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Effect of sex on group 2 innate lymphoid cells in the airways of mild and severe asthmatics

To the Editor,

Asthma is predominately observed in males in childhood but during and after puberty there is a shift favoring females.¹ Consequently, sex hormones have been implicated in the pathogenesis of asthma. Group 2 innate lymphoid cells (ILC2s) are critical modulators of allergic asthma, secreting large quantities of type 2 cytokines, interleukin 5 (IL-5), and interleukin 13 (IL-13).² Greater numbers of lung-extracted ILC2s are found in female compared to male allergen-sensitized mice.³ Testosterone upregulates ILC2 expression of killer cell lectin-like receptor G1 (KLRG1) that binds to E-cadherin inhibiting cell proliferation and activation.^{3,4} Additionally, androgen signaling downregulates CD25, an important ILC2 proliferation signal.⁵ Androgen-dependent regulation of ILC2s may mediate differences in asthma prevalence and severity between the sexes.

In a retrospective study of consenting adult mild atopic asthmatics (MA) and severe eosinophilic asthmatics (SA), we assessed the effect of sex on sputum and blood levels of canonical type-2 cells, ILC2s, and CD4+ T lymphocytes. We included nonsmoking MA (n = 38 [15 male, 23 female], FEV₁ ≥ 80% predicted, methacholine

PC₂₀ ≤ 16 mg/mL, dual-phase response to whole lung allergen challenge, intermittent β₂ agonist use) and SA (n = 30 [13 male, 17 female], FEV₁ < 80% predicted, methacholine PC₂₀ ≤ 8 mg/mL, sputum eosinophilia > 3%, mean ICS/LABA > 880 µg/day; mean prednisone > 7.5 mg/day). Blood and sputum were collected at baseline for all asthmatics and sputum at 24 hours postallergen, for a subgroup of MA (n = 19) (Table S1). By flow cytometry, we gated on ILC2 and CD4+ T cells phenotypes that are upregulated in eosinophilic asthmatic responses within the airways (Figure S1).⁶ Procedures were reviewed and approved by the institutional ethics board. See Data S1 for methodological details.

For MA, all demographic characteristics were comparable between the sexes except for BMI, which was higher in males. Both sexes demonstrated similar allergen-induced changes in FEV₁, methacholine airway hyperresponsiveness, total leukocyte counts, and sputum eosinophilia (Table S2). At baseline, we found significantly higher absolute ILC2 numbers in sputum from females compared to male with MA (Figure 1A). When expressed as a percentage of CD45+ cells, the between-sex difference in total ILC2s was lost (Figure S2A). No significant sex differences in type 2 cytokine-positive sputum ILC2s (Figure 1B-D) or blood ILC2s (Figure 1E-H) were found at baseline. Total sputum ILC2 numbers in all MA increased significantly 24 postallergen (Figure 1I) but no significant sex

Abbreviations: BMI, body mass index; EAR, early asthmatic response; FEV₁, forced expiratory volume in one-second; ICS, inhaled corticosteroid; ILC2, type 2 innate lymphoid cell; IL, interleukin; LABA, long-acting beta agonist; LAR, late asthmatic response; MA, mild asthmatic; Methacholine PC₂₀, provocative concentration of methacholine inducing a 20% fall in FEV₁; SA, severe asthmatic; TCC, total cell count.

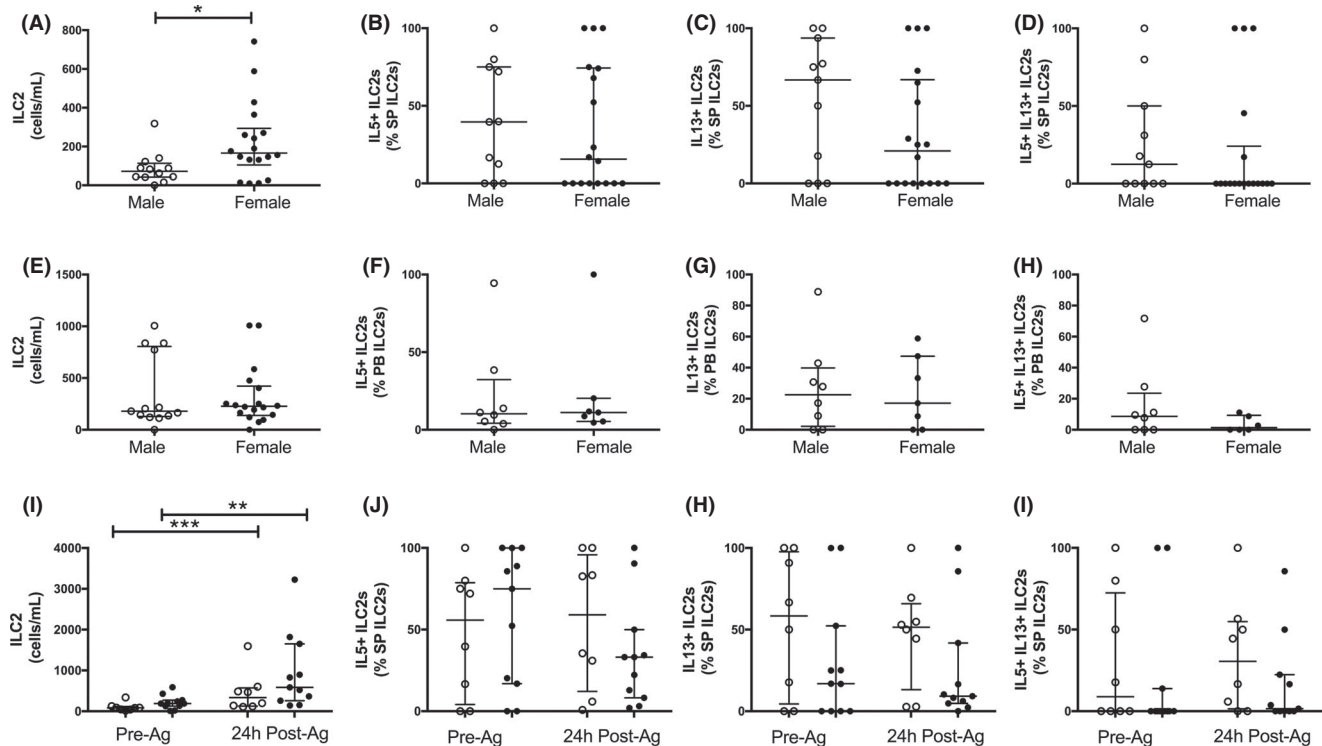


FIGURE 1 Absolute Sputum ILC2s at Baseline and 24-h Post-Allergen in Mild Asthmatics. A, Absolute numbers of sputum ILC2s are significantly greater in females (filled circles) compared to males (open circles), and (B–D) no between-sex differences in cytokine+ ILC2s were observed. E–H, In blood, no significant differences were found in total ILC2s or IL-5+, IL-13+, and IL-5+ IL-13+ ILC2s when cells were expressed as a percentage of total ILC2s (E–H). I–L, No significant allergen-induced changes in sputum ILC2 numbers were found between the sexes. Horizontal bars represent medians and error bars—the interquartile range of the data. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

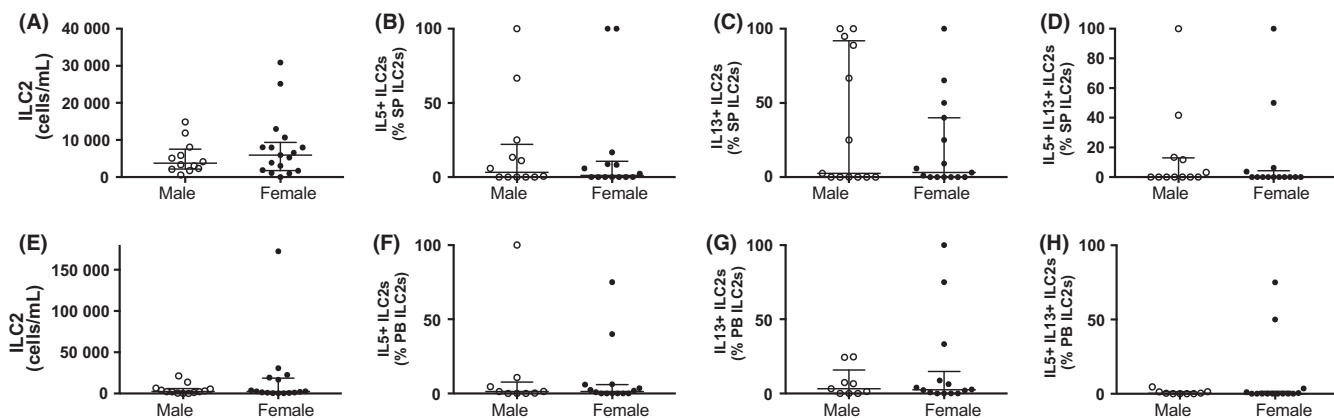


FIGURE 2 Total and cytokine-positive ILC2s in sputum and blood of severe eosinophilic asthmatics. There were no sex differences in either total ILC2 or cytokine-positive ILC2 in sputum (A–D) or blood (E–H). IL-5+, IL-13+, and IL-5+ IL-13+ ILC2s are expressed as a percentage of total ILC2. Horizontal bars represent the medians and error bars—the interquartile range of the data sets

differences were observed in allergen-induced changes for total or type 2 cytokine-positive ILC2s (Figure 1I–L). No between-sex differences in sputum levels of total or type 2 cytokine+ CD4+ T cells were found at baseline or 24 hours postallergen (Figure S3A–H).

Although there were significantly greater sputum ILC2 numbers in females with MA, this did not correspond to any airway functional or inflammatory cell differences at baseline (Table S2). This suggests that an alternate subpopulation of ILC2s, not driving eosinophilic

inflammation and not phenotyped in this study, may contribute to sex-associated differences reported for these cells.^{3–5}

For SA, all baseline demographics were comparable between sexes (Table S2). Total and cytokine-producing ILC2s were significantly greater in SA than MA as previously reported by us.⁷ In SA, there were no significant between-sex differences in absolute sputum (Figure 2A–D) or blood ILC2s (Figure 2E–H). We report similar findings for ILC2s and CD4+ T cells when expressed as a percent of

leukocytes in sputum and blood (CD45+ cells; Figure S2I-P; and Figure S3I-P, respectively). These observations may be explained by prolonged prednisone use in the SA cohort. Prednisone is known to decrease circulating testosterone levels.⁸ Consequently, this may have minimized between-sex differences of this hormone and possibly mitigated testosterone-driven dissimilarities in ILC2 levels amongst severe eosinophilic asthmatics.

Age influences circulating sex steroid levels whereby menopause is associated with decreases in female sex hormones and testosterone wanes with age in males. Moreover, ILC2 numbers are negatively influenced by obesity.⁹ Therefore, using generalized linear models, whereby sex was a predictor and age or BMI were covariates, we investigated age and BMI effects on ILC2s. We found no significant age-sex interactions for total and cytokine-producing ILC2s in either MA or SA. In addition, no significant BMI-sex interactions for sputum ILC2s in mild or severe asthmatics were found.

Our findings are in contrast to Cephuss et al,⁵ who reported significantly higher blood ILC2s (% CD45+ cells) in females compared to males with asthma, but did not report levels in sputum. Compartmental sampling differences may account for these differences, as the current study is the first to compare airway levels of ILC2s in humans. Additionally, Cephuss et al studied atopic moderate to severe asthmatics who were not as clearly phenotyped compared to this investigation, which only included eosinophilic asthmatics. Moreover, they exclusively studied premenopausal subjects, while this study included individuals beyond the average menopausal age, even though age was not found to influence sex differences in ILC2s.⁵ Finally, our gating of ILC2s (Lin-CD45+ CD127+ CRTH2+) was broader than Cephuss et al, who identified ILC2s as Lin-CD45+ CD127+ CRTH2+ CD161+ CD25+ cells, where CD25 may be influenced by androgen signaling. However, the argument against this is that the authors found similar results even when ILC2s were more generally gated as Lin-CD45+ CD127+ GATA3+ cells.⁵

The retrospective nature of this project limited our ability to extrapolate additional information pertinent to sex such as menopausal status, sex steroid levels, and hormonal birth control use. Since ILC2s are regulated negatively by androgens, examining the relationship between sputum ILC2s and circulating testosterone levels should be assessed. Additionally, comparisons between prepubescent, premenopausal, postmenopausal females and prepubescent and adult males are warranted to further our understanding of the effect of sex on airway levels of ILC2s in asthma.

In conclusion, this is the first detailed between-sex comparison of cytokine-producing ILC2s in the airways and blood eosinophilic asthmatics. We found no significant differences in type 2 cytokine-positive ILC2s in severe asthma and no effect of sex on allergen-induced increases in sputum ILC2s and CD4+ T cells in mild asthmatics. While we do not refute the influence of sex on prevalence and severity of disease, the current data suggest that in clinically similar individuals, sex does not influence differences in broadly phenotyped ILC2 and CD4+ T cells in asthma.

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CONFLICTS OF INTEREST

M Aw, B Salter, R Chen, K Machida, M Inman have no conflicts of interest. P Nair has received honoraria for lectures/consultancy from AstraZeneca, Teva, Sanofi, Roche, Knopp, Novartis, and Merck and research grants from AZ, Sanofi, Novartis, BI, Roche and Teva. PM O'Byrne is a member on the Joint Oversight Board for LABA safety and has research grants from AstraZeneca, GlaxoSmithKline, Merck, Genentech and Boehringer. GM Gauvreau holds research grants from AstraZeneca, Novartis, Regeneron, BioGaia, and Genentech, and consults for AstraZeneca and Novartis. R Sehmi has received research grants from AstraZeneca, Genentech and Teva.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Effect of tacrolimus on skin microbiome in atopic dermatitis

To the Editor,

Atopic dermatitis (AD) is a common skin disease in which genetics and environment influence the AD pathogenesis. Recently, evidence has emerged to support the notion that skin microbiota can modulate development and exacerbation of this disease. Meta-analysis reports estimated that prevalence of *Staphylococcus aureus* colonization in AD skin lesion was 70%.¹ Beside the colonization of *S aureus*, dysbiosis of the skin microbiome via reduction of commensal microbes such as *Staphylococcus epidermidis* and *Corynebacterium* spp. has been evident in AD.²⁻⁵ Nowadays, several therapeutic approaches exist for AD. Tacrolimus, a topical calcineurin inhibitors, has been recommended as a maintenance therapy as they are low risk of triggering adverse events. To date, there is no information on the effect of tacrolimus on skin microbiome in AD and our study seeks to address this. Our aim is a comprehensive comparison between healthy and AD skin microbiome following the introduction of tacrolimus.

Nine Thai AD patients and 12 healthy subjects participated in this study. All patients with AD were reported significant improvement of all clinical scores (SCORAD, EASI, and IGA) after 4-week monotherapy with tacrolimus (Table S1).

Samples of normal skin from normal subjects (Control, C) and lesional skin from patients with AD (D) at cubital fossa before (Bf) and after 4-week monotherapy with tacrolimus (Af) were characterized using 16S ribosomal RNA gene sequencing (NCBI accn.no. SRP155450) (Data S1).⁶ The 16S rRNA gene sequencing yielded

an average of 146922 clean sequences for OTU classification per sample, and high Good's coverage indices of 98.45%-99.93% (avg. 99.26%) at genus level diversity (Tables S2 and S3). The latter estimated the data coverage of the true community diversity. We found that the bacterial diversity (OTUs, Chao, Shannon) was relatively higher in the Control than the D groups. The variance box plot analyses by Chao and Shannon alpha-diversity indices were especially low in the Bf (Figure 1A and Table S3). Supportively, the taxonomic profiles demonstrated the diversity that differ among the Control and the AD: phylum Actinobacteria (class Actinobacteria) was relatively high followed by Proteobacteria (class Gammaproteobacteria) in control, whereas phylum Firmicutes (class Bacilli) was generally higher in the AD, in particular the Bf (Figure 1B,C and Figure S1). The number of the overlapping OTUs between the Control and D groups was thereby low between the Control and the Bf and relatively higher between the control and the Af (Figure 1D: Control-Bf overlapped 71.62%, Control-Af overlapped 81.66%). The nonmetric multidimensional scaling (NMDS) based on Morisita-Horn dissimilarity indices, along an analysis of molecular variance (AMOVA) and a homogeneity of molecular variance (HOMOVA) statistics, was constructed to visualize the relative dissimilarity among the microbiota structures, and the Control and the D groups were discrete, albeit the D8 as an exception showing close to C11, C12, and C7, in orderly. Analysis without the D8 showed even more prominent the community structure difference between the Control and the D groups with the

Online Repository: Supplementary methods: Effect of Sex on Group 2 Innate Lymphoid Cells in the Airways of Mild and Severe Asthmatics

Subjects and study design: This is a retrospective analyses of data collected in previously reported studies for ILC2 cells in the sputum and blood of adult mild (MA) (1, 2) and severe (SA) asthmatics (3,4). MA provided sputum and blood at baseline (n=38), and a subset of MA subjects underwent an allergen challenge and sputum was sampled pre and 24 h post-allergen or diluent challenge (n=19). SA participants provided sputum and blood samples at baseline only. Baseline demographics are presented in Table E1 for mild asthmatics and Table E2 for severe asthmatics. All subjects were current nonsmokers (<10 pack-years). All procedures were reviewed and approved by institutional research ethics boards.

Immunofluorescence Staining and Flow Cytometric Gating Strategy: Sputum-extracted cells and mononuclear cells isolated from blood were stained for ILC2 and CD4-enumerations with antibodies to Lin-eFluor450, FcεR1-eFluor450, CD45-BV786, CRTH2- BV421 , IL7Rα (CD127)-PE-cy7, and CD4-AF700 or relevant isotype controls (BD Bioscience, Canada; eBiosciences, San Diego, CA; R&D Systems, MN, USA). Lin-cocktail antibodies to (CD2, CD3, CD14, CD16, CD19, CD56, CD235a) plus FcεR1 excluded T and B cells, monocytes, eosinophils, NK cells and basophils. Cells were then incubated in Perm/Fix Buffer and intracellular stains for anti-IL-5-FITC and anti-IL-13 PerCP-Cy5.5 (BD Biosciences) were added. After incubation, cells were washed and resuspended in PBS with 1% paraformaldehyde and analyzed by a FACS LSRII flow cytometer within 24 h. Gating in the lympho-mononuclear region (low side scatter/low forward scatter) and following acquisition of 300,000 events, data were analyzed using FlowJo program to enumerate intracellular IL-5 and IL-13 levels in ILC2s (lin-CD45+CD127+ST2+/CRTH2+) and CD3+CD4+ T lymphocytes. Gating strategies for

ILC2s detailed in Figure E1. Absolute cell numbers were calculated using percent positive population and the total white cell count.

Statistical Analyses: All data were Log₁₀ transformed, distribution evaluated by D'Agostino & Pearson normality test. For between-group comparisons an unpaired T Test with Welch's correction or a Mann-Whitney ranked test for normally and non-normally distributed data, respectively were used. For within-group comparisons a paired T Test or Wilcoxon ranked test for normally and non-normally distributed data, respectively was used. A generalized linear model considering age as a covariate and sex as a predictor was performed for ILC2 and CD4 data in MA and SA cohorts. Statistical analyses were performed using PRISM, version 7 and co-variate analyses using SPSS Statistics 23. Alpha was set at 0.05. Sample size was determined from previously data where a 365 cell/mL mean increase in sputum ILC2s post-allergen challenge was associated with a significant increase in sputum eosinophils in MA (1). The effect size was set at half this mean difference. Sample size is estimated at 5 subjects per group. $N = (2(Z_{\alpha/2} + Z_{\beta})^2(\sigma^2))/(\delta^2)$; $N = (2(1.96 + 0.84)^2(94.36^2))/(182.5^2)$; $N = 5$ per group.

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Table E1: Allergen Challenge for Mild Asthmatics

Sex	Age (years)	FEV ₁ (% predicted)	MCh PC ₂₀ mg/mL	Allergen	Allergen Dilution
M	30	76.1	3.17	HDMSP	1:128
M	68	100.3	10.01	Cat	1:8
M	27	110.0	53.87	HDMDf	1:128
M	23	81.8	9.81	HDMDP	1:128
M	54	92.9	3.72	HDMDP	1:64
M	51	103.0	0.42	Cat	1:64
M	37	85.2	16.34	Grass Mix	1:1024
M	23	80.3	0.28	Cat	1:256
F	45	81.0	1.52	Grass	1:64
F	50	87.8	1.57	Cat	1:64
F	70	88.51	2.35	Cat	1:8
F	46	97.9	10.66	HDMDf	1:16
F	21	98.8	1.30	HDMDf	1:64
F	23	90.5	10.00	HDMDP	1:64
F	22	88.5	0.26	Cat	1:256
F	22	105.5	2.59	Cat	1:64
F	21	89.5	1.71	Tree	1:16
F	23	98.2	3.62	Horse	1:256
F	22	99.2	15.20	Ragweed	1:4
M/F 8:11	21	90.5	3.17		
	(21 – 70)	(85.2 – 99.2)	(1.52 – 10.01)		

Baseline characteristics of non-smoking mild asthmatics (MA) with a prebronchodilator forced expiratory volume in 1 second (FEV₁) $\geq 80\%$ predicted, dual phase response to allergen, with a fall in FEV₁ of $\geq 20\%$ by 2 h and a fall in FEV₁ of $\geq 15\%$ by 7 h post-challenge (1, 2). Subjects were skin prick positive to common aeroallergens, had a baseline methacholine PC₂₀ ≤ 16 mg/ml, only used inhaled short-acting bronchodilators as reliever therapy, were asymptomatic for 4 weeks prior to study. Subjects avoided exposure to sensitizing allergens apart from house dust mite and refrained from β_2 -agonist and caffeinated beverages before laboratory visits. Data are presented as mean $\pm$ SEM. Definition of abbreviations: FEV₁ - forced expiratory volume in 1 second. Allergen – antigen (at a specified dilution) used for inhalation challenge.

Table E2: Baseline Patient Demographics

	Sex			
	Mild Asthmatics		Severe Asthmatics	
	Male	Female	Male	Female
N	15	23	13	17
Age	27.0 (20.0 – 67.0)	23.5 (21.0 – 70.0)	50.0 (20.0 – 61.0)	55.0 (32.0 – 70.0)
BMI (Kg/m²)	27.5* (20.5 – 34.9)	24.2 (18.7 – 33.8)	29.9 (23.1 – 38.6)	33.5 (20.9 – 47.3)
Prednisone Dose Equivalent (mg/day)	N/A	N/A	12.5 (5.0 – 25.0)	10.0 (5.0 – 23.5)
ACQ	N/A	N/A	2.7 (1.2 – 3.3)	2.0 (0.9 – 2.7)
FEV1 (%Predicted)	91.3 (81.1 – 104.2)	94.4 (88.9 – 99.4)	57.0 (41.5 – 67.8)	66.0 (48.1 – 71.5)
EAR (maximal fall in FEV₁)	-36.6 (-60.3 – -32.3)	-37.6 (-48.4 – -31.6)	N/A	N/A
LAR (maximal fall in FEV₁)	-19.9 (-26.3 – -11.2)	-27.2 (-36.2 – -17.2)	N/A	N/A
Methacholine PC₂₀ (mg/mL)	3.7 (0.3 – 16.3)	2.6 (1.6 – 10.0)	N/A	N/A
Doubling Dose Shift Methacholine PC₂₀	3.0 (1.8 – 5.3)	1.67 (1.1 – 41.0)	N/A	N/A
Baseline Sputum TCC (x10⁶ cells/ml)	5.2 (2.8 – 7.6)	3.5 (1.5 – 5.7)	5.4 (1.9 – 14.7)	5.5 (2.3 – 9.8)
Baseline Sputum Eosinophils (% of TCC)	1.8 (0.5 – 5.1)	1.5 (0.8 – 6.3)	6.2 (2.8 – 28.9)	7.8 (1.3 – 34.8)
Sputum TCC 24h post-Ag (x10⁶ cells/mL)	4.7 (2.2 – 9.7)	8.0 (1.5 – 11.0)	N/A	N/A
Sputum Eosinophils 24h post-Ag (% of TCC)	3.8 (1.2 – 18.5)	6.8 (2.3 – 17.1)	N/A	N/A
Baseline Peripheral Blood TCC (x10⁶ cells/mL)	5.4 (4.7 – 7.6)	6.4 (5.6 – 7.9)	9.7 (8.0 – 10.7)	7.8 (7.4 – 10.2)
Baseline Peripheral Blood Eosinophils (x10⁶ cells/mL)	0.3 (0.1 – 0.4)	0.2 (0.0 – 0.6)	0.3 (0.2 – 0.6)	0.4 (0.2 – 0.6)

Demographic data are presented as median and interquartile range, except age and BMI which are presented as median and range. All data were comparable between sexes except for BMI where MA males had a higher BMI than females (* p<0.05). Abbreviations: BMI=body mass index; ACQ=asthma control questionnaire; FEV₁=forced expiratory volume in 1 second; EAR=early asthmatic response; LAR=late asthmatic response; PC₂₀=provocative concentration of methacholine inducing a 20% fall in FEV₁; post-AG= post allergen challenge; TCC=total cell count.

Severe asthmatics had prebronchodilator FEV₁ <80% predicted; postbronchodilator reversibility ≥12% and ≥200 mL in FEV₁, or a remote PC₂₀ ≤8 mg/mL; were on high-dose ICS/LABA (>880 µg/day); had ≥6 months history of treatment with OCS (7.5–40 mg daily prednisone or equivalent); and eosinophilic inflammation determined by blood eosinophil counts ≥150 cells/µL currently or ≥300 cells/µL in the past 12 months (3, 4).

SUPPLEMENTARY FIGURES

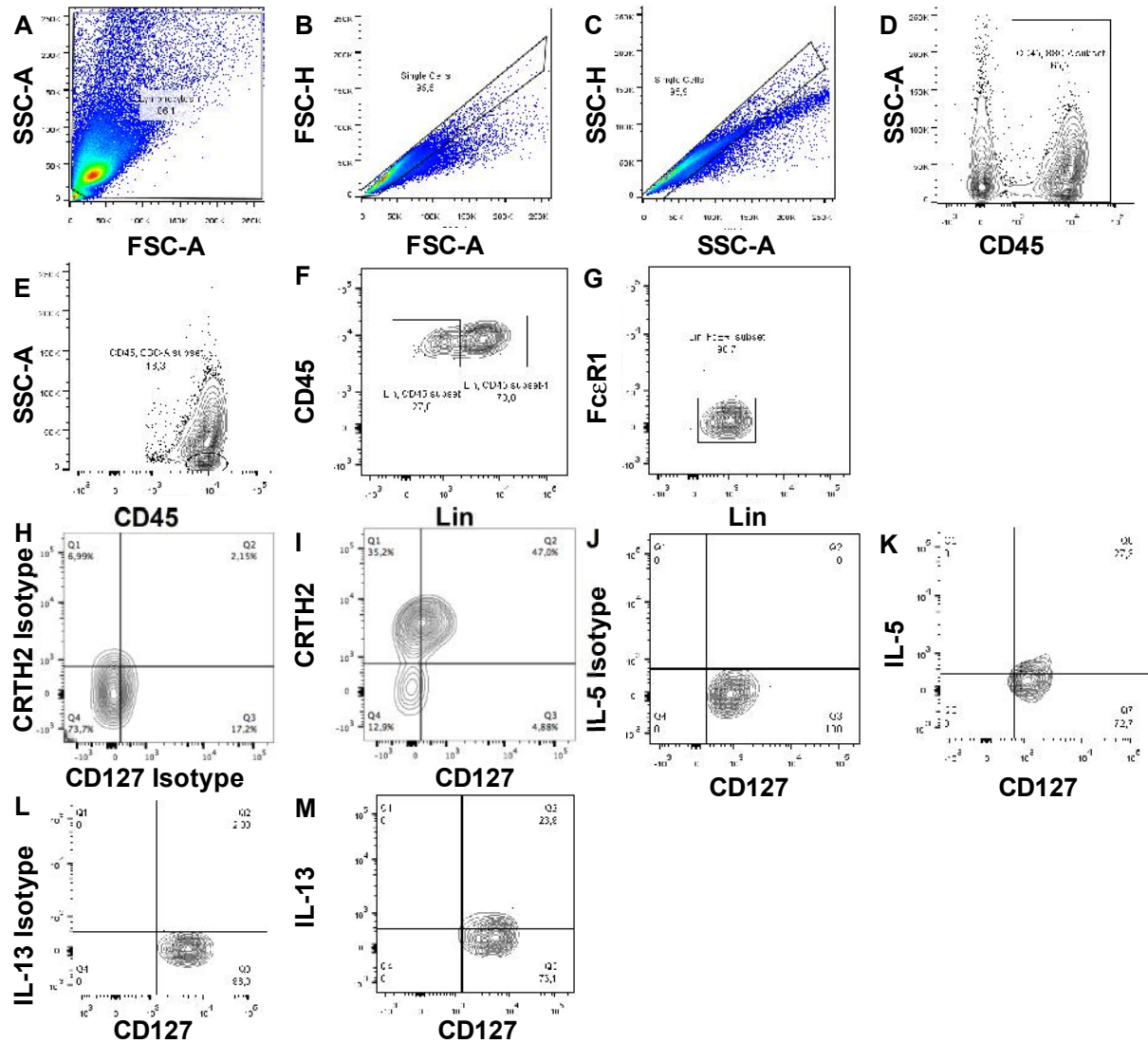


FIG E1: Representative gating strategy for absolute and ILC2 with isotype controls in sputum. (A-C) Representative plots from a patient show sequential gating of live, singlet sputum cells to identify (D-F) Lineage-negative and CD45+ region, (H) Isotype control for CRTH2, and (I) specific staining of ILC2 population: CD127+ CRTH2+ cells. Compared to relevant isotype controls, intracellular staining for (J-K) IL-5 and (L-M) IL-13 were assessed for ILC2s using a 98% confidence limit. Similar strategies were used for blood-derived cell samples.

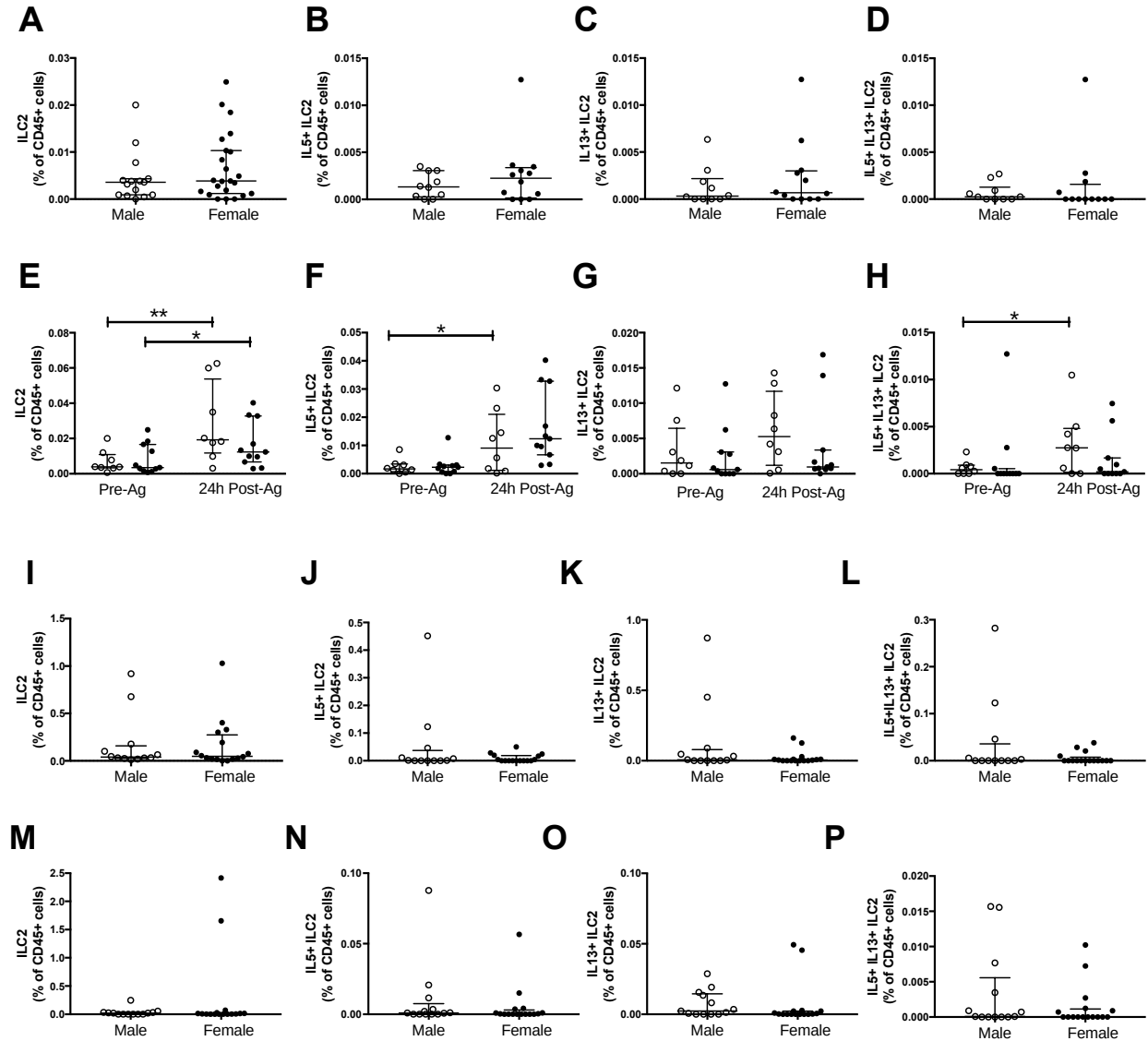


FIG E2: ILC2s in Male and Female Asthmatics Sputum ILC2s (% of total WBC) in mild asthmatics at baseline (A-D) and pre and post-allergen challenge (E-H) in males (open circles) and females (closed circles). In mild asthmatics, no significant differences were found in either total or type 2 cytokine+ ILC2s at baseline or in allergen-induced changes. In severe asthmatics, no significant differences were found between the sexes in sputum (I-L) or blood (M-P) for % ILC2. Horizontal bars represent the medians and error bars – the interquartile range of the data sets.

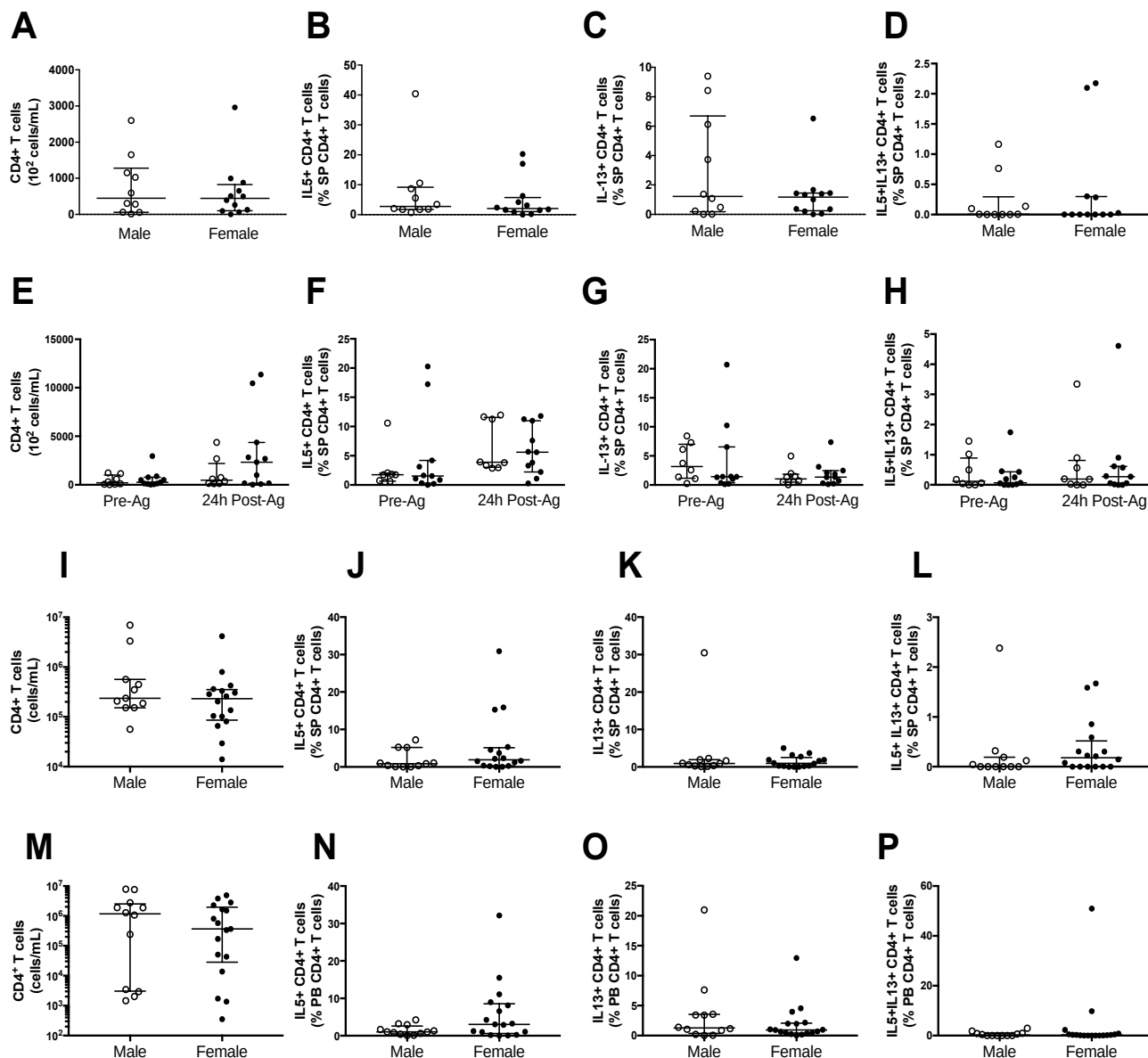


FIG E3: Absolute CD4⁺ T Cells in Male and Female with Mild Asthmatics. No significant differences in total, IL-5⁺, IL-13⁺ and IL-5+IL-13⁺ CD4⁺ T cells in sputum from male (open circles) and female (closed circles) mild asthmatics were observed, at baseline (A-D) or 24h post-allergen challenge (E-H). In severe asthmatics, no significant difference was found in total and type 2 cytokine⁺ CD4⁺ T cells in the sputum (I-L) or blood (M-P) between male and female asthmatics. Horizontal bars represent the medians and error bars – the interquartile range of the data sets.