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Elevated circulating Th2 but not group 2 innate lymphoid cell responses characterize canine atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) is an allergic skin disease that causes significant morbidity and affects multiple species. AD is highly prevalent in companion dogs, and the clinical management of the disease remains challenging. An improved understanding of the immunologic and genetic pathways that lead to disease could inform the development of novel treatments. In allergic humans and mouse models of AD, the disease is associated with Th2 and group 2 innate lymphoid cell (ILC2) activation that drives type 2 inflammation. Type 2 inflammation also appears to be associated with AD in dogs, but gaps remain in our understanding of how key type 2-associated cell types such as canine Th2 cells and ILC2s contribute to the pathogenesis of canine AD. Here, we describe previously uncharacterized canine ILC2-like cells and Th2 cells *ex vivo* that produced type 2 cytokines and expressed the transcription factor Gata3. Increased circulating Th2 cells were associated with chronic canine AD. Single-cell RNA sequencing revealed a unique gene expression signature in T cells in dogs with AD. These findings underline the importance of pro-allergic Th2 cells in orchestrating AD and provide new methods and pathways that can inform the development of improved therapies.

1. Introduction

Atopic dermatitis (AD) is a chronic skin allergy that occurs in multiple species including humans and dogs and is associated with significant morbidity in canine patients (Marsella and De Benedetto, 2017). Genetic, environmental, and immunological factors contribute to disease pathogenesis, but our understanding of the factors that are critical for disease development and progression remain unclear (Agler et al., 2019; Bizikova et al., 2015; Hakanen et al., 2018; Marsella and De Benedetto, 2017; Nødtvedt et al., 2007; Plager et al., 2012). AD occurs with high prevalence in companion dogs and significantly impacts the quality of life of affected individuals and their human caretakers (Hillier and Griffin, 2001; Linek and Favrot, 2010). Exposure to environmental or food-associated allergens causes allergic skin inflammation that is characterized by pruritus, marked skin lesions and aggravating secondary infections (Bizikova et al., 2015; Griffin and DeBoer, 2001). While many therapeutic approaches are available to treat the symptoms of acute and chronic AD, diagnosis and management of the disease in dogs remains challenging (Hensel et al., 2015; Saridomichelakis and Olivry, 2016). Allergen avoidance is not always feasible and current therapeutic options have limitations in efficacy and/or are associated with considerable side effects (Saridomichelakis and Olivry, 2016). A better understanding of the complex progression and pathogenesis of canine AD could inform the development of improved therapies.

Excessive type 2 immune responses are a hallmark of AD in humans and mouse models of disease (Czarnowicki et al., 2015; Kim et al., 2013; Licona-Limón et al., 2013; Mashiko et al., 2017; Salimi et al., 2013). Studies of induced type 2 inflammation in mice and naturally occurring human AD show that Th2 cells and group 2 innate lymphoid cells (ILC2s) express the master transcription factor Gata3 and produce the type 2 cytokines IL-4, IL-5 and IL-13 (Czarnowicki et al., 2015; Kim

Abbreviations: AD, atopic dermatitis; ILC2, group 2 innate lymphoid cell; ILC, innate lymphoid cell; rh, recombinant human; rc, recombinant canine; DEG, differentially expressed genes; M, media condition

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et al., 2013; Mashiko et al., 2017; Mjösberg et al., 2012; Pelly et al., 2016; Salimi et al., 2013). These mediators elicit pruritus and skin inflammation, IgG1 and IgE production, and decreased skin barrier integrity that drive AD pathogenesis (Tait Wojno and Artis, 2016). Several studies suggest that type 2 inflammation is also the predominant effector response in atopic dogs. Transcripts for type 2 cytokines *IL4, IL13*, *IL31* and *IL33* are upregulated in the skin and the peripheral blood of allergic dogs (Majewska et al., 2016; Marsella et al., 2006; Nuttall et al., 2002; Olivry et al., 2016). Likewise, type 2 effector cells, including eosinophils, mast cells, and $CD4^+$ T cells infiltrate the inflamed epidermis and dermis in atopic dogs (Olivry et al., 1997; Sinke et al., 1997). Aside from these data, the immunological mechanisms, including pathways, cell types and genes that underpin allergic conditions systemically in dogs are still unclear. Specifically, ILC2s have not been identified in dogs, nor can canine Th2 cells be reliably identified *ex vivo*. These limitations have previously made the study of the cellular immunologic and genetic factors that drive AD pathogenesis in the companion dog more difficult.

To address this challenge, for the first time, we have developed a novel approach to identify canine ILC2s and Th2 cells *ex vivo*, allowing us to quantify, purify, and characterize these cells. Notably, there was a significant increase in $Gata3^+$ Th2 cells but not ILC2-like cells in the blood of dogs with chronic AD compared to non-allergic dogs. We complemented this cellular immune analysis with single-cell RNA sequencing, revealing a unique differentially expressed gene set in peripheral blood T cells of allergic dogs. Together, these data identify a peripheral immune cell phenotype and gene expression signature associated with chronic canine AD, providing a foundation for further study of the atopic dog as a powerful naturally occurring disease model for chronic human AD and critically, for dissecting new pathways that could be targeted to develop novel treatments for canine AD.

2. Materials and methods

2.1. Canine allergy cohort

Age- and sex matched blood samples from predominantly neutered or spayed (38 of 41 total) healthy and allergic companion dogs were collected at the Cornell University Hospital for Animals Dermatology Service (CUHA Derm), or in Houston, Texas (West Highland White Terriers) by licensed veterinary health care professionals. Additional blood samples for cell culture experiments were obtained from Marshall BioResources (healthy dogs only). Dogs diagnosed with AD (characterized by Food Hypersensitivity or Environmental AD) were included in the allergic group. All diagnoses were based on standard clinical diagnostic procedures that included serological testing, intradermal testing, food trials, patient history assessment and assessment of allergic signs. Dogs with neoplasia or receiving systemic corticosteroid treatment were excluded. This study was reviewed and approved by the Cornell University Institutional Care and Use Committee (IACUC) and informed dog owner consent was obtained for all samples.

2.2. Flow cytometry, cell sorting and cytospin

PBMCs were isolated from canine blood using density gradient centrifugation with Ficoll-Paque Premium (GE Healthcare, Chicago, IL, USA) and washed in flow cytometry buffer. For surface staining, PBMCs were incubated with canine FcR Binding Inhibitor Polyclonal Antibody (Thermo Fisher Scientific (TFS), Waltham, MA, USA) for 30 min at 4 °C. Cells were then incubated for 30 min at 4 °C with Aqua Live/Dead Fixable Dye (TFS) and monoclonal antibodies (mAbs) phycoerythrin (PE)-Cyanine 7-labeled rat anti-canine CD4 (YKIX302.9), peridinin chlorophyll protein complex (PerCP)-eFluor 710-labeled rat anti-canine CD5 (YKIX322.3), eFluor 450-labeled rat anti-canine CD45 (YKIX716.13), fluorescein isothiocyanate (FITC)-labeled mouse anti-canine CD25 (P4A10) (TFS), Alexa Fluor 700-labeled rat anti-mouse CD11b (M1/70) (BD Biosciences, Franklin Lakes, NJ, USA), RPE-labeled mouse anti-canine CD21 (MCA1781PE) (Bio Rad, Hercules, CA, USA) or PE-labeled rat anti-canine CD5 (YKIX322.3) (TFS). For transcription factor staining, cells were fixed and permeabilized using the Transcription Factor Staining Set kit (TFS) according to manufacturer instructions and stained with the mAb PE-eFluor 610- or Alexa Fluor 488-labeled rat anti-human/mouse GATA3 (TWAJ) and PE-eFluor 610-labeled IgG2b kappa isotype control (eB149/10H5) (TFS). Single-cell RNA staining was performed using a commercially available kit according to the manufacturer's instructions (PrimeFlow RNA Assay Kit, TFS). RNA was detected with PrimeFlow RNA Assay Kit target-specific probes in combination with specific fluorescence-conjugated label probes, including an Alexa Fluor 750-conjugated Type 6 probe with target probe against canine *Actβ* or *β2m* and an Alexa Fluor 647-conjugated Type 1 probe with target probe against canine *GATA3*. Samples were run on a 3 laser LSR II (BD Biosciences), a 3 laser Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA), or a 4 laser Attune flow cytometer (TFS) and analyzed using FlowJo 10.5.3 (FlowJo, LLC, Ashland, OR, USA). Gates were set using fluorescence minus one and isotype controls with less than 3 % background accepted.

For sorting, freshly isolated PBMCs were surface stained as above and sorted using a 4 laser BD FACSAria Fusion (BD Biosciences) with an 80 μm nozzle. Post-sorting, a small aliquot of cells in suspension was spun on Fisherbrand Superfrost Microscope Slides (TFS) via Cytospin, stained with a Differential Quik Stain Kit (Electron Microscopy Sciences, Hatfield, PA, USA) according to the manufacturer's instructions and imaged on a Axio Oberserver Z1 microscope (Zeiss, Oberkochen, Germany).

2.3. In vitro cultures of $CD4^+$ T cells and $CD25^+$ ILCs

All PBMC samples for *in vitro* cultures were freshly isolated from whole blood in EDTA tubes. Sort-purified $CD4^+$ T cells (> 95 % purity) were cultured at 5×10^4 cells/well and $CD25^+$ ILCs (avg. 95 % purity) were cultured at $2-5 \times 10^3$ cells/well in DMEM with 10 % FBS (Corning, Corning, NY, USA), 1 % L-Glutamine (Corning), 1 % penicillin/streptomycin (Corning), 25 mM HEPES buffer (Corning) and 55 μM 2-β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) for 5 days at 37 °C. T cells were stimulated with 2.5 μg/mL Concanavalin A (Sigma-Aldrich) (all conditions), 50 ng/mL rcIL-6 (Th17), 10 ng/mL rcIL-1β (Th17), 100 ng/mL rcIL-4 (Kingfisher Biotech, Saint Paul, MN, USA) (Th2), 100 ng/mL rcIL-12 (R & D Systems, Minneapolis, MN, USA) (Th1), 2.5 μg/mL anti-canine IFN-γ (Kingfisher Biotech) (Th17, Th2) and/or 2.5 μg/mL anti-canine IL-4 (Kingfisher Biotech) (Th17, Th1). ILCs were stimulated with 50 ng/mL rhIL-2 (R & D Systems) and 50 ng/mL rcIL-33 (Sino Biological, Beijing, China). Fresh media with fresh cytokines was added to the cultures on day 3.

2.4. ELISA and quantitative realtime PCR

Cell culture supernatants from Th cell polarization cultures and ILC cultures were analyzed using ELISA DuoSets for canine IL-17A, IL-4 and IFN-γ (R & D Systems) on an infinite M200 pro TECAN ELISA plate reader (Tecan Group Ltd., Männedorf, Switzerland). RNA was isolated from the cells using a low input RNA purification kit (Norgen Biotek Corp., Thorold, ON, Canada). cDNA was generated using a Superscript II reverse transcription kit (TFS) and realtime PCR was performed using SYBR green master mix (TFS) and commercially available primer sets (Qiagen, Hilden, Germany). Realtime PCR was run on an ABI 7500 system (TFS).

2.5. Drop-seq single-cell RNA sequencing and data analysis

PBMCs from canine blood samples were isolated as described above and stored in 10 % DMSO (TFS) in heat inactivated FBS at −80 °C prior to analysis. Cells were washed twice in 0.01 % BSA (Millipore Sigma,

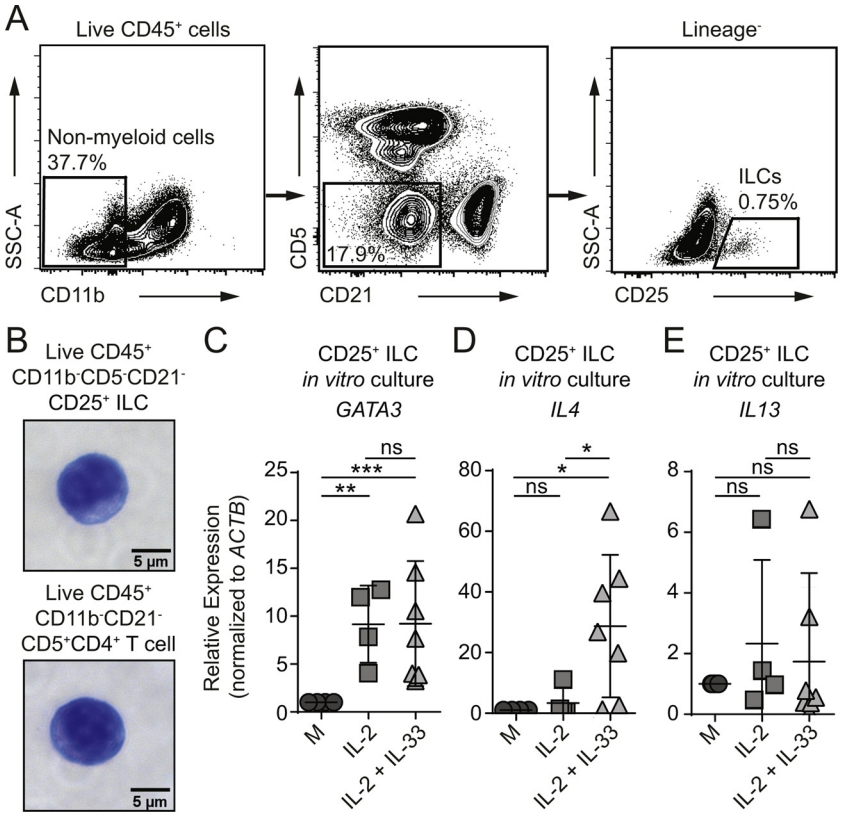

Fig. 1. Identification of canine ILCs in PBMCs. (A) Flow cytometry of canine PBMCs. CD25$^+$ canine ILC-like cells were identified in the Live CD45$^+$lineage (CD11b/CD5/CD21)$^-$ cell fraction. **(B)** Canine ILCs and CD4$^+$ T cells were sort-purified and imaged (100x, scale bar = 5 µm) after cytospin and Diff-Quik staining. Sort-purified ILCs were cultured for 5 days with or without rhIL-2 and rcIL-33 and assessed for **(C)** *GATA3*, **(D)** *IL4* and **(E)** *IL13* by realtime PCR (normalized to *ACTβ* and relative to the media condition (M)). Mean ± SD; *, P ≤ 0.05; ***, P ≤ 0.001; **(A, B)** representative of > 3 independent experiments, male, healthy dog shown; **(C, D and E)** 3–5 independent experiments, 3 females and 4 males included.

Burlington, MA, USA) in PBS (Corning). Drop-seq was performed as described previously (Macosko et al., 2015) using the Drop-seq Laboratory Protocol (version 3.1, 12/28/2015) by the MacCarroll lab (http://mccarrolllab.org/dropseq/). Drop-seq beads were purchased from ChemGenes (Cat # Macosko-2011-10, Wilmington, MA, USA). Libraries were prepared using a Nextera XT Library Preparation Kit and sequenced on a NextSeq500 (Illumina, San Diego, CA, USA).

Illumina reads were processed as described in the Drop-seq Core Computational Protocol (version 2.0.0, 9/28/18, https://github.com/broadinstitute/Drop-seq/releases) using Drop-seq_tools-1-12, Picard-tools-2.8.2 and STAR-2.7.0f to create the digital gene expression matrix (Dobin et al., 2013; Macosko et al., 2015). The canine genome assembly CanFam3.1 with annotation CanFam3.1.96 was downloaded from the ensembl browser.

Clustering and differentially expressed gene (DEG) analysis were performed in RStudio 1.2.1335 with R 3.6.0 and Seurat 3.0 (Butler et al., 2018; Stuart et al., 2019). Seurat objects were created (min.cells = 3, min.features = 200 (150 for Westie samples)) and cells were filtered (features > 100, mitochondrial genes < 5 %, ribosomal proteins < 17 %). To perform clustering, each sample was normalized using SCTransform (Hafemeister and Satija, 2019) (mitochondrial mapping percentage regressed out) and samples from healthy and allergic dogs were integrated separately using FindIntegrationAnchors and IntegrateData (dims = 1:30). Both datasets were then integrated (dims = 1:20) to a single object and standard Seurat integrated analysis was performed (ScaleData, RunPCA (npcs = 30), RunUMAP (dims = 1:20), FindNeighbours (dims = 1:20), FindClusters (res = 0.5). Thirteen clusters were identified, with markers identified using the FindConservedMarkers function on the (unintegrated) "RNA" slot of the data. Marker genes were then used to determine the cell type identities in each cluster. For cluster 6, DEGs between healthy and allergic dogs were determined with FindMarkers using the default Wilxocon Rank Sum test. DEGs were plotted using EnhancedVolcano (Blighe, 2018). Gene expression averages for each cluster were calculated using AverageExpression and plotted for the "count" slot.

2.6. Statistical analysis

Statistical analysis was carried out using JMP software (V. 14.0.0 SAS Institute, Cary, NC, USA) and R. Data were analyzed using linear mixed effects models with a fixed effect of experimental group and a random effect of experiment day. Model assumptions of normality and homogeneous variance were assessed by a visual analysis of the raw data and the model residuals. Right skewed data was log or square root transformed as indicated by the residuals. Experimental group was considered statistically significant if the fixed effect F test *p*-value was less than or equal to 0.05, with results in graphs shown as mean ± SD. Post-hoc pairwise comparisons between experimental groups were made using Tukey's HSD multiple-comparison test. FACS data from the allergy cohort was analyzed using an unpaired two-sample t-test; right-skewed variables were log or square root transformed and left-skewed variables were cubed as necessary for t-tests. Chi-square tests of association, Fisher's exact tests, Spearman correlations, t-tests and linear models were applied to determine the effect of different variables on immune parameters, including age, sex and season of blood draw. Statistical analysis could not be performed on the T cell polarization assay comparing healthy *vs* allergic dogs (Fig. 4D) due to low power associated with a small sample size. Summary statistics are presented on the untransformed data.

3. Results

3.1. ILCs in canine PBMCs

Based on the key role that ILC2s play in human skin allergy (Kim et al., 2013; Mashiko et al., 2017; Salimi et al., 2013), we hypothesized that ILC2s are key mediators of canine AD and that their activation or accumulation would be associated with the disease. To date however, ILCs have not been described in dogs. Therefore, we developed a novel flow cytometry panel that stains for canine T cells (CD5, CD4), B cells (CD21), myeloid cells (CD11b), and the IL-2Rα, CD25, a cytokine

receptor expressed by murine and human ILCs (Monticelli et al., 2011). This analysis revealed a previously uncharacterized $CD45^+CD11b^-CD5^-CD21^-CD25^+$ ILC-like cell population present in canine PBMCs that displayed a surface marker phenotype similar to that of human and mouse ILCs (Fig. 1A) (Kim et al., 2013; Monticelli et al., 2011).

To assess whether this ILC-like population included an ILC2-like subset, lineage $(CD11b/CD5/CD21)^-CD25^+$ cells were sort-purified (Fig. 1B and Fig. S1) and assessed for ILC2-like phenotype and function (Mjösberg et al., 2011; Roediger et al., 2013; Salimi et al., 2013). In response to stimulation with recombinant human (rh)IL-2 and recombinant canine (rc)IL-33, canine ILC-like cells upregulated *GATA3* expression (Fig. 1C), suggesting that there is a canine ILC2-like population that expresses the master transcriptional regulator Gata3 (Mjösberg et al., 2012; Wang et al., 2013). While canine ILC-like cells did not secrete detectable IL-4 protein (data not shown), they highly upregulated *IL4* expression in response to rhIL-2 and further with the addition of rcIL-33 (Fig. 1D), consistent with previous reports that murine ILC2s can express *Il4* but do not secrete IL-4 when stimulated with IL-2, IL-25 and IL-33 (Pelly et al., 2016). Unfortunately, to date no ELISA assays are available for the detection of dog IL-5 and IL-13 protein, but we did find upregulated expression of *IL13* in canine ILCs in some dogs in response to IL-2 and IL-33 (Fig. 1E). Thus, we report for the first time the existence of ILC-like cells in the dog that can express *GATA3* and have the capacity to express type 2 cytokine genes, establishing that ILC2s exist in dogs.

3.2. Gata3 expression can be used to identify canine ILC2s and Th2 cells in PMBCs

To further characterize canine ILC2s and understand their significance in canine AD, we sought to identify canine ILC2s as well as their adaptive counterparts, Th2 cells, *ex vivo* in PBMCs using flow cytometry using an anti-mouse/human monoclonal antibody that is cross-reactive against canine Gata3 (Mjösberg et al., 2012; Wang et al., 2013). We were able to identify both ILC2s $(CD45^+lineage^-CD25^+Gata3^+)$ and a small population of Th2 cells $(CD45^+CD11b^-CD21^-CD5^+CD4^+Gata3^+)$ in the PBMCs of a healthy donor dog (Fig. 2A) and in the PBMCs of a dog with AD (Fig. 2B). To our knowledge, this anti-Gata3 mAb has not been used previously to stain canine cells. Therefore, we validated the specificity of the anti-Gata3 mAb for staining of canine Gata3 using various approaches. Consistent with the expression patterns of Gata3 in mice and humans (Mjösberg et al., 2012; Wang et al., 2013), detectable Gata3 staining was observed in ILC-like cells and $CD4^+$ T cells but not in B cells and myeloid cells in a healthy dog and a dog with AD (Fig. 2A, B). In addition, single-cell fluorescence *in situ* RNA hybridization for *GATA3* coupled with standard Ab labeling of proteins measured using flow cytometry showed that there was overlap between Gata3 and *GATA3* expression in canine $CD4^+$ T cells (Fig. 2C, left) but no staining for either in myeloid cells (Fig. 2C, right). Together, these approaches validate that the cross-reactive anti-Gata3 antibody appears to specifically label Gata3-expressing canine immune cells, suggesting that Gata3 staining and flow cytometry can be used to identify canine ILC2s and Th2 cells *ex vivo*.

3.3. Allergic dogs have elevated frequencies and total numbers of Th2 cells in the blood

With a new tool in hand to identify canine ILC2s and Th2 cells *ex vivo*, we hypothesized that elevated canine ILC2 responses would be associated with atopy in dogs, as has been described in human AD (Mashiko et al., 2017). To test this hypothesis, we recruited a cohort of 20 healthy dogs and 21 dogs with signs of chronic allergic disease in the skin from the patient population of companion dogs seen at the Cornell University Hospital for Animals (Table S1). Canine allergies triggered by environmental or food allergens both manifest in the skin and appear almost indistinguishable (Marsella and De Benedetto, 2017); thus, our cohort includes animals with diagnosed hypersensitivities associated with skin signs of AD caused by diverse stimuli and are clinically designated as having food hypersensitivity with skin signs or environmental AD (atopy). Blood was drawn from all participants, and we then compared frequencies and numbers of ILC2s, as well as Th2 cells, in the PBMCs of animals from allergic (with AD) and non-allergic groups. Surprisingly, dogs with AD and healthy dogs had similar frequencies and total numbers per mL blood of ILC-like cells (Fig. 3A), the ILC2 subset (Fig. 3B), $CD11b^+$ myeloid cells (Fig. S2A), $CD21^+$ B cells (Fig. S2B) and total $CD4^+$ Th cells (Fig. 3C). The lack of differences in these cell populations in healthy compared to allergic dogs is consistent with data in humans, with the exception that ILC2s appear to be elevated in at least some cases in humans with AD (Mashiko et al., 2017; Salimi et al., 2013; Villanova et al., 2014).

Strikingly however, the average frequency and the total number of $CD4^+$ $Gata3^+$ T cells were increased in the peripheral blood more than threefold in dogs with AD compared to healthy dogs (Fig. 3D). Importantly, these samples were taken from dogs in a chronic AD disease state. In addition, the differences in the frequency and number of Th2 cells between healthy and allergic dogs as well as the frequencies and numbers of most other immune cell types measured in healthy or allergic dogs were not associated with sex, age or season of blood draw (data not shown). These differences also did not appear to be confined to a single breed, though notably, in a separate cohort of healthy and allergic West Highland White Terriers (Westies, WHWT), which are highly susceptible to the development of allergic disease (DeBoer, 1999; Mazrier et al., 2016), peripheral blood $CD4^+$ as well as $CD8^+$ T cells had high expression levels of Gata3 protein in both non-allergic and allergic individuals (Fig. S3). These data in Westies reveal interesting breed-specific differences in T cell Gata3 expression that will require further study to determine whether baseline Gata3 expression contributes to the predisposition toward allergy in this breed. Overall however, in most breeds and mixed breed dogs sampled, elevated canine Th2 cell but not ILC2 responses were associated with chronic allergic skin inflammation in the peripheral blood.

3.4. Canine $CD4^+$ T cells differentiate readily into the Th2 effector cell subset

We next wanted to explore potential mechanisms that could explain why dogs with AD had elevated Th2 cells in the peripheral blood compared to healthy dogs. To do so, we developed an *in vitro* T cell polarization assay for canine T helper subsets that would allow us to test how various conditions impacted Th2 differentiation in $CD4^+$ T cells from healthy and allergic dogs. $CD4^+$ T cells sort-purified from healthy canine PBMCs were stimulated with the lectin Concanavalin A (ConA), a potent T cell activator (Ritt et al., 2015), along with cytokines to induce polarization of T cells to Th17, Th1 and Th2 effector cell subsets. Cells in Th2 culture conditions were more likely to display a highly elevated frequency of $Gata3^+$ cells (Fig. 4A) and increased *GATA3* expression (Fig. 4B), and they produced increased amounts of IL-4 but not IFN-γ and IL-17 compared to cells in Th1 or Th17 conditions (Fig. 4C). Thus, these culture conditions drove differentiation to the Th2 fate in $CD4^+$ T cells from healthy dogs.

One possibility for why dogs with AD could have increased Th2 cells in the blood is that their $CD4^+$ T cells are more responsive to Th2 polarizing signals compared to $CD4^+$ T cells from healthy dogs, being more likely to differentiate to the Th2 fate. Therefore, we used our T cell polarization assay to test whether a higher frequency of $CD4^+$ T cells from the PBMCs of allergic dogs upregulated Gata3 expression in response to Th2 polarizing cytokines than PBMC T cells from healthy dogs. In samples from 3 pairs of age- and sex-matched healthy and allergic dogs, there was no difference in the ability of $CD4^+$ T cells from healthy *vs*. allergic dogs to upregulate Gata3 expression in response to Th2 stimulatory signals (Fig. 4D). Though these data are from only 3

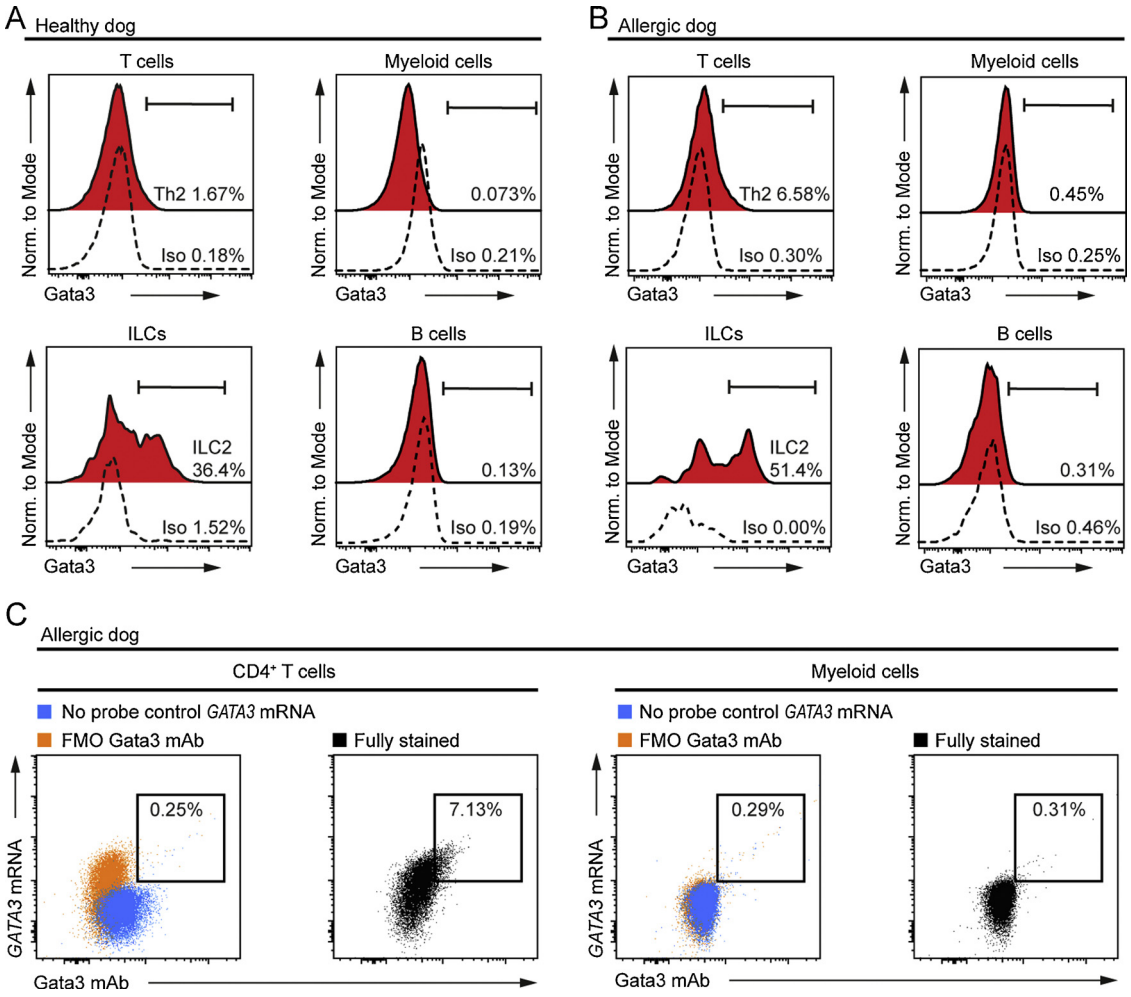

Fig. 2. Gata3 as a specific marker for canine Th2 cells and ILC2s. PBMCs were extracted for flow cytometric analysis. Anti-human/mouse Gata3 mAb labeling of ILC (Live $CD45^+CD11b^-CD21^-CD5^-CD25^+$) and $CD4^+$ T cell (Live $CD45^+CD11b^-CD21^-CD5^+CD4^+$) subsets, but not myeloid cells (Live $CD45^+CD11b^-$) and B cells (Live $CD45^+CD11b^-CD5^-CD21^+$) in **(A)** a healthy dog and **(B)** a dog with AD. **(C)** Co-staining for *GATA3* mRNA by single-cell fluorescence RNA *in situ* hybridization and Gata3 protein in $CD4^+$ T cells (Live $CD45^+\beta2M^+CD11b^-CD5^+CD4^+$, left) but not in myeloid cells ($CD45^+\beta2M^+CD5^-CD11b^+$, right). Representative plots shown; **(A, B)** representative of 9 independent experiments, male, healthy dog and female allergic dog shown, red shaded = fully stained; **(C)** representative of 2 independent experiments; female, allergic dog shown; FMO = fluorescence-minus-one control.

pairs of subjects, they suggest that in the presence of potent signals, $CD4^+$ T cells from healthy and allergic animals both readily become Th2 cells. Thus, elevated Th2 cells in the blood of allergic dogs in our study (Fig. 3) may not necessarily be due to an intrinsic ability of $CD4^+$ T cells in allergic dogs to polarize preferentially to the Th2 fate. However, elevated baseline levels of Gata3 could play a role in predisposition to allergy in certain breeds such as Westies (Fig. S3). In addition, this study did not address how $CD4^+$ T cells from healthy and allergic dogs polarize to Th2 cells in the presence of antigen. Thus, more studies are required to assess why allergic compared to healthy dogs in our cohort have elevated Th2 cell frequencies and numbers.

3.5. Single-cell RNA sequencing reveals a unique gene expression profile in GATA3-expressing T cells in allergic dogs

We next sought to better understand the functional potential of canine Th2 cells *in vivo* during chronic allergic disease. Unfortunately, sort-purification of canine Th2 cells *ex vivo* for use in functional assays is not currently feasible, as intracellular Gata3 staining requires fixation and permeabilization of the cells, and the surface marker CCR4, which is associated with the Th2 phenotype (Maeda et al., 2002) only stains a subset of Gata3-expressing Th2 cells (Fig. S4 and Fig. 4A). Thus, we took an unbiased single-cell RNA sequencing approach (Macosko et al., 2015) to analyze gene expression profiles, with a focus on assessing genes in pathways that dictate T cell functionality, in PBMCs from 5 pairs of matched healthy dogs and dogs diagnosed with AD. Unsupervised UMAP clustering (Butler et al., 2018; Hafemeister and Satija, 2019; Stuart et al., 2019) identified 13 clusters of peripheral blood immune cells common to healthy and allergic dogs, including clusters with gene expression profiles consistent with those of cytotoxic cells (#11) and T cells (#6) (Fig. 5A, Table S2, Fig. S5).

Analysis of specific genes enriched in the T cell cluster showed that this cluster was enriched for expression of *GATA3* in both healthy and allergic dogs (Table S2, Fig. S5). Intriguingly, cells from dogs with AD in the T cell cluster also had generally higher levels of *GATA3* transcripts compared to cells in the T cell cluster from healthy dogs (Fig. 5B). Additional analysis of differentially expressed genes between cells from healthy dogs and those with AD within the *GATA3*-expressing T cell cluster revealed 95 ribosomal and non-ribosomal genes that were differentially expressed with an adjusted p-value < 0.05 (Fig. 5C, Table S3). These included a number of genes that have previously been associated with allergies in human and mouse studies (but not in dogs). Expression of the gene encoding cysteine rich protein 1 (CRIP1), which can be hypo-methylated in atopic humans and is more highly expressed in asthmatic humans (Stefanowicz et al., 2012), as well as the S100 proteins S100A6 and S100A4, calcium binding proteins associated with

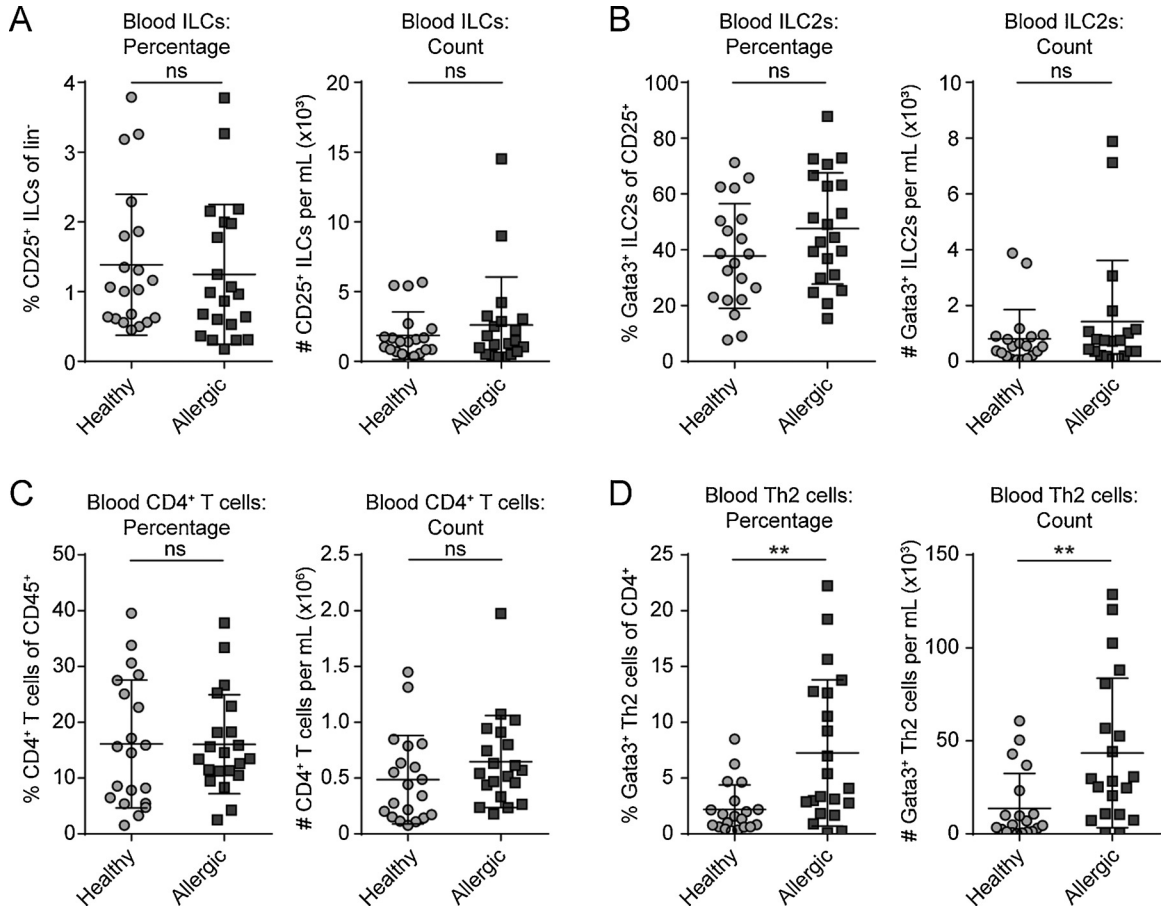

Fig. 3. Systemic increase of Gata3$^+$ Th2 cells but not ILC2s in atopic dogs. PBMCs were extracted from healthy and allergic dogs and analyzed by flow cytometry. Frequencies (left) and numbers (right) of **(A)** Lineage$^-$CD25$^+$ ILCs, **(B)** Gata3$^+$ ILC2s, **(C)** CD4$^+$ T cells and **(D)** Gata3$^+$ Th2 subsets. Mean ± SD; **, P ≤ 0.01; **(A–D)** n = 20 (healthy) or n = 21 (allergic) dogs (n = 20 for the counts) (Table S1); analyzed over 9 independent experiments.

asthma and seasonal allergic rhinitis, respectively (Bruhn et al., 2014; Calvo et al., 2009), were enriched in the T cell cluster in allergic dogs. The gene encoding the poly(A)-binding protein PABPC1, which can regulate posttranscriptional modification in murine lymphoid cells to tune protein function (Peng et al., 2017), was also enriched in this cluster in allergic dogs. This raises questions regarding the role of such factors in coordinating T cell function during allergy. Finally, the gene encoding HLA-DRB was strongly upregulated in the T cell cluster from allergic dogs. As human T cells upregulate MHC II upon activation (Holling et al., 2004), these data illustrate the similarity between canine and human T cell responses. Taken together, these data establish that single-cell RNA sequencing can be used to analyze gene expression profiles in canine T cells that are uniquely associated with the allergic condition and that could potentially highlight phenotypic and functional differences in T cells in the PBMCs of healthy *vs*. allergic dogs.

4. Conclusion

The studies described here utilize new approaches to study naturally occurring atopic disease in companion dogs and to dissect pathways associated with AD. We identified a canine immune cell type with a phenotype and function similar to that of human and murine ILC2s and canine Th2 cells *ex vivo* for the first time using an anti-Gata3 antibody. Elevated Th2 cell but not ILC2 frequencies and numbers in the peripheral blood were associated with chronic canine atopy. We further show that T cells from healthy and allergic donors readily polarize to the Th2 fate. Finally, single-cell RNA sequencing allowed for identification of immune cell clusters in the peripheral blood of healthy and allergic dogs and characterization of a cluster with an activated T cell-like gene expression profile that was enriched for *GATA3* expression in allergic dogs.

5. Discussion

Similar to human allergic disease, canine allergy is characterized by increased expression of type 2 cytokines IL-4, -5 and -13, specifically in the skin and blood of dogs with natural or experimental AD (Majewska et al., 2016; Marsella et al., 2006; Nuttall et al., 2002; Olivry et al., 2016). Our identification of canine ILC2s and Th2 cells supports the idea that these cells may be a rich cellular source of type 2 cytokines in dogs, as they are in humans and mice (Mjösberg et al., 2012, 2011; Pelly et al., 2016; Roediger et al., 2013; Salimi et al., 2013). The tools and approaches described here could facilitate further studies in canine breeds predisposed to allergy that could track ILC2 and Th2 cell responses from initiation to maintenance of the allergic state to dissect how these cell types contribute to the pathogenesis of allergic disease.

In particular, further studies are needed to assess canine ILC2 biology, how canine ILC2s contribute to allergic responses and whether canine ILC2s act similarly to human and murine ILC2s. Due to limited cell numbers, we were only able to assess how canine ILC-like cells responded to IL-2 or IL-2 + IL-33, observing results comparable to what is reported in humans and mice (Tait Wojno and Artis, 2016). However, numerous other factors regulate ILC2 responses in humans and mice, and murine and humans ILC2s possess a large range of functions, producing cytokines and directly interacting with other innate and adaptive cells (Tait Wojno and Artis, 2016). Additional *in vitro* and *ex vivo* studies will be needed to fully characterize the pathways that regulate diverse functions of canine ILC2s. Moreover, previous studies have

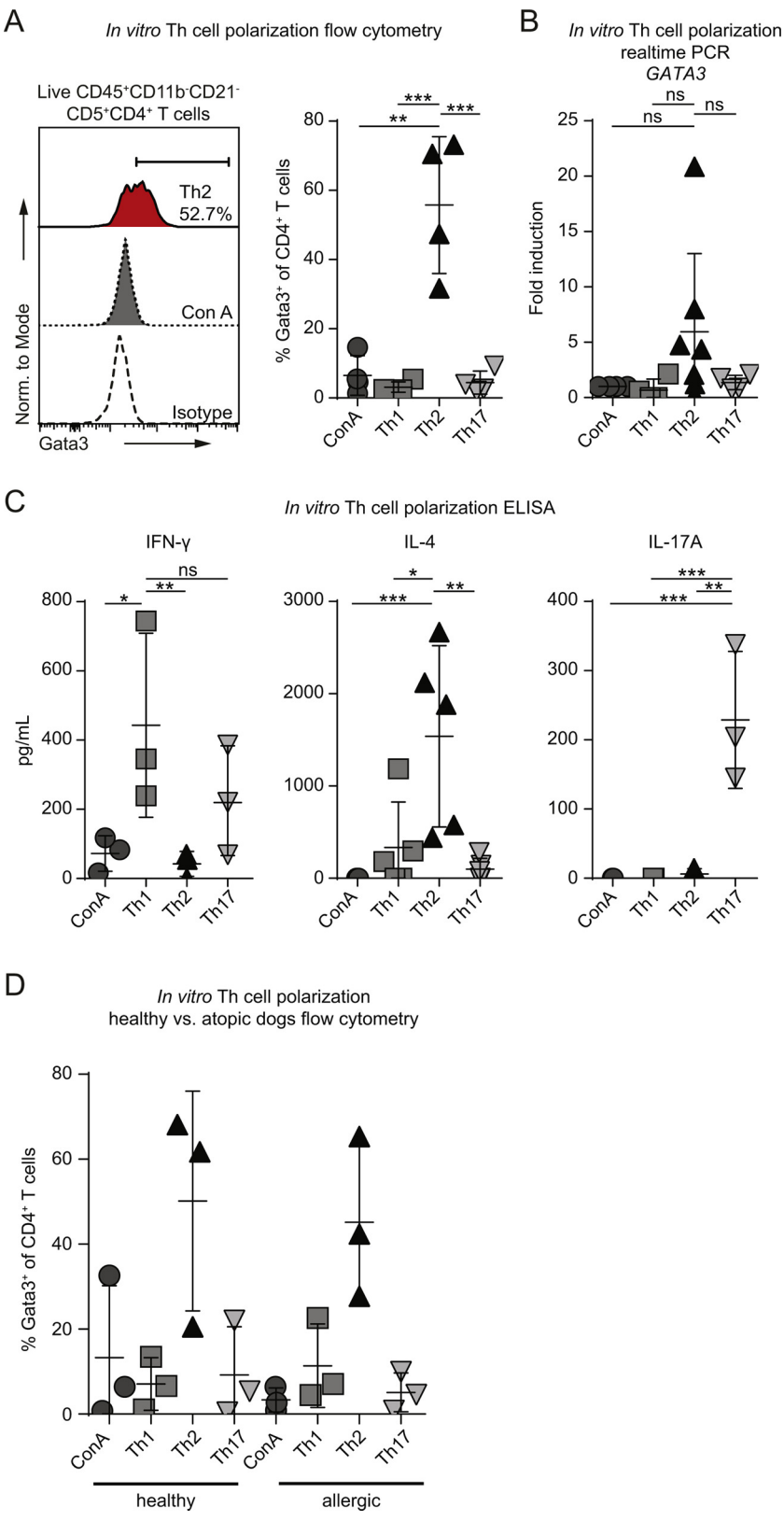

Fig. 4. Activated CD4$^+$ T cells polarize to the Th2 fate and secrete IL-4. CD4$^+$ T cells were sort-purified from PBMCs of healthy **(A–D)** and allergic **(D)** dogs, gated as live CD45$^+$CD11b$^-$CD21$^-$CD5$^+$CD4$^+$ cells, cultured for 5 days and differentiated to Th1, Th2 and Th17 subsets. **(A)** Gata3 protein determined by flow cytometry, **(B)** *GATA3* expression determined by realtime PCR (normalized to *ACTβ* and relative to the ConA condition), and **(C)** cytokine secretion measured by ELISA in cell culture supernatants (IL-4 background corrected; net IL-4 in Th2 condition (detected - added IL-4)) in polarized Th subsets. Mean ± SD; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; **(A)** representative of 3 independent experiments, 1 female, 3 males, red shaded = fully stained; **(B)** 3–5 independent experiments, 3 females, 4 males; **(C)** 3–5 independent experiments, 4 females, 6 males; **(D)** 2 independent experiments that included 3 age- and sex-matched pairs of 2 female pairs and 1 male pair (statistical comparison not performed due to lack of power).

shown that sex, age, spay/neuter status and availability of sex hormone exposure can play a role in allergy development in part by impacting the function of immune cells (Cephus et al., 2017; Fuseini and Newcomb, 2017; Laffont et al., 2017; Lambert et al., 2005; Mathä et al., 2019). Future studies will be required to determine if these factors affect ILC2 and Th2 responses in dogs or are associated with particular immune cell responses during canine allergy. While we did not find that these variables were associated with ILC2 or Th2 responses in our study, our participants were age- and sex-matched as much as possible and included mostly neutered or spayed animals. Thus, additional cohorts of dogs will need to be studied to determine how sex, age, or spay/neuter status impacts ILC2 or Th2 responses in healthy or allergic

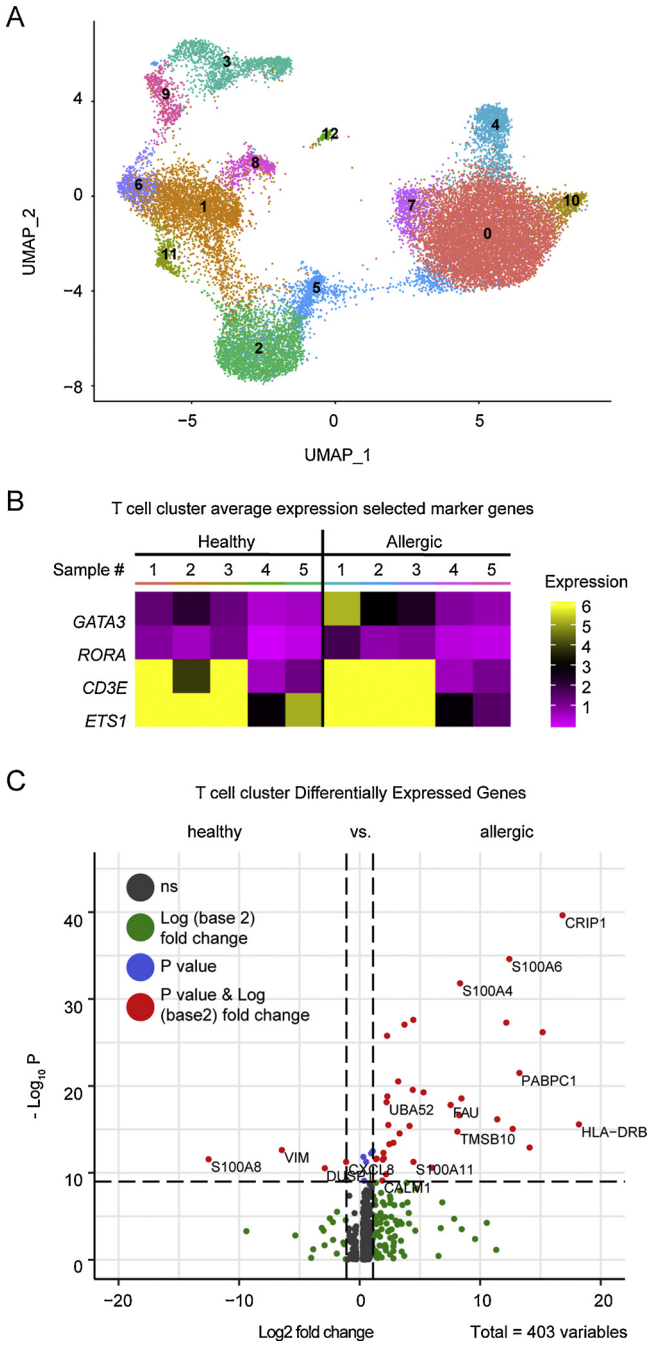

Fig. 5. T cells enriched for *GATA3* expression differentially express a suite of genes in allergic dogs. Single-cell RNA sequencing analysis of PBMCs from 5 healthy and 5 allergic dogs identified **(A)** 13 immune cell clusters common to all samples based on unsupervised clustering, displayed by UMAP plot. Analysis of differential gene expression in these clusters revealed **(B)** elevated expression of *GATA3* compared to selected marker genes in the T cell cluster (#6) of allergic compared to healthy dogs and **(C)** other DEGs between cells isolated from healthy and allergic dogs in the T cell cluster (#6) (genes of interest labeled); p cutoff = 10e-10, $\log_2$ cutoff = 1.1; colors indicate whether the gene meets the cutoff values for p value and/or log(2) fold-change. **(A–C)** 5 healthy and 5 allergic samples, 2 females, and 8 males.

dogs.

In our study ILC2 numbers in the PBMCs of allergic compared to healthy dogs were not increased, while Th2 cell numbers were. These data suggest that ILC2s may display different migratory or tissue localization patterns compared to Th2 cells in chronic canine allergy, or that ILC2s play a less prominent role than Th2 cells. The temporal and spatial organization of ILCs and how this affects their function remains a major open question in multiple species. In mice, ILC precursors in the bone marrow and blood can seed peripheral tissues early in life and replenish tissue resident populations in homeostasis and inflammation (Constantinides et al., 2014; Kim et al., 2016; Moro et al., 2016; Roediger et al., 2013). In addition, mature murine ILC subsets with tissue homing receptors in the blood mobilize during inflammation, with some subsets migrating to tissues (Huang et al., 2018; Kim et al., 2015; Stier et al., 2018). As we were only able to assess ILC2 responses in the peripheral blood and not the inflamed skin, further work assessing canine ILC2, as well as Th2, responses in the skin will be critical to fully understand how these cells are distributed during health and disease in dogs and the roles of these cell types in canine AD pathogenesis. Of note, as we were not able to identify an ILC-like cluster in the blood using an unsupervised single-cell sequencing approach (perhaps not surprising as identifying ILC2s in PBMCs can require presorting or targeted approaches (Björklund et al., 2016; Suffiotti et al., 2017)), such future studies should employ both flow cytometry-based and single-cell sequencing-based approaches to dissect immune pathways and cell types associated with AD in the blood and skin.

The significant increase in Th2 cell frequency and number in the peripheral blood of dogs with AD suggests that this population is strongly associated with chronic canine allergy. The potential importance of Th2 cells in driving or maintaining allergy in dogs was also highlighted by unbiased single cell RNA sequencing data. This analysis revealed immune cell clusters including a cluster containing T cells that on average had higher expression levels of *GATA3* in the allergic compared to healthy dogs, as well as 95 other differentially expressed genes. While some of these genes have previously been associated with AD in humans and dogs, including S100 calcium binding proteins (Dyjack et al., 2018; Guttman-Yassky et al., 2009; Majewska et al., 2016; Merryman-Simpson et al., 2008; Olivry et al., 2016; Plager et al., 2012; Salzmann et al., 2011; Suárez-Fariñas et al., 2015; Wood et al., 2009), others appear to be novel. Further studies are needed to determine the precise role of these genes in canine AD and how these factors influence the pro-allergic Th2 cell population. Excitingly, the Th cell polarization assay described here could provide an *in vitro* platform to test how such factors are regulated in canine Th2 cells and to better understand how canine Th2 polarization occurs in allergic and healthy dogs (Hammad et al., 2001).

$CD4^+$ T cells from allergic and healthy dogs similarly polarized to the Th2 fate *in vitro*, and while a larger sample size is called for in this analysis, these data suggest that $CD4^+$ T cells from allergic dogs are not poised to preferentially adopt the Th2 fate per se, especially in the presence of strong polarizing signals that may override normal differentiation. *In vivo*, allergic dogs may have higher type 2 cytokine levels that drive strong Th2 polarization. In addition, our studies do not address how $CD4^+$ T cells are primed to the Th2 fate by antigen-presenting cells in allergic or healthy dogs. Of note, we did find a breed-specific increase of Gata3 expression in Westies, a breed with a predisposition for the development of AD (DeBoer, 1999). Future work will be needed to determine how elevated baseline Gata3 expression in Westies in immune- and non-immune cells could contribute to this predisposition. In particular, Gata3 in keratinocytes regulates epidermal differentiation and expression of filaggrin, whose disruption by genetic defect or inflammatory signals can lead to atopic skin disease in humans and mouse models (Zeitvogel et al., 2017; Cabanillas and Novak, 2016; Marsella et al., 2011; de Guzman Strong et al., 2006). Filaggrin gene expression is upregulated in the epidermis and filaggrin protein is distributed discontinuously in atopic dogs (Santoro et al., 2013; Theerawatanasirikul et al., 2012), but while null mutations in filaggrin are prevalent in atopic humans, the filaggrin locus was not associated with AD in Westies (Barros Roque et al., 2009). Thus, increased Gata3 in Westie T cells may alter other factors to predispose to allergy, though the significance of the Gata3-filaggrin axis and the role of elevated Gata3 expression in $CD4^+$ T cells are unknown.

Intriguingly, the Gata3^{+} Th2 cell frequencies in our canine groups resembled the frequencies of Th2 cells in the blood of healthy humans and human patients with intrinsic AD (Czarnowicki et al., 2015; Tokura, 2010). In addition, dogs with chronic allergic disease, similar to adult humans with intrinsic AD, had largely normal frequencies and numbers of other leukocytes in the peripheral blood (Salimi et al., 2013; Villanova et al., 2014; Czarnowicki et al., 2017, 2015). Further, in both species, disease does not correlate with elevated IgE (DeBoer and Hillier, 2001; Tokura, 2010). Thus, dogs with naturally occurring chronic canine AD that have remarkable similarities in the cellular immunology in the blood to humans with intrinsic AD could be used as a relevant model of this human disease, as is the case for other complex human diseases such as cancer, autoimmune diseases and allergies (Marsella and Girolomoni, 2009; Rowell et al., 2011; Wilbe et al., 2010). Critically, AD is a major concern in canine health. AD is often difficult to manage in clinics due to disease chronicity and heterogeneity, and an incomplete understanding of the complex underlying mechanisms that contribute to pathogenesis. Taken together, the new methods presented here and the further characterization of Th2 and ILC2 responses in the atopic dog will facilitate a better understanding of both canine and human AD that can ultimately inform clinical management.

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Authors' contributions

SPF and EDTW designed the project, carried out experimental work, analyzed data and wrote the manuscript, with valuable input from the other authors. MS carried out experimental work and analyzed single-cell RNA sequencing data. OOO, LMW, SAP, and RLC carried out experimental work. JE, CAM, JEM, JMC and WHM coordinated and performed sample acquisition and clinical evaluation of canine patients. CGD assisted in single-cell RNA seq analysis. EDTW conceived the project and supervised the research. All authors read and approved the final manuscript.

Availability of data

Sequencing data are available in NCBI's GEO, a publicly accessible data repository, under accession number GSE144730. All other data are included in this published article and its supplementary information files.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2020.110015.

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