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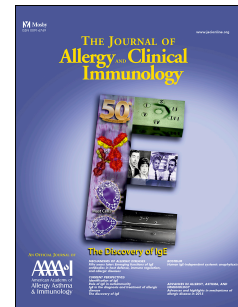
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Circulating allergen-specific Th2 lymphocytes: CCR4⁺ rather than CLA⁺ is the predominant phenotype in peanut allergic subjects

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Capsule summary

The skin and airway homing chemokine CCR4, but not CLA, emerged as the primary discriminating homing factor, of Th2 lymphocytes, suggesting that peanut sensitization occurs both via the skin and airways.

Key words

CD154, CLA, CCR4, Peanut-specific Th cells, Th2 cells

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25 **To the Editor:**

26 Early exposure to peanut through a dysfunctional skin barrier may result in sensitization to peanut
 27 while oral exposure is suggested to promote tolerance induction ⁽¹⁾. A study on circulating peanut-
 28 specific Th cells from peanut allergic (PA) subjects have supported this hypothesis by
 29 demonstrating stronger proliferation of skin homing cutaneous lymphocyte-associated antigen
 30 (CLA) expression compared to gut homing ($\alpha 4\beta 7$) Th cells ⁽²⁾. Moreover, high levels of the Th2
 31 subtype associated skin and lung homing C-C motif chemokine receptor 4 (CCR4) have been
 32 described on Ara h 1 specific Th cells of PA subjects ⁽³⁾. It is well recognized that Th2 cells
 33 dominate peanut-responsive Th cells in PA patients. To better understand the mechanism of food
 34 allergen sensitization and the importance of exposure route we aimed to investigate the gut and skin
 35 homing phenotype of circulating human peanut-specific Th cells of PA and non-allergic (NA)
 36 subjects.

37 PA subjects all had IgE to Ara h 2 and the median year of latest allergic episode to peanuts was 3
 38 (1-5 years). All PA subjects had concomitant atopic dermatitis (AD) and allergic rhino-
 39 conjunctivitis. Moreover, eight out of the nine PA subjects was diagnosed with asthma (Table E1 in
 40 Repository).

41 Circulating Th cells of PA and NA subjects showed similar expression of the investigated skin
 42 markers CLA, CCR4, and CCR10 as well as the gut homing antigen integrin $\alpha 4\beta 7$ ($\beta 7$) (see Fig E1,
 43 A and C and this article's Methods section in the Repository). Moreover, no differences were found
 44 in the frequency of circulating memory ($CD161^+$) Th cells and conventional ($CRTH2^+CD161^-$) ⁽⁴⁾ or
 45 pathogenic ($CRTH2^+CD161^+$) Th2 cell subpopulations in PA and NA subjects (Fig E1, D and E).

46 Peanut-responsive Th cells were identified by increased CD154 expression after short-term *ex-vivo*
 47 stimulation with whole peanut extract of PBMCs from PA and NA subjects. PA subjects showed
 48 stronger peanut-specific $CD154^+$ Th cell responses than NA subjects ($P = 0.0252$, mean frequencies

of 113 vs 35 per million Th cells and ratios compared to the unstimulated control of 15 vs 8, Fig 1, A and B). In line with our findings, one study⁽⁵⁾ reported comparable frequency of peanut-specific Th cells (100-200 per million Th cells) in PA subjects.

Peanut-responsive Th cells of PA subjects had increased expression of the Th2 cell type associated skin and airway homing chemokine receptor CCR4 compared to NA subjects ($P = 0.0042$, 24% vs 8%), but neither the signature skin homing antigen CLA (16% vs 14%) nor the other skin homing chemokine receptor CCR10 (13% vs 7%, Fig 1, D and E). In agreement with our data, another study characterizing Ara h 1-specific Th cells of PA subjects reported expression of CLA in ~10% of the cells⁽³⁾. Furthermore, no difference in expression of $\beta 7$ was found in the peanut-responsive Th cells of PA and NA subjects (2.5% vs 3.1%, Fig 1, D and E), an indication of non-gut priming. As expected, the largest subpopulation of the peanut-responsive Th cells of PA but not NA subjects was Th2 cells (CRTh2+) (44% vs 9%, Fig 1, E). Also, most CRTh2 positive peanut-specific Th cells of PA subjects co-expressed CD161, indicative of a pathogenic Th2 cell profile with production of IL-5 (Fig 1, F and G)⁽⁴⁾. Finally, peanut-responsive Th cells of PA but not of NA subjects was characterized by high expression of the newly described type 2 immune response associated receptor CD200R (median fluorescence intensity, 1602 vs 633, Fig 1, H and I)^(E1).

We next characterized the cytokine profile of the peanut-responsive Th cells (Fig E2, A). The peanut-specific Th cells of PA subjects had a larger fraction of conventional ($IL-4^+IL-5^-$, $P = 0.0002$, 20% vs 1.6%) and pathogenic ($IL-4^+IL-5^+$, $P = 0.0002$, 29% vs 1.5%) Th2 cells compared with the NA subjects (Fig E2, B and C). This is in agreement with a previous publication, reporting the two largest subpopulations of peanut-responsive Th2 cells as $IL-5^-Th2$ ($IL-4^+IL-5^-$) and $IL-5^+Th2$ ($IL-4^+IL-5^+$) cells in PA subjects⁽⁵⁾. Furthermore, the peanut-specific Th cells of PA subjects had a greater percentage of single positive $IL-5^+IL-4^-$ cells (9.6% vs 1.4%) as well as IL-31, another Th2 subtype associated cytokine (2% vs 0.6%) than NA subjects (Fig E2, B and C). In contrast,

compared to the PA subjects, the majority of peanut-reactive Th cells of NA subjects showed a Th1 profile with more IFN- γ positive cells (10% vs 45%, Fig E2, *B* and *C*).

Having established that the majority of the peanut-responsive Th cells of PA subjects were of a Th2 profile, we failed to find different expression of the canonical skin homing marker CLA in the PA compared to the NA subjects. Chan and co-workers⁽²⁾ showed that the isolated CLA⁺Th cell fraction of PA subjects primarily produced Th2 cytokines while a Th1 profile was the main population observed of isolated β 7 cells in peanut-tolerant subjects⁽²⁾. However, the same authors later reported in an elegant transcriptomic study⁽⁶⁾, of sorted CD69⁺ peanut-specific CLA⁺ and β 7⁺ Th cells, that Th2 and Th9 associated cytokines were equally expressed in the CLA⁺ and β 7⁺ homing Th cells. By analyzing the skin and gut homing potential of the largest cytokine cell populations of IL-4⁺IL-5⁻, IL-4⁺IL-5⁺ and IFN- γ ⁺ peanut-specific Th cells of PA subjects; unbiased grouping analysis using t-SNE revealed an association of CCR4 but not CLA to peanut-specific Th2 cells in the PA subjects (Fig 2, *A*). Interestingly, even though all PA subjects had AD, the main subpopulation of peanut-specific pathogenic (IL-4⁺IL-5⁺) Th2 cells lacked CLA (<1%) and weakly (11%) co-expressed CCR4 (Fig 2, *B-D*). These findings were unexpected, since the main (>90%) skin-homing T cell population in subjects with AD is characterized by CLA-expression, a surrogate marker for cutaneous T cells⁽⁷⁾. More studies are needed to verify whether the phenotype of circulating peanut-specific Th cells mirrors peanut-specific Th cells located in the airway and skin. Furthermore, CCR4 but not CLA was in particular expressed by the conventional IL-4⁺IL-5⁻ Th2 cells, with 21% expressing CCR4 (CCR4⁺CLA⁻) in the PA subjects (Fig 2, *B-D*). However, it would be interesting to study PA subjects without asthma to elucidate whether the CCR4 expression by the conventional Th2 cells is asthma related. On an allergen-specific level, we confirm the link between CCR4 expression of the IL-4⁺IL-5⁻ Th cells, but not the IL-4⁺IL-5⁺ Th cells in agreement with another study⁽⁸⁾. The IL-4 single positive CCR4 expressing Th cells have been suggested to

represent a less differentiated Th2 cell type seen in allergic subjects with none or few re-exposures to the allergen and it is thought to drive IgE allergic inflammation⁽⁸⁾. If correct, the IL-4 single positive CCR4 expressing cells should represent the newly primed peanut-specific Th2 cells, and therefore it is interesting that most of these cells lacked co-expression of the signature skin homing antigen CLA. As CCR4 expression has been associated with homing to the airways⁽⁹⁾, this could indicate that the IL-4 single positive peanut-specific Th2 cells could originate from the airways. In support of the hypothesis of airway priming of the conventional peanut-responsive Th cells in PA subjects, the less frequent Bet v 1-specific Th cells of birch sensitized subjects, also being PA, showed a conventional Th2 phenotype with comparable expression of CCR4 (Fig E3, A-D).

On the level of allergen-specific T cells, this is the first investigation to break down the skin homing potential of the Th2 cytokine producing cells in PA patients. Using the tissue homing potential of the circulating pool of peanut-responsive Th2 cells from PA subjects as a surrogate of the tissue located Th cells, we could find indications that peanut sensitization might occur through both the skin and the airways.

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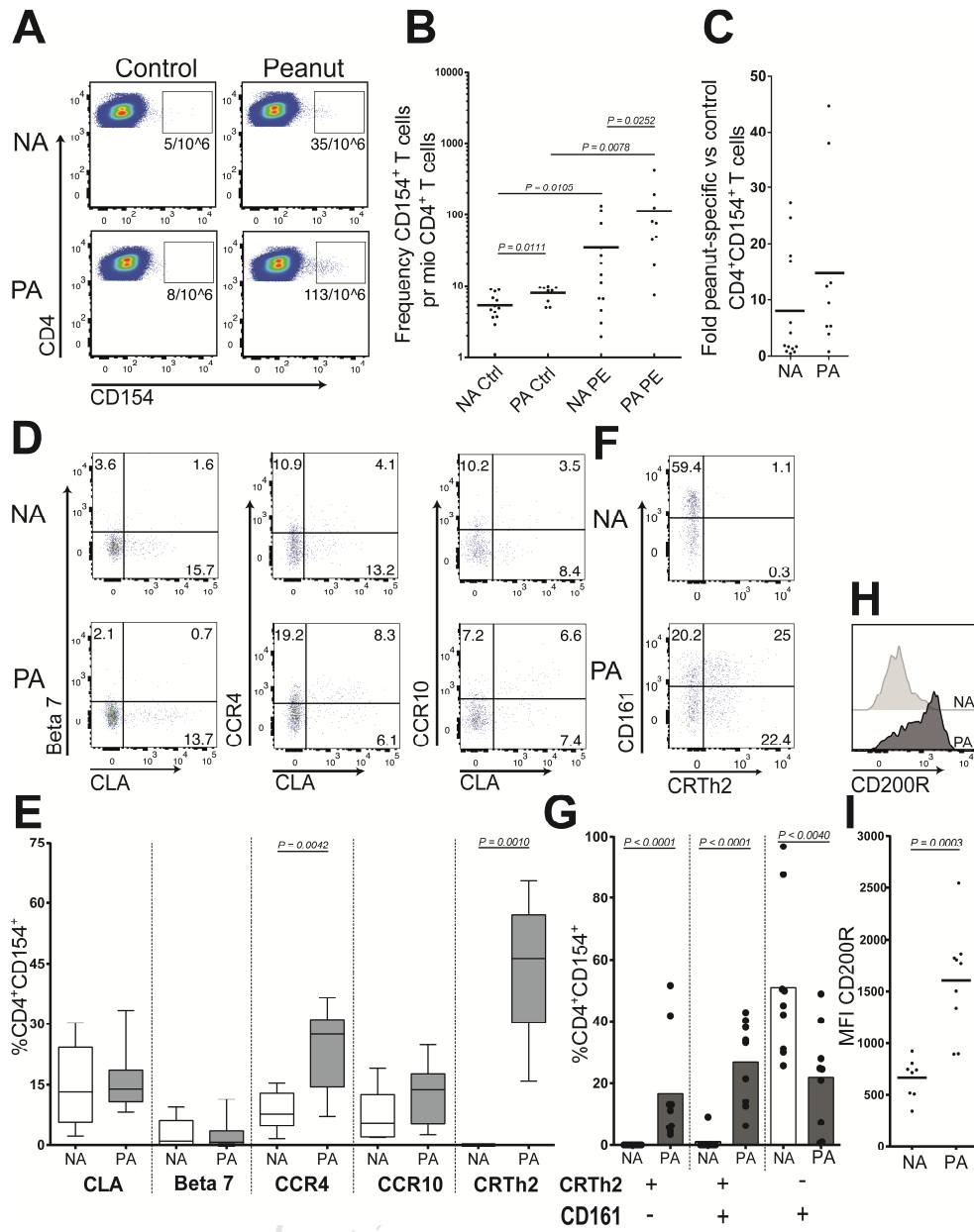
132 **Figure Legends**

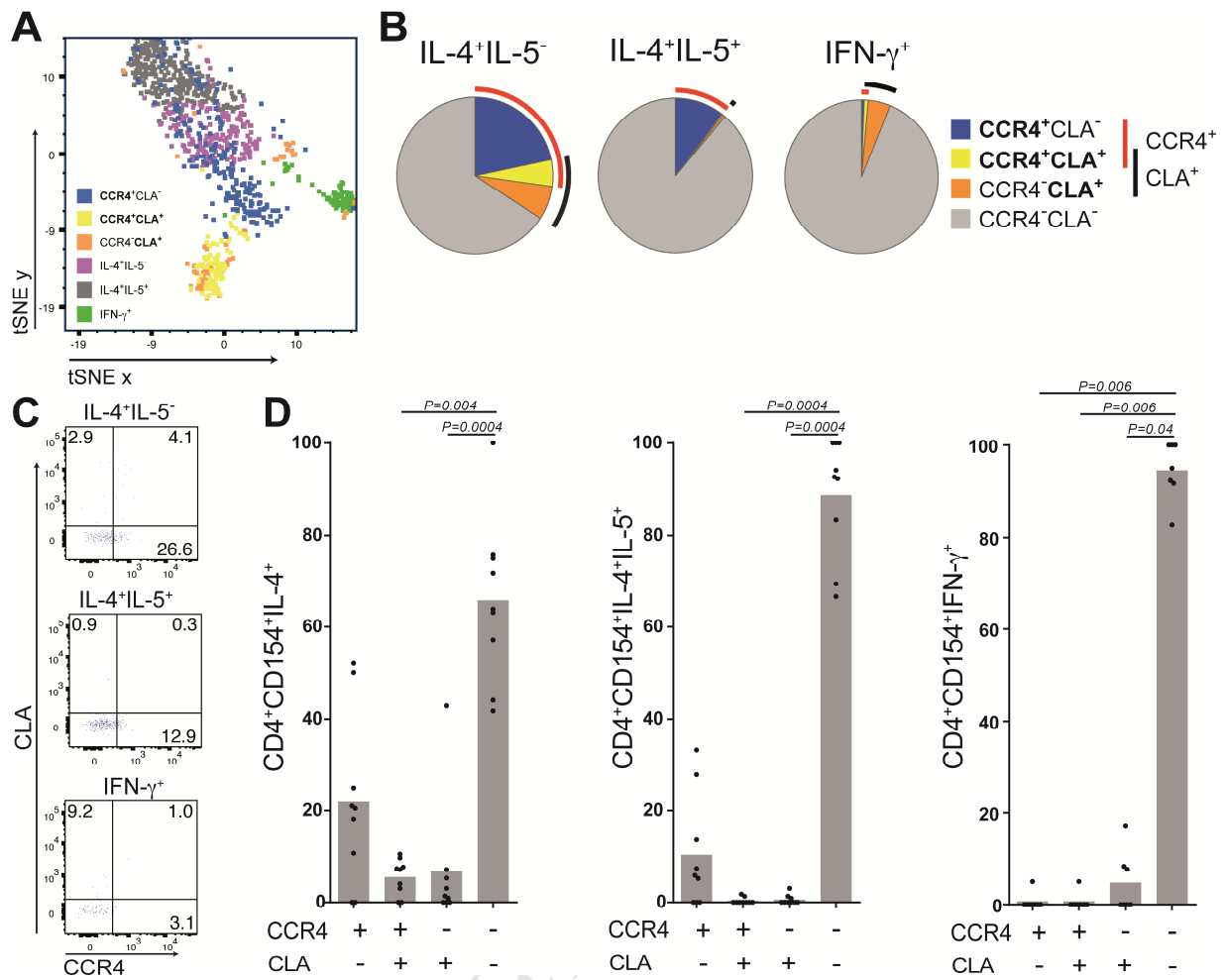
133 **Fig 1. Peanut-specific Th cells from peanut-allergic subjects are characterized by expression of CCR4,**
 134 **CRTh2, and CD200R but not CLA.** Peanut-specific CD154⁺Th cells from PA (n = 9) and NA (A-C) (n =
 135 13) (D-I) (n = 9) subjects. A-C) Representative dot plots (A), frequencies (B) and ratios compared to the
 136 unstimulated control (C) of CD154⁺Th cells. D-I) Representative merged dot-plots (D, F), histogram (H) and
 137 percentage (E, G) of peanut-specific Th cells expression of CLA and β7, CCR4 or CCR10 (D, E) as well as
 138 CRTh2 and CD161(F, G) or CD200R (H-I). Significant differences were identified using the Mann-Whitney
 139 U test.

141 **Fig 2. Pathogenic peanut-specific Th2 cells of peanut allergic subjects lack the canonical skin homing**
 142 **antigen CLA.** Peanut-specific CD154⁺Th cells from PA (n = 9) subjects. A) t-SNE plot of CCR4⁺CLA⁻,
 143 CCR4⁺CLA⁺, CCR4⁻CLA⁺, IL-4, IL-4⁺IL-5⁺, and IFN-γ grouping in the two-dimensional t-SNE
 144 representation. B) Pie charts demonstrate the average percentage of CCR4⁺CLA⁻, CCR4⁺CLA⁺, CCR4⁻
 145 CLA⁺, and CCR4⁻CLA⁻ in the peanut-specific Th2 cells positive for IL-4⁺, IL-4⁺IL-5⁺, and IFN-γ⁺. C, D)
 146 Representative merged dot-plots (C) and percentage (D) of CLA and CCR4 in IL-4⁺, IL-4⁺IL-5⁺, and IFN-γ⁺
 147 cell populations. Significant differences were identified using the Mann-Whitney U test.

Demographic features	Non allergic (n = 13)	Peanut allergic (n = 9)	P value
Age (years)	25 (20-32)	20 (19-27)	0.06
Female sex. no. (%)	9 (69)	8 (88)	0.3602
Latest allergic episode	0 (0)	3 (1-5 years)	
Total IgE (kU _A /L)	40.2 (15 – 127.2)	339 (228-2875)	0.0011
sIgE to Peanut - whole nut extract, no.	<0.35, 0	56.7 (19.7-88.9), 9	<0.0001
sIgE to Ara h 2 (peanut), no	<0.35, 0	19.5 (17.65-34.3), 9	<0.0001
sIgE to grass pollen, no.	<0.35, 0	1.6 (<0.35-29.5), 5	0.0048
sIgE to birch pollen, no.	<0.35, 0	1.36 (<0.35-71.2), 8	0.0002
Other plant food allergies. no. (%)	0 (0)	7 (78)*	0.0084
Atopic dermatitis no. (%)	0 (0)	9 (100)	<0.0001
Asthma no. (%)	0 (0)	8 (88)	<0.0001
Rhino-conjunctivitis no. (%)	0 (0)	9 (100)	<0.0001

Table E1. Values are shown as numbers (percentages) or medians (range). Bold indicate statistically significant differences. * Unknown in the remaining two cases (avoids other nuts). IgE < 0.35 = 0.34 in statistical testing (range = minimum and maximum value). Fisher's exact test or Mann-Whitney *U* test were used for determining statistical significance.





Repository

Repository methods

Subjects

Nine PA subjects and thirteen NA subjects were recruited as previously described^(E1). The study was approved by the committees on health research ethics in the capital region of Denmark (H-15001321). PA and NA subjects with detectable peanut-specific CD154⁺ T cells were also included in another study^(E1). Specific IgE towards common inhalation allergens, whole peanut, and Ara h 2 were analysed by the Phadia ImmunoCAP immuno- enzymatic assay (ThermoFisher Scientific, Uppsala, Sweden). Peanut allergy was diagnosed by oral food challenge and/or by convincing medical history of an immediate and severe allergic reaction with unambiguous objective symptoms upon peanut ingestion.

Cell cultures

PBMCs were purified as previously reported^(E2). For detection of CD154⁺ allergen-specific Th cells, 45×10^6 PBMCs ($5-7.5 \times 10^6$ cells/mL) were stimulated with or without 100 µg/mL peanut extract or 5 µg/mL rBet v 1a (Biomay, Vienna, Austria) for 14-16 h. All cells were cultured in RPMI 1640 supplemented with penicillin (100 U/mL), streptavidin (100 µg/mL), L-glutamine (1 mM), β-mercaptoethanol (50 mM) (all from Sigma), and 5% heat-inactivated sterile filtered human AB serum (Invitrogen).

Flow cytometry

Prior harvest, the cultures were incubated with Brefeldin A (10 µg/mL) over the whole stimulation period. Post-harvest, all cells were stained with a viability dye (Fixable Viability Dye eFluor® 780, ebiosciences) for 20 min at 4°C. Phenotypic staining was for 20 min at 4°C with or without BV421–conjugated anti-CRTh2 (BM16, BD), PerCP-Cy5.5–conjugated anti-CCR10 (1B5), PE-

Cy7–conjugated anti-CCR4 (1G1), and BV711–conjugated anti-CD161 (DX12) (all from BD), PerCP-Cy5.5–conjugated anti-CD200R (OX108, eBiosciences). The residual erythrocytes were lysed with PharmLyse (BD) for 5 min at room temperature prior to permeabilization. Cells were then treated with IntraPrep (Beckman Coulter, Fullerton, USA) and intracellularly stained for 20 min at room temperature with or without BV650–conjugated anti-Integrin β 7 (FIB504), BV510–conjugated anti-CD4 (SK3), BV786–conjugated anti-IL-17A (N49-653), PE–conjugated anti-IL-5 (JES1-39D10), FITC–conjugated anti-CD154 (TRAP1), BV650–conjugated anti-CD3 (UCHT1), PE-CF594–conjugated anti-IL-10 (JES3-19F1), and Alexa Fluor 700–conjugated anti-IFN- γ (B27) (all from BD Biosciences), BV605–conjugated anti-IL-4 (MP4-25D2, Biolegend, San Diego, USA), eFluor660–conjugated anti-IL-31 (31SNEZE), and PE-Cy7–conjugated anti-IL-22 (22URTI) (all from eBioscience). APC-eFluor780–conjugated anti-CD14 (61D3), and APC-eFluor780–conjugated anti-CD19 (HIB19) (both from eBioscience) were used as dump channel reagents in the detection of CD154⁺ Th cells. Data acquisition was performed with a Fortessa flow cytometer (BD). A total of $5-9 \times 10^6$ events were collected for each of three antibody panels in the characterization of CD154⁺ T cells. Data were analyzed with FlowJo 10.1 software (TreeStar, Ashland, USA). Merged representative files of CD154⁺ positive Th cells were created by down-sampled (Flowjo) to 100 events of CD154⁺ positive cells (Flowjo). Down-sampled data files samples of peanut-specific cells were merged in one file of PA or NA subjects (Flowjo). The t-SNE analysis was run on the merged files with the parameters: CLA, CCR4, B7, IL-4, IL-5, IL-10, IL-17A, IL-31, and IFN- γ (FlowJo). Analysis and presentation of distributions were performed using SPICE version 5.1^(E3).

Peanut extract

Peanut extract was prepared as previously described ^(E1). LPS levels were assessed by the Limulus amoebocyte lysate (LAL) assay (Lonza, Basel, Switzerland) and the peanut extract exhibited levels <0.001 endotoxin units/mL in culture.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6.0 (GraphPad Software, La Jolla, USA). Mann-Whitney U test, Kruskal-Wallis test with Dunn's correction, Fisher's exact test or 2-way Wilcoxon matched-pair signed rank test were used for determining statistical significance. For box-and-whisker plots, midline indicates the median; upper and lower edges of the box plot are the 75th and 25th percentiles; whiskers are min and max values. Bar graphs are represented as means. The horizontal bar represents group means.

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Repository figure legends

Fig E1. PBMCs from PA (n = 9) and NA (n = 13) subjects. **A)** Gating strategy for Th cells: after exclusion of doublets, dead and non T cells, live CD3⁺CD4⁺ cells were selected. **B-E)** Representative merged dot-plots (**B, D**) and percentage (**C, E**) of Th cells expression of (**B, C**) CLA and β 7, CCR4 or CCR10 as well as (**D, E**) CRTh2 and CD161. Significant differences were identified using the Mann-Whitney U test. For box-and-whisker plots, midline indicates the median; upper and lower edges of the box plot are the 75th and 25th percentiles; whiskers designate min and max values. Bars represent means.

Fig E2. Peanut-specific CD4⁺CD154⁺ T cells have a Th2 dominated phenotype with production of IL-4 and IL-5. Peanut-specific Th cells were identified, from NA (n = 9) and PA (n = 9) subjects, by expression of CD154 after 14-16 h of ex-vivo stimulation with and without peanut extract. **A)** Pie charts demonstrate the relative percentage of the Th2 related cell subsets; conventional and pathogenic Th2 cells other cytokine expressions are indicated independently of IL-4 and IL-5 status in the peanut-responsive Th cells of NA and PA subjects. **B)** Representative merged dot-plots of peanut-specific Th cells expression of IL-4, IL-5, IL-31, IL-17A, IL-22, IFN- γ , and IL-10 in NA and PA subjects. **C)** Percentage peanut-specific Th cells that express combinations of IL-4, IL-5, IL-31, IL-17A, IL-22, IFN- γ , and IL-10 in NA and PA subjects. Significant differences were identified using the Mann-Whitney U test. Bars represent means.

Fig E3. Bet v 1-responsive CD4⁺CD154⁺ T cells of birch sIgE positive subjects show a conventional Th2 phenotype with homing to the airways. Peanut-specific Th cells from NA (n = 9) and PA (n = 9) subjects and Bet v 1-specific Th cells from birch sIgE negative ($kU_A/L < 0.35$, n = 15, 2 of which were PA) and positive ($kU_A/L > 0.35$, n = 8 (A), n = 7 (B-D) all of which were PA) subjects were identified by expression of CD154⁺ after 14-16 h of ex-vivo stimulation with and without PE or Bet v 1. **A)** Frequencies of CD154⁺Th cells in unstimulated control and Bet v 1 conditions. **B)** Representative merged dot-plots of the largest cytokine populations of Bet v 1-specific Th cells of birch sIgE⁺ subjects. **C)** Percentages peanut- and Bet v 1-specific Th cells expressing IL-4, IL-5, IL-31, IL-17A and IFN- γ in of NA, PA and sIgE positive subjects. **D)** Representative merged dot-plots of CLA, $\beta 7$, CCR4, CCR10, CRTh2 and CD161 in Bet v 1-specific Th cells of birch sIgE⁺ subjects. **E)** Percentages peanut- and Bet v 1-specific Th cells expressing CLA, $\beta 7$, CCR4, CCR10 and CRTh2 in NA, PA and sIgE positive subjects. Significant differences were identified using the Kruskal-Wallis test with Dunn's correction. For box-and-

whisker plots, midline indicates the median; upper and lower edges of the box plot are the 75th and 25th percentiles; whiskers designate min and max values. Bars represent means.

