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Increased density of intraepithelial mast cells in exercise-induced bronchoconstriction regulated via epithelial-derived TSLP and IL-33

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Abstract

Background—Exercise-induced bronchoconstriction (EIB) is a prototypical feature of indirect airway hyperresponsiveness (AHR). Mast cells are implicated in EIB, but the characteristics, regulation, and function of mast cells in EIB are poorly understood.

Objectives—To examine mast cell infiltration of the airway epithelium in EIB, and the regulation of mast cell phenotype and function by epithelial-derived cytokines.

Methods—Endobronchial biopsies, epithelial brushings, and induced sputum were obtained from asthmatics with and without EIB, and normal controls. Mast cell proteases were quantified by

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qPCR, and mast cell density by design-based stereology. Airway epithelial responses to wounding and osmotic stress were assessed in primary airway epithelial cells and *ex vivo* murine lung tissue. Mast cell granule development and function were examined in cord blood-derived mast cells.

Results—Tryptase and carboxypeptidase A3 (CPA3) expression in epithelial brushings and epithelial mast cell density were selectively elevated in the asthma group with EIB. A *in vitro* scratch wound initiated the release of TSLP that was greater in epithelial cells derived from asthmatics. Osmotic stress induced the release of IL-from explanted murine lung that was increased in allergen-treated mice. TSLP combined with IL-33 increased tryptase and CPA3 immunostaining in mast cell precursors, and selectively increased cysteinyl leukotriene formation by mast cells in a manner that was independent of *in vitro* sensitization.

Conclusions—Mast cell infiltration of the epithelium is a critical determinant of indirect AHR, and the airway epithelium may serve as an important regulator of the development and function of this mast cell population.

Keywords

Asthma; Airway Hyperresponsiveness, Eicosanoid; Epithelial Cell; Leukotriene

INTRODUCTION

Mast cells are present in the conducting airways of the lung, and have important roles in host defense, repair, and allergen-induced inflammation;[1, 2] however, the type of mast cells infiltrating the airways in asthma, and their function remains incompletely understood. Mast cells are phenotypically divided into MC_T and MC_{TC} types based on the composition of their secretory granules that contain tryptase in both types of cells, but with the addition of carboxypeptidase (CPA3) and chymase in the MC_{TC} phenotype.[3] Initial studies found that mast cells in mucosal surfaces of the lung are predominantly MC_T type cells.[4, 5] A recent genomic study identified an increase in tryptase and CPA3 in the airway epithelium of asthmatics suggesting a novel mast cell phenotype in asthma,[6] particularly in patients with "Th2 high" asthma,[7] but the full physiological significance of this mast cell population is not fully established. In a genome-wide expression study among patients with asthma, we found that the expression of tryptase and CPA3 are specifically increased in induced sputum cells of patients with exercise-induced bronchoconstriction (EIB) a prototypical feature of indirect airway hyperresponsiveness (AHR) in asthma.[8] We have previously demonstrated mast cell degranulation following exercise challenge in patients with EIB.[9] Thus, we hypothesized that this novel tryptase- and CPA3-expressing mast cell population may play a key role in EIB. As recent genome-wide association studies have highlighted the potential importance of the IL-33 and thymic stromal lymphopoietin (TSLP) genes that are avidly expressed in the airway epithelium,[10–12] we tested a potential contribution from epithelial-derived cytokines towards the accumulation and activation of these EIB-associated mast cells.

We conducted endobronchial biopsies, epithelial brushings, and induced sputum in patients with asthma and EIB, and made comparisons to asthmatics that have AHR to methacholine (i.e. direct AHR), but do not have EIB, and to normal controls. To appreciate the origin of

this mast cell population, we examined the response to wounding *in vitro* using organotypic cultures of primary epithelial cells from subjects with and without asthma, and an *ex vivo* model of osmotic stress in lung tissue derived from mice with and without allergen-induced inflammation. As these model systems led to the release of TSLP and IL-33, we examined the effects of these epithelial-derived cytokines on mast cell granule development and mast cell production of eicosanoids. The results support a potential role of this novel mast cell population in indirect AHR, and that the airway epithelium may regulate the development and function of this mast cell population through TSLP and IL-33.

METHODS

Full experimental details are provided in the Methods section in this article's Online Repository at www.jacionline.org.

Study Subjects and Study Protocol

We used endobronchial biopsies, epithelial brushings and induced sputum from a repository of samples collected at the University of Washington designed to examine differences between asthmatics with and without EIB and non-asthmatic controls.[13] Induced sputum and research bronchoscopy were conducted 2–10 days apart. Written informed consent was obtained from all participants and the University of Washington Institutional Review Board approved the study protocol. Patients with asthma, based on a positive methacholine challenge, were characterized as EIB(+) or EIB (−) based on the response to exercise challenge.[14]

Either epithelial brushings or endobronchial biopsy samples were available from 10 controls, 12 EIB (−) asthmatics, and 19 EIB (+) asthmatics. Endobronchial biopsy tissue was inadequate for stereology assessment in 1 control, 2 EIB (−) asthmatics, and 1 EIB (+) asthmatic. Insufficient RNA was available from the epithelial brushings for the PCR analysis in 1 control, 2 EIB (−) asthmatics, and 2 EIB (+) asthmatics.

Copy number quantitative PCR

Real-time PCR analysis was conducted using TaqMan primer probe sets with FAM probes for *TPSAB1* (Hs02576518_gH), *CPA3* (Hs00157019_m1), *CMA1* (Hs01095979_g1), *IL33* (Hs00369211_m1), *TSLP* (Hs00263639_m1), and when applicable a primer-limited VIC probe for *HPRT1* (4326321E) as an endogenous control.[15] In some samples, the PCR amplification of HPRT1 was low, and these samples were excluded. The number of samples with accurate PCR data for each group is noted in the figures.

Immunohistochemistry and Design-based Stereology

We used the physical disector method to enumerate the density of mast cells in the airway epithelium relative to the volume of the epithelium (*Nv MC, epi*).[7] The surface area of the basal lamina to the volume of epithelium (*Sv bala, epi*) was quantified to calculate the number of mast cells relative to the surface area of the basal lamina (*Ns MC, bala*).

Primary airway epithelial cell culture and scratch wound model

Primary bronchial epithelial cells isolated during bronchoscopy were expanded in culture and cryopreserved. Cryopreserved primary epithelial cells were differentiated in air-liquid-interface (ALI) organotypic cultures.[8] A series of scratch wounds were created with a pipet tip on the apical surface of the ALI culture. The levels of TSLP and IL-33 were assayed in the basolateral medium by ELISA.

Murine osmotic stress model and allergen-induced airway inflammation model

The murine pre-B cell line Ba/F3 cells were stably transfected with full length ST2L and a NF-kB-luciferase reporter. Conditioned supernatants from C57Bl/6 mouse lung explants were evaluated for murine IL-33 and for ST2-activating biological activity using the Ba/F3 cell assay. Osmotic stress was applied to mouse lung tissue by adding sorbitol or mannitol at concentrations ranging from 0.06 to 0.5 M for 48 hours. To examine the effects of allergen-induced airway inflammation, cockroach extract (100 ug) was administered by intranasal delivery every three days for 2 weeks prior to removal of lung explants. All animal studies were reviewed and approved by the Amgen Animal Care and Use Committee.

Human cord blood-derived mast cell (CBMC) culture

Human umbilical cord blood was obtained from anonymous donors to the Puget Sound Blood Center. CD34+ cells were isolated and treated with IL-3 (30 ng/ml), IL-6 (100 ng/ml), and SCF (100 ng/ml) during the first week and transferred to a new flask containing IL-6 (100 ng/ml), and SCF (100 ng/ml) in subsequent weeks.[16]

Flow cytometry and immunocytochemistry of granule development

Granule development in CD34-selected cord blood cells was assessed at one week and three weeks of culture media alone or with IL-33 (10 ng/ml), TSLP (10 ng/ml), or both IL-33 and TSLP (both at 10 ng/ml).[17] Intracytoplasmic staining was evaluated by flow cytometry using primary antibodies directed against CPA3, tryptase, and chymase. Immunocytochemistry for CPA3 was conducted on cytospin preparations using the DAB technique.

Assessment of eicosanoid formation by mature CBMCs

Mature CBMCs were passively sensitized with human IgE for 7 days and activated with the murine monoclonal CRA1 (clone AER-37) antibody that activates high affinity IgE receptor (FcεR1) for 1 hour.[18] Prior to activation, CBMCs were treated with or without IL-13, IL-25, IL-33, TSLP, or both IL-33 and TSLP (all at 10 ng/ml) for 7 days.[16] ELISA assays were used to measure LTC_4, cysteinyl leukotrienes (CysLTs), and prostaglandin D_2 (PGD_2).

Statistical Analysis

Differences in the characteristics of the groups were assessed with a chi-square, ANOVA or Kruskal-Wallis test. Differences in mast cell gene expression and density were tested with a Kruskal-Wallis test. Associations between mast cell markers and the severity of EIB were assessed by linear regression. Differences across multiple cell culture conditions were

assessed with a one-way ANOVA. Differences in cell phenotype as well as treatment condition (i.e. wounding) were assessed with a two-way ANOVA.

RESULTS

Subject Characteristics

The groups of subjects were similar with respect to age, gender, ethnicity, race, and baseline lung function measured by FEV_1 and FVC (Table 1). There were notable differences between the groups for baseline airflow obstruction reflected in the FEV_1/FVC ratio and direct AHR to methacholine challenge with overall differences between the three groups, as well as differences between the two asthma groups regarding the FEV_1/FVC ratio (*P=0.03*) and methacholine PC_{20} (*P=0.03*). The severity of EIB was markedly greater in the EIB (+) group than either of the other groups with marked differences between the two asthma groups with respect to the maximum fall in FEV_1 after exercise challenge (*P<0.001*) and area under the FEV_1-time curve (AUC30, *P<0.001*).

Epithelial mast cell protease gene expression is increased in asthmatics with EIB

The expression of the tryptase (*TPSAB1*, Fig 1A) and CPA3 (Fig 1B) genes in epithelial brushings were increased in the EIB (+) asthma group relative to the EIB (−) asthma group and the control group, but there was no difference between the controls and the EIB (−) group. Immunohistochemistry of airway biopsies revealed leukocytes in the epithelium that contain tryptase and CPA3 consistent with intraepithelial mast cells as the origin of these findings in epithelial brushings (Fig S1). The expression of chymase (*CMA1*) was more than 1000 times lower than the expression of either *TPSAB1* or *CPA3* in the epithelium. The expression of *CMA1* was increased in the EIB (+) asthma group relative to the control group, but not relative to the EIB (−) group (Fig 1C). Gene expression analysis of induced sputum cells confirmed our prior genomic findings in a separate cohort of subjects.[8] The expression of *TPSAB1* in induced sputum cells was increased in the EIB (+) asthma group relative to controls, while the expression of *CPA3* was increased in the EIB (+) group relative to the EIB (−) asthma group and to the control group (Figs 1D & E). There was no difference in *CMA1* expression in induced sputum cells between the groups (Fig 1F). The severity of EIB measured by the maximum fall in FEV_1 after exercise was associated with the number of copies of *TPSAB1* (r^2=0.31, *P=0.0006*) and *CPA3* in the airway epithelium (r^2=0.34, *P=0.0004*), but less associated with the number of copies of Chymase (r^2=0.11, *P=0.08*, Fig S2).

Intraepithelial mast cell density is specifically increased in EIB (+) phenotype of asthma

Differences in epithelial mast cell density were quantified by design-based stereology, a technique that avoids the usual sources of bias encountered in two-dimensional sections.[7, 19] The density of mast cells per volume of the epithelium was greater in the EIB (+) group relative to the normal control, and relative to the EIB (−) asthma group (Fig 2A). The surface area of the basal lamina (bala) relative to the volume of the epithelium was not altered in asthma (*P=0.74*, Fig S3). The number of mast cells relative to the surface area of the basal lamina was greater in the EIB (+) group relative to the control group (Fig 2B). The severity of EIB measured by the maximum fall in FEV_1 after exercise was associated with

the density of mast cells relative to the epithelial volume (r^2=0.24, *P=0.002*) and epithelial mast cells relative to the area of the basal lamina (r^2=0.12, *P=0.03*, Fig S4).

TSLP and IL-33 are generated in response to physiologically relevant stressors implicated in asthma

To further understand the origin of the intraepithelial mast cell population that we identified, we conducted studies examining the release of IL-33 and TSLP by epithelial cells. We postulated that IL-33 and TSLP might influence mast cell development as both mature mast cells and early CD34+ progenitor cells express the IL-33 receptor (ST2L) and the TSLP receptor (TSLPR).[17] Although the gene expression for IL-33 and TSLP in epithelial brushings was similar among the three different groups (Fig S5), we further examined the release of TLSP and IL-33 in response to mechanical wounding and osmotic stress. These stimuli are relevant to the pathogenesis of indirect AHR as epithelial shedding is increased in patients with EIB.[20] Also, as the minute ventilation increases during exercise challenge, osmotic stress occurs leading to acute bronchoconstriction and an injury syndrome linked to asthma in athletes training at high levels.[21] A scratch wound in fully differentiated primary airway epithelial cells in organotypic culture caused a time-dependent release of TSLP that was greater in epithelial cells derived from EIB (+) asthmatic donors (n=3) relative to cells from normal controls (n=2, Fig 3). Although IL-33 release presumably occurs in response to injury and mechanical stress, extracellular IL-33 was below the ELISA detection limit in this model.

We used an *ex vivo* murine model to examine the release of IL-33 in response to epithelial stress initiated by osmotic agents. Ba/F3 cells stably transfected with murine ST2L and an NF-kB-luciferase reporter were used to detect IL-33 activity (see Online Repository). Lung explants exposed to increasing concentrations of sorbitol from 0.06 to 0.5 M for 48 hours caused a dose-dependent increase in ST2 activity in the culture medium (Fig 4A). Lung explants exposed to mannitol had a similar concentration-dependent increase in ST2 activity in the supernatant that reached a maximum at about 0.3 M possibly due to the limited solubility of mannitol (Fig 4B). A comparison of ST2 activity in response to 0.5 M concentrations of sorbitol or mannitol from the same lung explants revealed a similar increase in ST2 activity (Fig 4C). Immunoprecipitation (IP) of lung supernatant revealed IL-33 bands from the lungs treated with osmotic agents, but not in the supernatant from lung explants exposed to medium alone (Fig S6C).

Lung tissue from allergen-challenged mice had increased ST2 activity compared to explants from naïve or saline-challenged mice (*P<0.001*). Osmotic stress applied to allergen-challenged lung tissue caused a substantially greater release of ST2 activity than the same osmotic stress applied to explants from either naïve or saline challenged mice (Fig 4D).

The development of the mast cell granule phenotype is influenced by TSLP and IL-33

As TSLP and IL-33 were released from the epithelium, we examined the effects of these cytokines on the development of mast cell proteases in CD34+ cord blood cells after one and three weeks of *in vitro* mast cell differentiation. Following the first week, there was no discernable effect of TSLP, IL-33 or both cytokines combined on the intracytoplasmic

staining for tryptase (Fig S7A) or CPA3 (Fig S7B) by flow cytometry. After three weeks of culture with the same cytokines, there was an overall increase in mean intensity of immunostaining for tryptase (*P=0.02*, Fig S7C) and CPA3 (*P=0.05*, Fig S7D) in the cells treated with the TSLP and/or IL-33. As the intracellular immunostaining for chymase could not be identified in fully mature CBMCs, we did not assess chymase by flow cytometry. We further quantified CPA3 immunostaining by immunocytochemistry on the same cord-blood derived cells after three weeks of culture, demonstrating that there was an increase in the percentage of CPA3-positive cells after treatment with TSLP and/or IL-33 (*P=0.05*, Figs S7E&F).

TSLP and IL-33 alters mast cell formation of CysLTs and PGD_2

We further examined the effects of IL-33 and/or TSLP on the function of MCs. Treatment of CBMCs with IL-33 and/or TSLP for 7 days prior to activation via FcεR1 increased CysLT formation when both IL-33 and TSLP were administered together, but not IL-33 or TSLP alone (Fig 5A). When CBMCs were passively sensitized with human polyclonal IgE, there was an increase in CysLT formation in response to the FcεR1-activating antibody (*P=0.06*). In passively sensitized CBMCs, treatment with IL-33 and TSLP together increased CysLT formation, while treatment with either IL-33 or TSLP alone did not (Fig 5B). Although activation by the FcεR1-activating antibody initiated PGD_2 synthesis by unsensitized and passively sensitized CBMCs, there was no increase in PGD_2 formation mediated by IL-33 and/or TSLP (Figs 5C&D).

We also examined the effects of IL-25 on CBMCs as epithelial cells are a prominent source of IL-25, and the IL-25 receptor is expressed on airway mast cells.[22] Treatment of CBMCs with IL-25 did not alter CysLT or PGD_2 formation with or without passive sensitization (Fig 6A&B). We also found that IL-13 did not alter CysLT or PGD_2 formation in unsensitized CBMCs; however, after passive sensitization there was an increase in CysLT but not PGD_2 formation in IL-13 treated CBMCs following FcεR1-mediated activation (Fig 6C&D). Collectively, these results indicate that IL-33 and TSLP together have a selective influence on the 5-LO pathway that is independent of sensitization, while IL-13 selectively alters the 5-LO pathway only during *in vitro* sensitization.

DISCUSSION

Our findings demonstrate that intraepithelial mast cells with high CPA3 and tryptase expression, but low chymase expression recently identified in asthma,[6, 7, 23] are restricted to patients with indirect AHR in the form of EIB. We found that airway epithelial cells release TSLP and IL-33 in response to wounding and osmotic stress respectively, and that these two cytokines are generated in increased quantities by epithelial cells from patients with asthma and from allergen-sensitized airways of mice respectively. TSLP and IL-33 enhanced the EIB-associated granule phenotype and increased IgE receptor-mediated CysLT formation by mast cells with or without passive sensitization. These results highlight an important function of the airway epithelium to regulate mast cell phenotype and function, particularly in the setting of indirect AHR.

Among subjects with asthma, approximately 30–50% of subjects have EIB, a feature of asthma that is closely tied to the severity of indirect AHR.[24] Asthmatics with EIB have epithelial shedding into the airway lumen and increased production of CysLTs.[20] Mast cell degranulation and sustained generation of both CysLTs and PGD_2 occurs following exercise challenge in subjects with EIB.[9, 25] Recent work demonstrates that the density of intraepithelial mast cells is increased in asthma,[7] especially in patients with a "Th2 high" genomic signature.[6, 7] Another study found the epithelial expression of both tryptase and CPA3 were elevated in asthma, but similar across differing levels of asthma severity.[23] We found that tryptase and CPA3 expression in epithelial brushings and mast cell infiltration of the epithelium in endobronchial tissue are selectively increased in the group of asthmatics that have EIB, revealing an important physiological implication of this mast cell population. The group of asthmatics without EIB in our study all had positive methacholine challenge tests, but had mast cell density and gene expression that were no different than in the control group suggesting that differences in direct and indirect hyperresponsiveness may be mast cell mediated.

Recent studies demonstrate that the epithelium can regulate AHR in a murine model through mast cells,[26] and that epithelial cells alter the activation of mast cells.[27] Cross talk between mast cells and airway epithelial cells in culture has also been described.[28] We focused on the response to epithelial injury and osmotic stress as these factors have been implicated in the "injury syndrome" that is associated with the development of asthma and EIB in athletes who train at high levels in cold and dry environments.[29] We found that epithelial cells isolated from asthmatics released more TSLP following a scratch wound, suggesting that disruption of susceptible epithelium may bias immunity towards allergen sensitization.[30] As prior work revealed that TSLP expression is elevated in the skin following trauma,[31] and plays an important role in wound repair,[32] these data further indicate that airway epithelial cells of asthmatics exhibit features of a chronic wound.

Osmotic stress during exercise or through inhalation of a solution with high osmolarity such as mannitol causes acute bronchoconstriction in asthmatics with indirect AHR.[24] We demonstrate here that osmotic stress applied to murine lung tissue generates IL-33 activity *in vitro*, and that allergen-induced airway inflammation primes the lung tissue for greater osmotic stress-induced IL-33 release. Although we do not demonstrate that the IL-33 was of epithelial origin *per se*, small airway epithelial immunostaining for IL-33 was lost rapidly following osmotic stress (not shown). The form of IL-33 released from lung explants was ~24 kDa, indicating that proteolytic processing occurred, possibly coincident with recently described processed and more active forms of IL-33 (i.e. $IL\text{-}33_{95–270}$, $IL\text{-}33_{99–270}$, or $IL\text{-}33_{109–270}$).[33] As IL-33 can be both activated and inactivated by post-translational processing, our assay based on ST2 activity suggests that osmotic stress leads to the release of an active form of IL-33.[34, 35] As osmotic stress does not lead directly to cell death, IL-33 may be released by an active but still incompletely defined pathway.[36] The release of IL-33 in murine airways is provoked by ATP release,[37] and the nuclear to cytoplasmic export of IL-33 is also ATP-dependent,[36] suggesting that ATP release in response to water loss or osmotic stress may initiate IL-33 release.

The effects of IL-33 and TSLP on granule development have not been studied in detail. A prior study indicated that conditioned media from IL-13 treated epithelial cells reduces the expression of chymase without changes in tryptase and CPA3 in CBMCs.[7] In CD34+ progenitor cells, IL-33 increased tryptase immunostaining at three weeks of culture during *in vitro* mast cell differentiation.[31] Our results suggest that IL-33 in combination with TSLP increases both tryptase and CPA3 early during *in vitro* CBMC differentiation, but the effects were modest in magnitude.

The formation of CysLTs is important during the sensitization phase of allergen-induced inflammation,[38] and CysLTs and PGD_2 are implicated in the development of bronchoconstriction following exercise or osmotic challenges to the airways.[21] Although IL-33 induces the generation of PGD_2 in murine bone marrow-derived mast cells (BMMCs),[39] IL-33 did not generate PGD_2 [31, 40] or the CysLT LTC_4 in human CD34+ progenitor-derived mast cells.[31] However, IL-33 in combination with TSLP had prominent effects on IL-5 and IL-13 release in human mast cells,[31] and TSLP-mediated effects were enhanced in the presence of an IL-1 family member including IL-33.[41] Supernatant from necrotic murine structural cells induces CysLT generation by BMMC at least in part via IL-33.[42] Our work demonstrates that IL-33 in combination with TSLP augmented IgE receptor-mediated CysLT generation in CBMCs, and this "priming" effect occurred with or without passive sensitization. In contrast, IL-13 augmented the production of CysLTs only in the context of passive sensitization.

We conclude that mast cell infiltration of the airway epithelium is a key feature of indirect AHR, and that the epithelium may play a important role in the retention and activation of mast cells via the generation of TSLP and IL-33 in response to epithelial stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AHR	Airway hyperresponsiveness

ALI	Air liquid-interface
CBMC	Cord blood-derived mast cell
CPA3	Carboxypeptidase A3
EIB	Exercise-induced bronchoconstriction
FEV_1	Forced expiratory volume in one second
$cPLA_2$	Cytosolic phospholipase A_2
CysLT	Cysteinyl leukotriene
$CysLTR_1$	Cysteinyl leukotriene 1 receptor
LT	Leukotriene
PG	Prostaglandin
$sPLA_2$	Secreted phospholipase A_2
TSLP	Thymic stromal lymphopoietin

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Key Messages

- Infiltration of the airway epithelium with tryptase- and CPA3-positive mast cells is selectively increased in patients susceptible to exercise-induced bronchoconstriction (EIB).
- Epithelial cells release TSLP and IL-33 in response to mechanical wounding and osmotic stress.
- TSLP in combination with IL-33 increases the mast cell formation of eicosanoids that are important in EIB.

Capsule Summary

Intraepithelial mast cells are a critical determinant of indirect airway hyperresponsiveness (AHR). Epithelial cells release TSLP and IL-33 in response to injury and osmotic stress that alter the phenotype and function of human mast cells.

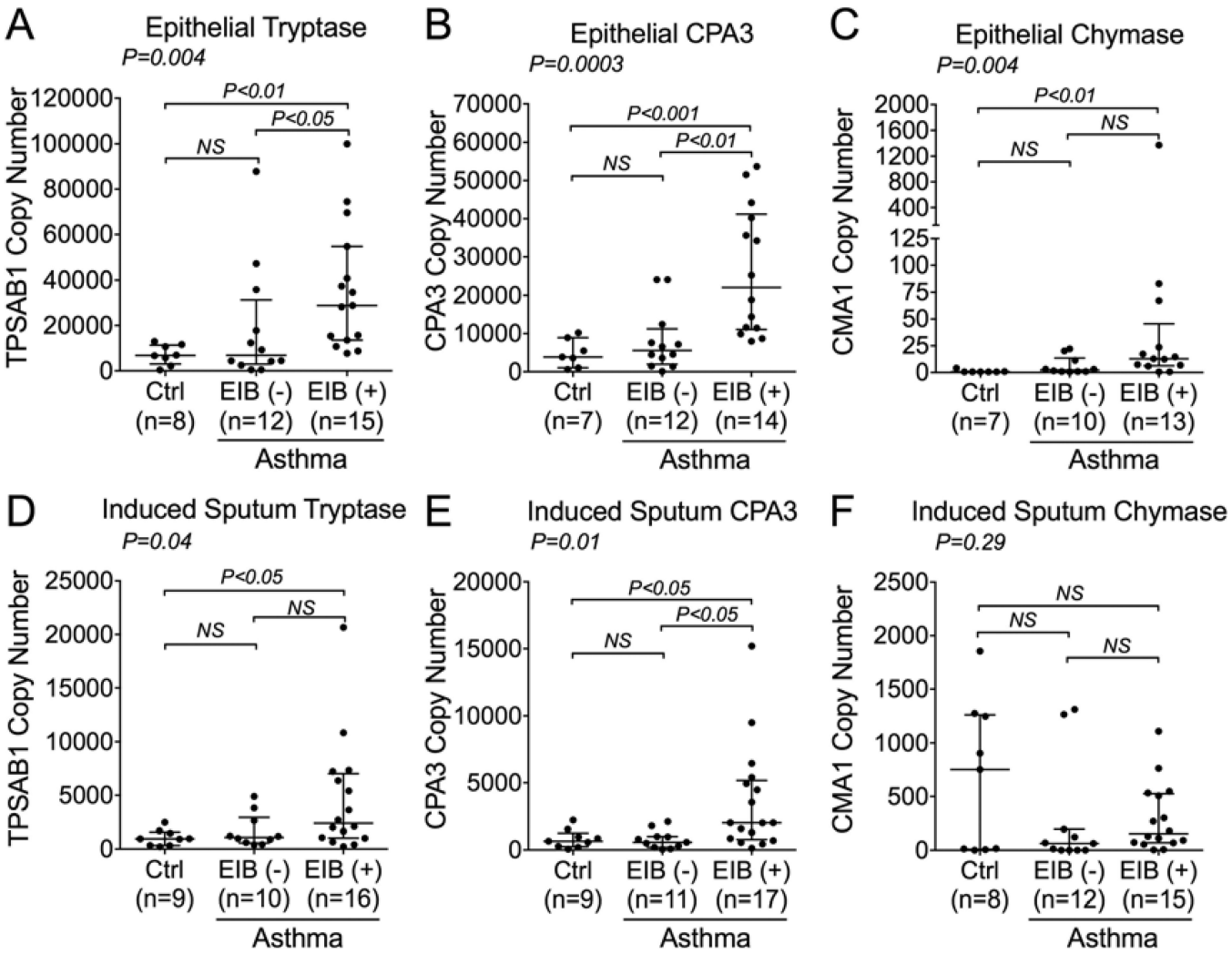

Figure 1. Airway gene expressions of specific mast cell proteases are increased in a precise phenotype of asthma

Quantitative PCR of airway epithelial brushings (A–C) and induced sputum cells (D–F) for *TPSAB1*, *CPA3*, and *CMA1* gene expression. The overall *P value* for the Kruskal-Wallis test is shown in the upper left of each pane, and the *P values* for post hoc tests are shown above the horizontal bars.

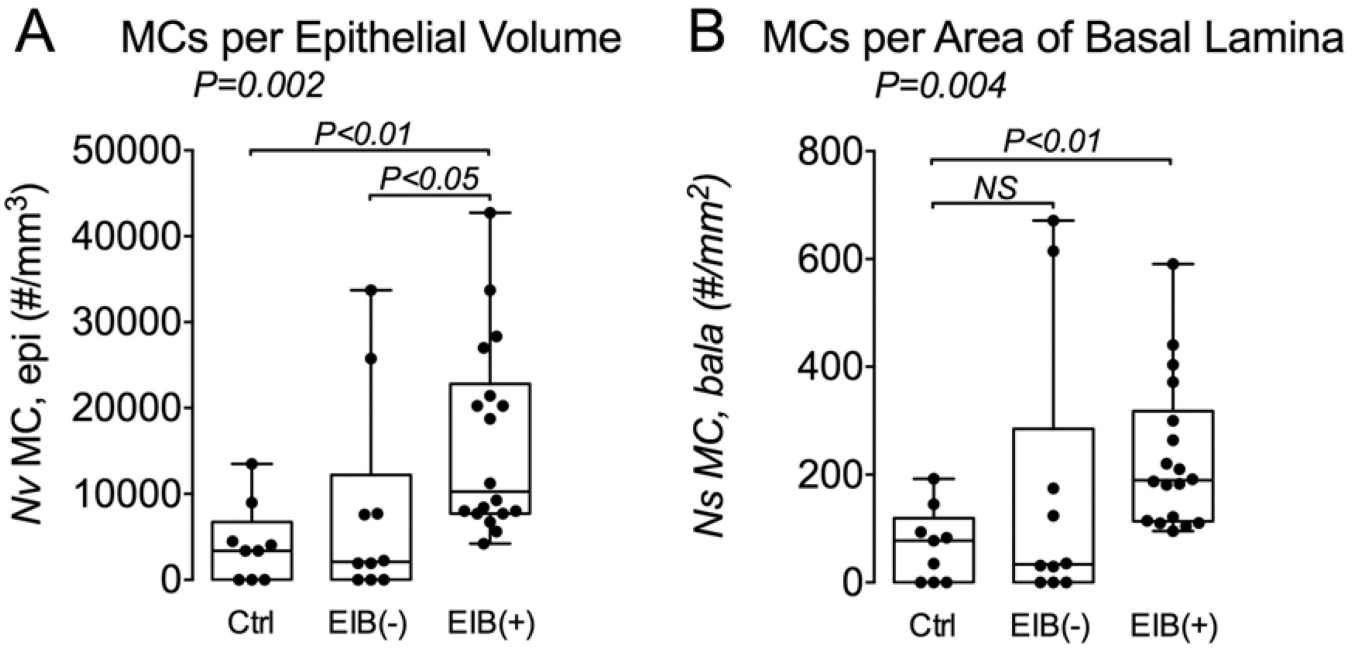

Figure 2. Quantification of the density of intra-epithelial mast cells by design-based stereology

The density of intraepithelial mast cells was quantified by the numeric density of intraepithelial mast cells relative to the volume of the airway epithelium (*Nv MC, epi*) (**A**), and the numeric density of mast cells relative to the surface area of the basal lamina (*Ns MC, bala)* (**B**).

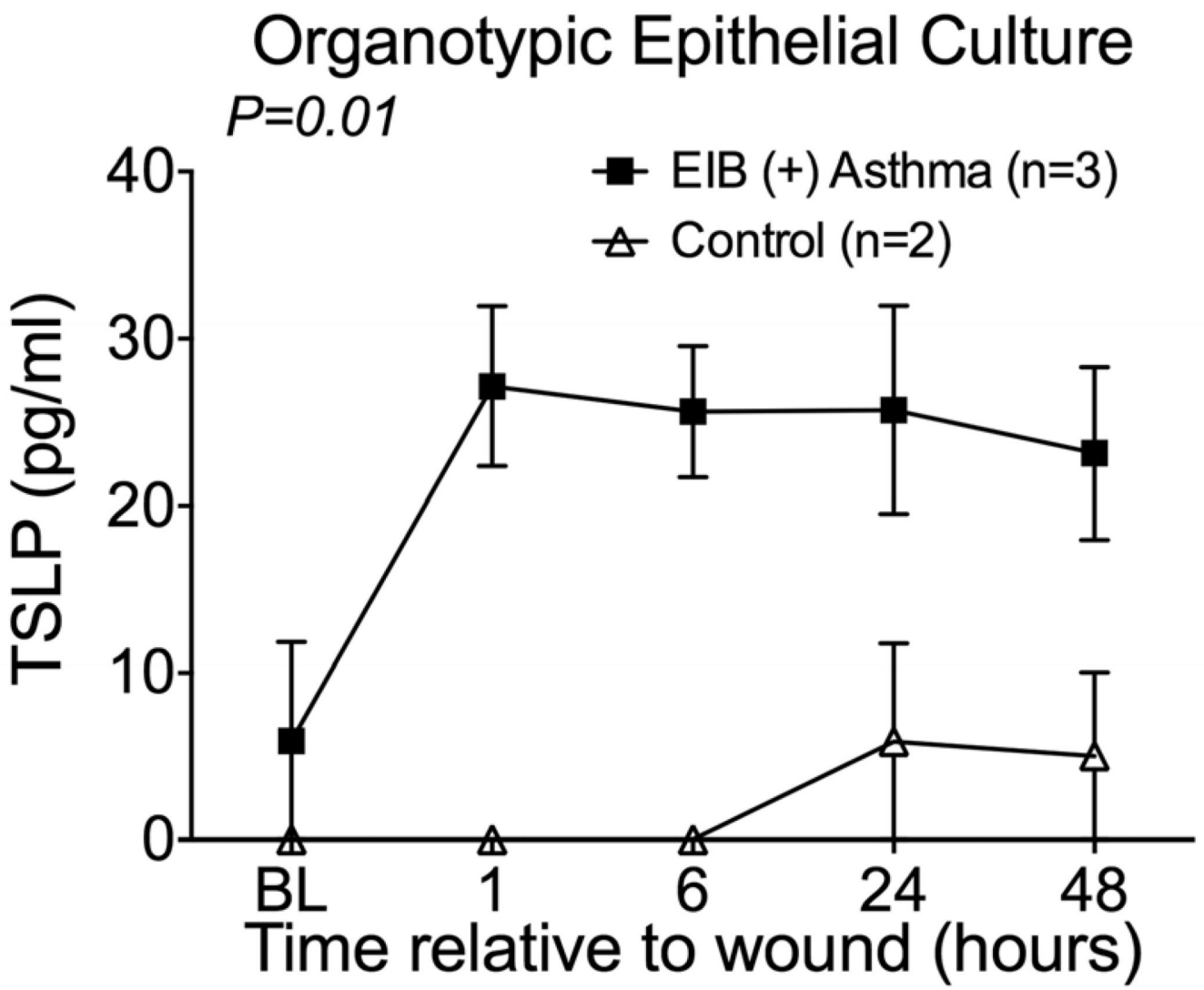

Figure 3. A mechanical scratch wound initiates TSLP release from primary airway epithelial cells in organotypic culture

Airway epithelial cells at passage 2 from asthmatic and control subjects differentiated in organotypic culture release TSLP protein into the basolateral media following a series of mechanical scratch wounds. The release of TSLP was greater from epithelial cells derived from patients with asthma.

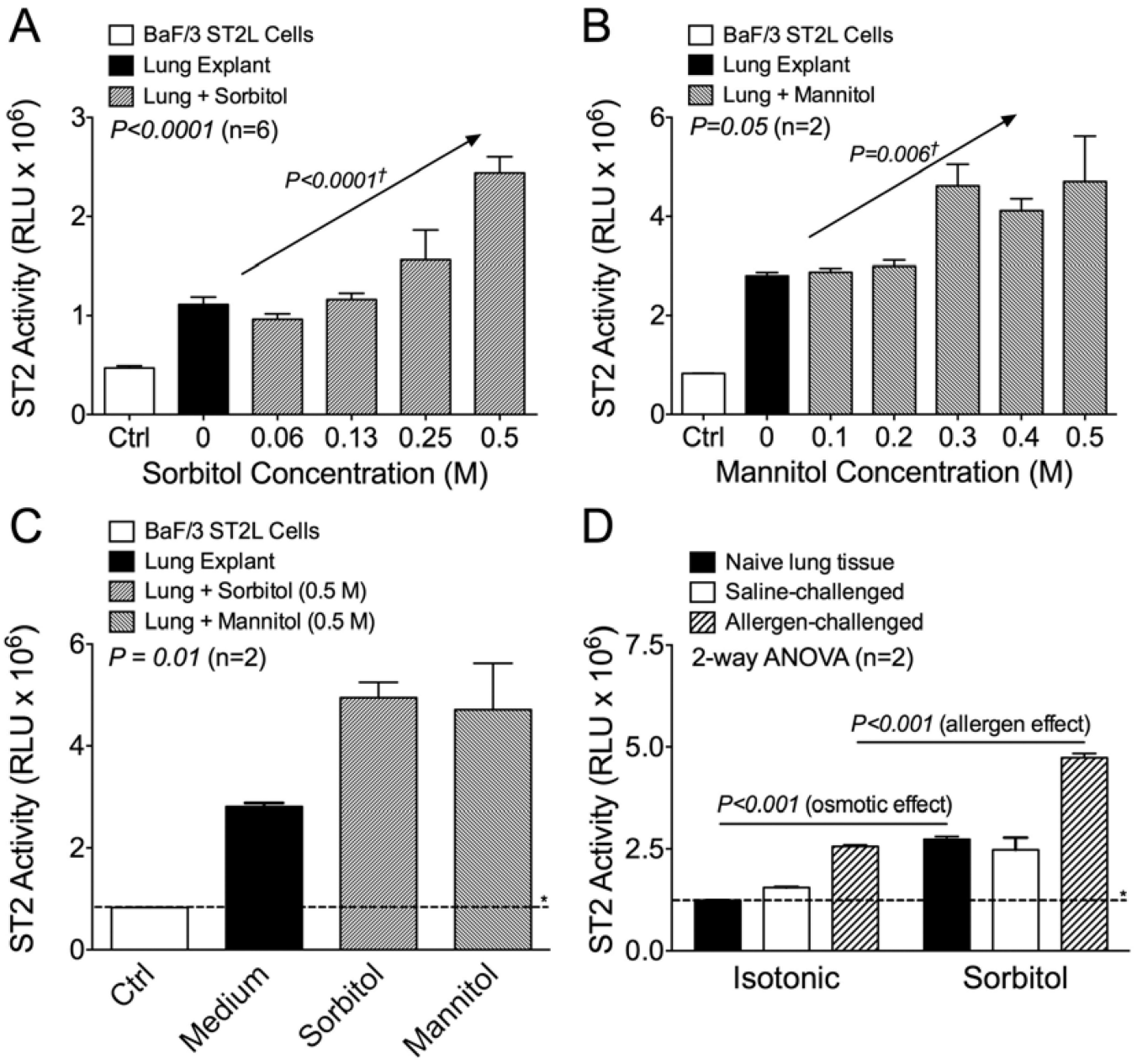

Figure 4. Osmotic stress in murine lung tissue initiates the release of biologically active IL-33

Conditioned medium from explanted lung tissue exposed to osmotic stress in the form of sorbitol (A) or mannitol (B) increased ST2 reporter activity in Ba/F3 cells that was related to the amount of osmotic stress († test for linear trend). Release of ST2 activity was similar for equal molar concentrations of sorbitol or mannitol (**C**). Allergen challenge increased the basal release of ST2-activating activity, and increased the release of this activity in response to osmotic stress (**D**).

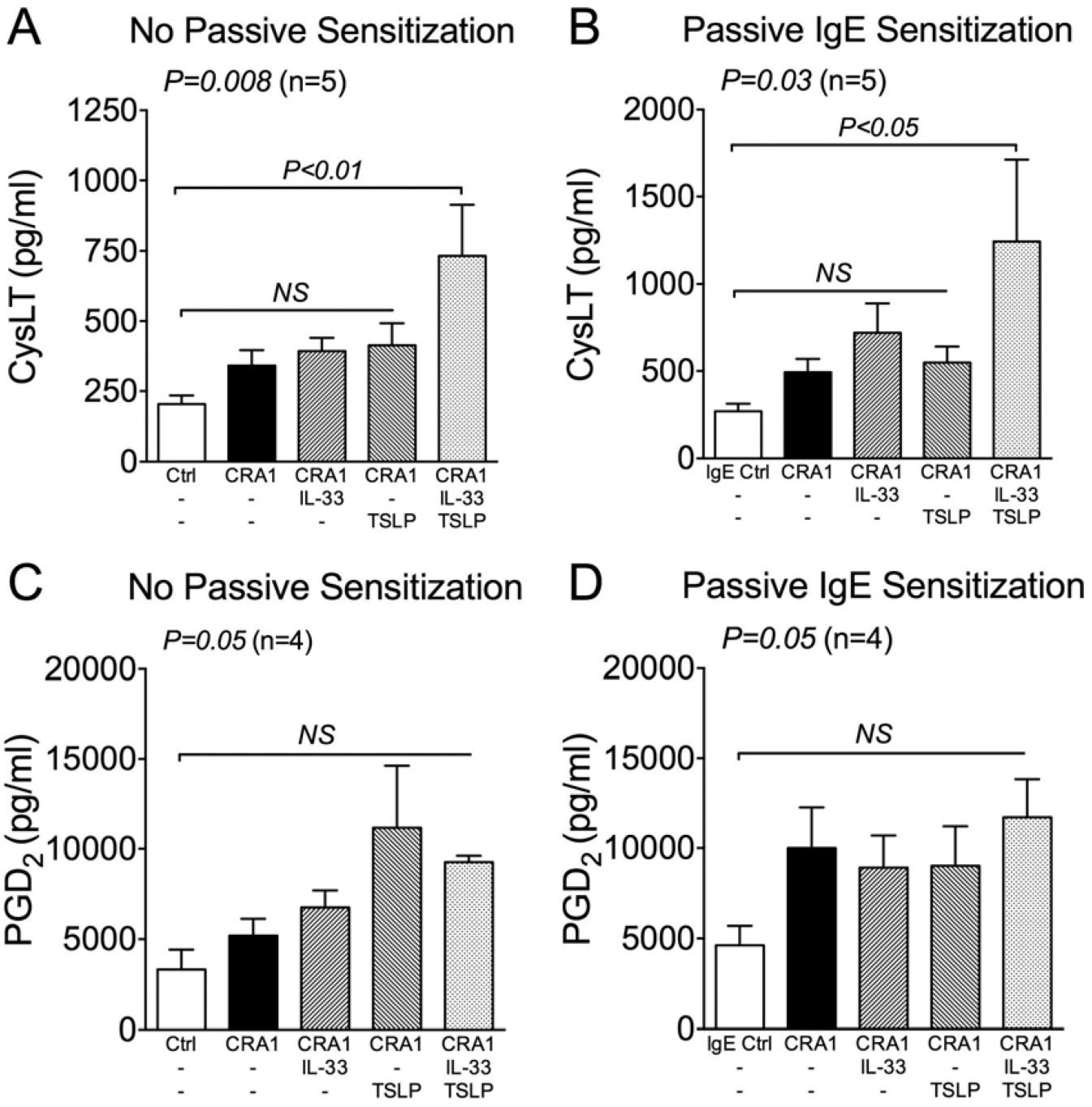

Figure 5. Influence of IL-33 and TSLP on IgE-dependent mast cell production of CysLTs and PGD_2

Treatment of CBMCs for 7 days with IL-33 in combination with TSLP increased CysLT formation in response to the high-affinity IgE receptor-activating antibody CRA1 both without passive sensitization (**A**) and following passive sensitization with polyclonal IgE for 7 days (**B**). There was no increase in PGD_2 formation in response to IL-33 and/or TSLP without passive sensitization (**C**) or following passive sensitization (**D**).

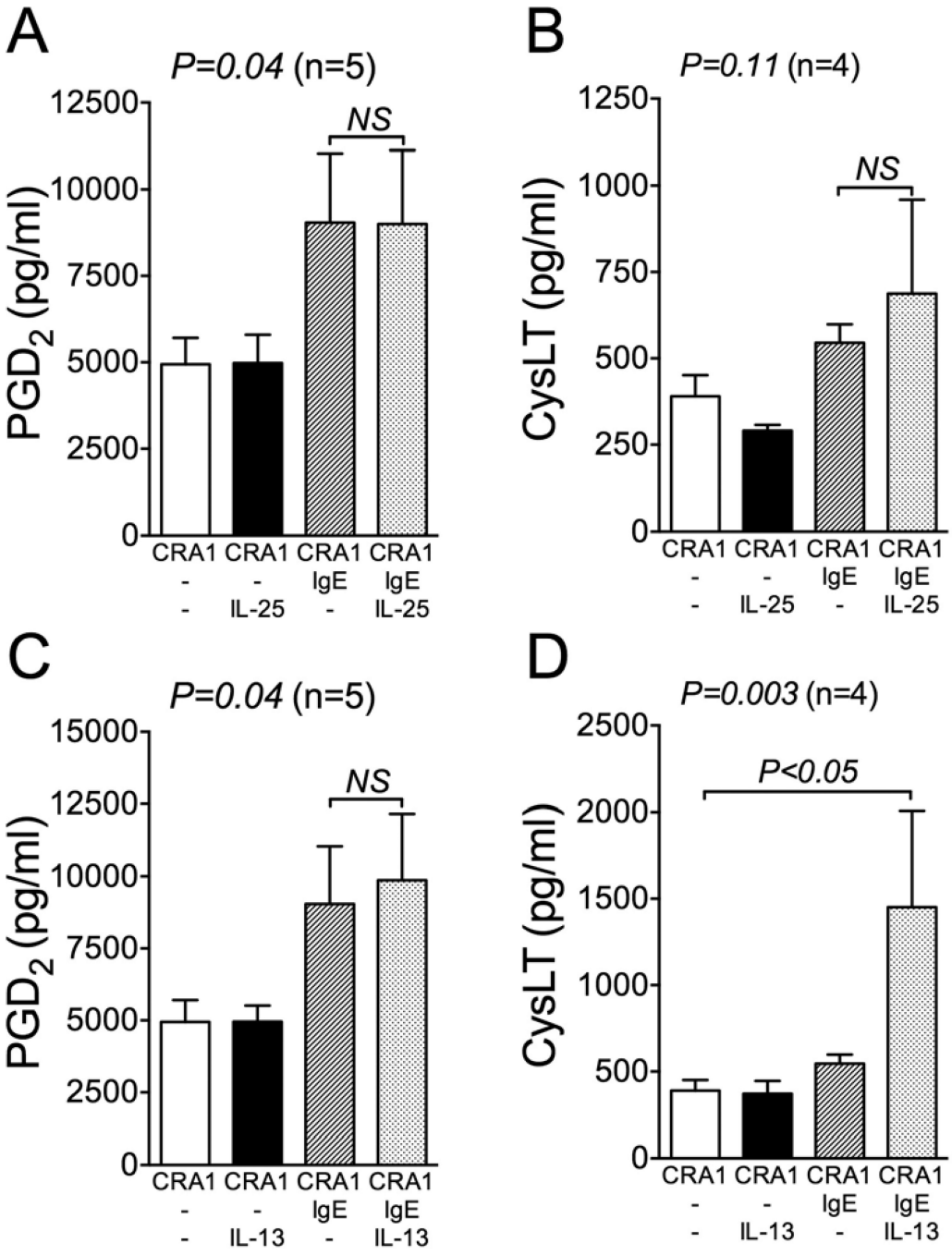

Figure 6. Effects of IL-25 and IL-13 on IgE-dependent mast cell CysLTs and PGD_2 formation

Treatment of mature CBMCs with IL-25 did not alter the formation of IgE receptor-mediated (CRA1) formation of PGD_2 (**A**) or CysLTs (**B**) with or without passive sensitization. Treatment of CBMCs with IL-13 did not alter the formation of PGD_2 either with or without passive sensitization (**C**). The formation of CysLTs was increase by IL-13 following passive sensitization, but not without passive sensitization of the CBMCs (**D**).

Table 1

Study population

Characteristic	Asthma			
	Control (10)	EIB Neg (12)	EIB Pos (19)	*P value*
Age (yrs)	30.4 ± 12.7	24.8 ± 5.0	26.8 ± 8.6	*0.35*
Gender - Male (%)	20.0%	25.0%	31.6%	*0.79*
Ethnicity – White (%)	70.0%	91.7%	84.2%	*0.46*
FEV_1 (% Pred)	96.5 ± 11.3	90.7 ± 9.7	88.8 ± 10.9	*0.19*
FVC (% Pred)	95.7 ± 13.5	96.2 ± 8.9	103.3 ± 9.6	*0.10*
FEV_1/FVC Ratio	0.87 ± 0.06	0.80 ± 0.09	0.73 ± 0.09	*<0.001**
Methacholine PC_{20}	>8 ± 0	1.8 ± 1.3	0.6 ± 1.5	*<0.001**
Exercise Challenge				
Max Fall in FEV_1 (%)	1.7 ± 2.1	2.3 ± 2.6	27.7 ± 9.5	*<0.001*†
AUC30 FEV_1	−7.4 ± 58.1	−13.4 ± 69.5	624.7 ± 295.7	*<0.001*†

The P value represents the overall comparison between the three groups. Comparisons between the asthma groups:

* *P=0.03*

† *P<0.001*.