Online Repository Materials

Th1 Cells and Atypical Cytokine Signatures Are Present in the Lower Airways of Children with Severe Asthma, Regardless of Allergic Status.

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Methods

Human Subjects and Bronchoscopy.

BAL fluid samples were obtained from 52 children. The indications for bronchoscopy were poorly controlled asthma despite treatment with high dose corticosteroids (**Table EI**), recurrent health care access for care, and recurrent or persistent abnormality on chest film. Participants received specialty care at a regional academic center, and treatment was adjusted by a senior clinician. Adherence to treatment was confirmed by analysis of prescription re-fill pharmacy records, and inhaled medication technique was evaluated regularly in the clinic. Oral corticosteroid usage was obtained from medical record electronic monitoring. Participants were treated with asthma control medications per the EP-3 guidelines with treatment adjusted in follow up visits to the clinic according to the level of symptom control and lung function. Bronchoscopies were done under conscious sedation or general anesthesia via a laryngeal mask airway trans-orally inserted to avoid contamination via the nasopharynx.

Serum Antibody Assays.

Serum total IgE and specific IgE (dust mite (*Dermatophagoides pteronyssinus*), cat, egg, cow's milk, peanut, ryegrass, ragweed, *Alternaria alternata*, and *Aspergillus fumigatus*) were measured by ImmunoCAP assay (Phadia US, Portage, MI) using the ImmunoCAP 250 system. Allergic status was assigned based on the presence of specific IgE using a low assay threshold (>0.1kU$_A$/L, n=37) or elevated total IgE according to age (>50 IU/ml for children ≤5 yrs., n=1). Serum IgG and IgA antibodies were measured by the University of Virginia Medical Laboratories using Rate Nephelometry (IMMAGE^R 800 Immunochemistry System, Beckman Coulter).

Detection of Respiratory Microbes.

Bacteria were detected by culture and respiratory viruses were assessed by multiplex PCR analysis for the following: Influenza A, subtype seasonal H1 or seasonal H3; Influenza B; Parainfluenza 1,2,3; Metapneumovirus; Rhinovirus/Enterovirus; Adenovirus; and Respiratory Syncytial Virus subtypes A and B. Tests were performed through the University of Virginia Medical Laboratories.

Flow Cytometry Antibodies and Other Reagents.

Mouse IgG used to block Fc receptors prior to cell staining was obtained from Lampire Biological Laboratories (Pipersville, PA). Antibodies used to identify cell populations, including lineage cocktails, are listed in **Table EII**. Fixable Aqua Live/Dead Staining Kit was obtained from Invitrogen (Eugene, OR).

Flow Cytometry Analysis.

To detect intracellular cytokines in ILCs and T cells and ILCs, cells were restimulated with phorbol 12-myristate 13-acetate (50ng/ml; Fisher Scientific) and 2µg/ml ionomycin (Invitrogen) in the presence of Brefeldin A (BD Biosciences) for 4 hours (E1). Cells were then stained for surface and intracellular markers and analyzed using an LSRII flow cytometer (BD Biosciences). Dead cells were excluded by Aqua staining. For all multicolor analyses, compensation controls (single stains, 1 for each fluorochrome) and gating controls (cells stained with all reagents minus 1) were included (E2, E3). To analyze T-cell populations, a lineage cocktail was used to exclude $CD14^+$ monocytes, $CD16^+$ NK cells, and $CD19^+$ B cells. After counterstaining for CD8, live $CD3^+CD8^-$ T cells were analyzed in fresh BAL and PBMC specimens in order to capture total $CD4^+$ T cell events (**Fig. E3**)(E1, E4, E5). Staining for CD4 confirmed the identity of $CD4^+$ T cells. Basophils, mDC and pDC were identified within lineage-negative CD3-negative cells based on differential expression of HLA-DR and CD123 as follows: basophils ($HLA\text{-}DR^-CD123^+$), mDC ($HLA\text{-}DR^+CD123^{mid}$ and $HLA\text{-}DR^+CD123^{neg}$), and pDC ($HLA\text{-}DR^+CD123^+$)(**Fig. E8**). ILCs were identified within lineage-negative CD3-negative CD4-negative cells, based on a $CD45^{hi}CD127^+$ phenotype. ILC2s were defined as those ILCs positive for IL-4 and/or IL-13 based on FMO controls (**Fig. E8**). The Th2 lineage-defining transcription factor GATA-3 was not used to identify ILC2s, based on its downregulation in a Th1-associated inflammatory milieu (E6).

Cytometric Bead Assay for Secreted Cytokines.

Cytokines included in the cytometric bead assay (Millipore, Billerica, MA) included 25

markers: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28A (IFN-λ2), IL-31, IL-33, IFN-γ, GM-CSF, MIP-3α, TNF-α and TNF-β.

Statistical Analysis.

Differences in cell numbers or percentages were analyzed within groups by the nonparametric Wilcoxon rank sign test for paired data, and between groups by the 2 sample Wilcoxon rank sum test. Owing to the limited sample size, allergy was evaluated as a continuous variable, as judged by serum total IgE. Associations between cell percentages/ratios, total IgE, age and ICS dose, were examined using univariate and multivariate Spearman correlations. In the multivariate setting, partial correlations were determined after adjustment for age, ICS dose and total IgE where applicable. Between group differences in the prevalence of IgE and antibody levels were analyzed by chi-square test and Mann-Whitney *U* test respectively. Heat maps for secreted cytokines were generated as follows: samples were assayed in at least duplicates and computed concentrations were averaged prior to plotting. Unsupervised hierarchical clustering was performed for samples using Euclidean distance and average linkage, and for cytokines using correlation distance and average linkage. For linear mixed-effects modeling, MFI values were used to assess differential expression of cytokines because of the increased sensitivity compared to using derived concentration values (E7, E8). Background corrected median fluorescence intensity (MFI) values were obtained from the luminex csv files and imported into the R statistical programming environment (version 3.3) (E9). MFI values were log10 transformed and the MFI values from the standards and quality control beads for each assay run were used to remove batch effects between different assays. Linear models for each cytokine were generated using batch corrected MFI values and subject source was treated as a random effect to account for variation between subjects. The R package Limma was used to remove batch effects and implement the linear models (E10). Moderated t tests were used to determine the significance of differential

95 expression between groups examined. Owing to the lack of previous data, no power calculation
96 was performed.

Figure Legends

Figure E1. Serum Antibodies and Neutrophil Counts in BAL Fluid of Children with Severe Asthma. (**A**) Serum IgE, IgA and IgG levels among asthmatic children according to bacterial culture status. Closed symbols denote children >6 years of age and open symbols denote children 6 months to 6 years of age. Bars denote geometric means with 95% confidence intervals. Grey boxes denote the range of normal antibody levels across all ages >6 months. (**B**) Neutrophil counts in BAL fluid (% of total cells). No neutrophils were detected in 3 specimens. NS, not significant.

Figure E2. Specimens Available for Flow Cytometry Studies and Luminex Cytokine Assays. (**A**) Numbers of specimens available for analysis. (**B**) Comparison of the characteristics of children included in flow cytometry studies with the asthma cohort.

Figure E3. Gating Strategy and Surface Signature of T Cells in Blood and BAL Fluid of Asthmatic Children. (**A**) $CD4^+$ T cells were identified by gating on live $CD3^+$ T cells after excluding B cells, monocytes, NK cells, and $CD8^+$ T cells by counterstaining with anti-CD19, anti-CD14, anti-CD16 antibodies, and anti-CD8 antibodies. The presence of $CD8^+$ T cells was confirmed based on a $CD3^+CD8^+$ signature. (**B**) Percentages of $CD4^+$ and $CD8^+$ T cells within total live T cells in BAL fluid from asthmatic subjects. (**C**) Comparison of the percentage of total $CD8^+$ T cells expressing CD45RO and CCR5 in blood and BAL fluid specimens obtained within the same subject. ****$p<0.0001$.

Figure E4. Expression of IL-4 and IFN-γ in $CD4^+$ and $CD8^+$ T Cells in BAL Fluid. Representative plots are shown for $CD4^+$ T cells (A) and $CD8^+$ T cells (B) in 3 asthmatic subjects across a broad range of serum total IgE levels.

Figure E5. T-Cell Skewing in Relation to Age and Inhaled Corticosteroid Dose.

Correlation between the percentages and ratio of cytokine-positive $CD4^+$ T-cell types in BAL fluid with age (A) and inhaled corticosteroid dose (B). Th1/Th2 ratio was defined as the ratio of total IFN-γ^+ to total Th2 (IL-$4^+$and/or IL-5^+) cells. in BAL fluid. Univariate and partial (parentheses) correlation values adjusted for total IgE and inhaled corticosteroid dose (A) and age and total IgE (B). A, allergic; NA, non-allergic.

Figure E6. Comparison of Cytokine Profiles In BAL Fluid Obtained from Different Lung Regions. BAL specimens were obtained from different lung regions in 20 asthmatics. Heat maps were positioned side by side to visualize variations in cytokine profiles between lung regions. Green bars at the top of the heat map denote specimens from allergic subjects. LLL, left lower lobe; RLL, right lower lobe; LUL, left upper lobe; RUL, right upper lobe; RML, right middle lobe; LING, lingular lobe.

Figure E7. Relationship Between IL-5 Levels in BAL Fluid and Eosinophils. (**A**) Correlation between IL-5 levels and blood eosinophil counts in 67 BAL specimens from 47 subjects. (**B**) IL-5 levels in subjects with and without detectable eosinophils in BAL fluid. Black and blue symbols denote allergic and non-allergic subjects respectively. The filled symbols denote specimens obtained from different lung regions within the same subject, with each shape representing a different subject.

Figure E8. Identification of Type 2 Innate Lymphoid Cells in BAL Fluid. (**A**) Gating strategy for ILC2s. An extensive cocktail of antibodies was used to exclude lineage-positive cells and type 1 innate lymphoid cells based on the following markers: CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, CD235a. Further exclusion of $CD4^+$ T cells yielded $CD45^{hi}CD127^+$ innate lymphoid cells. (**B**) Percentages of ILC2s (IL-4^+/IL-13^+) within total live single-cell events in BAL fluid obtained from 7 asthmatics. (**C**) Representative scatter plots showing intracellular cytokine expression in innate lymphoid cells from one allergic and one

non-allergic subject. Red dots denote cells expressing IL-4 or IL-13, or both. Inset plots show fluorescence minus one (FMO) controls for IL-4^{+} and IL-13^{+} cells using PBMCs from an allergic subject. (**D**) Expression of the ILC2 marker CRTH2 on IL4^{+}/IL13^{+} innate lymphoid cells (red histogram) compared with IL-4^{neg}/IL-13^{neg} innate lymphoid cells (blue histogram) and FMO (grey histogram).

Figure E9. Gating Strategy and Phenotypic Characteristics of Dendritic Cells and Basophils in BAL Fluid. **(A)** Gating strategy used to identify basophils and DC subsets. After excluding cells expressing other lineage markers, cell populations were identified based on differential expression of HLA-DR and CD123 as follows: basophils (HLA-DR^{-}CD123^{+}), mDC (HLA-DR^{+}CD123$^{neg/mid}$), and pDC (HLA-DR^{+}CD123^{hi}). **(B)** Representative histograms showing expression of characteristic markers on basophils (FcεRI), mDC (CD11c) and pDC (CD303) in blood and BAL.

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Table EI
Asthma Definitions, Severity Assignment, and Symptom Control by Age

Asthma Definitions	
Pre-school (6 months to 5 years); all present: • Recurrent episodes of cough, wheeze, and respiratory distress. • Current asthma medications • Positive modified asthma predictive index (E10, E11)	School-age and Adolescent (6 -17 years); all present: • Physician diagnosis • Current asthma medications • Airflow limitation present and/or a ≥12% increase in FEV_1 % predicted after bronchodilator.
Severe Asthma Definitions	
Pre-school • Modification of the ERS/ATS definition as asthma requiring treatment with high-dose ICS + a second controller: - ≥ 160 mcg/day fluticasone equivalents - 2^{nd} controller	School-Age and Adolescent • Asthma requiring treatment with high dose ICS plus a second controller (and/or systemic corticosteroids) to prevent it from becoming un-controlled despite this therapy (E12). High-dose ICS thresholds: - 6-11 years ≥ 440 mcg/day fluticasone equivalents - 12-17 years ≥ 880 mcg/day fluticasone equivalents
Poor Symptom Control Definitions	
Pre-school • Prolonged respiratory symptoms for > 3 days on 3 or more occasions in the previous calendar year	School-Age and Adolescent (any one) • Score < 20 units on the ACT/cACT validated asthma control questionnaire. • 2 or more prednisone bursts in the previous calendar year • Recurrent un-scheduled health care access for asthma treatment.

Table EII. List of Flow Cytometry Antibodies.

Marker	Fluorochrome	Source (Clone[1])
CD3	Alexa 700	BD (UCHT1)
	APC-H7	BD (UCHT1)
	APC-H7	BD (SK7)
	PE-Cy7	BioLegend (SK7)
CD4	PerCP	Invitrogen (S3.5)
	PE-Cy5	BD (RPA-T4)
	BB515	BD (RPA-T4)
CD8	FITC	Invitrogen (3B5)
CD11c	PE	BD (B-ly6)
CD45	PerCP	BioLegend (2D1)
CD45RA	PerCP-eF710	eBioscience (GRT22)
CD45RO	PB/eFlur 700	BioLegend (UCHL1)
	PerCP-eF710	eBioscience (UCHL1)
CD123	PerCP Cy5.5	eBioscience (6H6)
CD127	PE-Dazzle	BioLegend (A019D5)
CD303a	PECy7	eBioscience (201A)
CCR5	PECy7	BD (2D7)
CRTH2	BV421	BioLegend (BM16)
ICOS	BV650	BD (DX29)
CD161	BV785	BioLegend (HP-3G10)
Lineage cocktail (CD14, CD16, CD19)	PETR	Invitrogen (TuK4, 3G8, SJ25-C1)
Lineage cocktail (CD14, CD19)	PerCP	BD (MΦP9, SJ25C1)
Lineage cocktail (CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCRγ/δ, CD235a)	Biotin	Miltenyi Biotec (CD4+ T-Cell Isolation Kit)
IgE	FITC	KPL
FcεRI	PerCP	BioLegend (AER-37)
TSLP receptor	APC	BioLegend (1B4)
HLA-DR	APC Cy7	BD (L243)

IL-4	PerCP Cy5.5	BioLegend (MP4-25D2)
	PE-Cy7	BD (8D48)
	BV605	BioLegend (MP4-s5D2)
IL-5	APC	Miltenyi (JES1-39D10)
IL-13	FITC	eBioscience (PVM13-1)
	BV711	BD (JES10-5A2)
IL-17A	APC-eFluor 780	eBioscience (eBio64DEC17)
	AF488	BioLegend (BL168)
	APC-Cy7	BioLegend (BL168)
IFN-γ	PE	BD (4S.B3)
	AF700	BioLegend (4S.B3)

[1]Provided where available

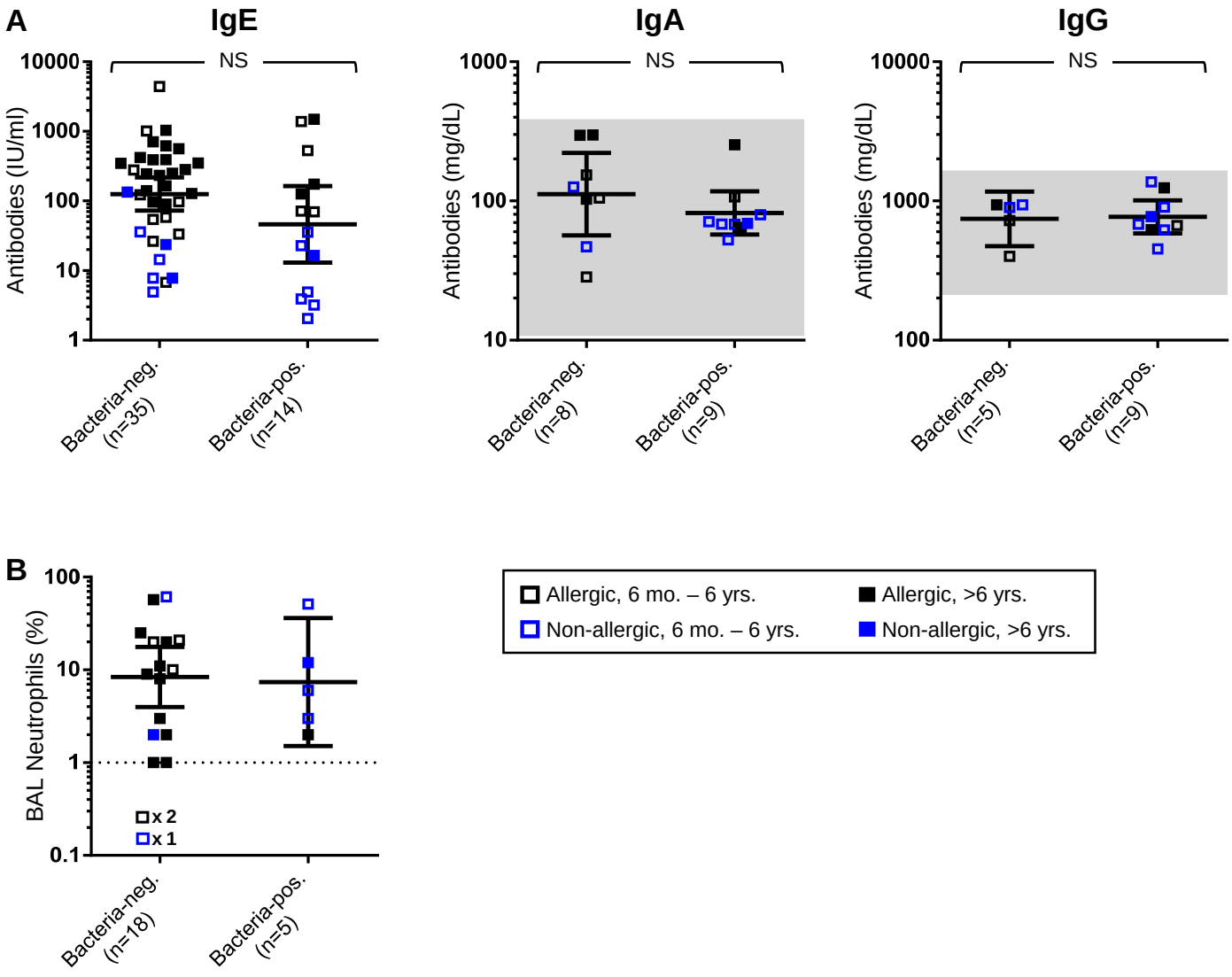
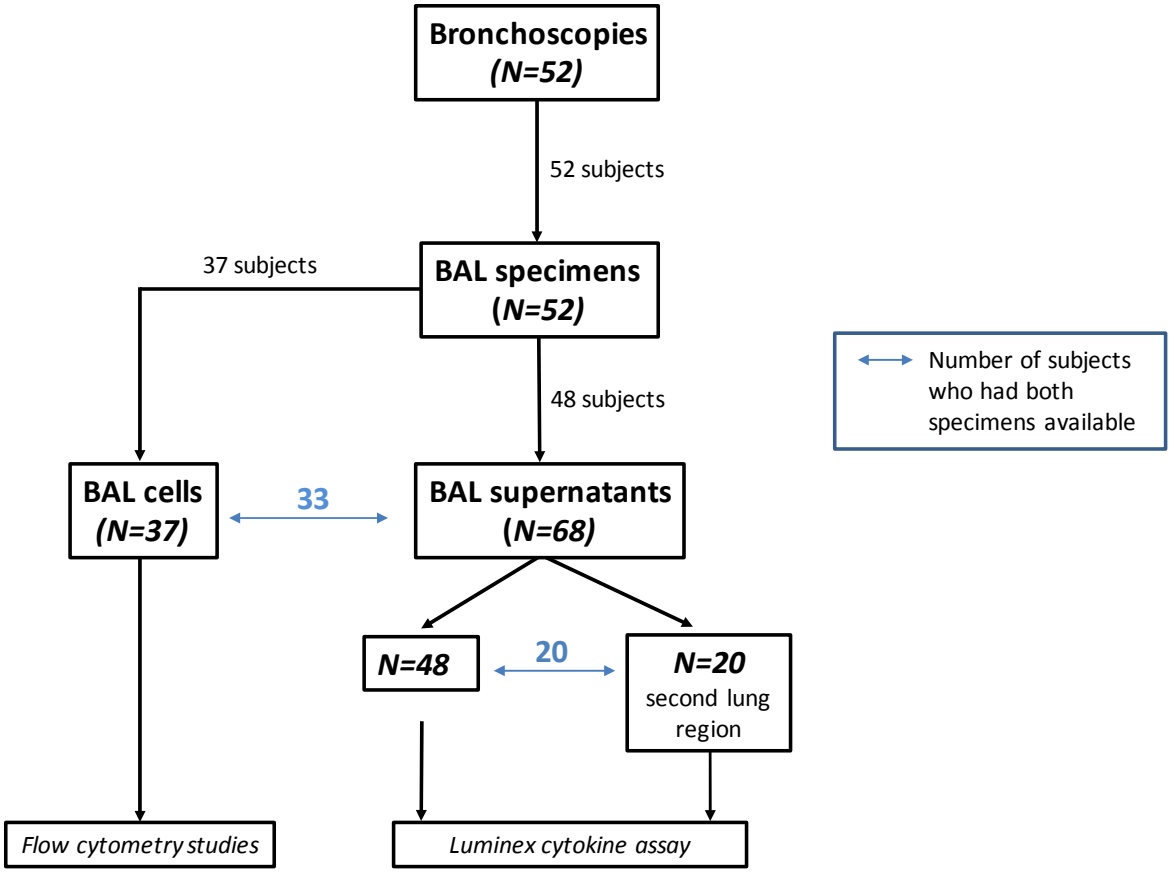

A
IgE
IgA
IgG
NS
NS
NS
Antibodies (IU/ml)
Antibodies (mg/dL)
Antibodies (mg/dL)
10000
1000
100
10
1
1000
100
10
10000
1000
100
Bacteria-neg. (n=35)
Bacteria-pos. (n=14)
Bacteria-neg. (n=8)
Bacteria-pos. (n=9)
Bacteria-neg. (n=5)
Bacteria-pos. (n=9)
B
BAL Neutrophils (%)
100
10
1
0.1
x 2
x 1
Bacteria-neg. (n=18)
Bacteria-pos. (n=5)
Allergic, 6 mo. – 6 yrs.
Allergic, >6 yrs.
Non-allergic, 6 mo. – 6 yrs.
Non-allergic, >6 yrs.

Figure E1

A



[1] Four subjects had no supernatants available for analysis and 15 subjects had no cells available for analysis

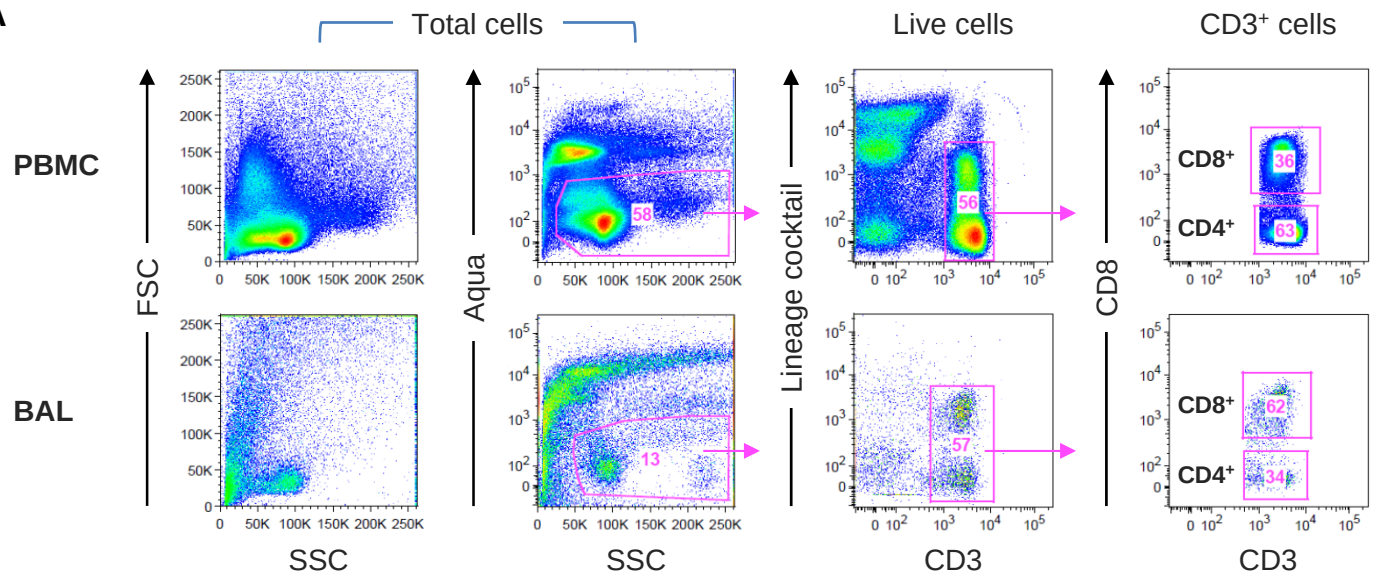
B **Comparison of the Characteristics of Subjects Included in Flow Cytometry Studies with the Asthma Cohort.**

	Flow Cytometry Studies	Asthma Cohort	
Numbers of subjects	37	52	P value
Age[1]	4.8 [3.6-6.3]	5.7 [4.5-7.2]	0.26
Male[2]	65% (24/37)	63% (33/52)	0.89
African American[2]	27% (10/37)	29% (15/52)	0.85
Total IgE[1]	79 [40-160]	111 [66-188]	0.38
ICS dose (mcg)[1]	433 [333-565]	479 [382-602]	0.55
EV/HRV-pos[2]	46% (16/35)	38% (19/50)	0.48
Bacteria-pos[2]	34% (12/35)	29% (14/49)	0.57
Fungal sensitization[2,3]	27% (10/37)	36% (19/52)	0.34
Allergic[2,4]	68% (25/37)	73% (38/52)	0.57

[1]Geometric mean [95% confidence interval]. [2]Percentage and (prevalence).
[3]IgE >0.1 kU_A/L to *Alternaria alternata* and/or *Aspergillus fumigatus*. [4]IgE >0.1 kU_A/L to food or inhalant allergens. EV, enterovirus; HRV, human rhinovirus.

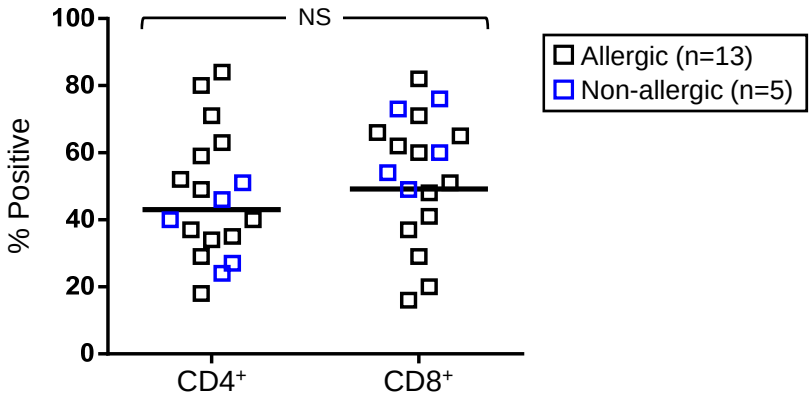
Figure E2

A



B

Live Lymphocytes in BAL



C

CD8⁺ T cells

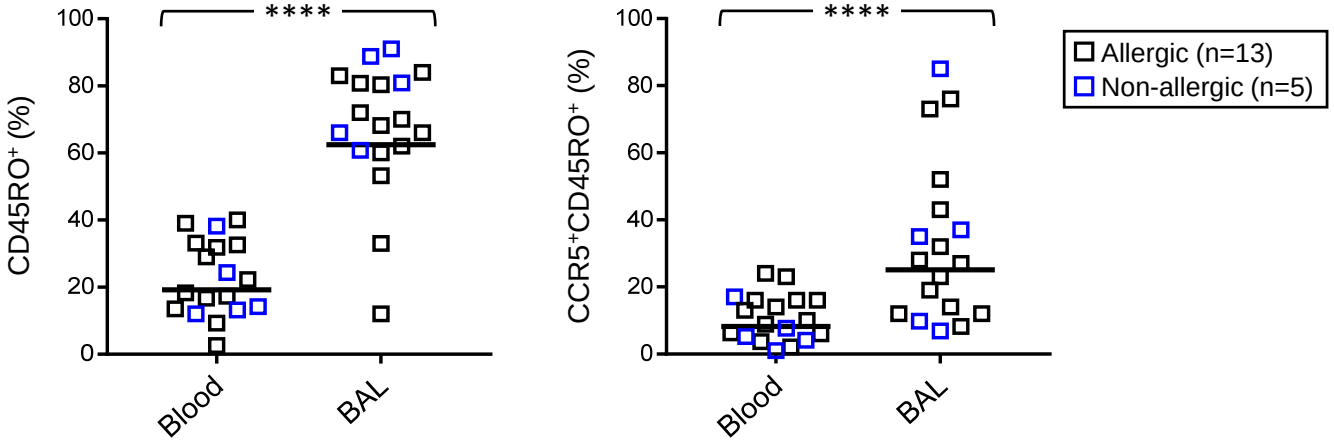


Figure E3

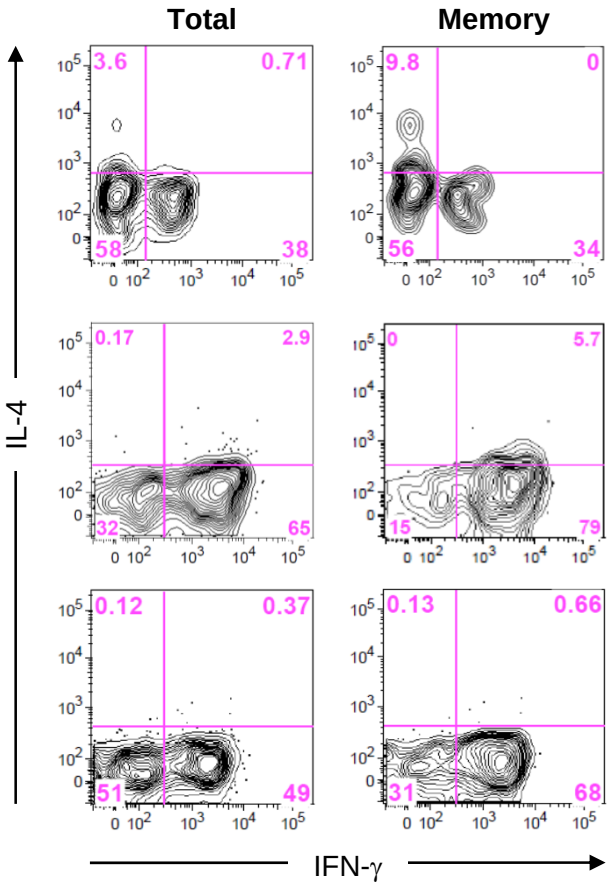
A

CD4⁺ T cells in BAL

Subject #10, age 3.8 yrs.
Total IgE 3,263 IU/ml

Subject #50, age 5.4 yrs.
Total IgE 531 IU/ml

Subject #51, age 4.3 yrs.
Total IgE 4.9 IU/ml



B

CD8⁺ T cells in BAL

Subject #10, age 3.8 yrs.
Total IgE 3,263 IU/ml

Subject #50, age 5.4 yrs.
Total IgE 531 IU/ml

Subject #51, age 4.3 yrs.
Total IgE 4.9 IU/ml

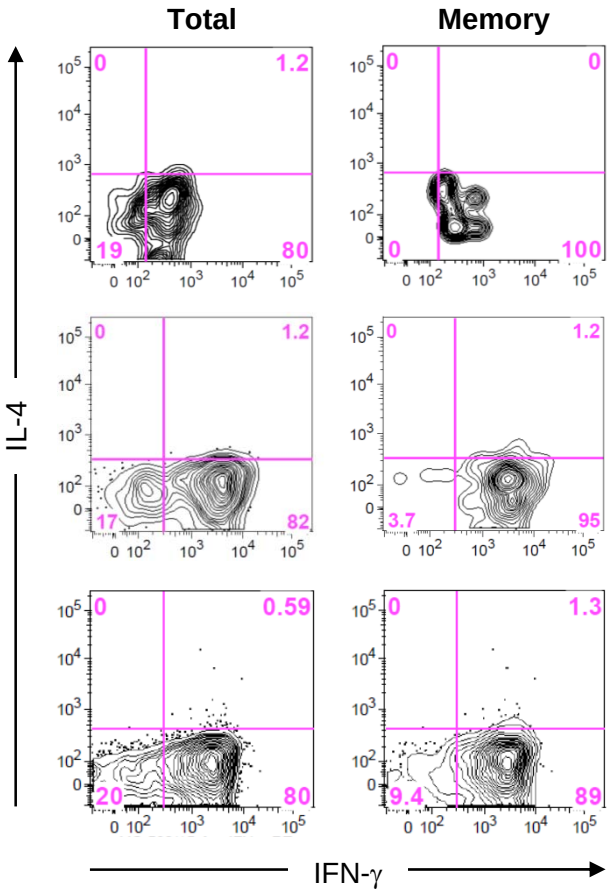


Figure E4

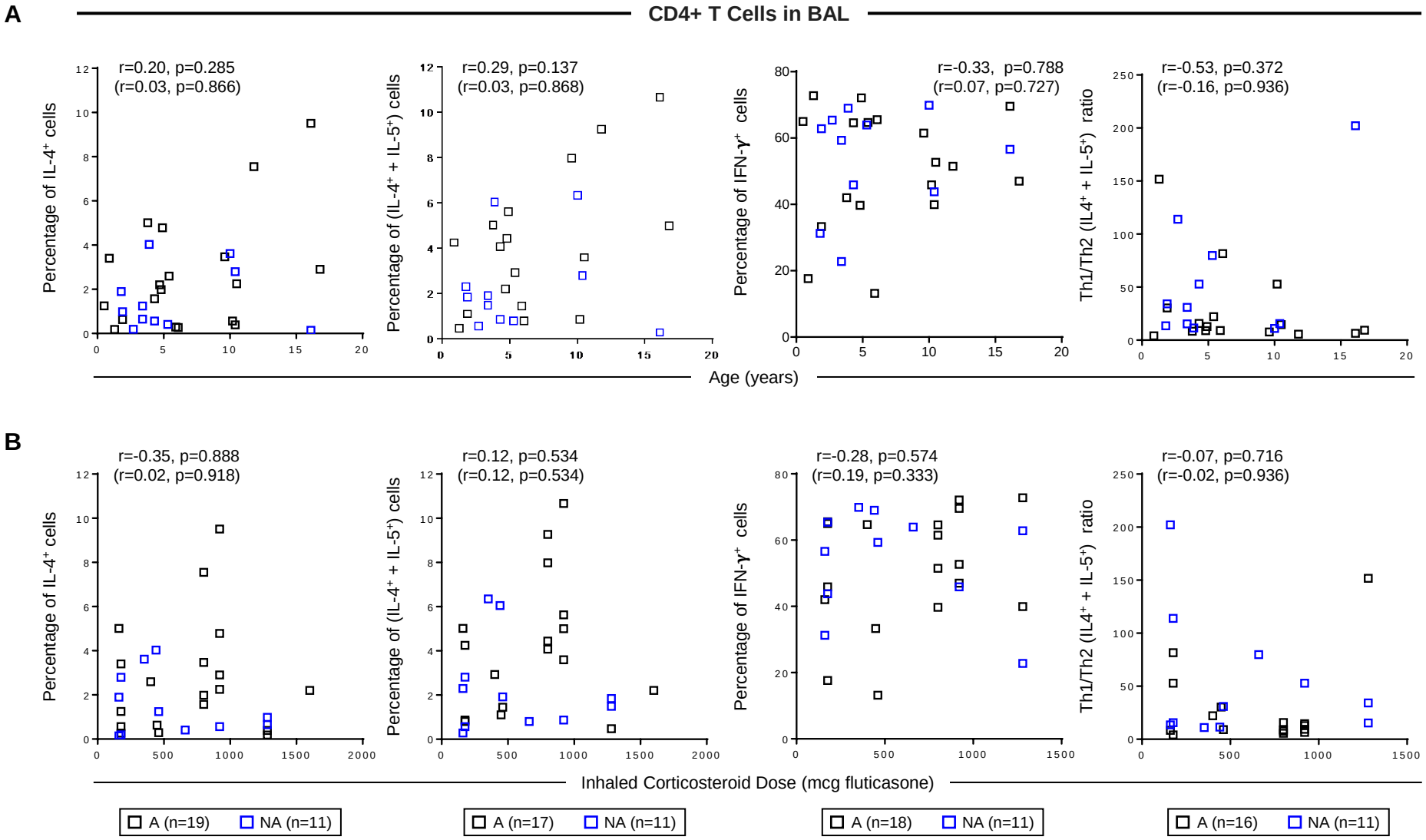
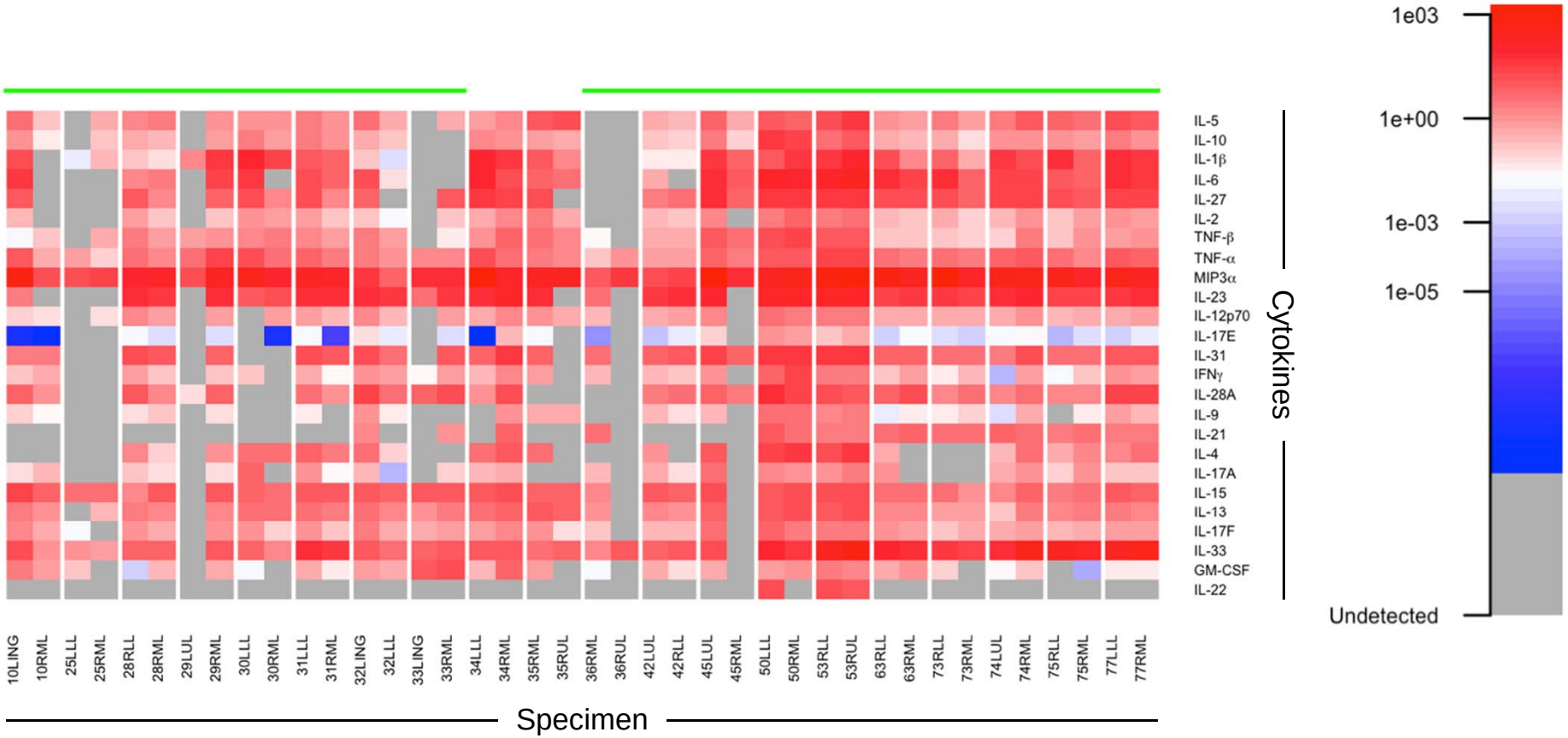

A
CD4+ T Cells in BAL
r=0.20, p=0.285
(r=0.03, p=0.866)
Percentage of IL-4+ cells
r=0.29, p=0.137
(r=0.03, p=0.868)
Percentage of (IL-4+ + IL-5+) cells
r=-0.33, p=0.788
(r=0.07, p=0.727)
Percentage of IFN-γ+ cells
r=-0.53, p=0.372
(r=-0.16, p=0.936)
Th1/Th2 (IL4+ + IL-5+) ratio
Age (years)
B
r=-0.35, p=0.888
(r=0.02, p=0.918)
Percentage of IL-4+ cells
r=0.12, p=0.534
(r=0.12, p=0.534)
Percentage of (IL-4+ + IL-5+) cells
r=-0.28, p=0.574
(r=0.19, p=0.333)
Percentage of IFN-γ+ cells
r=-0.07, p=0.716
(r=-0.02, p=0.936)
Th1/Th2 (IL4+ + IL-5+) ratio
Inhaled Corticosteroid Dose (mcg fluticasone)
A (n=19) NA (n=11)
A (n=17) NA (n=11)
A (n=18) NA (n=11)
A (n=16) NA (n=11)

Figure E5


IL-5
IL-10
IL-1β
IL-6
IL-27
IL-2
TNF-β
TNF-α
MIP3α
IL-23
IL-12p70
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IL-31
IFNγ
IL-28A
IL-9
IL-21
IL-4
IL-17A
IL-15
IL-13
IL-17F
IL-33
GM-CSF
IL-22
Cytokines
1e03
1e+00
1e-03
1e-05
Undetected
10LING
10RML
25LLL
25RML
28RLL
28RML
29LUL
29RML
30LLL
30RML
31LLL
31RML
32LING
32LLL
33LING
33RML
34LLL
34RML
35RML
35RUL
36RML
36RUL
42LUL
42RLL
45LUL
45RML
50LLL
50RML
53RLL
53RUL
63RLL
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74RML
75RLL
75RML
77LLL
77RML
Specimen

Figure E6

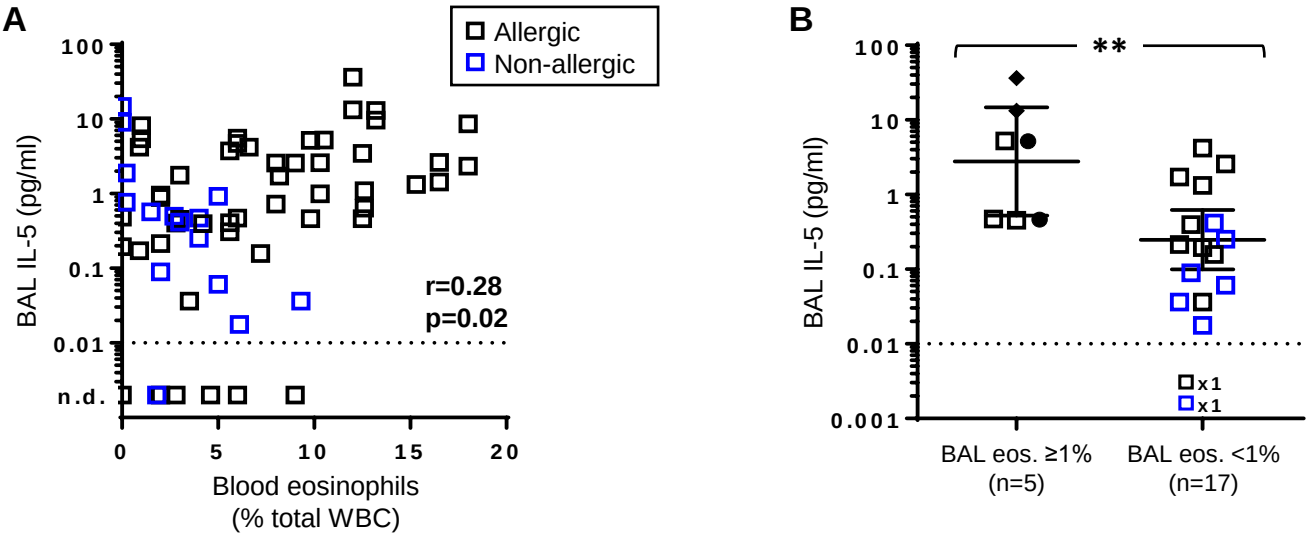
A
Allergic
Non-allergic
BAL IL-5 (pg/ml)
100
10
1
0.1
0.01
n.d.
r=0.28
p=0.02
0
5
10
15
20
Blood eosinophils
(% total WBC)
B
**
BAL IL-5 (pg/ml)
100
10
1
0.1
0.01
0.001
x1
x1
BAL eos. ≥1%
(n=5)
BAL eos. <1%
(n=17)

Figure E7

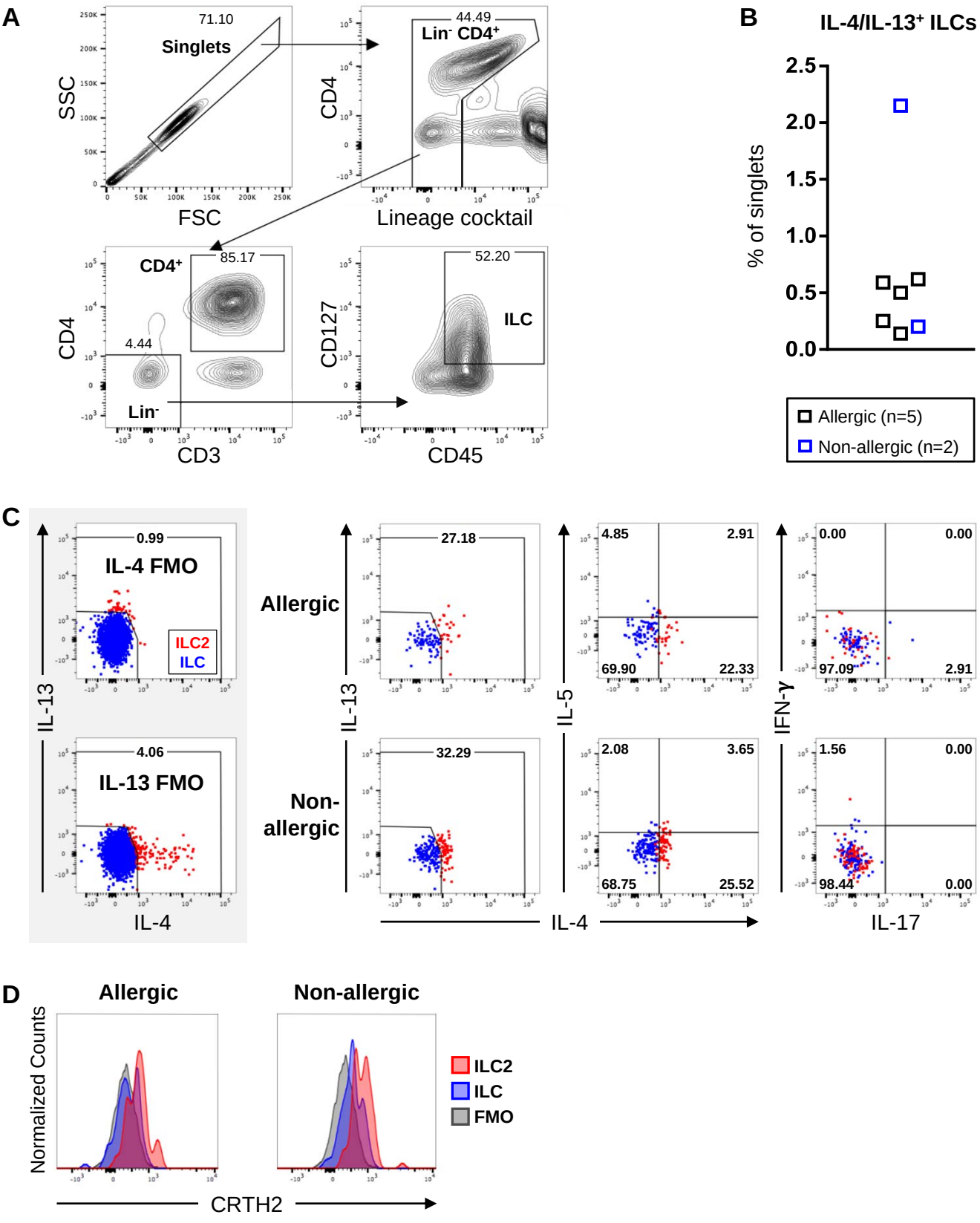
A
71.10
Singlets
SSC
FSC
44.49
Lin- CD4+
CD4
Lineage cocktail
CD4+
85.17
4.44
Lin-
CD4
CD3
52.20
ILC
CD127
CD45
B
IL-4/IL-13+ ILCs
% of singlets
2.5
2.0
1.5
1.0
0.5
0.0
Allergic (n=5)
Non-allergic (n=2)
C
0.99
IL-4 FMO
ILC2
ILC
IL-13
4.06
IL-13 FMO
IL-4
Allergic
Non-allergic
27.18
32.29
IL-13
4.85
2.91
69.90
22.33
2.08
3.65
68.75
25.52
IL-5
IL-4
0.00
0.00
97.09
2.91
1.56
0.00
98.44
0.00
IFN-γ
IL-17
D
Allergic
Non-allergic
Normalized Counts
ILC2
ILC
FMO
CRTH2

Figure E8

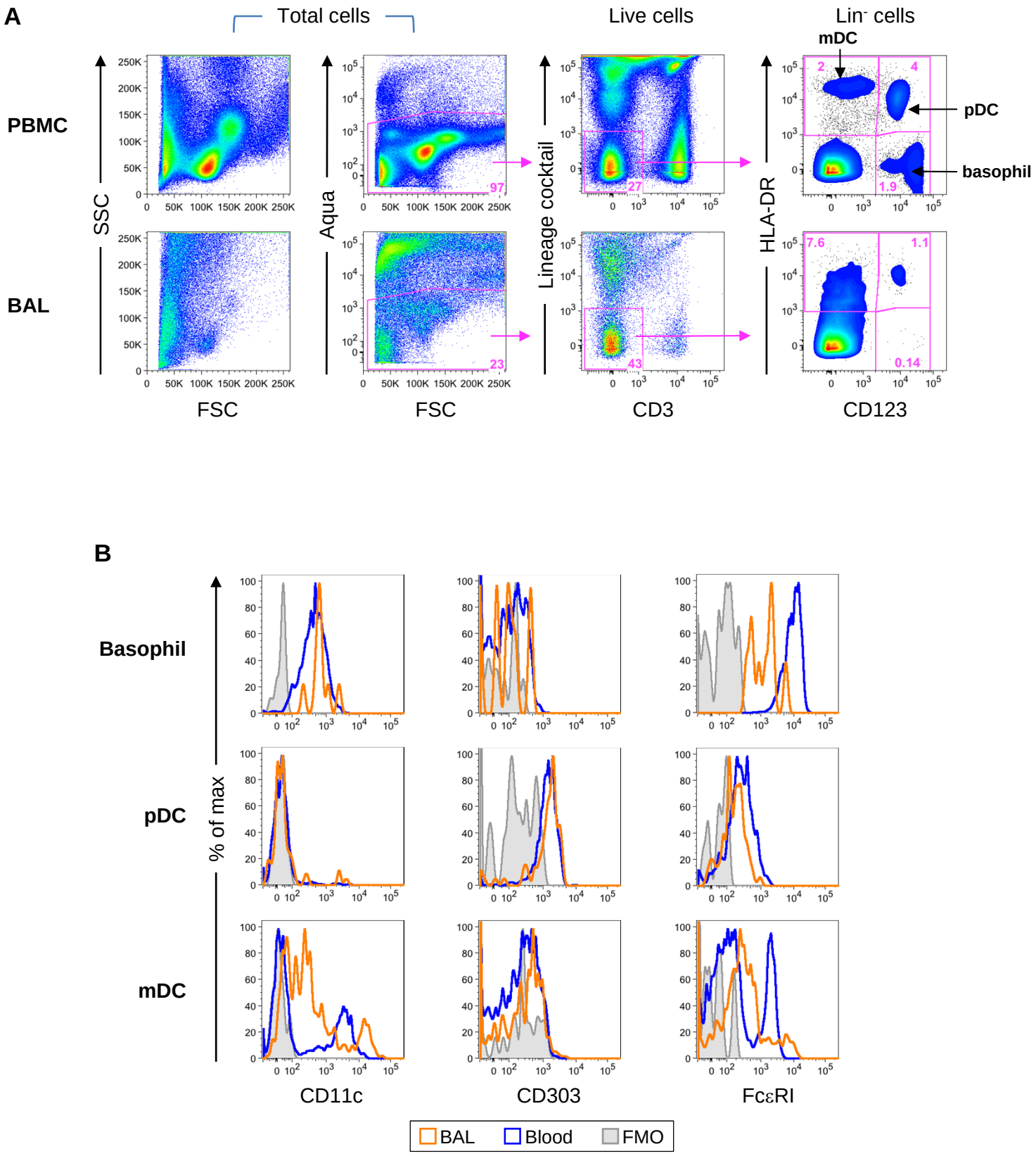
A
Total cells
Live cells
Lin- cells
PBMC
BAL
SSC
Aqua
Lineage cocktail
HLA-DR
FSC
FSC
CD3
CD123
mDC
pDC
basophil
97
27
2.
4
1.9
23
43
7.6
1.1
0.14
B
Basophil
pDC
mDC
% of max
CD11c
CD303
FcεRI
BAL
Blood
FMO

Figure E9