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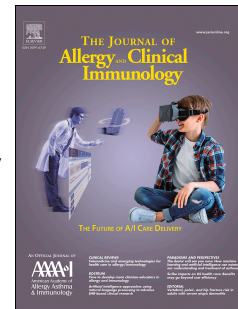
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Single-cell characterization of dog allergen-specific T cells reveals Th2 heterogeneity in allergic individuals

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ABSTRACT

Background. Allergen-specific type 2 CD4⁺ T helper (Th2) cells are critically involved in the pathogenesis of IgE-mediated allergic diseases. However, the heterogeneity of the Th2 response has only recently been appreciated.

Objective. To characterize at the single-cell level the *ex vivo* phenotype, transcriptomic profile and TCR repertoire of circulating CD4⁺ T cells specific to the major dog allergens Can f 1, Can f 4 and Can f 5 in subjects with and without dog allergy.

Methods. Dog allergen-specific memory CD4⁺ T cells were detected *ex vivo* by flow cytometry using a CD154-based enrichment assay and single-cell sorted for targeted gene expression analysis and TCR sequencing.

Results. Dog-allergen-specific T-cell responses in allergic subjects were dominantly of Th2-type. Th2 cells could be phenotypically further divided into three subsets, which consisted of Th2-like (CCR6⁺CXCR3⁻CRTH2⁻), Th2 (CCR6⁺CXCR3⁻CRTH2⁺CD161⁻) and Th2A (CCR6⁺CXCR3⁻CRTH2⁺CD161⁺CD27⁻) cells. All these subsets were non-existent within the allergen-specific T-cell repertoire of healthy subjects. Single-cell transcriptomic profiling confirmed the Th2-biased signature in allergen-specific T cells from allergic subjects and revealed a Th1/17 signature in nonallergic subjects. TCR repertoire analyses showed that dog allergen-specific T cells were diverse and allergic subjects demonstrated less clonality compared to non-allergic donors. Finally, TCR and transcriptomic analyses revealed a close relationship between Th2-like, Th2 and Th2A cells, the last ones representing the most terminally differentiated and highly polarized subtype.

Conclusions. Our study demonstrates heterogeneity within allergen-specific Th2 cells at the single-cell level. The results may be utilized for improving immune monitoring after allergen immunotherapy and for designing targeted immunomodulatory approaches.

KEY MESSAGES

- Dog allergen-specific blood T cells in allergic subjects are dominantly Th2-polarized, whereas in nonallergic subjects they are Th1/17-polarized.
- Dog allergen-specific Th2 cells in allergic subjects are heterogeneous but clonally related, likely representing different stages of differentiation.
- Dog allergen-specific T cells in allergic patients appear to be clonally more diverse than those in nonallergic subjects

CAPSULE SUMMARY

Allergen-specific blood Th2 cells in allergic patients are heterogeneous. Different Th2 subsets may be utilized as a biomarker in allergy or could represent separate targets for immunotherapy.

KEYWORDS: T cell, Th2, Th2A cell, CD154, dog allergy, Can f 1, Can f 4, Can f 5

ABBREVIATIONS

CPM	Counts per minute
hPGDS	Hematopoietic prostaglandin D synthase
InfA	Influenza A
MCA	Multiple correspondence analysis
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PPD	Tuberculin purified protein derivative
SEB	Staphylococcus Enterotoxin B
TCR	T-cell receptor
Treg	Regulatory T cell
t-SNE	t-Distributed Stochastic Neighbor Embedding

INTRODUCTION

Th2-type CD4⁺ T cells have a central role in the pathogenesis of allergic diseases. Through the production of IL-4, IL-5 and IL-13 they regulate the synthesis of IgE by B cells and promote eosinophilic inflammation in the tissues. Consequently, interrogating the specific nature of these Th2-type T-cell responses in allergic individuals, as well as the protective T-cell responses in nonallergic individuals, is of central importance for understanding the pathogenesis of allergic diseases.

In recent years, it has become evident that the Th2 response observed in allergic individuals is more heterogeneous than previously appreciated¹ Early studies suggested the presence of a separate subpopulation within Th2 cells that could be characterized by a reduced expression of CD27 but elevated expression of CCR4, IL-4, IL-5, IL-13 and GATA3.^{2,3} More recent studies have confirmed the presence of this highly differentiated, polyfunctional subset, coined either Th2A or pTh2 cells.⁴⁻⁶ Compared to more conventional Th2 cells, these cells express high levels of the surface markers CD161 and CD49d and Th2 cytokines, in particular IL-5 and IL-9, but low levels of CD27 and CD45RB.⁴⁻⁶ Moreover, they express high levels of genes encoding for hematopoietic prostaglandin D synthase (*hPGDS*), and for IL-25 (*IL17RB*) and IL-33 (*IL1RL1*) receptors that are associated with the Th2 response.⁴⁻⁶ Recent studies have confirmed that a large fraction of circulating allergen-specific T cells in allergic individuals have a Th2A signature,^{5,7-9} and that these cells are preferentially deleted during immunotherapy.^{5,10} Moreover, Th2A cells are present in the skin of patients with atopic dermatitis but are absent in healthy individuals.⁶

Despite this progress, phenotypic and transcriptomic studies performed at the level of polyclonal cells may still fail to capture the true underlying cellular heterogeneity that can only be resolved at the single-cell level. For example, a recent study employing single-cell RNA-sequencing in a mouse model of asthma

revealed substantial heterogeneity within CD4⁺ T-cell subsets and detected a previously unidentified subset responding to type 1 interferons.¹¹ To our knowledge, only two human studies so far have analyzed the transcriptome of allergen-specific T cells at the single-cell level. The first study analyzed a limited number of around 200 T cells specific to peanut allergens⁸ and the second, larger study >50,000 T cells specific to house dust mite allergens.¹² In line with earlier studies, both studies also demonstrated considerable heterogeneity within the allergen-specific T cell population from allergic individuals, and detected a signature consistent with a Th2A phenotype within these cells.^{8,12}

Dog-derived proteins are a common cause of respiratory hypersensitivity.¹³ To date, seven of them have been designated as allergens, among which the lipocalin proteins Can f 1 and Can f 4, as well as the prostatic kallikrein Can f 5 are important sensitizers.¹³ We have previously reported using both *in vitro* and *ex vivo* assays that circulating CD4⁺ T cells specific to Can f 1, 4 and 5 are dominantly Th2-biased, detectable at a higher frequency, and are of higher functional avidity in allergic compared to nonallergic individuals.^{14–16}

In the current study, we set out to investigate the heterogeneity of CD4⁺ T cell responses against dog allergens at the single-cell level. For this, the surface phenotype, transcriptome and T-cell receptor (TCR) repertoire of single circulating allergen-specific memory CD4⁺ T cells was analyzed *ex vivo* in dog-allergic patients and in healthy nonallergic individuals.

METHODS

Study subjects

Peripheral-blood samples were collected from twelve clinically diagnosed dog-allergic patients (age mean $\pm$ SD 41 ± 18 y, range 19-69 y) and eight nonallergic donors (age 42 ± 15 y, range 26-64 y) (Table E1). The allergic subjects were characterized at the Pulmonary Clinic of Kuopio University Hospital. They exhibited positive skin prick tests to dog dander extract, and Can f 1, Can f 4 and/or Can f 5-specific IgE reactivity in ELISA, measured as described previously.^{15,16} The nonallergic control subjects reported no allergic symptoms when exposed to dogs and did not exhibit IgE reactivity to Can f 1, Can f 4 and/or Can f 5 (Table E1). The study was approved by the ethics committee of Kuopio University Hospital. Signed informed consent was provided by all participants prior to sampling, as mandated by the Declaration of Helsinki.

Antigens

Recombinant (r) Can f 1, Can f 4 and Can f 5 proteins (produced as previously described¹⁷⁻¹⁹), Staphylococcus Enterotoxin B (SEB; Sigma-Aldrich), Influenza A (H1N1) MP1 peptide pool (InfA MP1, Miltenyi Biotec), tuberculin purified protein derivative (PPD; Statens Serum Institute, Denmark) and heat-inactivated *Candida albicans*²⁰ were used as antigens. Peptides containing T cell epitopes from Can f 1, Can f 4 and Can f 5 were synthesized at a minimum of 80% purity by GL Biochem (China).^{15,16,21}

CD154 enrichment assay

The experimental approach is summarized in Fig E1. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood with Ficoll density gradient centrifugation. The *ex vivo* analysis of dog allergen- and influenza-specific CD4⁺ T cells was performed by stimulating 50 to 75x10⁶ freshly isolated PBMCs for 16h at +37°C on 6-well plates with the rCan f protein pool (10 µg/mL of rCan f 1, 4 and 5) or

InfA MP1 peptide pool (1 µg/mL), as previously described.^{16,22} An equal number of PBMCs was seeded on identical plates with no antigen to serve as unstimulated controls. Moreover, 1x10⁶ PBMCs were seeded on 96-well plates and stimulated with SEB (1 µg/mL), PPD (10 µg/mL) or *Candida albicans* (at a ratio of 1 inactivated *C. albicans* per 10 PBMCs) as positive controls. The culture medium used was RPMI 1640 supplemented with 2 mM L-glutamine, 20 µM 2-mercapthoethanol, 1 mM sodium pyruvate, nonessential amino acids, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (all from Lonza), and 5% inactivated human AB serum (Sigma-Aldrich). For optimal detection of CXCR3 and CCR2 expression, the cells were stained with anti-CXCR3-BV510 and anti-CCR2-BV421 for 20 min at +37°C before the 16h stimulation (Fig E2). After stimulation, antigen-specific T cells were enriched with the CD154 Microbead kit (Miltenyi) according to the manufacturer's instructions. Surface staining of the cells was performed in a total volume of 100 µL for 20 min at room temperature with a panel of monoclonal antibodies (Table E2). Counting beads (Sphero AccuCount Fluorescent Particles; Spherotech Inc) were added to each sample for absolute cell counts. The samples were acquired and sorted with a FACSARIA III cell sorter (BD Biosciences) and the data was analyzed with the FlowJo software v10 (FlowJo).

Targeted gene expression analysis and sequencing of single cell paired TCR alpha and beta chains

Following the CD154 enrichment assay, memory CD4⁺CD69⁺CD154⁺ T cells from Can f-, InfA MP1-, SEB-, PPD- or *Candida albicans*-stimulated PBMCs were single-cell sorted on FACSARIA III using the index-sorting mode to 96-well PCR plates (4titude) containing 10 µL of 1x One-Step RT-PCR buffer (Qiagen). The expression of 16 mRNA transcripts as well as paired TCR α and β sequences were simultaneously assessed by a series of nested PCR reactions, as previously described²³ (Table E3). The nested amplification protocol was modified from the original version, reducing the reaction volumes to 18 µl and separating TCRα and TCRβ amplification steps to improve efficiency. Finally, the generated amplified sequences were combined, gel purified and sequenced using 500 cycle v2 MiSeq reagents (Illumina). Demultiplexing data

for individual wells and counting effector gene transcripts were performed using previously described algorithms.²³ Multiple correspondence analysis (MCA) was performed using FactoMiner.²⁴ The evenness of the paired TCR beta or TCR alpha-beta CDR3 repertoires was quantified using Shannon equitability index (E_H), which is the Shannon diversity index divided by the maximum diversity (<https://www.itl.nist.gov/div898/software/dataplot/refman2/auxillar/shannon.htm>). This normalizes the Shannon diversity index to a value between 0 and 1, with 1 being complete evenness. The Venn diagrams were generated by <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

Generation of Can f- and InfA MP1-specific CD4⁺ T-cell clones

Following CD154 enrichment assay, memory CD4⁺CD69⁺CD154⁺ cells from Can f- or InfA MP1-stimulated PBMCs were single-cell sorted on FACSaria III using the index sorting mode to 96-well U-bottom plates containing 5×10^4 γ -irradiated (3000 rad) allogeneic PBMCs and 5×10^3 γ -irradiated allogeneic EBV-transformed B cells. T-cell clones were expanded in the presence of 1 μ g/mL phytohemagglutinin (PHA; Oxoid) and 50 IU/mL IL-2 (Miltenyi Biotec) for 2 weeks. Thereafter, growing T-cell clones were expanded and maintained by stimulating them every 2 to 3 weeks with anti-CD2/CD3/CD28 beads (T Cell Activation and Expansion kit, Miltenyi Biotec) at a 1:2 bead to cell ratio in the presence of IL-2.

Proliferation and cytokine assays for T-cell clones

Thymidine incorporation-based proliferation assays for T-cell clones were set up in duplicate wells in 96-well U-bottom plates with 2.5×10^4 T cells and 5×10^4 γ -irradiated (3000 rad) autologous PBMCs, together with recombinant proteins (10 μ g/mL) or peptides (1 μ g/mL), as previously described.^{15,16,21} Stimulation with anti-CD2/CD3/CD28 beads at a 1:1 bead to cell ratio was used as a positive control. Supernatants from duplicate anti-CD2/CD3/CD28 bead-stimulated wells were collected from the proliferation assay plates after 72h. The production of 13 cytokines was measured by the LEGENDplex™ Human Th Cytokine

Panel kit (BioLegend) according to the manufacturer's instructions. Samples were acquired with the NovoCyte Quanteon flow cytometer (ACEA Biosciences) and analyzed with the LEGENDplex™ Data Analysis software (BioLegend).

Statistical analyses

Data were analyzed with the Prism software version 8 (GraphPad). Tests used to establish statistical significance are indicated in the figure legends. Values of $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Dog allergen-specific memory CD4⁺ T cells exhibit phenotypic heterogeneity within the Th2 compartment

To study the phenotypic heterogeneity of the T cell response to dog allergens, we used the CD154-enrichment method^{16,22} to identify rare antigen-specific CD4⁺ T cells *ex vivo*. Specifically, freshly isolated PBMCs from clinically diagnosed dog-allergic patients and nonallergic healthy controls (Table E1) were stimulated with a pool consisting of rCan f 1/rCan f 4/rCan f 5 allergens. A peptide pool consisting of peptides from the influenza A virus MP1 protein (InfA MP1 peptide pool) and the superantigen staphylococcal enterotoxin B (SEB) were used as controls. After a 16h stimulation, antigen-specific memory T cells (CD4⁺CD45RA⁻CD69⁺CD154⁺) were magnetically enriched and analyzed by flow cytometry (Table E2 and Figs E1 and E2). The expression of CCR6, CXCR3, CCR4, CD161, CCR7 and CD27 was used to analyze the polarization and differentiation status of the antigen-specific T cells.^{5,16,25-27}

As a first approach, we visualized the multiparameter cytometry data with t-Distributed Stochastic Neighbor Embedding (t-SNE) and analyzed it using an unsupervised clustering algorithm, FlowSOM.²⁸ In two representative allergic subjects, both dog allergen-specific and influenza-specific memory CD4⁺ T cells could be mapped to distinct clusters within total memory CD4⁺ T cells (Fig 1A and B, Fig E3). Compared to SEB-stimulated T cells, representing diverse polyclonal memory T cells, dog allergen-specific T cells were enriched in cell clusters 1 and more strikingly in clusters 2 and 3, whereas influenza-specific T cells predominantly localized in cluster 4 (Fig 1C). Cells in cluster 2 displayed a CXCR3⁻CCR6⁻CCR4⁺CD161⁻ and cells in cluster 3 a CXCR3⁻CCR6⁻CCR4⁺CD161⁺CD27⁻ phenotype (Fig 1D), which correspond, respectively, to the classification of conventional Th2 and pathogenic effector Th2A subsets, as recently described.^{4,5} Cluster 1 displayed a CXCR3⁻CCR6⁻CCR4⁻CD161⁻ phenotype, which we designated “Th2-like”, since Th2

cells do not uniformly express CRTH2²⁹ and the CXCR3⁺CCR6⁻ T cell fraction has previously been shown to contain T cells with Th2 characteristics.^{16,25-27} Finally, cluster 4, that contained the majority of influenza-specific cells, displayed a CXCR3⁺CCR6⁻CRTH2⁻CD161⁻ Th1 phenotype (Fig 1D).^{16,25-27} In contrast to allergic subjects, in a representative nonallergic healthy donor dog allergen-specific T cells were enriched in clusters displaying either a CXCR3⁺CCR6⁺ Th17 or CXCR3⁺CCR6⁺ Th1/17 phenotype (Fig E3). In conclusion, dog allergen-specific memory CD4⁺ T cells in allergic donors can be subdivided to at least three phenotypically differing subsets, designated Th2-like, Th2 and Th2A.

Frequencies of dog allergen-specific Th2 cells are elevated in allergic patients

Next, using traditional biaxial gating, we determined the *ex vivo* frequencies of total, Th2-like (CXCR3⁺CCR6⁻CRTH2⁻), Th2 (CXCR3⁺CCR6⁻CRTH2⁺CD161⁻), Th2A (CCR6⁺CXCR3⁺CRTH2⁺CD161⁺CD27⁻) and Th1 (CCR6⁻CXCR3⁺) dog allergen- and influenza-specific memory CD4⁺ T cell frequencies within PBMCs from twelve allergic patients and eight nonallergic healthy donors (Fig 2 and Figs E2 and E4). Dog allergen-specific T cells were rare in both allergic and nonallergic donors, with only 5/12 allergic patients and 1/8 healthy subjects exceeding 100 dog allergen-specific T cells per 1 million memory CD4⁺ T cells (Fig 2A). Importantly, however, allergic patients had markedly elevated frequencies of Th2-like, Th2 and Th2A dog allergen-specific T cells compared to healthy donors who virtually had no dog allergen-specific T cells within these Th2 subsets (mean frequencies per million memory CD4⁺ T cells: 72 vs 6 cells for Th2-like cells; 34 vs 0 cells for Th2 cells; 17 vs 0 cells for Th2A cells, respectively) (Fig, 2B-D).

The frequencies of influenza-specific T cells were higher than those of dog allergen-specific T cells but similar between the allergic and healthy subject groups (mean frequencies per million memory CD4⁺ T cells: 225 vs 268 cells, respectively). These cells almost completely resided within the Th1 subset (Fig, 2A and E).

Transcriptomic profiling at the single-cell level confirms the differences between dog allergen-specific T cells from allergic and nonallergic donors

Next, we determined whether the differences in the expression patterns of cell-surface markers observed on dog allergen-specific memory CD4⁺ T cells between allergic patients and healthy donors extended to their transcriptomic profiles. For this, we sorted single CD4⁺CD69⁺CD154⁺ memory T cells from Can f- or InfA MP1-stimulated PBMCs of 9 allergic patients and 8 healthy donors to assess the expression of gene transcripts of 16 major T-cell subset-defining transcription factors and cytokines (*BCL6*, *FOXP3*, *GATA3*, *RORC*, *TBX21*, *IFNG*, *IL2*, *IL4*, *IL9*, *IL10*, *IL13*, *IL17A*, *IL21*, *IL22*, *TGFB1* and *TNFA*) through a plate-based targeted expression analysis.²³ In total, we analyzed 1402 dog allergen-specific and 1006 influenza-specific single T cells (Table E4). For three patients, we also separately sorted *Candida albicans*- and PPD-stimulated memory CD4⁺CD154⁺CD69⁺ T cells to validate the capacity of the assay to distinguish different prototypical T-helper cell profiles (Fig E5). The expression of cell surface markers and transcriptomic data were combined and subjected to multiple correspondence analysis (MCA) to examine the relationships of all single cells analyzed in a reduced dimensional space (Fig 3A and Fig E5). On the MCA map, we observed that influenza-specific memory CD4⁺ T cells from allergic patients and healthy donors almost completely overlapped, suggesting a high degree of similarity between them. In contrast, dog allergen-specific memory CD4⁺ T cells from allergic patients and healthy donors occupied largely different regions of the reduced dimensionality space. A row-normalized heatmap was also used to visualize the proportion of single cells expressing each marker within the different groups analyzed, demonstrating a preferential expression of Th2-associated markers by dog allergen-specific T cells from allergic and Th1/Th17-associated markers by Can f-specific T cells from healthy subjects, respectively (Fig 3B). We observed that the expression of *CRT2* as well as *IL4*, *IL13* and *IL9* transcripts was higher, and the expression of *CXCR3* and *CCR6* as well as *IFNG*, *IL17A*, *IL22*, *TNFA*, *TBX21* and *RORC* transcripts was lower in Can f-specific T cells

from allergic patients compared to healthy subjects (Fig 3C). The other transcripts analyzed were not differentially expressed between dog allergen-specific T cells from allergic and healthy subjects, although there was a nonsignificant tendency for higher expression of *FOXP3*, *IL10* and *TGFB1*, markers associated with regulatory T cells, by dog-allergen specific T cells from healthy subjects (Fig 3C and Fig E5). As expected, influenza-specific T cells predominantly expressed Th1-associated markers, such as *CXCR3*, *IFNG* and *TBX21* (Fig 3B and C). In conclusion, we show that the transcriptomic profile of dog allergen-specific memory CD4⁺ T cells also differs at the single-cell level, with allergic subjects demonstrating a Th2-associated signature compared to the Th1/Th17 signature observed in healthy, nonallergic subjects.

Transcriptomic heterogeneity within dog allergen-specific Th2 cells in allergic subjects

Next, we separately analyzed the expression of the Th2-associated transcripts *GATA3*, *IL4*, *IL13* and *IL9* among Th2-like, Th2 and Th2A dog allergen-specific memory CD4⁺ T cells from allergic patients (Fig 4A). We observed that the Th2 and Th2A subsets displayed a general trend for increasingly higher proportions of cells expressing the Th2-associated transcripts. This was particularly evident for *GATA3* and *IL13*, which were both expressed by a significantly higher proportion of Th2A than Th2-like cells (Fig 4A). Moreover, the expression of *IL9* was observed only within the Th2 and Th2A subsets. The Th2A cells also displayed the highest polyfunctional potential, with 35.9% of single Th2A cells expressing at least two and 3.2% expressing all three Th2 cytokine transcripts compared to 25.7% and 0% of Th2-like cells, respectively ($p < 0.0001$ for both, Fisher's test; Fig 4B). Finally, Th2A cells displayed reduced expression of *IL2* and *CCR7* compared to Th2-like cells, suggestive of a more terminally differentiated state (Fig 4C). No differences in the expression of the other analyzed transcripts were observed between the Th2-like, Th2 and Th2A dog allergen-specific T cells from allergic patients (Fig E5).

Allergic patients have a more diverse dog allergen-specific T cell repertoire

As part of our single-cell analysis pipeline, we also performed TCR alpha and beta sequencing (Fig 5). On average, we recovered the TCR alpha or beta sequence from 75% and paired alpha and beta TCR sequences from 60% of the T cells sequenced (Table E5). The detected TCR sequences were largely private, i.e., a particular alpha or beta sequence, or combination, was only detected in Can f- or influenza-specific T cells from a single individual. Only 5 out of 550 (0.9%) unique TCR sequences detected in Can f-stimulated samples and 2 out of 589 (0.3%) unique TCR sequences detected in InfA MP1-stimulated samples were shared between two different individuals, i.e., represent public clones.

Due to the better amplification efficiency of single TCR chain sequences compared to paired alpha-beta sequences, we primarily used TCR beta sequences for analyses of clonality. Clonal identity is indicated with a high confidence between T cells with identical TCR beta sequences in an antigen-stimulated culture from a single individual, even in cases where the alpha chain sequence could not be resolved. This approach increased the robustness of the data analyses by increasing the number of individual TCRs included in the analyses by 25%, as compared with paired sequences.

We considered identical TCR beta chain sequences expressed by at least two separate T cells sorted from the same stimulation to represent clonal expansions *in vivo*. Expanded T-cell clones (sequences expressed by at least two cells) represented 34% and 33% of the total TCR sequences obtained from Can f-stimulated cells from allergic and nonallergic individuals, and 17% and 14% from InfA MP1-stimulated cells, respectively (Fig 5A). Among expanded dog allergen-specific TCR sequences, the proportion of sequences shared by at least 5 five separate cells was higher in healthy donors (10.5% of sequences) than in allergic patients (4.7% of sequences, $p < 0.05$, Fisher's exact test; Fig 5A). Analogously, the frequency of the most abundant T-cell clone (as a percentage of all TCR sequences) was higher in Can f-stimulated cells from healthy donors compared to allergic patients (Fig 5B). In InfA MP1-stimulated cells, highly expanded T-cell

clones (at least five separate cells), in contrast, were extremely rare (Fig 5A). Consequently, calculation of Shannon's equitability indexes (value of 1 indicating complete diversity) demonstrated that dog allergen-specific TCR sequences were less diverse than influenza-specific sequences (Fig 5C). Largely similar results were obtained through the analysis of paired alpha-beta TCR sequences, although statistical significance was lost due to reduced power in this smaller dataset (Fig E6)

Collectively our TCR sequencing analyses suggested that the dog allergen-specific T-cell repertoire appears to be less diverse than the influenza-specific repertoire. Moreover, the dog allergen-specific T-cell repertoire of allergic individuals appears to be slightly more diverse, i.e., contains less mono/oligoclonal TCR expansions, than that of nonallergic individuals.

Dog allergen-specific T cells within different Th2 cell subsets are clonally related

We also examined the developmental relationship between Th2-like, Th2 and Th2A dog allergen-specific T cells by assessing whether expanded TCR beta sequences were shared between these subsets. In three allergic patients who had enough T cells within each of these subsets for the analysis (patients AP1, AP9 and AP21), we observed that the majority of expanded TCR sequences were shared between 2 or 3 subsets (AP1: 63%, AP9: 58%, AP21: 63% of total expanded TCR beta sequences; Fig 5D). In all, these results demonstrate that *in vivo*-expanded clonal dog allergen-specific T cells reside within multiple Th2 subsets, providing direct support for a common developmental origin of Th2-like, Th2 and Th2A cells.

***In vitro*-expanded dog allergen-specific T-cell clones recognize diverse epitopes and exhibit a Th2-type cytokine secretion profile**

To further confirm the specificity of the dog allergen-specific memory CD4⁺ T cell detected *ex vivo* with the CD154 enrichment assay, we resampled two allergic patients (AP1 and AP9) at a second time point 8 to 11

months apart. In addition to repeating TCR sequencing *ex vivo*, we expanded *in vitro* half of the sorted single T cells to generate T cell clones for downstream analyses (Table E6). Using a proliferation assay, we first tested whether the clones that had expanded *in vitro* were specific to one of the three recombinant dog allergens that were used to stimulate PBMCs *ex vivo* (Fig 6A and Fig E7). In both patients, most of the T-cell clones responded to either to rCan f 1, rCan f 4 or rCan f 5, confirming the specificity of the cells detected *ex vivo* with the CD154-based enrichment assay (Tables E6 and E7). We observed that T-cell clones from AP9 predominantly responded against rCan f 5 (7 of 9 clones), with single clones recognizing rCan f 1 and rCan f 4 (Table E7). Most of the clones from AP1 (11 of 14), in turn, responded to rCan f 4 with three clones recognizing rCan f 1 (Table E7). For some clones, we were further able to determine their exact epitope specificity (Table E7 and Fig E7). In summary, these analyses confirm that cells detected *ex vivo* with the CD154-based enrichment assay are highly antigen-specific and that the dog allergen-specific clones generated from both allergic patients recognized a wide diversity of different epitopes within Can f 1, Can f 4 or Can f 5 allergens.

We further analyzed the cytokine secretion profile of the dog allergen- as well as influenza-specific T-cell clones generated (Fig 6B and Fig E8). Dog allergen-specific T-cell clones secreted significantly higher levels of the Th2-associated cytokines IL-4, IL-5, IL-13 and IL-9, while influenza-specific cells were characterized by the secretion of IFN- γ and TNF- α , the hallmarks of Th1 cells. Of note, allergen-specific T-cell clones deriving *ex vivo* from Th2-like, Th2 or Th2A subsets all produced comparable level of the Th2 cytokines IL-4, IL-5, IL-13 and IL-9 after *in vitro* culture (Fig 6C). This finding demonstrates that Th2-like and Th2 cells have a similar potential as Th2A cells for highly efficient Th2 cytokine production after *in vitro* activation and expansion. Of note, clones deriving from Th2-like cells produced more IL-2 than those deriving from the Th2 or Th2A subsets (Fig E8), which may reflect their less differentiated state *ex vivo* (Fig 4C).

371 Finally, we assessed whether dog allergen-specific TCR beta sequences were shared between the *in vitro*-
372 expanded T-cell clones and T cells sampled *ex vivo* from the same donors at the two different timepoints
373 (Fig 6D). Several of the TCR beta sequences from the established T-cell clones matched sequences
374 detected *ex vivo* (Fig 6D). We also observed that only few TCR beta sequences sampled *ex vivo* were
375 shared over time, suggesting a large clonal diversity of dog allergen-specific CD4⁺ T cell responses in
376 allergic subjects. Of note, these shared sequences belonged exclusively to *in vivo* expanded clones
377 (expressed by at least two cells at a single time point) (Fig 6D).

DISCUSSION

In this study, we comprehensively characterized human CD4⁺ T-cell responses to dog allergens at the single-cell level in allergic and nonallergic individuals. We observed Th2-biased T-cell responses exclusively in allergic patients and demonstrated considerable heterogeneity within these cells. Using unsupervised clustering based on surface marker expression we observed that the Th2-biased allergen-specific T-cell responses in allergic individuals clustered into at least three distinct subsets, which we designated Th2-like, Th2 and Th2A cells (Fig 1). Combining the surface phenotype to transcriptomic data, we confirmed that the Th2A subset bears characteristics of highly polarized, terminally differentiated effector cells, as previously reported.^{4,5} Compared to the other Th2 subsets, Th2A cells expressed higher levels of *GATA3* and *IL13* transcripts, higher Th2 cytokine coexpression and lower *CCR7*, *CD27* and *IL2* expression (Fig 3). Of note, our targeted transcriptome panel regrettably did not include primers for *IL5*, which has previously been shown to be highly expressed by Th2A cells and the expression of which appears to distinguish Th2A cells better than *IL4* and *IL13* from the other Th2 subsets.^{4,5}

Multiple studies have demonstrated an association between the Th2A signature and activity of allergic inflammation in patients with allergic diseases.^{4,6,9,12,30,31} It is therefore conceivable that Th2A cells could only be present in individuals undergoing chronic allergen exposure and inflammation. Our data is consistent with this hypothesis, as we observed Th2A cells only in some but not all patients with dog allergy (Fig. 2), and consequently the strongest Th2 signature was detected in these same individuals (Fig.3). Since individuals allergic to animal danders generally avoid exposure to the allergen source, it is possible that Th2A cell numbers in blood have waned in some of the allergic subjects studied here due to the lack of continuing or recurrent allergen exposure. Indeed, only 3/12 of the allergic subjects and 2/8 of the nonallergic subjects studied here reported recurrent contact with dogs (Table E1), which could be

one explanation for the low frequency of circulating allergen-specific T cells detected within both subject groups (Fig 2).

One unresolved question thus far has been whether Th2A cells are related to other Th2 subsets or represent a unique developmental subset. Using single-cell TCR-sequencing, we can directly demonstrate here TCR sharing between allergen-specific T cells residing *in vivo* within the Th2-like, Th2 and Th2A subsets (Fig 5.) This result supports the hypothesis that these different subsets share a common developmental origin and potentially represent different stages of Th2 differentiation. The demonstration that *in vitro*-expanded T-cell clones from Th2-like, Th2 and Th2A subsets all produced comparable levels of the Th2 signature cytokines IL-4, IL-5, IL-9 and IL-13 (Fig 6) further supports the idea that cells belonging to each of these subsets *in vivo* have a similar Th2 effector potential once activated.

The dog allergen-specific T-cell repertoire of nonallergic individuals was devoid of Th2 cells (Fig 2), and consequently these cells clustered separately from T cells from allergic individuals based on surface marker expression as well as transcriptomic signature (Fig 3). Interestingly, dog-allergen specific T cells from nonallergic subjects also clustered differently from influenza-specific T cells that had a very distinct Th1-phenotype (Fig 3). Allergen-specific T cells in nonallergic individuals rather displayed a mixed Th1/Th17 signature with higher expression of *CXCR3*, *TBX21*, *IFNG* but also *CCR6*, *RORC*, *IL17A*, *IL22* and *TNFA*. Our findings are largely consistent with those of previous studies that have detected higher *CXCR3* and IFN- γ expression by allergen-specific T cells from nonallergic than from to allergic individuals.^{3,15,16,32}

Single-cell TCR sequencing revealed that the dog-allergen specific T-cell repertoire in both allergic and nonallergic individuals was largely polyclonal and diverse. There was no evidence of public TCRs, i.e., TCR sequence sharing between different individuals, and most of the sequences observed within an

individual were unique. It is important to note, however, that due to the rarity of dog allergen-specific T cells in peripheral blood, we likely undersampled considerably the dog allergen-specific T-cell repertoire by analyzing a limited number of single T cells (23 to 271) per individual. The diversity of the dog allergen-specific T-cell repertoire was also supported by the finding that only a minority of the different TCR sequences were repeatedly detected when two allergic subjects were sampled at two different time points, and the shared sequences belonged exclusively to clones that had undergone expansion *in vivo* (expressed by at least two distinct T cells; Fig 6). Our analyses of *in vitro*-expanded T-cell clones also support a wide polyclonal response to dog allergens, since the clones derived from the two allergic subjects studied in detail recognized multiple different epitopes on all the three dog allergens assessed (Table E7). Furthermore, our results suggest a higher TCR diversity of dog allergen-specific T-cells in allergic compared to nonallergic subjects (Fig 5). Studies on TCR diversity of allergen-specific T cells are so far scarce, and to our knowledge, no previous studies at the single-cell level have been performed. One previous study demonstrated that allergen-specific blood T cells in peanut-allergic patients with heightened clinical sensitivity exhibited a more diverse TCR repertoire as compared to hyporeactive patients.⁹ Together with our findings, these results could be interpreted to reflect a wider range of clonal reactivity in highly allergic than in less reactive patients, or nonallergic individuals, potentially through epitope spreading.

Our results provide proof of concept that using the CD154-enrichment method together with index sorting, allergen-specific T cells can be analyzed at the single-cell level both *ex vivo* and after expansion *in vitro*. *In vitro*-expanded T-cell clones demonstrated a shared clonal origin with the T cells analyzed *ex vivo* (Fig 6) and they maintained their Th2 phenotype upon culture. The easy generation of T-cell clones representative of true *in vivo* memory T-cell responses through this approach allows further functional

characterization of the disease-associated T cells *in vitro* and could also be utilized for T-cell epitope mapping studies.

One caveat of our study is that we utilized a predefined, targeted transcriptomic approach²³ rather than unbiased RNA sequencing. This potentially limits our capacity to capture the whole transcriptomic heterogeneity of the CD4⁺ T-cell response to dog allergens. However, it is important to note that our panel included most of the critical markers observed to contribute to Th2 heterogeneity in previous studies employing unbiased RNA-sequencing.^{4,8,9,12,31} The targeted approach also has certain benefits, in particular a better resolution to detect low-abundance transcripts, such as those for cytokines, that may fail to be detected at the single-cell resolution using RNA sequencing.³³ Also, the extremely low frequency of dog allergen-specific T cells in the blood of both allergic and nonallergic donors (at maximum a few hundred cells isolated from >50 million PBMCs) largely prevents the use of currently available commercial single-cell RNA-sequencing platforms. Another caveat to note is that the CD154-based enrichment assay utilized here does not allow the detection of antigen-specific FOXP3⁺ regulatory T cells (Tregs), as these cells upregulate CD137 instead of CD154 upon activation.³⁴ However, previous investigations have not demonstrated any quantitative or qualitative differences in allergen-specific FOXP3⁺ Tregs between allergic and nonallergic subjects.^{14,34}

In summary, we present here one of the first studies comprehensively characterizing allergen-specific T cells at the single-cell level, and, to our knowledge, the first one to combine transcriptomic analyses and TCR sequencing to the phenotype of single T cells assessed by surface marker expression. We demonstrated a Th2-biased T-cell response to dog allergens in allergic subjects and a Th1/17-biased response in nonallergic subjects. The Th2 response in allergic subjects was also shown to be heterogeneous and the different Th2 subsets appeared to be developmentally related and likely

reflected different stages of Th2 polarization. Increased understanding of the single-cell heterogeneity of CD4+ T-cell responses is instrumental for deciphering the pathogenesis of allergic diseases. Different Th2 subsets could potentially be utilized as a biomarker in allergy or be separately targeted by immunotherapeutic approaches.

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FIGURE LEGENDS

Figure 1: Dog allergen-specific memory CD4⁺ T cells from allergic subjects exhibit phenotypic heterogeneity within the Th2 compartment. (A) tSNE plot displaying memory CD4⁺CD154⁺CD69⁺ T cells from Can f- (blue dots), influenza A MP1- (red dots) and SEB-stimulated (gray dots) PBMCs from a representative dog-allergic patient (AP1). The phenotypic markers CCR6, CXCR3, CRTH2, CD161, CCR7 and CD27 were used for dimensional reduction. (B) The same tSNE plot overlaid with the clusters automatically identified by the FlowSOM algorithm. (C) Distribution of FlowSOM clusters among Can f-, influenza- and SEB-stimulated memory CD4⁺CD154⁺CD69⁺ T cells. (D) Heatmap representing row-normalized marker expression (median fluorescence intensities; MFI) within each FlowSOM cluster.

Figure 2: Frequencies of dog allergen-specific Th2 cells are elevated in allergic patients. *Ex vivo* frequencies of total (A), Th2-like (B), Th2 (C), Th2A (D) and Th1 (E) dog allergen (Can f)- and influenza (InfA)-specific memory CD4⁺ T cells from 12 allergic patients (AP) and 8 healthy donors (HD). The background from unstimulated cells (Fig E4) was subtracted. Th1 cells were defined as CCR6⁻CXCR3⁺, Th2-like cells as CCR6⁻CXCR3⁻CRTH2⁻, Th2 cells as CCR6⁻CXCR3⁻CRTH2⁺CD161⁻ and Th2A cells as CCR6⁻CXCR3⁻CRTH2⁺CD161⁺CD27⁻. The four allergic patients with the highest frequencies of Th2-polarized cells, in particular Th2A cells, are indicated with red color. Mann-Whitney U-test was used for statistical analyses. ***p<0.001; ****p<0.0001.

Figure 3: Single-cell transcriptomic profiling confirms phenotypic differences between dog allergen-specific T cells from allergic and nonallergic donors. Memory CD4⁺CD154⁺CD69⁺ T cells from dog allergen (Can f)- and influenza (InfA)-stimulated PBMCs from 9 allergic patients (AP) and 8 healthy donors (HD) were single-cell sorted with the index sort mode to record the expression of surface markers. The expression of 16 transcription factors and cytokines was assessed through plate-based

targeted mRNA sequencing. **(A)** Multiple correspondence analysis (MCA) plot of all sorted cells. Both transcriptomic and cell surface marker expression data were used to calculate the MCA map. Colored confidence ellipses for the different groups are depicted. **(B)** Heatmap representing the row-normalized marker expression on dog allergen- and influenza-specific memory CD4⁺ T single cells from AP and HD. **(C)** Percentages of dog allergen- and influenza-specific single cells expressing the indicated surface and transcriptomic markers. The four allergic patients with the highest frequencies of Th2-polarized cells (Fig. 2) are indicated with red color. Mann-Whitney U-test was used for statistical analyses. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

Figure 4: Transcriptomic heterogeneity within dog allergen-specific Th2 cells in allergic subjects. (A) Percentages of dog allergen-specific memory CD4⁺ T cells from different *ex vivo* Th2 subsets expressing Th2-associated transcripts. **(B)** Frequencies of single dog allergen-specific memory CD4⁺ T cells within different *ex vivo* Th2 subsets expressing 0 to 3 Th2 cytokine (IL4, IL13 and IL9) transcripts. **(C)** Percentages of dog allergen-specific memory CD4⁺ T cells from different *ex vivo* Th2 subsets expressing IL2 or CCR7. The four allergic patients with the highest frequencies of Th2-polarized cells (Fig. 2) are indicated with red color. Kruskal-Wallis test with Dunn's multiple comparisons test was used for statistical analyses. * $p < 0.05$. ** $p < 0.01$.

Figure 5: Single-cell TCR sequencing reveals increased diversity within dog allergen-specific T cell repertoire from allergic patients and a clonal relationship between different dog allergen-specific Th2 subsets. (A) TCR beta clonality of dog allergen (Can f)- and influenza (InfA)-specific memory CD4⁺ T cells in allergic (AP) and nonallergic (HD) individuals. The proportion of clones with a unique TCR beta sequence detected once or more within an individual is indicated in the plot. **(B)** Quantification of the most abundant (max) TCR beta clone out of all dog allergen- or influenza-specific T cells analyzed from

each individual donor. Samples lacking clonal cells were scored as zero. (C) Shannon's equitability index calculated in dog allergen- and influenza-specific T cells from each individual donor. The four allergic patients with the highest frequencies of Th2-polarized cells (Fig. 2) are indicated with red color. Mann-Whitney U-test was used for statistical analyses. * $p < 0.05$.

(D) TCR beta sequence sharing of dog allergen-specific memory CD4⁺ T cells between different *ex vivo* Th2 subsets in three allergic patients.

Figure 6: *In vitro*-expanded dog allergen-specific T-cell clones recognize diverse allergen epitopes and exhibit a Th2-type cytokine secretion profile. Memory CD4⁺CD154⁺CD69⁺ T cells from dog allergen (Can f)- and influenza (InfA)-stimulated PBMCs from two allergic patients were single-cell sorted using the index sort mode and expanded *in vitro*. (A) Representative proliferative responses, plotted as counts per minute (CPM), of an *in vitro*-expanded dog allergen-specific CD4⁺ T cell clone (clone B2 from patient AP9) in response to the rCan f proteins (rCan f 1, rCan f 4, rCan f 5) and to its cognate peptide (Can f 5 p148-163). Medium was used as a negative control and anti-CD2/CD3/CD28 beads as a positive control. Bars represent the mean of three replicate wells with SEM. (B) Cytokine levels in the supernatants of anti-CD2/CD3/CD28-stimulated Can f- or influenza-specific CD4⁺ T-cell clones. Each data point represents an individual clone. (C) Th2 cytokine production by dog allergen-specific T-cell clones originally sorted from different *ex vivo* Th2 subsets. (D) TCR β sequence sharing of dog allergen-specific memory CD4⁺ T cells between two *ex vivo* analyses at different time points (sample 1 and 2) and between *in vitro*-expanded dog allergen-specific CD4⁺ T cell clones established at the second time point. Top panel: all *ex vivo*-detected TCR beta sequences included in the analysis. Bottom panel: only *in vivo* expanded TCR beta sequences (the same TCR sequence expressed by at least two separate cells) were included in the analysis. Mann-Whitney U-test was used for statistical analyses. * $p < 0.05$. *** $p < 0.001$. **** $p < 0.0001$.

Table E1: Demographic data of donors included in the study

Study group	ID	Sex	Age (years)	Serum IgE ELISA (OD at 450nm)			Skin prick test (mm)	Dog Dust Exposure	Asthma	Allergic Symptoms	Exacerbation*	FC	Seq
				Can f 1	Can f 4	Can f 5	Dog						
Allergic patients	AP1	F	41	2.878	2.913	3.086	9 +++	+	+	+ CRUW	+	x	x
	AP5	F	53	2.032	0.890	1.323	++	-	+	+ RW	-	x	x
	AP7	F	52	1.150	0.019	0.598	++	-	+	+ UW	-	x	x
	AP9	M	69	3.100	0.754	2.736	6 +++	-	+	+ W	+	x	x
	AP12	F	69	0.011	0.009	1.827	++	-	+	+ R	+	x	x
	AP13	M	27	0.898	1.984	0.550	++	-	+	+ RW	-	x	x
	AP14	M	19	0.261	0.395	2.864	NA	+	-	+ CR	-	x	x
	AP16	F	56	2.705	2.594	2.755	7 +++	+	+	+ CRU	-	x	
	AP18	F	29	2.377	0.009	0.041	5 +++	-	-	+ U	-	x	x
	AP19	F	26	3.159	1.847	0.050	6 +++	-	+	+ UW	+	x	
	AP20	F	23	2.877	0.315	0.364	7 +++	-	-	+ CRU	-	x	
	AP21	M	24	2.855	3.064	2.678	8 +++	-	-	+ CRU	-	x	x
Healthy donors (non-allergic)	HD01	M	41	0.062	0.038	0.042	ND	-	-	-	-	x	x
	HD04	M	38	0.001	0.005	0.020	ND	-	-	-	-	x	x
	HD22	M	26	0.038	0.033	0.032	ND	-	-	-	-	x	x
	HD24	F	55	0.001	0.019	0.009	ND	-	-	-	-	x	x
	HD27	F	64	0.000	0.000	0.000	ND	-	-	-	-	x	x
	HD29	F	57	0.004	0.000	0.000	ND	-	-	-	-	x	x
	HD31	F	29	0.000	0.000	0.000	ND	+	-	-	-	x	x
	HD44	M	28	0.002	0.009	0.018	ND	+	-	-	-	x	x

C = conjunctivitis, R = rhinitis, U = urticaria, W = wheezing

* = self-reported exacerbation of symptoms upon dog exposure

FC = flow cytometry analysis

Seq = Transcriptomic and TCR repertoire analysis

NA = not available. ND = not done

Table E2: Antibodies used for flow cytometry analysis

Marker	Fluorochrome	Manufacturer	Clone	Dilution
7-AAD	NA	BioLegend	NA	NA
CD14	PerCP-Cy5.5	BioLegend	HCD14	1/50
CD16	PerCP-Cy5.5	BioLegend	3G8	1/50
CD19	PerCP-Cy5.5	BioLegend	HIB19	1/50
CD56	PerCP-Cy5.5	BioLegend	HCD56	1/50
CD4	APC-F750	BioLegend	RPA-T4	1/50
CD45RA	PE-CF594	BD	HI100	1/50
CD154	PE	Miltenyi	5C8	1/50
Anti-biotin	PE	Miltenyi	Bio3-18E7	1/50
CD69	APC	BioLegend	FN50	1/50
CCR6	PE-Cy7	BioLegend	G034E3	1/50
CXCR3	BV510	BioLegend	G025H7	1/50
CRTH2	BV421	BioLegend	BM16	1/50
CD161	BV605	BioLegend	HP-3G10	1/50
CD27	APC-R700	BD	M-T271	1/50
CCR7	BB515	BD	3D12	1/50

NA = not applicable

Table E3: Primers used for targeted gene expression analysis of transcription factors and cytokines in PCR reactions 1 and 2. All other primers used (TCR families and PCR reaction 3 primers) are listed in ref. 23.

	Gene	Primer A	Primer B
Reaction 1	<i>BCL6</i>	GCCAAACCAGAGGGCCTGAG	GAGAGCCGCAGGACGTGCACTT
	<i>FOXP3</i>	GGCTCCTGCTGCATCGTAGCTGCT	GTCCGCTGCTTCTCTGGAGCCT
	<i>GATA3</i>	GACGCGGCGCAGTACCCGCT	GGAGAAGGGGCTGAGATTCCAG
	<i>RORC</i>	CCCGGGAGGAAGTGACTGGCTA	CCATGCCACCGTATTTGCCTTCAA
	<i>TBX21</i>	GCCTGTACGTCCACCCGACT	CTGGGTTTCTTGAAAGTAAAGATAT
	<i>IFNG</i>	GGCTTTTCAGCTCTGCATGTTTT	GGATGCTCTGGTCATCTTTAAAGTT
	<i>IL2</i>	CTCACATTTAAGTTTTACATGCCAA	GACAAAAGGTAATCCATCTGTTGAG
	<i>IL4</i>	CCTTCTGCAGGGCTGCGACTG	CAGCTCGAAGACTTTGAATATTCT
	<i>IL9</i>	GCAGTGCTAATGTGACCAAGTGTG	CCAGAAGACTCTTCAGAAATGTCA
	<i>IL10</i>	CCAGTTTTACCTGGAGGAGGTGA	GTAGGCTTCTATGTAGTTGATGAAGA
	<i>IL13</i>	CCCAGAACAGAAGGCTCCGCT	CCCTCGCGAAAAAGTTTCTTTAAAT
	<i>IL17A</i>	GACAAGAAGTCCCGGACTG	GGACCAGGATCTCTGCTGGAT
	<i>IL21</i>	GGTCAAGATCGCCACATGATTAGA	CCCTGCATTTGTGGAAGGTGGTT
	<i>IL22</i>	CCTCTTGGTACAGGGAGGAGCA	CCTATCAGATTGAGGGAACAGCA
	<i>TGFB</i>	GCATATATATGTTCTTCAACACATCA	CCCTCCACGGCTCAACCACT
	<i>TNFA</i>	CATGATCCGGGACGTGGAGCT	GGGCTACAGGCTTGCACTCG
Reaction 2	<i>BCL6</i>	CCAGGGTTTTCCAGTCACGACCCTACACGGCCCCACCTGCCT	AGCGGATAACAATTTACACAGGAGGGTGCATGTAGAGTGGTGAGTG
	<i>FOXP3</i>	CCAGGGTTTTCCAGTCACGACGGCAGCCAAGGCCCTGTCGT	AGCGGATAACAATTTACACAGGACCAGGATGGCCAGCGGATGA
	<i>GATA3</i>	CCAGGGTTTTCCAGTCACGACGCCGAGGAGGTGGATGTGCTT	AGCGGATAACAATTTACACAGGAGGGGAGGCGGTGTGGTGGCT
	<i>RORC</i>	CCAGGGTTTTCCAGTCACGACAGAGGAAGTCCATGTGGGAGATGT	AGCGGATAACAATTTACACAGGATCAGCATTGTAGGCCCGGCACATC
	<i>TBX21</i>	CCAGGGTTTTCCAGTCACGACCCCAACACAGGAGCGCACTGG	AGCGGATAACAATTTACACAGGACGTGTTGGAAGCGTTGCAGGCT
	<i>IFNG</i>	CCAGGGTTTTCCAGTCACGACGGGTTCTCTGGCTGTTACTGC	AGCGGATAACAATTTACACAGGAGTTTGAAGTAAAGGAGACAATTTG
	<i>IL2</i>	CCAGGGTTTTCCAGTCACGACCCACAGAAGTAAACATCTTCAGT	AGCGGATAACAATTTACACAGGATTCTACAATGGTTGCTGTCTCA
	<i>IL4</i>	CCAGGGTTTTCCAGTCACGACGGCAGTTCTACAGCCACCATGA	AGCGGATAACAATTTACACAGGACTCTCTCATGATCGTCTTTAGCCT
	<i>IL9</i>	CCAGGGTTTTCCAGTCACGACGGGATTCCCTCTGACAAGTGC	AGCGGATAACAATTTACACAGGAGCGGTTGCCTGCCGTGGTT
	<i>IL10</i>	CCAGGGTTTTCCAGTCACGACCCCAAGCTGAGAACCAAGACCCA	AGCGGATAACAATTTACACAGGAGTCAAACTCACTCATGGCTTTGTA
	<i>IL13</i>	CCAGGGTTTTCCAGTCACGACGGTATGGAGCATCAACCTGACAG	AGCGGATAACAATTTACACAGGAGGTCCTTTACAACTGGGCCAC
	<i>IL17A</i>	CCAGGGTTTTCCAGTCACGACCAACTGAACATCCATAACCGGAA	AGCGGATAACAATTTACACAGGAGGGGACAGAGTTTCATGTGGTAGT
	<i>IL21</i>	CCAGGGTTTTCCAGTCACGACCGTCAACTTATAGATATTGTTGATCA	AGCGGATAACAATTTACACAGGACCTCTTCAGCTTTTAATTGATA
	<i>IL22</i>	CCAGGGTTTTCCAGTCACGACCGCCCATCAGCTCCCACTGCA	AGCGGATAACAATTTACACAGGAGGGTGAAGTTCAGCACCTGCTT
	<i>TGFB</i>	CCAGGGTTTTCCAGTCACGACCCGAGAAGCGGTACCTGAACC	AGCGGATAACAATTTACACAGGACCGCACAACCTCCGGTGACATCA
	<i>TNFA</i>	CCAGGGTTTTCCAGTCACGACGGAGGCGCTCCCAAGAAGAC	AGCGGATAACAATTTACACAGGACGAGAAGATGATCTGACTGCCTG

Table E4: Number of single cells with transcriptomic data analyzed

Donor	Can f	InfA MP1
AP1	271	140
AP5	48	32
AP7	81	63
AP9	239	42
AP12	36	29
AP13	62	47
AP14	99	45
AP18	59	23
AP21	94	47
Total AP	989	468
HD01	74	53
HD04	26	33
HD22	23	13
HD24	41	34
HD27	108	75
HD29	61	143
HD31	30	127
HD44	50	60
Total HD	413	538
Total	1402	1006

Table E5: Number of single cells with TCR sequencing data analyzed

	Can f			InfA MP1		
Donor	TCR β	TCR α	TCR $\alpha\beta$	TCR β	TCR α	TCR $\alpha\beta$
AP1	202	190	145	124	123	113
AP5	48	41	41	30	31	29
AP7	51	52	40	44	51	35
AP9	191	190	154	35	39	34
AP12	29	27	27	25	26	25
AP13	51	49	43	30	37	25
AP14	63	83	56	24	36	17
AP18	11	35	3	11	13	2
AP21	59	59	30	25	32	16
Total AP	705	726	539	348	388	296
HD01	56	43	38	43	50	42
HD04	20	14	8	26	24	19
HD22	20	14	14	9	10	7
HD24	39	37	37	34	32	31
HD27	92	103	94	68	45	52
HD29	39	37	26	95	104	71
HD31	0	0	0	111	88	83
HD44	46	41	38	47	50	39
Total HD	312	289	255	433	403	344
Total	1017	1015	794	781	791	640

Table E6: Dog allergen-specific T-cell clone growth according to *ex vivo* phenotype

Donor	<i>Ex vivo</i> subset	Sorted cells within subset	Expanded cells from sorted subset	Can f-reactive cells from expanded subset
AP1	Non-Th2	17/83 (20.5%)	4/17 (23.5%)	0/4 (0.0%)
	Th2-like	32/83 (38.6%)	6/32 (18.8%)	5/6 (83.3%)
	Th2	18/83 (21.7%)	8/18 (44.4%)	7/8 (87.5%)
	Th2A	16/83 (19.3%)	2/16 (12.5%)	2/2 (100.0%)
AP9	Non-Th2	15/60 (25.0%)	1/15 (6.7%)	0/1 (0.0%)
	Th2-like	24/60 (40.0%)	7/24 (29.2%)	7/7 (100.0%)
	Th2	5/60 (8.3%)	2/5 (40.0%)	2/2 (100.0%)
	Th2A	16/60 (26.7%)	0/16 (0.0%)	-

Table E7: Epitope specificities of *in vitro*-expanded dog allergen-specific T-cell clones

Patient	Clone	Allergen	Epitope
AP9	B2	Can f 5	p148-163
	B10	Can f 4	p43-58
	C8	Can f 1	p73-88
	C11	Can f 5	p214-229
	D3	Can f 5	p58-73
	D10	Can f 5	ND
	E5	Can f 5	p31-46
	F4	Can f 5	p97-112
	G5	Can f 5	p31-46
AP1	P1 B8	Can f 4	p43-58
	P1 B9	Can f 4	p43-58
	P1 B11	Can f 1	ND
	P1 C4	Can f 4	p128-142
	P1 C6	Can f 4	ND
	P1 E7	Can f 1	ND
	P1 G7	Can f 1	ND
	P1 G9	Can f 4	ND
	P2 B5	Can f 4	p46-64
	P2 B11	Can f 4	ND
	P2 C2	Can f 4	ND
	P2 C5	Can f 4	p43-58
	P2 C10	Can f 4	ND
	P2 D4	Can f 4	ND

ND = not done

Figure E1: Graphical representation of the workflow used to analyze dog allergen- and influenza-specific CD4⁺ T cells from allergic patients and healthy donors. The figure was created with Biorender.com.

Figure E2: Gating strategies used in the CD154 enrichment assay analyses and optimization of CXCR3 and CRTH2 staining (A) Gating strategy used to sort memory CD4⁺CD45RA⁻CD154⁺CD69⁺ T cells after *ex vivo* stimulation. Lymphocytes were first gated based on FSC and SSC properties, followed by elimination of doublets through FSC-A and FSC-H gating and exclusion of dead cells and non-T cells. The percentages of CD69⁺CD154⁺ events within the enriched Can f-stimulated and unstimulated samples are shown (two last panels). The calculated frequencies within total memory CD4⁺ T cells are indicated in parentheses (B) Example gating strategy used to analyze Th2 subsets within CD4⁺CD45RA⁻CD154⁺CD69⁺ T-cells in Can f-stimulated PBMCs from a dog-allergic patient. (C) Example of Th1 subset gating in influenza A MP1-stimulated PBMCs from the same dog-allergic patient. (D) Similar frequencies of CD4⁺CD45RA⁻CD154⁺CD69⁺ T cells were observed after a 16h stimulation with SEB both when PBMCs were stained with anti-CXCR3 and anti-CRTH2 antibodies before (top row) or after (bottom row) the stimulation. The intensity of CXCR3 and CRTH2 staining was better when the staining was performed before the stimulation (top row).

Figure E3: Dog allergen-specific memory CD4⁺ T cells from allergic subjects exhibit phenotypic heterogeneity within the Th2 compartment. tSNE plots displaying memory CD4⁺CD154⁺CD69⁺ T cells from Can f- (blue dots), influenza A MP1- (red dots) and SEB-stimulated (gray dots) PBMCs from one representative dog-allergic patient (AP9) (A) and one representative nonallergic healthy subject (HD27) (E). The phenotypic markers CCR6, CXCR3, CRTH2, CD161, CCR7 and CD27 were used for dimensional reduction. (B, F) The same tSNE plots overlaid with the clusters automatically identified by the FlowSOM algorithm. (D, G) Distribution of FlowSOM clusters among Can f-, influenza- and SEB-stimulated memory CD4⁺CD154⁺CD69⁺ T cells. (E, H) Heatmaps representing row-normalized marker expression (median fluorescence intensities; MFI) within each FlowSOM cluster.

Figure E4: Minimal background is observed in unstimulated samples in the CD154 enrichment assay. *Ex vivo* frequencies of total (A), Th2-like (B), Th2 (C), Th2A (D) and Th1 (E) CD69⁺CD154⁺ memory CD4⁺ T cells in unstimulated samples from 12 allergic patients (AP) and 8 healthy donors (HD). These values were subtracted from the antigen-stimulated samples (Fig 2). Th1 cells were defined as CCR6⁻CXCR3⁺, Th2-like cells as CCR6⁻CXCR3⁻CRTH2⁺, Th2 cells as CCR6⁻CXCR3⁻CRTH2⁺CD161⁻ and Th2A cells as CCR6⁻CXCR3⁻CRTH2⁺CD161⁺CD27⁻.

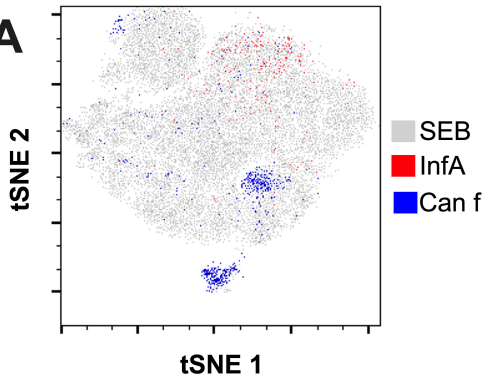
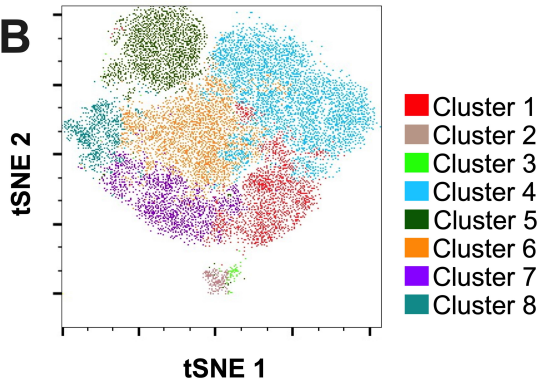
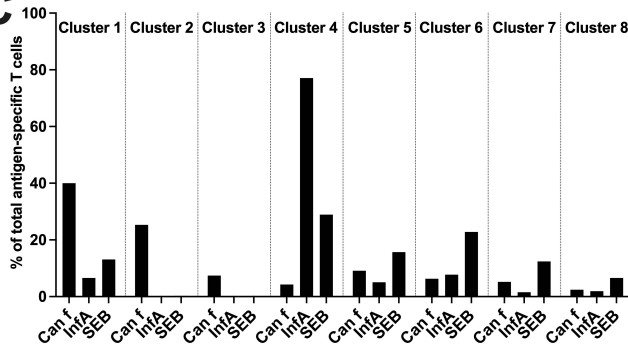
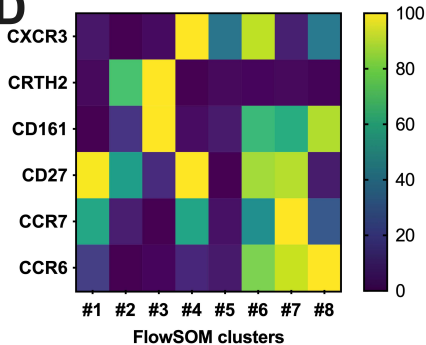
Figure E5: Single-cell transcriptomic profiling of antigen-specific memory CD4⁺ T cells. (A) Single-cell transcriptomic profile of memory CD4⁺CD154⁺CD69⁺ single T cells sorted with the index sort mode from the PBMCs of three dog-allergic patients stimulated with Influenza A MP1 peptides (245 cells), rCan f proteins (591 cells), PPD (85 cells) or *Candida albicans* (109 cells). The expression of 16 transcription factors and cytokines was assessed through plate-based targeted mRNA sequencing. Each data point represents a single donor. A typical expression pattern of predominantly Th1-associated transcripts (*TBX21*, *IFNG*) within influenza-specific T cells, Th2-associated transcripts (*GATA3*, *IL4*, *IL9*, *IL13*) within dog allergen-specific T-cells and Th17-associated transcripts (*RORC*, *IL17A*, *IL22*) within *C. albicans*-specific T cells was observed. (B) Multiple correspondence analysis (MCA) factor map demonstrates that Th1 (*IFNG*, *TBET*, *CCXR3*), Th2 (*IL4*, *IL9*, *IL13*, *CrTh2*) and Th17 (*IL17A*, *IL22*, *CCR6*) markers explain most of the detect variance between single cells. (C) Percentages of dog allergen (Can f)- and influenza (InfA)-specific single cells expressing the indicated surface and transcriptomic markers. (D) Percentages of dog allergen-specific memory CD4⁺ T cells from different *ex vivo* Th2 subsets expressing different transcripts. The four allergic patients with the highest frequencies of Th2-polarized cells (Fig. 2) are indicated with red color.

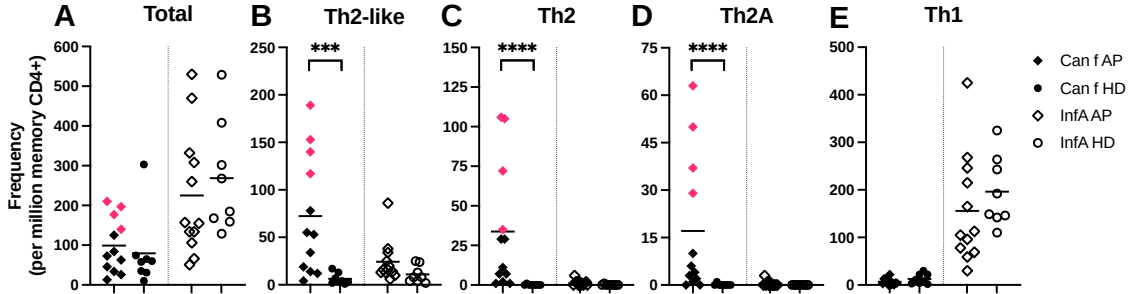
Figure E6: Analysis of paired TCR alpha and beta sequences of dog allergen- and influenza-specific T-cells at the single-cell level.

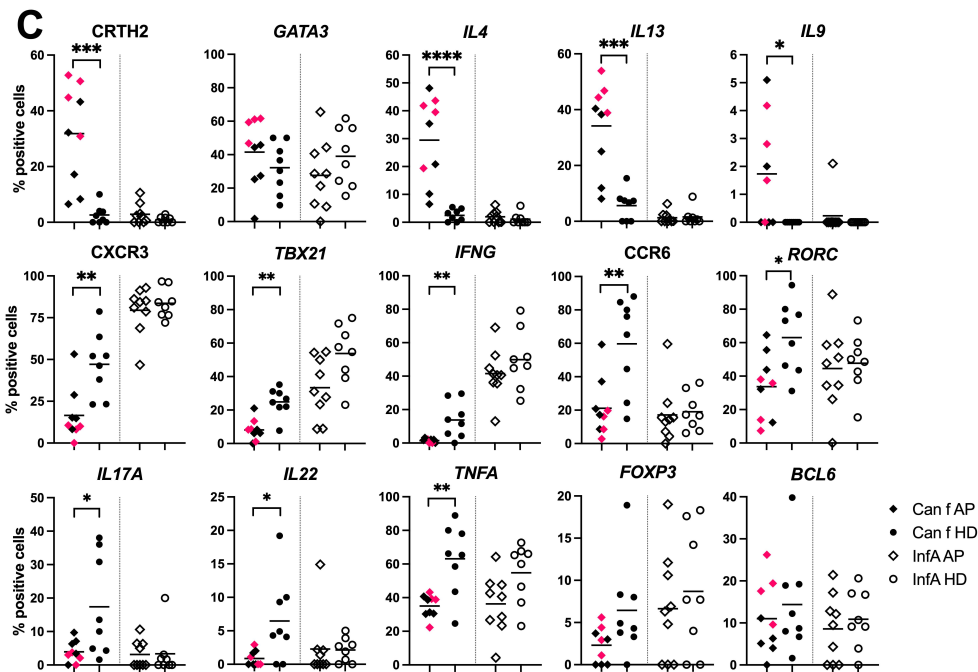
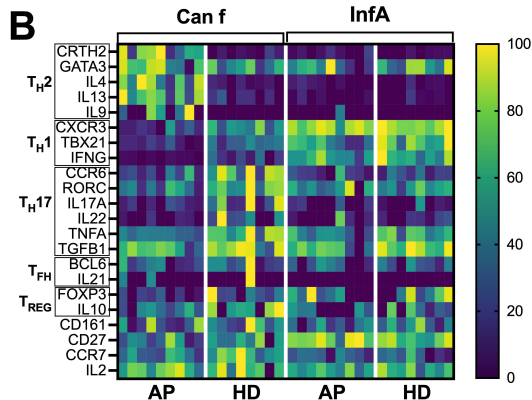
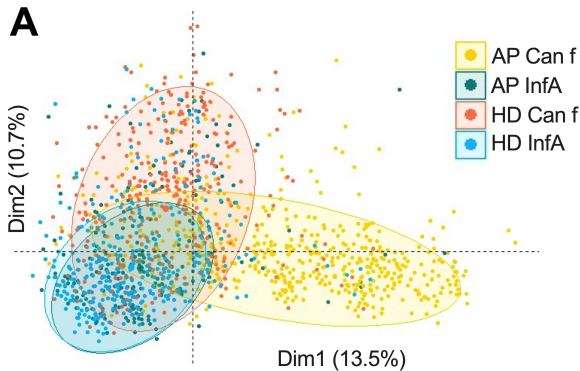
(A) TCR alpha-beta clonality of dog allergen- and influenza-specific memory CD4⁺ T cells in allergic (AP) and nonallergic (HD) individuals. The proportion of clones with a unique TCR alpha-beta sequence detected once or more within an individual is indicated in the plot. (B) Quantification of the most abundant (max) TCR alpha-beta clone out of all dog allergen- or influenza-specific T cells analyzed from each individual donor. Samples lacking clonal cells were scored as zero. (C) Shannon's equitability index for TCR alpha-beta sequences calculated in dog allergen- and influenza-specific T cells from each individual donor. The four allergic patients with the highest frequencies of Th2-polarized cells (Fig. 2) are indicated with red color.

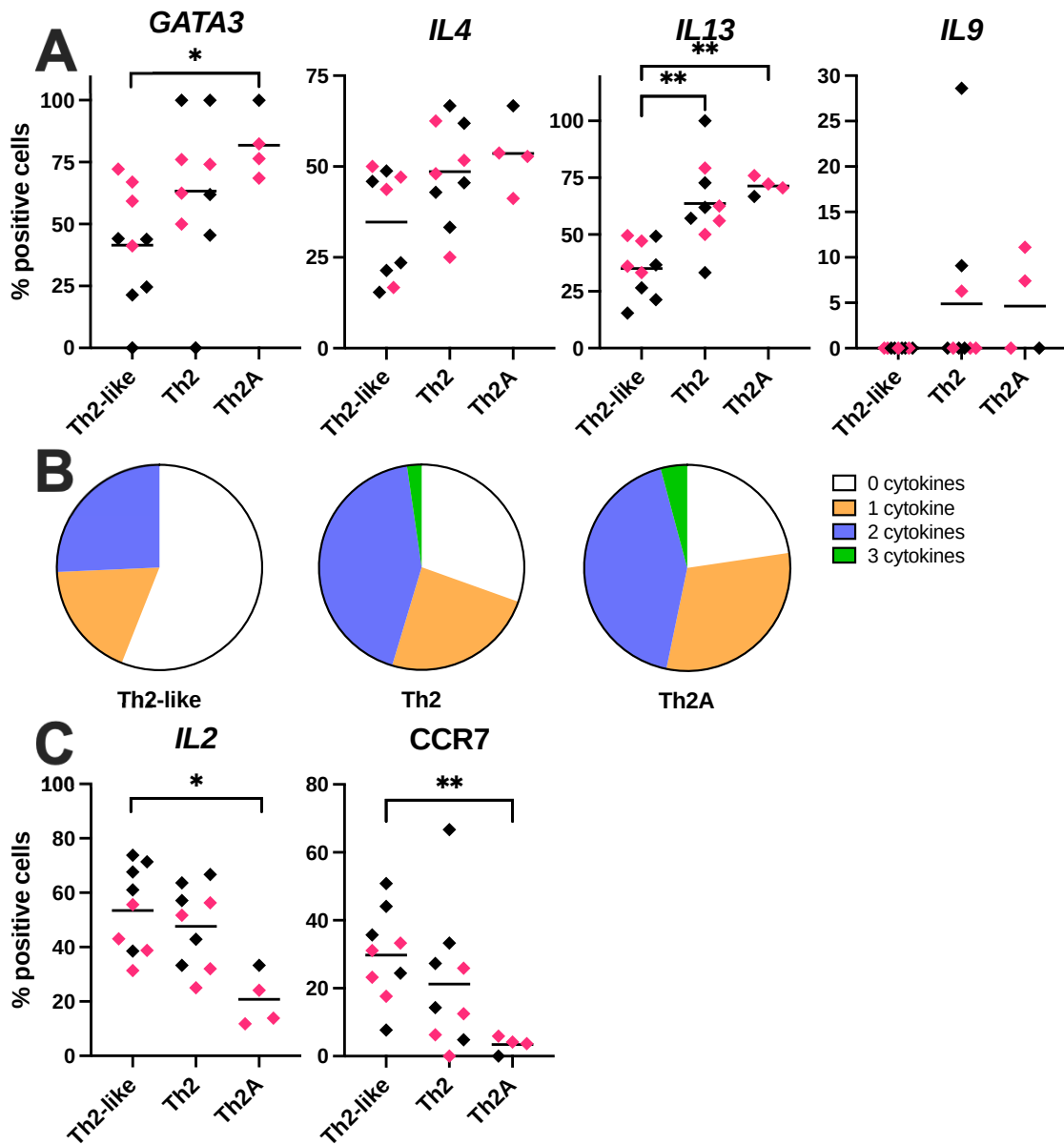
Figure E7: T-cell epitopes recognized by *in vitro*-expanded dog allergen-specific T-cell clones. Proliferative responses, plotted as counts per minute (CPM), of *in vitro*-expanded dog allergen-specific CD4⁺ T cell clones in response to dog allergens (rCan f 1, rCan f 4, rCan f 5) and to peptides containing T-cell epitopes of these allergens. Medium was used as a negative control. Bars represent the mean of three replicate wells with SEM.

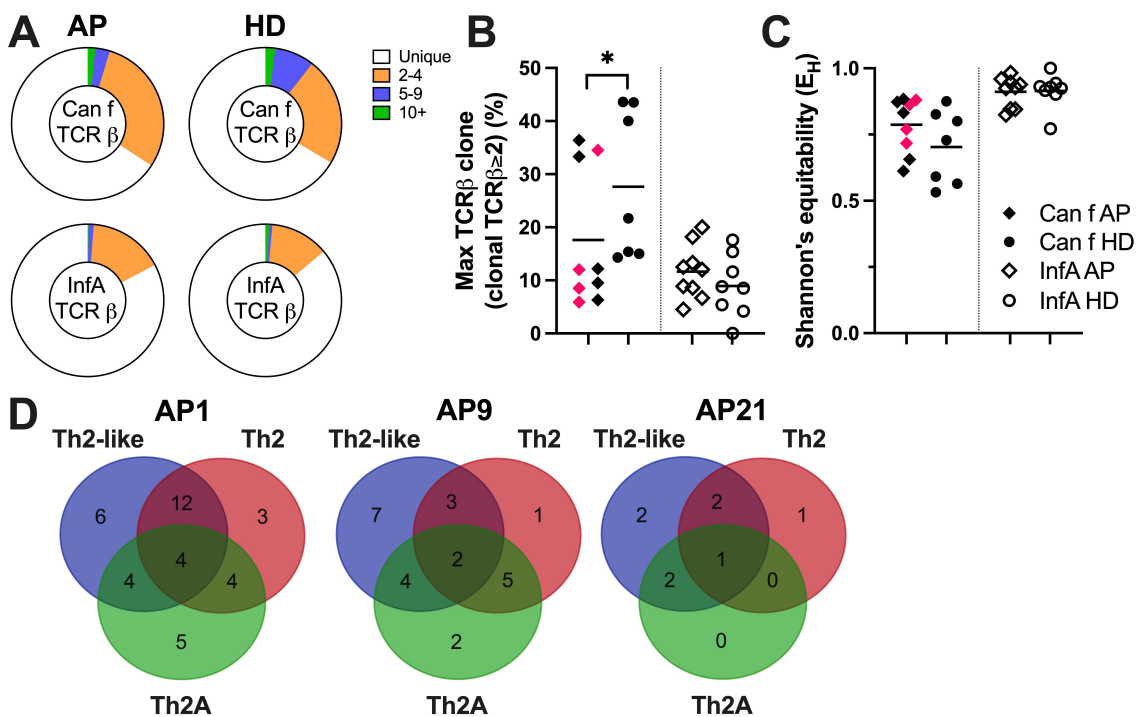
Figure E8: Cytokine secretion by antigen-specific T-cell clones. (A) Cytokine levels in the supernatants of anti-CD2/CD3/CD28-stimulated dog allergen (Can f)- or influenza (InfA)-specific CD4⁺ T-cell clones. (B) Cytokine production by dog allergen-specific T-cell clones originally sorted from different *ex vivo* Th2 subsets. Each data point represents an individual clone. Mann-Whitney U-test and Kruskal-Wallis test with Dunn's multiple comparisons test were used for statistical analyses. *p<0.05. **p<0.01.

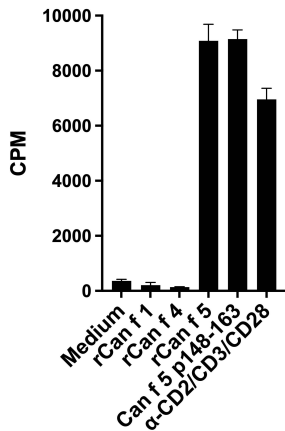
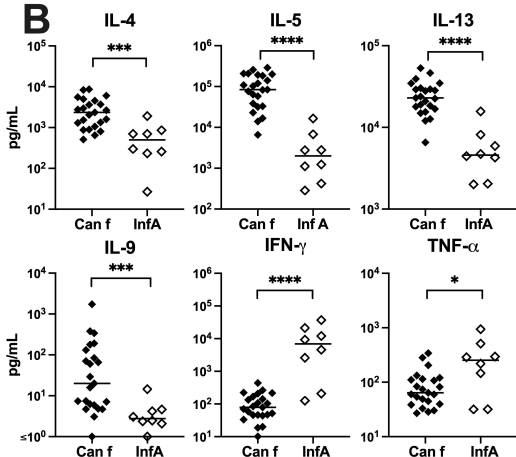
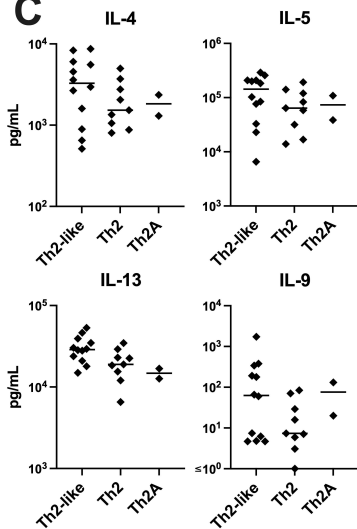
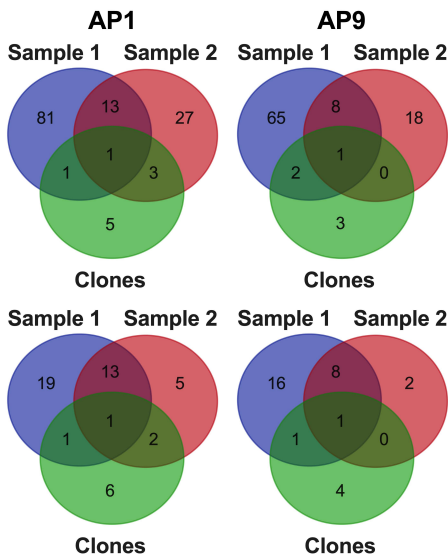
A**B****C****D**









A**B****C****D**

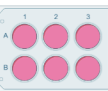
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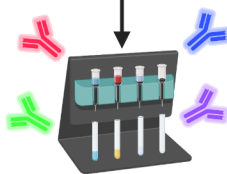
Healthy subjects



PBMC isolation

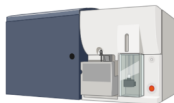


PBMC stimulation (16h)
Can f or InfA MP1

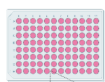


CD154-based magnetic enrichment
and fluorescent antibody staining

Single-cell
index sorting



Single-cell
index sorting

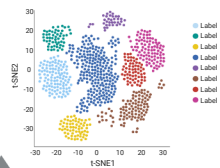


In vitro
expansion

Sequencing



Transcriptome

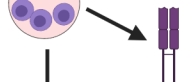


Immunophenotype

Epitope specificity



TCR repertoire

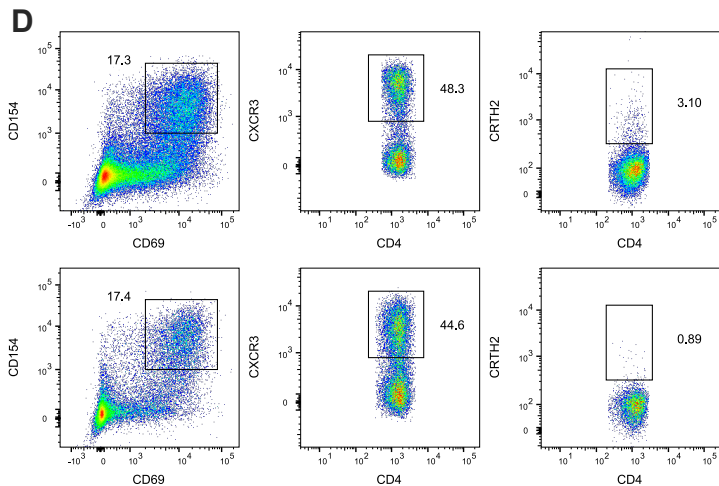
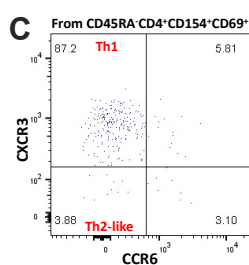
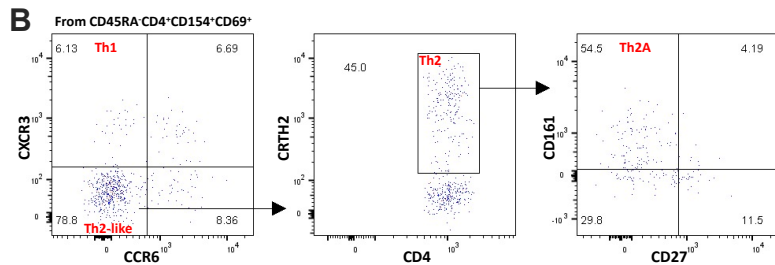
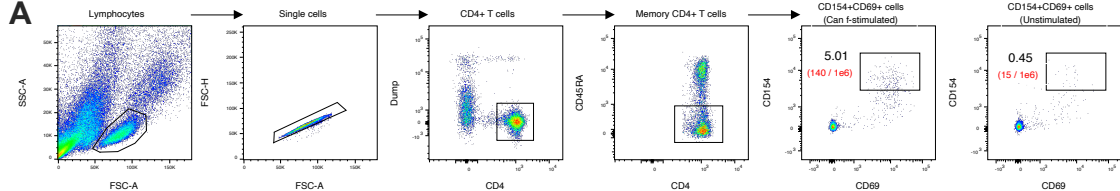


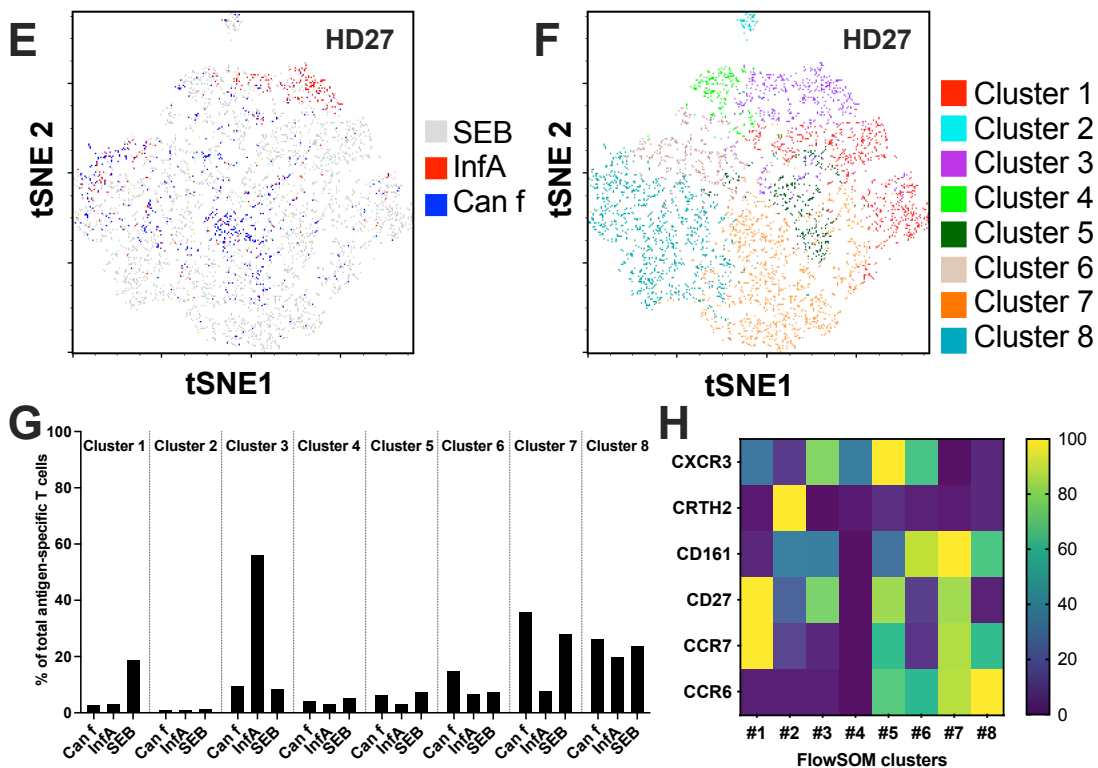
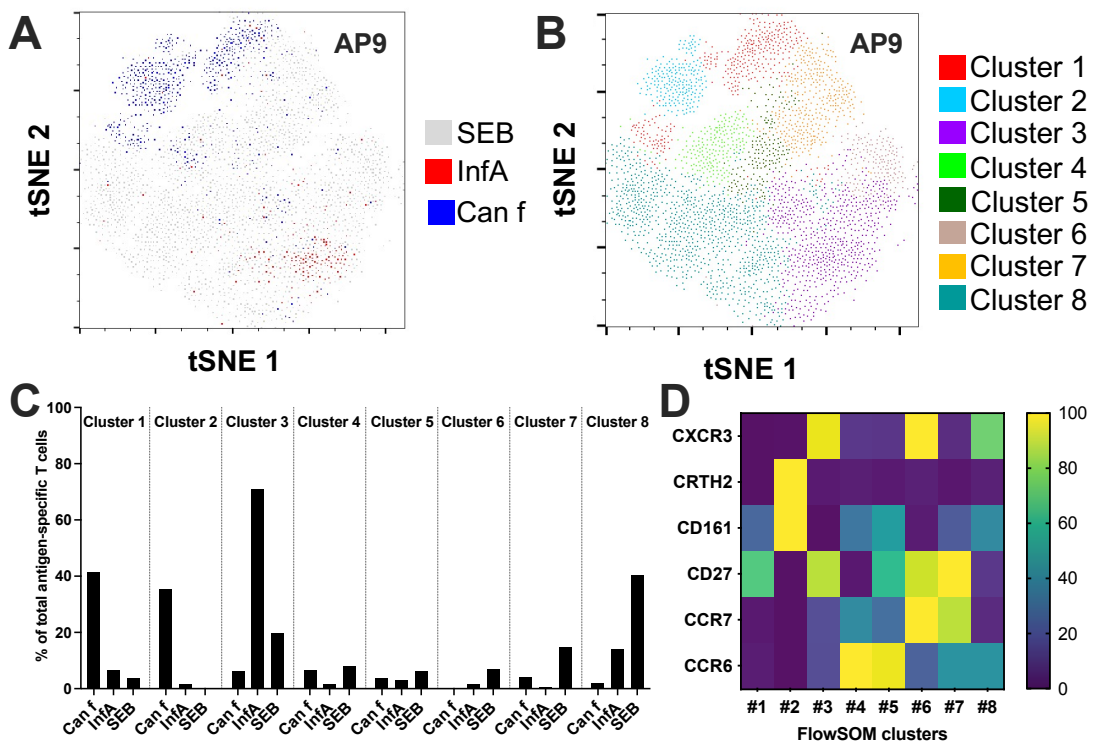
Cytokine secretion

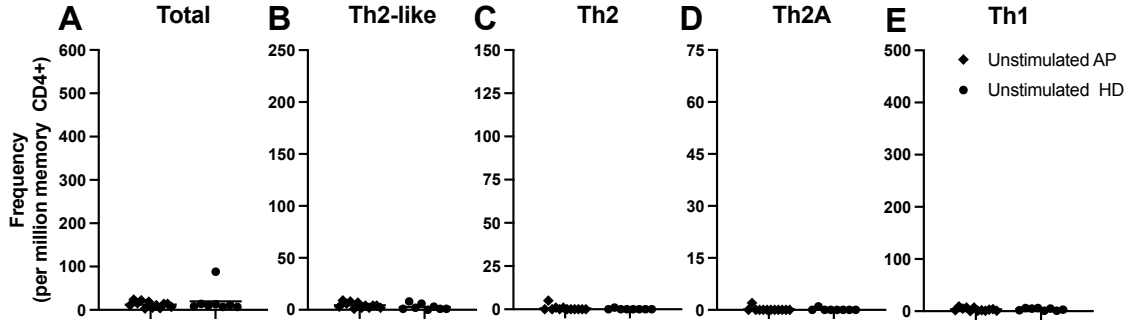


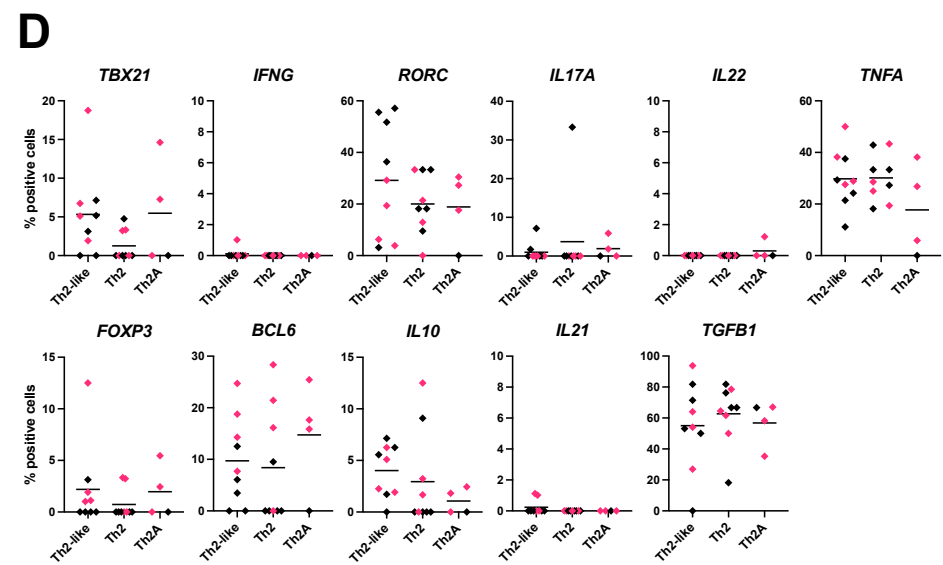
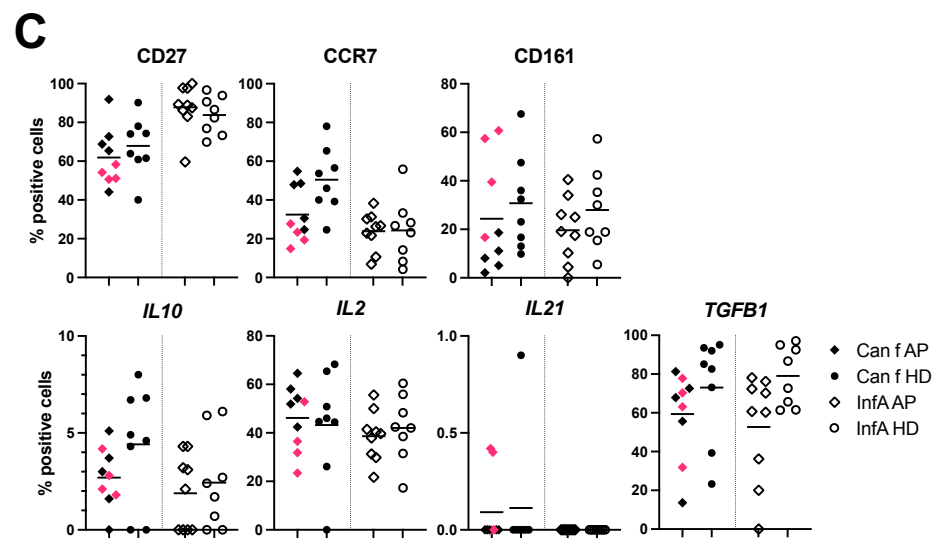
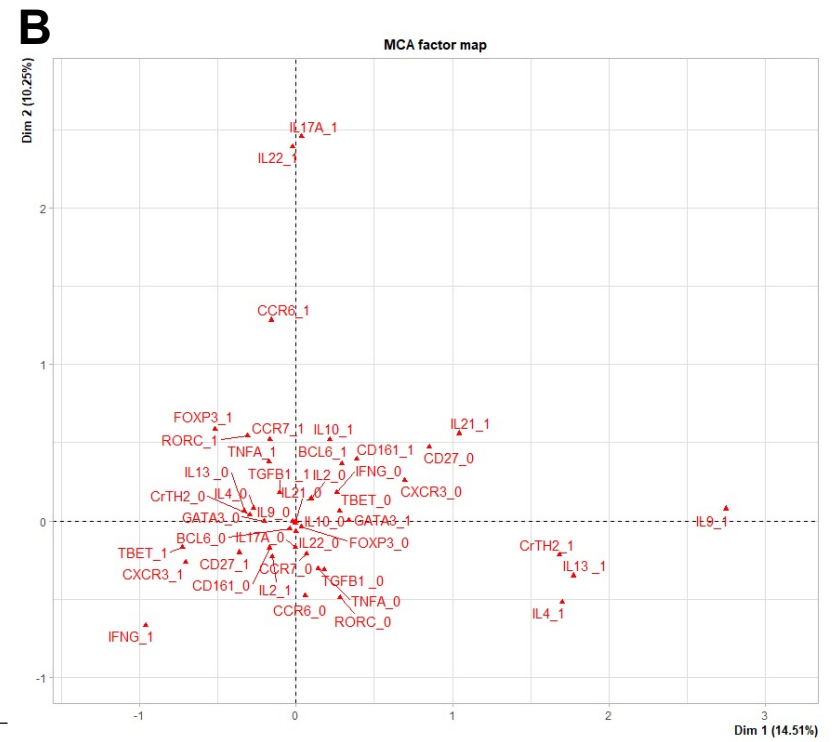
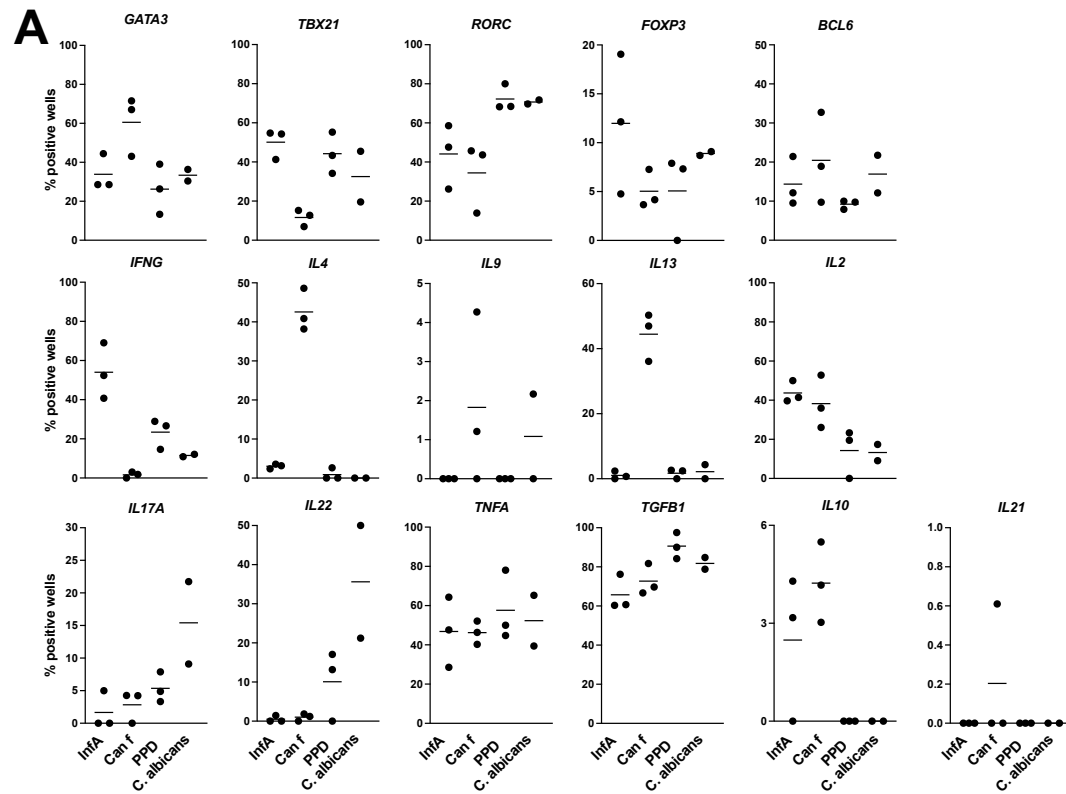
TCR repertoire

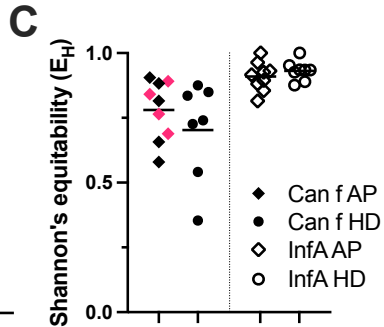
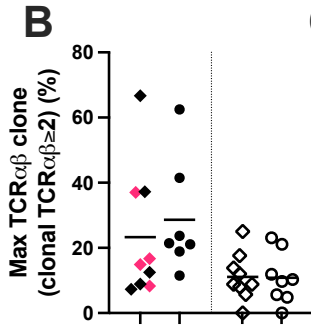
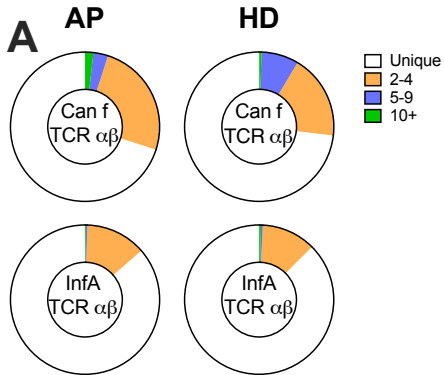


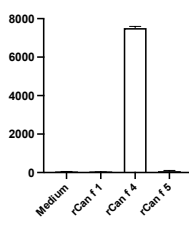
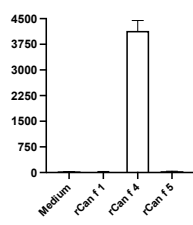
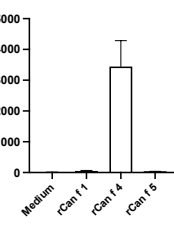
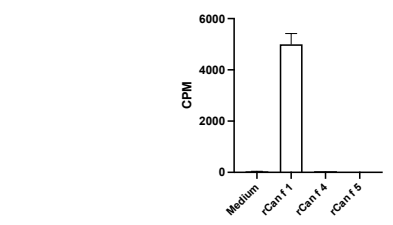
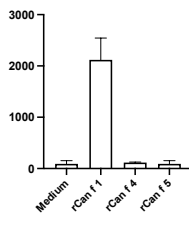
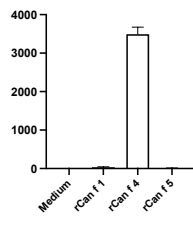
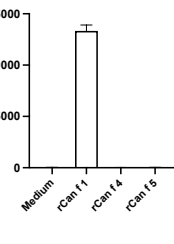
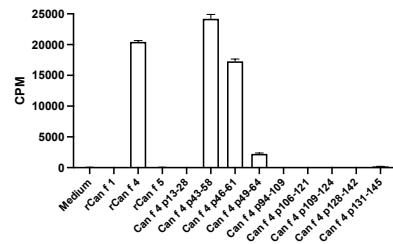
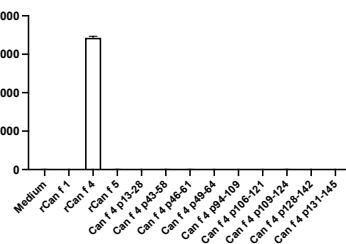
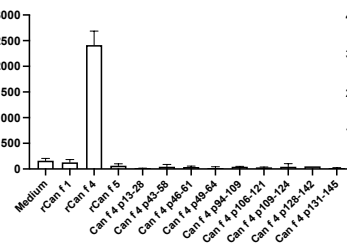
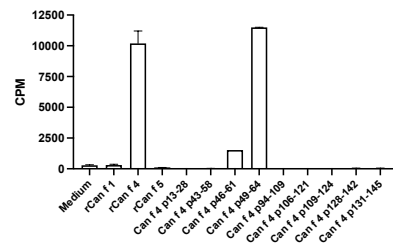
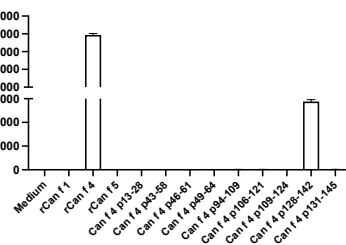
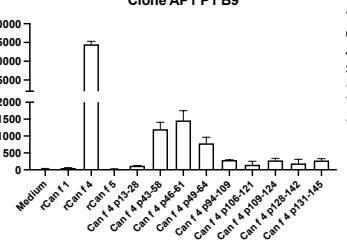
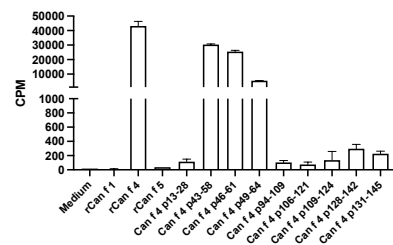
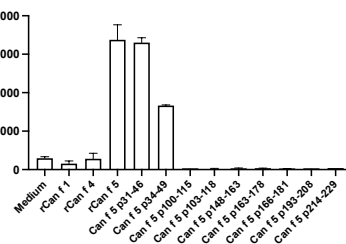
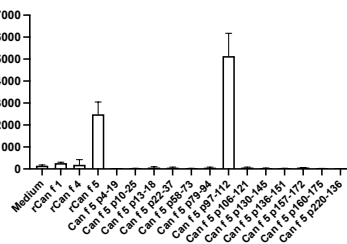
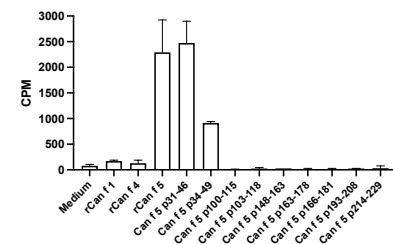
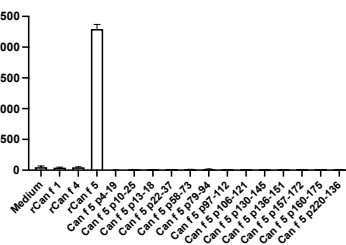
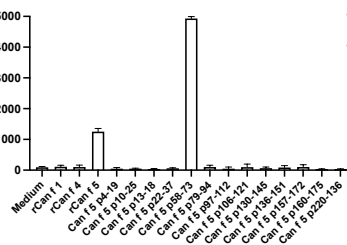
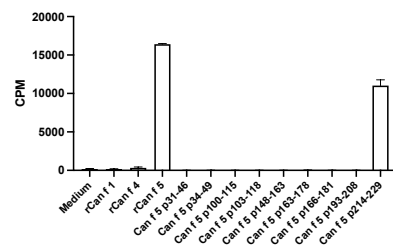
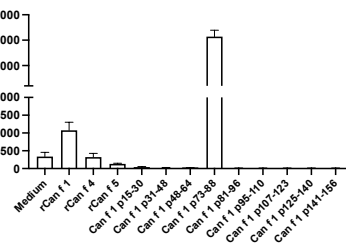
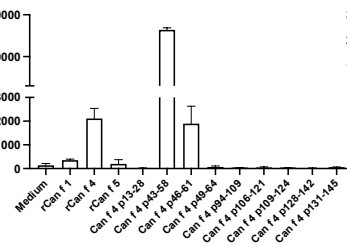
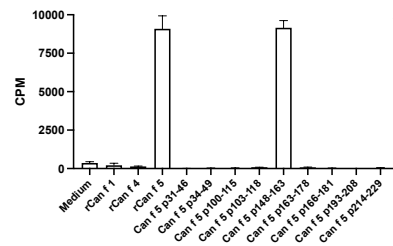


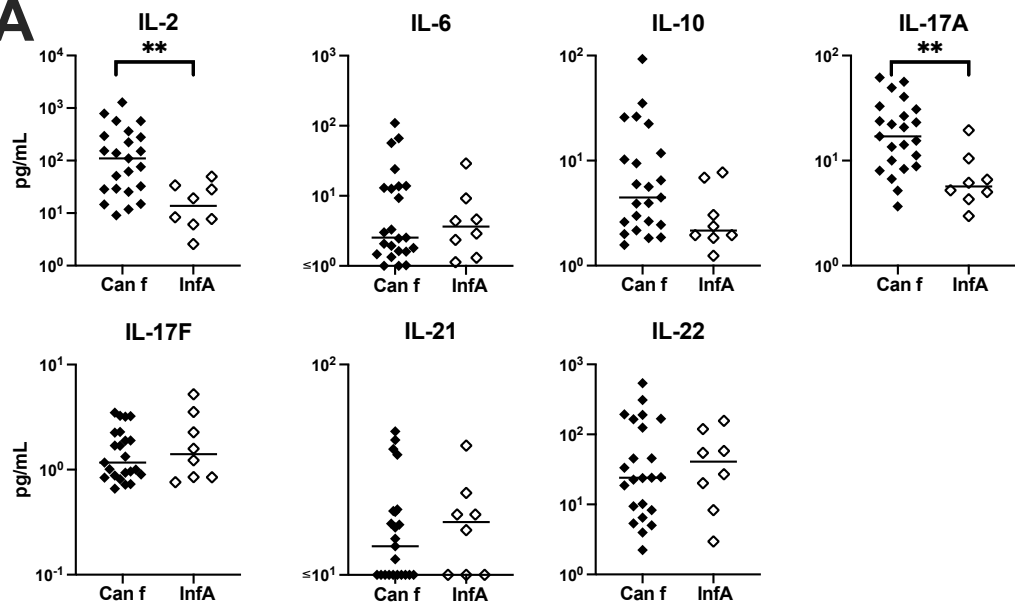










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