

DR. PAUL K KEITH (Orcid ID : 0000-0002-5644-1855)

DR. ROMA SEHMI (Orcid ID : 0000-0003-0968-6304)

Article type : Original article

Title: Effect of Intranasal corticosteroid treatment on allergen-induced changes in group 2 innate lymphoid cells in allergic rhinitis with mild asthma

Manuscript Acceptance Date: 10-Feb-2021

Authors: *Yanqing Xie^{1,2}, *Xiaotian Ju¹, Suzanne Beaudin¹, Lesley Wiltshire¹, John Paul^{1,3} Oliveria, Jonathan MacLean⁴, Doron D. Sommer⁴, Ruth Cusack¹, Olga Li⁵, Prajna Banerjee⁵, Paul K. Keith¹, Paul M O’Byrne¹, Rebecca N. Bauer⁵, Tracy Staton⁵, Gail M Gauvreau¹, Roma Sehmi

* These authors contributed equally to the development of this manuscript

Address: ¹Department of Medicine, McMaster University, Hamilton, Ontario, Canada; ² State Key Laboratory of Respiratory Disease, National Clinical Research Center for Respiratory Disease, Guangzhou Institute of Respiratory Health, the First Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong, China; ³Department of Pathology, Stanford University, Palo Alto, California, United States; ⁴ Department of Surgery, Otolaryngology, Head & Neck Surgery Division,

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14835](#)

This article is protected by copyright. All rights reserved

McMaster University, Hamilton, Ontario, Canada. ⁵Genentech Inc, South San Francisco, California, United States.

Key Words: Allergic rhinitis, asthma, nasal challenge, type 2 inflammation, innate lymphoid cells

Declaration of Funding Sources: This research was supported by an educational grant from Genentech Inc. and funding from AllerGen Network of Centres of Excellence.

Statement of contribution: Conception and design: YX, XJ, SB, LW, JPO, JM, DDS, OL, PB, PKK, PMO, RNB, TS, GMG, RS; Patient Recruitment, Sample Collection and Conducting Experiments: YX, XJ, SB, JPO, JM, DDS, RC; Analysis and interpretation: YX, XJ, PMO, RNB, TS, GMG, RS; Drafting the manuscript for important intellectual content: XJ, JPO, PMO, RNB, GMG, RS. All authors approved the manuscript.

Correspondence: Address correspondence and reprint requests to Drs. Gail Gauvreau and Roma Sehmi, Cardiorespiratory Research, Department of Medicine, McMaster University, Hamilton, Ontario, Canada

Email: gauvreau@mcmaster.ca; sehmir@mcmaster.ca;

Word Count: 3500

ABSTRACT(250 words)

Background: Allergic rhinitis is characterized by rhinorrhea, nasal congestion, sneezing and nasal pruritis. Group 2 innate lymphoid cells (ILC2s), CD4⁺ T cells and eosinophils in nasal mucosa are increased significantly after nasal allergen challenge (NAC). Effects of intranasal corticosteroids (INCS) on ILC2s remains to be investigated.

Methods: Subjects (n=10) with allergic rhinitis and mild asthma were enrolled in a single-blind, placebo-controlled, sequential treatment study and treated twice daily with intranasal triamcinolone acetonide (220µg) or placebo for 14 days, separated by a 7-day washout period. Following treatment, subjects underwent NAC and upper airway function was assessed. Cells from the nasal mucosa and blood, sampled 24 hours post-NAC, underwent flow cytometric enumeration for ILC2s, CD4⁺ T and eosinophil progenitor (EoP) levels. Cell differentials and cytokine levels were assessed in nasal lavage.

Results: Treatment with INCS significantly attenuated ILC2s, IL-5⁺/IL-13⁺ ILC2s, HLA-DR⁺ ILC2s and CD4⁺ T cells in the nasal mucosa, 24h post-NAC. EoP in nasal mucosa were significantly increased, while mature eosinophils were significantly decreased, 24h post-NAC in INCS versus placebo treatment arm. Following INCS treatment, IL-2, IL-4, IL-5 and IL-13 were significantly attenuated 24h post-NAC accompanied by significant improvement in upper airway function.

Conclusion: Pre-treatment with INCS attenuates allergen-induced increases in ILC2s, CD4⁺ T cells and terminal differentiation of EoP in the nasal mucosa of allergic rhinitis patients with mild asthma, with little systemic effect. Attenuation of HLA-DR expression by ILC2s may be an additional mechanism by which steroids modulate adaptive immune responses in the upper airways.

INTRODUCTION

Allergic rhinitis and asthma are chronic, heterogeneous airways diseases, with similar underlying inflammatory mechanisms, pathobiology and shared treatment approaches. There is increasing recognition of a link between allergic rhinitis and asthma, with the concept of a “united airway”¹. Epidemiological investigations have shown that 40% of patients with allergic rhinitis have co-morbid allergic asthma, whereas 80 to 90% of allergic asthmatics develop allergic rhinitis. This suggests that inflammatory processes in allergic rhinitis and allergic asthma manifest with similar cellular signaling and activation pathways².

Inherent to the pathogenesis of allergic rhinitis and asthma are type 2 immune responses, which contribute to upper and lower airway eosinophilia, tissue remodeling, and bronchial hyperresponsiveness. Group 2 innate lymphoid cells (ILC2s) are lineage negative lymphomononuclear cells that predominantly reside in mucosal tissues³⁴. The maintenance and activation of ILC2s is mediated by a triad of epithelial-cell derived cytokines namely IL-25, IL-33, and TSLP in both an allergen-specific and non-allergic specific manner (e.g. pollution, smoking, viral infection)³⁴⁵. ILC2s have been implicated as key players in type 2 immune responses, through the production of copious amounts of IL-5 and IL-13. ILC2s work co-operatively with CD4⁺ T cells to induce a maximal type 2 immune response during the first 24 hours (h) post-allergen challenge in mild asthmatics⁶⁷. The greatest numbers of IL-5⁺IL-13⁺ILC2s have been identified in the airways of prednisone-dependent severe asthmatics with >3% sputum eosinophilia, indicating that these may be steroid insensitive cells that drive persistent airway eosinophilia⁵⁸. The production of type 2 cytokines by ILC2s contributing to local eosinophilia by promoting activation, recruitment of mature eosinophils and driving *in situ* differentiation of eosinophil progenitor cells (EoPs) within the airway mucosa⁹¹⁰.

Nasal allergen challenge (NAC) is a validated, reproducible clinical model to investigate the pathophysiology of allergic rhinitis and assess the kinetics of nasal inflammatory responses including eosinophilia which is sustained to 24 h post-NAC¹¹¹². Nasal curettage is a minimally invasive method for repeated sampling of the upper airways to assess inflammatory cell changes in mucosal tissue¹³. ILC2s have been identified in the nasal mucosa of allergic rhinitis by immunofluorescence staining¹⁴. In addition, following NAC in moderately severe asthmatics with allergic rhinitis, significant

Accepted Article

increases in nasal mucosa-extracted ILC2s enumerated by flow cytometry were shown to correlate with the level of tissue eosinophilia¹⁵. Although studies have shown that intranasal corticosteroids (INCS) can attenuate the early (0-2 h) and late phase (2-7 h) response following NAC¹⁶, the changes in ILC2 number, phenotype and activation level within in the nasal mucosa and steroid sensitivity of these cells remains largely unknown. The aim of the current study was to determine whether type 2 inflammation in the nasal mucosa, induced by NAC can be attenuated by intranasal corticosteroid treatment in allergic rhinitis patients with mild asthma, and whether this effect is detected in the systemic circulation.

METHODS

Subjects

Ten non-smokers with allergic rhinitis and mild asthma were enrolled. Subjects (aged 18-65 years) were asymptomatic and not on corticosteroid treatment for 4 weeks prior to study commencement. All subjects had a baseline predicted forced expiratory volume (percent) in one second (FEV₁) of $\geq 70\%$; methacholine PC20 (concentration causing a 20% fall in FEV₁) ≤ 16 mg/mL; positive skin-prick test to common aeroallergens; total nasal symptom score (TNSS) < 3 with each symptom < 2 at baseline. Subjects met eligibility if their peak nasal inspiratory flow (PNIF) value was reduced by $\geq 50\%$ from the baseline and TNSS score increased above 10 following NAC at screening. Baseline subject characteristics are summarized in **(Table 1)**. The study (NCT03431961) was reviewed and approved by the Hamilton Integrated Research Ethics Board and all subjects provided informed consent.

Study Design

A single-blind, placebo-controlled sequential treatment study detailed in **Figure E1** was used to compare 14-days treatment with triamcinolone acetonide (220 μ g bid) to placebo (saline nasal spray, bid) with a 7-day washout period. A sequential order of treatment, where placebo always preceded the INCS treatment arm to eliminate possible carryover effects of steroids on the post-NAC cellular response. On day 13 of each treatment arm, NAC was performed. TNSS and PNIF were measured at baseline and 5, 15, 30, 60 min and then hourly until 7 h post-NAC; blood and nasal lavage were collected at baseline, 7 h and 24 h post-NAC for cell differentials, cytokine and chemokine levels. Nasal curettage was performed 24h post-NAC (day 14) to enumerate tissue-extracted inflammatory cells by flow cytometry. Negligible cell recovery by nasal curettage at baseline (pre-NAC) precluded the use of this time point. Blood was collected at 1, 4, 7 and 24h post-NAC for cell differentials and 24h post-NAC for enumeration of type 2 cells by flow cytometry. Subjects blinded to the treatment type, recorded drug self-administration in a diary, and compliance was confirmed by weighing the placebo/steroid dispenser before and after the treatment arms.

Nasal Allergen Challenge

NAC was performed using the Aptar VP7 device (Aptar Pharma) and 100 μ l of allergen was delivered per nostril. The choice of allergen was based on skin prick test positivity and the challenge cumulative

challenge dose was determined by the post-NAC response that caused a reduction in PNIF by $\geq 50\%$ from the baseline and a total nasal symptom score (TNSS) increase >10 at screening (Table 1).

Peak Nasal Inspiratory Flow (PNIF) and Total Nasal Symptom Scores (TNSS)

PNIF was performed with peak nasal inspiratory flow meter (GM Instrument) and provided an objective measurement of nasal airflow obstruction. TNSS provided a subjective measurement of symptoms scored by subjects ¹⁷.

Nasal Lavage

Pre-warmed physiological saline (0.9%; 10 ml) was instilled using a syringe into each nostril sequentially. A sponge adapter blocking the opposite nostril prevented leakage and up to 7 mL of lavage fluid was recovered. Cytospins were prepared, stained with Diff-Quik and cell differential cell counts performed to assess percentage of eosinophils, macrophages, neutrophils and lymphocytes. Protease inhibitor was added to the supernatants and stored at -70°C till assayed for inflammatory mediators using the Meso-Scale Discovery (MSD) and QuickPlex SQ120 Imager.

Nasal Curettage and Peripheral Blood Collection for Flow Cytometry

Nasal mucosal samples were collected from both nostrils by scraping the inferior nasal turbinate in each nostril (two-four deep scrapes) using a sterile thermoplastic curette (Rhino-Probe) with zero-degree rigid nasoendoscopy visualization. The tissue sample was resuspended in sterile RPMI + 10% FBS with benzonase nuclease on cold beads, washed and then resuspended in RPMI + 10% FBS. Heparinized blood (40 mL) was layered by LymphoprepTM density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMCs).

Immunofluorescence staining and Flow Cytometric Gating Strategy

Nasal mucosa-extracted cells and PBMCs were subject to immunofluorescence staining for enumeration of inflammatory cells as detailed in the online supplement.

Mesoplex Assays

Using the Meso-Scale Discovery (MSD) and a QuickPlex SQ120 Imager, nasal lavage samples were assayed for pro-inflammatory cytokines and chemokines as detailed in the online supplement.

Statistical analysis

Data were analyzed using GraphPad Prism 6 software. Statistical analysis of cells, cytokines, and chemokines was performed using two-way ANOVA and *post-hoc* Bonferroni test. Non-parametric paired data from flow cytometric analyses was analyzed using a Wilcoxon test for within group comparisons. A P-value <0.05 was considered as statistically significant.

RESULTS

Effect of INCS treatment on nasal symptoms and inflammatory response

Ten subjects (5 males, 5 female) with allergic rhinitis and mild asthma completed the study. Following NAC, mean changes in PNIF (% change from baseline) and TNSS during the early response (0-2 h), late airway response (2-7 h and 24 h post-NAC) are presented in **Figure 1A&B**, (individual subject data shown in supplemental **Figure E2A&B**). Compared to the placebo treatment, PNIF was significantly improved at 6 h post-NAC following 14-day INCS treatment (**Figure 1A**). Similarly, there was a significant improvement in TNSS at 5 min, 7 h and 24 h post-NAC following 14-day INCS treatment compared to placebo treatment (**Figure 1B**). Similar findings were obtained when PNIF and TNSS data were analysed as area under the curve (**Figure E2C&D**).

In nasal lavage, a significant increase in the proportion of eosinophils was detected at 7h and 24 h post-NAC compared to baseline in the placebo- treatment arm and at 24h post NAC in the INCS-treatment arm (**Figure 1C**). A significant reduction in percent eosinophils in the INCS versus placebo treatment arm was observed at 24 h post-NAC (**Figure 1C**). Absolute eosinophil numbers in blood did not change following NAC in the placebo treatment arm and there was no effect of INCS treatment (**Figure 1D**). Although the proportion of macrophages, lymphocytes and neutrophils in nasal lavage did not change at 7 h and 24 h post-NAC compared to baseline in the placebo treated group, between treatment comparisons showed a significant increase in the proportion of macrophages and lymphocytes at 7 h and 24h post-NAC and a reduction in neutrophils at baseline (supplemental **Figure E3**).

Effect INCS pre-treatment on inflammatory cells in nasal mucosa and blood following NAC

Type 2 innate lymphoid cells were identified by flow cytometry and sequential gating as live, singlet cells with $SSA^{\text{low}}\text{Lin}^-\text{CD45}^+\text{CD127}^+\text{CRTH2}^+$ (**Figure 2A and Table E1**). Due to low cell recovery pre-NAC, pro-inflammatory cells in the nasal mucosa were only enumerated 24h post-NAC in each treatment arm (supplemental **Figure E4**). There was a significant reduction in total ILC2s and $\text{IL-5}^+/\text{IL-13}^+$ ILC2s in the nasal mucosa, 24 h post-NAC in the INCS versus placebo treatment arm (**Figure 2B**) but not in blood (**Figure 2C**). Of note, we detected expression of HLA-DR on ILC2s extracted from the nasal mucosa (**Figure 3A**). Levels of $\text{HLA-DR}^+\text{ILC2s}$ expressed either as a

proportion of CD45⁺ cells or as a proportion of total ILC2 cells, showed a significant reduction in the INCS compared to the placebo treatment arm, 24 h post NAC(**Figure 3B**).

Data from *in vitro* cultures show that HLA-DR expression is increased on ILC2s from blood following overnight incubation with optimal concentrations of either IL-2 (10 ng/ml), TSLP (10 ng/ml) or IFN- γ (50 ng/ml) but not IL-4 (10 ng/ml) (**Figure 3C & D**).Pre-incubation with dexamethasone (10⁻⁷M) significantly attenuated IL-2 mediated up-regulation of HLA-DR expression, *in vitro* (**Figure 3Ei**). Although dexamethasone showed a trend for attenuating TSLP mediated up-regulation of HLA-DR on ILC2s, this effect was not significant(**Figure 3Eii**).

Recent reports have described unconventional ILC2s (uncILC2s) which compared to conventional ILC2s (cILC2s:Lin⁻CD45⁺CD127⁺CRTH2⁺cells) are identifiable as Lin⁻CD45⁺cells that are non-CD127⁺(CD127⁻CRTH2⁺) or non-CRTH2⁺(CD127⁺CRTH2⁻) cells (supplemental **Figure E5A**)¹⁸. Gating on these populations, we found that IL-5/IL-13⁺and HLA-DR⁺cell numbers were comparable between all ILC2 groups. However, proportionally INCS treatment had a significant inhibitory effect on IL-5/IL-13 and HLA-DR expression on cILC2s but not on uncILC2s, in particular non-CD127⁺cells (supplemental **Figure E5B&C**).

The proportion of total CD4⁺ T cells in the nasal mucosa was significantly reduced at 24 h post-NAC in the INCS compared to placebo treatment arm (supplemental**Figure E6A& B**). In contrast, the proportion of IL-5⁺/IL-13⁺ CD4⁺ T cells,remained unchanged between the two treatment arms (supplemental**FigureE6B**). No significant difference was found in total CD4⁺ T cells or IL-5⁺/IL-13⁺ CD4⁺T cells in blood, 24h post NAC between the treatment arms(supplemental**Figure E6C**).

Eosinophil progenitor cells (EoPs; CD34⁺CD45^{dim}CD125⁺) (**Figure 4A**), expressed as a proportion of total CD45⁺ cells were significantly increased, 24 h post-NAC in the INCS versus placebo treatment arm. In contrast, INCS treatment had no effect on IL-5⁺/IL-13⁺EoPs24h post-NAC (**Figure 4B**). No significant difference between the treatment arms was found in blood EoPs(**Figure 4C**).We found no effect of steroid treatment compared to placebo on NAC induced changes in total numbers of hematopoietic progenitor cellsor IL-5⁺/IL-13⁺ HPCsin either nasal mucosa or blood (supplemental**Figure E7**).

Effect of INCS on NAC-induced cytokines and chemokines levels in nasal lavage

There were significant increases in IL-2, IL-4, IL-5, IL-13 and eotaxin levels in nasal lavage 7 h post-NAC compared to baseline in the placebo treatment arm which was significantly attenuated by INCS treatment (**Figure 5A-E**). At 24 h post-NAC, IL-2 and IL-4 significantly declined relative to 7 h post-NAC. IL-13 and eotaxin were sustained up to 24 h post-NAC compared to baseline, with trends for an inhibitory effect of INCS although this was not significant. IL-25 levels were increased 7 h post-NAC in the placebo treatment arm with a trend for decrease compared to INCS treatment group ($P=0.07$), although this was not significant; no treatment effect was detected for IL-7 (**Figure 5F&G**). IL-33 and TSLP were below the lower limit of detection in all samples (data not shown). Changes in additional cytokines and chemokines in the nasal lavage are summarized in **Table E2**.

DISCUSSION

This study investigated the inflammatory response within the nasal mucosa of allergic rhinitis patients with mild asthma. Our data show the induction of a local type 2 inflammatory response in the nasal mucosa, characterized by increases in ILC2s, CD4⁺ T cells and associated increases in eosinophils in the nasal lavage in response to intranasal allergen challenge consistent with previous findings¹¹¹⁵. Treatment with intranasal steroids attenuated allergen-induced levels of ILC2s, CD4⁺ T cells and type 2 cytokines generation by these cells which may have attenuated terminal differentiation of EoPs in the nasal mucosa causing a reduction in tissue eosinophilia. Of note, this study detected HLA-DR expression on ILC2s in the nasal mucosa 24h post-NAC which was significantly attenuated in the INCS treatment group. We propose that this may be a potential mechanism by which steroids attenuate local ILC2 mediated adaptive immune responses to allergen challenge in the upper airway.

Our data from subjects with allergic rhinitis and mild asthma shows that compared to placebo, INCS treatment caused a significant inhibition of allergen-induced nasal symptom severity (TNSS) during the early and late response post-NAC, but only had an inhibitory effect on PNIF in the late response. The early response is mediated by the crosslinking of the pre-loaded IgE on the mast cell surface followed by a degranulation of histamine¹⁹. This result is consistent with a study by Liu *et al* who noted that three-day prednisone treatment did not inhibit increases in mast cell numbers or mast cell-derived mediators (histamine, PGD₂ and thromboxane) in the bronchoalveolar lavage samples five minutes after allergen challenge²⁰. Similarly, Schleimer *et al.* demonstrated that incubation with dexamethasone did not inhibit the release of mediators from human mast cells, *in vitro*²¹. Therefore, the significant modulatory effect of INCS on the TNSS but not the PNIF early response may, in part, be explained by 14 days of triamcinolone acetonide therapy not effectively attenuating allergen-induced mast cell activation in the upper airways. A limitation of the current study was that changes in the proportion of mast cells following treatment with INCS, particularly in the early phase response to NAC were not assessed.

Our findings showed a significant increase in nasal lavage eosinophils, 24 h post-NAC in the placebo treatment arm which was significantly attenuated in the INCS treatment group. This is consistent with findings from Benson *et al.*, who reported that the eosinophils in nasal lavage was significantly reduced after 1 week of intranasal budesonide treatment during pollen season in allergic

rhinitis patients ²². Importantly, we found no effect of NAC in blood eosinophil numbers, indicating the lack of a systemic effect of our NAC in this study. Raised numbers of EoPs have previously been detected in sputum and airway tissue of allergic asthmatics and rhinitics compared to normal healthy controls⁹¹⁰²³²⁴. The capacity of these cells to undergo *insitu* differentiation in an IL-5 dependent manner to form mature eosinophils has been demonstrated in *ex-vivo* nasal explant tissue from allergic rhinitics⁹. In a clinical trial, where severe eosinophilic asthmatics were treated with mepolizumab, EoP numbers increased in response to anti-IL-5 treatment with an associated decline in mature eosinophil numbers¹⁰. Similarly, here we suggest that steroid mediated attenuation of tissue IL-5 levels reduces local differentiative processes resulting in a build-up of precursor cells i.e. EoPs, within the nasal mucosa which may in part contribute to the observed attenuation of eosinophilia in the nasal lavage 24h post-NAC in the INCS versus placebo treatment arm. While steroid pre-treatment may reduce eotaxin and IL-13 levels that ameliorate tissue recruitment of both mature eosinophils and EoPs, it is plausible that other EoP chemoattractants such as stromal cell derived factor -1 may not have been affected by the drug treatment additionally contributing to the tissue build-up of EoPs post-allergen challenge.

ILC2s are central players in type 2 immune response that produce copious amounts of IL-5 and IL-13 ⁶. We have previously shown significantly increased numbers of ILC2s in the lower airways of patients with severe steroid-dependent eosinophilic asthma, compared to mild asthmatics, with the highest level of activated ILC2s being found in the sputum of those with uncontrolled eosinophilia (>3% sputum eosinophils) ⁸. The steroid insensitivity of ILC2s has been further implicated by Liu *et al.*, demonstrating that ILC2s from BAL of severe asthmatics, in the presence of IL-7 and TSLP, are resistant to inhibitory effects of dexamethasone ¹⁸. In the context of chronic rhinosinusitis (CRS), ILC2s within nasal polyp tissue have been shown to significantly decrease after oral steroid treatment ²⁵. Likewise, blood ILC2s were decreased in asthmatics with allergic rhinitis after a three-month treatment with intranasal and inhaled corticosteroids ²⁶. An attenuation of IL-5 and IL-13 production by alarmin-stimulated ILC2s in the presence of steroids *in vitro* has been reported²⁶²⁷. In mild asthmatics, total numbers of ILC2s and IL-5⁺/IL-13⁺ ILC2s in sputum are increased significantly during the first 24 h after inhalation allergen challenge in mild asthmatics, which then declined to baseline levels by 48 h post-allergen challenge ⁶. We therefore selected the 24 h time point post-NAC,

with a view to capturing maximal change in ILC2 numbers post-NAC and assessing the effects of INCS on these cells in the nasal mucosa. In the INCS treatment arm, there were significantly lower total numbers of ILC2s and IL-5⁺/IL-13⁺ILC2s, and significantly lower CD4⁺T cells in nasal mucosa, compared to the placebo treatment. In line with this, there was a decrease in type 2 cytokines, including IL-5 and IL-13, within the nasal lavage in the INCS treatment arm. These findings suggest that INCS has the potential to attenuate the ability of ILC2s to drive type 2 inflammation locally within the nasal mucosa. Due to the low numbers of ILC2s in the nasal curettage samples detected by flow cytometry the current data should be interpreted with caution. Supportive data from more rigorous techniques such as CyTOF (cytometry by time of flight)²⁸ and multiplexed ion beam imaging (MIBI)²⁹ will be essential to confirm the current findings and to exclude the possibility of ILC2s from blood vessels contaminating the mucosal scraping taken in this study. IL-2 maintains ILC2 survival and cytokine production^{30,31}, whereas IL-4 is important for ILC2 proliferation^{31,32}. The alarmin cytokines, IL-33, IL-25 and TSLP are important activators of ILC2s, stimulating IL-5 and IL-13 production that drive type 2 inflammatory responses³¹. Furthermore, TSLP and IL-7 produced in high levels within the airways of severe asthmatics are proposed to confer steroid insensitivity to airway ILC2s in this patient population¹⁸. In this study, the NAC stimulated an increase in nasal lavage levels of IL-2, IL-4 and IL-25 at 7 h, which were attenuated (not significantly for IL-25) in the INCS treatment arm. As such, a decline in the signals for ILC2 survival and proliferation may have, in part, resulted in lower ILC2 numbers within the INCS treatment arm compared to placebo. In addition low TSLP levels, consistent with findings from other studies in allergic rhinitis challenged with house dust extract³³ may have facilitated the inhibitory effects of steroid on ILC2 numbers and function in the nasal mucosa.

With respect to novel pathways by which ILC2s drive adaptive immune responses, the expression of major histocompatibility complex II (MHCII) has recently been reported on ILC2s, suggesting a possible antigen presenting cell (APC) function of these cells in mice^{30,34}. Similarly, human leukocyte antigen (HLA-DR) has also been identified on ILC2s from COPD patients following an acute exacerbation³⁵. The role of HLA-DR on human ILC2s has not been well studied in the context of allergic airways disease. Murine and human *in vitro* models have shown that the interaction between ILC2s and CD4⁺T cells through MHC II results in significant proliferation of both cell types,

as well as IL-5 and IL-13 production³⁰³⁴³⁵³⁶. A novel aspect of this study is the detection of HLA-DR expression on ILC2s. A previous study which reported that ILC2s from non-pathological tissues (lung, skin, tonsil, colon) do not express HLA-DR³⁷. Here we show that HLA-DR expression is upregulated on ILC2s from chronically inflamed tissue post-allergen challenge and that pre-treatment with intranasal steroids attenuates allergen-induced up-regulation of this receptor. This is supported by our *in vitro* data where overnight culture with either IL-2, TSLP or IFN- γ significantly up-regulated HLA-DR expression on ILC2s. The IL-2 but not TSLP mediated effect was reversed in the presence of steroids. We therefore propose that steroids attenuate ILC2 mediated activation of T2 inflammatory responses, in part by reducing the antigen presenting capacity of these cells within the nasal mucosa. Further research is needed to determine the relevance of HLA-DR expression in mediating the antigen presenting capacity of ILC2s compared to other antigen presenting cells in human allergic airways disease.

In summary, our data show that nasal allergen challenge induces a local type 2 inflammatory response within the nasal mucosa which is modulated by pre-treatment with intranasal corticosteroids. Increases in ILC2s numbers and activation level in the nasal mucosa was associated with type 2 inflammation, contributing to allergic rhinitis symptoms and inflammation in the nose. The lack of predominance of TSLP or IL-7 within the local upper airways may facilitate steroid sensitivity of ILC2s. Whether the relative proportion of conventional versus non-conventional ILC2s and the steroid sensitivity of these populations varies with severity of allergic rhinitis disease warrants further investigation.

Acknowledgements

Dr. Xie has nothing to disclose. Mr. Ju has nothing to disclose. Ms. Beaudin has nothing to disclose. Ms. Wiltshire has nothing to disclose. Dr. Oliveria has nothing to disclose. Dr. Maclean has nothing to disclose. Dr. Sommer reports other from Medtronic, other from GSK, other from Sanofi, outside the submitted work. Dr. Cusack has nothing to disclose. Dr. Li reports current employee of Genentech, a member of the Roche group, and holder of Roche stock or stock options. Dr. Banerjee reports current employee of Genentech, a member of the Roche group, and holder of Roche stock or stock options. Dr. Keith has nothing to disclose. POB has grants in aid and speakers fees from AstraZeneca,

Accepted Article

and GSK, grants in aid from Bayer, Medimmune, and Novartis, and has received speakers fees from Chiesi and Menarini, all outside the scope of this work. Dr. Bauer reports current employee of Genentech, a member of the Roche group, and holder of Roche stock or stock options. Dr. Staton reports and current employee of Genentech, a member of the Roche group, and holder of Roche stock or stock options. Dr. Gauvreau reports grants and other from AstraZeneca, grants and other from Novartis, grants from Regeneron, grants from BioGaia, grants from Genentech, outside the submitted work; Dr. Sehmi reports grants and personal fees from Genentech Inc, grants and personal fees from Astra Zeneca, grants and personal fees from Teva Pharmaceuticals, personal fees from GSK, outside the submitted work;

REFERENCES

1. Meltzer EO, Szwarcberg J, Pill MW. Allergic Rhinitis, Asthma, and Rhinosinusitis: Diseases of the Integrated Airway. *J Manag Care Pharm*. 2004;10(4):310-317.
2. Bourdin A, Gras D, Vachier I, et al. Upper airway · 1: Allergic rhinitis and asthma: United disease through epithelial cells. *Thorax*. 2009;64(11):999-1004.
3. Kim CH, Hashimoto-Hill S, Kim M. Migration and Tissue Tropism of Innate Lymphoid Cells. *Trends Immunol*. 2016;37(1):68-79.
4. Martinez-Gonzalez I, Steer CA, Takei F. Lung ILC2s link innate and adaptive responses in allergic inflammation. *Trends Immunol*. 2015;36(3):189-195.
5. Christianson CA, Goplen NP, Zafar I, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J Allergy Clin Immunol*. 2015;136(1):59-68.
6. Chen R, Smith SG, Salter B, et al. Allergen-induced increases in sputum levels of group 2 innate lymphoid cells in subjects with asthma. *Am J Respir Crit Care Med*. 2017;196(6):700-712.
7. Winkler C, Thomas H, Israelsson E, et al. Activation of group 2 innate lymphoid cells after allergen challenge in asthmatic patients. *J Allergy Clin Immunol*. 2019;144(1):61-69.
8. Smith SG, Chen R, Kjarsgaard M, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol*. 2016;137(1):75-86.
9. Cameron L, Christodoulopoulos P, Lavigne F, et al. Evidence for Local Eosinophil Differentiation Within Allergic Nasal Mucosa: Inhibition with Soluble IL-5 Receptor. *J Immunol*. 2000;164:1538-1545.
10. Sehmi R, Smith SG, Kjarsgaard M, et al. Role of local eosinophilopoietic processes in the development of airway eosinophilia in prednisone-dependent severe asthma Experimental Allergy. *Clin Exp Allergy*. 2015;(46):793-802.
11. Soliman M, Steacy LM, Thiele J, et al. Repeatability of nasal allergen challenge results – further validation of the allergic rhinitis clinical investigator collaborative (AR-CIC) protocols. *Ann Allergy, Asthma Immunol*. 2018;120(6):607-613.

12. Leaker BR, Malkov VA, Mogg R, et al. The nasal mucosal late allergic reaction to grass pollen involves type 2 inflammation (IL-5 and IL-13), the inflammasome (IL-1 β), and complement. *Mucosal Immunol*. 2017;10(2):408-420.
13. Graaf-in't V, Garrelds I, AP J, et al. Effect of intranasal fluticasone propionate on the immediate and late allergic reaction and nasal hyperreactivity in patients with a house dustmite allergy. *Clin ExpAllergy*. 1995;(25):966-973.
14. Peng YQ, Qin ZL, Fang S Bin, et al. Effects of myeloid and plasmacytoid dendritic cells on ILC2s in patients with allergic rhinitis. *J Allergy Clin Immunol*. 2020;145(3):855-867.
15. Dhariwal J, Cameron A, Trujillo-Torralbo M-B, et al. Mucosal Type 2 Innate Lymphoid Cells Are a Key Component of the Allergic Response to Aeroallergens. *Am J Respir Crit Care Med*. 2017;195(12):1586-1596.
16. Mygind N, Nielsen P, Hoffmann H, et al. Mode of action of intranasal corticosteroids. *J Allergy Clin Immunol*. 2001;108(1):16-25.
17. Badorrek P, Müller M, Koch W, et al. Specificity and reproducibility of nasal biomarkers in patients with allergic rhinitis after allergen challenge chamber exposure. *Ann Allergy, Asthma Immunol*. 2017;118(3):290-297.
18. Liu S, Verma M, Michalec L, et al. Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: The role of thymic stromal lymphopoietin. *J Allergy Clin Immunol*. 2018;141(1):257-268.
19. Gauvreau GM, El-Gammal AI, O'Byrne PM. Allergen-induced airway responses. *Eur Respir J*. 2015;46(3):819-831.
20. Liu MC, Proud D, Lichtenstein LM, et al. Allergy , rhinitis , other respiratory diseases Effects of prednisone on the cellular responses and release of cytokines and mediators after segmental allergen challenge of asthmatic subjects. *J Allergy Clin Immunol*. 2001;108(1):29-38.
21. Schleimer P. Robert, Peters SP, Hayes EC, et al. Effects of Dexamethasone on Mediator Release from Human Lung Fragments and Purified Human Lung Mast Cells. *JClinInvest*. 1983;71:1830-1835.
22. Benson M, Strannegård I, Strannegård Ö. Topical steroid treatment of allergic rhinitis decreases nasal

fluid Th2 cytokines , eosinophils , eosinophil cationic protein , and IgE but has no significant effect on IFN- γ , IL-1 β , TNF- α , or neutrophils. *J Allergy Clin Immunol.* 2000;106:307-312.

23. Dorman SC, Efthimiadis A, Babirad I, et al. Sputum CD34+IL-5R α + Cells Increase after Allergen Evidence for In Situ Eosinophilopoiesis. *Am J Respir Crit Care Med.* 2004;169(5):573-577.
24. Robinson DS, Damia R, Zeibecoglou K, et al. CD34+/interleukin-5R α messenger RNA+ cells in the bronchial mucosa in asthma: Potential airway eosinophil progenitors. *Am J Respir Cell Mol Biol.* 1999;20(1):9-13.
25. Walford HH, Lund SJ, Baum RE, et al. Increased ILC2s in the eosinophilic nasal polyp endotype are associated with corticosteroid responsiveness. *Clin Immunol.* 2014;155(1):126-135.
26. Yu.Q.N, Guo.Y.B, Li.X, et al. ILC2 frequency and activity are inhibited by glucocorticoid treatment via STAT pathway in patients with asthma. *Allergy.* 2018;73:1860-1870.
27. Ogasawara N, Poposki JA, Klingler AI, et al. IL-10,TGF β and glucocorticoid prevent the production of type 2 cytokines in human group 2 innate lymphoid cells. *J Allergy Clin Immunol.* 2018;141(3):1147-1151.
28. Vivanco Gonzalez N, Oliveria JP, Tebaykin D, et al. Mass Cytometry Phenotyping of Human Granulocytes Reveals Novel Basophil Functional Heterogeneity. *iScience.* 2020;23(11):101724.
29. Keren L, Bosse M, Thompson S, et al. MIBI-TOF: A multiplexed imaging platform relates cellular phenotypes and tissue structure. *Sci Adv.* 2019;5(10):1-17.
30. Oliphant CJ, Hwang YY, Walker JA, et al. MHCII-mediated dialog between group 2 innate lymphoid cells and CD4+ T cells potentiates type 2 immunity and promotes parasitic helminth expulsion. *Immunity.* 2014;41(2):283-295.
31. Kabata H, Moro K, Koyasu S. The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms. *Immunol Rev.* 2018;286(1):37-52.
32. Symowski C, Voehringer D, Symowski C, et al. Th2 cell-derived IL-4/IL-13 promote ILC2 accumulation in the lung by ILC2-intrinsic STAT6 signaling in mice. *EurJImmuno.* 2019;49:1421-1432.

- Accepted Article
33. Tojima I, Matsumoto K, Kikuoka H, et al. Evidence for the induction of Th2 inflammation by group 2 innate lymphoid cells in response to prostaglandin D2 and cysteinyl leukotrienes in allergic rhinitis. *Allergy*. 2019;74(12):2417-2426.
 34. Mirchandani AS, Besnard A, Yip E, et al. Type 2 Innate Lymphoid Cells Drive CD4 + Th2 Cell Responses. *J Immunol*. 2014;192:2442-2448.
 35. Jiang M, Liu H, Li Z, et al. ILC2s Induce Adaptive Th2-Type Immunity in Acute Exacerbation of Chronic Obstructive Pulmonary Disease. *Mediators Inflamm*. 2019;2019:1-13.
 36. Lin L, Chen Z, Dai F, et al. CD4 + T cells induce productions of IL-5 and IL-13 through MHCII on ILC2s in a murine model of allergic rhinitis. *Auris Nasus Larynx*. 2018;46(4):533-541.
 37. Simoni Y, Fehlings M, Kløverpris HN, et al. Human Innate Lymphoid Cell Subsets Possess Tissue-Type Based Heterogeneity in Phenotype and Frequency. *Immunity*. 2017;46(1):148-161.

Table1. Baseline Characteristics of Study Subjects

Sex	Age (yr)	FEV ₁ (% Predicted)	FEV/FVC	Allergen	AllergenDilution	PNIF(L/min)	TNSS
M	37	79.74	0.65	HDMDP	1:128	100	0
F	30	89.59	0.90	Cat	1:8	70	2
M	28	92.51	0.80	Grass	1:2	170	0
F	24	121.35	0.84	HDMDP	1:8	130	1
M	57	85.86	0.62	HDMDP	1:2	150	0
F	22	109.06	0.87	HDMDP	1:8	160	1
M	37	107.82	0.72	Ragweed	1:8	170	2
M	23	98.60	0.85	Horse	1:32	150	0
F	19	91.09	0.75	HDMDP	1:32	130	0
F	21	110.31	0.88	HDMDP	1:8	190	0

Definition of abbreviation: FEV₁= forced expiratory volume in one second; HDMDP= house dust mite protein.

Baseline characteristics of mild allergic rhinitis with asthma (n=10) that were enrolled in the study. Non-smoking subjects aged 21 to 57 were enrolled in this study. Subjects were skin-prick positive to common aeroallergens, FEV₁ greater or equals to 70% of the predicted value. Subjects had no asthma exacerbation and respiratory tract infection during the previous 8 weeks. Subjects had no using of β_2 -agonist treatment, corticosteroids, aspirin or anti histamine medications prior to the laboratory visit. Choice of allergen was based on largest skin prick test positivity and challenge dilution of the allergen was determined based on a post-nasal allergen challenge that caused a peak nasal inspiratory flow (PNIF) reduction by $\geq 50\%$ from the baseline and a total nasal symptom score (TNSS) increase above 10.

Accepted Article

FIGURE LEGENDS

Figure 1: Nasal symptoms (PNIF –peak nasal inspiratory flow and TNSS: Total nasal symptom score) and eosinophils in the nasal lavage and blood detected pre- and post- nasal allergen challenge (NAC) in the placebo (open circles) and intranasal corticosteroid (INCS) (closed circles) treatment arms. There was a significant improvement in (A) PNIF in INCS treatment arm 6 h post-NAC (n=9) and (B) TNSS at 5 min, 4 h, 7 h and 24 h post NAC in the INCS treatment arm (n=10). (C) NAC-induced increase in percent eosinophils in nasal lavage which was attenuated in the INCS treatment arm 24 h time post (n=10). (D) Absolute numbers of eosinophils in blood were not affected by NAC in the placebo or INCS treatment arms (n=10). Data are presented as mean $\pm$ SD; For statistical significance, a Wilcoxon test (A& B) and ANOVA test with Bonferroni post-hoc test (C & D) was used. (# $P < 0.05$ for within and * $P < 0.05$ for between treatment arm comparisons); BL= baseline.

Figure 2: (A) Representative FACS plot of ILC2s gated as live, singlet SSC^{low} Lin^{-} $CD45^{+}$ $CD127^{+}$ $CRTH2^{+}$ cells from nasal mucosa. A significant reduction in nasal mucosa levels of (B)(i) total ILC2s and (B)(ii) $IL-5^{+}/IL-13^{+}$ ILC2s expressed as percent of total $CD45^{+}$ cells or (B)(iii) total ILC2s was found 24h post-NAC in the INCS versus placebo treatment arm. (C) No significant change in total ILC2s or $IL-5^{+}/IL-13^{+}$ ILC2s was found in blood, 24h post-NAC in placebo versus INCS treatment arm. Data (n=8 of 10 as 2 subjects had insufficient cell recovery by nasal curettage) were analyzed for statistical significance using a Wilcoxon test; horizontal bars represent median values of each data set. (* $P < 0.05$ and ** $P < 0.01$).

Figure 3: Representative FACS plot of HLA-DR expression on (A)(i) ILC2s (Lin^{-} $CD45^{+}$ $CD127^{+}$ $CRTH2^{+}$) compared to a positive control - monocytes (Lin^{+} $CD45^{+}$ $CD16^{+}$ cells) extracted from nasal mucosa (A)(ii). HLA-DR expression was significantly attenuated on ILC2s 24h post NAC in INCS vs placebo treatment arm when expressed as a proportion of (B)(i) $CD45^{+}$ cells or (B)(ii) total ILC2s. (C) FACS gating on PBMCs for HLA-DR expression on ILC2s cultured in PBS vs TSLP (10 ng/ml) overnight. (D) $HLA-DR^{+}$ ILC2s were increased significantly following overnight culture with optimal concentrations of IL-2 (10ng/ml), TSLP (10 ng/ml) and IFN- γ (50 ng/ml) but not

IL-4 (10 ng/ml) (n=5). (E) Upregulation of HLA-DR expression on ILC2s by IL-2 but not TSLP was significantly attenuated in the presence of dexamethasone (10^{-7} M; n=7).

Statistical analyses of data in (B) used a Wilcoxon test for non-parametric paired data; horizontal bars represent median values of each data set, (D) one-way ANOVA with Bonferroni post-hoc and (E) paired T-test.

Figure 4: Enumeration of Eosinophilic progenitor cells (EoPs) in nasal mucosa and peripheral blood 24 hours after NAC in placebo and INCS treatment arms. (A) EoPs were identified as CD34⁺CD45^{dim}CD125⁺ cells by FACS. There was a significant increase in (B)(i) total EoPs in nasal mucosa 24h post-NAC in the INCS versus placebo treatment group. No significant change was found for (B)(ii) IL-5⁺/IL-13⁺EoPs in nasal mucosa, (C)(i) total blood EoPs or (C)(ii) IL-5⁺/IL-13⁺EoPs cells in blood when 24h post-NAC samples were compared in the INCS versus placebo treatment arms. Data (n=8) were analyzed for statistical significance using a Wilcoxon test for non-parametric paired data; Horizontal bars represent median values of each data set.

Figure 5: Cytokine and chemokines in nasal lavage samples from patients with mild allergic rhinitis and asthma. Subjects were treated with placebo (solid circles) or intranasal steroids (solid squares) for 14 days before NAC. At baseline (BL), 7h and 24h post-nasal Ag challenge, levels of (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-13, (E) eotaxin (F) IL-25 and (G) IL-7 were determined by mesoplex assays on the MSD Platform. A two-way ANOVA test with Bonferroni correction (α/m) was used to determine statistical significance of parametric paired data. Data was presented as mean and SD (n=9). * indicates the significance within the placebo group and # indicates the significance between placebo and INCS groups.

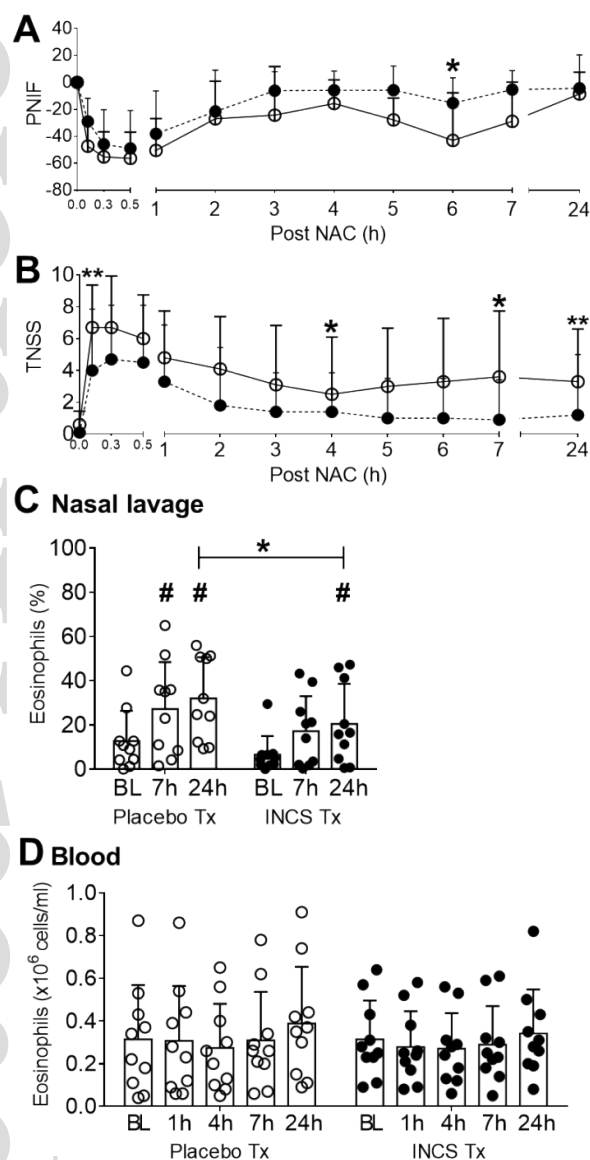
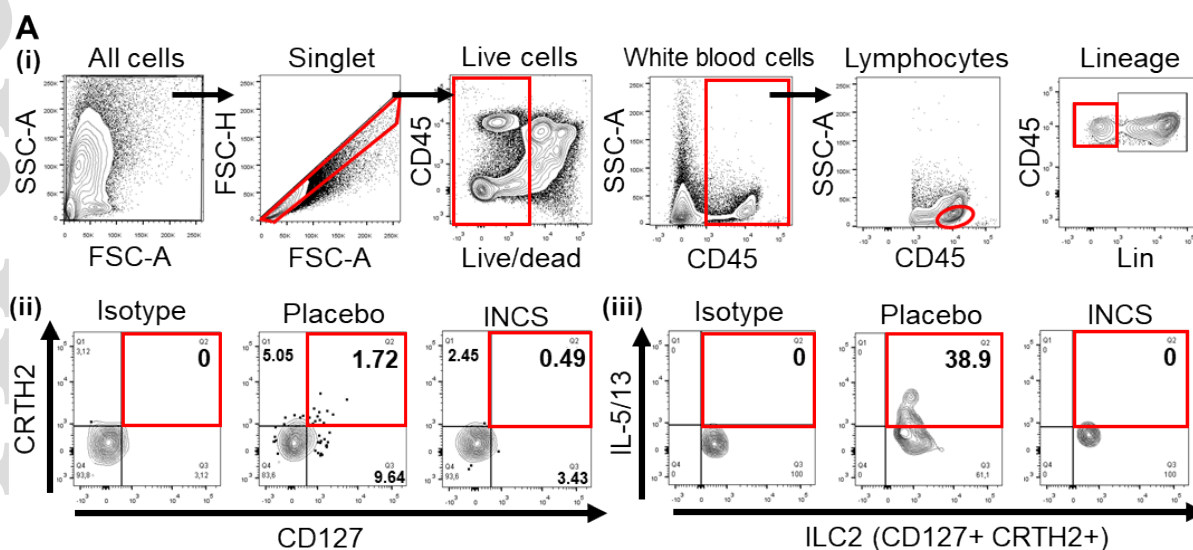
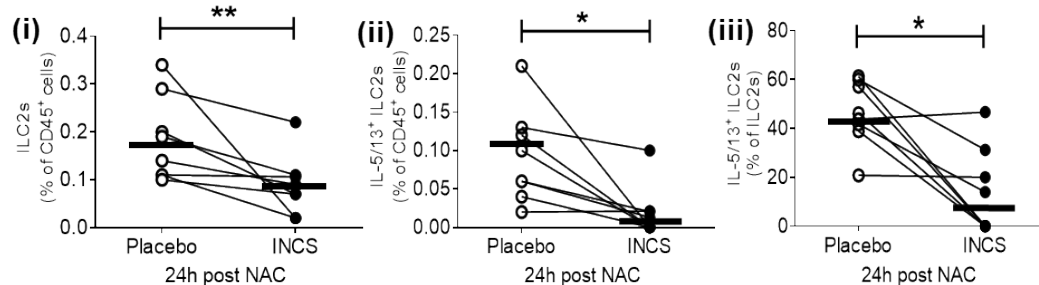


Figure 1. Nasal symptoms (PNIF –peak nasal inspiratory flow and TNSS: Total nasal symptom score) and eosinophil levels in the upper and blood detected pre and post nasal allergen challenge (NAC) in the placebo (open circles) and intranasal corticosteroid (INCS) (closed circles) treatment arms. There was a significant improvement in (A) PNIF in INCS treatment arm 6 h post-NAC (n=9) and (B) TNSS at 5 min, 4 h, 7 h and 24 h post NAC in the INCS treatment arm (n=10). (C) NAC-induced increase in the percentage of eosinophils in nasal lavage was attenuated in the INCS treatment arm 24 h time post (n=10). (D) Absolute numbers of eosinophils in blood were not affected by NAC in the

placebo or INCS treatment arms (n=10). Data are presented as mean $\pm$ SD; A Wilcoxon test for non-parametric paired data was used for (A& B) and ANOVA test with Bonferroni post-hoc test was used to detect significance for (C & D). (# P<0.05 for within and * P<0.05 for between treatment arm comparisons); BL= baseline.



B Nasal mucosa



C Blood

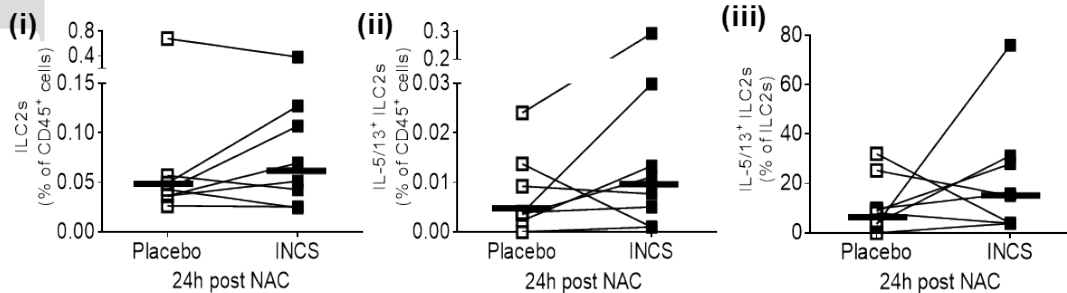


Figure 2. (A) Representative FACS plot of ILC2s gated as live, singlet $\text{SSC}^{\text{low}} \text{Lin}^- \text{CD45}^+ \text{CD127}^+ \text{CRTH2}^+$ cells from nasal mucosa. A significant reduction in nasal mucosa levels of (B)(i) total ILC2s and (B)(ii) $\text{IL-5}^+/\text{IL-13}^+$ ILC2s expressed as percent of total CD45^+ cells or (B)(iii) total ILC2s was found 24h post-NAC in the INCS versus placebo treatment arm. (C) No significant change in total ILC2s or $\text{IL-5}^+/\text{IL-13}^+$ ILC2s was found in blood, 24h post-NAC when placebo was compared to INCS treatment arm. Data (n=8 of 10 as 2 subjects had insufficient cell recovery by nasal curettage) were analyzed for statistical significance using a Wilcoxon test for non-parametric paired data; horizontal bars represent median values of each data set. (* $P < 0.05$ and ** $P < 0.01$).

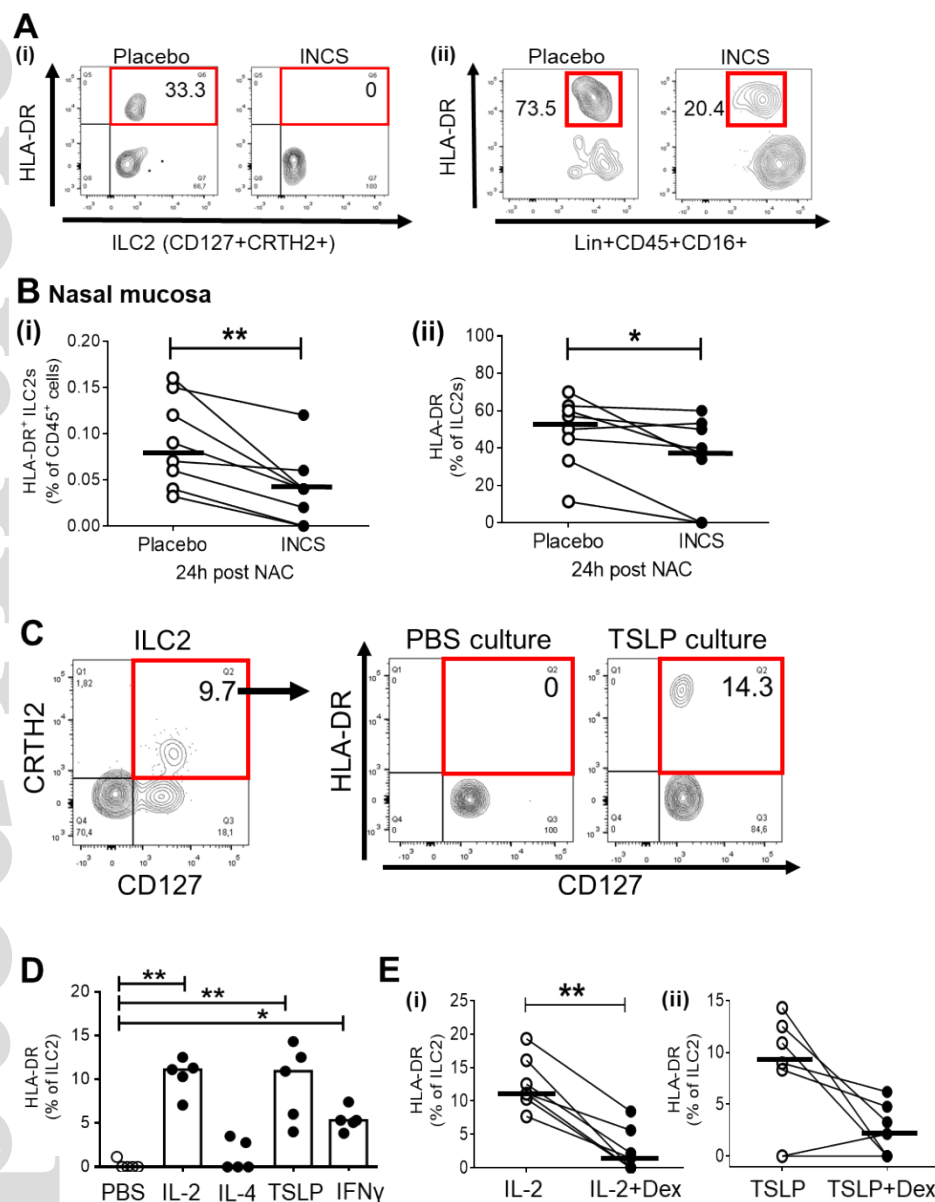
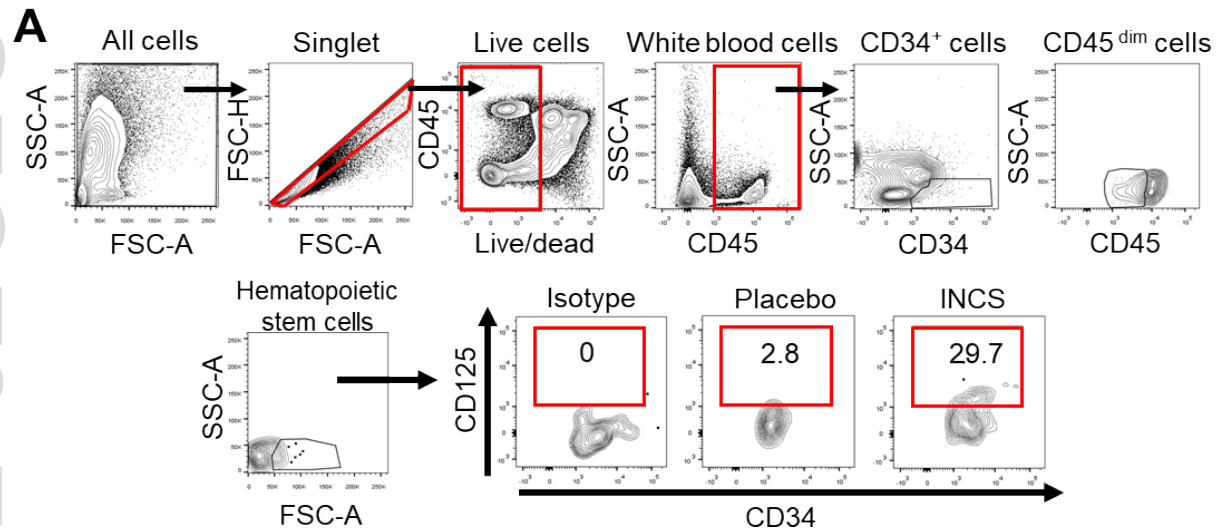
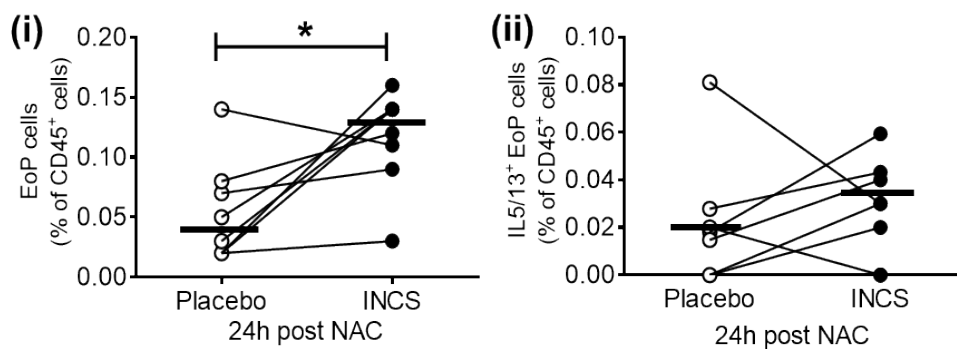


Figure 3. Representative FACS plot of HLA-DR expression on (A)(i) ILC2s (Lin⁻CD45⁺CD127⁺CRTH2⁺) compared to the positive control (A) (ii) monocytes (Lin⁺CD45⁺CD16⁺cells) extracted from nasal mucosa. (B) (i) HLA-DR expression was significantly attenuated on ILC2s detected at 24h post NAC in INCS vs placebo treatment arm when data were expressed as a proportion of (B)(ii)CD45⁺ cells or (B)(iii) as a proportion of total ILC2s. A similar trend was observed with monocytes although this was not significant. (C) FACS gating on PBMCs for HLA-DR expression on ILC2s cultured in PBS vs TSLP (10 ng/ml) overnight. (D) HLA-DR⁺ILC2s were

increased significantly following overnight culture with optimal concentrations of IL-2, TSLP (10 ng/ml) and IFN- γ (50 ng/ml) but not IL-4 (10 ng/ml). (E) Upregulation of HLA-DR expression by ILC2s was attenuated in the presence of dexamethasone (10^{-7} M). Statistical analyses of data in (B) used a Wilcoxon test for non-parametric paired data; horizontal bars represent median values of each data set, (D) one-way ANOVA with Bonferroni post-hoc and (E) paired T-test.



B Nasal mucosa



C Blood

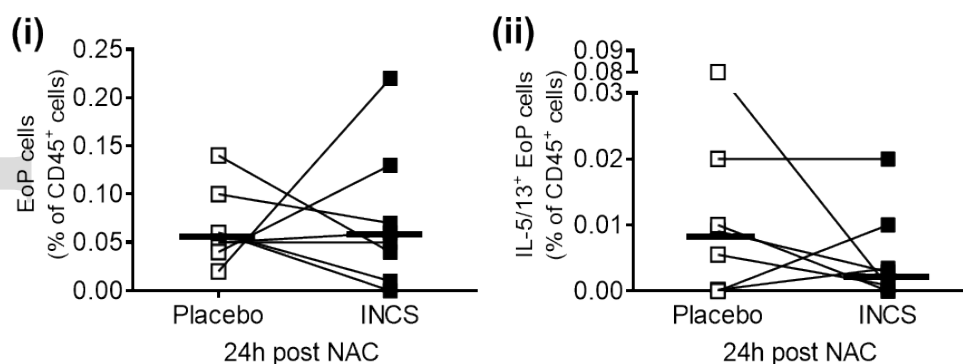


Figure 4. Enumeration of Eosinophilic progenitor cells (EoPs) in nasal mucosa and peripheral blood 24 hours after NAC in placebo and INCS treatment arms. (A) EoPs were identified as CD34⁺CD45^{dim}CD125⁺ cells by FACS. There was a significant increase in (B)(i) total EoPs in nasal mucosa 24h post-NAC in the INCS versus placebo treatment group. No significant change was found

for (B)(ii) IL-5⁺/IL-13⁺EoPs in nasal mucosa, (C)(i) total blood EoPs or (C)(ii) IL-5⁺/IL-13⁺EoPs cells in blood when 24h post-NAC samples were compared in the INCS versus placebo treatment arms. Data (n=8) were analyzed for statistical significance using a Wilcoxon test for non-parametric paired data; Horizontal bars represent median values of each data set.

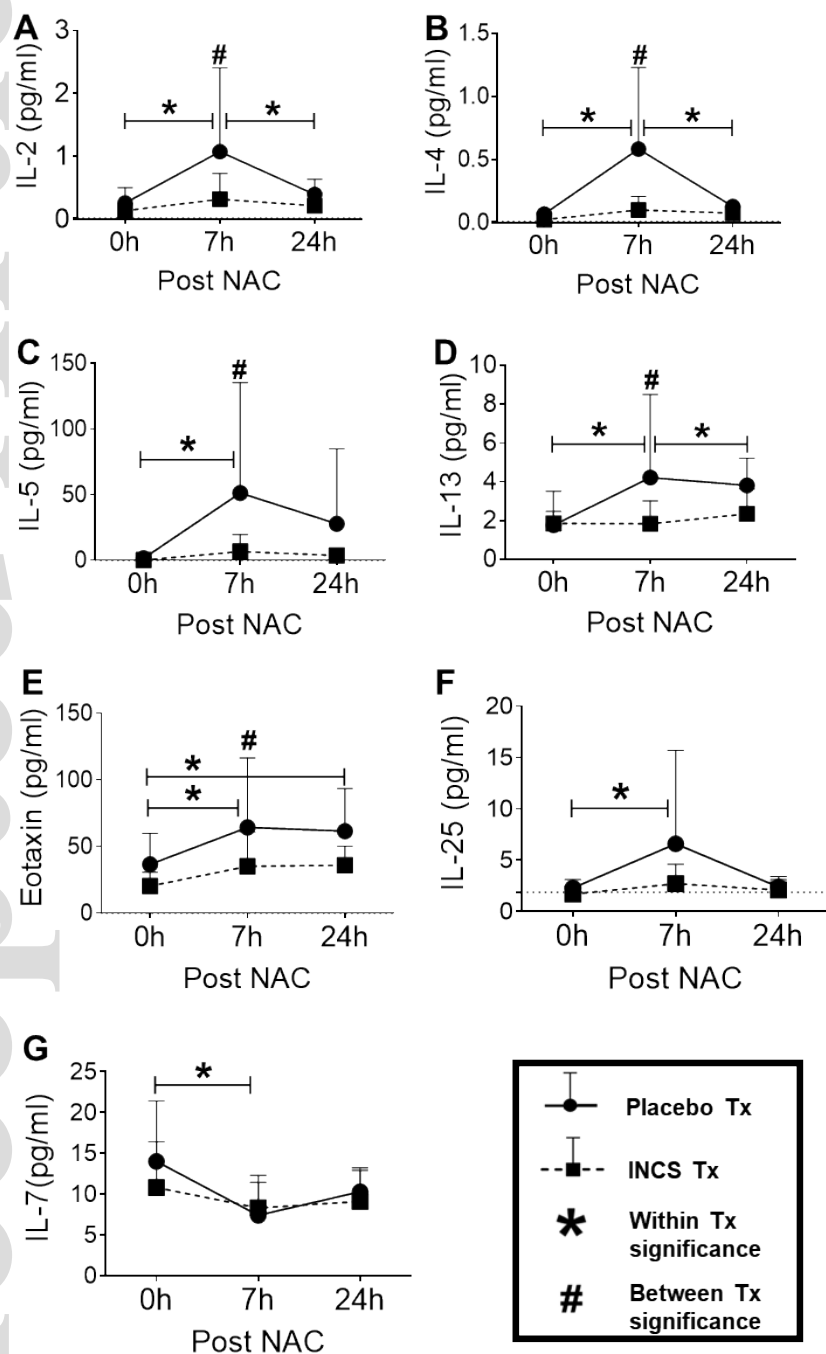


Figure 5. Cytokine and chemokines in nasal lavage samples from patients with mild allergic rhinitis and asthma. Subjects were treated with placebo (solid circles) or intranasal steroids (solid squares) for 14 days before NAC. At baseline (BL), 7h and 24h post-nasal Ag challenge, levels of (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-13, (E) eotaxin (F) IL-25 and (G) IL-7 were determined by mesoplex assays on the MSD Platform. A two-way ANOVA test with Bonferroni correction (α/m) was used to determine statistical significance of parametric paired data. Data was presented as mean and SD (n=9). * indicates the significance within the placebo group and # indicates the significance between placebo and INCS groups.

ONLINE SUPPLEMENTAL DATA

Title: Effect of Intranasal corticosteroid treatment on allergen-induced changes in group 2 innate lymphoid cells in allergic rhinitis with mild asthma

Authors: *Yanqing Xie^{1,2}, *Xiaotian Ju¹, Suzanne Beaudin¹, Lesley Wiltshire¹, John Paul^{1,3} Oliveria, Jonathan MacLean⁴, Doron D. Sommer⁴, Ruth Cusack¹, Olga Li⁵, Prajna Banerjee⁵, Paul K. Keith¹, Paul M O'Byrne¹, Rebecca N. Bauer⁵, Tracy Staton⁵, Gail M Gauvreau¹, Roma Sehmi

Immunofluorescence Staining for Flow Cytometric Analyses

Nasal-extracted cells and peripheral blood mononuclear cells were stained with viability Dye (AF700) and incubated in Perm/Fix Buffer (BD Bioscience). Cells were then stained with antibodies for both surface and intracellular markers Lin-FTIC, CD45-APC-H7, IL-5/13-PE, PerCP-Cy5.5-HLA-DR, CRTH2-BV421, CD127-BV605, and CD4-BV510, CD34-BV786 and CD125-APC or relevant isotype controls (BD Bioscience, Mississauga, ON, Canada; eBiosciences, San Diego, CA; R&D Systems, MN, USA). Lin-cocktail antibodies to (CD2, CD3, CD14, CD19, CD20 and CD94) plus FcεR1 excluded T and B lymphocytes, B-cells, monocytes, eosinophils, NK cells and basophils. After incubation, cells were washed and resuspended in fixed in PBS with 1% paraformaldehyde and analyzed by a FACS LSRII flow cytometer (BD Biosciences, CA, US) within 24 h. As cells were treated with Perm/Fix immediately after isolation from the nasal mucosa, a protein transport inhibitor was not required to maintain intracellular cytokine. Gating in the lympho-mononuclear region (low side scatter/low forward scatter) and following acquisition of 300,000 events, data were analyzed using Flow-Jo software (Tree Star, CA, US) where ILC2 were defined as live, singlets with $SSA^{low}Lin^{-}CD45^{+}CD127^{+}CRTH2^{+}$ phenotype expressing intracellular type 2 cytokines, IL-5/IL-13+ (Figure 2) or surface HLA-DR

(Figure 3). Gating for CD4⁺ T cells as live, singlet cells with SSA^{low} Lineage⁺ [CD2, 3, 14, 16, 19, 20, 94, FcεRI] CD45⁺CD4⁺) phenotype is described in Figure E6 with a 98% confidence limit. Data are expressed as a proportion of the total CD45⁺ (white cell population) cells gated unless otherwise stated. Gating for eosinophilic progenitor cells (EoPs; CD34⁺CD45^{dim} CD125⁺) and hematopoietic progenitor cells (HPCs; CD34⁺ CD45^{dim}) is described in Figure 4.

Mesoplex Assays

Using the Meso-Scale Discovery (MSD) and a QuickPlex SQ120 Imager, nasal lavage samples were assayed using (i) Human V-PLEX 36-cytokine panel to detect chemokines, pro-inflammatory and Th17 cytokines and, (ii) Uplex kit to detect alarmin cytokines (TSLP, IL-17E/IL-25 and IL-33). The lower limit of detection (LLOD) values were IL-2, IL-5 (0.02 pg/ml); IL-4 (0.01 pg/ml); IL-7 (0.06 pg/ml); IL-13 (0.14 pg/ml); IL-25 (1.88 pg/ml); TSLP (1.96 pg/ml); Eotaxin (3.29 pg/ml). Any value below LLOD value was replaced by LLOD/2.

41

Subjects	CD45 ⁺ cells (Events)	ILC2s (Events)	IL5/13 ⁺ ILC2s (Events)	ILC2s (%CD45 ⁺ cells)	IL5/13 ⁺ ILC2s (%CD45 ⁺ cells)	IL5/13 ⁺ ILC2s (% ILC2s)
01 Placebo INCS	5465 6740	16 15	7 7	0.29 0.22	0.13 0.1	43.8 46.7
02 Placebo INCS	16132 4003	18 1	7 0	0.11 0.02	0.04 0	38.9 0
03 Placebo INCS	21728 4704	24 5	5 1	0.11 0.11	0.02 0.02	20.8 20
04 Placebo INCS	17293 7854	24 7	10 1	0.14 0.09	0.06 0.01	41.7 14.3
05 Placebo INCS	3465 5605	7 4	4 0	0.20 0.07	0.12 0	57.1 0
06 Placebo INCS	15704 4729	32 5	16 0	0.20 0.11	0.10 0	50 0
07 Placebo INCS	4977 24425	5 16	3 5	0.10 0.07	0.06 0.02	60 31.3
08 Placebo INCS	13054 4773	44 1	27 0	0.34 0.02	0.21 0	61.4 0

42

43 **Table E1.** Raw data for FACS gating for total and cytokine positive ILC2 from nasal curettage
44 samples taken from each patient in the current study. These raw data from 24h post nasal allergen
45 challenge (NAC) samples taken from the placebo and intranasal corticosteroid (INCS) arms are
46 presented as number of gated events and percent of gated CD45⁺ cells (white blood cells). These
47 data are also presented in figure 2 of the manuscript.

48

49

Not significant	Placebo						INCS					
(pg/mL)	BL		7h		24h		BL		7h		24h	
Cytokines	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Eotaxin-3	359.5	811.1	649.2	1477.1	269.2	254.3	45.9	69.5	44.1	34.6	184.2	385.8
IL-1 β	3.2	3.3	1.4	1.1	3.2	2.2	2.2	2.0	1.5	1.6	2.7	1.9
IL-6	1.3	0.9	2.8	2.5	3.4	3.0	1.2	0.9	4.0	5.2	3.0	3.4
IL-8	513.0	368.1	267.8	112.0	693.6	330.9	349.0	224.6	279.7	193.6	654.5	730.7
IP-10	993.3	1136.1	449.5	227.4	769.0	513.4	674.1	1046.9	435.2	279.2	421.8	234.8
MCP-1	24.2	8.7	41.9	28.2	35.2	15.3	29.9	9.8	42.2	27.2	32.7	12.9
MCP-4	5.7	8.1	3.9	3.0	5.1	4.4	1.6	1.2	2.4	1.6	2.8	1.9
MDC	28.7	18.6	42.2	34.8	65.4	58.0	13.1	13.1	15.4	4.9	82.1	184.1
MIP-1 α	10.9	6.1	15.5	14.8	15.0	9.5	7.9	4.1	8.9	2.9	19.7	27.0
MIP-1 β	12.9	17.3	32.4	42.2	46.6	66.9	5.6	3.5	13.2	15.9	28.4	42.1
IL-16	62.7	79.8	71.9	84.8	79.1	71.8	22.0	21.7	31.7	34.7	53.8	36.9
IL-1 α	15.8	10.9	11.0	7.3	15.2	10.8	19.1	11.6	10.8	5.1	12.5	7.2
TARC	15.2	11.9	57.0	66.9	92.6	115.6	8.7	4.1	14.3	7.7	47.4	59.2
VEGF	288.0	124.1	232.9	88.8	236.9	66.1	263.0	103.1	247.8	57.4	268.1	106.0

50

51 **Table E2. Additional cytokine and chemokine levels in nasal lavage samples from patients**
52 **with mild allergic rhinitis and asthma.** Subjects were treated with placebo followed by intranasal
53 steroid for 14 days before NAC. At baseline (BL), 7h and 24h post-NAC. Cytokines that
54 demonstrated no significant change after INCS treatment are listed above. Data was presented as
55 mean with SD. A two-way ANOVA test with Bonferroni correction (α/m) was used to determine
56 statistical significance of parametric paired data.

57 *A number of cytokines were below the limit of detection of the assay kit including GM-CSF,
58 IFN- γ , IL-10, IL-12, IL-12p70, IL-15, IL-17, IL-17A/F, IL-33, TSLP, TNF α and TNF- β

59

60

61

62

63

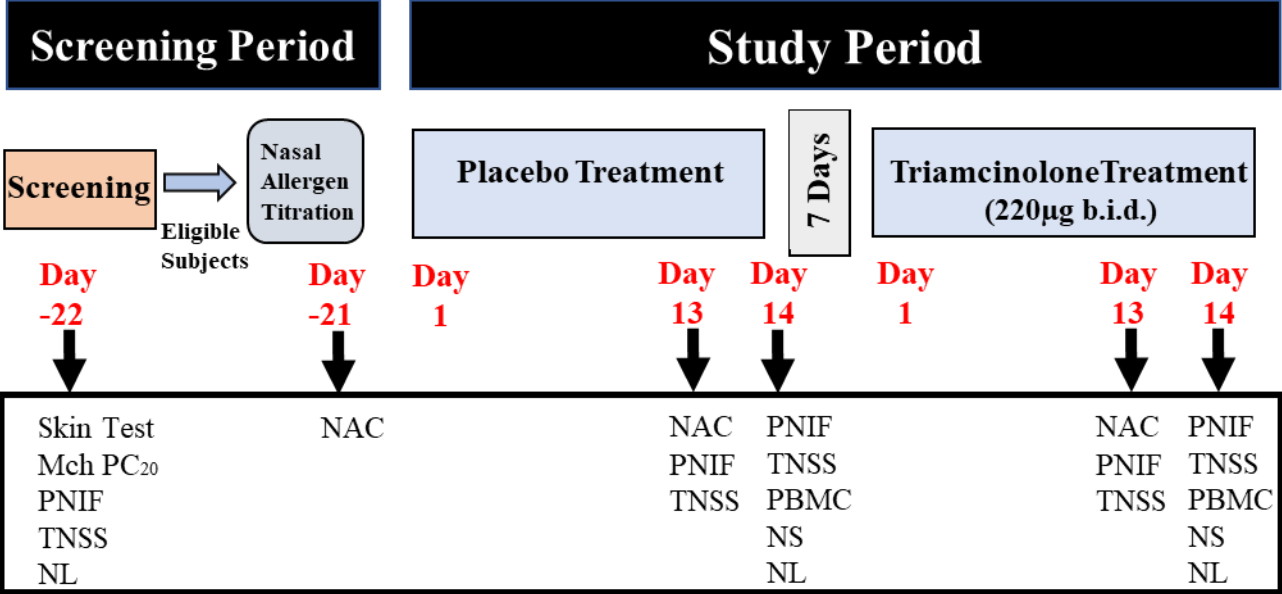


Figure E1. Study Design: Eligible subjects (allergic rhinitis patients with asthma) inhaled placebo by aqueous nasal spray twice a day for 14 days before nasal allergen challenge (NAC). Blood and nasal sampling were made 24 hours after NAC. Following a 7 day washout period, subjects inhaled 220 µg triamcinolone acetonide twice daily by aqueous nasal spray for 14 days before the NAC. Blood and nasal sampling were repeated as indicated. NAC= nasal allergen challenge; Mch PC₂₀ = concentration of methacholine required to reduce FEV₁ by 20%; INCS= intranasal corticosteroid; PNIF= peak nasal inspiratory flow; TNSS= total nasal symptom score; NL= nasal lavage; PBMC= peripheral blood mononuclear cells; NS= nasal scraping.

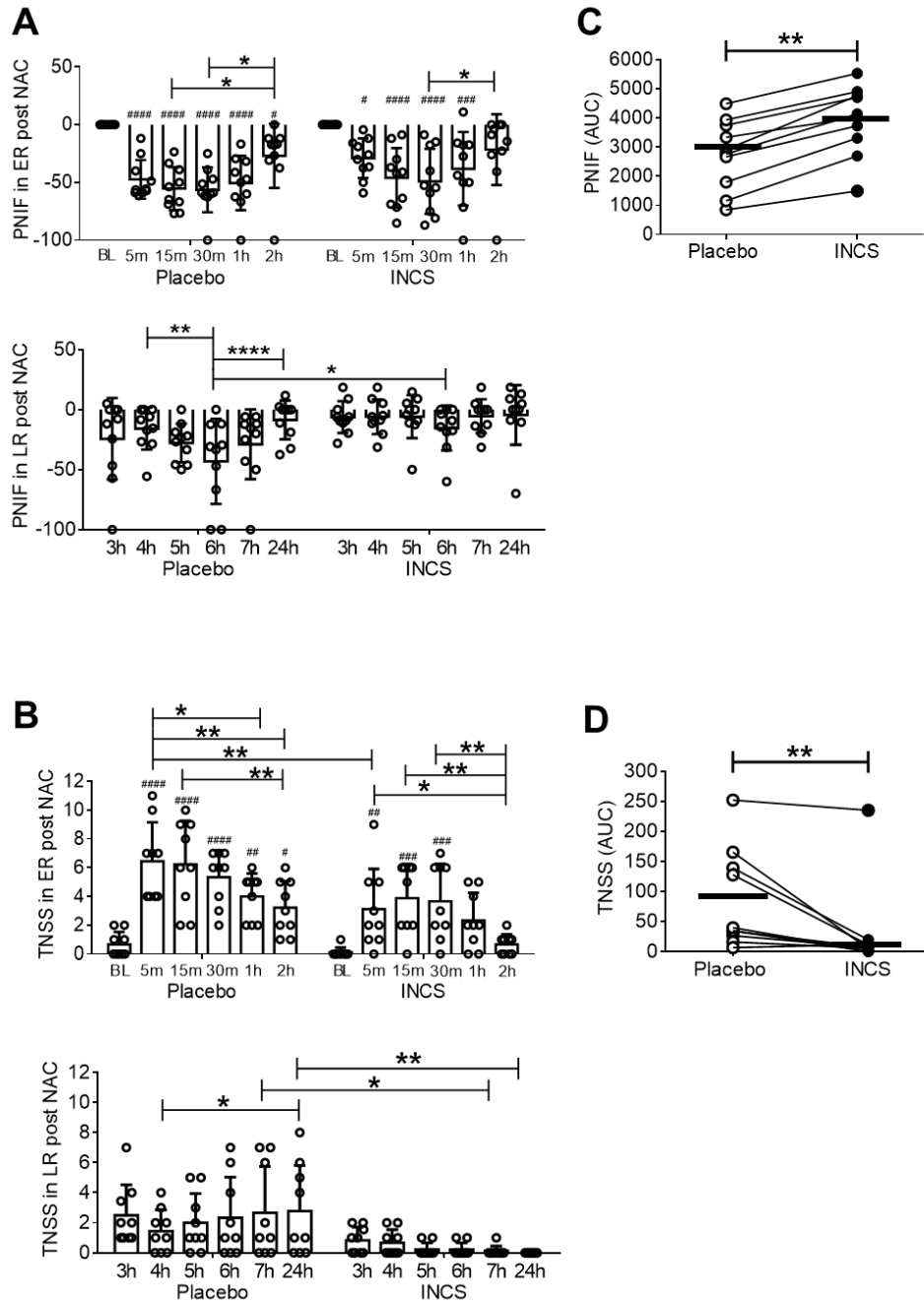
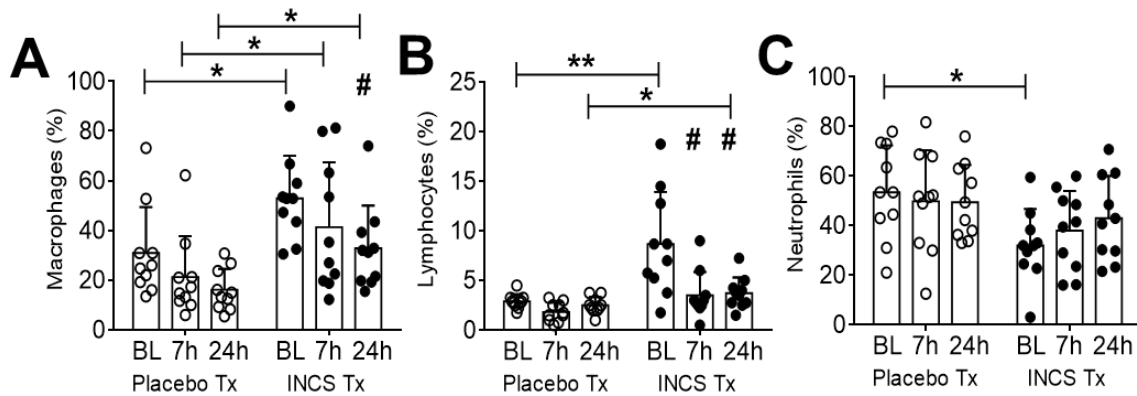


Figure E2. Nasal symptoms score (PNIF and TNSS) at baseline and post NAC in placebo and INCS Treatment Arms. Data are subdivided based on early and late responses. **(A)** PNIF is presented as % change from baseline (n=10), **(B)** TNSS at baseline and different time points after NAC in placebo and INCS arms (n=10). Nasal symptoms - PNIF **(C)** and TNSS **(D)** were analysed from 0 min to 24 hours post NAC in each treatment arm and data are presented as area under the curve (AUC) for each patient with horizontal bars showing median levels. A two-way ANOVA test with Bonferroni correction (α/m) was used to determine statistical significance of parametric paired data. A Wilcoxon test was used for between placebo and INCS treatment arm comparisons. BL= baseline. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$; # indicates comparison to baseline.

105



106

107

108

109

110

111

112

113

114

115

Figure E3. Cellular frequencies in nasal lavage fluid taken pre and post allergen challenge in the placebo and INCS treatment arms. INCS pre-treatment resulted in a significant decrease in (A) macrophages and (B) lymphocytes but not (C) neutrophils, 24h post NAC versus pre-NAC in the INCS treatment arm but not in the placebo treatment. Data were presented as mean $\pm$ SD; a two-way ANOVA test with Bonferroni correction (α/m) was used to assess significance of parametric paired data. BL= baseline.

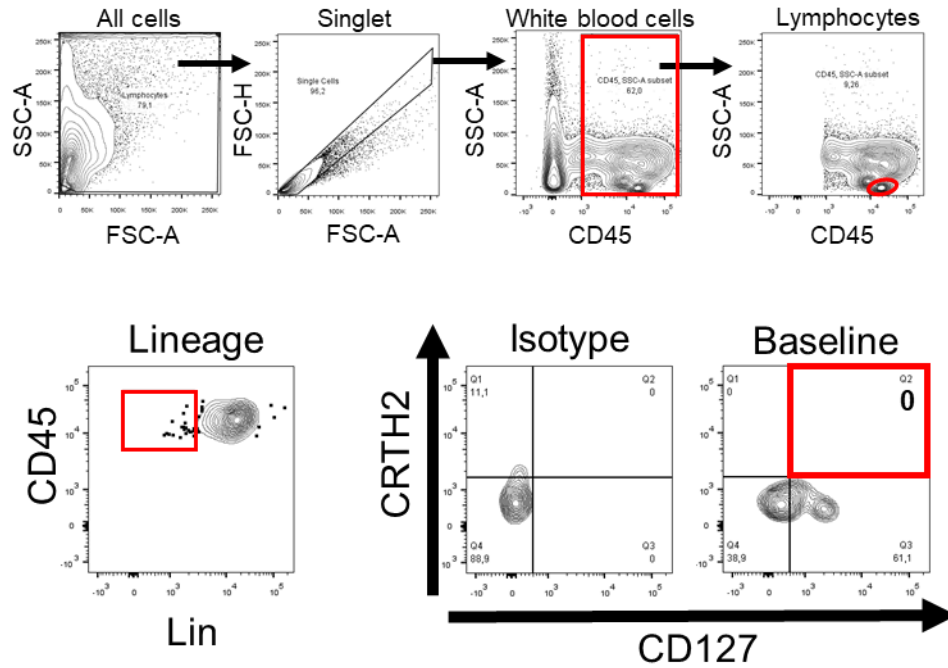


Figure E4. FACS gating of a representative nasal curettage sample taken pre-allergen challenge to identify ILC2s as live, singlet cells that are lin-CD45+CRTH2+CD127+. Data show that negligible numbers of ILC2s were detected in pre-allergen challenge samples from mild allergic rhinitis with asthma. This precluded the use of pre-allergen challenge samples and only 24h post-nasal allergen challenge samples were collected in the placebo and steroid treatment arm of this study.

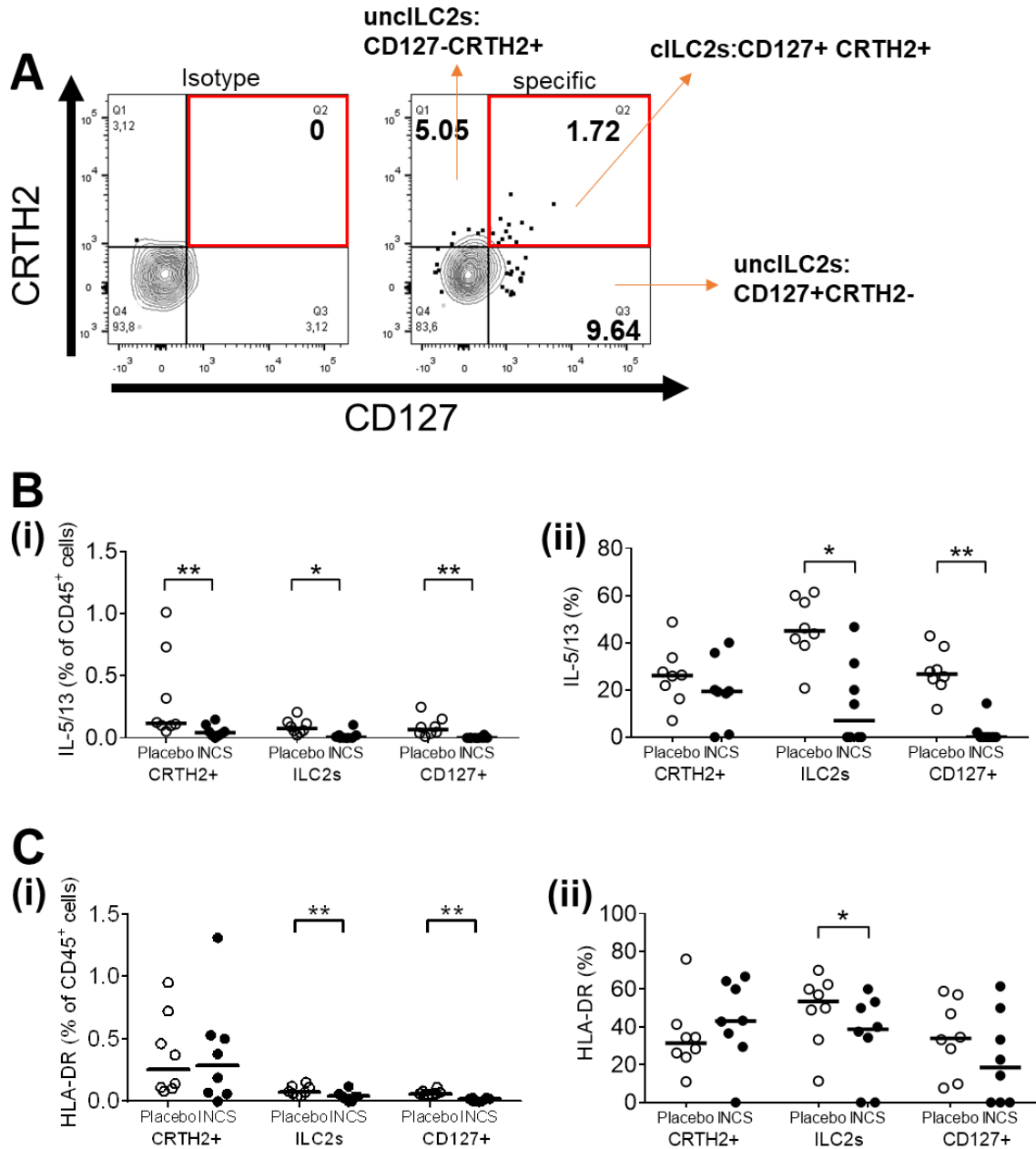


Figure E5: (A) Flow cytometric enumeration of nasal mucosa extracted cells identified as conventional ILC2s (cIL2s: lin-CD45+CD127+CRTH2+) compared to unconventional ILC2s (uncILC2s: lin-CD45+CD127-CRTH2+ and lin-CD45+CD127+CRTH2- cells). Expressed as (B) (i) a percent total CD45+ cells, comparable expression levels of IL-5/13 in all ILC2s sub-groups and all were significantly attenuated by INCS treatment, (ii) a percent of each ILC2 population, INCS treatment had differential inhibitory effects on type 2 cytokine expression. For HLA-DR+ILC2s expressed as (C)(i) percent CD45 or (C)(ii) percent ILC2 population – although comparable levels of expression were found, INCS treatment only significantly attenuated HLA-DR expression 24h post-NAC in the cILC2 population and not uncILC2s. Data (n=8) were analyzed for statistical significance using a Wilcoxon test for non-parametric paired data; horizontal bars represent median values of each data set. (*P<0.05 and ** P<0.01).

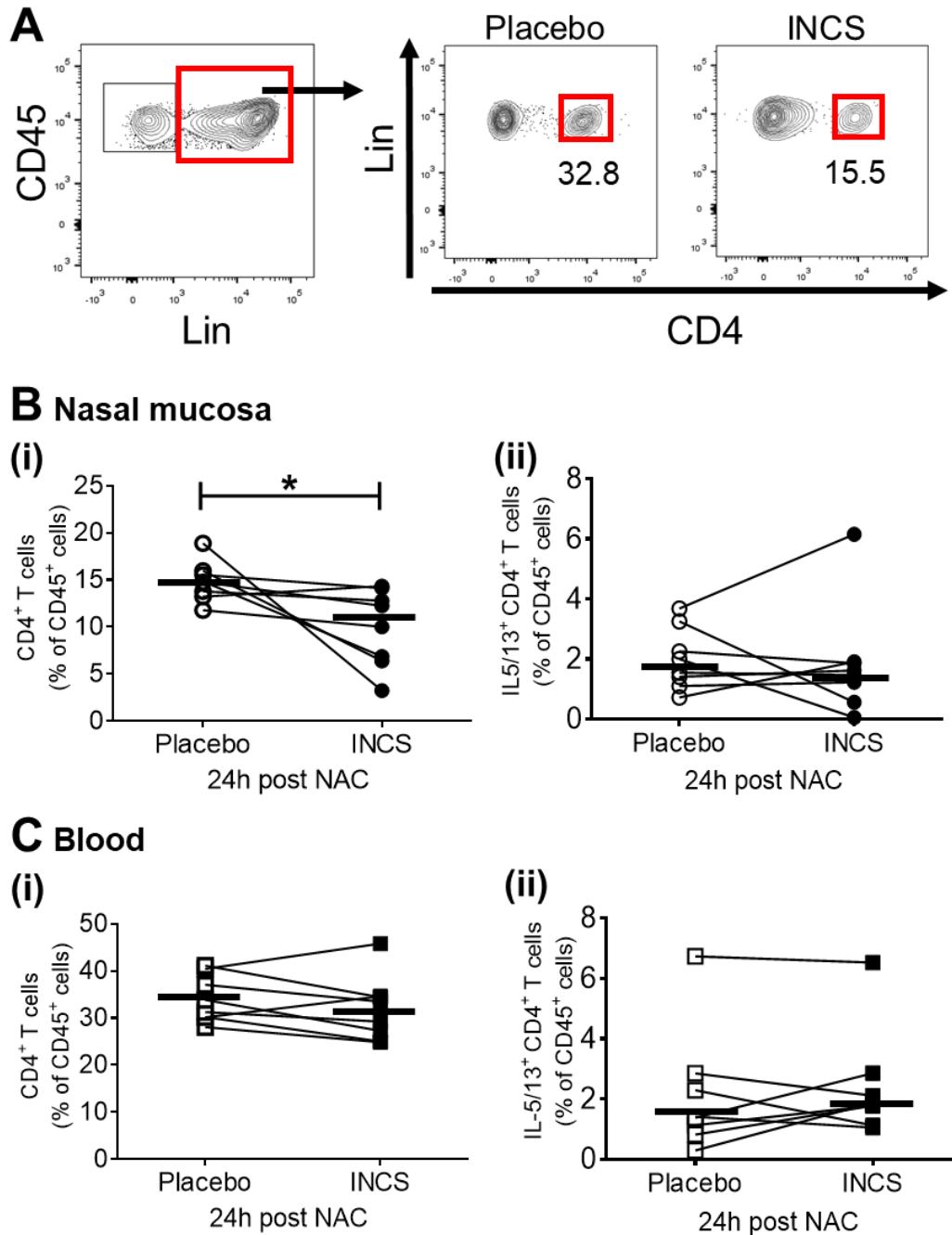


Figure E6. CD4⁺ T cells in nasal mucosa and peripheral blood 24 hours after NAC in placebo and INCS treatment groups. (A) FACS gating of live, singlet CD45⁺ Lin⁺CD4⁺ T cells in nasal mucosa extracted cells (B) There was a significant reduction of total CD4⁺ cells in nasal mucosa 24h post NAC in INCS treatment arm versus placebo (n=8). (C) No significant change was detected in IL-5⁺/IL-13⁺ CD4⁺ cells in nasal mucosa, total or IL-5⁺/IL-13⁺ CD4⁺ cells in blood (n=8). Data were analyzed for statistical significance using a Wilcoxon test; horizontal bars represent median values of each data set.

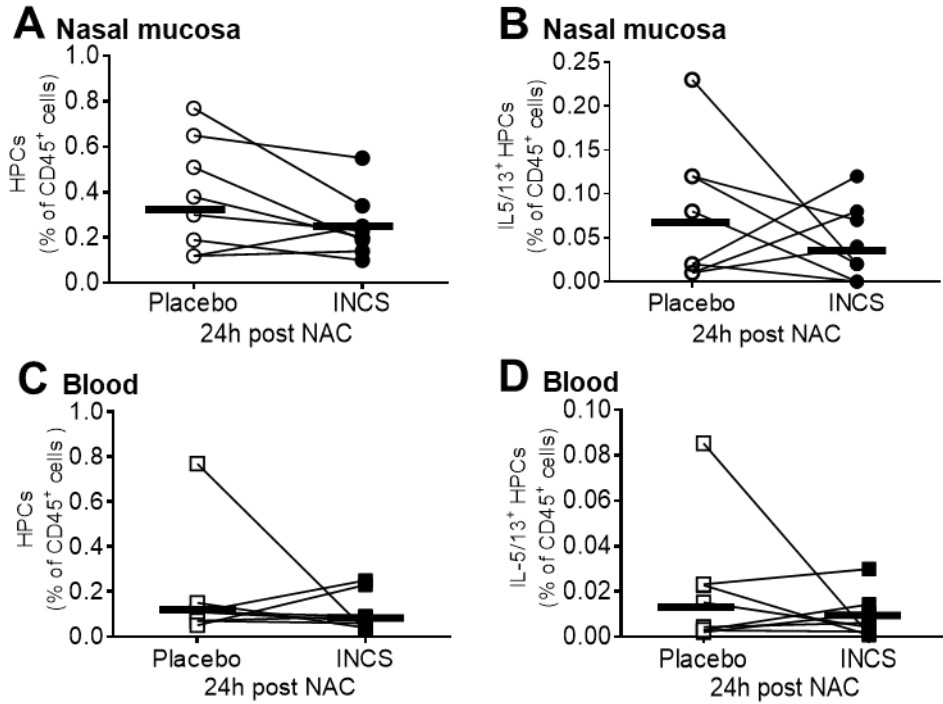
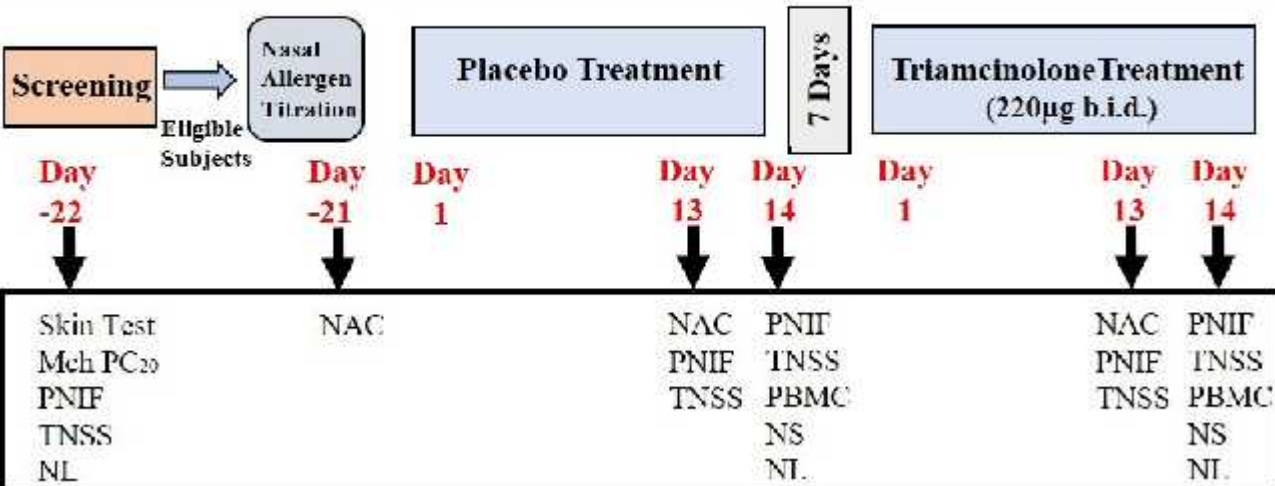
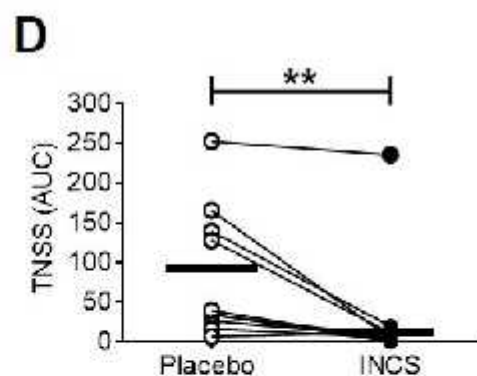
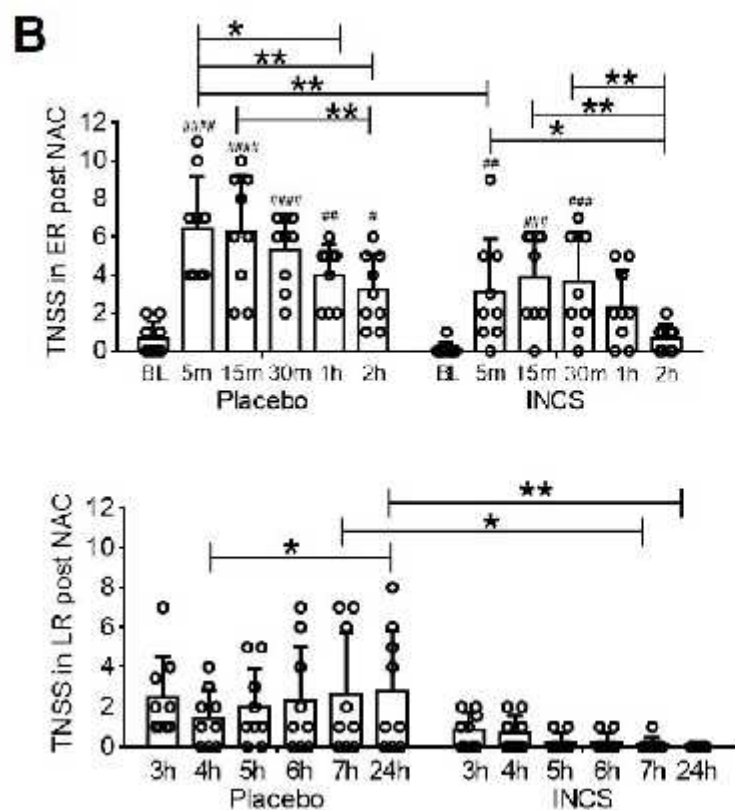
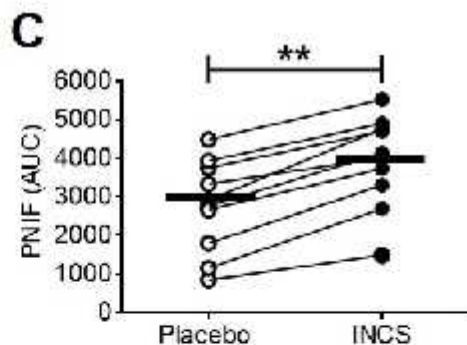
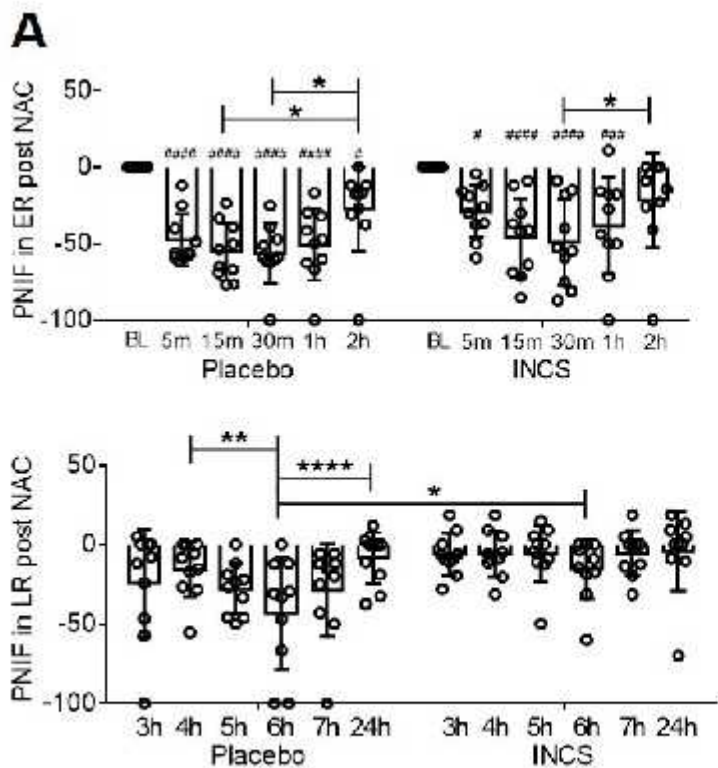


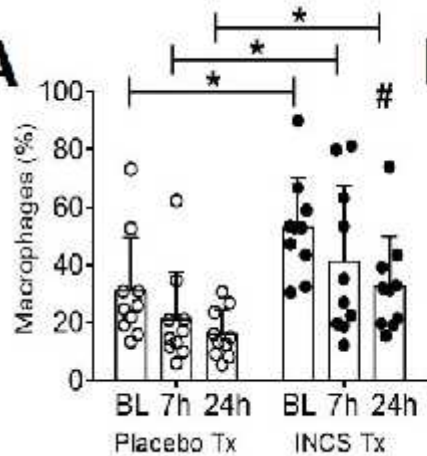
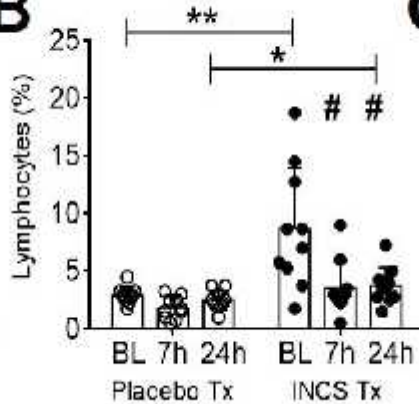
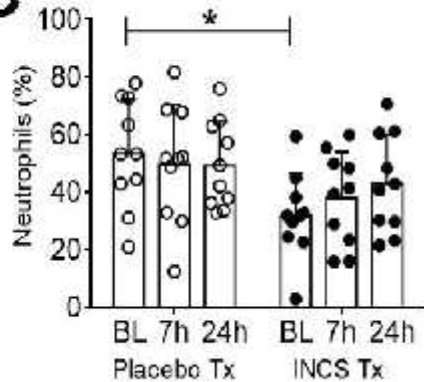
Figure E7. Hemopoietic progenitor cell (HPCs) levels in nasal mucosa and peripheral blood 24 hours after NAC in placebo and INCS treatment groups. (A-D) No significant change was detected in HPCs or IL-5⁺/IL-13⁺ HPCs in nasal mucosa or blood (n=8) after INCS treatment. Data were analyzed for statistical significance using a Wilcoxon test and the horizontal bars represent median values of each data set.

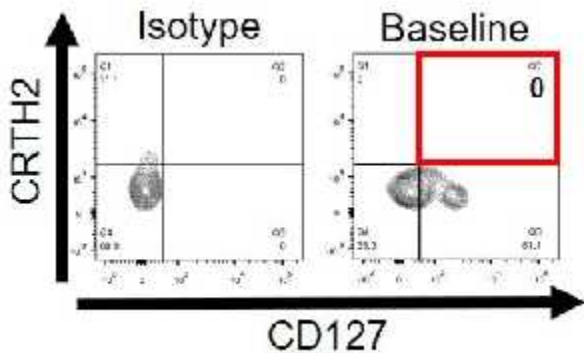
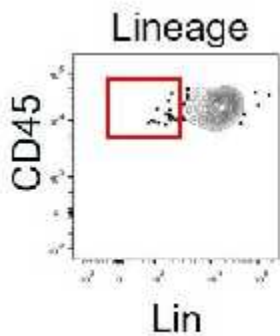
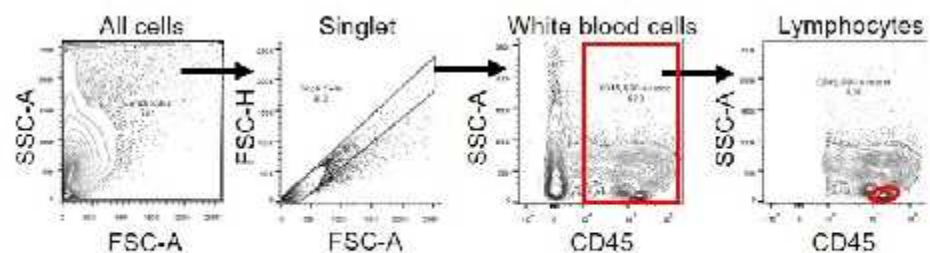
Screening Period

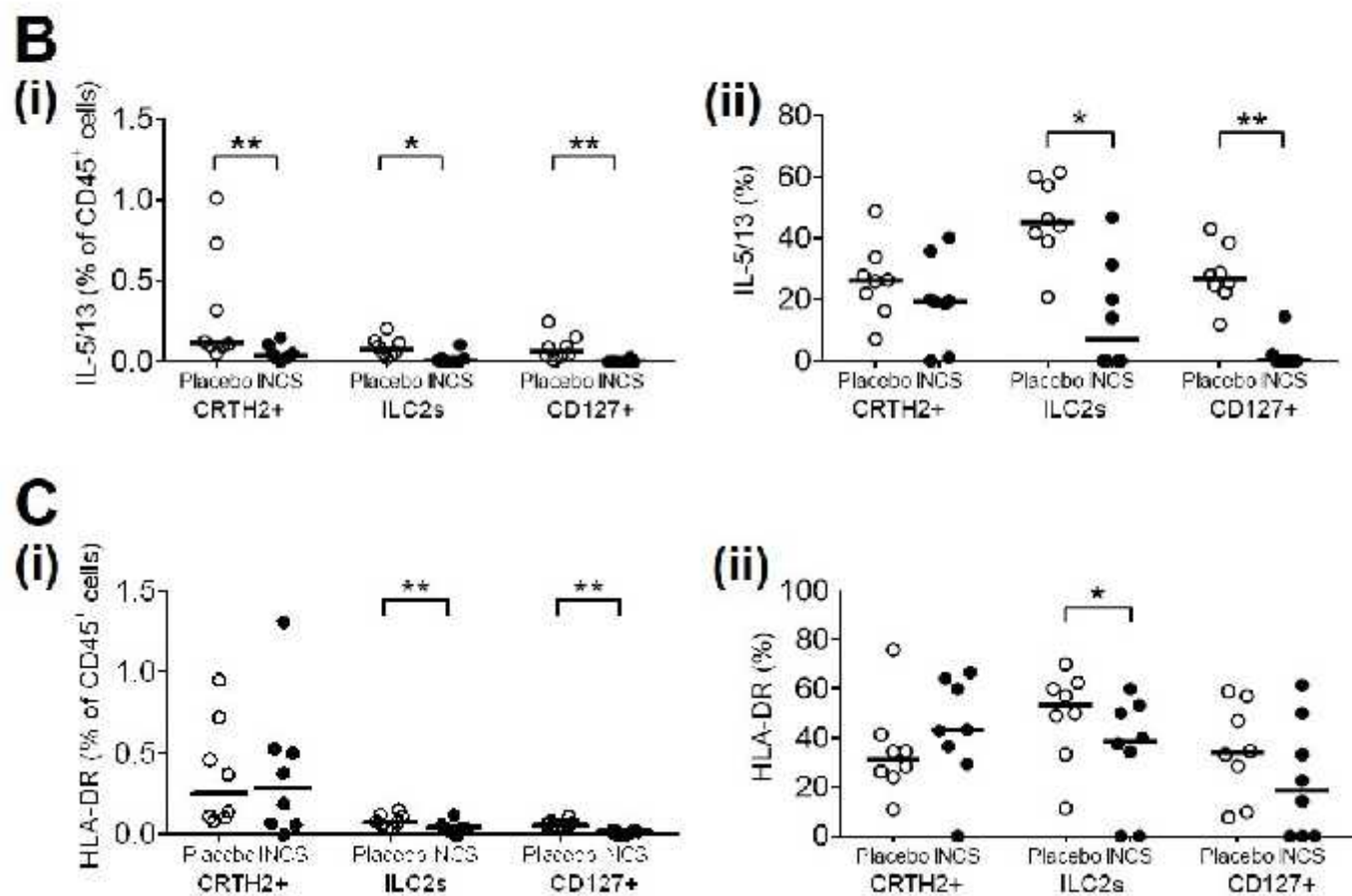
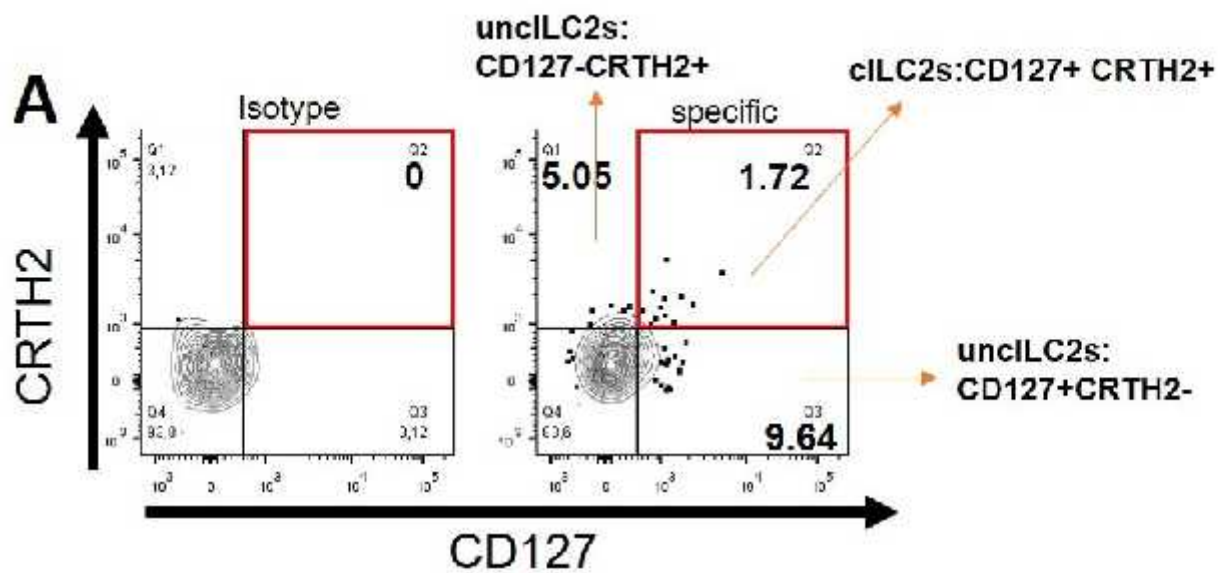
Study Period

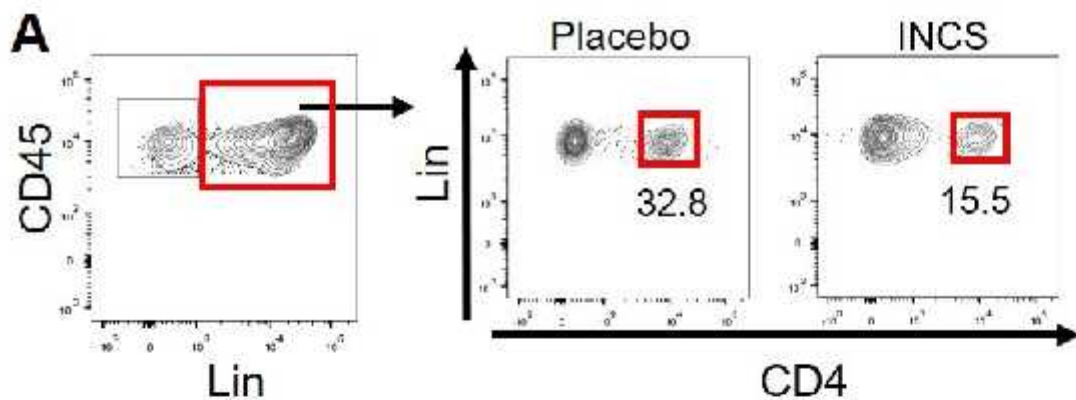




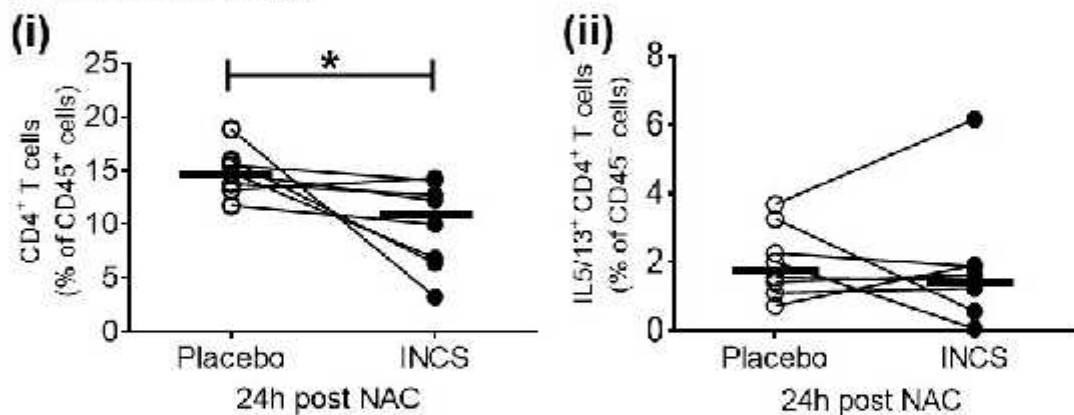
A**B****C**



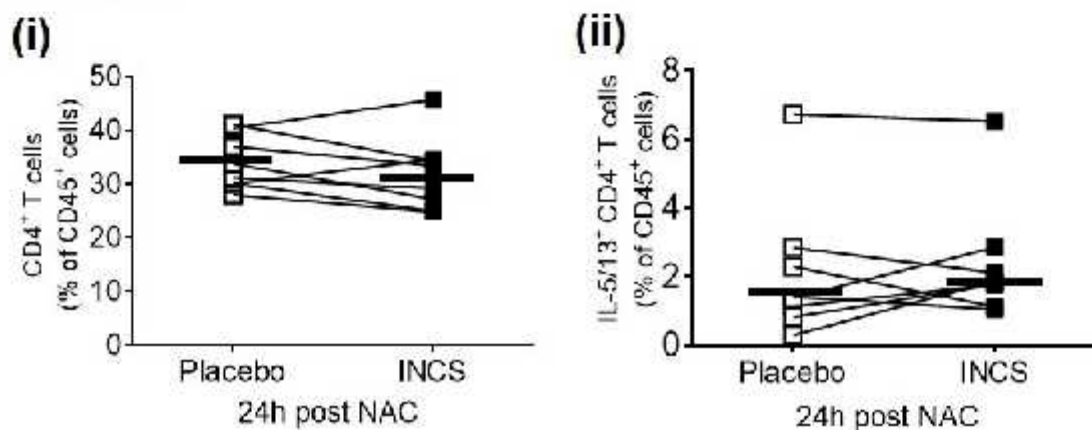


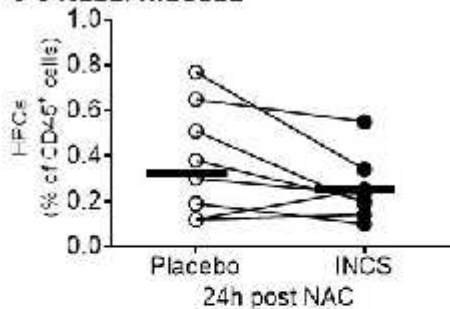
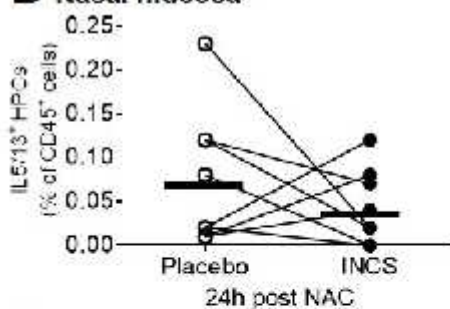
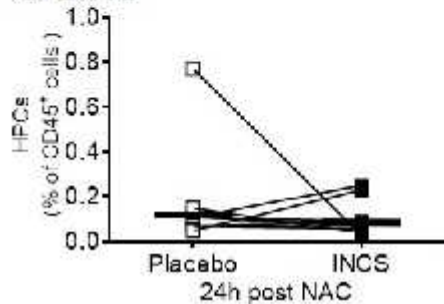


B Nasal mucosa



C Blood



A Nasal mucosa**B Nasal mucosa****C Blood****D Blood**