Single-cell characterization of dog allergen–specific T cells reveals T_H2 heterogeneity in allergic individuals

Céline Vandamme, PhD,[a]* Marja Rytkönen-Nissinen, PhD,[a] Tapio Lönnberg, PhD,[b,c] Jukka Randell, MD, PhD,[d] Rauno J. Harvima, MD, PhD, DSc,[e] Tuure Kinnunen, MD, PhD,[a,f] and Tuomas Virtanen, MD, PhD[a] *Kuopio and Turku, Finland*

Background: Allergen-specific type 2 $CD4^+$ T_H2 cells are critically involved in the pathogenesis of IgE-mediated allergic diseases. However, the heterogeneity of the T_H2 response has only recently been appreciated.

Objective: We sought to characterize at the single-cell level the *ex vivo* phenotype, transcriptomic profile, and T-cell receptor (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 T_H2 type. T_H2 cells could be phenotypically further divided into 3 subsets, which consisted of T_H2-like ($CCR6^-CXCR3^-CRTH2^-$), T_H2 ($CCR6^-CXCR3^-CRTH2^+CD161^-$), and T_H2A ($CCR6^-CXCR3^-CRTH2^+CD161^+CD27^-$) cells. All these subsets were nonexistent within the allergen-specific T-cell repertoire of healthy subjects. Single-cell transcriptomic profiling confirmed the T_H2-biased signature in allergen-specific T cells from allergic subjects and revealed a T_H1/T_H17 signature in nonallergic subjects. TCR repertoire analyses showed that dog allergen–specific T cells were diverse and allergic subjects demonstrated less clonality compared to nonallergic donors. Finally, TCR and transcriptomic analyses revealed a close relationship between T_H2-like, T_H2, and T_H2A cells, with the last ones representing the most terminally differentiated and highly polarized subtype.

Conclusions: Our study demonstrates heterogeneity within allergen-specific T_H2 cells at the single-cell level. The results may be utilized for improving immune monitoring after allergen immunotherapy and for designing targeted immunomodulatory approaches. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

***Key words:** T cell, T_H2, T_H2A cell, CD154, dog allergy, Can f 1, Can f 4, Can f 5*

T_H2-type $CD4^+$ T cells play 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 T_H2-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 T_H2 response observed in allergic individuals is more heterogeneous than previously appreciated.[1] Early studies suggested the presence of a separate subpopulation within T_H2 cells that could be characterized by a reduced expression of CD27 but elevated expression of CRTH2, IL-4, IL-5, IL-13, and GATA3.[2,3] More recent studies have confirmed the presence of this highly differentiated, polyfunctional subset, termed either T_H2A or peT_H2 cells.[4-6] Compared to more conventional T_H2 cells, these cells express high levels of the surface markers CD161 and CD49d and T_H2 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 T_H2 response.[4-6] Studies have confirmed that a large fraction of circulating allergen-specific T cells in allergic individuals has a T_H2A signature,[5,7-9] and that these cells are preferentially deleted during immunotherapy.[5,10] Moreover, T_H2A 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 study that used single-cell RNA sequencing in a mouse model of asthma revealed substantial heterogeneity within $CD4^+$ T-cell subsets and

From [a]the Department of Clinical Microbiology, Institute of Clinical Medicine, University of Eastern Finland, Kuopio, [b]the Turku Bioscience Centre, University of Turku and Åbo Akademi University, Turku, [c]the InFLAMES Research Flagship Center, University of Turku, Turku, [d]the Department of Pulmonary Diseases, Kuopio University Hospital, Kuopio, [e]the Department of Dermatology, Kuopio University Hospital and University of Eastern Finland, Kuopio, and [f]Eastern Finland Laboratory Centre (ISLAB), Kuopio.

*C. Vandamme, PhD, is currently at Janssen Pharmaceutica NV, Beerse, Belgium.

The last 2 authors contributed equally to this article, and both should be considered senior author.

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Corresponding author: Tuure Kinnunen, MD, PhD, Department of Clinical Microbiology, Institute of Clinical Medicine, University of Eastern Finland, Yliopistonranta 1C, FIN-70210, Kuopio, Finland. E-mail: tuure.kinnunen@uef.fi.

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Abbreviations used
InfA: Influenza A
PBMC: Peripheral blood mononuclear cell
PPD: Tuberculin purified protein derivative
SEB: *Staphylococcus* enterotoxin B
TCR: T-cell receptor

detected a previously unidentified subset responding to type 1 interferons.[11] To our knowledge, only 2 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 allergens,[8] and the second, larger study analyzed >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 T_H2A phenotype within these cells.[8,12]

Dog-derived proteins are a common cause of respiratory hypersensitivity.[13] To date, 7 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, Can f 4, and Can f 5 are dominantly T_H2 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 12 clinically diagnosed dog-allergic patients (age mean ± SD 41 ± 18 years, range 19-69 years) and 8 nonallergic donors (age 42 ± 15 years, range 26-64 years) (see Table E1 in this article's Online Repository at www.jacionline.org). The allergic subjects were characterized at the Pulmonary Clinic of Kuopio University Hospital. They exhibited positive skin prick tests to dog dander extract as well as 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, 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 before 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 described[17-19]), *Staphylococcus* enterotoxin B (SEB; Sigma-Aldrich, St Louis, Mo), influenza A (H1N1) MP1 peptide pool (InfA MP1; Miltenyi Biotec, Bergisch Gladbach, Germany), tuberculin purified protein derivative (PPD; Statens Serum Institute, Copenhagen, 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 (Shanghai, China).[15,16,21]

CD154 enrichment assay

The experimental approach is summarized in Fig E1 in the Online Repository available at www.jacionline.org. 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 75 × 10^6 freshly isolated PBMCs for 16 hours 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, 1 × 10^6 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 mmol L-glutamine, 20 μmol 2-mercapthoethanol, 1 mmol sodium pyruvate, nonessential amino acids, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 mmol HEPES (all from Lonza, Basel, Switzerland), and 5% inactivated human AB serum (Sigma-Aldrich). For optimal detection of CXCR3 and CRTH2 expression, the cells were stained with anti–CXCR3-BV510 and anti–CRTH2-BV421 for 20 minutes at 37°C before the 16-hour stimulation (see Fig E2 in the Online Repository available at www.jacionline.org). 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 minutes at room temperature with a panel of monoclonal antibodies (see Table E2 in this article's Online Repository at www.jacionline.org). Counting beads (Sphero AccuCount Fluorescent Particles; Spherotech, Lake Forest, Ill) were added to each sample for absolute cell counts. The samples were acquired and sorted with a FACSAria III cell sorter (BD Biosciences, San Jose, Calif), and data were analyzed by FlowJo v10 software (FlowJo, Ashland, Ore).

Targeted gene expression analysis and sequencing of single-cell paired TCR αβ chains

After the CD154 enrichment assay, memory $CD4^+CD69^+CD154^+$ T cells from Can f–, InfA MP1–, SEB-, PPD-, or *C albicans*–stimulated PBMCs were single-cell sorted on FACSAria III using the index-sorting mode to 96-well PCR plates (4titude, Dorking, UK) containing 10 μL of 1× One-Step RT-PCR buffer (Qiagen, Hilden, Germany). The expression of 16 mRNA transcripts as well as paired TCR αβ sequences were simultaneously assessed by a series of nested PCR reactions, as previously described[23] (see Table E3 in this article's Online Repository at www.jacionline.org). The nested amplification protocol was modified from the original version, reducing the reaction volumes to 18 μL and separating TCR α and TCR β amplification steps to improve efficiency. Finally, the generated amplified sequences were combined, gel purified and sequenced using 500 cycle v2 MiSeq reagents (Illumina, San Diego, Calif). Demultiplexing data for individual wells and counting effector gene transcripts were performed using previously described algorithms.[23] Multiple correspondence analysis was performed by FactoMiner.[24] The evenness of the TCR β or paired TCR αβ CDR3 repertoires was quantified using Shannon equitability index (EH), which is the Shannon diversity index divided by the maximum diversity (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 an online tool (bioinformatics.psb.ugent.be/webtools/Venn/).

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

After CD154 enrichment assay, memory $CD4^+CD69^+CD154^+$ T 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 Epstein-Barr virus–transformed B cells. T-cell clones were expanded in the presence of 1 μg/mL phytohemagglutinin (Oxoid, Basingstoke, UK) and 50 IU/mL IL-2 (Miltenyi) 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) 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 μg/mL) or peptides (1 μg/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 72 hours. The production of 13 cytokines was measured by the LEGENDplex Human Th Cytokine Panel kit (BioLegend, San Diego, Calif) according to the manufacturer's instructions. Samples were acquired with the NovoCyte Quanteon flow cytometer (Agilent, Santa Clara, Calif) and analyzed with the LEGENDplex Data Analysis software (BioLegend).

Statistical analyses

Data were analyzed by GraphPad Prism v8 (GraphPad Software, San Diego, Calif). Tests used to establish statistical significance are indicated in the figures. Values of $P < .05$ were considered to indicate statistical significance.

RESULTS

Dog allergen–specific memory CD4$^+$ T cells exhibit phenotypic heterogeneity within the T$_H$2 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 SEB were used as controls. After a 16-hour 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, CRTH2, 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 and analyzed it using an unsupervised clustering algorithm, FlowSOM.[28] In 2 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 1, *A* and *B,* and see Fig E3 in the Online Repository available at www.jacionline.org). 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 1, *C*). Cells in cluster 2 displayed a CXCR3$^-$CCR6$^-$CRTH2$^+$CD161$^-$ and cells in cluster 3 a CXCR3$^-$CCR6$^-$CRTH2$^+$CD161$^+$CD27$^-$ phenotype (Fig 1, *D*), which correspond, respectively, to the classification of conventional T$_H$2 and pathogenic effector T$_H$2A subsets, as described elsewhere.[4,5] Cluster 1 displayed a CXCR3$^-$CCR6$^-$CRTH2$^-$CD161$^-$ phenotype, which we termed T$_H$2-like because T$_H$2 cells do not uniformly express CRTH2[29] and the CXCR3$^-$CCR6$^-$ T-cell fraction has previously been shown to contain T cells with T$_H$2 characteristics.[16,25-27] Finally, cluster 4, which contained the majority of influenza-specific cells, displayed a CXCR3$^+$CCR6$^-$CRTH2$^-$CD161$^-$ T$_H$1 phenotype (Fig 1, *D*).[16,25-27] In contrast to allergic subjects, in a representative nonallergic healthy donor, dog allergen–specific T cells were enriched in clusters displaying either a CXCR3$^-$CCR6$^+$ T$_H$17 or CXCR3$^+$CCR6$^+$ T$_H$1/T$_H$17 phenotype (Fig E3). In conclusion, dog allergen–specific memory CD4$^+$ T cells in allergic donors can be subdivided to at least 3 phenotypically differing subsets, designated T$_H$2-like, T$_H$2, and T$_H$2A.

Frequencies of dog allergen–specific T$_H$2 cells are elevated in allergic patients

Next, using traditional biaxial gating, we determined the *ex vivo* frequencies of total, T$_H$2-like (CXCR3$^-$CCR6$^-$CRTH2$^-$), T$_H$2 (CXCR3$^-$CCR6$^-$CRTH2$^+$CD161$^-$), T$_H$2A (CCR6$^-$CXCR3$^-$CRTH2$^+$CD161$^+$CD27$^-$), and T$_H$1 (CCR6$^-$CXCR3$^+$) dog allergen– and influenza-specific memory CD4$^+$ T cell frequencies within PBMCs from 12 allergic patients and 8 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 of 12 allergic patients and 1 of 8 healthy subjects exceeding 100 dog allergen–specific T cells per 1 million memory CD4$^+$ T cells (Fig 2, *A*). Importantly, however, allergic patients had markedly elevated frequencies of T$_H$2-like, T$_H$2, and T$_H$2A dog allergen–specific T cells compared to healthy donors, who virtually had no dog allergen–specific T cells within these T$_H$2 subsets (mean frequencies per million memory CD4$^+$ T cells: 72 vs 6 cells for T$_H$2-like cells; 34 vs 0 cells for T$_H$2 cells; and 17 vs 0 cells for T$_H$2A cells) (Fig 2, *B-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 T$_H$1 subset (Fig 2, *A* 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

FIG 1. Dog allergen–specific memory $CD4^+$ T cells from allergic subjects exhibit phenotypic heterogeneity within the T_H2 compartment. (**A**) t-Distributed stochastic neighbor embedding (tSNE) plot displaying memory $CD4^+CD154^+CD69^+$ T cells from Can f– (*blue dots*), InfA 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 overlayed 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**) Heat map representing row-normalized marker expression (median fluorescence intensities [MFI]) within each FlowSOM cluster.

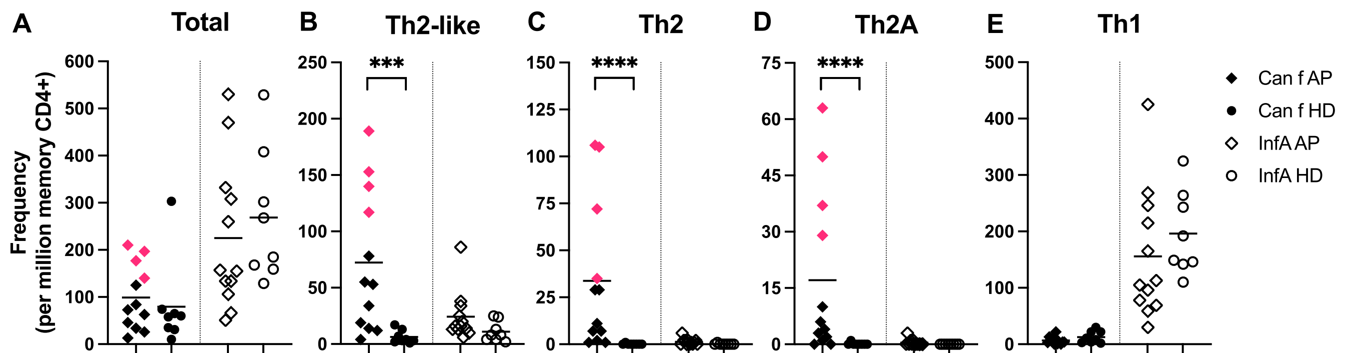

FIG 2. Frequencies of dog allergen–specific T_H2 cells are elevated in allergic patients. *Ex vivo* frequencies of total (**A**), T_H2-like (**B**), T_H2 (**C**), T_H2A (**D**), and T_H1 (**E**) dog allergen (Can f)- and InfA-specific memory $CD4^+$ T cells from 12 allergic patients (AP) and 8 healthy donors (HD). The background from unstimulated cells (Fig E4) was subtracted. T_H1 cells were defined as $CCR6^-CXCR3^+$, T_H2-like cells as $CCR6^-CXCR3^-CRTH2^-$, T_H2 cells as $CCR6^-CXCR3^-CRTH2^+CD161^-$, and T_H2A cells as $CCR6^-CXCR3^-CRTH2^+CD161^+CD27^-$. The 4 allergic patients with the highest frequencies of T_H2-polarized cells, in particular T_H2A cells, are indicated in *red*. Mann-Whitney *U* test was used for statistical analyses. $^{***}P < .001$, $^{****}P < .0001$.

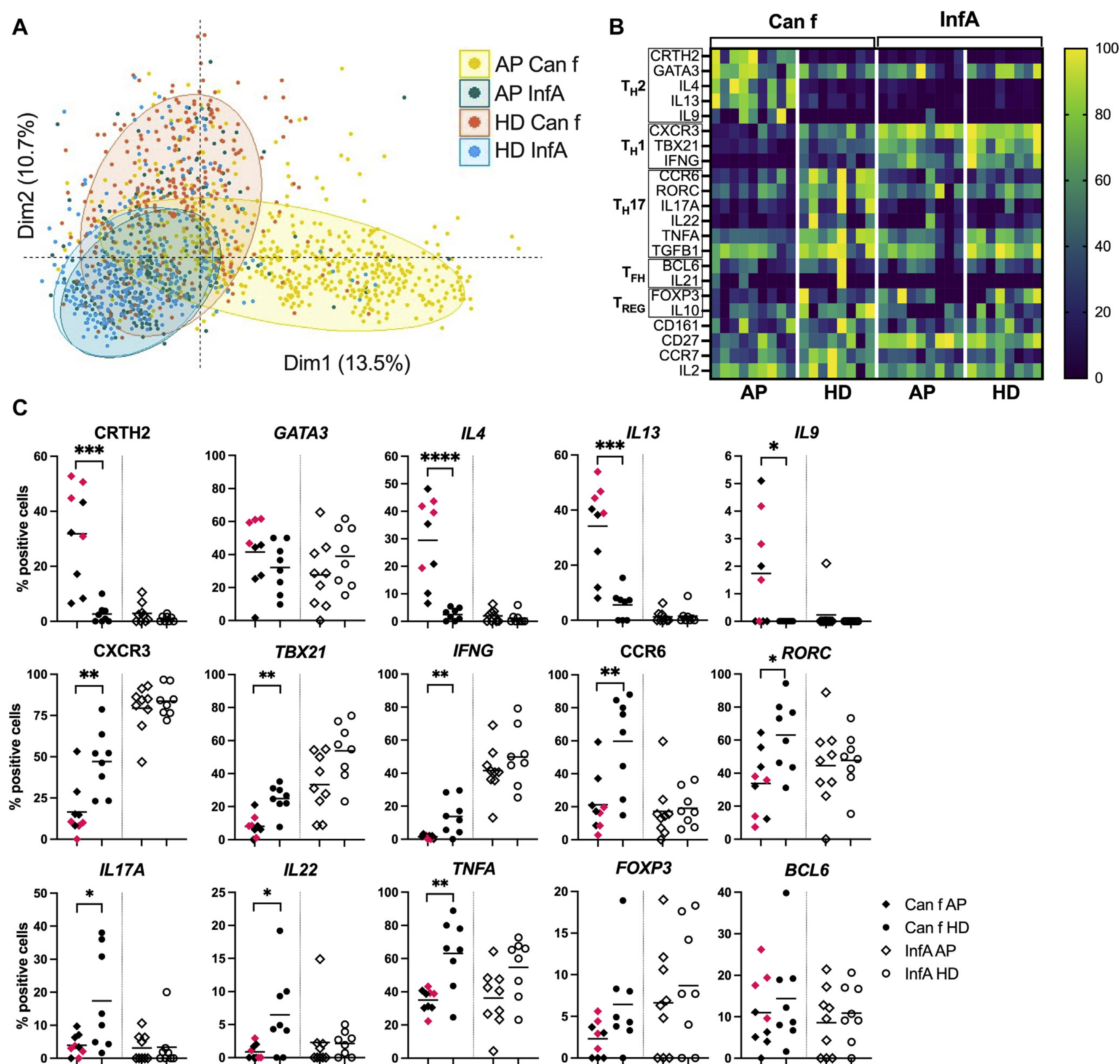

FIG 3. Single-cell transcriptomic profiling confirms phenotypic differences between dog allergen–specific T cells from allergic and nonallergic donors. Memory CD4$^+$CD154$^+$CD69$^+$ T cells from dog allergen (Can f)- and InfA-stimulated PBMCs from 9 allergic patients (AP) and 8 healthy donors (HD) 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**) Heat map 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 4 allergic patients with the highest frequencies of T_H2-polarized cells (Fig 2) are indicated in *red.* Mann-Whitney *U* test was used for statistical analyses. $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

TNFA) through a plate-based targeted expression analysis.[23] In total, we analyzed 1402 dog allergen–specific and 1006 influenza-specific single T cells (see Table E4 in this article's Online Repository at www.jacionline.org). For 3 patients, we also separately sorted *C 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 (see Fig E5 in the Online Repository available at www.jacionline.org). The expression of cell surface markers and transcriptomic data were combined and subjected to multiple correspondence

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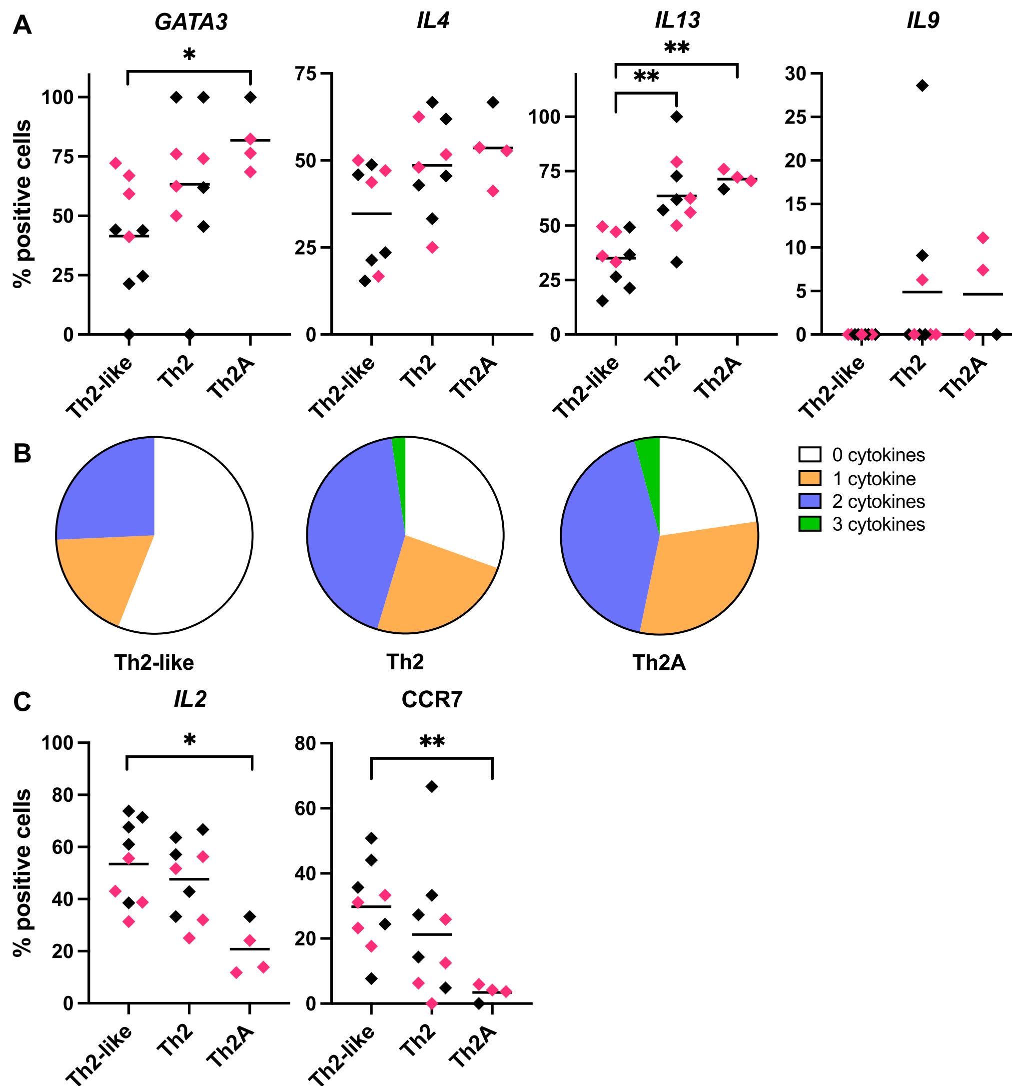

FIG 4. Transcriptomic heterogeneity within dog allergen–specific T_H2 cells in allergic subjects. (**A**) Percentages of dog allergen–specific memory $CD4^+$ T cells from different *ex vivo* T_H2 subsets expressing T_H2-associated transcripts. (**B**) Frequencies of single dog allergen–specific memory $CD4^+$ T cells within different *ex vivo* T_H2 subsets expressing 0 to 3 T_H2 cytokine (*IL4, IL13,* and *IL9*) transcripts. (**C**) Percentages of dog allergen–specific memory $CD4^+$ T cells from different *ex vivo* T_H2 subsets expressing *IL2* or *CCR7.* The 4 allergic patients with the highest frequencies of T_H2-polarized cells (Fig 2) are indicated in *red.* Kruskal-Wallis test with Dunn multiple comparisons test was used for statistical analyses. **P* < .05, ***P* < .01.

analysis to examine the relationships of all single cells analyzed in a reduced dimensional space (Fig 3, *A,* and Fig E5). On the multiple correspondence analysis 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 heat map was also used to visualize the proportion of single cells expressing each marker within the different groups analyzed, demonstrating a preferential expression of T_H2-associated markers by dog allergen–specific T cells from allergic and T_H1/T_H17-associated markers by Can f–specific T cells from healthy subjects, respectively (Fig 3, *B*). We observed that the expression of CRTH2 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 lower, in Can f–specific T cells from allergic patients compared to healthy subjects (Fig 3, *C*). 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 3, *C,* and Fig E5). As expected, influenza-specific T cells predominantly expressed T_H1-associated markers, such as CXCR3, *IFNG,* and *TBX21* (Fig 3, *B* 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 T_H2-associated signature compared to the T_H1/T_H17 signature observed in healthy, nonallergic subjects.

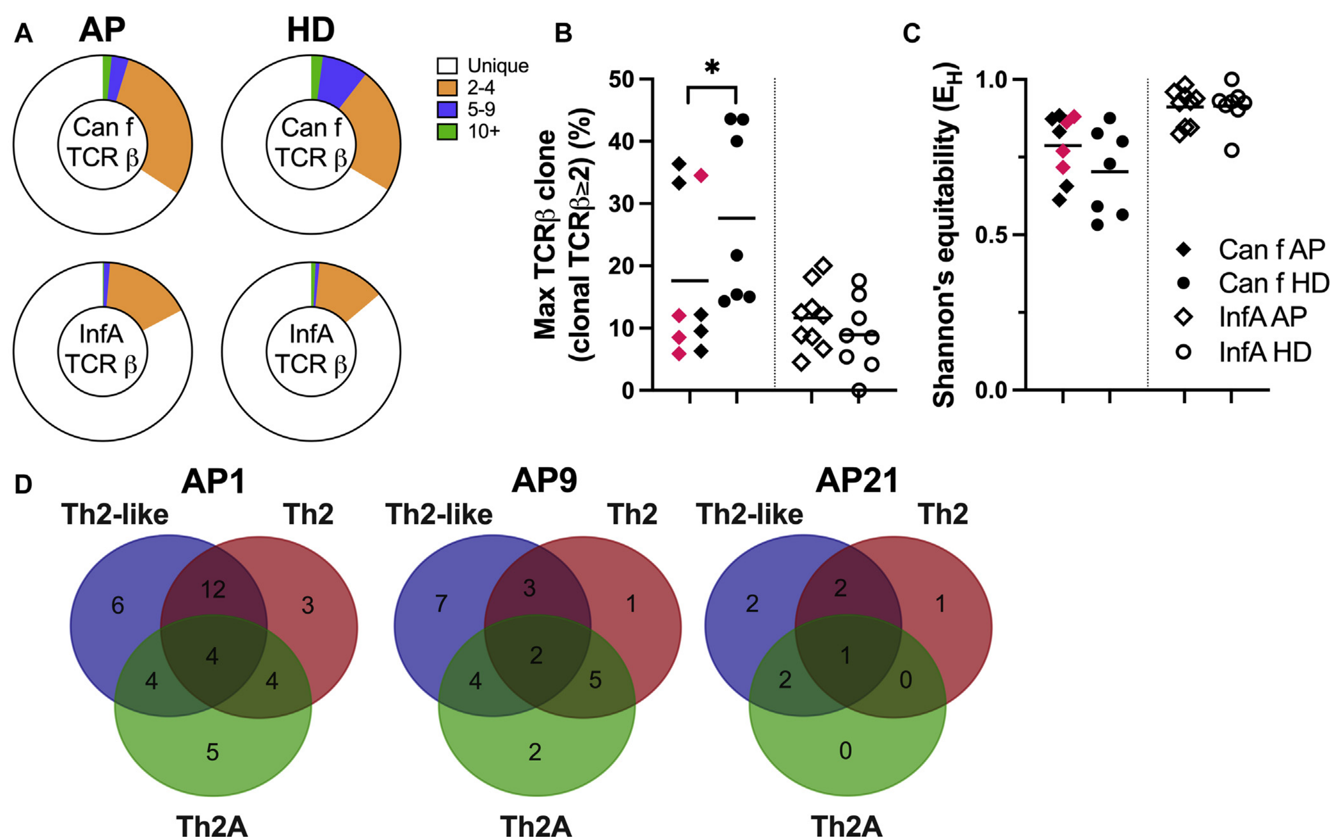

FIG 5. Single-cell TCR sequencing reveals increased diversity within dog allergen–specific T-cell repertoire from allergic patients and a clonal relationship between different dog allergen–specific T_H2 subsets. (**A**) TCR β clonality of dog allergen (Can f)- and InfA-specific memory $CD4^+$ T cells in allergic (AP) and nonallergic (HD) individuals. The proportion of clones with a unique TCR β sequence detected once or more within an individual is indicated. (**B**) Quantification of the most abundant (max) TCR β clone out of all dog allergen– or influenza-specific T cells analyzed from each individual donor. Samples lacking clonal expansions were scored as zero. (**C**) Shannon equitability index calculated in dog allergen– and influenza-specific T cells from each individual donor. The 4 allergic patients with the highest frequencies of T_H2-polarized cells (Fig 2) are indicated in *red*. Mann-Whitney *U* test was used for statistical analyses; $*P < .05$. (**D**) TCR β sequence sharing of dog allergen–specific memory $CD4^+$ T cells between different *ex vivo* T_H2 subsets in 3 allergic patients.

Transcriptomic heterogeneity within dog allergen–specific T_H2 cells in allergic subjects

Next, we separately analyzed the expression of the T_H2-associated transcripts *GATA3*, *IL4*, *IL13,* and *IL9* among T_H2-like, T_H2, and T_H2A dog allergen–specific memory $CD4^+$ T cells from allergic patients (Fig 4, *A*). We observed that the T_H2 and T_H2A subsets displayed a general trend for increasingly higher proportions of cells expressing the T_H2-associated transcripts. This was particularly evident for *GATA3* and *IL13*, which were both expressed by a significantly higher proportion of T_H2A than T_H2-like cells (Fig 4, *A*). Moreover, the expression of *IL9* was observed only within the T_H2 and T_H2A subsets. The T_H2A cells also displayed the highest polyfunctional potential, with 35.9% of single T_H2A cells expressing at least 2 and 3.2% expressing all 3 T_H2 cytokine transcripts compared to 25.7% and 0 of T_H2-like cells, respectively ($P < .0001$ for both, Fisher test; Fig 4, *B*). Finally, T_H2A cells displayed reduced expression of *IL2* and CCR7 compared to T_H2-like cells, suggestive of a more terminally differentiated state (Fig 4, *C*). No differences in the expression of the other analyzed transcripts were observed between the T_H2-like, T_H2, and T_H2A 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 αβ sequencing (Fig 5). On average, we recovered the TCR α or β sequence from 75% and paired α and β TCR sequences from 60% of the T cells sequenced (see Table E5 in this article's Online Repository at www.jacionline.org). The detected TCR sequences were largely private—that is, a particular α or β sequence or combination was only detected in Can f– or influenza-specific T cells from a single individual. Only 5 (0.9%) of 550 unique TCR sequences detected in Can f–stimulated samples and 2 (0.3%) of 589 unique TCR sequences detected in InfA MP1–stimulated samples were shared between 2 different individuals (ie, represent public clones).

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

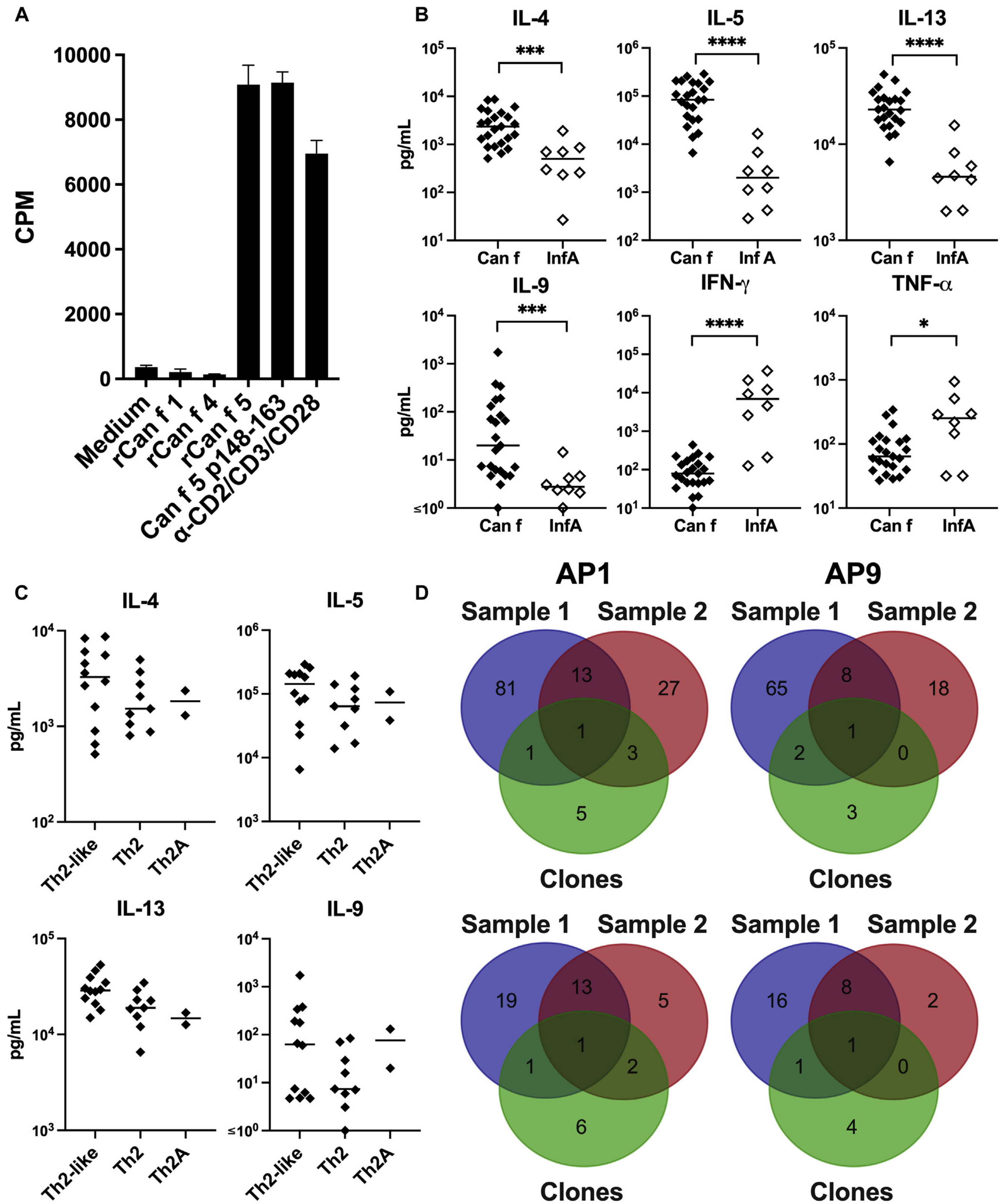

FIG 6. *In vitro*–expanded dog allergen–specific T-cell clones recognize diverse allergen epitopes and exhibit a T_H2-type cytokine secretion profile. Memory $CD4^+CD154^+CD69^+$ T cells from dog allergen (Can f)- and InfA-stimulated PBMCs from 2 allergic patients were single-cell sorted using the index sort mode and expanded *in vitro.* (**A**) Representative proliferative responses, plotted as counts per minute, 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

individual TCRs included in the analyses by 25% compared to paired sequences.

We considered identical TCR β chain sequences expressed by at least 2 separate T cells sorted from the same stimulation to represent clonal expansions *in vivo.* Expanded T-cell clones (sequences expressed by at least 2 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 5, *A*). Among expanded dog allergen–specific TCR sequences, the proportion of sequences shared by at least 5 separate cells was higher in healthy donors (10.5% of sequences) than in allergic patients (4.7% of sequences, $P < .05$, Fisher exact test; Fig 5, *A*). 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 5, *B*). In InfA MP1–stimulated cells, highly expanded T-cell clones (at least 5 separate cells), in contrast, were extremely rare (Fig 5, *A*). Consequently, calculation of the Shannon equitability indexes (with a value of 1 indicating complete diversity) demonstrated that dog allergen–specific TCR sequences were less diverse than influenza-specific sequences (Fig 5, *C*). Largely similar results were obtained through the analysis of paired αβ TCR sequences, although statistical significance was lost as a result of the reduced power in this smaller data set (see Fig E6 in the Online Repository available at www.jacionline.org)

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 (ie, contains fewer mono- or oligoclonal TCR expansions) than that of nonallergic individuals.

Dog allergen–specific T cells within different T_H2 cell subsets are clonally related

We also examined the developmental relationship between T_H2-like, T_H2, and T_H2A dog allergen–specific T cells by assessing whether expanded TCR β sequences were shared between these subsets. In 3 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 β sequences; Fig 5, *D*). In all, these results demonstrate that *in vivo*–expanded clonal dog allergen–specific T cells reside within multiple T_H2 subsets, providing direct support for a common developmental origin of T_H2-like, T_H2, and T_H2A cells.

In vitro–expanded dog allergen–specific T-cell clones recognize diverse epitopes and exhibit a T_H2-type cytokine secretion profile

To further confirm the specificity of the dog allergen–specific memory $CD4^+$ T cells detected *ex vivo* with the CD154 enrichment assay, we resampled 2 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 (see Table E6 in this article's Online Repository at www.jacionline.org). Using a proliferation assay, we first tested whether the clones that had expanded *in vitro* were specific to 1 of the 3 recombinant dog allergens that were used to stimulate PBMCs *ex vivo* (Fig 6, *A,* and see Fig E7 in the Online Repository available at www.jacionline.org). In both patients, most of the T-cell clones responded to one of rCan f 1, rCan f 4, or rCan f 5, thus 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 (see Table E7 in this article's Online Repository at www.jacionline.org). Most of the clones from AP1 (11 of 14), in turn, responded to rCan f 4 with 3 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 6, *B,* and see Fig E8 in the Online Repository available at www.jacionline.org). Dog allergen–specific T-cell clones secreted significantly higher levels of the T_H2-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 T_H1 cells. Of note, allergen-specific T-cell clones deriving *ex vivo* from T_H2-like, T_H2, or T_H2A subsets all produced comparable level of the T_H2 cytokines IL-4, IL-5, IL-13, and IL-9 after *in vitro* culture (Fig 6, *C*). This finding demonstrates that T_H2-like and T_H2 cells have a similar potential as T_H2A cells for highly efficient T_H2 cytokine production after *in vitro* activation and expansion. Of note, clones deriving from T_H2-like cells produced more IL-2 than those deriving from the T_H2 or T_H2A subsets (Fig E8), which may reflect their less differentiated state *ex vivo* (Fig 4, *C*).

Finally, we assessed whether dog allergen–specific TCR β sequences were shared between the *in vitro*–expanded T-cell clones and T cells sampled *ex vivo* from the same donors at the 2 different

used as a negative control and anti-CD2/CD3/CD28 beads as a positive control. *Bars* represent the means of 3 replicate wells with SEMs. (**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**) T_H2 cytokine production by dog allergen–specific T-cell clones originally sorted from different *ex vivo* T_H2 subsets. (**D**) TCR β sequence sharing of dog allergen–specific memory $CD4^+$ T cells between 2 *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* lists all *ex vivo*–detected TCR β sequences included in the analysis; *bottom panel,* only *in vivo* expanded TCR β sequences (the same TCR sequence expressed by at least 2 separate cells) were included in the analysis. Mann-Whitney *U* test was used for statistical analyses. $^*P < .05$, $^{***}P < .001$, $^{****}P < .0001$.

time points (Fig 6, *D*). Several of the TCR β sequences from the established T-cell clones matched sequences detected *ex vivo* (Fig 6, *D*). We also observed that only a few TCR β sequences sampled *ex vivo* were shared over time, suggesting a large clonal diversity of dog allergen–specific $CD4^+$ T-cell responses in allergic subjects. Of note, these shared sequences belonged exclusively to *in vivo* expanded clones (expressed by at least 2 cells at a single time point) (Fig 6, *D*).

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 T_H2-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 T_H2-biased allergen-specific T-cell responses in allergic individuals clustered into at least 3 distinct subsets, which we designated T_H2-like, T_H2, and T_H2A cells (Fig 1). Combining the surface phenotype to transcriptomic data, we confirmed that the T_H2A subset bears characteristics of highly polarized, terminally differentiated effector cells, as previously reported.[4,5] Compared to the other T_H2 subsets, T_H2A cells expressed higher levels of *GATA3* and *IL13* transcripts, higher T_H2 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 T_H2A cells, and the expression of which appears to distinguish T_H2A cells better than *IL4* and *IL13* from the other T_H2 subsets.[4,5]

Many studies have demonstrated an association between the T_H2A signature and activity of allergic inflammation in patients with allergic diseases.[4,6,9,12,30,31] It is therefore conceivable that T_H2A cells could only be present in individuals undergoing chronic allergen exposure and inflammation. Our results are consistent with this hypothesis, as we observed T_H2A cells only in some but not all patients with dog allergy (Fig 2), and consequently the strongest T_H2 signature was detected in these same individuals (Fig 3). Because individuals allergic to animal danders generally avoid exposure to the allergen source, it is possible that T_H2A cell numbers in blood have waned in some of the allergic subjects studied here because of the lack of continuing or recurrent allergen exposure. Indeed, only 3 of 12 of the allergic subjects and 2 of 8 of the nonallergic subjects studied here reported recurring 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 T_H2A cells are related to other T_H2 subsets or represent a unique developmental subset. Using single-cell TCR sequencing, we can directly demonstrate TCR sharing between allergen-specific T cells residing *in vivo* within the T_H2-like, T_H2, and T_H2A subsets (Fig 5). This result supports the hypothesis that these different subsets share a common developmental origin and potentially represent different stages of T_H2 differentiation. The demonstration that *in vitro*–expanded T-cell clones from T_H2-like, T_H2, and T_H2A subsets all produced comparable levels of the T_H2 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 T_H2 effector potential once activated.

The dog allergen–specific T-cell repertoire of nonallergic individuals was devoid of T_H2 cells (Fig 2), and consequently these cells clustered separately from T cells from allergic individuals on the basis of 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 T_H1 phenotype (Fig 3). Allergen-specific T cells in nonallergic individuals rather displayed a mixed T_H1/T_H17 signature with higher expression of CXCR3, *TBX21,* and *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 rather than 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—that is, TCR sequence sharing between different individuals—and most of the sequences observed within an individual were unique. It is important to note, however, that as a result of the rarity of dog allergen–specific T cells in peripheral blood, we likely considerably undersampled 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 2 allergic subjects were sampled at 2 different time points, and these shared sequences belonged exclusively to clones that had undergone expansion *in vivo* (expressed by at least 2 distinct T cells; Fig 6). Our analyses of *in vitro*–expanded T-cell clones also support a wide polyclonal response to dog allergens because the clones derived from the 2 allergic subjects studied in detail recognized multiple different epitopes on all the 3 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 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 by 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 T_H2 phenotype when cultured. 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 approach[23] 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 T_H2 heterogeneity in previous studies that used 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 $FOXP3^+$ Treg cells, 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 $FOXP3^+$ Treg cells 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 to combine transcriptomic analyses and TCR sequencing to the phenotype of single T cells assessed by surface marker expression. We demonstrated a T_H2-biased T-cell response to dog allergens in allergic subjects and a T_H1/T_H17-biased response in nonallergic subjects. The T_H2 response in allergic subjects was also shown to be heterogeneous, and the different T_H2 subsets appeared to be developmentally related and likely reflected different stages of T_H2 polarization. Increased understanding of the single-cell heterogeneity of $CD4^+$ T-cell responses is instrumental for deciphering the pathogenesis of allergic diseases. Different T_H2 subsets could potentially be utilized as a biomarker in allergy or could be separately targeted by immunotherapeutic approaches.

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Key messages

- **Dog allergen–specific blood T cells in allergic subjects are dominantly T_H2 polarized, whereas in nonallergic subjects they are T_H1/T_H17 polarized.**
- **Dog allergen–specific T_H2 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.**

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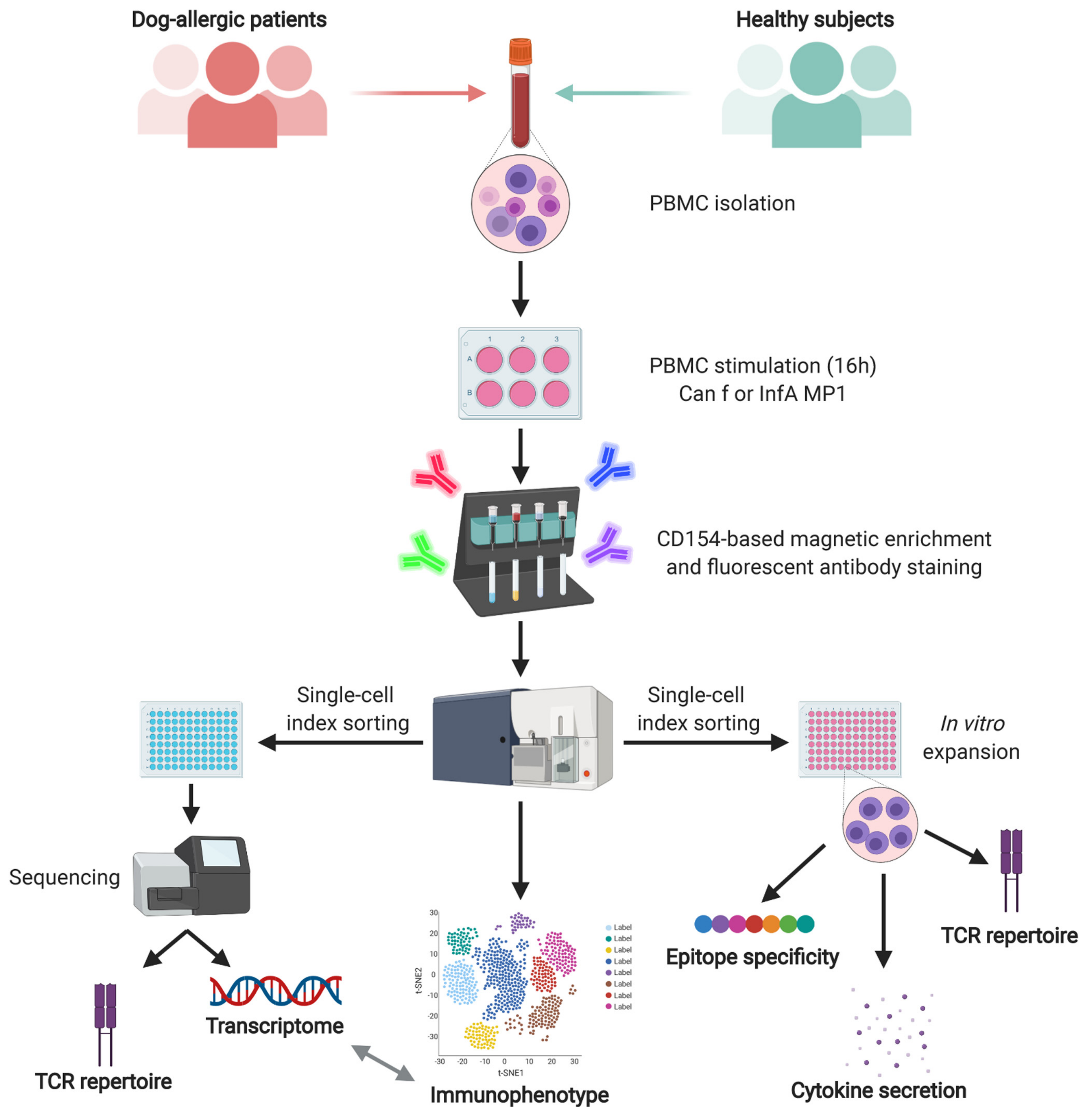

FIG E1. Graphical representation of the work flow used to analyze dog allergen– and influenza-specific $CD4^+$ T cells from allergic patients and healthy donors. Created with Biorender.com.

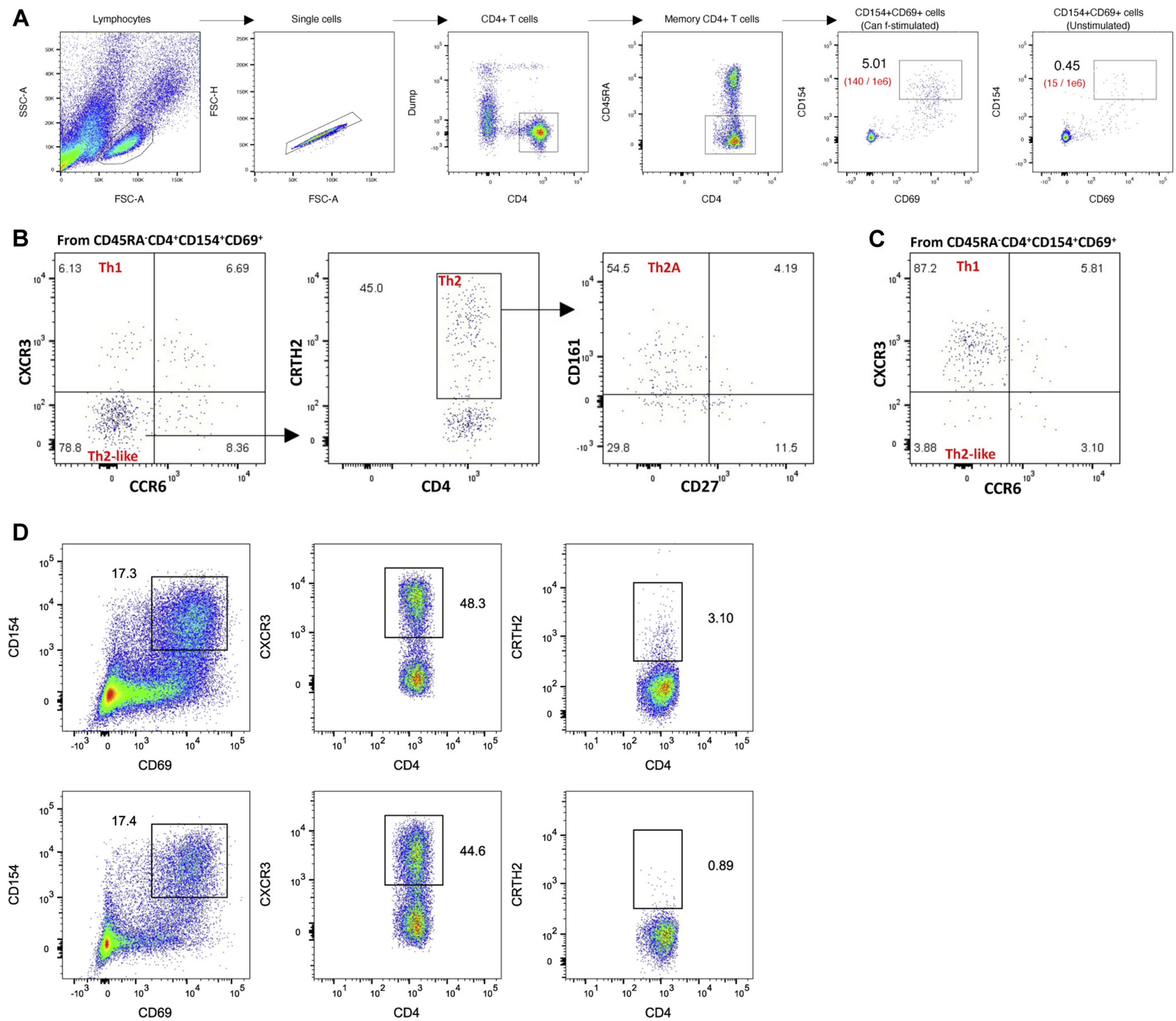

FIG E2. Gating strategies used in the CD154 enrichment assay analyses and optimization of CXCR3 and CRTH2 staining (**A**) Gating strategy used to sort memory $CD4^+CD45RA^-CD154^+CD69^+$ T cells after *ex vivo* stimulation. Lymphocytes were first gated on the basis of forward scatter (FSC) and side scatter (SSC) properties, followed by elimination of doublets through FSC-area (A) and FSC-height (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 in the *last 2 panels.* The calculated frequencies within total memory $CD4^+$ T cells are indicated in *parentheses.* (**B**) Example gating strategy used to analyze T_H2 subsets within $CD4^+CD45RA^-CD154^+CD69^+$ T cells in Can f–stimulated PBMCs from a dog-allergic patient. (**C**) Example of T_H1 subset gating in InfA MP1–stimulated PBMCs from the same dog-allergic patient. (**D**) Similar frequencies of $CD4^+CD45RA^-CD154^+CD69^+$ T cells were observed after a 16-hour 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*).

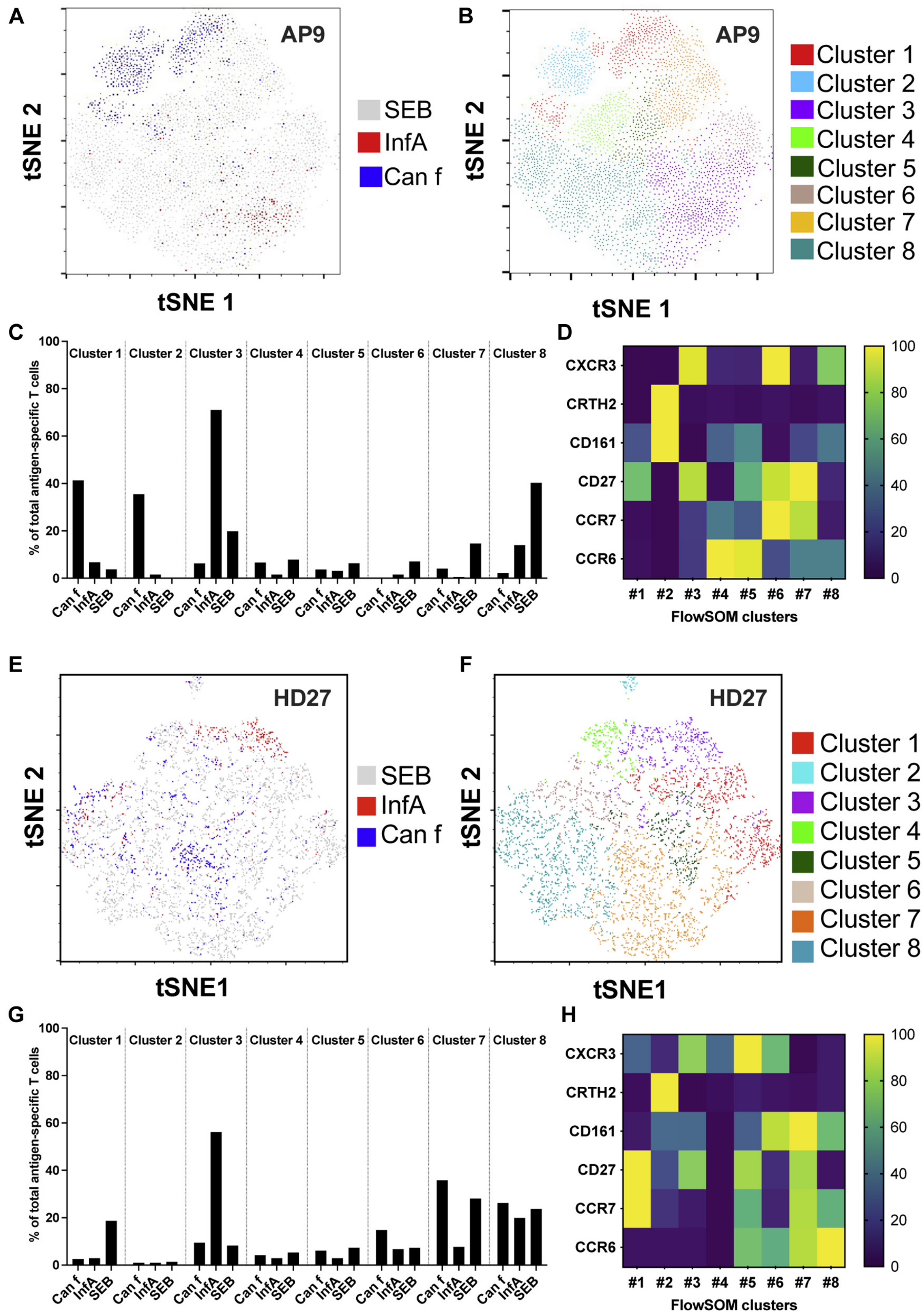

FIG E3. Dog allergen–specific memory CD4$^+$ T cells from allergic subjects exhibit phenotypic heterogeneity within the T$_H$2 compartment. t-Distributed stochastic neighbor embedding (tSNE) plots displaying memory CD4$^+$CD154$^+$CD69$^+$ T cells from Can f– (*blue dots*), InfA MP1– (*red dots*), and SEB-stimulated (*gray dots*) PBMCs from a representative dog-allergic patient (AP9) (**A**) and a 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 overlayed with the clusters automatically identified by the FlowSOM algorithm. (**C, G**) Distribution of FlowSOM clusters among Can f–, influenza-, and SEB-stimulated memory CD4$^+$CD154$^+$CD69$^+$ T cells. (**D, H**) Heat maps representing row-normalized marker expression (median fluorescence intensities, MFI) within each FlowSOM cluster.

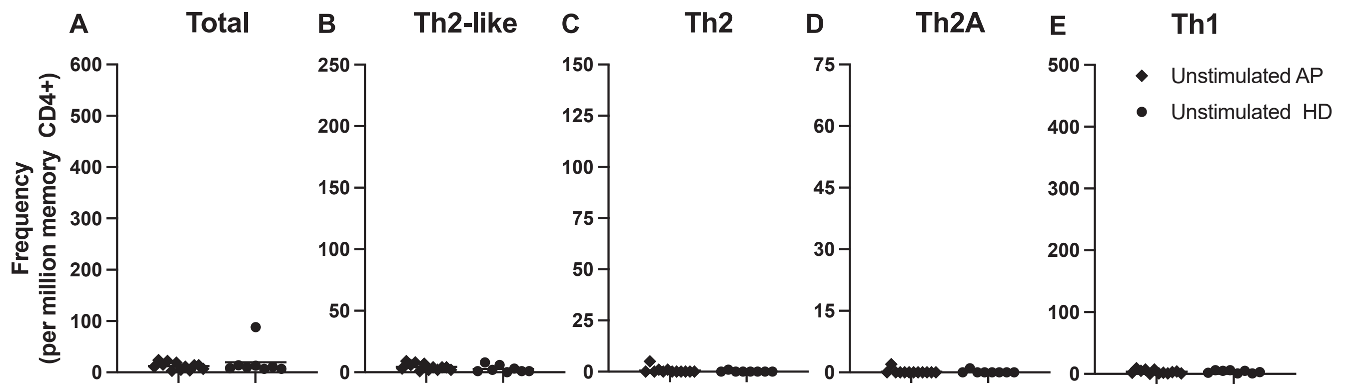

FIG E4. Minimal background is observed in unstimulated samples in the CD154 enrichment assay. *Ex vivo* frequencies of total (**A**), T_H2-like (**B**), T_H2 (**C**), T_H2A (**D**), and T_H1 (**E**) $CD69^+CD154^+$ memory $CD4^+$ T cells in unstimulated samples from 12 allergic patients (AP) and 8 healthy donors (HD). These values were subtracted from the antigen-stimulated samples (Fig 2). T_H1 cells were defined as $CCR6^-CXCR3^+$, T_H2-like cells as $CCR6^-CXCR3^-CRTH2^-$, T_H2 cells as $CCR6^-CXCR3^-CRTH2^+CD161^-$, and T_H2A cells as $CCR6^-CXCR3^-CRTH2^+CD161^+CD27^-$.

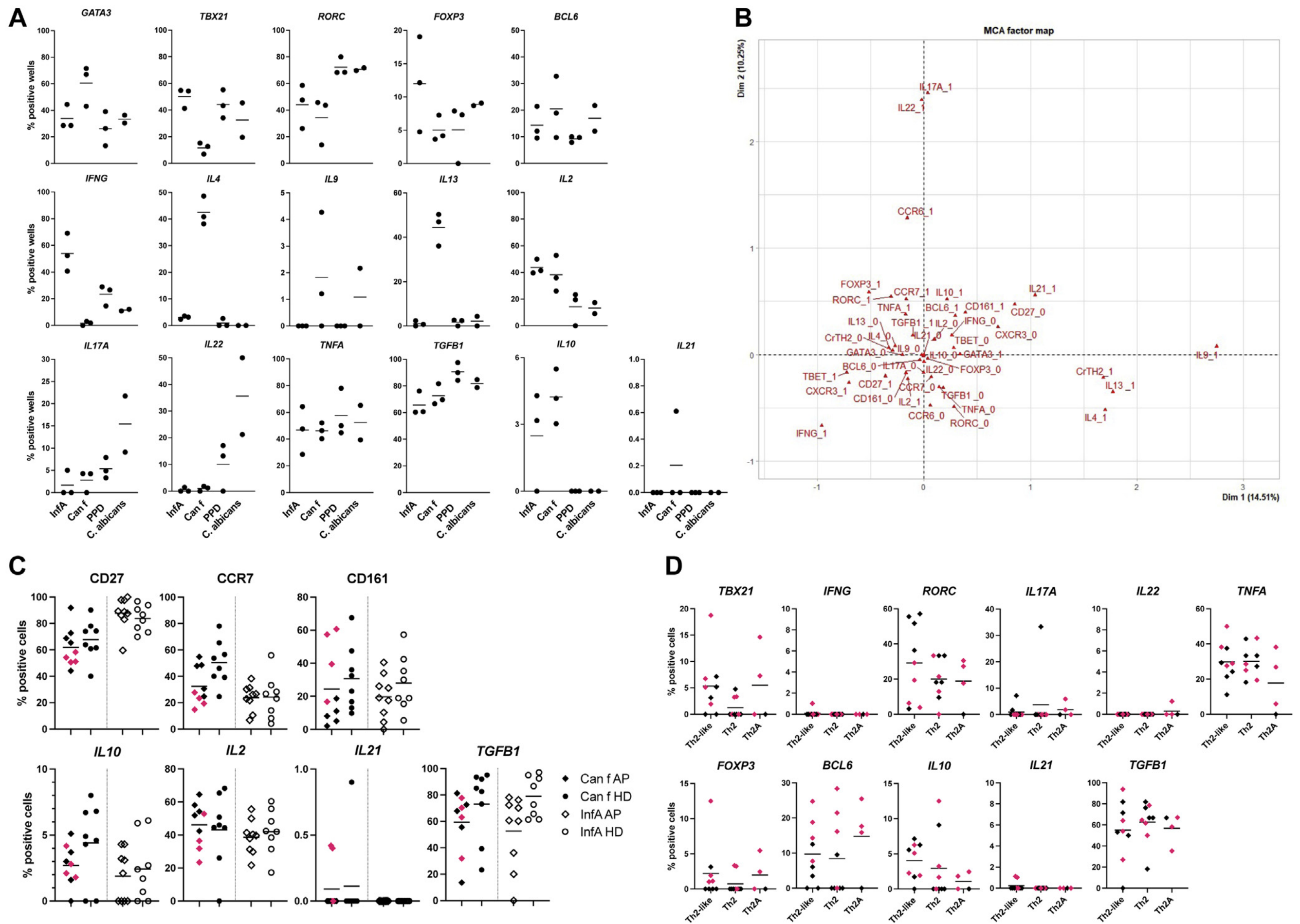

FIG E5. Single-cell transcriptomic profiling of antigen-specific memory CD4^{+} T cells. (**A**) Single-cell transcriptomic profile of memory CD4^{+}CD154^{+}CD69^{+} single T cells sorted with the index sort mode from the PBMCs of 3 dog-allergic patients stimulated with InfA MP1 peptides (245 cells), 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 T$_H$1-associated transcripts (*TBX21, IFNG*) within influenza-specific T cells, T$_H$2-associated transcripts (*GATA3, IL4, IL9, IL13*) within dog allergen–specific T cells, and T$_H$17-associated transcripts (*RORC, IL17A, IL22*) within *C albicans*–specific T cells was observed. (**B**) Multiple correspondence analysis (MCA) factor map demonstrates that T$_H$1 (*IFNG, TBET,* CXCR3), T$_H$2 (*IL4, IL9, IL13,* CRTH2), and T$_H$17 (*IL17A, IL22,* CCR6) markers explain most of the detected variance between single cells. (**C**) Percentages of dog allergen (Can f)- and InfA-specific single cells expressing the indicated surface and transcriptomic markers. (**D**) Percentages of dog allergen–specific memory CD4^{+} T cells from different *ex vivo* T$_H$2 subsets expressing different transcripts. The 4 allergic patients with the highest frequencies of T$_H$2-polarized cells (Fig 2) are indicated in *red.*

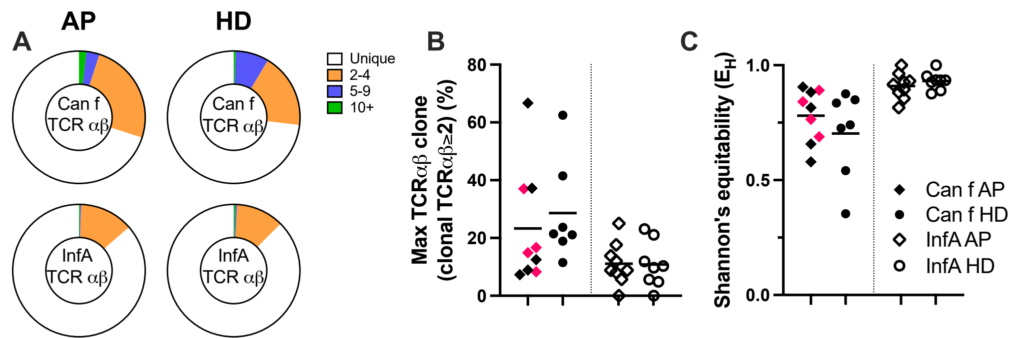

FIG E6. Analysis of paired α and β TCR sequences of dog allergen– and influenza-specific T cells at the single-cell level. (**A**) TCR αβ clonality of dog allergen– and influenza-specific memory $CD4^+$ T cells in allergic (AP) and nonallergic (HD) individuals. The proportion of clones with a unique TCR αβ sequence detected once or more within an individual is indicated in the plot. (**B**) Quantification of the most abundant (max) TCR αβ clone out of all dog allergen– or influenza-specific T cells analyzed from each individual donor. Samples lacking clonal expansions were scored as zero. (**C**) Shannon equitability index for TCR αβ sequences calculated in dog allergen– and influenza-specific T cells from each individual donor. The 4 allergic patients with the highest frequencies of T_H2-polarized cells (Fig 2) are indicated in *red.*

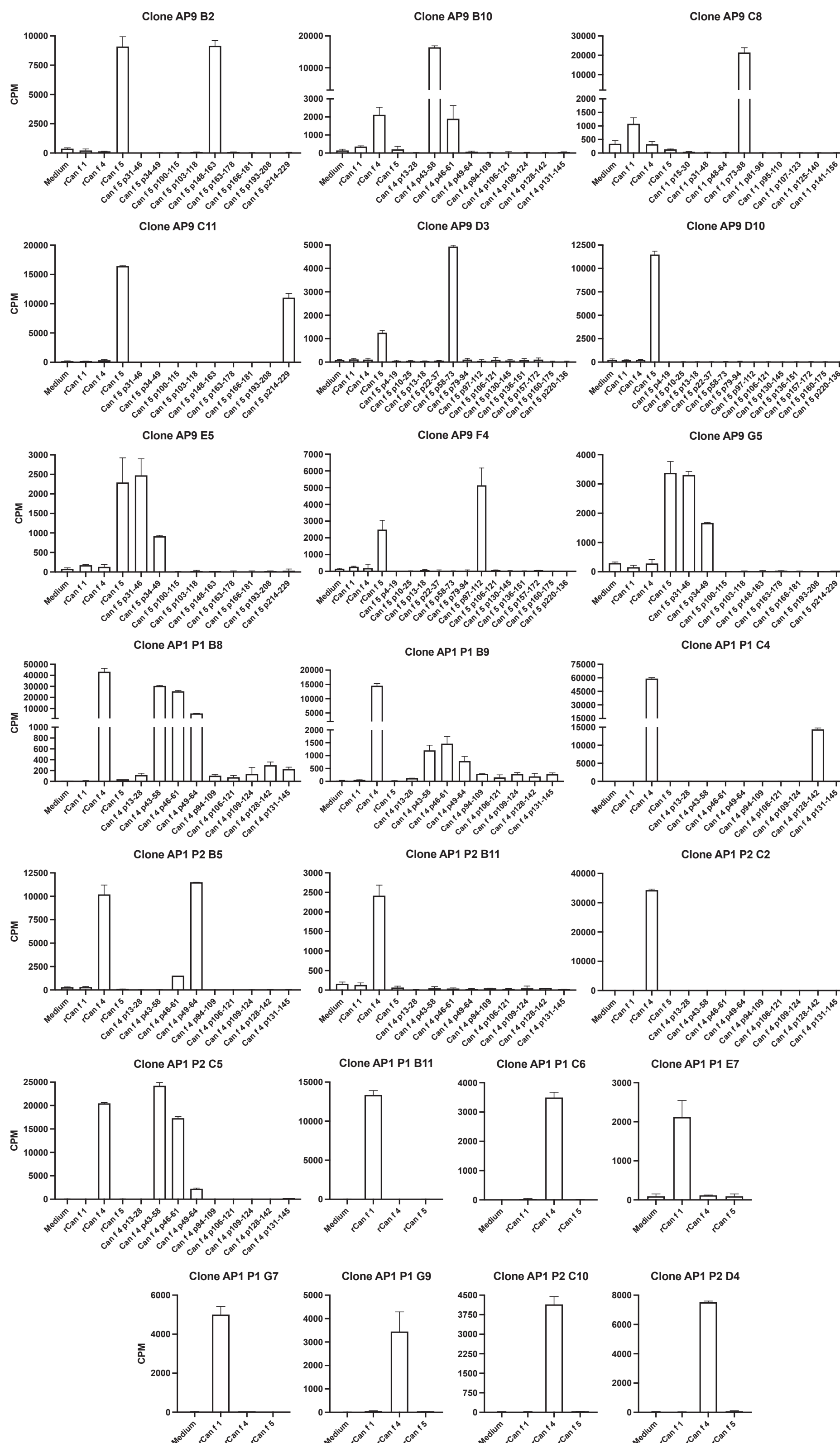

FIG E7. T-cell epitopes recognized by *in vitro*–expanded dog allergen–specific T-cell clones. Proliferative responses, plotted as counts per minute (CPM), of *in vitro*–expanded dog allergen–specific CD4$^+$ T-cell clones in response to dog allergens (rCan f 1, rCan f 4, rCan f 5) and to peptides containing T-cell epitopes of these allergens. Medium was used as a negative control. *Bars* represent the means of 3 replicate wells with SEMs.

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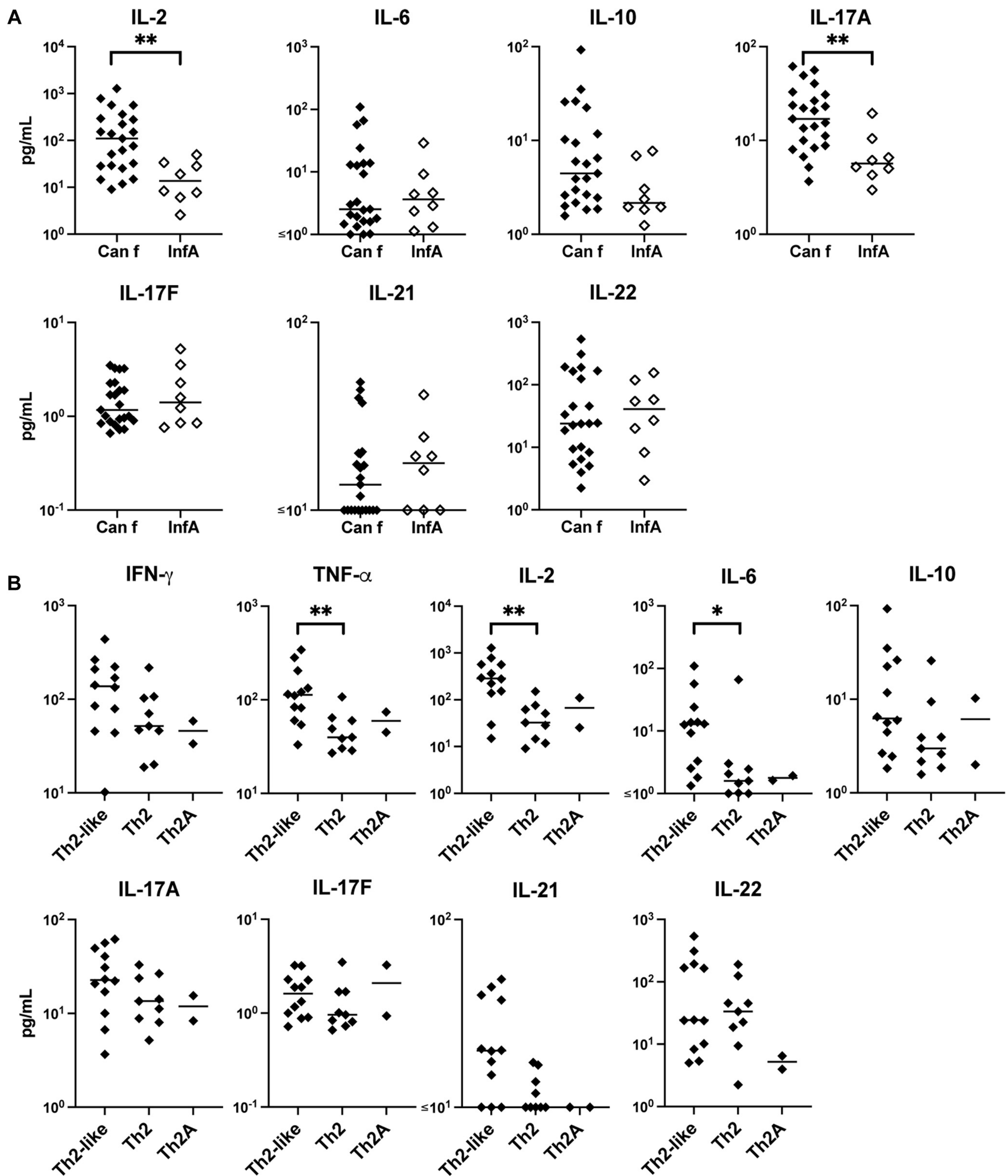

FIG E8. Cytokine secretion by antigen-specific T-cell clones. (**A**) Cytokine levels in the supernatants of anti-CD2/CD3/CD28–stimulated dog allergen (Can f)- or InfA-specific CD4$^+$ T-cell clones. (**B**) Cytokine production by dog allergen–specific T-cell clones originally sorted from different *ex vivo* T$_H$2 subsets. Each data point represents an individual clone. Mann-Whitney *U* test and Kruskal-Wallis test with Dunn multiple comparisons test were used for statistical analyses. *$P < .05$, **$P < .01$.

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TABLE E1. Demographic data of donors included in the study

Patient ID*	Sex	Age (years)	Serum IgE ELISA (OD at 450nm)			Skin prick test (mm)	Dog dust exposure	Asthma	Allergic symptoms	Exacerbation†	FC	Seq
			Canf1	Canf4	Canf5	Dog						
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
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; *FC,* flow cytometry analysis; *NA,* not available; *ND,* not done; *R,* rhinitis; *Seq,* transcriptomic and TCR repertoire analysis; *U,* urticaria; *W,* wheezing.
*Patient IDs prefixed AP indicate allergic patients; HD, healthy donors (nonallergic).
†Self-reported exacerbation of symptoms when exposed to dogs.

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TABLE E2. Antibodies used for flow cytometry analysis

Marker	Fluorochrome	Manufacturer	Clone	Dilution
7-AAD	Not applicable	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

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TABLE E3. Primers used for targeted gene expression analysis of transcription factors and cytokines in PCR reactions 1 and 2

Reaction	Gene	Primer A	Primer B
Reaction 1	*BCL6*	GCCAAACCAGAGGGGCCTGAG	GAGAGCCGCAGGACGTGCACTT
	FOXP3	GGCTCCTGCTGCATCGTAGCTGCT	GTCCGCTGCTTCTCTGGAGCCT
	GATA3	GACGCGGCGCAGTACCCGCT	GGAGAAGGGGCTGAGATTCCAG
	RORC	CCCGGGAGGAAGTGACTGGCTA	CCATGCCACCGTATTTGCCTTCAA
	TBX21	GCCTGTACGTCCACCCGGACT	CTGGGTTTCTTGGAAAGTAAAGATAT
	IFNG	GGCTTTTCAGCTCTGCATCGTTTT	GGATGCTCTGGTCATCTTTAAAGTT
	IL2	CTCACATTTAAGTTTTACATGCCCAA	GACAAAAGGTAATCCATCTGTTCAG
	IL4	CCTTCTGCAGGGCTGCGACTG	CAGCTCGAACACTTTGAATATTTCT
	IL9	GCAGTGCTAATGTGACCAGTTGTC	CCAGAAGACTCTTCAGAAATGTCA
	IL10	CCAGTTTTACCTGGAGGAGGTGA	GTAGGCTTCTATGTAGTTGATGAAGA
	IL13	CCCAGAACCAGAAGGCTCCGCT	CCCTCGCGAAAAAGTTTCTTTAAAT
	IL17A	GACAAGAACTTCCCCCGGACTG	GGACCAGGATCTCTTGCTGGAT
	IL21	GGTCAAGATCGCCACATGATTAGA	CCCTGCATTTGTGGAAGGTGGTT
	IL22	CCTCTTGGTACAGGGAGGAGCA	CCTATCAGATTGAGGGAACAGCA
	TGFB	GCATATATATGTTCTTCAACACATCA	CCCTCCACGGCTCAACCACT
	TNFA	CATGATCCGGGACGTGGAGCT	GGGCTACAGGCTTGTCACTCG
Reaction 2	*BCL6*	CCAGGGTTTTCCCAGTCACGACC CTACACGGCCCCACCTGCCT	AGCGGATAACAATTTCACACAGGAGGGTGCATGTAGAGTGGTGAGTG
	FOXP3	CCAGGGTTTTCCCAGTCACGACGG CAGCCAAGGCCCTGTCGT	AGCGGATAACAATTTCACACAGGACCAGGATGGCCCAGCGGATGA
	GATA3	CCAGGGTTTTCCCAGTCACGACGCC GGAGGAGGTGGATGTGCTT	AGCGGATAACAATTTCACACAGGAGGGGAGGCGGTGTGGTGGCT
	RORC	CCAGGGTTTTCCCAGTCACGACAGA GGAAGTCCATGTGGGAGATGT	AGCGGATAACAATTTCACACAGGATCAGCATTGTAGGCCCGGCACATC
	TBX21	CCAGGGTTTTCCCAGTCACGACCCC AACACAGGAGCGCACTGG	AGCGGATAACAATTTCACACAGGACGTGTTGGAAGCGTTGCAGGCT
	IFNG	CCAGGGTTTTCCCAGTCACGACGGG TTCTCTTGGCTGTTACTGC	AGCGGATAACAATTTCACACAGGAGTTTGAAGTAAAAGGAGACAATTTG
	IL2	CCAGGGTTTTCCCAGTCACGACCCA CAGAACTGAAACATCTTCAGT	AGCGGATAACAATTTCACACAGGATTCTACAATGGTTGCTGTCTCA
	IL4	CCAGGGTTTTCCCAGTCACGACGG CAGTTCTACAGCCACCATGA	AGCGGATAACAATTTCACACAGGACTCTCTCATGATCGTCTTTAGCCT
	IL9	CCAGGGTTTTCCCAGTCACGACGG GCATTCCCTCTGACAACTGC	AGCGGATAACAATTTCACACAGGAGCGCGTTGCCTGCCGTGGTT
	IL10	CCAGGGTTTTCCCAGTCACGACCCC AAGCTGAGAACCAAGACCCA	AGCGGATAACAATTTCACACAGGAGTCAAACTCACTCATGGCTTTGTA
	IL13	CCAGGGTTTTCCCAGTCACGACGG TATGGAGCATCAACCTGACAG	AGCGGATAACAATTTCACACAGGAGGTCCTTTACAAACTGGGCCAC
	IL17A	CCAGGGTTTTCCCAGTCACGACCA ACCTGAACATCCATAACCGGAA	AGCGGATAACAATTTCACACAGGAGGGGACAGAGTTCATGTGGTAGT
	IL21	CCAGGGTTTTCCCAGTCACGACCG TCAACTTATAGATATTGTTGATCA	AGCGGATAACAATTTCACACAGGACCTCTTCAGCTTTTTAATTGATA
	IL22	CCAGGGTTTTCCCAGTCACGACCG CCCATCAGCTCCCACTGCA	AGCGGATAACAATTTCACACAGGAGGGTGAAGTTCAGCACCTGCTT
	TGFB	CCAGGGTTTTCCCAGTCACGACCCG AGAAGCGGTACCTGAACC	AGCGGATAACAATTTCACACAGGACCGCACAACTCCGGTGACATCA
	TNFA	CCAGGGTTTTCCCAGTCACGACGGA GGCGCTCCCCAAGAAGAC	AGCGGATAACAATTTCACACAGGACGAGAAGATGATCTGACTGCCTG

All other primers used (TCR families and PCR reaction 3 primers) are listed in Han et al.[23]

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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

Donor	Can f			InfA MP1		
	TCR β	TCR α	TCR αβ	TCR β	TCR α	TCR αβ
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

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TABLE E6. Dog allergen-specific T-cell clone growth according to *ex vivo* phenotype

Donor	*Ex vivo* subset	Sorted cells within subset	Expanded cells from sorted subset	Can f–reactive cells from expanded subset
AP1	Non-T_H2	17/83 (20.5%)	4/17 (23.5%)	0/4 (0)
	T_H2-like	32/83 (38.6%)	6/32 (18.8%)	5/6 (83.3%)
	T_H2	18/83 (21.7%)	8/18 (44.4%)	7/8 (87.5%)
	T_H2A	16/83 (19.3%)	2/16 (12.5%)	2/2 (100.0%)
AP9	Non-T_H2	15/60 (25.0%)	1/15 (6.7%)	0/1 (0)
	T_H2-like	24/60 (40.0%)	7/24 (29.2%)	7/7 (100.0%)
	T_H2	5/60 (8.3%)	2/5 (40.0%)	2/2 (100.0%)
	T_H2A	16/60 (26.7%)	0/16 (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.