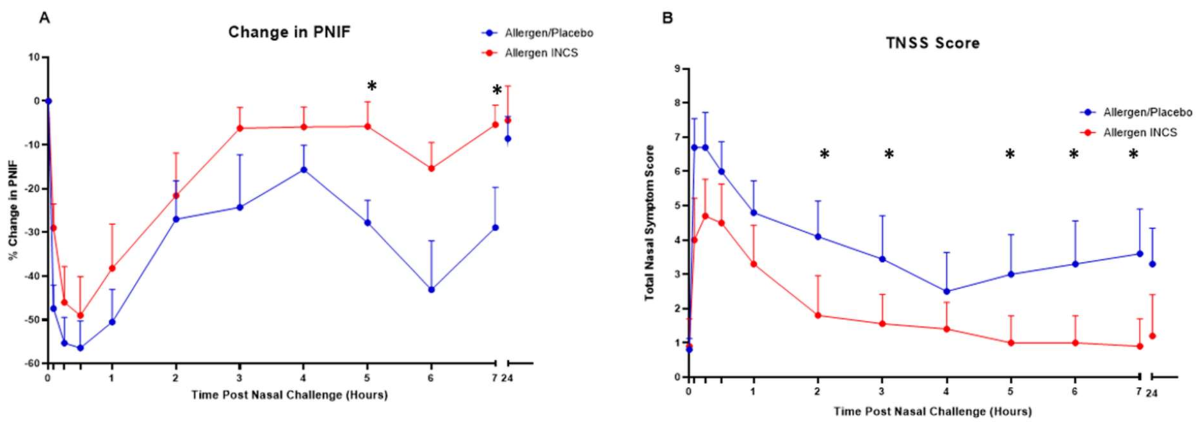


Figure 12: Effect of intranasal corticosteroid (INCS) treatment on upper airway physiology after nasal allergen challenge (NAC) measured by change in peak nasal inspiratory flow (PNIF) and Total Nasal Symptom Score (TNSS). Data were analyzed using Wilcoxon matched-pairs signed rank test (two-tailed) test, (* $p<0.05$, intranasal corticosteroid (INCS) versus placebo).

Effect of INCS on eosinophil levels in nasal lavage before and after nasal allergen challenge

INCS treatment significantly attenuated allergen-induced changes in nasal lavage eosinophils at 24 hours post-NAC ($p=0.0229$). The effect of INCS treatment on eosinophils levels in nasal lavage before and after NAC is shown in **Figure 13.**

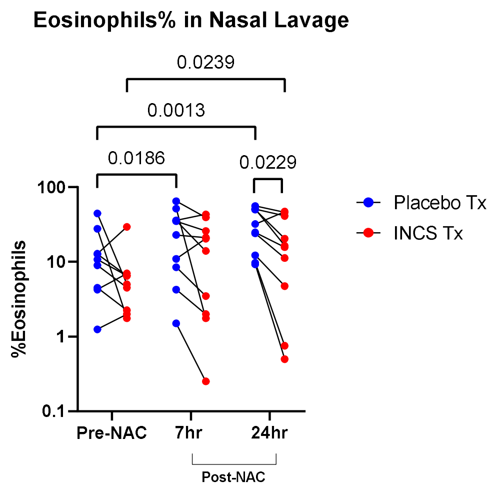


Figure 13: The effects of 14 days treatment of intranasal corticosteroid (INCS) (red) compared to placebo (blue) on percent eosinophils in nasal lavage before and after nasal allergen challenge (NAC). Raw data values are shown on log10 scale. Data analyzed using repeated measures 2-way ANOVA with Bonferroni's multiple comparisons test. Between group analysis of percent eosinophils at 24h was conducted using a paired t-test.

<u>Effect of INCS on mast cell, basophil and eosinophil numbers in nasal tissue after nasal allergen challenge</u>

INCS treatment had no significant effect on the total number of mast cells or basophils in nasal tissue measured 24h post-NAC, or in the number of mast cells or basophils in the epithelium and submucosa regions of nasal tissue ($p>0.05$). The effect of INCS treatment on mast cell, basophil, and eosinophil levels in nasal tissue before and after NAC is shown in **Figure 14.**

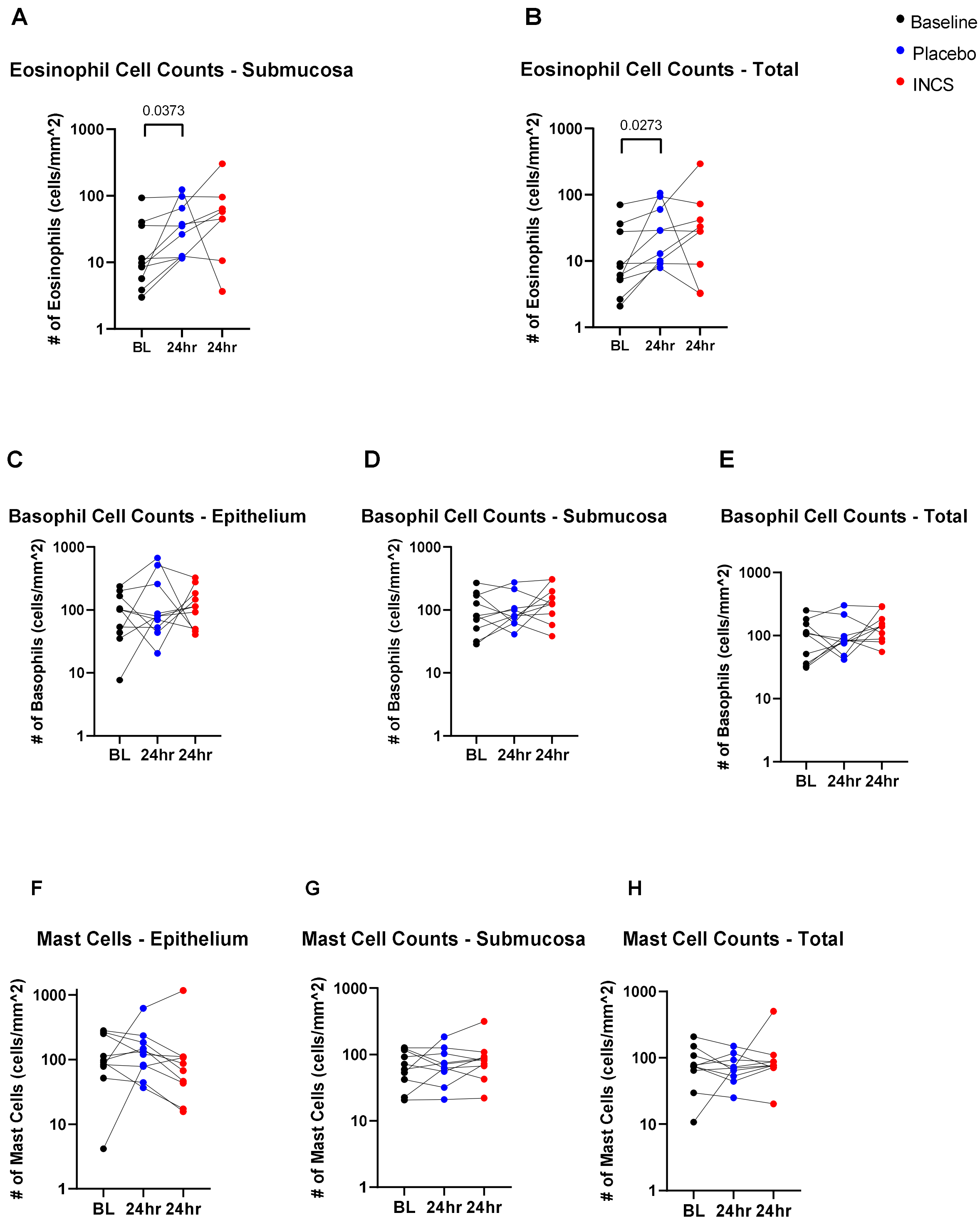


Figure 14: Total number of eosinophils (identified as cells that were eosin-positive and had a bi-lobed nucleus) in tissue epithelium (A), and submucosa (B) mast cells (identified as cells

immunopositive for tryptase) in tissue epithelium (C), submucosa (D), and combined total (E), and basophils (identified as cells immunopositive for 2D7) in tissue epithelium (F) submucosa (G), and combined total (H), per mm^2 in the inferior turbinate 24 hours post nasal allergen challenge (NAC) compared to pre-treatment baseline (BL). Results from patients are reported at pre-treatment baseline and 24h post-NAC using corresponding colour (Black= baseline, Blue = placebo, Red = intranasal corticosteroid (INCS)). Raw data values on log10 scale are shown. Mast cells and eosinophils data was non-parametric prior to analysis and log10 transformed. All data analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test. The number of eosinophils in tissue epithelium was too low for separate statistical analysis.

Effect of INCS on alarmin levels in nasal lavage before and after NAC

Allergen challenge induced a numerical but not statistically significant increase in both IL-25 and TSLP at 7 hr post-challenge in nasal lavage fluid. INCS treatment consistently and numerically lowered IL-25 and TSLP levels in nasal lavage at 7 hr after allergen challenge however this inhibition was not statistically significant ($p=0.42$ and $p=0.28$, respectively). The effect of INCS on alarmin levels in nasal lavage before and after NAC is shown in **Figure 15.**

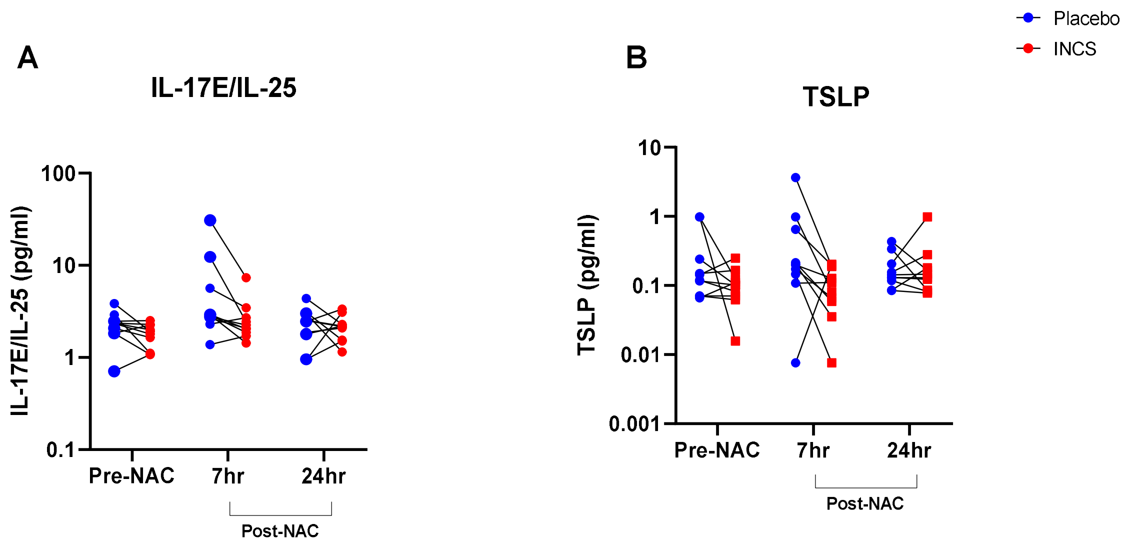


Figure 15: Expression of alarmins IL-25 (A) and TSLP (B) in nasal lavage pre-nasal allergen challenge (NAC) and at 7 hours (7hr) and 24 hours (24hr) post-NAC during placebo (blue) and intranasal corticosteroid (INCS) (red) treatment periods. Raw data values on log10 scale are shown. Data was non-parametric prior to analysis and log10 transformed. All data analyzed using repeated measures two-way ANOVA with Bonferroni multiple comparisons test.

<u>Effect of INCS on cytokines in nasal lavage before and after NAC</u>

In nasal lavage fluid, allergen challenge significantly increased the level of chemokines MIP-1α and TARC (all $p<0.05$) at 24h post-NAC, with trends for increase in eotaxin, MCP-1, and MDC ($p<0.10$). Compared to placebo, INCS treatment had trends for attenuation in eotaxin and MDC ($p<0.10$). The effect of INCS on chemokine levels in nasal lavage before and after NAC is shown in **Figure 16.**

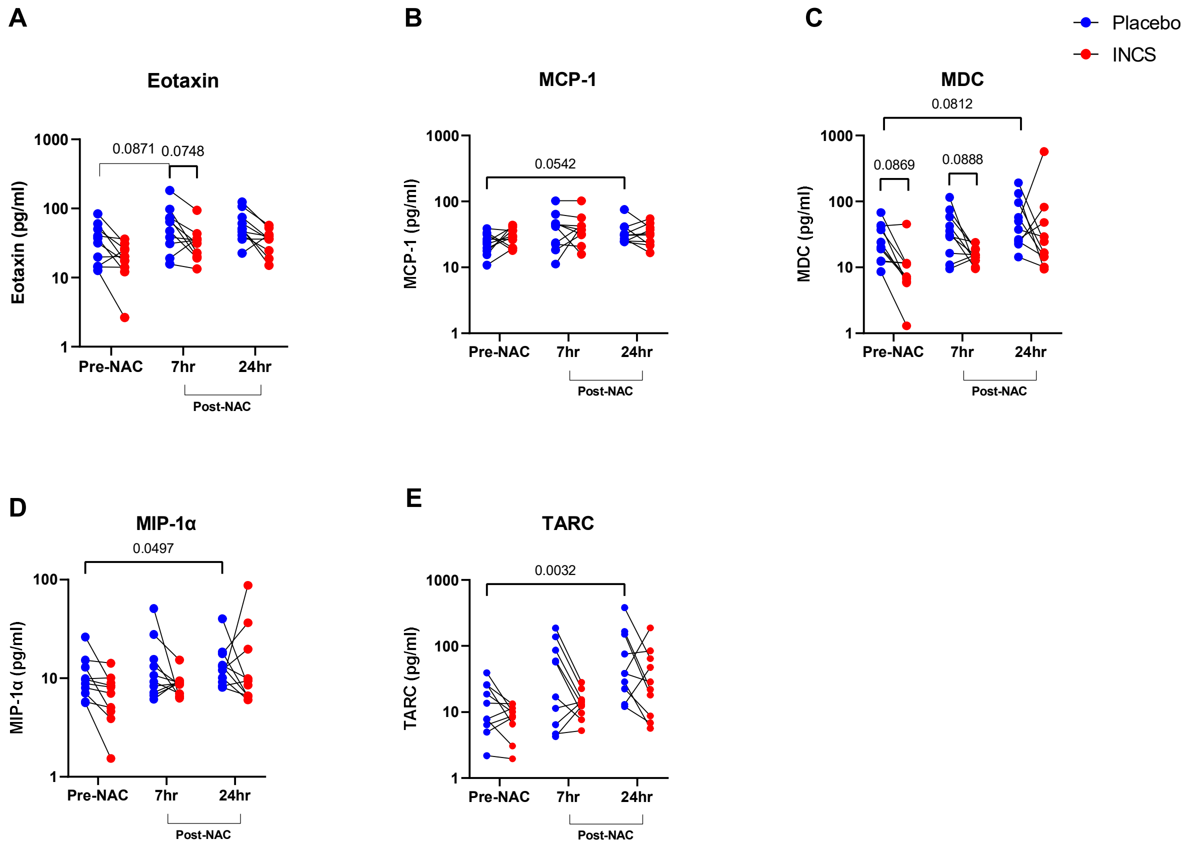


Figure 16: Expression of chemokines eotaxin (A), MCP-1 (B), MDC (C), MIP-1α (D), and TARC (E) in nasal lavage measured at 7 hours (7hr) and 24 hours (24hr) post nasal allergen challenge (NAC) compared to pre-challenge after treatment with placebo (blue) and intranasal corticosteroid (INCS) (red). Raw data values on log10 scale are shown. All data analyzed using repeated measures two-way ANOVA with Bonferroni multiple comparisons test.

In nasal lavage fluid, allergen challenge significantly decreased the level of growth factor IL-7 ($p=0.0264$), at 7h post-NAC and had no significant effect on GM-CSF ($p>0.05$). Compared to placebo, INCS treatment had no significant attenuation for either growth factor but showed a trend for attenuation in GM-CSF ($p<0.10$). The effect of INCS on growth factor levels in nasal lavage before and after NAC is shown in **Figure 17.**

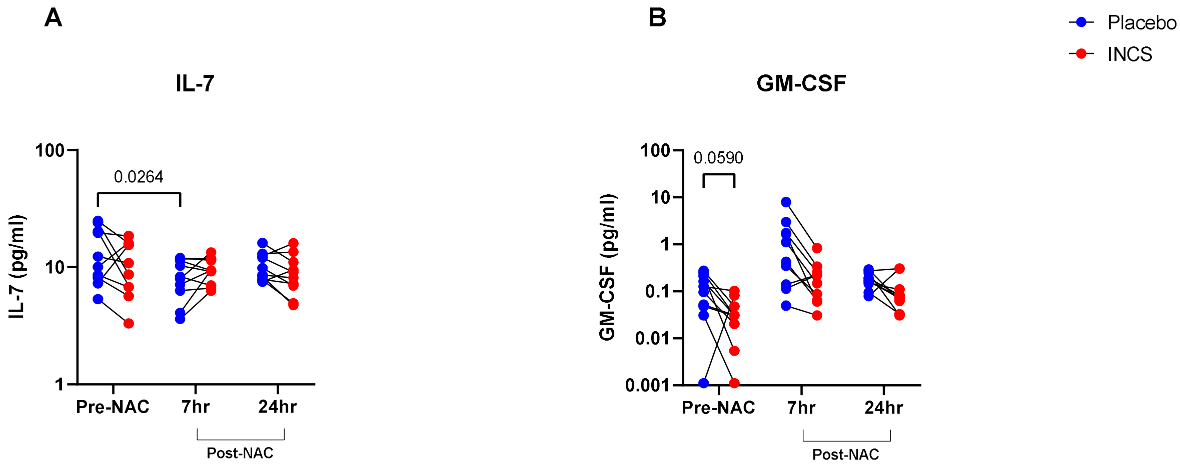


Figure 17: Expression of growth factors IL-7 (A), and GM-CSF (B) in nasal lavage measured at 7 hours (7hr) and 24 hours (24hr) post nasal allergen challenge (NAC) compared to pre-challenge after treatment with placebo (blue) and intranasal corticosteroid (INCS) (red). Raw data values on log10 scale are shown. All data analyzed using repeated measures two-way ANOVA with Bonferroni multiple comparisons test.

In nasal lavage fluid, allergen challenge significantly increased the level of T1 cytokines IL-10 ($p=0.0272$) and TNF-α ($p=0.0011$). Compared to placebo, INCS treatment had trends for attenuation in TNF-α ($p<0.10$). The effect of INCS on T1 cytokine levels in nasal lavage before and after NAC is shown in **Figure 18.**

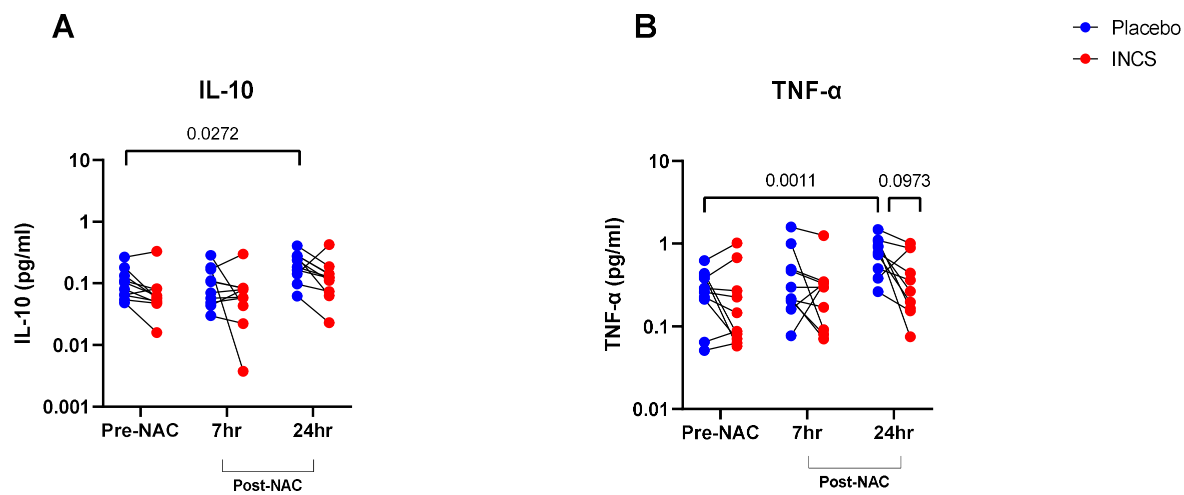


Figure 18: Expression of T1 cytokines IL-10 (A), and TNF-α (B) in nasal lavage measured at 7 hours (7hr) and 24 hours (24hr) post nasal allergen challenge (NAC) compared to pre-challenge after treatment with placebo (blue) and intranasal corticosteroid (INCS) (red). IL-10 data was non-parametric prior to analysis and was log-transformed. Raw data values on log10 scale are shown. All data analyzed using repeated measures two-way ANOVA with Bonferroni multiple comparisons test.

In nasal lavage fluid, allergen challenge significantly increased the level of T2 cytokines IL-4, IL-5 and IL-13 (all $p<0.05$). Compared to placebo, INCS treatment attenuated allergen-induced increase of T2 cytokines IL-4, IL-5 and IL-13 ($p<0.05$). The effect of INCS on T2 cytokine levels in nasal lavage before and after NAC is shown in **Figure 19.**

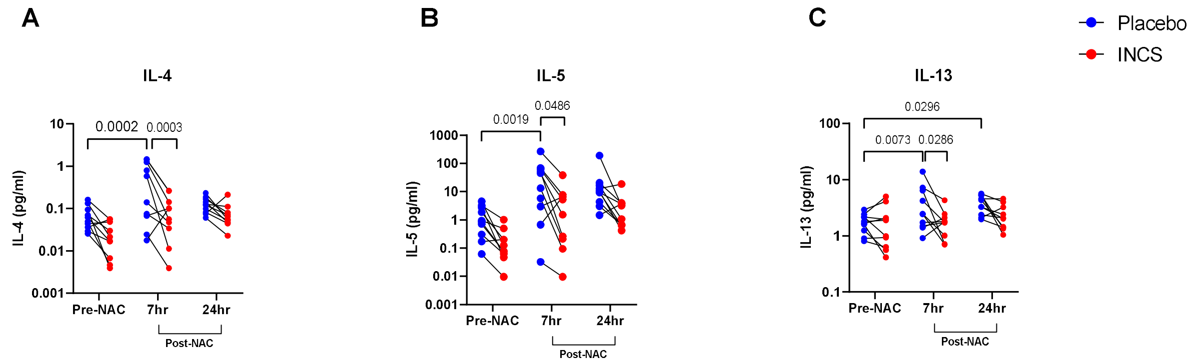


Figure 19: Expression of T2 cytokines IL-4 (A), IL-5 (B), and IL-13 (C) in nasal lavage measured at 7 hours (7hr) and 24 hours (24hr) post nasal allergen challenge (NAC) compared to pre-challenge after treatment with placebo (blue) and intranasal corticosteroid (INCS) (red). Raw data values on log10 scale are shown. All data analyzed using repeated measures two-way ANOVA with Bonferroni multiple comparisons test.

In nasal lavage fluid there was a trend for a decrease in IL-1α ($p<0.10$). Compared to placebo, INCS treatment significantly attenuated IL-15 at 24h post-NAC ($p=0.0113$) effect of INCS on pro-inflammatory cytokine levels in nasal lavage before and after NAC is shown in **Figure 20.**

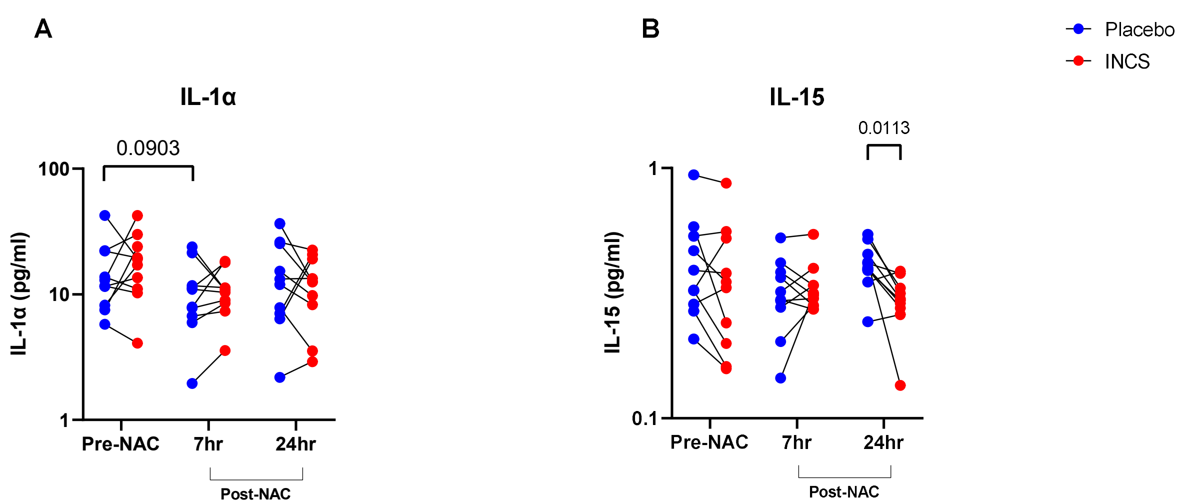
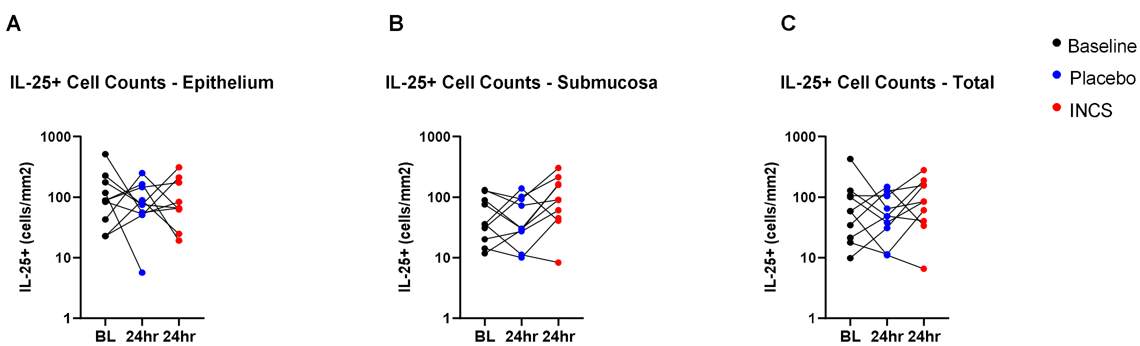


Figure 20: Expression of pro-inflammatory cytokines IL-1α (A), and IL-15 (B) in nasal lavage measured at 7 hours (7hr) and 24 hours (24hr) post nasal allergen challenge (NAC) compared to pre-challenge after treatment with placebo (blue) and intranasal corticosteroid (INCS) (red). Raw data values on log10 scale are shown. All data analyzed using repeated measures two-way ANOVA with Bonferroni multiple comparisons test.

Effect of INCS on IL-25, TSLP, and IL-33 numbers in nasal tissue after nasal allergen challenge

INCS treatment had no significant effect on the total number of cells immunoreactive for IL-25 or TSLP in nasal tissue measured 24h post-NAC in the epithelium, submucosa, or combined total regions of nasal tissue ($p>0.05$). There was a significant increase in cells immunoreactive for IL-33 in the submucosa regions (Figure 21H). Allergen challenge significantly decreased the total number of cells expressing IL-33 in the combined total regions of nasal biopsies (Figure 21I) ($p<0.05$). The effect of INCS treatment on cells expressing IL-25, TSLP and IL-33 in nasal tissue compared to placebo is shown in **Figure 21.**



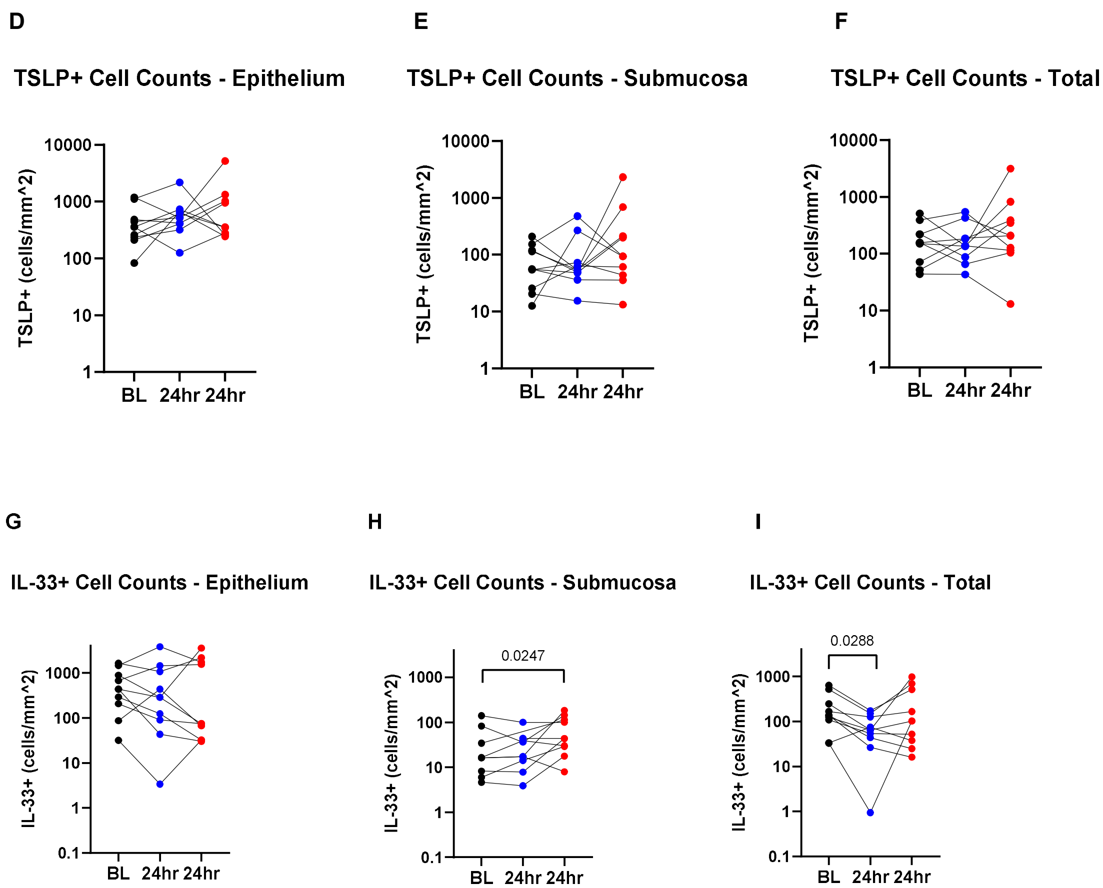
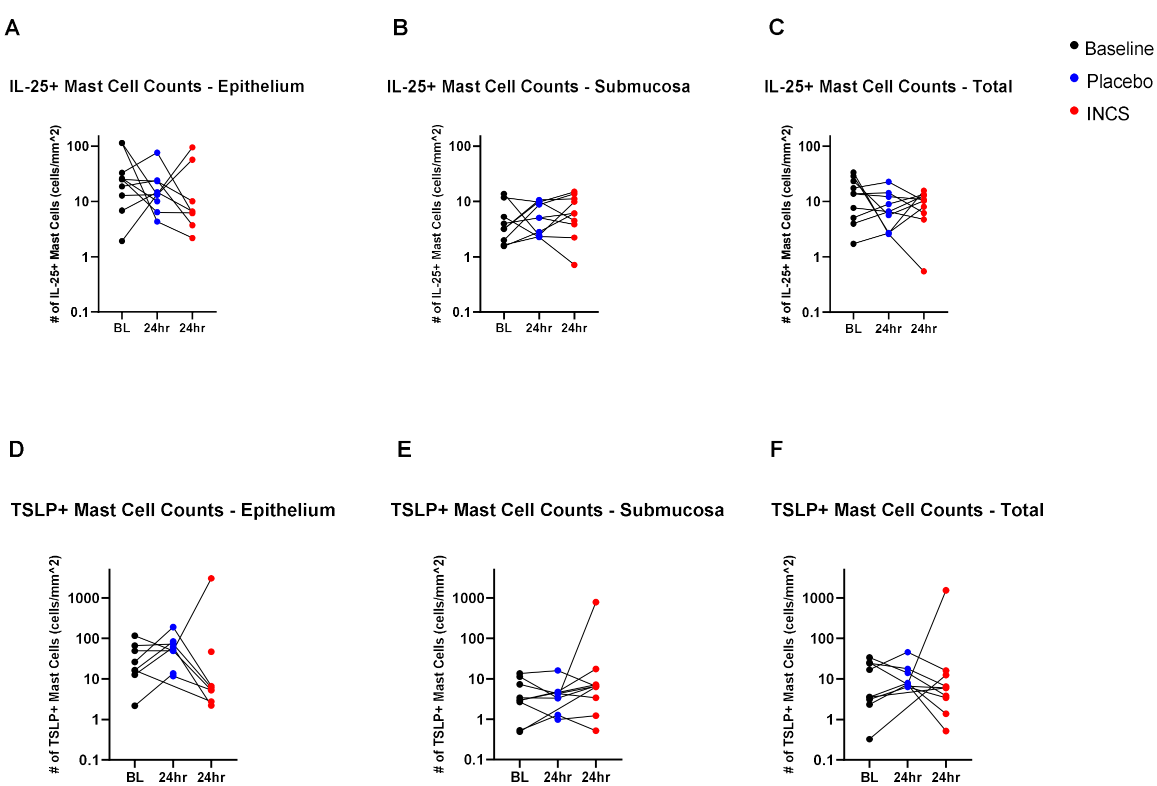


Figure 21: Total number of cells immunopositive for IL-25 in tissue epithelium (A), submucosa (B) and combined total regions (C); total number of cells immunopositive for TSLP in tissue epithelium (D), submucosa (E), and combined total regions (F); total number of cells immunopositive for IL-33 in tissue epithelium (G), submucosa (H), and combined total regions (I) per mm^2 in the inferior turbinate 24 hours (24hr) post nasal allergen challenge (NAC) compared to pre-treatment baseline (BL). Results are reported using corresponding colour (Black= baseline, Blue = placebo, Red = intranasal corticosteroid (INCS)). Raw data values on log10 scale are shown. TSLP (epithelium, submucosa, and combined regions) and IL-33 (epithelium, submucosa, and combined regions) data was non-parametric prior to analysis and

log10 transformed. All data analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test.

<u>Effect of INCS on mast cells expressing IL-25, TSLP, and IL-33 in nasal tissue after nasal allergen challenge</u>

INCS treatment had no significant effect on the total number of mast cells immunoreactive for IL-25, TSLP, or IL-33 in nasal tissue measured 24h post-NAC in the epithelium, submucosa, or combined total regions of nasal tissue ($p>0.05$). There was no significant increase in mast cells immunoreactive for the alarmins in the epithelium, submucosa, or combined total regions (**Figure 22**). The effect of INCS treatment on mast cells expressing IL-25, TSLP and IL-33 in nasal tissue compared to placebo is shown in **Figure 22.**



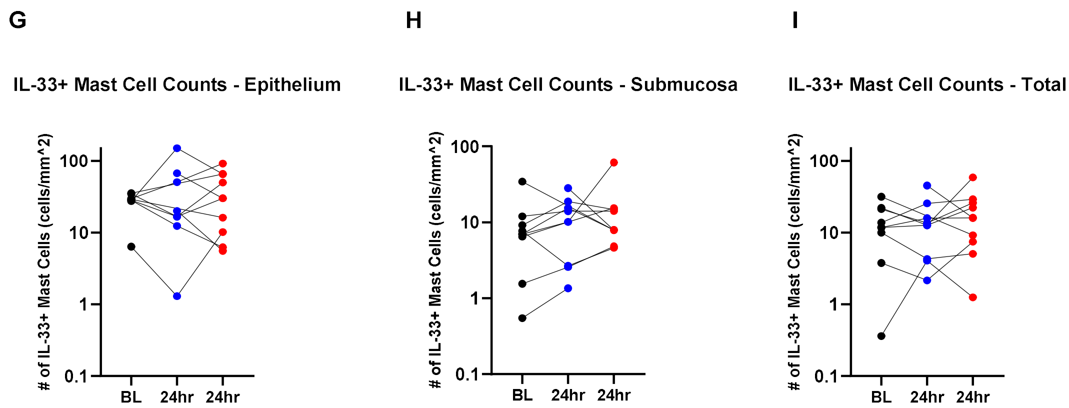


Figure 22: Total number of mast cells positive for IL-25 (identified as cells immunopositive for IL-25 and tryptase) in tissue epithelium (A), submucosa (B) and combined total regions (C); total number of mast cells positive for TSLP (identified as cells immunopositive for TSLP and tryptase) in tissue epithelium (D), submucosa (E), and combined total regions (F); total number of mast cells positive for IL-33 (identified as cells immunopositive for IL-33 and tryptase) in tissue epithelium (G), submucosa (H), and combined total regions (I) per mm^2 in the inferior turbinate 24 hours (24hr) post-nasal allergen challenge (NAC) compared to pre-treatment baseline (BL). Results are reported using corresponding colour (Black= baseline, Blue = placebo, Red = intranasal corticosteroid (INCS)). Raw data values on log10 scale are shown. IL-25 (epithelium and submucosa), TSLP (epithelium, submucosa, and combined regions) and IL-33 (epithelium and submucosa) data was non-parametric prior to analysis and log10 transformed. All data analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test.

Effect of INCS on basophils expressing IL-25 and TSLP in nasal tissue after nasal allergen challenge

INCS treatment had no significant effect on the total number of basophils immunoreactive for IL-25 or TSLP in nasal tissue measured 24h post-NAC in the epithelium, submucosa, or combined total regions of nasal tissue ($p>0.05$). There was no significant increase in basophils immunoreactive for the alarmins in the epithelium, submucosa, or combined total regions (**Figure 23**). The effect of INCS treatment on mast cells expressing IL-25 or TSLP in nasal tissue compared to placebo is shown in **Figure 23.**

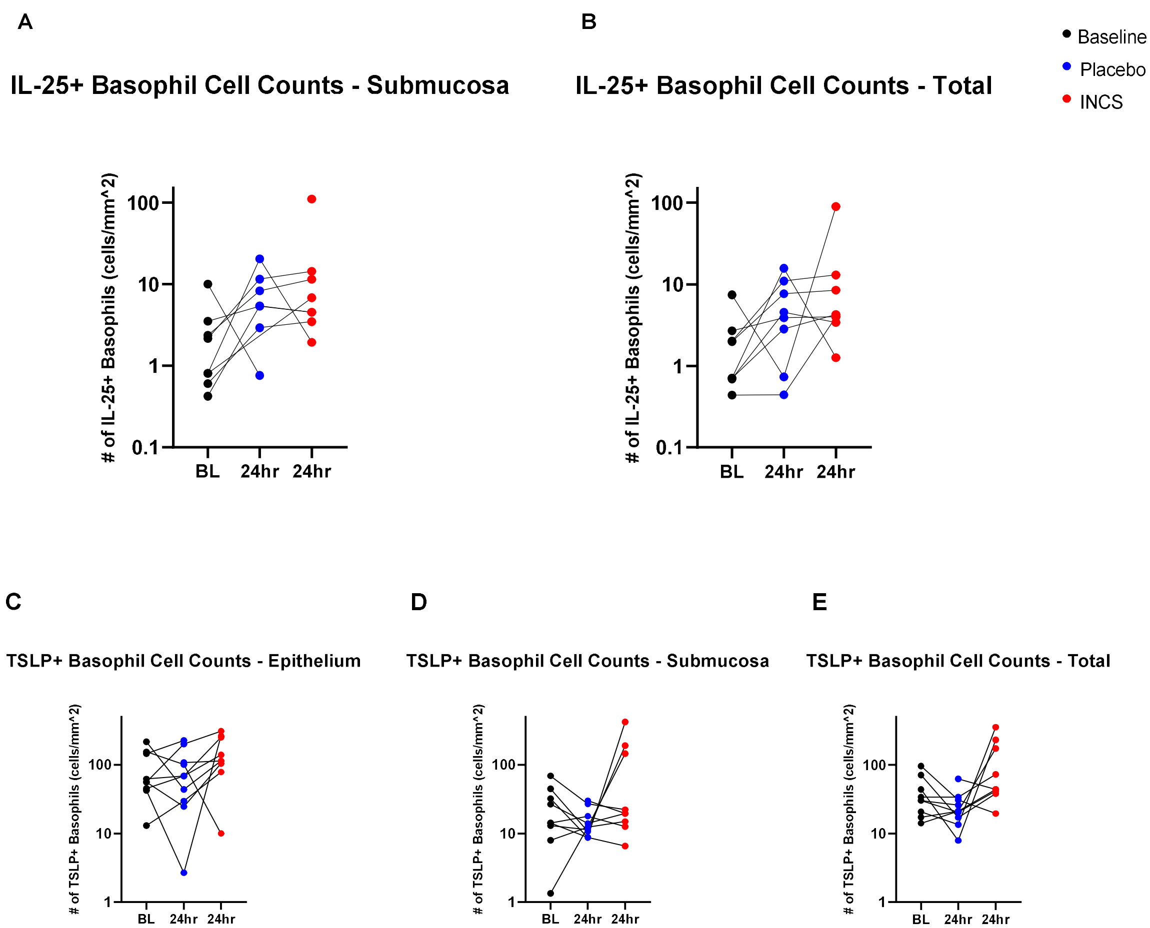


Figure 23: Total number of basophils positive for IL-25 (identified as cells immunopositive for IL-25 and basogranulin) in tissue epithelium (A), submucosa (B) and combined total regions (C);

total number of basophils positive for TSLP (identified as cells immunopositive for TSLP and basogranulin) in tissue epithelium (D), submucosa (E) per mm^2 in the inferior turbinate 24 hours (24hr) post-nasal allergen challenge (NAC) compared to pre-treatment baseline (BL). Results are reported using corresponding colour (Black= baseline, Blue = placebo, Red = intranasal corticosteroid (INCS)). Raw data values on log10 scale are shown. Basophils expressing IL-25 (submucosa and combined total regions) and basophils expressing TSLP (submucosa and combined total regions) data was non-parametric prior to analysis and log10 transformed. Values for basophils expressing IL-33 and IL-25+ basophils in the epithelium were too low to be statistically analyzed separately. All data analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test.

Relationship between eosinophil levels and alarmin levels (TSLP and IL-25) in nasal samples

The relationship between levels of eosinophils and alarmins in nasal lavage were analyzed using both multivariate analysis and individual correlations. There was no significant relationship between the alarmins and the percent of eosinophils in the multivariate analysis in both treatment arms across all timepoints ($p>0.05$) (**Table 5**).

Table 5: Multivariate analysis between alarmins in nasal lavage and percent of eosinophils

	Coefficient of Determination (r^2)	p-value
IL-25	0.044	0.127
IL-33	-(0.001)	0.804
TSLP	0.011	0.537

To assess for a relationship between percent eosinophil levels with alarmin levels in nasal lavage, correlations were drawn between each separate alarmin with percent eosinophils with all study samples. A numerically significant but weak positive correlation exists between the % of eosinophils and IL-25 levels (r^2=0.1041, p=0.0820) in nasal lavage fluid of subjects with mild allergic asthma and allergic rhinitis during the placebo period. There was no significant relationship between the levels of IL-25 during the placebo period with percent eosinophils, nor was there any significance between TSLP levels and percent eosinophil at any treatment period. The relationship between eosinophil levels and alarmin levels in nasal lavage with each treatment and after NAC is shown in **Figure 24.**

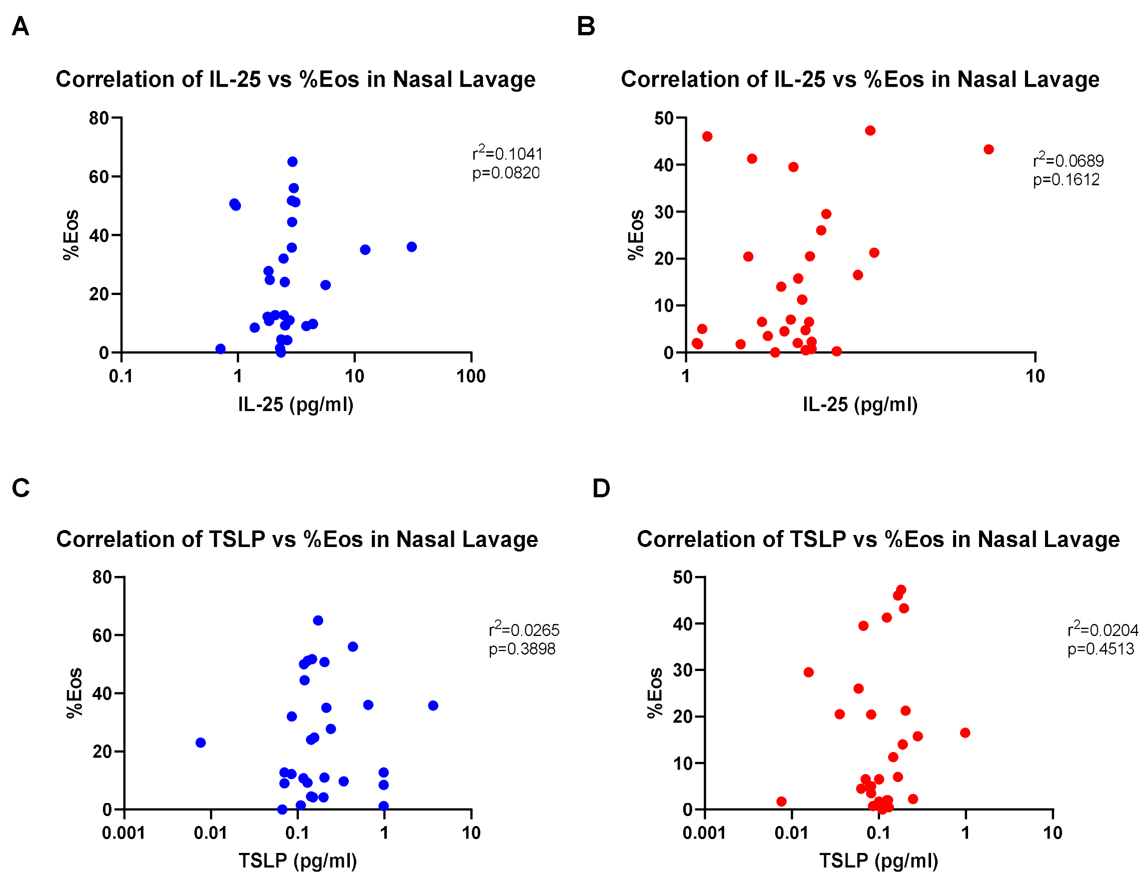


Figure 24: Correlation between percent eosinophils and IL-25 during placebo (blue) (A) and INCS (red) treatment period (B); between percent eosinophils in nasal lavage and TSLP during placebo (C) and intranasal corticosteroid (INCS) treatment period (D). Data was non-parametric and analyzed using Spearman's test. R-values were squared to determine coefficient of determination.

Relationship between eosinophil levels and type 2 cytokines (IL-4, IL-5, and IL-13) in nasal samples

The relationship between eosinophils and T2 cytokines in nasal lavage were analyzed using both multivariate analysis and individual correlations. IL-4 (r^2=0.160, p=0.043) and IL-5 (r^2=0.177, p=0.022) had positive, moderate, and significant association between the alarmins and the percent of eosinophils in the multivariate analysis (**Table 6**). IL-13 and GM-CSF did not demonstrate any significant association.

Table 6: Multivariate analysis between eosinophils and T2 cytokines

	Coefficient of Deterrmination (r^2)	p-value
IL-4	**0.160**	**0.043**
IL-5	**0.177**	**0.022**
IL-13	0.109	0.797
GM-CSF	0.099	0.104

To further assess for a relationship between percent eosinophil levels with type 2 cytokines in nasal lavage, correlations were drawn between each separate cytokine at each treatment period with percent eosinophils. Significant and moderate to strong correlation exists between the % of eosinophils and IL-4 during placebo (r^2=0.2394, p=0.0061) and INCS (r^2=0.2125, p=0.0104) periods. Even stronger statistical significance is present for the relationship between IL-5 levels and percent eosinophils at placebo (r^2=0.5744, p<0.0001) and INCS (r^2=0.3672, p=0.0004) treatments in nasal lavage fluid of subjects with mild allergic asthma and allergic rhinitis. A weaker and significant relationship exists between the percent of eosinophils and IL-13 (r^2=0.2533, p=0.0044) levels in nasal lavage fluid of subjects at the placebo treatment. The relationship between eosinophil levels and type 2 cytokine levels in nasal lavage before and after NAC is shown in **Figure 25.**

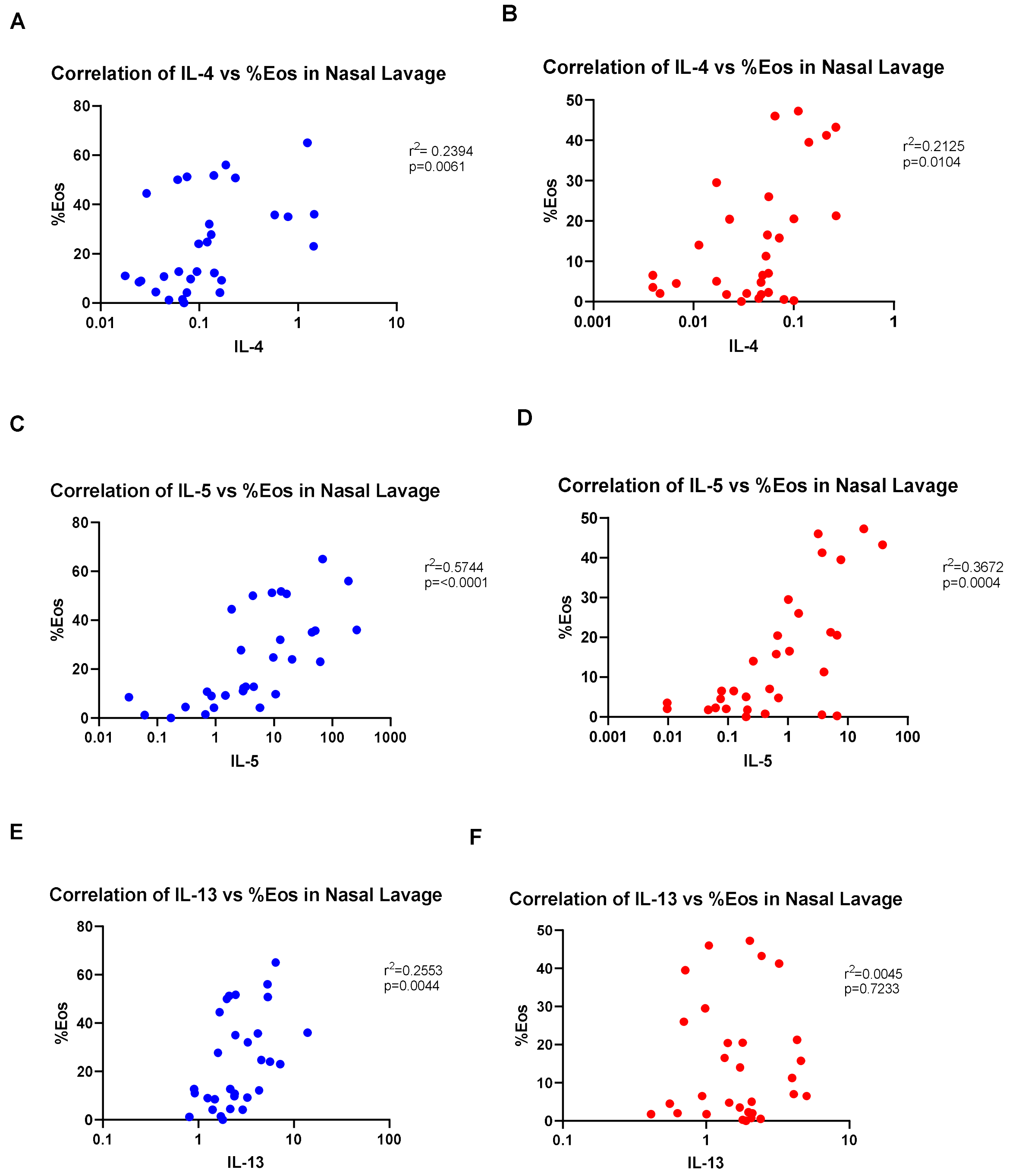


Figure 25 Correlation between percent eosinophils in nasal lavage and IL-4 during placebo (blue) (A) and intranasal corticosteroid (INCS) (red) treatment period (B), between percent eosinophils in nasal lavage and IL-5 during placebo (C) and INCS treatment (D), between

percent eosinophil levels in nasal lavage during placebo (E) and INCS treatment period (F) from subjects with mild allergic asthma and allergic rhinitis. Data was non-parametric and analyzed using Spearman's test. R-values were squared to determine coefficient of determination.

CHAPTER 4: DISCUSSION

There are numerous pathophysiological consequences of inflammation of the upper airways in allergic rhinitis. These include the compromised epithelial integrity and increased numbers of inflammatory cells that are hallmark indications of rhinitis-induced damage in the nasal mucosa (189). The first aim of this study was to investigate the effects of intranasal allergen challenge on cellular inflammation and protein expression of alarmins IL-25, IL-33 and TSLP in the nasal mucosa and nasal lavage of subjects with mild allergic asthma and allergic rhinitis.

NAC-induced Changes on Upper Airway Physiology and Symptoms

During this study, nasal allergen challenge caused a decrease in PNIF and an increase in TNSS. These findings align with existing literature, reproducing what previous studies have shown. As an example, cat allergen exposure has been shown to induce significant changes in TNSS between 5 minutes to 4 hours post-challenge when compared to diluent, in a dose-dependent manner. Similarly, NAC induced a significant drop in PNIF between a time course of 5 minutes to 8 hours post-lavage when compared to diluent (190).

NAC-induced Changes in Cellular Inflammation

This study has demonstrated that nasal allergen challenge increased eosinophil levels at 7h and 24h post-challenge in nasal lavage, and in nasal tissue at 24h post-challenge. These findings are consistent with the change of eosinophil levels in other studies using nasal allergen challenge in allergic subjects. Recent studies report similar findings in which eosinophil levels in nasal lavage increase post-NAC during the late asthmatic response. Participants with allergic rhinitis and/or asthma reported an increase in nasal lavage eosinophils and sputum eosinophils 6 hours post-nasal provocation test when challenged with *Dermatophagoides pteronyssinus* (191). Similarly, allergic participants sensitized to Bermuda grass have reported significantly increased levels of nasal lavage eosinophils at 1 and 6 hours post-NAC (192). Recently published studies also support our findings from the nasal tissues. In nasal biopsies, repetitive NAC results in tissue eosinophilia, and augmented IL-5 expression (193). Other studies have investigated eosinophilic infiltration in the allergic upper airways. The activated and total number of eosinophils is significantly heightened in the epithelium and laminal propria of nasal biopsies when measured in the early and late-phase allergic reaction (194). Taken together, these findings, along with our current study results, confirm that allergen exposure induces an inflammatory type 2 eosinophilic response in the upper airways. Subsequently, this has allowed us to examine the effect of NAC-induced AR on the inflammatory profiles of the upper airways in patients with mild allergic asthma and allergic rhinitis.

In the current study, there was no significant increase in mast cells or basophils in nasal tissue at 24 hours post-NAC. These findings are in contrast to what has already been published regarding mast cell levels in the nasal mucosa post-allergen challenge. When examining the immunohistology of the nasal airways, patients with seasonal allergic rhinitis who have natural exposure to environmental aeroallergens show increases in tryptase-only positive mast cells within the nasal epithelium (195). Other studies have reported an increase in basophil accumulation in the epithelium and lamina propria within the first hour post-allergen provocation, and prolonged increase of basophils in the lamina propria at 24h and 1 week post-allergen provocation (196). Therefore, natural and continuous allergen exposure during the allergy season may provide a more realistic representation of mast cell activity in the airways over allergen challenges that induce local allergen provocation. Another possible reason for the discrepancies between our findings and what has been previously reported could be due to the differences in study design. Our study collected nasal biopsies only at 24h post-NAC, therefore only the late-allergic response was assessed in the nasal tissue. This makes it difficult to determine whether the epithelium or submucosa undergo any inflammatory changes during the early allergic response. If nasal biopsies were collected at timepoints during both the early and late allergic response, additional comparisons between the early and late phase allergic response could be made.

Differences in the anatomy/ histology across the respiratory mucosa may explain why contrasting results are seen between cellular inflammation in the upper and lower airways. Existing evidence suggests that bronchial tissue undergoes a significantly higher level of basophil influx in the subepithlium compared to that of nasal tissue (197). Similarly, mast cells with the tryptase-only phenotype are known as the least common cell type in the nasal tissue, with the most abundant being tryptase-chymase mast cells. In bronchial tissue, tryptase-only mast cells are the most abundant cell type amongst atopic patients with allergic rhinitis (197). The current study would benefit from examining all mast cell phenotypes in the nasal tissue to better account for the mast cell profile in the upper airways.

NAC-induced Changes in T2 Cytokines, and Effect on Alarmins

This study has extended the current literature by showing changes in other chemokines, growth factors, T1 cytokines, T2 cytokines, and pro-inflammatory cytokines that have not been previously reported pre- and post-NAC. While the cytokines analysis have been reported previously (151), both analyses of data were derived from the same clinical trial. Of particular relevance to the current study is the allergen-induced effects on T2 cytokines. In nasal lavage, the data from the present study shows a significant allergen-induced increase of IL-4 and IL-5 at 7h post-NAC compared to baseline, and a significant increase in IL-13 at 7h and 24h post-NAC compared to baseline. These findings suggest that IL-4, IL-5, and IL-13 contribute to the T2 allergic response and are consistent with previously published literature. Studies using a similar crossover study design and nasal allergen challenge model demonstrate a significant increase in nasal lavage fluid levels in IL-5 from 6h to 24h compared to baseline, and in IL-13 from 4h to 24h post-NAC compared to baseline (198). Correspondingly in other studies, nasal biopsies collected 6h post-NAC show an increase in the number of cells that express IL-4 and IL-5 mRNA. The majority of these cells positive for the mRNA expression were eosinophils, and immunohistology reveals that expression of IL-4 was also expressed at the protein level (199). As such, the current study has confirmed that the nasal allergen challenge effectively induced a T2 allergic response, and successfully upregulated the expression of classical T2 cytokines in nasal lavage samples.

IL-25 levels did not increase either at 7h or 24h post-challenge. Previously published data has reported no change from baseline in IL-25 immunoreactivity in epidermal cells of bronchial and skin biopsies post-allergen inhalation challenge, but there was a significantly higher expression of IL-25 immunoreactivity in the submucosa (61). TSLP was also not significantly changed by nasal allergen challenge in either nasal lavage or nasal tissue in our study, although both IL-25 and TSLP showed trends to be higher at 7h post-challenge compared to baseline. IL-33 protein levels in nasal lavage were below the level of detection and could therefore not be measured. Interestingly, IL-33 expression in nasal tissue decreased post-challenge. These findings are in contrast to current literature in which bronchial allergen challenge has been shown to induce an increase of alarmin expression in the respiratory mucosa (62,200). Specifically, IL-33, IL-25, and their respective receptors are more highly expressed post-allergen challenge in bronchial biopsies (200). Additionally studies examining bronchial biopsies demonstrate similar outcomes regarding bronchial mucosal expression of TSLP in asthmatic airways, with increased levels of TSLP mRNA in both the epithelium and submucosa of asthmatic participants compared to controls (107). Bronchoalveolar lavage fluid collected from asthmatic participants demonstrate a significantly increased expression of IL-33 and TSLP, but not IL-25, when compared to controls (157). TSLP expression in skin is present post-allergen exposure in allergic patients, whereas the role of dendritic cells in the allergic response was suggested by TSLPR co-localized with CD11c+ dendritic cells in the early- and late-phase response (201). Further contribution of dendritic cells in the alarmin-induced inflammation is in cells observing co-expression of TSLPR and dendritic cell antigens (201). The lack of significant allergen-induced increase in alarmins in nasal tissue in the present study may be due to our methodology, or the study design with single timepoint of biopsy collection, with biopsies obtained 24h post-NAC, whereas previous studies have examined both the early and late-phase response (201). Additionally, much of the previously literature reporting any alarmin-induced pathogenesis did so in respect to the bronchial mucosa and skin, not the nasal mucosa, and our data may reflect underlying differences between upper and lower airways epithelial cell responses to allergen exposure.

Sample size calculations conducted using mean and standard deviation values of IL-25 and TSLP measured in nasal lavage at pre-allergen baseline and 7h post-NAC suggest that this study was underpowered to detect changes in alarmin levels. According to these calculations, approximately 34 patients would be needed to achieve 80% power and a significant difference ($p<0.05$) in IL-25 levels in nasal lavage. Moreover, 51 patients would be needed to ensure the same parameters in observing a statistically significant difference in TSLP levels in nasal lavage between pre-allergen baseline and 7h-post NAC timepoints. As such our negative data is subject to a type 2 statistical error, and results must be interpreted with caution.

ILC2 cells are also implicated in asthma and allergic rhinitis pathogenesis. Studies have shown that ATP activates mast cells to produce IL-33 and subsequently promote the activation of ILC2 cells (122). Furthermore, TSLP and IL-33 assist in ILC2 survival and activation, respectively, whereas all three alarmins have been found to support ILC2 plasticity (202). Therefore, current literature suggests that alarmins promote ILC2 survival and function. We have previously reported from the present study a NAC-induced increase in ILC2 cells in nasal lavage post-NAC (151). Thus, an allergen-induced increase of ILC2 cells would suggest alarmins may be acting upstream of ILC2 activation. The absence of allergen-induced increase of alarmins in the nasal mucosa in the present study suggest that the mechanism of disease surrounding airway inflammation may differ between the upper and lower airways.

<u>Effect of triamcinolone treatment for two weeks on alarmin expression in nasal lavage and nasal tissue</u>

The second aim of this study was to study the effects of INCS treatment on alarmin expression in nasal tissue post-allergen challenge in subjects with mild allergic asthma and allergic rhinitis. The current study hypothesized that treatment with intranasal triamcinolone for two weeks would reduce allergen-induced inflammation through a reduction in expression of epithelial-derived alarmins. In the present study, INCS treatment attenuated the allergen-induced increase in percent eosinophils, and T2 cytokine levels, with trends for suppression of alarmins and T1 cytokines in nasal lavage. In nasal biopsies, INCS treatment did not have an effect on measures of cellular inflammation with eosinophils, mast cells, or basophils, and neither with alarmins after NAC. Though the data is negative and inconclusive, sample size calculations show that the present study was underpowered, and that a larger sample size would allow for more accurate conditions to determine a difference between INCS and placebo treatment on alarmin expression in nasal mucosa.

Effect of triamcinolone treatment for two weeks on upper airway physiology and symptoms

The present study showed intranasal triamcinolone treatment for two weeks significantly attenuated allergen-induced changes in peak nasal inspiratory flow and total nasal symptom scores. These findings align with what has been previously reported in studies investigating corticosteroid efficacy in grass pollen and ragweed-induced allergic rhinitis. When examining the efficacy of a novel nasal steroid, all tested dosing regimens significantly improved nasal symptoms in patients with grass pollen-induced seasonal allergic rhinitis at day 15-16 from baseline compared to placebo (203). Similarly, patients suffering from ragweed-induced allergic rhinitis underwent significant improvement in TNSS scores at day 14 from baseline with all tested dosing regimens when challenged in an environmental exposure unit (EEU) (204). Other glucocorticoids, such as fluticasone furoate have demonstrated equally effective results in reducing nasal and ocular symptoms associated with AR when used alone, as well as in reducing TNSS in a combination-therapy approach when used in conjunction with levocabastine as a once daily dosing regimen (205,206). Taken together, our findings confirm what has been already proven in literature, thus further substantiating how INCS effectively reduces but does completely prevent allergen-induced nasal symptoms associated with allergic rhinitis.

Effect of triamcinolone treatment for two weeks on cellular inflammation in nasal lavage and nasal tissue

In the present study, triamcinolone treatment for two weeks has attenuated allergen-induced changes in nasal lavage eosinophils. Eosinophil infiltration in the nasal mucosa is amongst the classic signs of allergic pathophysiology. Our findings parallel those that have been previously reported with INCS studies investigating the attenuation of inflammatory cells and cytokines. These studies have demonstrated that a single dose of intranasal budesonide significantly decreased eosinophils in nasal lavage post-allergen challenge (198). Additionally, fluticasone furoate nasal spray (FFNS) reduced the nasal-ocular reflex associated with allergic rhinitis when patients underwent nasal antigen challenge (182). Overall, our results reaffirm the anti-inflammatory benefit of topical corticosteroids on regulating eosinophilic cellular inflammation.

There was, however, no effect of two-week intranasal triamcinolone treatment on the number of allergen-induced mast cells, basophils or eosinophils in nasal tissue at 24 hours challenge. Another clinical trial evaluating the INCS fluticasone propionate investigated the effect of topical corticosteroid on inflammatory cell profiles in nasal biopsies. After six weeks of treatment, nasal provocation was performed and biopsy results in patients with grass pollen-induced allergic rhinitis reflected attenuated mast cell levels in the laminal propria of the nasal mucosa when compared to placebo (207). Other studies examining the cellular infiltrate in endobronchial biopsies report significant attenuation of epithelial and mucosal mast cells and eosinophils in patients who received a 6-week course of beclomethasone diproprionate (208).

A notable difference between the study design of these studies and the present study is the duration of treatment given to patients. This implies that longer courses of glucocorticoid use could be more efficacious in ameliorating cellular inflammation local to the nasal mucosa. Additionally, the contrast between the current findings and those of previous reports suggests that while inhaled corticosteroids may exhibit anti-inflammatory properties in the bronchial mucosa, perhaps alternate mechanisms in the upper airways prevents the complete reduction of cellular inflammation in response to allergen exposure.

Effect of triamcinolone treatment for two weeks on T2 cytokines and alarmins in nasal lavage and nasal tissue

The release of classical T2 cytokines propagates allergic inflammation in atopic disease. As the current gold standard in immunomodulatory agents in regulating allergic rhinitis symptoms, current literature concerning INCS efficacy focuses on its effects in mediating T2 cytokine-induced and cellular inflammation. This study has demonstrated that INCS treatment reduced allergen-induced levels of T2 cytokines in nasal lavage. In particular, INCS was most effective in attenuating IL-4, IL-5, and IL-13 levels at 7h post-NAC. With the nasal allergen challenge model, Linden et al demonstrated an increase in IL-5 and GM-CSF in the late phase response being attenuated when pretreated with budesonide (149). Similarly, fluticasone propionate 100 µg twice daily for 7 days, has been shown to selectively reduce levels of IL-4, IL-5, and IL-13 in the upper allergic airways amongst patients with grass pollen-induced seasonal allergic rhinitis (150). Glucocorticoids are known for their anti-inflammatory properties, especially for suppression of eosinophilic inflammation.

The current study found that INCS treatment did not attenuate alarmin cytokines IL-25 and TSLP in nasal lavage after allergen challenge, nor had any effect on the number of cells expressing IL-25, IL-33 or TSLP in nasal inferior turbinate tissue in epithelium, submucosa, or the combined total regions. In the submucosa, IL-33 levels underwent a significant increase after patients were pre-treated with triamcinolone for two weeks. Though the current findings demonstrate that receiving triamcinolone for two weeks did not attenuate alarmin levels in the upper airways, these findings are somewhat consistent in what is seen in severe asthma patients, in which levels of IL-33 and TSLP persist in the airways despite the use of intensive corticosteroid therapy (157). Taken together with the current findings, our data may substantiate the concept of alarmins being steroid-resistant, and that intranasal corticosteroids may be better suited to reducing eosinophilia and other contributors of airway inflammation. Additionally, the absence of reduced alarmin expression might reflect outcomes from anti-TSLP studies showing additional improvement in asthma control in patients when added tezepelumab was added to their maintenance corticosteroid therapy. Tezepelumab is a monoclonal antibody that prevents human TSLP from binding to its receptor (136). Patients from studies evaluating the efficacy tezepelumab were all taking maintenance ICS prior to, and during the study. Tezepelumab has improved asthma control and exacerbation risk in these patients with severe, uncontrolled asthma treated with maintenance corticosteroid therapy (209). This suggests that topical corticosteroid use alone may not be effective in attenuating allergen-induced alarmin expression. However this study was not powered to draw definitive conclusions; the consistent numerical reduction in IL-25 and TSLP levels in nasal lavage by INCS supports the need to re-evaluate this question in a larger clinical trial.

<u>The relationship between eosinophils and alarmins, eotaxin, and T2 cytokines</u>

The final aim of our study was to evaluate the relationship between eosinophil levels and alarmin levels in nasal samples from subjects with mild allergic asthma and allergic rhinitis. Additionally, the current study examined the effect of eotaxin on eosinophil levels in nasal lavage. The positive relationship between eosinophil levels and alarmin levels of TSLP and IL-25 collected from nasal lavage suggest these cytokines may be contributing to eosinophilic inflammation in allergic rhinitis. The correlation between IL-25 and the percentage of eosinophils is a weak yet positive association and is statistically significant. This is consistent with previously published literature in which IL-25 enhances the survival and expression of eosinophilic adhesion molecules, suggesting that IL-25 inhibits eosinophil apoptosis and drives T2 inflammation (129).

In particular, ICAM-1, ICAM-3, and L-selectin operate via intracellular signalling mechanism pathways to maintain and regulate eosinophilia (129). Therefore, the current findings are consistent with the notion of IL-25 inhibiting eosinophil apoptosis and contributing to allergic inflammation. Additionally, IL-25-induced eotaxin production in primary human nasal epithelial cells indicates that IL-25 has the capacity to promote eosinophil migration (210). It is important to note that these findings were from patients who had not been treated with oral corticosteroids or asthma medications for 4 weeks prior to assessment, demonstrating the inhibition of eosinophil apoptosis by IL-25 in this study occurred in steroid-free tissue. Furthermore, our current analysis reveals a weak positive but not statistically significant, association between TSLP and eosinophilia. TSLP has been described as a "master switch" for eosinophil survival and hinders eosinophil apoptosis via extracellular signal-regulated protein kinase, NF-κB signalling pathway, and p38 mitogen-activated protein kinase (135). Its regulatory effects facilitation, in part, the survival of eosinophils and subsequent Th2 response in allergic inflammation.

Eotaxin is a C-C chemokine and eosinophil chemoattractant that contributes to mechanisms of eosinophil infiltration and migration (33). Eotaxin works in conjunction with CCR3 to induce eosinophil infiltration in the bronchial mucosa of atopic asthma patients (34). Our multivariate analysis determined the effect of known chemoattractants (eotaxin, IL-5, and GM-CSF) in relation to the percent of eosinophils in the nasal lavage. From this, the analysis shows a weak to moderately strong (r^2=0.219) association between eotaxin and NAC-induced increase in percent eosinophils. When determining the individual effect of eotaxin on the percent of eosinophils via a Spearman's correlation, a strong association was present between eotaxin and the percent of eosinophils collected in nasal lavage across baseline, 7h post-NAC and 24h post-NAC in the placebo arm (r^2=0.3799, p=0.0003). These findings are consistent with existing literature which demonstrates that eotaxin is directly chemotactic for eosinophil accumulation and recruitment. Early *in* vivo guinea pig studies investigating the role of eotaxin in allergic inflammation of the lower airways highlighted how eotaxin induces selective eosinophil accumulation in the BAL fluid via intracellular-free calcium concentration (211). Additional studies have identified upregulated levels of eotaxin at both the mRNA and protein level in nasal tissue from allergic rhinitis patients. Specifically, constitutive expression of eotaxin at the mRNA and immunoreactive protein levels were observed in the epithelium and subepithelium of nasal biopsies (212). Therefore, the current findings further substantiate that eotaxin is critically important for chemotaxis leading to eosinophilic inflammation in allergic rhinitis.

Since other cytokines also support eosinophilia, we investigated both the combined and individual associations of type 2 cytokines with the percent of eosinophils in the nasal lavage. In the current study, the combined association of type 2 cytokines (IL-4, IL-5, IL-13, and GM-CSF) was assessed through a multivariate analysis. Our results revealed a moderately strong and strong statistically significant association between IL-4 and IL-13 with eosinophils, and with IL-5. However, on an individual basis, IL-4, IL-5, and IL-13 all demonstrated significant associations with percent eosinophils in nasal lavage. In particular, IL-4 and IL-13 had significant moderately strong (r^2=0.2394 and r^2=0.2553) and IL-5 had strong (r^2=0.5744) associations with the percent of eosinophils, respectively.

<u>Clinical Implications</u>

Previous studies that have used an anti-TSLP therapeutic in patients with mild allergic asthma demonstrate a decrease in sputum eosinophils and fraction of exhaled nitric oxide at baseline and after bronchial allergen challenge, indicating that targeting TSLP during an asthmatic response attenuates clinically significant biomarkers of airway inflammation (136). In adults with uncontrolled asthma, anti-TSLP treatment resulted in reduced fraction exhaled nitric oxide and serum IgE levels by week 52 of treatment. Collectively, these results indicate that a anti-TSLP monoclonal antibody can reduce inflammatory biomarkers in asthma, while also altering type 2 cytokine pathways (213). Correspondingly, if the pathobiology of upper airways is similar to lower airways, these findings from patients with asthma could support the addition of an anti-TSLP therapeutic to a intranasal corticosteroid for patients with allergic rhinitis, potentially resulting in a larger combined anti-inflammatory effect to control responses to allergen exposure. The present study saw no attenuation in alarmins levels post-corticosteroid treatment, suggesting that additional treatment such as anti-alarmins therapies are required for effective for regulation of alarmin levels.

Limitations

A limitation of the present study is the timepoint at which the nasal biopsies were performed post-allergen challenge. Biopsies collected 24h after nasal allergen challenge provide limited insight into the early allergic response. The early asthmatic response develops approximately 10 minutes post-allergen exposure and is monitored over three hours, whereas bronchoconstriction in the late asthmatic response reoccurs at 3-4h and can be monitored for up to 12 hours (165–167). As the nasal biopsies in the present study were taken at 24h, the window in which the nasal tissue could be assessed was limited. While our current study design accounted for changes in the nasal lavage within the first 24h post-allergen challenge, this only accounts for changes in the inflammatory biomarker profile. Further investigations of the presence of alarmins in nasal tissue in response to topical corticosteroid treatment would benefit from biopsies being collected at multiple times between baseline and 24h post-NAC, as this would offer researchers a compartmentalized perspective of the inflammatory infiltrate change occurring in the epithelium and submucosa over time.

Immunofluorescence staining and imaging relies on antigen-specific staining methods to identify the target in tissue biopsies. While the current study was able to use antibodies that identified alarmins and inflammatory cells such as basophils and mast cells, it is important to consider the conditions under which antibodies are developed, and if they are exclusively immunoreactive to the targets in question. For example, the antibody that was used to identify IL-33 in the biopsies is designed to detect both latent and bioactive forms of the protein. The bioactivity of IL-33 is determined by full-length IL-33 undergoing proteolytic cleavage. However, once a cytokine is cleaved, it is important to verify whether the antibody used to detect it via immunofluorescence staining can bind to both the truncated and normal form of the cytokine. Without sufficient information indicating whether the antibody is binding all forms of IL-33, the epitopes that are being identified under immunofluorescent imaging remain unclear. While the antibody that was used for staining purposes was polyclonal, to the best of our knowledge, no references in literature have reported use of the IL-33 antibody to verify which epitopes it identifies.

Additionally, the present study consisted of ten participants, which is a small sample size and is a limitation of the current study. The crossover design of the current clinical trial improved the statistical power by allowing for each participant to receive both placebo and triamcinolone treatment, thus each participant acted as their own control. When dealing with low sample sizes, crossover designs minimize patient variation and thus prove to be more efficient than a parallel study design (214). Future studies investigating the same objectives of the present study would benefit from having a larger sample size that may allow for a parallel study design that has a placebo group that is separate to the topical corticosteroid treatment group.

Finally, this study selected triamcinolone as the corticosteroid to be provided to study participants during the corticosteroid treatment period. When comparing the pharmacokinetics of different corticosteroids, triamcinolone has a much shorter half-life (4 hours) compared to others, such as fluticasone (>10 hours) or beclomethasone (7.5 hours) (215). In the present study, triamcinolone was administered twice a day during the treatment period, as well as on the day of allergen challenge, and in the morning after allergen challenge. If the effects of triamcinolone begin to diminish approximately 4 hours after administration, this would imply there are periods of time in which the treatment is not acting as effectively on a T2 late-phase response. A suggested adjustment to future study designs would be to select pre-treatment with a corticosteroid that has a stronger potency, as triamcinolone may be less potent than other steroids.

Future Directions

The upper and lower respiratory mucosa vary in their respective anatomy and histology. The predominant cell type in the nasal airways is ciliated epithelial cells (216). Other studies investigating the effects of allergen exposure have used alternate models of allergen challenges to deliver allergen to the airways. There exists a multitude of allergen challenge models that can be considered for future studies. Segmental bronchoprovocation (SBP), for example, delivers allergen more directly and with greater precision to the lower lung (217). SBP challenges have also proven to induce both nasal and bronchial effects post-allergen exposure, making them an alternative option to investigating the allergic rhinitis mechanism of disease in the upper airways (197). By choosing to use an allergen challenge model that can ensure the accuracy of antigen exposure to the respiratory mucosa, the outcomes relevant to changes in tissue architecture over the acute and late phase response can be re-assessed in future studies.

Moreover, while the current NAC model is effective and reproducible, repeated allergen exposure studies can help better mimic the natural and consistent exposure to allergen that occurs to people with allergic rhinitis. For example, studies have shown that being repeatedly challenged with allergen exposure induces eosinophilia and eosinophilic degranulation in nasal lavage and nasal mucosa (218). Similarly, repeated allergen challenge result in increases in both the total and activated number of eosinophils from nasal biopsy and nasal brushing samples (194). Studies with a crossover design and repeated exposure allergen challenge model demonstrate responsiveness to dose-dependent effects of corticosteroids, namely budesonide (219). A two-week treatment of topical corticosteroids has shown to reduce symptoms in patients with allergic rhinitis who undergo daily nasal allergen challenge (148). Mucosal plasma exudation and systemic drug levels in patients with allergic rhinitis also reveal reduced symptoms after being treated with intranasal and oral cetirizine (220).

In addition to measuring alarmins alone, examining the level of alarmin receptors may provide more insight to the effect, if any, of topical corticosteroids on the pathways under which alarmins operate. Previous studies examining alarmin receptor staining in the lower airways reveals an increase in cells immunopositive for IL-33, ST2, and TSLP in asthmatic airways post-allergen challenge (200). Given that alarmin receptors are involved in downstream effects in allergic inflammation, this provides probably cause for future research to explore the possible effects of corticosteroids on receptor expression in the upper airways of allergic asthmatics.

<u>Summary</u>

In conclusion, in this study of 10 participants, nasal allergen challenge did not induce significant increases in alarmins (IL-33, TSLP, and IL-25) in the nasal lavage or nasal tissue. The observed increase in T2 cytokines and eosinophils in the nasal mucosa suggest that while these cytokines and inflammatory cells confirm a type 2 allergic response in the upper airways, alarmins may have a secondary effect on the T2 immune response, thus explaining an absence of NAC-induced increase. INCS (triamcinolone) treatment attenuated the level of eosinophils and T2 cytokine expression in the upper airways post-NAC, without having a significant effect on alarmin cytokines. A larger study will be required to determine whether lack of INCS inhibition of alarmin cytokines is due to a type 2 statistical error, or due to steroid-resistance of alarmin cytokines.

CHAPTER 5: REFERENCES

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APPENDIX

The following tables depict the p-values for the epithelial and submucosa analyses of the immunofluorescence-stained biopsies. Non-parametric data was log-transformed prior to analysis. All data was analyzed using repeated measures one-way ANOVA with Tukey's multiple comparisons test.

IL-33+ Epithelial Analysis

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.4878
Baseline (V2) vs. INCS (V10)	0.5569
Placebo (V7) vs. INCS (V10)	0.9969

IL-33+ Submucosa Analysis

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9627
Baseline (V2) vs. INCS (V10)	0.0247
Placebo (V7) vs. INCS (V10)	0.1310

IL-33+Tryptase+ Epithelium Analysis

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9782
Baseline (V2) vs. INCS (V10)	0.2856
Placebo (V7) vs. INCS (V10)	0.5195

IL-33+Tryptase+ Submucosa Analysis

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9916
Baseline (V2) vs. INCS (V10)	0.4538

Placebo (V7) vs. INCS (V10)	0.6604

*IL-33+2D7+ Epithelium and IL-33+2D7+ Submucosa analyses could not be performed, as median values for at least one (1) of three biopsy visits was 0.

IL-25+Epithelium

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.7509
Baseline (V2) vs. INCS (V10)	0.8048
Placebo (V7) vs. INCS (V10)	0.9889

IL-25+ Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9871
Baseline (V2) vs. INCS (V10)	0.1049
Placebo (V7) vs. INCS (V10)	0.1071

IL-25+Tryptase+ Epithelium

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.6602
Baseline (V2) vs. INCS (V10)	0.5133
Placebo (V7) vs. INCS (V10)	0.6449

IL-25+Tryptase Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9645
Baseline (V2) vs. INCS (V10)	0.9987
Placebo (V7) vs. INCS (V10)	0.9039

*IL-25+2D7+ Epithelium analysis could not be performed, as median values for at least one (1) of three biopsy visits was 0.

IL-25+2D7+ Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.6809
Baseline (V2) vs. INCS (V10)	0.4004
Placebo (V7) vs. INCS (V10)	0.7618

TSLP+ Epithelium

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.4775
Baseline (V2) vs. INCS (V10)	0.9576
Placebo (V7) vs. INCS (V10)	0.9240

TSLP+ Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9189
Baseline (V2) vs. INCS (V10)	0.1409
Placebo (V7) vs. INCS (V10)	0.5448

TSLP+2D7+ Epithelium

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9560
Baseline (V2) vs. INCS (V10)	0.4455
Placebo (V7) vs. INCS (V10)	0.4909

TSLP+2D7+ Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9626

Baseline (V2) vs. INCS (V10)	0.8189
Placebo (V7) vs. INCS (V10)	0.7148

TSLP+Trp+ Epithelium

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.4844
Baseline (V2) vs. INCS (V10)	0.9369
Placebo (V7) vs. INCS (V10)	0.4845

TSLP+Trp+ Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.3952
Baseline (V2) vs. INCS (V10)	0.4415
Placebo (V7) vs. INCS (V10)	0.2247

Trp+ Epithelium

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.8849
Baseline (V2) vs. INCS (V10)	0.9822
Placebo (V7) vs. INCS (V10)	0.7141

Trp+ Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.9847
Baseline (V2) vs. INCS (V10)	0.3121
Placebo (V7) vs. INCS (V10)	0.1914

2D7+ Epithelium

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	0.4444
Baseline (V2) vs. INCS (V10)	0.7591
Placebo (V7) vs. INCS (V10)	0.8108

2D7+ Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Baseline (V2) vs. Placebo (V7)	>0.9999
Baseline (V2) vs. INCS (V10)	0.2572
Placebo (V7) vs. INCS (V10)	0.3734

*H&E Eosinophil Epithelium analysis could not be performed, as median values for at least one (1) of three biopsy visits was 0.

H&E Eosinophil Submucosa

Tukey's multiple comparisons test	Adjusted P Value
Pre-NAC Baseline vs. Post-NAC Placebo	0.0373
Pre-NAC Baseline vs. Post-NAC INCS	0.2594
Post-NAC Placebo vs. Post-NAC INCS	0.9526

The following tables show the raw data from the H&E stained biopsies, divided by epithelium and submucosa sections.

H&E Eosinophil Counts (Epithelium)

	Total Epithelium ROI area (mm^2)	# of Eosinophils in Epithelium	# of Eosinophils / Total Epithelium ROI Area (mm^2)
NACHO-02 V2 Avg	0.76	11	14.47368421
NACHO-02 V7 Avg	0.3476	0	0
NACHO-02 V10 Avg	0.0856	8	93.45794393
NACHO-05 V2 Avg	0.782	3	3.836317136
NACHO-05 V7 Avg	0.17	2	11.76470588

NACHO-05 V10 Avg	0.19	0	0
NACHO-06 V2 Avg	1.369	1	0.73046019
NACHO-06 V7 Avg	1.164	11	9.450171821
*NACHO-06 V10 Avg	0	0	#DIV/0!
NACHO-07 V2 Avg	0.7603	17	22.3595949
NACHO-07 V7 Avg	0.063	0	0
NACHO-07 V10 Avg	0.529	9	17.01323251
NACHO-08 V2 Avg	1.13	2	1.769911504
NACHO-08 V7 Avg	0.5006	0	0
NACHO-08 V10 Avg	0.26	1	3.846153846
NACHO-11 V2 Avg	0.967	12	12.40951396
NACHO-11 V7 Avg	0.2612	1	3.82848392
NACHO-11 V10 Avg	0.82	0	0
NACHO-12 V2 Avg	0.3986	0	0
NACHO-12 V7 Avg	1.778	2	1.124859393
NACHO-12 V10 Avg	0.75	0	0
NACHO-13 V2 Avg	0.963	4	4.153686397
NACHO-13 V7 Avg	0.16	0	0
NACHO-13 V10 Avg	0.077	0	0
NACHO-18 V2 Avg	0.15	0	0
NACHO-18 V7 Avg	0.753	0	0
NACHO-18 V10 Avg	0.865	2	2.312138728
NACHO-24 V2 Avg	1.4507	1	0.689322396
NACHO-24 V7 Avg	0.3755	0	0
NACHO-24 V10 Avg	0.433	2	4.618937644

*Insufficient biopsy tissue

H&E Eosinophil Counts (Submucosa)

	Total Submucosa ROI area	# of Eosinophils in Submucosa	# of Eosinophils / Total Submucosa ROI Area
NACHO-02 V2 Avg	4.53	182	40.17660044
NACHO-02 V7 Avg	4	261	65.25

NACHO-02 V10 Avg	1.85	561	303.2432432
NACHO-05 V2 Avg	3.85	22	5.714285714
NACHO-05 V7 Avg	0.89	110	123.5955056
NACHO-05 V10 Avg	1.64	6	3.658536585
NACHO-06 V2 Avg	2	6	3
NACHO-06 V7 Avg	0.52	6	11.53846154
*NACHO-06 V10 Avg	0	0	#DIV/0!
NACHO-07 V2 Avg	1.67	155	92.81437126
NACHO-07 V7 Avg	1.74	170	97.70114943
NACHO-07 V10 Avg	1.262	121	95.87955626
NACHO-08 V2 Avg	1.18	10	8.474576271
NACHO-08 V7 Avg	1.02	12	11.76470588
NACHO-08 V10 Avg	0.05	0	0
NACHO-11 V2 Avg	1.87	67	35.82887701
NACHO-11 V7 Avg	1.08	38	35.18518519
NACHO-11 V10 Avg	1.57	100	63.69426752
NACHO-12 V2 Avg	2.03	20	9.852216749
NACHO-12 V7 Avg	5.77	216	37.43500867
NACHO-12 V10 Avg	1.25	56	44.8
NACHO-13 V2 Avg	2.1	24	11.42857143
NACHO-13 V7 Avg	0.59	7	11.86440678
NACHO-13 V10 Avg	0.33	0	0
NACHO-18 V2 Avg	0.34	3	8.823529412
NACHO-18 V7 Avg	0.72	19	26.38888889
NACHO-18 V10 Avg	1.07	62	57.94392523
NACHO-24 V2 Avg	2.33	9	3.862660944
NACHO-24 V7 Avg	1.05	13	12.38095238
NACHO-24 V10 Avg	1.13	12	10.61946903

*Insufficient biopsy tissue

The following figures depict sample size calculations for the appropriate number of participants needed in the current study to accurately account for a difference in IL-25 and TSLP levels 7h post-NAC in nasal lavage samples:

IL-25 Power Calculations:

Mean and standard deviation entered for Group 1 were the values from participants during the placebo treatment arm at pre-challenge baseline (Mean: 2.3 pg/mL, Stan. Dev.: 0.8), whereas mean and standard deviation entered for Group 2 were the values from patients during the placebo treatment arm at 7h post-NAC (Mean: 6.7 pg/mL, Stan. Dev.: 9.1). The sample size required to see a significant difference at (p=0.05) with 80% power is 34 participants, and with 90% power is 45 participants.

I - Sample Size Calculations for Means

	Anticipated Values	
	Mean	Stan. Dev
Group 1	2.3	0.8
Group 2	6.7	9.1

Difference in means= 191.304 %

Table for (Z1-alpha/2+Z1-beta)squared

	beta			
alpha	0.05	0.1	0.2	0.5
0.1	10.8	8.6	6.2	2.7
0.05	13	10.5	7.8	3.8
0.02	15.8	13	10	5.4
0.01	17.8	14.9	11.7	6.6

The cells in the table below show the estimated number of subjects needed in each group in order to demonstrate a statistically significant difference at "p" values ranging from 0.10 - 0.01 and at varying levels of "power". Power is the probability of finding a statistically significant difference at a given "P" value with the specified number of subjects in each group.

alpha level ("p" value)	Sample Size Needed in Each Group: Power 95%	90%	80%	50%
0.10	47	37	27	12
0.05	56	45	34	16
0.02	68	56	43	23
0.01	77	64	50	28

TSLP Power Calculations:

Mean and standard deviation entered for Group 1 were the values from patients during the placebo treatment arm at pre-challenge baseline (Mean: 0.1 pg/mL, Stan. Dev.: 0.1), whereas mean and standard deviation entered for Group 2 were the values from patients during the placebo treatment arm at 7h post-NAC (Mean: 0.53 pg/mL, Stan. Dev.: 1.1). The sample size required to see a significant difference at (p=0.05) with 80% power is 51 participants, and with 90% power is 69 participants.

D5

I - Sample Size Calculations for Means

	Anticipated Values	
	Mean	**Stan. Dev**
Group 1	0.1	0.1
Group 2	0.53	1.1

Difference in means= 430 %

Table for (Z1-alpha/2+Z1-beta)squared

	beta			
alpha	0.05	0.1	0.2	0.5
0.1	10.8	8.6	6.2	2.7
0.05	13	10.5	7.8	3.8
0.02	15.8	13	10	5.4
0.01	17.8	14.9	11.7	6.6

The cells in the table below show the estimated number of subjects needed in each group in order to demonstrate a statistically significant difference at "p" values ranging from 0.10 - 0.01and at varying levels of "power".

Power is the probability of finding a statistically significant difference at a given "P" value with the specified number of subjects in each group.

	Sample Size Needed in Each Group			
alpha level	**Power**			
("p" value)	**95%**	**90%**	**80%**	**50%**
0.10	71	57	41	18
0.05	86	69	51	25
0.02	104	86	66	36
0.01	117	98	77	44