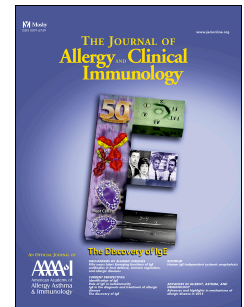


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Natural protective immunity against grass pollen allergy is maintained by a diverse spectrum of response types

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Supplementary Material

Material & Methods

Ethics Statement

Experiments involving human material were conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. Samples from human origin have been obtained from blood donations and use of residual cells was approved by the Institutional Review Board of Upper Austria (EK Nr.E-6-10 2.1.5.). All participants provided informed written consent. After the blood samples had been collected, each participant was allocated a study number, demographic data was collected, and all data was anonymized in a database.

Study Participants

The Red Cross Blood Transfusion Service Center Linz (Upper Austria, Austria) recruited a total of 228 non-allergic healthy adult individuals during grass pollen season (May-June 2012) and screened for individuals that live within a farming or urban environment. Non-allergic healthy donors reported no history of any atopic diseases or allergic symptoms and had no detectable IgE titers against Phl p 5. Donors from a farming environment had to answer a questionnaire regarding their working and living conditions on the farm. As control group, 11 allergic adult individuals, with symptoms or clinical history of allergic diseases and positive IgE titers against Phl p 5, were recruited during grass pollen season (June 2015). The demographic and lifestyle characteristics are reported in supplementary Table E1.

PBMC and plasma samples

Human peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn peripheral venous blood by density gradient centrifugation. Additionally, plasma samples from all donors were collected. PBMC and plasma samples were stored in aliquots in liquid nitrogen until analyzed.

Allergen

Recombinant Phl p 5 (rPhl p 5) was expressed in *E. coli* as previously described¹. Bacterial endotoxin was detected using the Limulus Amebocyte Lysate assay. LPS levels were below 1.5 pg/μg.

Luminometric ELISA

Phl p 5-specific plasma IgA, IgE, IgG1, and IgG4 were determined by enzyme linked immunosorbent assay (ELISA). High-binding half-area microtiter plates were coated with rPhl p 5 (1 µg/mL in DPBS) over night at 4°C and then blocked with bovine serum albumin (BSA) at 3% in DPBS for 1 hour at room temperature. Samples and reference sera were diluted and incubated for 2 hours at room temperature. Subsequently, plates were incubated with horseradish peroxidase (HRP) conjugated secondary anti-human IgE (Southern Biotech), anti-human IgA (BIO-RAD), anti-human IgG1 (Life technologies) or anti-human IgG4 (Life technologies) (all 1:500) antibodies for 1 hour at room temperature. The assay was developed by adding Luminol (BM Chemiluminescence ELISA substrate, Roche Diagnostics). Plates were measured on a Tecan Infinite 200 Pro microplate reader. All ELISA experiments were performed in triplicates. Reference curve was constructed from a pool of sera containing high amounts of IgA, IgE, IgG1, and IgG4 to Phl p 5 and units (corresponding to the specific titers of the reference serum pool) were calculated using reference line method as described elsewhere². Subjects were divided into "responder" (IgG > cut-off) and "non-responder" (IgG < cut-off) based on the calculated cut-off ($2 \times [\text{mean of blanks} + (2 \times \text{standard deviation of blanks})]$).

In vitro Expansion of Phl p 5-specific T Cells

Frozen PBMCs were thawed and resuspended in X-VIVOTM 15 medium (Lonza) supplemented with 5% heat-inactivated male human AB serum (Sigma-Aldrich), 100 U/mL penicillin and 0.1 mg/mL streptomycin (complete X-VIVOTM 15) at a concentration of 1×10^7 cells/mL in 6-well plates and stimulated with rPhl p 5 (30 µg/mL). Cells were kept at 37 °C in 5% CO₂ and 10 U/mL human rIL-2 (Peprotech) was added 6 days after culture setup. On day 12, cells were restimulated with rPhl p 5 (30 µg/mL) in the presence of autologous irradiated (60 Gy, 80kV, 26mA) PBMCs (feeder cells) and rIL-2 (10U/mL) for another 3 days. Finally, the non-adherent cells were harvested, washed, and cultured at a concentration of 1×10^7 cells/mL in 6-well plates in complete X-VIVOTM 15 and incubated for a resting phase of 13 days in order to enrich allergen-specific memory T cells.

Cytokine Production

The production of IFN-γ, IL-10, and IL-4 from cultured cells in response to antigen stimulation was detected by ELISPOT technique. 96-well-PVDF bottom plates (Millipore) were activated for 10 minutes using 70% ethanol, washed and coated with 4 µg/mL anti-human IFN-γ (clone NIB42, eBioscience), anti-human IL-10 (clone JES3-9D7, BioLegend) or anti-human IL-4 (clone 8D4-8, BioLegend) over night at 4°C. Then, the plates were incubated for 1 hour at 37°C with blocking solution (X-VIVOTM 15 medium, 10% human serum) and washed afterwards with DPBS. After the

resting phase, cells were harvested and 1×10^5 cells per well were cultured in the presence of 2×10^5 irradiated (60 Gy, 80kV, 26mA) autologous PBMCs with or without 30 $\mu\text{g}/\text{mL}$ rPhl p 5. After culture for 48 hours at 37°C in 5% CO_2 , cells were removed by extensive washing and cytokines were detected using a biotinylated anti-human IFN- γ (clone 4S.B3, BioLegend), anti-human IL-10 (clone JES3-12G8, eBioscience) or anti-human IL-4 (clone MP4-25D2, BioLegend), followed by HRP-conjugated streptavidin (BioLegend) and enzyme reaction with 3-amino-9-ethylcarbazole substrate. The reaction was stopped after 1 hour by washing with water, and plates were air-dried before spot counting using ImageJ software (Wayne Rasband, National Institutes of Health). Spot numbers in unstimulated wells (medium only) were subtracted from those obtained in stimulated wells (protein) and Spot-Forming Units (SFU) were calculated per 10^5 cells. According to their cytokine pattern subjects were attributed to TH1 (IFN- γ > IL-10 and IL-4), TH2 (IL-4 > IFN- γ and IL-10), Tr1 (IL-10 > IFN- γ and IL-4) and non-polarized (< 10 spots for all 3 cytokines) immune response types. Additionally, cytokines were measured in supernatants from identically prepared co-cultures after 72 hours incubation using a human cytokine 10-plex panel (Procarta Plex, eBioscience) (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IFN- γ , TNF- α , GM-CSF) or a quantitative luminometric ELISA (Human ELISA Ready-SET-GO!, eBioscience) (IL-2, IL-21, IL-22), according to the manufacturer's protocol. The 5 parameter logarithmic curve fit (5PL) was used to create the standard curves using Procarta Plex Analyst (eBioscience). The lower Limit of Quantitation (LLOQ) was 3.9 pg/mL for IL-2, 11.7 pg/mL for IL-4, 9.4 pg/mL for IL-5, 9.9 pg/mL for IL-6, 7.2 pg/mL for IL-9, 1.9 pg/mL for IL-10, 11.4 pg/mL for IL-13, 8.3 pg/mL for IL-17A, 15.6 pg/mL for IL-21 and IL-22, 13.8 pg/mL for IFN- γ , 32.1 pg/mL for TNF- α , and 57.2 pg/mL for GM-CSF. Values from unstimulated cultures (medium only) were subtracted from stimulated cultures (protein) and concentrations are shown as pg/mL. Supernatants for the assays were stored in aliquots at -70°C until analyzed. Samples were analyzed on a Luminex MAGPIX instrument or a Tecan Infinite 200 Pro microplate reader.

Flow cytometric analysis

After the resting phase, cells were harvested and washed with DPBS and surface staining was performed by incubation for 30 minutes with various combinations of anti-human CD3-APC (OKT3, eBioscience), anti-human CD4-BV510TM (OKT4, BioLegend), anti-human CD62L-PerCp-eFluor[®]710 (DREG-56, eBioscience), anti-human CD45RO-FITC (UCHL1, eBioscience) and viability Dye-eFluor[®] 780 (eBioscience) at 4°C . Next, cells were washed in FACS Buffer (DPBS, 0.5% BSA, 2mM EDTA), fixed and permeabilized using FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Intracellular staining was performed for 30 minutes with various combinations of anti-human T-bet-BV605TM (4B10, BioLegend), anti-human GATA3-BV421TM (16E10A23, BioLegend), anti-human FoxP3-PerCP-

Cy5.5 (PCH101, eBioscience), anti-human ROR- γ -PE (AFKJS-9, eBioscience), and anti-human Ki-67-PE-Cyanine7 (20Raj1, eBioscience). Isotype controls were performed and dead cells and doublets were excluded. Measurements were performed using a FACS Canto II flow cytometer or a FACS Aria III cell sorter (BD Biosciences) and analyzed using FACS Diva software. Stimulation indices were calculated as the ratio of percentage of Ki67⁺CD3⁺CD4⁺ cells under stimulating conditions (protein) to non-stimulating conditions (medium only).

T cell receptor repertoire analysis

Clonally expanded T cells were evaluated regarding their T cell receptor diversity by using next-generation sequencing. Cells before expansion, after expansion, and after the resting phase were stained with anti-human CD3-APC (OKT3, eBioscience), anti-human CD4-BV510TM (OKT4, BioLegend), and viability Dye-eFluor[®] 780 (eBioscience). Dead cells and doublets were excluded and 10⁶ CD3⁺CD4⁺ T cells were sorted using a FACS Aria III cell sorter. Afterwards, DNA was extracted using a genomic DNA SV kit (Promega), concentration was determined by UV absorption at 280 nm using a NanoDrop photometer, and integrity was estimated by agarose gel electrophoresis. The gDNA was amplified, sequenced and analyzed as previously described³. Briefly, we used a two-step multiplex PCR amplification of the rearranged T-cell receptor beta chain based on Biomed-2 primer panels. Final PCR products were purified using Agencourt AMPureXP beads (Beckman Coulter, Brea, CA) and subsequently sequenced using an Illumina Miseq (Illumina, San Diego, CA). Analysis of Illumina sequencing data was done by several sequential steps including filtering, trimming, sequence alignment, quantification of clonotypes, and diversity calculation⁴.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.01 software for Windows. Statistically significant differences in response type distributions (Fig. 1A and Fig 2C) were determined by chi-square test. Differences between IgG1 and IgG4 within a group were determined using paired Student's t test. Comparisons between multiple groups for a specific subclass were done by one-way ANOVA followed by Tukey's *post hoc* test (Fig. 1B). Statistical analysis of subclass distribution was performed using the Kruskal-Wallis nonparametric ANOVA test with *post hoc* analysis using Dunn's multiple comparison test (Fig. 1C). Statistically significant differences between the number of IFN- γ and IL-10 secreting cells were determined using paired Student's t test and comparison of the numbers of IL-4 secreting cells between the individual groups was done by one-way ANOVA followed by Tukey's *post hoc* test (Fig. 2A). Comparison of transcription factor

expression was done by unpaired (farmers vs. townspeople) or paired (comparison within one group) T-test (Fig. 2B). Statistically significant differences in cytokine secretion were determined using One-way ANOVA followed by Tukey's *post hoc* test (Fig. 2D-G). A P value <0.05 was considered statistically significant. Correlation matrices were computed using R version 3.2.1⁵. The correlations of antigen-specific antibodies and cytokines (ELISPOT and culture supernatants) were calculated for townspeople and farmers separately, using the pairwise.complete.obs option to handle missing values without discarding the whole sample⁶. Spearman's rho correlation coefficient was used and significance was determined by computing the P value of correlations using a confidence interval of 95%.

Supplementary Discussion

It is meanwhile well accepted that exposure to a farming environment in early childhood prevents from allergic diseases, and this effect is even enhanced if the mother is exposed during pregnancy^{7,8}. Representative molecules of this specific environment are bacterial compounds such as endotoxin or muramic acid, which have been proven to protect from allergy and asthma after prolonged exposure^{9,10}. Environmental microbes are not only found on farms but also in urban environments. Although we did not assess pet ownership or other microbial sources in townspeople, it is well established that the microbial diversity and also the overall endotoxin exposure is much lower in the city^{11,12}.

While townspeople are seasonally exposed to relatively low levels of pollen in the context of a low microbial load with limited diversity, the farmers in our study live in a microbially rich and diverse environment and are in contact with grass pollen allergens throughout the year. It has to be emphasized that the farming group in our study was comprised of selected individuals committed to a traditional type of farming on very small farms (closely resembling the farming style of the Amish people¹³). Due to their daily work, these farmers are perennially exposed to grass pollen and in daily close contact with farming animals and thus exposed to a vast microbial diversity originating from animal dander, dung, as well as soil.

Despite higher and perennial antigen exposure, farmers display a significantly higher proportion of IgG non-responders and a reduced percentage of IgG4 reactive individuals compared to townspeople (Fig.1A). This indicates that the farming environment triggers downregulation of IgG antibody responses.

19 % of townspeople (but only 2% of farmers) mounted a non-allergic IgG4 antibody response, with 3% of them exclusively producing IgG4. IgG4 in the absence of IgE antibodies has been termed a

“modified” Th2 immune response, and has been found in non-allergic animal owners and beekeepers¹⁴⁻¹⁷. The class switch to IgG4 is dependent on a combined TH2/Treg response type, but independent of IFN- γ and IL-6 (which downregulate IgG1 production)^{18,19}. Indeed, we have found more IL-4, IL-13, and IL-10 high responders, and higher levels of IFN- γ in townspeople (Fig. 2D,E and G), which might explain the observed bias towards IgG4 seroconversion. Season-independent allergen-specific IgG4 antibodies have been reported for the allergens PLA2 and Fel d 1^{20,21}, but these studies did not address the influence of the environment.

Maturation of allergen-specific antibody responses including the IgG, IgA and IgE subclasses implies the interaction with T helper cells, and allergen-specific T cell responses are commonly observed not only in allergic, but also in healthy individuals. Induction of IL-10-secreting Tr1 cells in the course of allergen immunotherapy^{15,22} and detection of IL-10-secreting regulatory T cells in non-atopics²²⁻²⁴ suggested “tolerance” as the general dominant mechanism underlying a healthy non-allergic status²⁵. However, in our current study we could not detect increased numbers of IL-10-secreting cells in non-allergic humans, compared to IFN- γ -secreting cells (Fig.2A). This finding is in line with other studies demonstrating that healthy donors express only low levels of IL-10, which are not elevated compared to allergic donors²⁶⁻³², but higher levels of IFN- γ than the latter^{26, 28-30, 33}. The TH2 suppressing capacity of IFN- γ is well described and successful immunotherapy against timothy grass antigen has been clearly correlated with skewing from TH2 to TH1³⁴. Several studies concerning the allergy-suppressive capacity of CD4+CD25+ regulatory T cells and the impact of IFN- γ in healthy individuals compared to those of allergic donors present contradictory results^{28, 35-39}. The high discrepancies between these studies likely reflect differences in antigen preparation, experimental protocols, and allergen exposure of the study population (in and out of season). Nevertheless, together these data clearly rule out IL-10-secreting Tregs as the only players defining the non-allergic status.

Our data also support our hypothesis that the environment influences the protective response type. We consistently found that townspeople preferentially mount a “more inflammatory” immune response type with both, increased levels of IFN- γ and expression of T-bet (Fig. 2A,B) as well as elevated levels of IL-17, IL-21, IL-22 (Fig. 2F) and expression of ROR γ t (Fig. 2B). It seems counterintuitive that farmers, who are exposed to a higher load of microbial compounds such as lipopolysaccharide (LPS), mount a less inflammatory immune phenotype. However, it has become clear that chronic exposure to low dose LPS reduces epithelial cell cytokine secretion important for the recruitment of DCs, resulting in suppression of type 2 immune responses⁴⁰. Similarly, microbiotas that produce poorly immunostimulatory LPS are associated with an increased risk of autoimmunity⁴¹. This is in line with the recently formulated “old friends hypothesis” stating that a reduced diversity

of intestinal microbiota in “westernized” countries and urban environments results in insufficient immune regulation, thus increasing TH17-based inflammatory diseases⁴². This may explain why we find a substantial proportion of townspeople who react with an unexpected TH17 response against the allergen Phl p 5. Similarly, IL-17-producing T cell lines specific for the dog allergen Can f 1 have been found in non-allergic humans⁴³, clearly indicating that induction and maintenance of the non-allergic state is a dynamic and active immunologic process employing multiple mechanisms besides Treg-based tolerance or TH 1-based immune deviation. In the context of allergy, TH17 responses have been mainly described for their potential to exacerbate lung inflammation. However, TH17/TH2 responses in the lung counteract each other and represent different asthmatic phenotypes⁴⁴. One could speculate that in a non-inflammatory healthy context, low-levels of antigen-specific TH17 cells may contribute to regulation of immune responses. Thus, a rich and diverse microflora is apparently essential for a regulatory T cell response^{45, 46}, as is indirectly also reflected by our data showing higher ratios of FoxP3⁺/T-bet and FoxP3⁺/ROR γ t expression in farmers (Fig. 2B). Similarly, the expression of IL-9 in individuals of both non-allergic groups is in agreement with data demonstrating antigen-dependent induction of IL-9 in non-atopics⁴⁷. However, the role of IL-9 remains unclear as it has also been correlated with allergic responses^{48, 49}.

Correlation analysis revealed a significant interaction of the TH2 cytokines IL-13 and IL-5 with the TH1 cytokines IFN- γ , TNF- α , GM-CSF, and also with IL-10 (IL-17 correlation reached no statistical significance level due to the low number of individuals). No such correlations were observed in allergic donors (Fig. E4 in this article’s online repository). This supports our hypothesis that beyond known reciprocal inhibitions/regulations^{29, 50}, TH2 type responses can be counterbalanced by several mechanisms, which also include feedback regulation (in both directions) via plasticity of T cells (e.g. IL-13 production in/together with TH1 and TH17 cells, suppl. Figs. E5 and E6)^{51, 52}.

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Fig E1. *In vitro* expansion of allergen-specific memory CD4⁺ T cells. In order to study the low frequency population of allergen-specific T cells in non-allergic subjects an *in vitro*, antigen-specific expansion is required. **A**, Scheme of *in vitro* expansion of allergen-specific memory CD4⁺ T cells. **B**, Gating strategy, isotype control and representative dot plots showing expression of CD45RO and CD62L of viable CD3⁺CD4⁺ T cells before expansion and after the resting phase. Dot plots illustrate log fluorescence intensity for all markers. T_{EM} = effector memory T cells, T_{CM} = central memory T cells, T_{EFF} = effector T cells, T_N = naïve T cells. **C**, Comparison of T cells at different stages of maturation before expansion (empty boxes) and after the resting phase (grey boxes) (n=26). The results are shown as percentages of CD3⁺CD4⁺Ki67⁺ cells. **D**, Antigen specificity of expanded cells from non-allergic subjects (n=5) was confirmed by measuring proliferation after restimulation with Phl p 5 or the

irrelevant antigen KLH. The results are shown as stimulation indices. The dashed line indicates the proliferation cut-off. Identical symbols correspond to the same subject. The line across indicates the mean. Statistically significant differences were determined using paired Student's t test (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

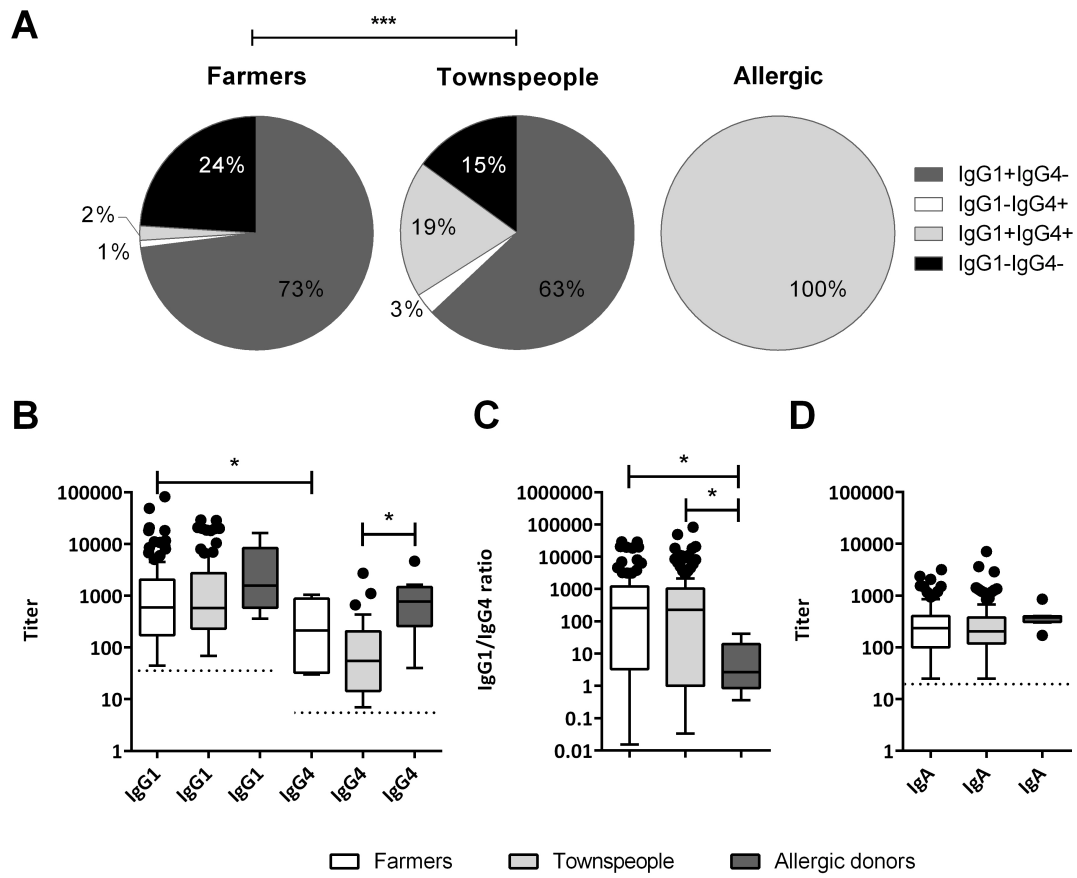
Fig E2. Clonotype analysis of *in vitro* expanded T cells. As an indicator of clonal expansion of circulating memory T cells from peripheral blood of the donors, the TCR repertoire of one non-allergic farmer (donor #1) and one non-allergic townsman (donor #2) was analyzed before and after *in vitro* expansion. Tables show percentage values of the top 10 clonotypes based on CDR3 sequence before expansion, after expansion and after resting phase. Color code indicates same amino acid (AA) sequences at different time points. The curve diagram summarizes the frequency (%) of top 10 clonotypes before expansion (dashed line), after expansion (dotted line), and after resting phase (solid line). Most of the top 10 clones found after the resting phase have already been present in the top 10 clones prior to the resting phase. Thus, they apparently represent Phl p 5-specific central memory T cells that survived in the absence of antigen. Beside the clear trend of clonal expansion, the data indicate that clonal proliferation of memory T cells after stimulation with Phl p 5 is limited to a few dominant clones per subject. **A**, Donor#1 primer set 1 **B**, Donor#1 primer set 2 **C**, Donor#2 primer set 1 **D**, Donor#2 primer set 2.

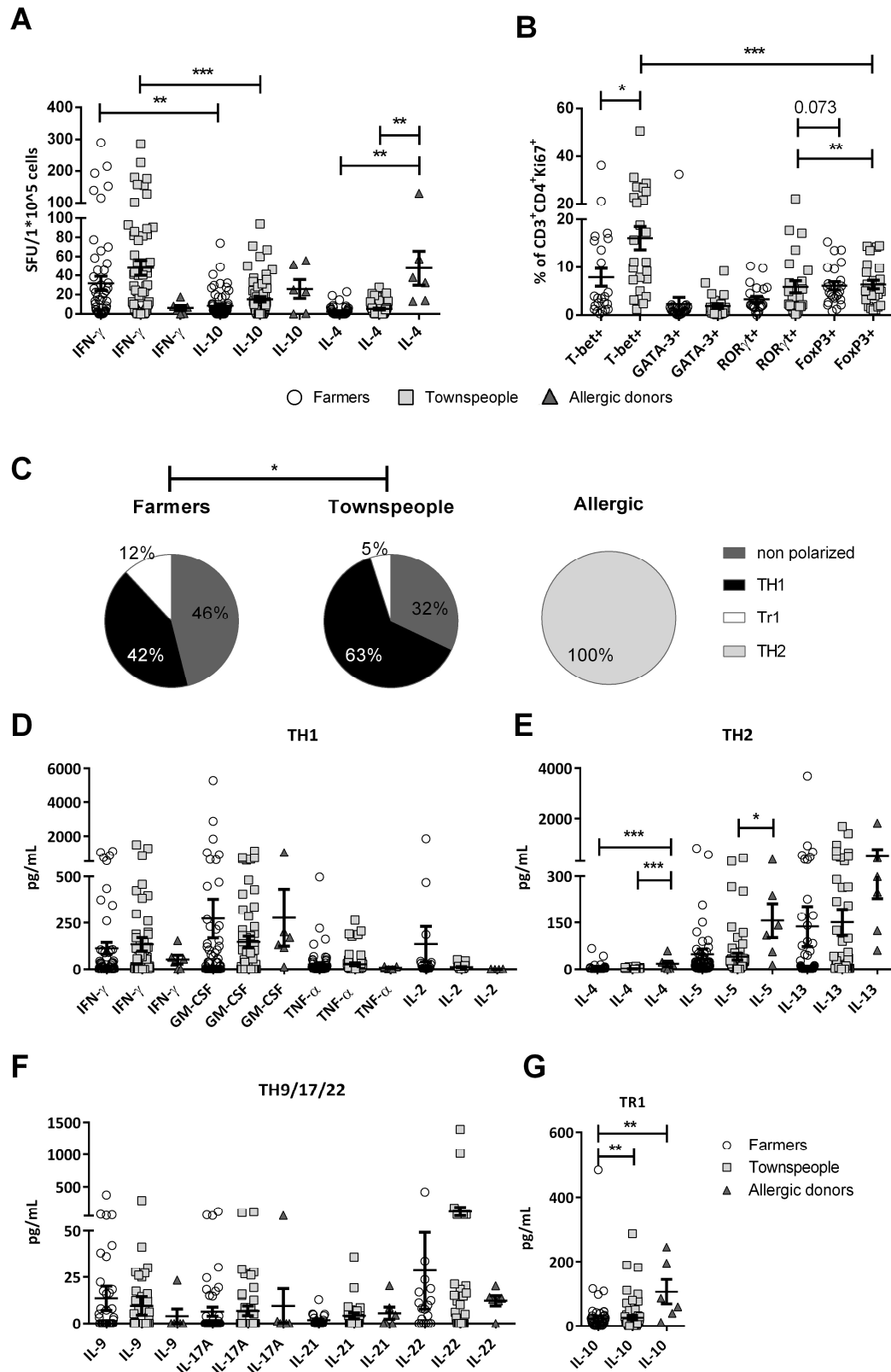
Fig E3. TH2 cytokine secretion in non-allergic individuals is compensated by elevated levels of pro-inflammatory cytokines. TH1/9/17/22-related cytokine levels in cultures from non-allergic farmers (circles, $n=8$) and non-allergic townspeople (squares, $n=11$) secreting elevated levels of TH2 cytokines (sum of IL-4/5/13 greater than 90pg/mL), and allergic subjects (triangles, $n=6$) was determined after 72 hours of restimulation with rPhl p 5 using a human cytokine 9-plex panel (IL-4, IL-5, IL-9, IL-10, IL-13, IL-17A, IFN- γ , TNF- α , GM-CSF) and a quantitative luminometric ELISA (IL-2, IL-21, IL-22).

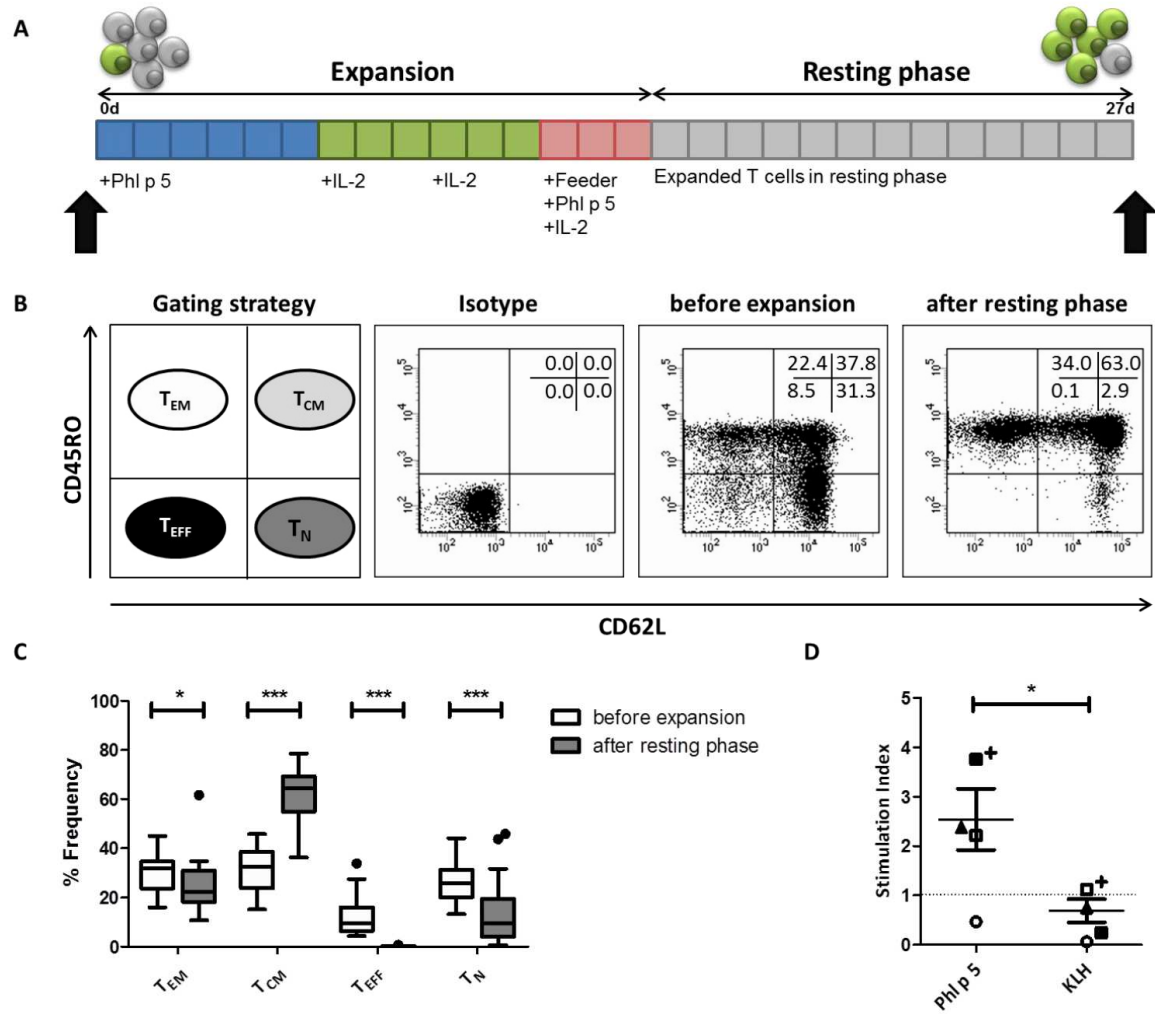
Fig E4: Correlogram of **A**, farmers, **B**, townspeople, and **C**, allergic donors. Correlation matrices were assembled with the data of 60 donors from each non-allergic group and 6 allergic donors, comprising a complete data set of the results from antibody, ELISPOT and cytokine measurements. Transcription factor data was only available for non-allergic donors ($n=22$ for farmers, $n=24$ for townspeople). The size of the circles and colors in the correlation plots represent the degree of correlation (spearman's rho) between two features. The significance level was set to < 0.05 . Non-significant coefficients were left blank.

Fig E5. Cytokine secretion pattern of "compensatory" cytokines in non-allergic farmers. The relative contribution of non-TH2 cytokines to the total amount of "pro-inflammatory" or regulatory cytokines is shown as pie chart. Amounts of cytokines in supernatants are displayed as pg/mL. Shown are the 8 farmers with the highest secretion of TH2 cytokines (sum of IL-4, 5, and 13).

Fig E6. Cytokine secretion pattern of “compensatory” cytokines in non-allergic townspeople. The relative contribution of non-TH2 cytokines to the total amount of “pro-inflammatory” or regulatory cytokines is shown as pie chart. Amounts of cytokines in supernatants are displayed as pg/mL. Shown are the 11 townspeople with the highest secretion of TH2 cytokines (sum of IL-4, 5, and 13).

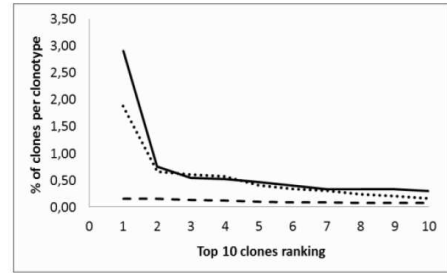






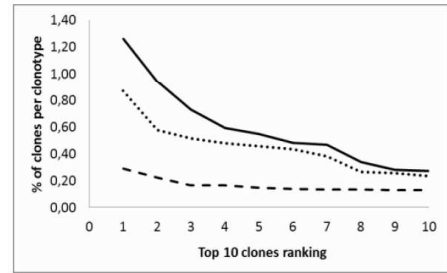
A

before expansion		after expansion		after resting phase	
CDR3 AA Sequence	%	CDR3 AA Sequence	%	CDR3 AA Sequence	%
ASSAGGNQPDH	0,15	ASSYAGQGVPGYT	1,88	ASSLGHQASPLH	2,91
ASSPAMSSYNPLH	0,15	AISEGNTAEF	0,66	AISEGNTAEF	0,75
ASTLQSNTEGELF	0,13	ASRETGWTEGELF	0,60	ASSGGRGQPDH	0,54
ASS	0,12	ASRTAATPLH	0,57	ASRETGWTEGELF	0,52
ASSSGANVLT	0,10	ASSLLQNTAEF	0,41	ASSYAGQGVPGYT	0,47
ASSWDRTYGYT	0,09	ASSLGHQASPLH	0,34	ASSQDGTGVGGYT	0,40
ASSLYGTANSPLH	0,08	ASSQDGTGVGGYT	0,30	ASSYGHSEYQY	0,33
ASRSTGTGTEGELF	0,08	ASSFSNQPDH	0,23	ASSLLQNTAEF	0,33
ASSGKGADGYT	0,08	ASSPLGQSGEYQY	0,20	ASSPAMSSYNPLH	0,33
AVQGLAGNTEGELF	0,07	ASTLQSNTEGELF	0,16	ASSLYGTANSPLH	0,30



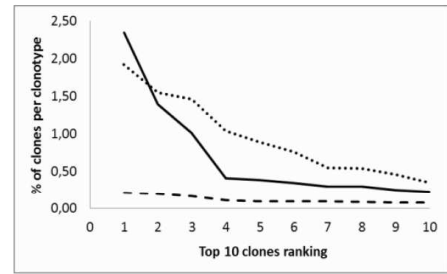
B

before expansion		after expansion		after resting phase	
CDR3 AA Sequence	%	CDR3 AA Sequence	%	CDR3 AA Sequence	%
AIRLSSGGSHQEF	0,29	SASESARWEQF	0,87	RASGAGVMTDTQY	1,26
ASSYTRQSGGYNEQF	0,22	ASTKERGIKDTQY	0,57	ASSILRQGYNEQF	0,94
ASTDRGQTNEQF	0,16	ASHPAGRRNEQF	0,51	SASESARWEQF	0,73
ASSLASGAYNEQF	0,16	SVGTSGVYNEQF	0,48	ASSPLMEQF	0,59
ASTNRAGGRTYNEQ	0,14	ASSLSDTQY	0,46	AISELAGGYAV	0,54
ASRRRGGGYNEQF	0,14	SVELR*GV	0,43	ASSLPVNEQF	0,48
SMGQEYEQF	0,13	ASSWAPEGPVQF	0,38	AISELSNEQF	0,46
SVELR*GV	0,13	ASSSHRAPRQF	0,26	ASSLASGAYNEQF	0,34
ASSLGGADNEQF	0,13	ASTPGGRTAYNEQF	0,26	ASSAPWGSEYQF	0,28
ASSLYNEQF	0,13	ASSLGGADNEQF	0,23	ASRRGADSYNEQF	0,27



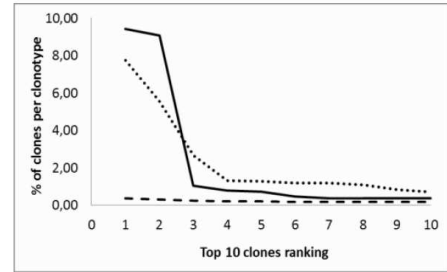
C

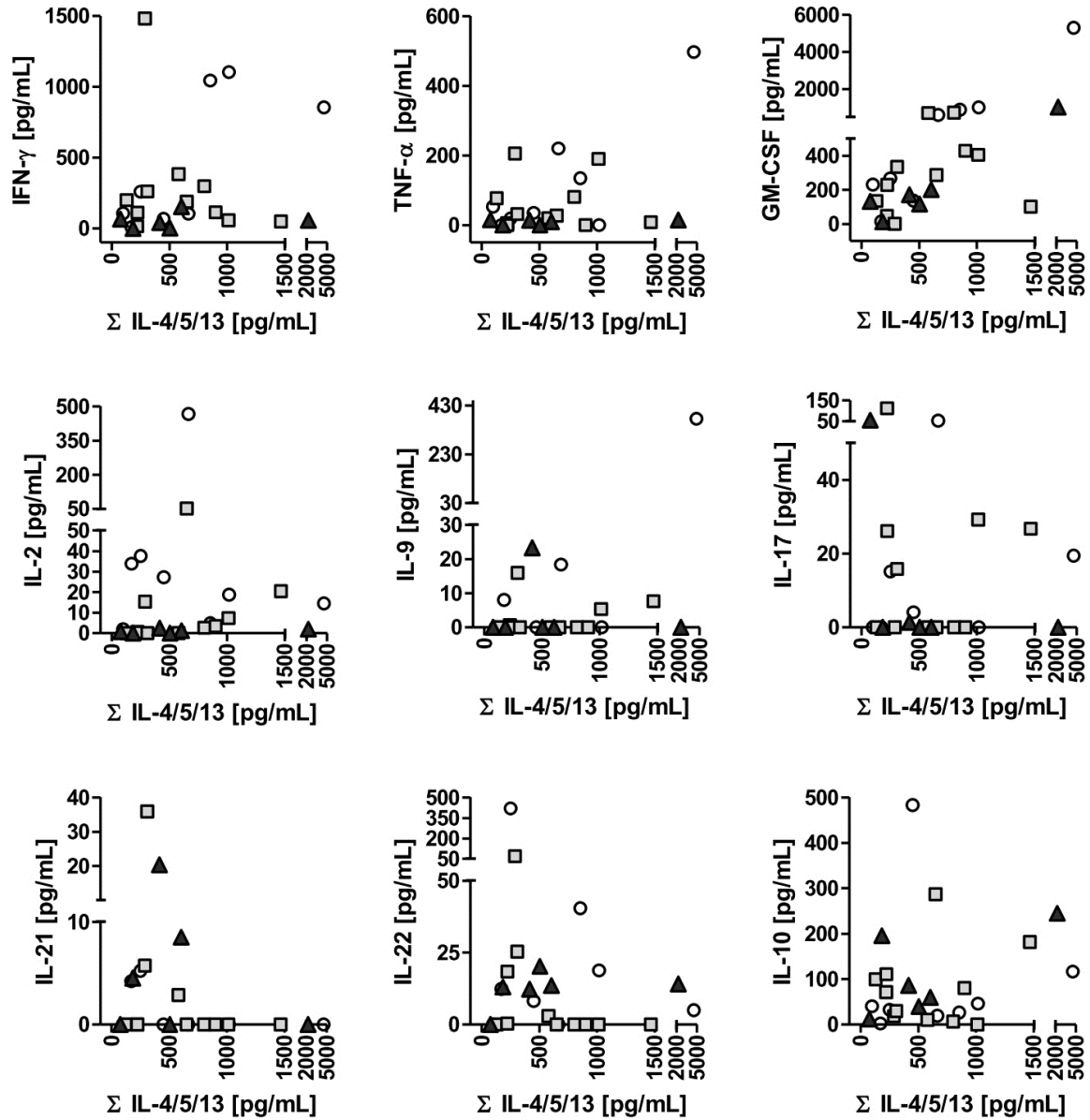
before expansion		after expansion		after resting phase	
CDR3 AA Sequence	%	CDR3 AA Sequence	%	CDR3 AA Sequence	%
AS	0,21	ASSPAGGATGANVLT	1,92	ATSDRDRAKGEGKL	2,34
ASSLR*GCNYGYT	0,19	ASRRGYSPLH	1,55	ASRRGYSPLH	1,39
ASSYSNLDYGYT	0,16	ASRHSTGNYGYT	1,46	ASRHSTGNYGYT	1,01
ASS	0,11	ATSDRDRAKGEGKL	1,04	ASSPAGGATGANVLT	0,41
ASSFDRAAGYT	0,09	SARGGGQVNTGELF	0,89	ASSPTHVGTGYNAAP	0,38
ASRQYNYGYT	0,09	ASSLGDVGYT	0,76	ASSFTRRRWLH	0,35
ASSRTGELF	0,09	ASSRDSVSNTAEF	0,55	ASTDRDRGLTNEKLF	0,30
ASSFDREGGYT	0,08	ASSPTHVGTGYNAAP	0,54	ASSPLEQGANSPLH	0,30
SVEAQGFYGYT	0,08	ASTPLRCGGQRPD	0,46	SVAATNTGELF	0,25
ASSPGTGVDGYT	0,08	AWSRGGGANVLT	0,35	SVAARRVGYT	0,23

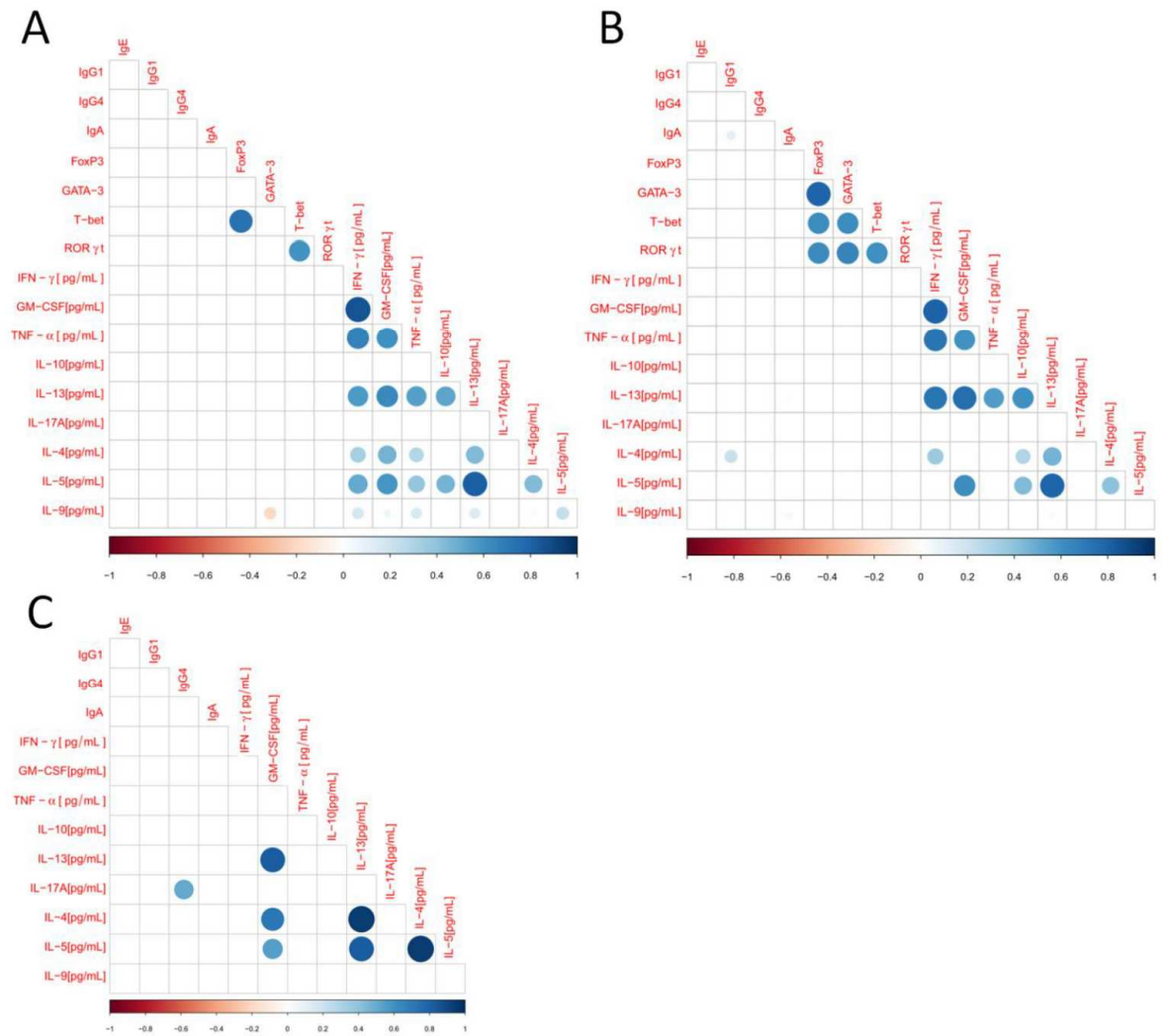


D

before expansion		after expansion		after resting phase	
CDR3 AA Sequence	%	CDR3 AA Sequence	%	CDR3 AA Sequence	%
ASSLRANEQF	0,36	ASSLHSSREGEQF	7,73	ASSLHSSREGEQF	9,43
ASSSSGTSGGALNEQ	0,31	ASTGRDRRPDTQY	5,57	ASTGRDRRPDTQY	9,08
ASKVYNEQF	0,24	ASRTTGATYNEQF	2,66	ASRTTGATYNEQF	1,03
ASSADRVNEQF	0,21	ASRQTDRLYNEQF	1,31	ASSPDRGGYNEQF	0,79
ASRMGQDGYNEQF	0,19	ASRLGWTGRSQ*AV	1,27	ASSPALANEQF	0,70
SPKE*AV	0,18	ASSYTDQY	1,19	ASSSGLAITEQF	0,46
ASSYTDQY	0,17	ASSFGPVYNEQF	1,17	ASSLTGGQF	0,37
ASSPRDTGIYNEQF	0,17	ASSPALANEQF	1,10	ASSYTDQY	0,37
ASSPDRVDNNEQF	0,16	ASSSGLAITEQF	0,82	ASSFHLADLQ*AV	0,36
ASSPMGIEQF	0,16	ASSLTGGQF	0,71	ASRQTDRLYNEQF	0,35







	TH1				TH9/17/22				Tr1	TH2			
	IFN- γ	GM-CSF	TNF-a	IL-2	IL-9	IL-17A	IL-21	IL-22	IL-10	IL-13	IL-4	IL-5	Σ
	106	230	53	2	0	0	0	0	40	76	13	8	97
	8	15	0	34	8	0	4	12	2	141	0	28	169
	260	267	19	38	0	15	5	420	32	226	3	19	248
	66	138	35	27	0	4	0	8	483	376	8	64	448
	103	615	220	467	18	53	0	0	19	536	3	124	663
	1045	900	134	5	0	0	0	40	26	671	43	139	853
	1104	1022	0	19	0	0	0	19	46	924	3	90	1016
	855	5295	497	15	376	19	0	5	116	3690	67	772	4529

	TH1				TH9/17/22				Tr1	TH2			
	IFN- γ	GM-CSF	TNF- α	IL-2	IL-9	IL-17A	IL-21	IL-22	IL-10	IL-13	IL-4	IL-5	Σ
	199	134	77	0	0	0	0	0	99	125	2	0	128
	15	47	5	1	1	112	0	0	71	219	1	1	220
	112	228	0	0	0	26	0	18	111	205	0	17	221
	1483	0	205	15	16	0	6	70	18	265	6	16	286
	260	335	32	0	0	16	36	25	29	266	0	41	307
	382	709	20	0	0	0	3	3	10	506	0	73	579
	187	286	27	52	0	0	0	0	287	501	5	144	650
	298	743	81	3	0	0	0	0	7	697	0	105	802
	113	428	0	4	0	0	0	0	80	633	8	259	899
	57	405	190	7	5	29	0	0	0	944	2	65	1011
	48	101	8	20	8	27	0	0	182	1404	7	56	1466

Natural protective immunity against grass pollen allergy is maintained by a diverse spectrum of response types

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Supplementary Tables

	Subjects sampled during pollen season May-July 2012		Subjects sampled during pollen season June 2015	
	Farmers	Townspeople	Allergic subjects	P-value
Mean age $\pm$ SD	42 $\pm$ 11.9	41 $\pm$ 11.1	38 $\pm$ 10.8	0.4869 ^a
Age range	21-62	18-62	22-62	
Males n (%)	76 (66.7%)	74 (64.9%)	7 (63.6%)	0.9515 ^b
Females n (%)	38 (33.3%)	40 (34.8%)	4 (36.4%)	
Mean titer Phl p 5 specific IgE	ND	ND	483	< 0.0001 ^a
Haying	100%	0%	0%	
Stable work	84.2%	0%	0%	
Hayloft work	86.8	0%	0%	
Cows	75.4%	0%	0%	
Sheep	22.8%	0%	0%	
Goats	10.5%	0%	0%	
n	114	114	11	

Table E1. Demographic characteristics, farm related exposures, and IgE titers of the study subjects. There was no significant difference in age or gender in the three groups. No IgE antibodies against Phl p 5 were detectable in non-allergic subjects, whereas 100% of allergic donors were positive for Phl p 5-specific IgE.

SD: standard deviation; ND: not detectable; ^aOne-way ANOVA; ^bChi-square test

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Fig E5. Cytokine secretion pattern of “compensatory” cytokines in non-allergic farmers. The relative contribution of non-TH2 cytokines to the total amount of “pro-inflammatory” or regulatory cytokines is shown as pie chart. Amounts of cytokines in supernatants are displayed as pg/mL. Shown are the 8 farmers with the highest secretion of TH2 cytokines (sum of IL-4, 5, and 13).

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