

Enhanced frequency and function of follicular T cells in the tonsils of house dust mite-sensitized children

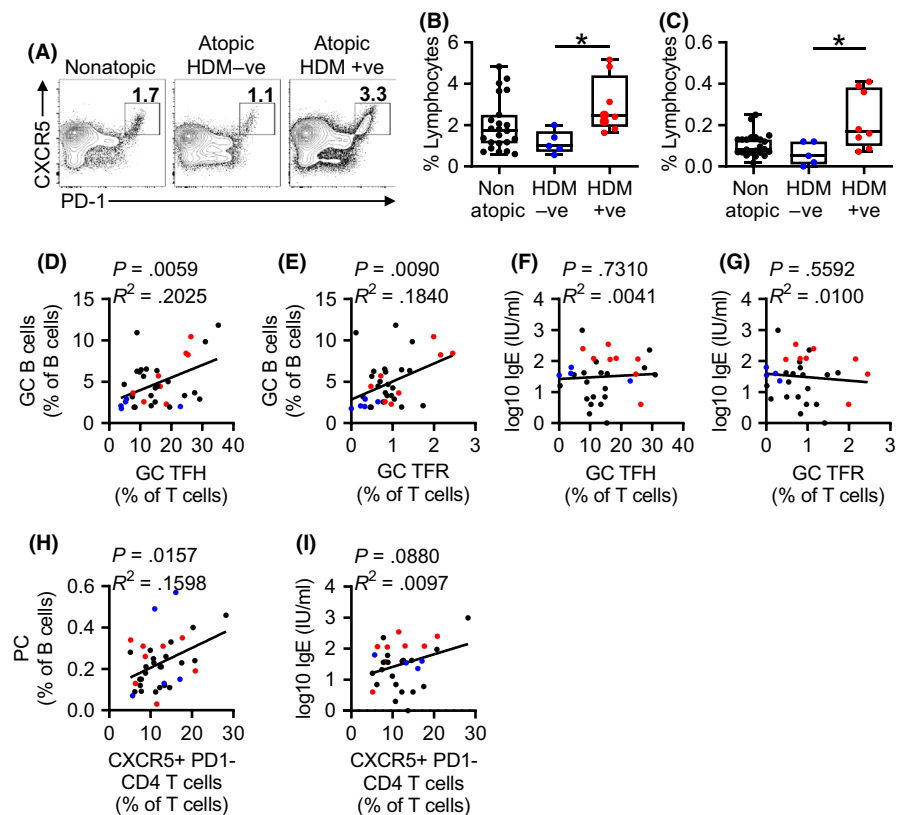
To the editor,

T follicular helper cells (TFH) are a specialized subset of CD4⁺T cells that regulate B cell responses within the germinal center (GC). Expression of the chemokine receptor CXCR5 allows TFH to migrate into and be retained within the B cell follicles of secondary lymphoid organs (SLOs). TFH regulate the survival, proliferation, and differentiation of B cells dictating the quality, quantity, and type of antibody¹; TFH are essential for the formation of GCs and antibody production in murine models of inflammation including allergy.² Particularly, large numbers of TFH are found in the tonsils, especially in early life, where they are associated with highly active GCs as a result of constant exposure to ingested and inhaled antigens.

Often the first SLOs to encounter environmental stimuli and infections, the tonsils can play an important role in the generation of protective immunity, and TFH dysfunction has recently been

associated with recurrent group A *Streptococcus* infection of the tonsil.³ Recurrent infection affects older patients primarily, with young children often presenting noninfectious tonsillar hyperplasia.⁴ Therefore, adenotonsillectomy remains one of the most common pediatric surgical procedures carried out worldwide, primarily as a result of diagnosis with pediatric obstructive sleep apnea (OSA). Recurrent bacterial or viral infection is commonly linked with tonsil hypertrophy, but there is increasing evidence of an association with atopic diseases. Children with OSA have a higher incidence of atopy than the general population and increased likelihood of developing atopic dermatitis or allergic asthma in later life,⁵ and adenotonsillectomy to treat OSA has been associated with improvements in a number of asthma outcomes.⁶ We therefore aimed to determine the relationship between the tonsil immune response, tonsillar enlargement, OSA, and atopy.

FIGURE 1 Germinal center TFH/TFR frequencies correlate with GC B cells and are increased in HDM-sensitized children. A, Representative flow cytometry plots gating tonsil stained for follicular helper cells, pregated on lymphocytes, numbers indicate (%). B & C, Quantification of tonsil GC TFH (B) and GC TFR (C). For B & C, the Kruskal-Wallis with Dunn's multiple comparison statistical test was used * $P < .05$. D & E, GC TFH + TFR frequency was compared to (D) GC B cells and (E) serum IgE. F & G, CXCR5⁺PD1⁺CD4 T cells were compared to (F) plasma cells (PC) and (G) serum IgE. For D-G linear regression analysis was used, with normality of data confirmed with the Shapiro-Wilk test. For (A-G), $n = 36$. * $P < .05$



Abbreviations: GC, germinal center; HDM, house dust mite; OSA, obstructive sleep apnea; TFH, T follicular helper cells; TFR, T follicular regulatory cells.

Thirty-six children undergoing routine adenotonsillectomy were recruited, eighteen children had an indication of OSA, previously confirmed by cardiorespiratory polygraph sleep study, and eighteen children recruited with other indications. The regional ethics committee approved the study, with written informed consent obtained. Clinical and demographics details are summarized in Table S1. Atopic status was determined by a positive ImmunoCAP IgE or skin prick test and was present in thirteen of thirty-six children, and other details such as recent viral illness and presence of bacteria on culture were also assessed. In this cohort, there was no significant association between atopic status and other diagnoses including OSA or infectious history.

Firstly, tonsil lymphocyte composition was analyzed by flow cytometry (Figure S1A). As expected, tonsil tissue was primarily composed of B cells (CD19⁺) and T cells (CD3⁺) including experienced (CD45RO⁺ CD45RA⁻) and naïve (CD45RO⁻ CD45RA⁺) T cells; there was no significant difference in the proportions of these cell types in

atopics versus nonatopics (Figure S1B-E). CD4⁺ T cells associated with B cell follicles were separated into those found within the GC, GC TFH (CXCR5^{hi}PD1^{hi}Foxp3⁻CD25⁻), and those that reside outside of the GC, non-GC TFH (CXCR5^{int}PD1^{int}Foxp3⁻CD25⁻). Suppressive, T follicular regulatory CD4⁺ T cells (TFR) could also be identified (CXCR5^{int/hi}PD1^{int/hi}Foxp3^{int/hi}CD25^{hi}) (Figure S2A).⁷ When analyzing atopic versus nonatopic status, OSA diagnosis or infectious history as a whole, there was no significant difference in the frequency of the different follicular T-cell populations (Figure S2B-E); however, in children sensitized to the common aeroallergen, house dust mite (HDM, *Dermatophagoides pteronyssinus*), there was an increase in CXCR5^{hi}PD1^{hi} GC-associated TFH and TFR compared with non-HDM-sensitized atopic children (Figures 1A-C); an effect that was not observed in any other CD4⁺ T-cell subpopulations (Figure S3). HDM-sensitized children had similar poly-sensitization profiles to non-HDM-sensitized atopic children suggesting this was the result of HDM sensitivity (Figure S4).

Tonsillar GC B cell frequency (gated in Figure S5) correlated with both GC TFH and GC TFR frequencies (Figure 1D-E). Total circulating IgE concentrations were elevated in HDM-sensitive individuals, but this did not directly correlate with GC TFH or GC TFR frequencies (Figure 1F-G). PD1⁻ CXCR5⁺ CD4 T cells that reside outside of the B cell follicle did however correlate with both the number of antibody-secreting cells in tonsils and circulating IgE concentrations (Figure 1H-I).

CCR4 expression is associated with T helper-2 skewed CD4 T cells, and individuals with allergic rhinitis have an increased frequency of circulating TFH expressing CCR4.⁸ A proportion of tonsil GC TFH were also CCR4⁺ (Figure S6A); however, while the frequency of these was significantly elevated in children recently reporting viral illness, it was similar between atopics and nonatopics (Figure S6B-C). TFH also produce high concentrations of the canonical TH2 cytokine IL-4, alongside IL-21, to promote B cell function.¹ Accordingly, GC TFH were the major source of IL-21 and among the most frequent producer of IL-4 in the tonsils after polyclonal stimulation (Figure 2A). Atopic children had increased frequencies of IL-4⁺ GC TFH, while IL-21⁺ GC TFH frequency was comparable to nonatopics, and responses were similar in HDM-positive and HDM-negative atopics (Figure 2B). IL-13, another canonical TH2 cytokine, could not be detected in any CD4⁺ T-cell population analyzed. Stimulation of tonsil cells with HDM also resulted in increased IL-4⁺, and to a lesser extent IL-21⁺, TFH in HDM-positive, but not HDM-negative, individuals (Figure 2C). Thus rather than having a type-2 phenotype, TFH from atopic individuals specifically enhanced IL-4 production potentially promoting IgE switch.

Recent studies have highlighted the altered frequency of circulating TFH in allergic individuals,⁸ but this is the first study to show both functionally and numerically altered TFH in the SLOs of allergen-sensitized individuals. One limitation is the absence of true healthy comparator tonsil tissue; however, aside from allergic status, no other pathological indicator, for example OSA or infection, was associated with increased TFH responses. The young age of this cohort highlights that TFH dysregulation can occur in early in life, often prior to diagnosis with allergic disease.

Importantly, allergen immunotherapy appears to suppress cTFH, potentially through a regulatory T-cell-dependent mechanism.⁸

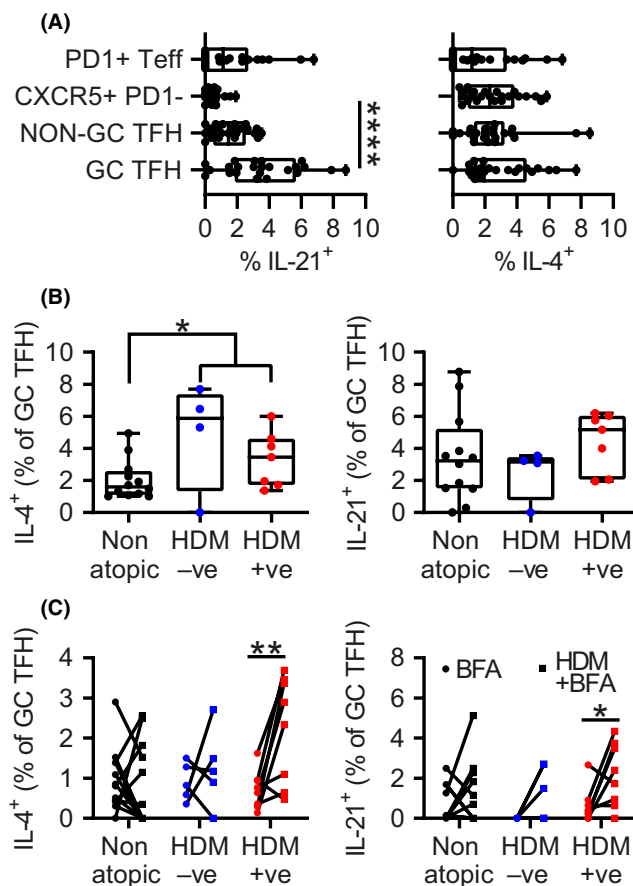


FIGURE 2 Germinal center TFH from atopic sensitized children produce more IL-4. A, Tonsil cells were polyclonally stimulated with phorbol myristate acetate (PMA) and Ionomycin in the presence of Brefeldin A for 4 h, with IL-4 and IL-21 production measured in CD4 T cells subpopulations by intracellular staining. B, IL-4 and IL-21 production by GC TFH in nonatopic and atopic patients was measured as in A. C, Tonsil cells were stimulated with whole HDM extract for 18 h and Brefeldin A for a final 4 h and IL-4 and IL-21 production by GC TFH was determined, as a control BFA, in the absence of HDM, was added. A Wilcoxon paired test was carried out. n = 22. *P < .05, **P < .01 ****P < .0001

Murine models have shown that sustained exposure to aeroallergens specifically promotes increased TFH frequency and function, while therapeutic blocking of TFH reduces IgE and ameliorates disease.⁹ Thus, targeting this pathway could prove effective in preventing or treating allergic disease.

1 | SUMMARY

We show that T follicular helper cells, central regulators of B cell responses, are dysregulated in the tonsils of atopic children, especially those sensitized to the common aeroallergen, house dust mite.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

CJG, H-L.T, and S.S organized patient recruitment and medical data collection. WSF, CJG, H-L, T, SS, GSW, CML, and JAH designed experiments. WSF and CJG carried out the experimental work. WSF analyzed the experimental work. WSF, CML, and JAH wrote the manuscript. CJG, H-L.T, GSW, MJR, G.C, S.S, CML, and JAH provided feedback.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Appendix S1**Methods****Patient recruitment**

Children were recruited from the Ear, Nose and Throat (ENT) surgical schedules at Royal Brompton Hospital (RBH) and Chelsea and Westminster Hospital (CW). Those undergoing adeno-tonsillectomy for treatment of OSA had previously undergone a cardiorespiratory polygraphy sleep study at RBH. Children undergoing adeno-tonsillectomy for other indications were recruited as a disease control group. Prior to surgery, recent viral illness was identified in some patients, from descriptions of the patient's health within the last two weeks, as described by their parents or guardian. For both groups, consent was obtained from the parent with the child involved in the process. Ethical approval was granted by the NRES committee London-Riverside on 10th February 2014 (Ref 14/LO/0001).

Atopy diagnosis

To establish prevalence of atopy, data was collected on previously performed skin-prick testing or specific immunoglobulin E (IgE) to a common aeroallergen panel (dog dander, cat dander, tree pollen, grass pollen, house dust mite and *aspergillus fumigatus*). Specific IgE to the aeroallergen panel was performed using the ImmunoCAP 250 analyzer. A positive result was defined as ≥ 0.35 IU/ml. If during the recruitment process, a known allergy was identified, this was added to the specific allergen panel, for example if the child was known to be or suspected to be allergic to peanuts. In addition to specific IgE testing, total serum IgE was recorded.

Tonsil processing for cryopreservation

Whole tonsil tissue was cut finely with a scalpel and then washed through a 70 μ M filter with warm RPMI (GIBCO, Carlsbad, CA). Cells were resuspended in 5mls Red cell lysis buffer, and incubated at room temperature for 5 minutes. The lysis buffer suspension was diluted with 45mls RPMI, and washed twice via centrifugation and resuspension in 50mls RPMI.

Cells were then counted using Trypan blue (Thermofisher, Waltham, MA) and suspended at 10^7 cells/ml in freezing media (90% FCS, 10% DMSO). The cells were aliquoted into 1.5ml cryovials, aliquots were placed into MrFrosty™ freezing containers (Thermofisher) and frozen at -80°C .

Tonsil cell recovery from cryopreservation

Aliquots were removed from -80°C storage onto ice, and resuspended in 5ml of warm complete RPMI media (RPMI, 10% fetal calf serum, 2mM L-glutamine, and 100 U/ml penicillin/streptomycin). The samples were washed twice by centrifugation and resuspension in 5ml of RPMI medium, before being finally resuspended in 5mls complete RPMI. Samples were then plated into 25 ml cell culture flasks and rested in a 37°C , 5% CO_2 incubator for 3 hours. After resting, the cells were removed, and the flasks washed with 5ml fresh RPMI medium to remove residual cells. The cells were then centrifuged and resuspended in 1 ml of PBS. Cells were counted using trypan blue, and up to 1million cells were plated into individual wells of a V bottom 96 well plate.

Stimulation of cells by PMA/Ionomycin

Following recovery, cells were centrifuged and resuspended into 200 μl of complete RPMI media containing 50 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich), 500 ng/ml Ionomycin (Emdchemicals) and 10 $\mu\text{g/ml}$ Brefeldin A (Sigma-Aldrich) for 4 hours at 37°C 5% CO_2 .

Stimulation of cells by House dust mite extract

Following recovery, cells were centrifuged and resuspended into 200 μl of complete RPMI media containing 50 $\mu\text{g/ml}$ house dust mite (HDM) extract (Greer Laboratories, NC, USA) and were cultured for 18 hours in a 37°C , 5% CO_2 incubator. As a negative control, cells from the same defrosted aliquots were cultured in complete RPMI media without HDM

extract in parallel. Then, 50 µl of complete RPMI containing of 50µg/ml Brefeldin A (Sigma-Aldrich) was added to both HDM stimulated and negative control cells, followed by an additional 4 hours of incubation.

Flow cytometry

All antibodies were purchased from Biolegend, except where otherwise specified.

Cells were first stained with 50 µl Fixable Viability Dye eFluor™ 780 (eBioscience) at a 1/1000 dilution for 30 minutes on ice. Cells were then washed twice with 150 µl flow cytometry buffer (PBS +2% FCS, 1mM EDTA), and stained for 30 minutes with the following antibodies: BV421 CD27 (M-T271), BV510 CD10 (HI10a), BV605 IgM (MHM-88), BV711 CD38 (HIT2), FITC IgG (G18-145) (BD biosciences), PerCpCy5.5 CD3 (UCHT1), PE CD138 (MI-15), PE-dazzle594 (ML5), PE-Cy7 IgD (IA6-2), APC CD19 (HIB19), A700 CD20 (2H7) diluted in 25 µl flow cytometry buffer. FC receptors were blocked simultaneously, using a 1/100 dilution of Human TruStain FcX. After staining, the cells were washed twice with 150 µl flow cytometry buffer, and then fixed with 1% paraformaldehyde (Sigma Aldrich) in PBS.

For staining of T cells, cell suspensions were stained with the following antibodies: BV421 CCR4 (L291H4), BV510 CD8 (SK1), BV605 CD4 (OKT1), BV650 CD45RO (UCHL1), BV711 CXCR3 (G025H7), BV785 CD45RA (HI100), BB515 CXCR5 (RF8B2) (BD biosciences), PerCpCy5.5 CD3 (UCHT1), PE-dazzle594 CD25 (M-A251), PE-Cy7 PD-1 (EH122H7), APC CD19 (HIB19) and APC-H700 CCR6 (11A9) (BD biosciences) in 25 µl flow cytometry buffer. After staining, the cells were washed twice with 150 µl flow cytometry buffer then fixed with eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set for 30 minutes on ice. Cells were then washed with PBS, and stained with PE Foxp3 (206D) in eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set permeabilisation buffer. Cells were then washed and resuspended in 150 µl flow cytometry buffer, with data being acquired immediately.

For intracellular cytokine staining, cells were stained with CD4, CD45RA, CD45RO, CD19, CXCR5, CD3, CD25, PD-1 as above, then fixed with 1% paraformaldehyde (Sigma Aldrich)

in PBS for 10 minutes at room temperature and then stained with the following antibodies, diluted in permeabilisation buffer (eBioscience): BV421 IL-4 (MP4-25D2), A647 IL-21 (3A3-N2). Data were acquired on an LSRFortessa (BD Biosciences) and analyzed using FACSDiva (BD Biosciences) and FlowJo (Treestar, La Jolla, CA) software.

Fluorescent antibodies used:

Target	Fluorophore	Clone	Dilution	Supplier
CCR4	BV421	L291H4	1/50	Biolegend
CD8	BV510	SK1	1/200	Biolegend
CD4	BV605	OKT1	1/100	Biolegend
CD45RO	BV650	UCHL1	1/100	Biolegend
CXCR3	BV711	G025H7	1/50	Biolegend
CD45RA	BV785	HI100	1/200	Biolegend
CXCR5	BB515	RF8B2	1/25	BD
CD3	PerCpCy5.5	UCHT1	1/50	Biolegend
FOXP3	PE	206D	1/25	Biolegend
CD25	PE-dazzle594	M-A251	1/50	Biolegend
PD1	PE-Cy7	EH122H7	1/50	Biolegend
CD19	A647	HIB19	1/100	Biolegend
CCR6	APC-H700	11A9	1/25	BD
CD27	BV421	M-T271	1/50	Biolegend
CD10	BV510	HI10a	1/50	Biolegend
IgM	BV605	MHM-88	1/50	Biolegend
CD38	BV711	HIT2	1/50	Biolegend
IgG	FITC	G18-145	1/10	BD
CD3	PerCpCy5.5	UCHT1	1/50	Biolegend

CD138	PE	MI-15	1/25	Biolegend
CD24	PEdazzle594	MIL5	1/50	Biolegend
IgD	PE-Cy7	IA6-2	1/50	Biolegend
CD19	APC	HIB19	1/100	Biolegend
CD20	A700	2H7	1/50	Biolegend
BV421	IL-4	MP4-25D2	1/50	Biolegend
A647	IL-21	3A3-N2	1/50	Biolegend

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90 **Statistical Analysis**

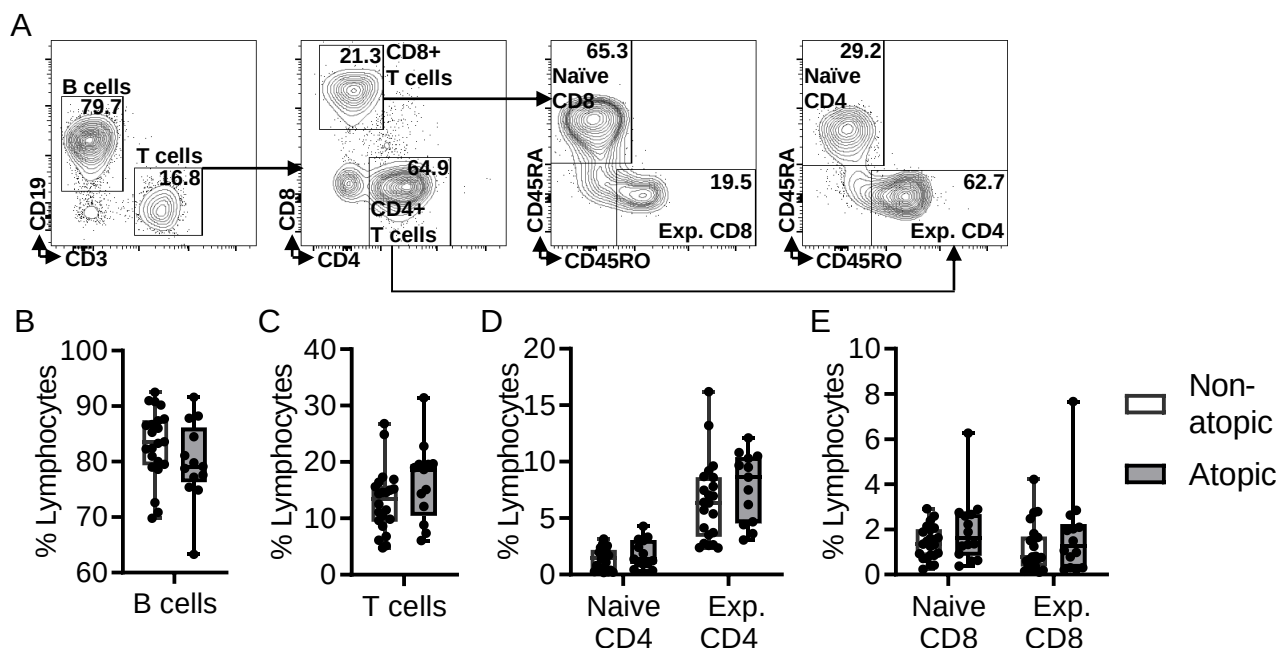
91 GraphPad 8 Prism (GraphPad Software Inc, La Jolla, CA) was used for data analysis. Two
 92 group non-parametric data was analyzed by Mann-Whitney test. Multiple group non-
 93 parametric data was analyzed with Kruskal-Wallis test and Dunn's multiple comparison test.
 94 For Figure 2C, the Wilcoxon-paired test was used. Data used in linear regression analysis
 95 was validated for normalcy with the Shapiro-Wilks test on scatterplot residuals.

96

Characteristic	Atopic (n = 13)	Non-Atopic (n = 23)	P value
Gender: Male:female	7:6	13:10	1
Age (years): Median (min-max)	5 (2-14)	4.5 (2-14)	0.7662
Tonsil grade (1-4): Mean	2.71	3.00	0.3309
Obstructive sleep apnea:	4/13	14/23	0.1642
Viral illness in past two weeks:	3/12	5/20	1
Food allergies:	4/12	3/18	0.3915
HDM sensitisation:	8/13	0/23	< 0.0001 ****
History of eczema:	5/12	6/19	0.7054
History of rhinitis:	4/12	4/19	0.6757
Pets at home:	3/12	4/19	1
Smoking relatives at home:	1/11	7/19	0.1082
Positive microbiota tonsil swab test	6/13	7/22	0.4741
Total serum IgE (IU/ml): Median (IQR)	63 (35-120)	17 (4-42)	0.0307 *
Sum of RAST: Median (IQR)	19.07 (0.78-27.48)	0.06 (0.01-0.08)	< 0.0001 ****
Weight of tonsil tissue (g): Median (IQR)	5.87 (4.11-6.98)	6.86 (5.31-8.67)	0.212

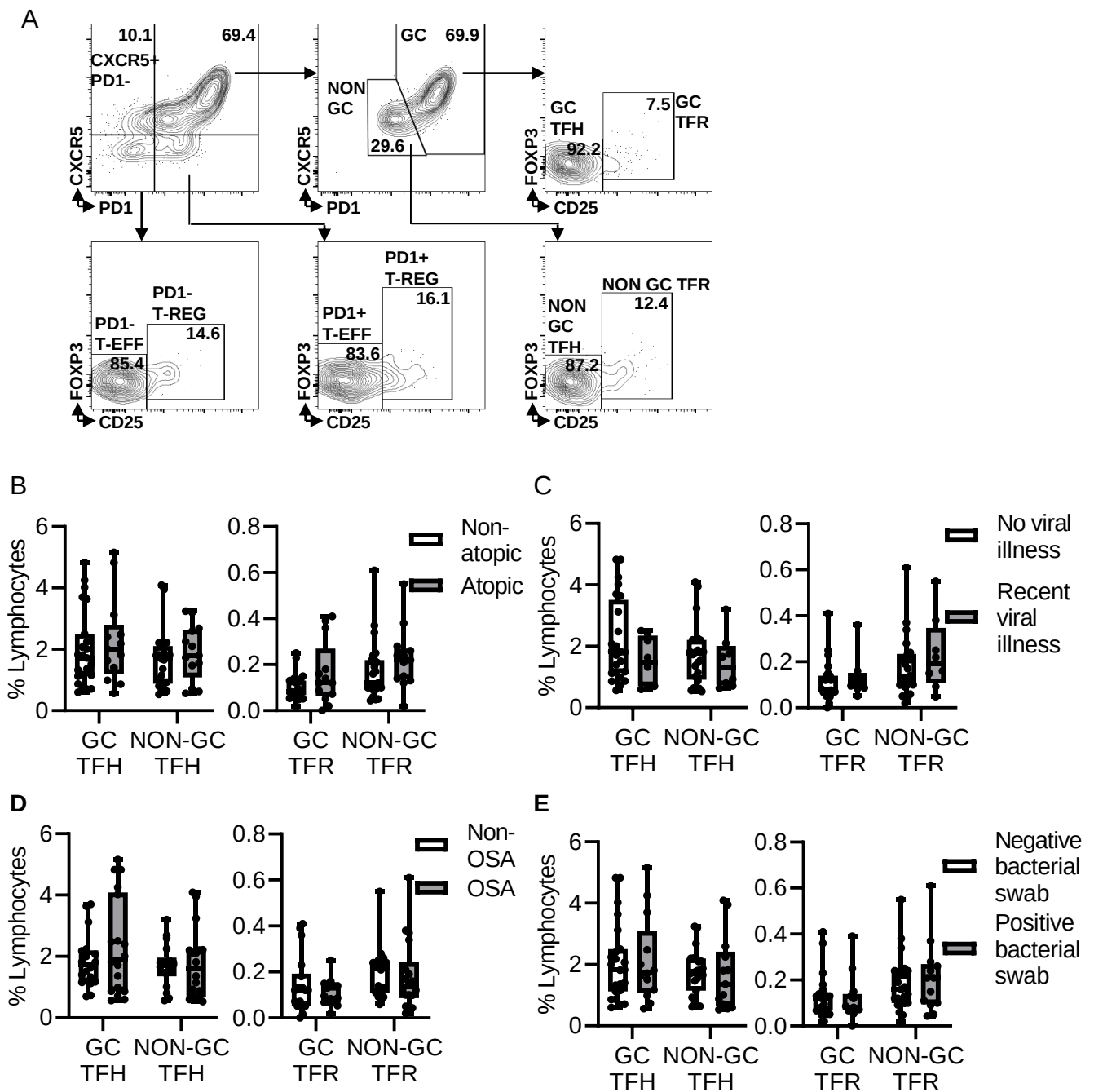
Extended Table 1 | Demographic characteristics of children undergoing tonsillectomy

Demographic data of patients undergoing tonsillectomy. Discrete data tested with Fishers exact test. Continuous data tested with Mann-Whitney test. Total n=36. For 5 of the patients, an incomplete medical history was available, and so have been excluded from the relevant table sections.



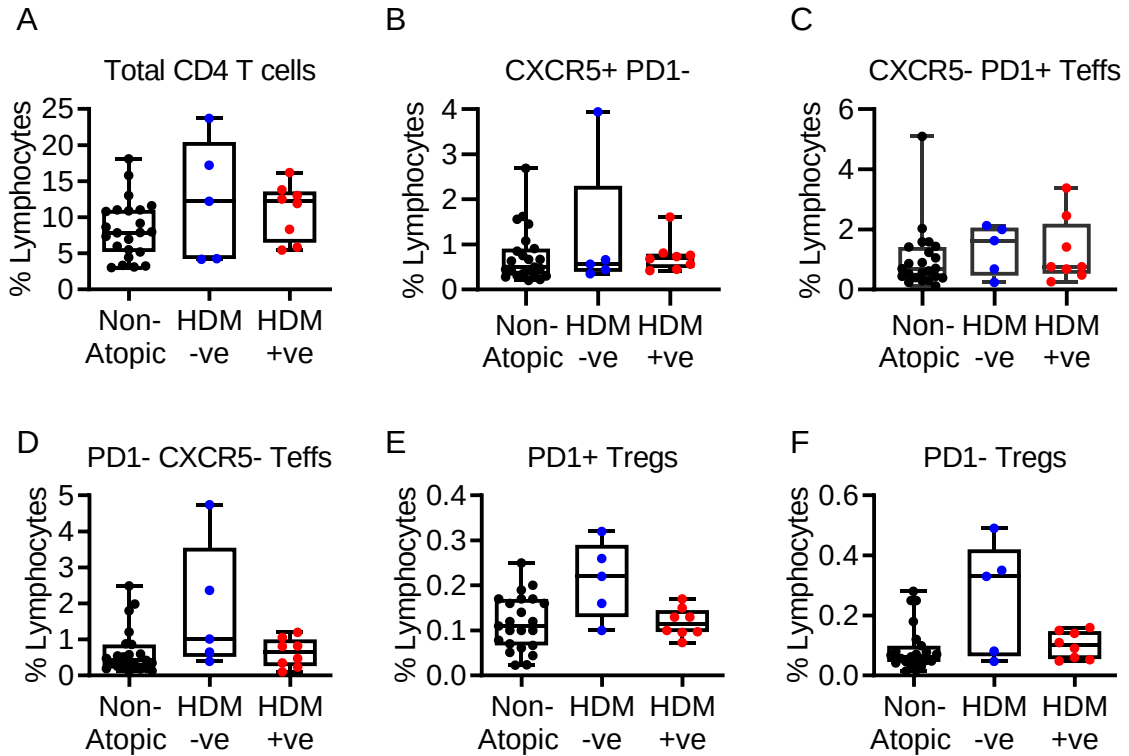
Extended Figure 1 | T cell composition is maintained in atopic tonsils

(A) Representative flow cytometry staining of Tonsil tissue stained for T pre-gated on live, single lymphocytes. Numbers in gates indicate percent cells in each gate. **(B)** B cell, **(C)** T cell **(D)** CD4 T cell subset and **(E)** CD8 T cell subset frequencies respectively, as a percentage of total lymphocytes analyzed. **(B-E)** Box plots depict median, IQR and range. Mann-Whitney statistical test was used. Empty box plots represent non-atopic patients, n=23. Filled box plots represent atopic patients, n=13.



Extended Figure 2 | T cell composition is maintained in OSA and infection

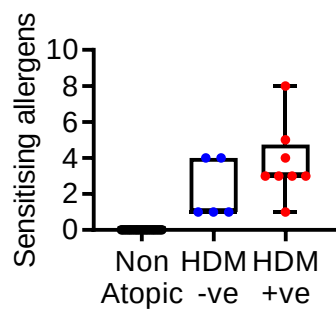
(A) Representative flow cytometry gating, pre-gated on Exp. CD4 T cells from Extended Figure 1. **(B-E)** TFH and TFR subsets, quantified using gating strategies and cell definitions from **(A)**, and quantified as proportion of total lymphocytes counted. for Atopy **(B)**, OSA diagnosis **(C)**, bacterial swab result **(D)**, and recent viral illness **(E)**. **(B-E)** Box plots depict median, IQR and range. Mann-Whitney statistical test was used. For **(B)** Non-Atopic n=23, Atopic n=13. For **(C)**, Non-OSA n=18, OSA n=18. For **(D)** -ve swab n=22, +ve swab n=13. For **(E)** -ve viral illness n=24, +ve viral illness n=8.



Extended Figure 3 | Non-GC T cell frequency is unchanged in HDM sensitised tonsils

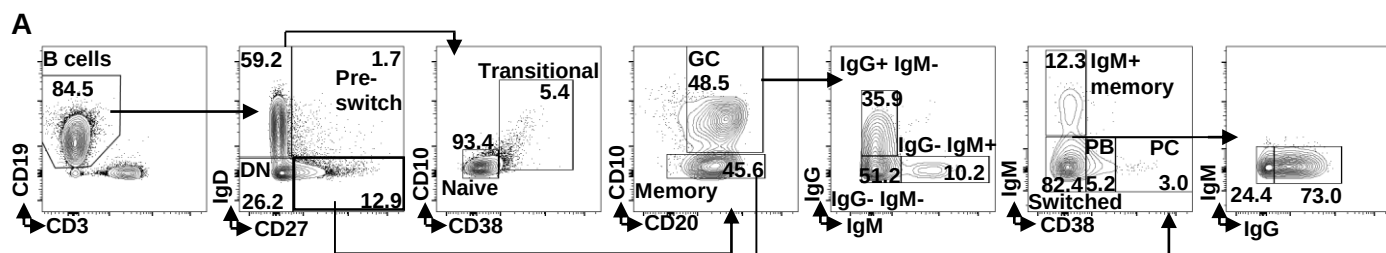
(A-F) show T cell subset frequencies, which were measured using gating strategies and cell definitions from Extended Figure 1, and quantified as proportion of total lymphocytes counted. (A) Total CD4 T cell frequency. (B) CXCR5+ PD1- subset frequency. (C) CXCR5- PD1+ T-effector frequency. (D) PD1- CXCR5- T-effector cell frequency. (E) PD1+ Treg frequency. (F) PD1- Treg frequency. (A-F) Error bars depict median, IQR and range. For C, Non-Atopic n=23, Atopic HDM -ve n = 5, Atopic HDM +ve n = 8.

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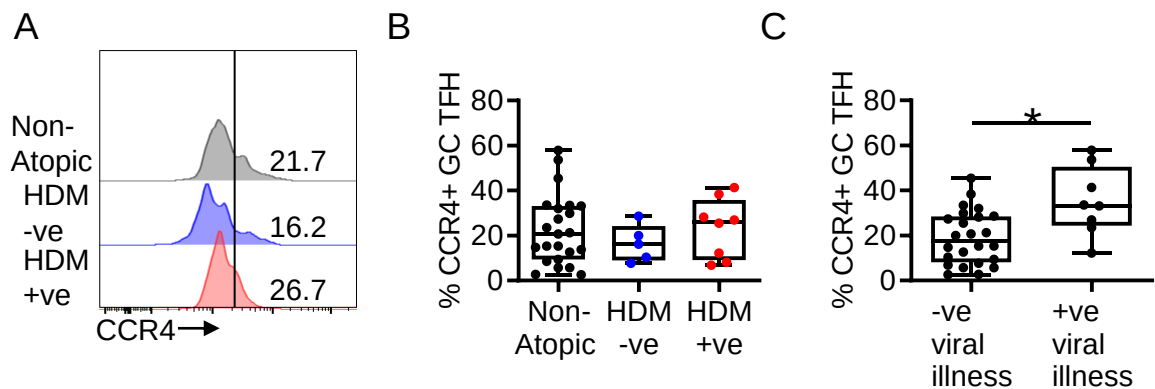
Extended Figure 4 | Extent of atopic-sensitisation with HDM sensitised patients

(A) Polysensitivity (no. of allergens above 0.35IU ImmunoCAP threshold) was quantified for each donor, and is shown versus their HDM-sensitive status. Non-Atopic n=23, Atopic HDM-ve n=5, Atopic HDM +ve n=8.



Extended Figure 5 | B cell subsets of the tonsil

(A) Representative flow cytometry staining of Tonsil tissue stained for B cell subsets, pre-gated on live, single lymphocytes. Numbers in gates indicate percent cells in each gate.



Extended Figure 6 | GC TFH CCR4 expression in children with recent viral illness, and unchanged in atopy

(A) Tonsillar TFH were identified by flow cytometry as in Figure E3A and stained for CCR4. **(B-C)** Tonsillar CCR4⁺ TFH were identified by flow cytometry and quantified against atopic status (HDM+ve/HDM-ve) (B) and recent viral illness symptoms (C), n=36.