Title: Sublingual allergen immunotherapy with recombinant dog allergens prevents airway hyperresponsiveness in a model of asthma marked by vigorous T_H2 and T_H17 cell responses

Julian M. Stark[1], Jielu Liu[2], Christopher A. Tibbitt[1], Murray Christian[1], Junjie Ma[1], Anna Wintersand[3], Ben Murrell[1], Mikael Adner[2], Hans Grönlund[3], Guro Gafvelin[3], Jonathan M. Coquet[1]

[1] Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, 171 65 Stockholm, Sweden

[2] Institute of Environmental Medicine and Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden

[3] Department of Clinical Neuroscience, Karolinska Institutet, Centre for Molecular Medicine, Stockholm, Sweden

Correspondence:

Jonathan.Coquet@ki.se

+46 8 524 86952

Abstract

Allergy to dogs affects around ten percent of the population in developed countries. Immune therapy of allergic patients with dog allergen extracts has shown limited therapeutic benefit. Herein, we established a mouse model of dog allergy and tested the efficacy of a recombinant protein containing Can f 1, f 2, f 4 and f 6 as a sublingual immune therapy (SLIT). Repeated inhalation of dog extracts induced infiltration of the airways by T_H2 cells, eosinophils and goblet cells, reminiscent of the house dust mite (HDM) model of asthma. However, dog allergen extracts also induced robust T_H17 cell responses, which was associated with a high neutrophilic infiltration of the airways and promoted airway hyperresponsiveness more potently than HDM allergens. scRNA-Seq analysis of T helper cells responding to dog allergens identified several unique clusters with T_H17 cells being hallmarked by the expression of several receptors including IL-17RE. Analysis of T cell receptors also depicted a high frequency of clones that were shared between T_H17, T_H2 and suppressive Treg cells, indicative of the plasticity of T helper cells in this model. Importantly, prophylactic SLIT reduced airway hyperresponsiveness and type 2-mediated inflammation in this model supporting the use of recombinant allergens in immune therapy.

Introduction

Asthma is a chronic inflammatory disorder of the airways affecting over 300 million people worldwide, leading to loss of quality of life and an estimated 420,000 deaths in 2016 (Network, 2018). Furthermore, the incidence of allergic airway disease is rising globally (Moorman et al., 2012).

A central player in the asthmatic and allergic response is the CD4 T cell. CD4 T cells respond to peptides presented in the context of MHC-II molecules. When activated by viruses, bacteria, or allergens including house dust mites, pollen and pet dander, CD4 T cells become activated and differentiate into several functionally-distinct T helper (T_H) cell subsets; T_H1 cells secrete the cytokine interferon-gamma (IFN-γ) and promote immunity to viruses, T_H2 cells secrete interleukin- (IL-) 4, IL-5 and IL-13 and mediate helminth clearance, T_H17 cells secrete IL-17 and promote immunity to fungi and Treg cells suppress inflammation through various mechanisms (Zhu and Paul, 2010).

In clinical and preclinical studies, CD4 T cells have repeatedly been shown to be central to the development of allergic and autoimmune disorders. The HLA-D locus, which codes for major histocompatibility class II molecules that present antigens to CD4 T cells, has repeatedly been implicated in the pathogenesis of asthma in genome-wide association studies (GWAS). GWAS have also pinpointed several genes associated with T_H2 cells in the development of allergies including asthma, such as *IL4*, *IL13*, *IL5*, *GATA3, IL33* and *IL33R* (Li et al., 2010; Michel et al., 2010; Moffatt et al., 2010). Cytokines produced by T_H2 cells have been shown to mediate several features of childhood-onset asthma including airway eosinophilia, goblet cell metaplasia and IgE secretion (Lambrecht and Hammad, 2015). However, roles for other T_H subsets including T_H1, Tfh, Treg and T_H17 cells have also been demonstrated (Coquet et al., 2015; Lambrecht and Hammad, 2015).

Adult-onset asthma, which is increasing in prevalence world-wide, is associated with higher levels of T_H17 cells and neutrophils in the airways and is more frequently resistant to standard treatments such as corticosteroids (Domvri et al., 2018). While severe treatment-resistant asthma only affects a minority of asthma patients, the medical costs per patient are disproportionally high (Breekveldt-Postma et al., 2008).

The only curative treatment available for allergic disease is allergen-specific immunotherapy (AIT). Patients are administered allergen in low doses over a long period with the aim of desensitization (Akdis and Akdis, 2011). Tolerance is thought to be achieved by the induction

of Treg cells, by decreasing the T_H2 cell response and by shifting the antibody balance from IgE to IgG1 and IgG4 (Sahin et al., 2017).

Over the last few decades, several preclinical animal models have been developed to mimic human allergy including models of house dust mite, cat, peanut, lupine and fungal (e.g. *Alternaria alternata*) allergies (Andreassen et al., 2018; Burton et al., 2017; Cates et al., 2004; Havaux et al., 2005; Neimert-Andersson et al., 2008). It has become apparent that allergens are highly diverse and elicit distinct immune responses, with some inducing strong innate allergic responses, while others induce primarily adaptive immune responses. Furthermore, the quality of innate or T helper cell response induced by allergens has been found to vary. For instance, *Aspergillus versicolor* induces strong T_H17 responses side-by-side with T_H2 cell responses, while house dust mite allergens induce primarily strong type 2 responses (Tibbitt et al., 2019; Zhang et al., 2017).

Dogs are not only a common pet but also serve various roles in society including working with police, in customs and in the health sector. Dog allergens are found in dander, hair, saliva and urine and can easily become airborne (Ownby et al., 2002; Polovic et al., 2013). Twelve percent of US citizens are sensitized to dog allergens and over 1 million asthma attacks are attributed to dog exposure every year in the US alone (Gergen et al., 2018). Eight dog allergens have been identified of which Can f 1, Can f 2, Can f 4 and Can f 6 are part of the lipocalin protein family. Members of the lipocalin superfamily can be found in all common mammalian allergen sources and have been proposed to promote T_H2 cell responses (Klaver et al., 2020). Cross reactivity is a common feature of pet allergies due to structural similarities of proteins from the lipocalin and serum albumin families. Approximately 95% of individuals sensitized to pet allergens are sensitive to multiple allergens (Uriarte and Sastre, 2016). Between 50-70% of dog-allergic people are sensitized to Can f 1 making it a major dog allergen. Approximately 25% of dog allergic people are sensitive to Can f 2 (Konieczny et al., 1997), 35-81% to Can f 4 (Mattsson et al., 2010; Rytkönen-Nissinen et al., 2015), 70% to Can f 5 (Mattsson et al., 2009) and 38% to Can f 6 (Nilsson et al., 2012). Sensitivity to other Can f antigens are also prevalent. Thus, dog-allergic individuals are typically sensitive to a number of known allergens found in dogs. Dog ownership is correlated with increased levels of airborne endotoxin (Park et al., 2001) and endotoxin exposure has been linked to an increased risk of neutrophilic asthma (Radon, 2006). Despite the prevalence of dog allergies, a mouse model to study dog allergen-induced airway inflammation has not been developed. Such a model would provide an important platform to test and develop novel therapeutics.

118 In this study, we show that intranasal administration of dog allergen extracts induced an
119 inflammatory response in the airways of mice. T_H2 cell responses and type 2-associated
120 inflammation hallmarked by airway eosinophilia was a prominent feature of dog allergen
121 administration, as it was in the house dust mite model. However, dog allergen extracts also
122 induced robust T_H17 cell responses and associated neutrophilia. scRNA-Seq analysis revealed
123 several transcriptionally-distinct T_H cell subsets and pinpointed several clones of T_H cells that
124 had expanded in response to dog allergens. Finally, prophylactic administration of a
125 recombinant protein combining Can f 1, f 2, f 4 and f 6 allergens ameliorated T_H2 cell responses
126 and airway hyperresponsiveness, but did little to alter the neutrophilic airway response. Thus,
127 we characterise a new model of allergic airway inflammation to dog allergen extracts and
128 propose that immunotherapy with recombinant allergens may be beneficial in individuals
129 sensitive to dogs.
130

131 **Methods**

132

133 **Experimental animals**

134 Wildtype C57BL/6J and *Rag1*$^{-/-}$ mice were bred and maintained at the Comparative Medicine
135 animal facility located at Karolinska Institutet. Mice were 8-10 weeks old at the start of
136 experiments and both female and male mice were used in experiments but only one gender
137 was used per experiment. All mice were housed in individually ventilated cages with food and
138 water *ad lib* and under specific pathogen-free conditions. Experiments were approved by
139 Stockholms jordbruskverket (8971/2017).

140

141 **Dog and house dust mite model**

142 Mice were intranasally sensitized with 1 µg of house dust mite (HDM) (Greer) extract in 40 µl
143 PBS, 1 µg each of dog dander and epithelial extract (Greer), referred to as dog allergen
144 extracts, in 40 µl PBS or with PBS as control. One week after sensitization, mice were
145 challenged with 5 daily administrations of 10 µg of HDM in 40 µl PBS or 5 µg each of dog
146 dander and dog epithelial extract in 40 µl PBS or with PBS as control. Mice were sacrificed
147 and organs harvested on day 15. Bronchoalveolar lavage was performed by cannulating the
148 trachea and flushing out the airways with 2x1 ml PBS (Tibbitt and Coquet, 2016). In
149 experiments aimed at quantifying airway neutrophilia, mice were challenged one additional
150 time 3h before sacrifice. All instillations were done under isoflurane anesthesia.

151

152 **Flow cytometry**

153 Flow cytometry was performed on a BD LSRII using combinations of the following antibodies:
154 from BD: B220 (RA3-6B2), CD3 (145-2C11), CD4 (RM4-5 and GK1.5), CD8 (53-6.7), CD44
155 (IM7), GR-1 (RB6-8C5), IFN-γ (XMG1.2), IL-4 (11B11), IL-17 (TC11-18H10), Siglec-F (E50-
156 2440) and FC-block; from Invitrogen: CD11c (N418), FOXP3 (FJK-16s), IL-13 (ebio13A); from
157 Biolegend: IL-5 (TRFK5). For intracellular staining, cells were fixed and permeabilized using
158 the eBioscience FOXP3/Transcription factor staining buffer set from Invitrogen.

159

160 **Restimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin**

161 For detection of cytokine-producing cells from the airways and lung tissue, cells were
162 stimulated with PMA and ionomycin in the presence of Brefeldin A and/or Golgistop (containing
163 Monensin, BD) for 3 hours at 37°C and analyzed by flow cytometry.

164

165 **Restimulation of lymph node cells and quantification of cytokine production by**
166 **cytometric bead array (CBA)**

167 $2.5x10^5$ cells from the mediastinal lymph node were cultured for 2 days in IMDM medium and
168 restimulated by either HDM (20 µg/ml), dog allergen extracts (20 µg/ml), Can f 1, Can f 2, Can
169 f 3, Can f 4 or Can f 6 (10µg/ml). The supernatant was collected and analyzed with the BD™
170 CBA Mouse Enhanced Sensitivity kit (BD). CBA samples were run on a CyAn™ ADP Analyzer
171 (Beckman Coulter).
172
173 **Quantification of endotoxin levels in allergen extracts**
174 Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermofisher) was used to measure
175 endotoxin content of HDM, dog dander and dog epithelium extracts.
176
177 **ELISA**
178 ELISA was performed by coating ELISA plates (nunc) either with unconjugated anti-IgE (R35-
179 92, BD,) allergen extracts (5 µg/ml) or recombinant Can f 1 (5 µg/ml). Plates were incubated
180 at 4 °C for twelve hours, washed with PBS and blocked with 2% milk in PBS. Serum was added
181 in three-fold or five-fold serial dilution and incubated for 2 h at room temperature. Plates were
182 washed and then incubated for one hour with secondary antibody, either HRP coupled anti-
183 IgG1 (SouthernBiotech) or biotin coupled anti-IgE (R35-72, BD) followed by streptavidin – HRP
184 (Mabtech). TMB substrate (KPL) followed by H_2SO4 were used to develop and stop the assay.
185 The Asys Expert 96 ELISA reader (Biochrom) was used to read OD at 450 nm.
186
187 **Histopathology**
188 Lungs were fixed with 10% Formalin for a minimum of 24 h before being embedded in paraffin.
189 Periodic acid-Schiff-diastase (PAS-D) and Hematoxylin & Eosin (H&E) stains were performed.
190 Complete airways in PAS-D stained lung sections were scored on a 0-4 point scale with points
191 awarded based on the percentage of the airway covered by positively stained cells; 0 points
192 for 0% of the airway affected, 1 point for 1-25%, 2 points for 26-50%, 3 points for 51-75% and
193 4 points for more than 75% PAS positive. 22-72 full airways were counted per mouse.
194
195 **Measurement of airway function**
196 Mice were sensitized and challenged following the standard regimen with one additional
197 challenge on day 14. Animals were anaesthetised with 10 ml/kg i.p. mixture of hypnorm
198 (Fentanyl 0.315 mg/ml, fluanisone 10 mg/ml, VetaPharma), midazolam (5 mg/ml, hameln) and
199 saline (Apoteket) in 1:1:2. After being tracheostomised and cannulated, mice were connected
200 to the *FlexiVent* apparatus equipped with module 1 (SCIREQ) where animals were ventilated
201 at respiratory rate of 150 breaths/minute, tidal volume of 10 ml/kg and positive end expiratory
202 pressure (PEEP) of 3 cmH_2O. Following stabilization, lung resistance was measured using
203 forced oscillation technique (FOT) at baseline and under increasing concentrations of

204 nebulised methacholine (Sigma). Respiratory mechanics parameters were calculated by
205 *flexiWare* version 8 (SCIREQ) based on a single compartment model and constant phase
206 model. These included total respiratory system resistance (Rrs), elastance (Ers) calculated
207 from single compartment model and Newtonian resistance (Rn), tissue damping (G), and
208 tissue elastance (H) from constant phase model.
209
210 **RNA-seq of single T helper cells from the BAL**
211 T helper cells were purified from the bronchoalveolar lavage of mice sensitized and challenged
212 with dog allergen extracts. The BAL was kept cold and processed rapidly. Cells were stained
213 for CD4, CD3, Siglec-F and B220. $CD4^+$ $CD3^+$ $SiglecF^-$ $B220^-$ cells were sorted into pure FCS
214 using a BD FACSAria Fusion. Cells were washed and resuspended in cold PBS. Single cells
215 were isolated with the droplet-based microfluidic system Chromium (10X Genomics). Libraries
216 were prepared by the Eukaryotic Single Cell Genomics national facility at SciLife Laboratory,
217 Stockholm. Gene expression matrices were preprocessed and filtered using Seurat v3 (Butler
218 et al., 2018; Stuart et al., 2019). TCR frequencies and expression patterns were analyzed and
219 graphed with Loupe V(D)J Browser (10x Genomics) and by combining the barcodes and
220 clusterfiles generated with Seurat v3. For each cluster, the clonotypes occurring in that cluster
221 were counted. To visualise the distribution of counts in each cluster, ball-packing plots (Fig.
222 6C) were created using the Julia package packBalls.jl (yet to be uploaded). To further
223 represent these distributions, the clonotype counts in each cluster were ordered from highest
224 to lowest and Lorenz curves plotted (Fig. 6D). The co-occurrence of clonotypes between
225 clusters is summarised by a plot of pairwise correlations of square-root-transformed counts of
226 all clonotypes (Fig. 6E).
227
228 **Sublingual immunotherapy**
229 Mice were anaesthetized with isoflurane and administered either 10 µg of Can f 1-2-4-6 protein
230 (Nilsson et al., 2014) in 20 µl PBS or PBS as control sublingually three times per week for four
231 weeks before the standard intranasal allergen instillations.
232
233 **Dexamethasone treatment**
234 To test for corticosteroid resistance mice were injected either with 1 mg/kg dexamethasone
235 intraperitoneal daily from day 7 to 14 after sensitization or with PBS as control.
236
237 **Quantification and statistical analysis**
238 Nonparametric Mann-Whitney U test was used to compare two groups. For multiple
239 comparisons, one-way analysis of variance (ANOVA) and Bonferonni's test were used. In fig.
240 1, to compare AHR of mice administered HDM or dog allergen extracts to PBS control mice,

ANOVA and Dunnett's multiple comparisons test were used. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$p<0.0001$.

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249 **Results**

250

251 **Intranasal administration of dog allergen extracts leads to immune cell infiltration of the**
252 **airways, goblet cell metaplasia and airway hyperresponsiveness**

253 A regimen of intranasal administration of dog allergen extracts was performed that was in line
254 with methods used for inducing airway inflammation with HDM allergens (Chen et al., 2017).
255 Briefly, 1 µg each of dander and epithelium extract was administered i.n. to sensitize mice
256 followed by five daily administrations of 5 µg of each extract (10 µg total) on days 7-11. On day
257 15, mice were sacrificed and analyzed for signs of airway and lung tissue inflammation (Fig.
258 1A). Instillations with HDM and dog allergen extracts led to a comparable influx of cells into the
259 airways (Fig. 1B), hallmarked by a high number of eosinophils (Fig. 1C and Fig. S1A for gating).
260 The number of alveolar macrophages in the lavage was not significantly different between
261 mice after HDM, dog allergen extracts or PBS instillation (Fig. 1D). A moderate increase in
262 neutrophil infiltration of the airways was observed in mice administered dog allergens
263 compared to those administered HDM (Fig. 1E). B cell numbers in the lavage were comparable
264 between mice administered dog allergen or HDM extracts (Fig. 1F), whereas more T cells were
265 present in the airways of mice administered dog allergen extracts (Fig. 1G). Inflammation of
266 the lungs and airways was confirmed by Hematoxylin and Eosin (H&E) staining. This also
267 depicted thickening of the basement membrane and smooth muscle cell layer surrounding the
268 airways (Fig. S1B). To assess the effect of dog allergen extracts on lung physiology, we
269 performed Periodic acid Schiff-diastase (PAS-D) staining of lung sections and invasive lung
270 function testing. Lungs of HDM and dog allergen extract-administered mice showed signs of
271 goblet cell metaplasia and appreciable mucus production (Fig. 1H,I). Furthermore, dog
272 allergen extract-sensitized and -challenged mice had higher levels of total airway resistance
273 (Rrs) and elastance (Ers) when compared to mice administered PBS mice, reacting more
274 strongly at most doses of methacholine (Fig. 1J,K). Newtonian resistance (Rn), which
275 measures the resistance of the large conducting airways, was increased in both HDM or dog
276 allergen extract-administered mice compared to control mice (Fig. 1L). Thus intranasal
277 administration of dog allergen extracts induced signs of allergic airway inflammation
278 reminiscent of that seen after instillation of HDM.

279 **Dog allergen extracts induce development of both T_H2 and T_H17 cells**

280 To investigate the role of adaptive immunity for the inflammatory response to dog allergens,
281 we administered dog allergen extracts to *Rag1*$^{-/-}$ mice. This failed to induce appreciable airway
282 eosinophilia such as that seen in WT mice (Fig. 2A). Thus, we analysed the immune response
283 of WT mice more comprehensively. Administration of either HDM or dog allergens to WT mice
284 led to increased numbers of effector (CD44^{+}) CD4 T cells in the airways and increased

285 proportions of Treg cells (% Foxp3$^+$ of CD4$^+$ cells) in the lungs (Fig. 2B,C). Cytokine production
286 was measured after stimulating airway or lung tissue cells with phorbol 12-myristate 13-acetate
287 (PMA) and ionomycin for 3 hours. In cells isolated from the airways, notable frequencies of T
288 helper cell expressing IL-5, IL-13 and IFN-γ were observed in mice administered dog allergen
289 extracts, comparable to the frequency in mice administered HDM (Fig. 2D). An average of 28%
290 of CD4$^+$ T cells from mice administered dog allergen extracts appeared to produce IL-17,
291 significantly higher than the frequency of IL-17-producing cells in HDM-sensitised and -
292 challenged mice (Fig. 2D). CD4$^+$ T cells from the lung showed a similar pattern of cytokine
293 expression, with the highest frequencies of IL-17-producing cells observed in dog-sensitized
294 and -challenged mice (Fig. 2E). High endotoxin levels have been reported to promote a strong
295 T_H17 response in murine asthma models (Zhao et al., 2017). While both HDM and dog allergen
296 extracts contained endotoxin, the levels in the dander extracts were around two orders of
297 magnitude higher than in the HDM extract and about 17-fold higher in epithelium extract
298 compared to HDM extract (Fig. S2A). Adminstration of dog epithelium or dander allergen
299 extracts separately, induced inflammation in the airways that was characterized by airway
300 eosinophilia and Th2, Th1 and Th17 cell cytokine production in the airways and lung tissue.
301 Although extracts of dander appeared to induce more inflammation, epithelial extracts also
302 promoted considerable inflammation and Th17 cell differentiation (Fig. S2B-D). The
303 abundance of Can f 1, Can f 2, Can f 3 and Can f 6 in each extract was also quantified, with
304 Can f 3 being detected at the highest concentration in both extracts (Fig. S2E).

305 Since T_H2 cell-driven allergic disease leads to class switching of B cells to IgG1 and IgE
306 production, we analyzed serum antibody levels by ELISA. Mice administered HDM or dog
307 allergens showed high levels of allergen-specific IgG1 antibodies (Fig. 2F) and showed
308 comparable levels of total IgE in serum. Whole dog allergen extract-specific IgE was not
309 detected in the serum of mice (not shown), potentially due to competition from IgG1, as has
310 been previously described (Lehrer et al., 2004). However, IgG1 and IgE specifically binding to
311 Can f 1 was detected from the serum of mice administered dog allergen extracts (Fig. 2F).

312 Strong T_H17 cell responses have been linked to the recruitment of neutrophils to sites of
313 inflammation and higher numbers of neutrophils were present in the BAL of mice administered
314 dog allergens (Fig. 1E), four days after the last allergen challenge. Since neutrophils are
315 typically recruited rapidly to a site of inflammation, we challenged mice an additional time on
316 day fifteen, three hours before sacrifice. Challenge on day 15 with dog allergens markedly
317 increased the infiltration of neutrophils into the airways, in a manner not observed with HDM
318 (Fig. 2G). In all, dog allergens induce strong type 2 mediated airway inflammation including
319 robust T_H2 cytokine production, airway eosinophilia, goblet cell metaplasia, airway
320 hyperresponsiveness and IgG1/IgE production. The response to dog allergen challenge

321 includes strong T_H17 cell responses and neutrophil recruitment into the airways, a feature more
322 commonly associated with adult-onset asthma in humans.

323 **Restimulation of lymph node cells with dog allergen extracts leads to production of IL-**
324 **13, IL-5, IL-10, IL-17 and IFN-γ**

325 To investigate the specificity of cytokine production in response to dog allergens, we
326 restimulated mediastinal lymph node cells from mice administered PBS, HDM or dog allergen
327 extracts for 48 hours in the presence of allergen extracts or recombinant Can f 1 or Can f 2
328 (Fig. 3A-E). HDM and dog allergen extracts induced comparable IL-13, IL-5 and IL-10
329 production in lymph node cell cultures from mice that had been sensitized and challenged with
330 the respective allergen (Fig. 3A-C). Only cells from mice administered dog allergen extacts
331 produced high levels of IL-17 and IFN-γ (Fig. 3D,E), whereas lymphocytes from mice
332 administered HDM produced very little of these cytokines. Recombinant Can f 1 appeared to
333 induce production of IL-5, IL-13, IL-10 and IFN-γ but did not appear to stimulate production of
334 IL-17. Thus, while Can f 1 induces considerable cytokine secretion from lymphocytes, other
335 antigens present in dog allergen extracts are also likely to promote responses from T cells.

336 **Dexamethasone reduces airway eosinophilia and cytokine production but not airway**
337 **neutrophila**

338 Production of IL-17 is a feature of adult-onset and steroid-resistant asthma. We used
339 dexamethasone to test the effect of corticosteroids on dog allergen-induced airway
340 inflammation treating mice daily from the first day of allergen challenge up to one day before
341 sacrifice (day 7 to day 14, Fig. 4A). Mice were administered an additional dose of allergen
342 extracts three hours before sacrifice in order to assess the effect of dexamethasone on the
343 levels of airway neutrophilia after dog allergen exposure (Fig. 4A). Dexamethasone reduced
344 the overall number of airway-infilitrating cells and the level of airway eosinophilia but did not
345 significantly reduce the number of airway neutrophils in the dog allergen model (Fig. 4B-D).
346 Both B cell and effector T helper cell numbers in the BAL were significantly reduced in
347 dexamethasone treated mice (Fig. 4E,F). Dexamethasone administration appeared to reduce
348 T_H2 and T_H17 cytokine-producing cells in the lungs of mice administered either HDM or dog
349 allergen extracts (Fig. 4G,H). Thus, corticosteroid treatment was effective in reducing several
350 parameters of airway inflammation in response to dog allergens, but failed to reduce airway
351 neutrophilia.

352 **Single cell RNA-Sequencing (scRNA-Seq) reveals several distinct T helper cell clusters**
353 **in the BAL**

354 To further probe the diversity and composition of T helper cell populations we purified
355 $CD3^+CD4^+$ cells from the airways of mice administered dog allergen extracts and analysed
356 these with the droplet-based microfluidic system, Chromium (10X Genomics). A total of 5,489
357 cells passed quality control. Unsupervised hierarchical clustering and visualization via Uniform
358 Manifold Approximation and Projection (UMAP) identified 8 distinct clusters (Fig. 5A,B). In all
359 clusters, the majority of cells expressed transcripts for *Cd3e*, *Cd3g* and *Cd3d* (Fig. 5C), while
360 *Cd4* was also detected at reasonable levels. Several clusters could be identified based on the
361 expression of genes with previously described functions. Cluster 7 expressed mRNAs
362 characteristic of naïve CD4 T cells such as *Sell* and *Ccr7* at high levels, but not *Cd44*. Cluster
363 4 expressed *Foxp3*, *Ctla4* and other genes typical of Treg cells. This cluster also expressed
364 high levels of *Ncmap* the gene encoding for Non-Compact Myelin Associated Protein, which a
365 recent *in silico* meta-analysis of Treg cell gene expression has suggested as a signature gene
366 (Marodon, 2019) (Fig. 5C and Table S1). Clusters 1 and 5 could be identified as T_H17 and T_H2
367 cells respectively based on the expression of cytokines, transcription factors and surface
368 markes (Fig. 5C, 5D, 5F and table S1). For instance, cells in cluster 1 were highly enriched for
369 the expression of *Il17a, Ccr6* and *Rorc* mRNA (Fig. 5C, 5D). Cluster 1 cells also expressed the
370 gene for the IL17C receptor *Il17re*. IL-17C:IL17RE interactions have previously been shown to
371 be required for the development of experimental autoimmune encephalomyelitis and to
372 promote T_H17 responses by inducing expression of IκBζ (Chang et al., 2011). Another gene
373 with a significantly higher expression in the T_H17 cluster was *Sdc4*. *Sdc4* encodes for
374 Syndecan4 which functions as a regulator of cell adhesion and the actin cytoskeleton, and
375 interacts with the extra cellular matrix. A role of Syndecan 4 in T_H17 cell biology has not been
376 described to our knowledge. *Smox*, the gene encoding for the enzyme spermidine oxidase
377 (SMOX) was also highly enriched in T_H17 cells (Fig. 5D). SMOX regulates polyamine levels
378 and has been implicated to play a role in IL-17 production in murine colitis models (Gobert et
379 al., 2018). Further highly enriched genes in the T_H17 cluster included *Aqp3* (Aquaporin-3),
380 *Ramp1* (Receptor activity modifying protein 1) and *Tmem176a*, all of which have recently been
381 described to promote T_H17 responses (Zhou, 2016 ; Mikami, 2012; Drujont, 2016). We
382 performed gene ontology (GO) analysis for the T_H17 cluster and identified several enriched
383 molecular processes in T_H17 cells related to ribonucleotide and purine ribonucleoside
384 metabolic processes, the cellular response to hypoxia and the T-helper 17 type immune
385 response (Fig. 5E).

386 Cells in cluster 5 resembled T_H2 cells and expressed classical T_H2 cell-associated genes
387 including *Gata3* and *Il1rl1* (Fig. 5F). Cluster 5 was also enriched for T_H2 cell-associated
388 cytokines including *Il13* and *Il5* (Table S1 and Fig. S3B). Several genes that have recently
389 been reported to be highly expressed in T_H2 cells in the HDM model (Tibbitt et al., 2019), such
390 as *Plac8*, *Zcchc10, Cd200r1* and *Il6* were also found to be enriched in the T_H2 cluster in this

391 model (Fig. 5F and table S1). GO analysis identified regulation of apoptotic pathways, type 2
392 immune response and regulation of lipid metabolism as enriched molecular processes in T_H2
393 cells (Fig. 5G), in line with molecular processes enriched in T_H2 cells from the HDM model
394 (Tibbitt, 2019). When the gene transcription profile of T_H2 cells in this model was compared to
395 that generated to HDM allergens (Tibbitt, 2019), a high overlap was observed (Fig. 5H).
396 Furthermore, T_H2 cells in the airways were enriched for the expression of genes related to fatty
397 acid oxidation (Fig. 5H), as they were in response to HDM. This suggests that T_H2 cells
398 generated in the HDM and dog allergen extract models have a high degree of similarity.

399 We noted that cluster 8 cells (Act4) expressed genes typically associated with Tfh cells,
400 including *Tox2*, *Il21*, *Bcl6* and *Cxcr5*. Gene set enrichment confirmed that these cells were
401 significantly enriched for markers typically associated with germinal centre Tfh cells (Fig. S3A).

402 Cells in cluster 6 (Act3) appeared to express some genes of recently described CD4 CTL
403 including *Ccl5*, *Gzmk*, *Ly6c2*, *Nkg7* and *Tbx21* (Fig. 5C and Table S1). Whether these cells
404 have true cytotoxic potential is unclear. Intriguingly, this cluster was not specifically enriched
405 for *Ifng* mRNA expression and this cytokine was seen to be expressed in many clusters (Fig.
406 S3B).

407 Expression of *Ifng*, *Il13* and *Foxp3* was also observed in the T_H17 cell cluster to some extent
408 (Fig. S3B). We thus analyzed the co-expression of T_H1, T_H2, T_H17 and Treg cell-associated
409 cytokines/transcription factors by flow cytometry. This depicted a significantly higher
410 percentage of IL-17$^+$ IFN-γ^+ expressing T helper effector cells in mice administered dog
411 allergen extracts compared to those administered HDM. Foxp3$^+$ IL-17$^+$ and IL-5$^+$ IL-13$^+$ IL-17$^+$
412 triple positive cells also appeared somewhat more frequent in mice sensitised and challenged
413 to dog allergens (Fig. S3B). Thus, T helper cells from mice administered dog allergens exhibit
414 greater breadth in cytokine production compared to cells from mice administered HDM.

415 Taken together, scRNA-Seq reveals exquisite gene expression profiles for several T helper
416 cell populations responding to dog allegen extracts. This depicts a highly conserved
417 transcriptional profile for T_H2 cells between the HDM and dog allergen extract models, but also
418 pinpoints several cell clusters that are enriched in the airways of mice exposed to dog allergen
419 extracts, including T_H17, Tfh and a putative CD4 CTL population.

420 **T cell antigen receptor (TCR) analysis depicts a high frequency of shared clones**
421 **between T_H17, T_H2 and Treg cells**

422 Side-by-side with transcriptomic analysis on the Chromium, TCRs were also sequenced from
423 single cells isolated from the airways of mice administered dog allergen extacts. This allowed
424 us to explore the relatedness of cells between different transcriptional clusters. Productive α/β

425 chains were called in most single cells analyzed and 1716 different clonotypes could be
426 identified. The most common TCR detected was found on around 4% of cells (Fig. 6A). There
427 was no indication that the response was biased towards specific TCR α or β chains since the
428 most common TRAV was detected in 3% of cells and the most common TRBV in around 6%
429 of cells (Fig. 6B). We plotted the clonotype size distribution onto the UMAP, which depicted
430 obvious clonal outgrowths in several clusters including the T_H17, T_H2, Treg clusters and in
431 clusters Act1 and Act2 (Fig. 6C). The cluster of naïve CD4 T cells, which makes up around 5%
432 of airway infiltrating cells, was the most diverse, with each cell expressing a different TCR. In
433 the Th17, Th2 and Act1 clusters, large clonal outgrowths were observed with 20% of clones
434 accounting for approximately 65% of the total cells in the cluster (Fig. 6D). Correlation analysis
435 showed that the clonal composition of most clusters overlapped, with the exception of naïve
436 cells, which was comoposed entirely of unique clones, and Act3 (putative CD4 CTL), which
437 also appeared clonally distinct (Fig. 6E). This suggests that Act3 cells follow a distinct
438 differentiation trajectory to cells in other effector clusters. Intriguingly, considerable clonal
439 overlap was observed between the Treg cell cluster and other effector clusters. This indicates
440 that many Treg cells may be 'induced' from naïve CD4 T cells in this model (so-called iTreg),
441 or may transdifferentiate from Treg cells into other effector subsets after activation. A high
442 degree of overlap was also observed between Th2 cells, Th17, Act1, Act2 and Act4 cells,
443 indicative of a shared differentiation trajectory. Thus, exposure to dog allergen extracts induces
444 the differentiation of several unique T helper cell subsets, highlighted by a strong T_H17 cell
445 cluster. Furthermore, analysis of TCR clonality indicates patterns of shared and restricted
446 clonality between transcriptionally-distinct subsets.

447 **Sublingual immunotherapy (SLIT) ameliorates airway hyperresponsiveness and T_H2 cell**
448 **responses to dog allergen extracts**

449 Several clinical trials have explored the use of dog allergen extracts in AIT. These trials have
450 had mixed results, partially attributed to varying quality and composition of allergen extracts
451 (Smith and Coop, 2016). The use of recombinant allergen proteins has been proposed as an
452 alternative to whole allergen extracts (Valenta et al., 2011). We thus analyzed whether
453 sublingual administration of recombinant Can f 1-2-4-6 (Nilsson et al., 2014) allergens could
454 prevent airway inflammation in mice administered dog allergen extracts. We administered mice
455 three sublingual challenges per week for four weeks of the recombinant Can f 1-2-4-6 protein
456 and thereafter, subjected mice to the dog allergen model over 15 days (Fig. 7A). Can f 1-2-4-
457 6 SLIT significantly reduced the total number of cells infiltrating the airways, in particular of
458 eosinophils, whereas the number of T helper cells in the lavage was not significantly reduced
459 (Fig. 7B-D). Mice administered Can f 1-2-4-6 had a higher frequency of $Foxp3^+$ Treg cells in
460 the lung (Fig. 7E) and strongly reduced proportions of T_H2 cytokine-producing cells in the

461 airways (Fig. 7F). Can f 1-2-4-6 however appeared to have little impact on the frequency of IL-
462 17-producing CD4 T cells and even significantly increased the frequency of IFN-γ^+ CD4 T cells
463 (Fig. 7F). To test cytokine production in response to specific dog allergens, we cultured cells
464 from mediastinal lymph nodes in the presence of whole dog allergen extracts or recombinant
465 Can f 1, f 2, f 3, f 4 or f 6 (Fig. S4). This confirmed that mice administered Can f 1-2-4-6
466 prophylactically produced less IL-5, IL-13 and IL-10 in response to restimulation with whole
467 dog allergen extracts or Can f 1 specifically. In contrast, IL-17 and IFN-γ secretion was
468 moderately enhanced in cells from mice administered Can f 1-2-4-6. Despite the use of several
469 Can f family members in SLIT, no obvious cytokine production was observed to these proteins,
470 which may indicate an overall lack of T cell immunogenicity for these allergens. We next tested
471 whether Can f 1-2-4-6 could reduce airway hyperresponsiveness in mice sensitized and
472 challenged with dog allergen extracts. Indeed, mice administered Can f 1-2-4-6 exhibited
473 reduced overall airway resistance, elastance and Newtonian resistance compared to PBS SLIT
474 controls (Fig. 7G-I). One aim of SLIT is to shift the balance of antibodies produced in response
475 to allergen exposure from IgE towards IgG1 and IgG4, thereby limiting the activation of mast
476 cells by allergen-specific IgE. Mice administered sublingual Can f 1-2-4-6 protein prior to dog
477 allergen extract sensitization and challenge exhibited much higher titres of dog allergen
478 extract- and Can f 1-specific IgG1 in serum, compared to mice given sublingual PBS (Fig. 7J).
479 A significant reduction in total serum IgE was observed in mice administered sublingual Can f
480 1-2-4-6 protein, although Can f 1-specific IgE was not significantly reduced (Fig. 7J). To test
481 whether the neutrophilic response was preserved in Can f 1-2-4-6-administered mice, airway
482 neutrophils were quantified in mice that received an additional challenge on day 15, three hours
483 before sacrifice. Mice administered Can f 1-2-4-6 developed an even stronger infiltration of
484 neutrophils into the airways (Fig. 7K) compared with mice that had received PBS. To determine
485 if SLIT with Can f 1-2-4-6 also alleviated airway hyperresponsiveness in a therapeutic setting,
486 mice were sensitized and challenged prior to SLIT and thereafter challenged for another week
487 (Fig. S5). In this therapeutic setting, SLIT with Can f 1-2-4-6 did not appear to significantly
488 reduce airway hyperresponsivness or the infiltration of lymphoyctes into the airways (Fig. S5).
489 Taken together, prophylactic SLIT with recombinant Can f 1-2-4-6 reduced AHR and type 2
490 responses, altered the antibody balance and increased the frequency of Treg cells in this
491 model of airway inflammation induced by dog allergens, indicating that recombinant allergen
492 immune therapy can reduce allergy-associated inflammation. The inability of SLIT to reduce
493 the frequency of T_H17 cells and its impact in different therapeutic regimens or as a
494 subcutaneous immune therapy could also be explored.

495

496 **Discussion**

497 Although the incidence of allergic disease is rising globally and a large proportion of allergic
498 patients are sensitized to allergens from furred animals, a mouse model to study allergy to
499 dogs has been lacking. The model proposed here uses natural extracts from dog dander and
500 epithelium without additional adjuvants in order to recapitulate the typical exposure of people
501 in society. The downsides of such an approach are the often varying levels of allergen content
502 between different batches and suppliers (Wintersand et al., 2019) and the potential effects of
503 unknown components of the extracts, which can make it difficult to compare results across the
504 world.

505 This study demonstrates that dog allergen extract instillations induce airway
506 hyperresponsiveness and airway inflammation marked by a mixed T_H2/T_H17 cell response,
507 eosinophilia and neutrophilia. The mixed cytokine response was further confirmed by allergen-
508 specific lymph node restimulation, where dog allergen extracts could induce production of T_H2
509 cytokines, IFN-γ and IL-17. A similarly mixed T_H cell profile in response to dog allergens was
510 also recently observed in one other study (Boute et al., 2021). This model of airway
511 inflammation induced by dog allergen extracts may more closely resemble severe types of
512 asthma, in which patients have been shown to have higher levels of T_H1/T_H17 cells and
513 increased airway neutrophilia (Domvri et al., 2018). A model using OVA demonstrated a strong
514 T_H1/T_H17 response and airway neutrophilia in mice with an unresolved *Chlamydia muridarum*
515 infection at the time of sensitization. But unlike instillation of dog allergen extracts, this model
516 did not result in high levels of airway eosinophilia (Horvat et al., 2010). Another model using
517 HDM and β-glucan reported high levels of both eosinophils and neutrophils in the BAL and a
518 mixed T_H2/T_H17 response, demonstrating how fungal components can skew the immune
519 response even without previous exposure (Zhang et al., 2017). Whether fungal components
520 are present in the dog allergen extracts alongside the endotoxin reported in this study will need
521 to be explored.

522 A higher proportion of adult-onset asthma also appears insensitive to corticosteroids,
523 compared to childhood asthma. It was notable that dexamethasone was able to reduce airway
524 eosinophilia, numbers of infiltrating B cells and T helper effector cells in the airways and
525 numbers of IL-17 producing T helper effector cells in the lung. T_H17 cells have been reported
526 to be resistant to the effects of corticosteroids (Banuelos and Lu, 2016). The reason for the
527 reduced number of T_H17 cells in dog allergen exposed mice injected with dexamethasone
528 might be due to early corticosteroid treatment preventing the proper priming of T_H17 cell
529 responses. Despite the reduction in IL-17 production and overall numbers of airway-infiltrating
530 cells, there was no significant reduction of airway neutrophilia. Whether this is due to

531 components of the dog allergen extracts triggering airway neutrophilia independently of T
532 helper cells remains to be explored.

533 A central tenet of allergic models of airway inflammation and atopic asthma is the induction of
534 IgE. Administration of dog allergen extracts induced total serum IgE levels similar to HDM, and
535 serum IgE specific to Can f 1 was detected in mice administered dog allergen extracts. As
536 observed previously, allergen-specific IgE was detected at much lower levels than IgG1, a
537 frequent observation in mouse models (Lehrer, 2004). Depletion of IgG1 may be a solution
538 that promotes the detection of IgE in more robust assays, although the magnitude of IgG1
539 responses makes this a difficult task.

540 In line with what we and others have shown previously, scRNA-Seq can be used to profile
541 diverse T helper cell responses (Tibbitt et al., 2019). scRNA-Seq defined several distinct
542 clusters including T_H2 and Treg cells, whose gene expression profiles largely matched
543 previously published data from the HDM model (Tibbitt et al., 2019). A distinct subset of T
544 helper cells responding to type-I interferons, described for mice administered HDM was not
545 observed in mice after dog allergen extract instillation, suggesting that the type-I IFN pathway
546 may be less active in this model. Several clusters pinpointed in this analysis contrasted from
547 our previous analysis of the HDM model. In particular, a cluster of T_H17 cells, which was not
548 elucidated in response to HDM was apparent in response to dog allergens. Several known and
549 many potentially novel regulators of T_H17 cells were identified, which could serve as targets to
550 block T_H17 cell-associated inflammation. The expression of these genes will need to be
551 confirmed by analysis of T_H17 cells in human asthma. One intriguing target may be the IL-17C
552 receptor, IL-17RE. Both IL-17C and IL-17RE have been described to be expressed by
553 epithelial cells, regulating the epithelial immune response in an autocrine manner (Ramirez-
554 Carrozzi et al., 2011). An antibody binding to IL-17C and thereby inhibiting receptor binding
555 has been shown to ameliorate disease in mouse models of psoriasis and atopic dermatitis
556 (AD) (Vandeghinste et al., 2018). A clinical trial targeting this cytokine:receptor pathway was
557 initiated in AD but results are still pending (ClinicalTrials.gov Identifier: NCT03568071).

558 The elucidation of a cell cluster that appeared to resemble Tfh cells was also in contrast to
559 results from the HDM model. Although IL-21-producing cells in the lung tissue and airways
560 have been shown to be a feature of HDM-induced inflammation (Coquet et al., 2015), those
561 cells did not appear to express canonical Tfh cell markers such as Bcl6 and Cxcr5. Given that
562 the cells profiled herein come from the airways, this indicates that administration of dog
563 allergen extracts may induce bronchus-associated lymphoid tissue (BALT) (Boute et al., 2021;
564 Hwang et al., 2016). BALT formation may be due to presence of higher levels of endotoxin in
565 dog allergen extracts. Since pets are known to contribute significantly to household endotoxin
566 levels (Mendy et al., 2018), Tfh cells and BALT may be a feature of pet-allergic asthmatics.

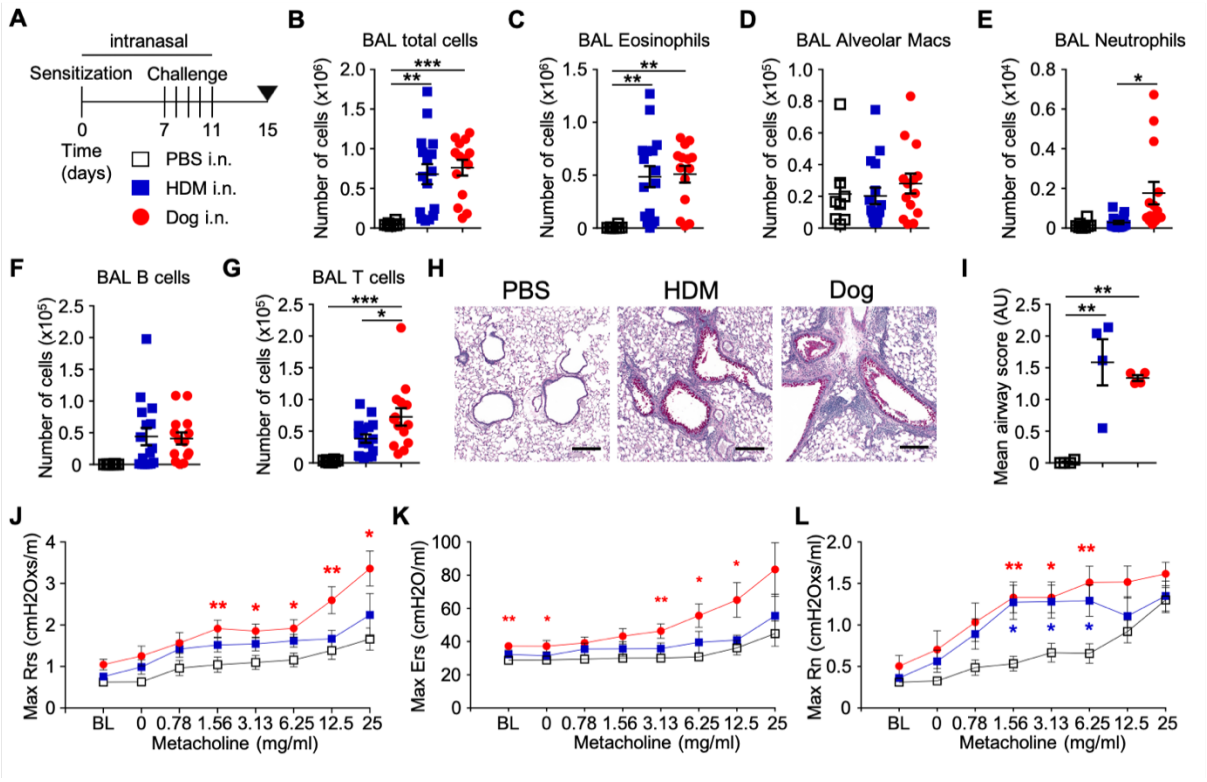
567 scRNA-seq also highlighted the plasticity of T helper cells by showing co-expression of key
568 cytokines and/or *Foxp3* between T_H17, T_H2, T_H1 and Treg cells. This plasticity was also evident
569 in the frequency of TCR clones shared between T_H17, T_H2 cells and Treg cells. T_H2/T_H17 dual
570 positive cells have been found in BAL fluid of patients with severe asthma (Irvin et al., 2014;
571 Wang et al., 2010). While this model used whole allergen extracts as opposed to purified
572 proteins, there was still a strong clonal response in T helper cells. This indicates that even
573 when mice are exposed to whole extracts, T cells may only react to a limited number of
574 antigenic peptides.

575 A goal of this study was to create a model whereby we could test the efficacy of recombinant
576 dog allergens as an immunotherapy. AIT can be performed with different administration routes;
577 most commonly subcutaneous (SC) or sublingual. Sublingual administration was chosen for
578 this study since SLIT has been proven to be efficacious (Calderón et al., 2011) and is attractive
579 as it can be self-administered by patients. Data from studies of AIT for pollen allergy also point
580 to a lower risk of systemic adverse reactions in SLIT compared to SCIT (James and Bernstein,
581 2017). Currently, AIT has focussed on treating allergic rhinitis and has proven to be beneficial
582 in this setting. There are some indications that AIT may also reduce asthmatic symptoms and
583 exacerbations, although the impact on lung function appears to be minimal (Abramson et al.,
584 2010; Costa et al., 2019)

585 SLIT using the recombinant Can f 1-2-4-6 protein reduced AHR and the T_H2 cell response but
586 did not affect the T_H17 cell response to dog allergen extracts. One possible explanation for this
587 observation is that the T_H17 cell response is not caused by these four allergens but instead
588 may be triggered by other dog allergens or unknown components of the allergen extracts.
589 Indeed, the lack of IL-17 in cultures of mediastinal lymph node cells with recombinant Can f
590 allergens points to other antigens being primarily responsible for eliciting this reponse.
591 However, the clonal overlap between T_H2, T_H17, Treg and other activated cell clusters
592 indicates a shared differentiation pathway between those subsets, making it unlikely that Th17
593 cells are responding to a completely independent set of antigens in the dog allergen extracts.
594 It is likely that the presence of endotoxin in dog allergen extracts helped to drive Th17 cell
595 responses and airway hyperresponsiveness (Caucheteux et al., 2017; Starkhammar et al.,
596 2012). However, it is worth noting that SLIT with only recombinant Can f 1-2-4-6 was able to
597 ameliorate AHR, suggesting that these allergens are responsible for at least part of the
598 hyperresponsiveness that occurs in the airways of mice in this model. SLIT in mice with already
599 established airway inflammation did not ameliorate cell infiltration in the airways or AHR. This
600 may be due to the rather short intervals used in our study or may suggest that SLIT is not
601 powerful enough to overcome an already fulminant response. Whether SCIT with recombinant
602 allergens can more potently suppress allergic inflammation should be investigated.

603 In all, we present a model of airway inflammation with dog allergen extracts that is
604 characterised by a mixed T_H2/T_H17 cell-mediated airway inflammation and which may be
605 useful in understanding adult-onset asthma. We provide comprehensive gene and TCR
606 profiling of T helper cells reacting to dog allergens in the airways and demonstrate that
607 recombinant Can f family allergens have the capacity to reduce airway hyperresponsiveness
608 and T_H2 cytokine production.

609 **Figures**



610

611 **Figure 1. Intranasal administration of dog allergen extracts leads to airway inflammation**
612 **and airway hyperresponsiveness.** A, regimen of intrasal administration of PBS (open
613 square), HDM (blue square) or dog allergen extracts (red circle). B-G, PBS n = 8, HDM n = 16,
614 dog allergen extracts n = 14. B, total number of cells in the BAL. C, number of eosinopils in the
615 BAL. D, number of alveolar macrophages in the BAL. E, number of neutrophils in the BAL. F,
616 number of B cells in the BAL. G, number of T cells in the BAL. H, Periodic acid-Schiff-diastase
617 staining of lung sections (line = 200 µm). I, mean airway score of lung sections (n = 4 per
618 group). J-L, airway resistance to increasing doses of methacholine as measured by FlexiVent
619 (PBS n = 6, HDM n = 6, dog allergen extracts n = 7). J, overall resistance Rrs. K, elastance
620 Ers. L, Newtonian resistance Rn. Red stars indicate a comparison between dog and PBS,
621 while blue stars indicate a comparison between HDM and PBS. In B-I, Bonferroni's test was
622 used to compare all groups. In J-L, ANOVA and Dunnett's test was used to compare dog- or
623 HDM-challenged mice to PBS.

624

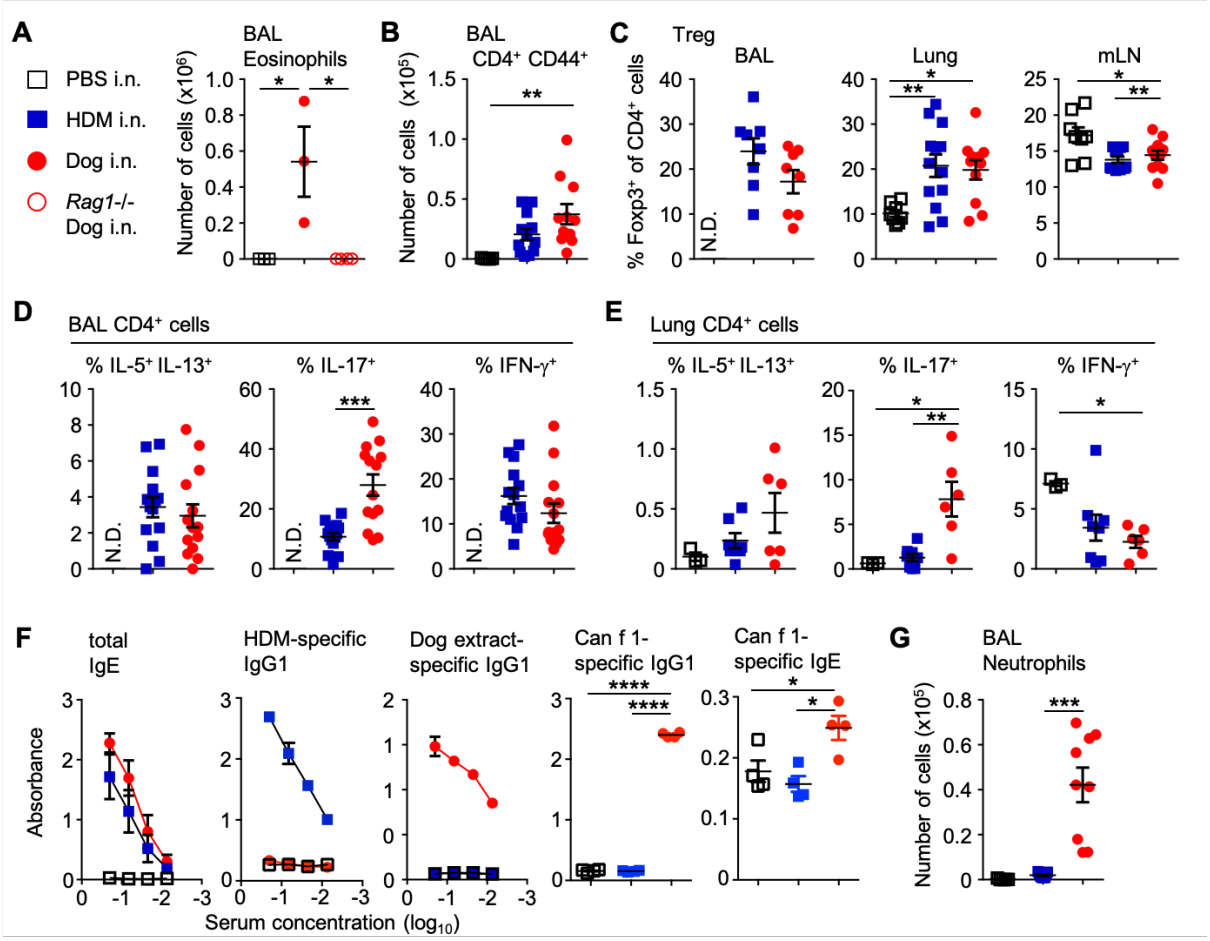
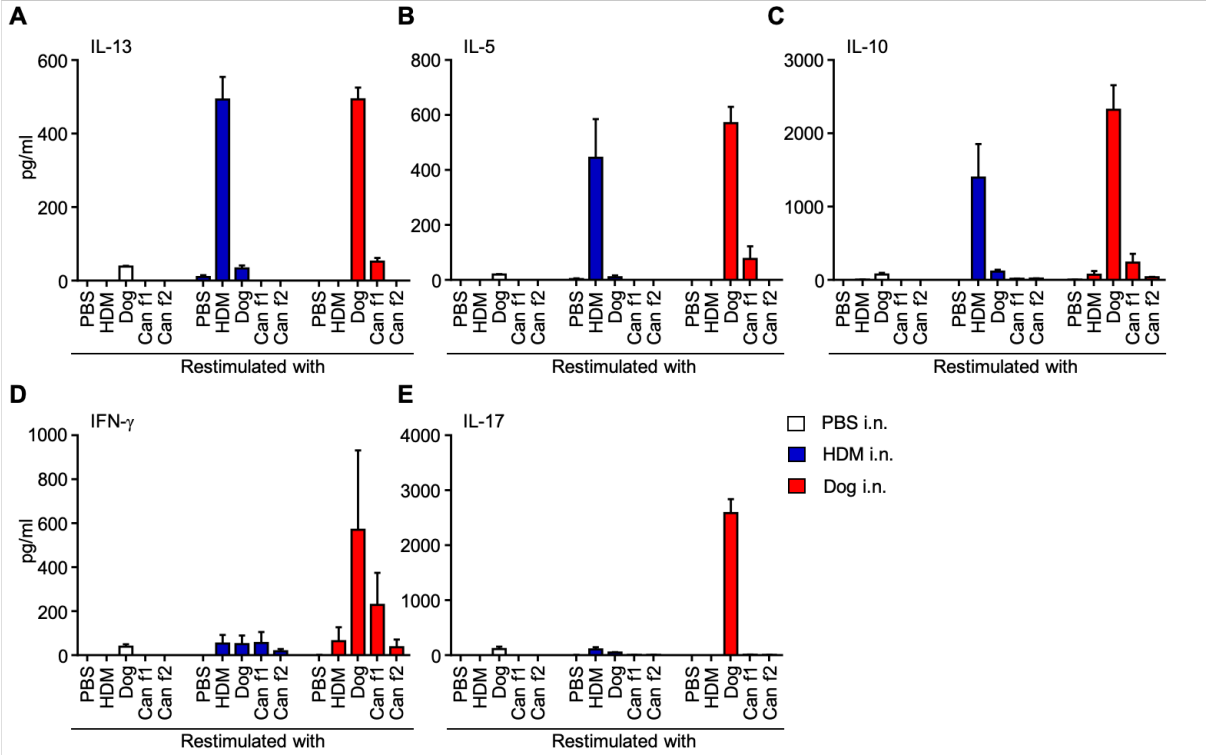


Figure 2. Intranasal administration of dog allergen extracts induces differentiation of both T_H2 and T_H17 cells. A, PBS (open square), HDM (blue square) or dog allergen extracts (red circle) in C57Bl6/J mice, dog (red open circle) in *Rag1*$^{-/-}$ mice, number of eosinophils (C57Bl6/J PBS n = 3, C57Bl6/J dog n = 3, *Rag1*$^{-/-}$ dog n = 4). B, number of CD4$^+$ CD44$^+$ cells in the BAL (PBS n = 5, HDM = 12, dog allergen extracts n = 11). C, graphs of frequency of Foxp3$^+$ cells among total CD4$^+$ cells from the BAL, lung and mLN (BAL: HDM = 8, dog allergen extracts n = 8; lung and mLN PBS n = 8, HDM = 13, dog allergen extracts n = 11). D, graphs of the frequency of IL-5$^+$ IL-13$^+$, IL-17$^+$ and IFN-γ$^+$ cells among total CD4$^+$ cells from the BAL (HDM = 14, dog allergen extracts n = 14). E, graphs of the frequency of IL-5$^+$ IL-13$^+$, IL-17$^+$ and IFN-γ$^+$ cells among total CD4$^+$ cells from the lung (PBS n = 3, HDM = 8, dog allergen extracts n = 6). F, ELISAs were performed on the serum of mice. Dog allergen-specific and HDM-specific IgG1 and total IgE graphs are representative of 3 experiments (PBS n = 3, HDM = 5, dog allergen extracts n = 3). Can f 1-specific IgG1 and IgE was also tested at a 1:3 dilution (PBS n = 4, HDM = 4, dog allergen extracts n = 4). G, regimen of intranasal administration with one additional challenge on day fifteen, three hours before harvesting organs, number of neutrophils from the BAL (PBS n = 5, HDM = 9, dog allergen extracts n = 9). N.D. Denotes not determined due to low cell numbers. Bonferroni's test was used to compare all groups, except to compare frequencies of cells in the airways, where Mann-Whitney U test was used.



644

645 **Figure 3. A specific cytokine response to dog allergens, in particular to the Can f 1**
646 **following dog allergen extract admnistration.** A-E, Concentration of the indicated cytokine
647 in supernatant from mediastinal lymph node cell cultures after 48 hours. Cells from mice
648 administered PBS n = 2 (white), HDM n = 3 (blue), dog allergen extracts n = 3 (red) were
649 restimulated with PBS, HDM, dog allergen extracts, Can f 1 or Can f 2. A, IL-13. B, IL-5. C, IL-
650 10. D, IFN-γ. E, IL-17. One representative of two independent experiments is shown.

651

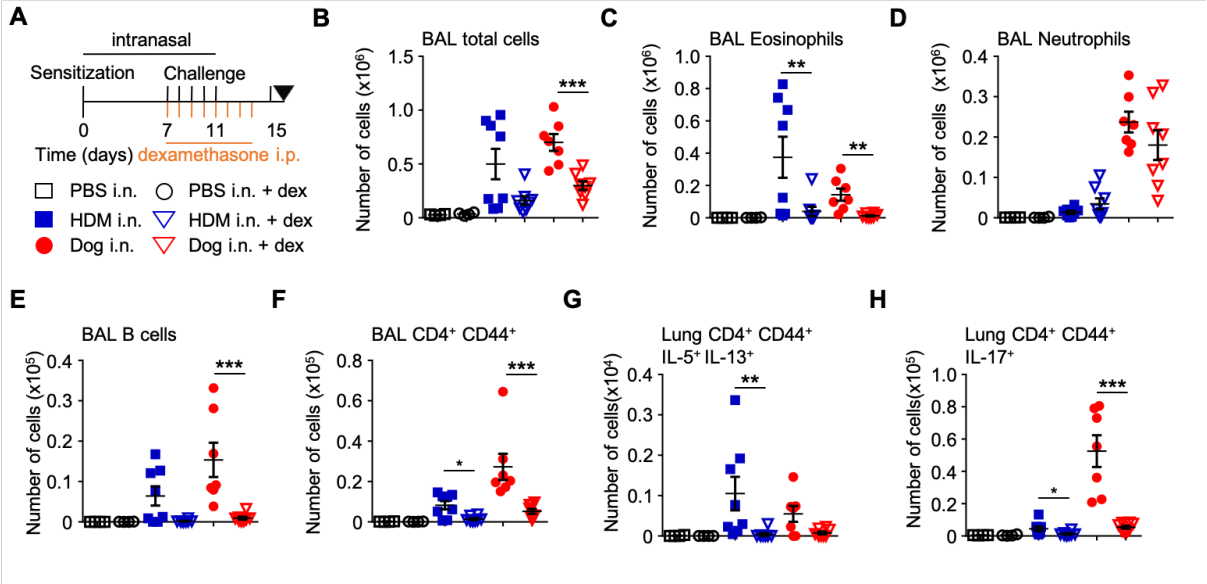


Figure 4. Dexamethasone reduces T_H cell responses and airway eosinophilia but not neutrophilia after administration of dog allegen extracts. A, regimen of intranasal allergen challenge with intraperitoneal dexamethasone/vehicle control administration: PBS i.n. vehicle i.p. n = 4 (open square), PBS i.n. dexamethasone i.p. n = 4 (open circle), HDM i.n. vehicle i.p. n = 8 (blue square), HDM i.n. dexamethasone i.p. n = 8 (open blue triangle), dog i.n. vehicle i.p. n = 8 (red circle), or dog i.n. dexamethasone i.p. n = 8 (open red triangle). B, total number of cells in the BAL. C, number of eosinophils in the BAL. D, number of neutrophils in the BAL. E, number of B cells in the BAL. F, number of effector T helper cells in the BAL. G, number of IL-5$^+$ IL-13$^+$ T helper cells in the lung. H, number of IL-17$^+$ T helper cells. Mann-Whitney U test was used throughout to compare mice receiving dexamethasone to those injected with vehicle.

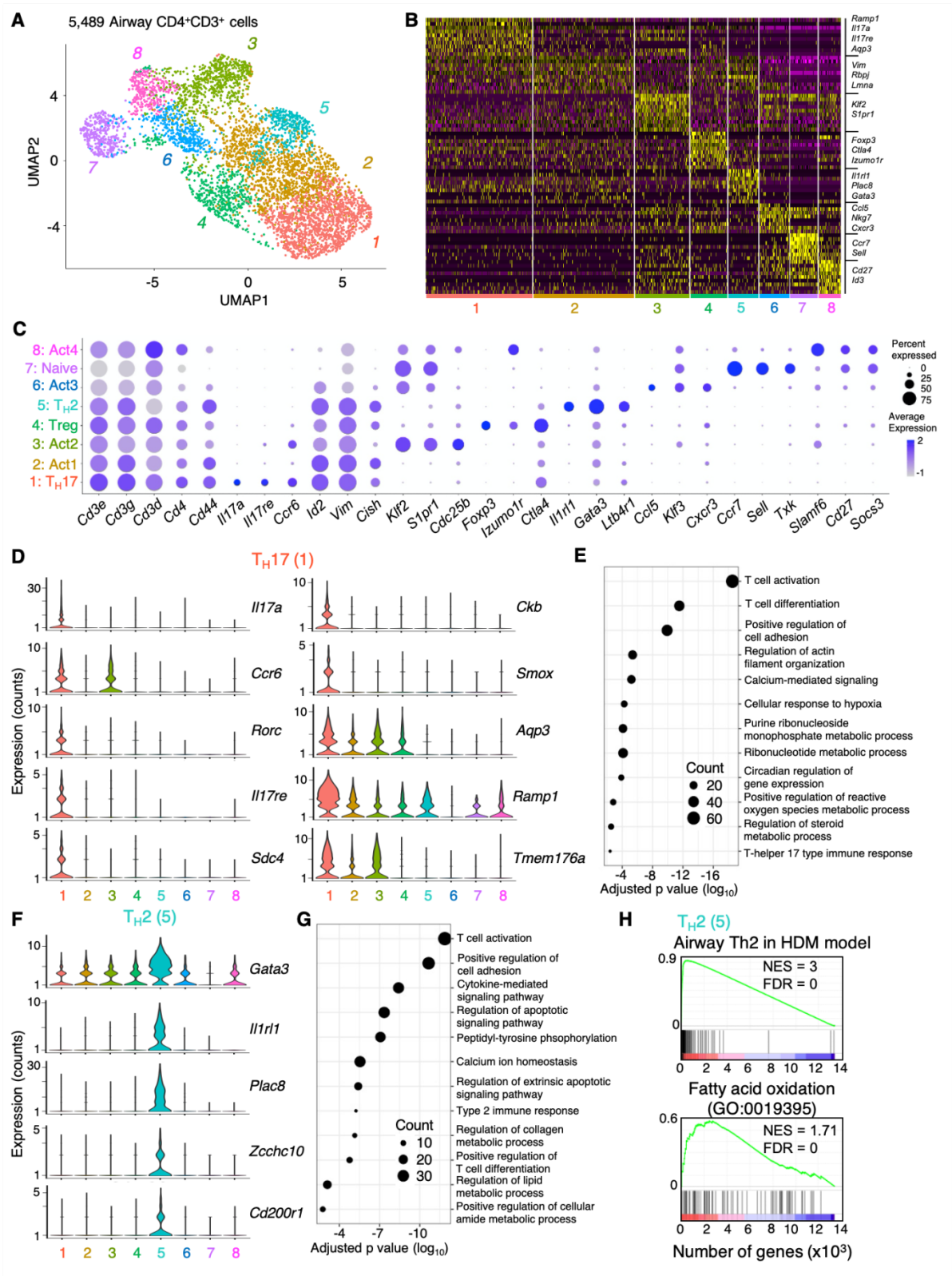


Figure 5. Analysis of T helper cells by scRNA-Seq identifies several distinct subsets including a prominent T_H17 cell cluster. A, UMAP representation of 5,489 single T_H cells from the airways of a mouse administered dog allergen extracts. B, heatmap of top 10 differentially expressed genes in each cluster. C, DotPlot of expression of key genes across all clusters. Clusters were assigned the following identities: Cluster 1: T_H17, Cluster 2:

activated cells (Act)1, Cluster 3: Act2, Cluster 4: Treg, Cluster 5: T_H2, Cluster 6: Act3, Cluster 7: naïve, Cluster 8: Act4. D, Violin plots of significant genes for the T_H17 (1) cluster. E, DotPlot showing gene ontology of biological processes in the T_H17 (1) cluster. F, Violin plots of significant genes for the T_H2 (5) cluster. G, DotPlot showing gene ontology of biological processes in the T_H2 (5) cluster. H, Gene set enrichment analysis of the Th2 cell cluster (5) for genes enriched in Th2 cells in the HDM model (Tibbitt et al., 2019) and for genes involved in fatty acid oxidation.

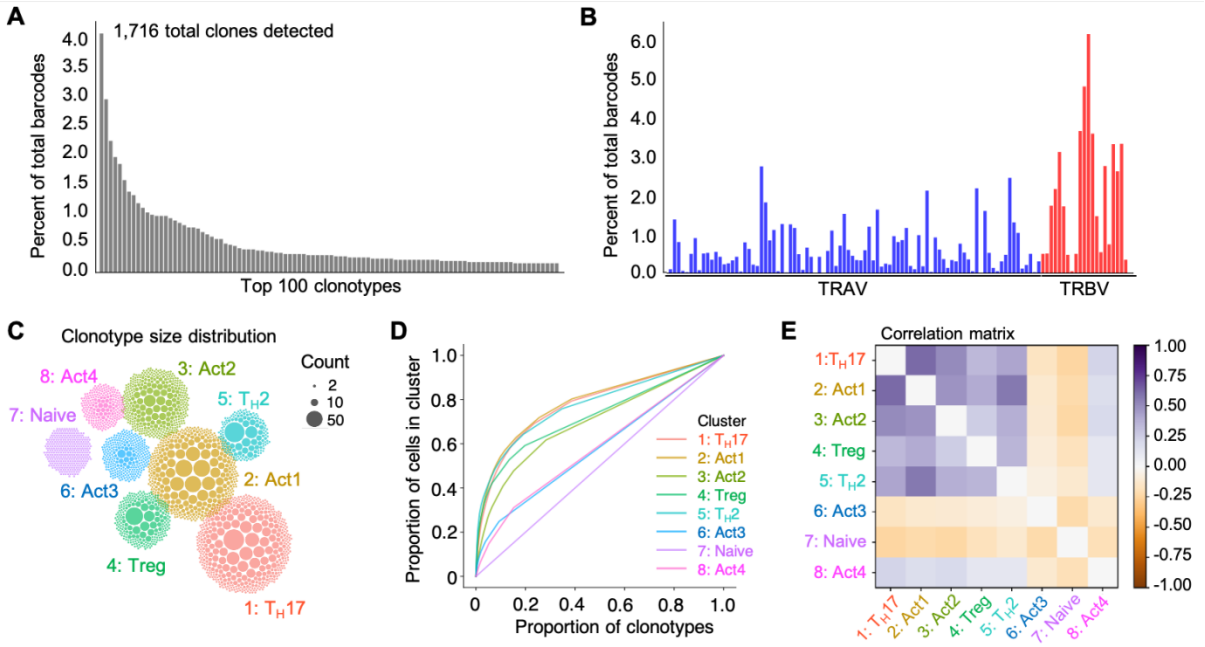
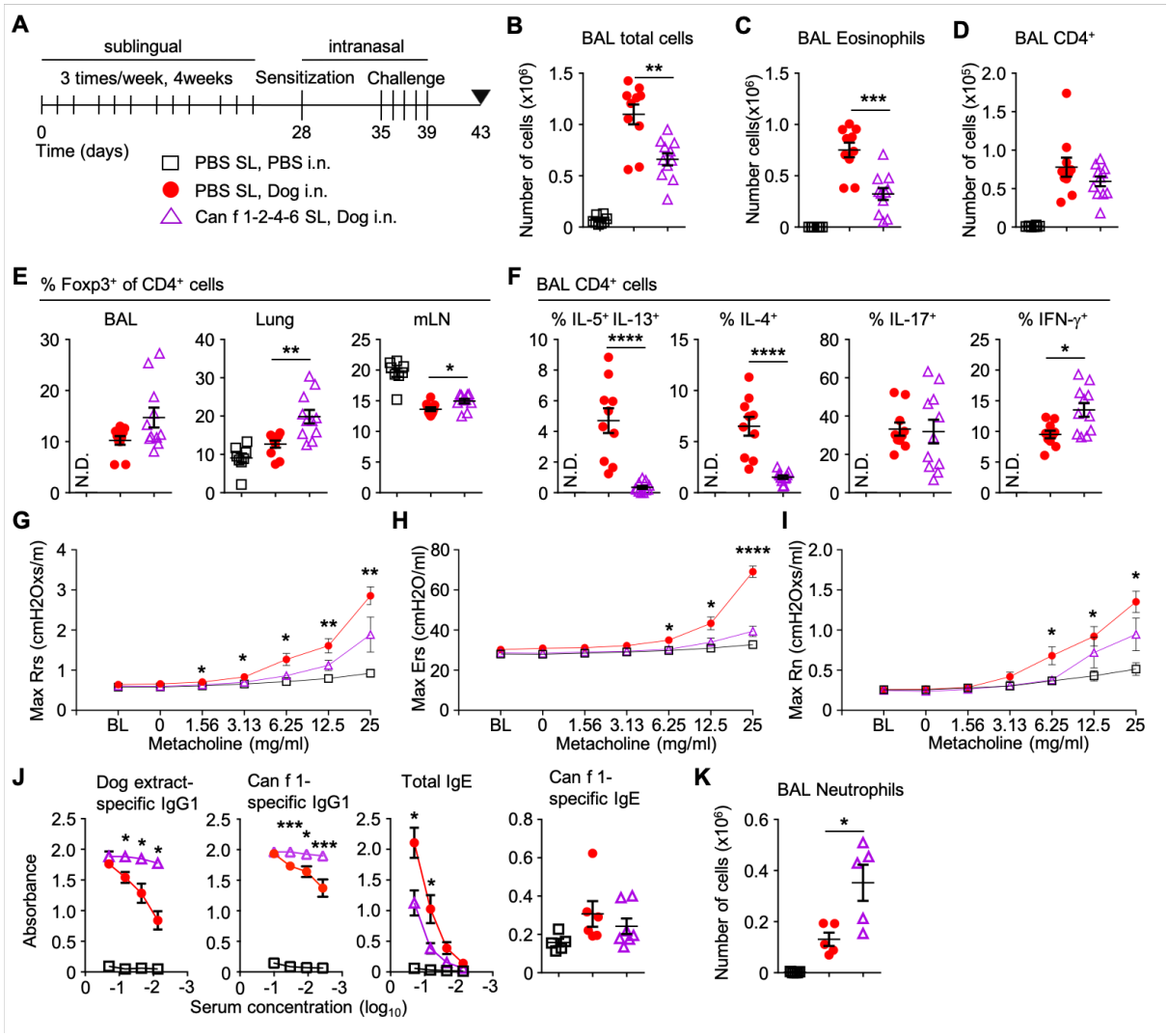


Figure 6. scRNA-Seq of airway T_H cells pinpoints large clonal outgrowths and a shared differentiation trajectory for T_H17, T_H2, Treg and other activated T_H cell clusters. Side-by-side with a 5' gene trascription library (Fig. 5), a VDJ library was amplified and sequenced on T_H cells isolated from the airways of mice. A, The frequency of the top 100 expressed TCRs is shown. B, The frequency of V gene usage across the TRAV and TRBV gene loci. C, Clonotype count distributions. Each ball corresponds to a clonotype in the given cluster, with the size of the ball proportional to the number of cells of that clone within the cluster. The clusters are placed in similar positions to the UMAP plot (Fig. 5A). D, Lorenz curves. A uniform size distribution (such as the naive cluster here) would fall along the diagonal, and progressively more uneven size distributions shift the curve up and to the left. E, Matrix representing the correlations of the number of cells expressing each TCR between each pair of clusters, after a variance stabilizing square root transformation.



694

695 **Figure 7. Sublingual immunotherapy ameliorates AHR and the T_H2 cell response, but**
696 **not the T_H17 cell response to dog allergen extracts.** A, Mice were sublingually administered
697 either PBS or Can f 1-2-4-6 protein 3 times per week for four weeks followed by i.n.
698 sensitization with either PBS or dog allergen extracts (1 µg) on day 28 and 5 i.n. challenges
699 (10 µg total) on days 35-39, mice were sacrificed on day 43; PBS sublingual, PBS i.n. (open
700 square, B-F n = 8), PBS sublingual, dog allergen extracts (red circle, B-F n = 10), Can f 1-2-4-
701 6 sublingual, dog allergen extracts (open purple triangle, B-F n =11). B, total number of cells
702 in the BAL. C, number of eosinophils in the BAL. D, number of T cells in the BAL. E, frequency
703 of $Foxp3^+$ cells among total $CD4^+$ cells from the BAL, lung and mLN. F, frequency of $IL\text{-}5^+$ IL-
704 13^+,$IL\text{-}4^+$, $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells among total $CD4^+$ cells from the BAL. G-I, airway resistance
705 to increasing doses of methacholine as measured by FlexiVent (PBS sublingual, PBS i.n. n =
706 6, PBS sublingual, dog allergen extracts n = 10, Can f 1-2-4-6 sublingual, dog allergen extracts
707 n = 10). G, overall resistance Rrs. H, elastance Ers. I, Newtonian resistance Rn. J, Dog
708 allergen-specific serum IgG1 and total serum IgE ELISA representative of 3 experiments (PBS
709 sublingual, PBS i.n. n = 3, PBS sublingual, dog allergen extracts = 5, Can f 1-2-4-6 sublingual,

710 dog allergen extracts n = 4). Can f 1-specific IgG1 (various dilutions) and IgE (1:3 dilution) was
711 also measured (PBS sublingual, PBS i.n. n = 5, PBS sublingual, dog allergen extracts = 6, Can
712 f 1-2-4-6 sublingual, dog allergen extracts n = 7). K, number of neutrophils in the BAL (PBS
713 sublingual, PBS i.n. n = 5, PBS sublingual, dog allergen extracts n =5 , Can f 1-2-4-6 sublingual,
714 dog allergen extracts n = 5). N.D. Denotes not determined. Mann-Whitney U test was used
715 throughout to compare values between mice receiving sublingual PBS and sublingual Can f 1-
716 2-4-6 in dog allergen extract sensitised and challenged mice.

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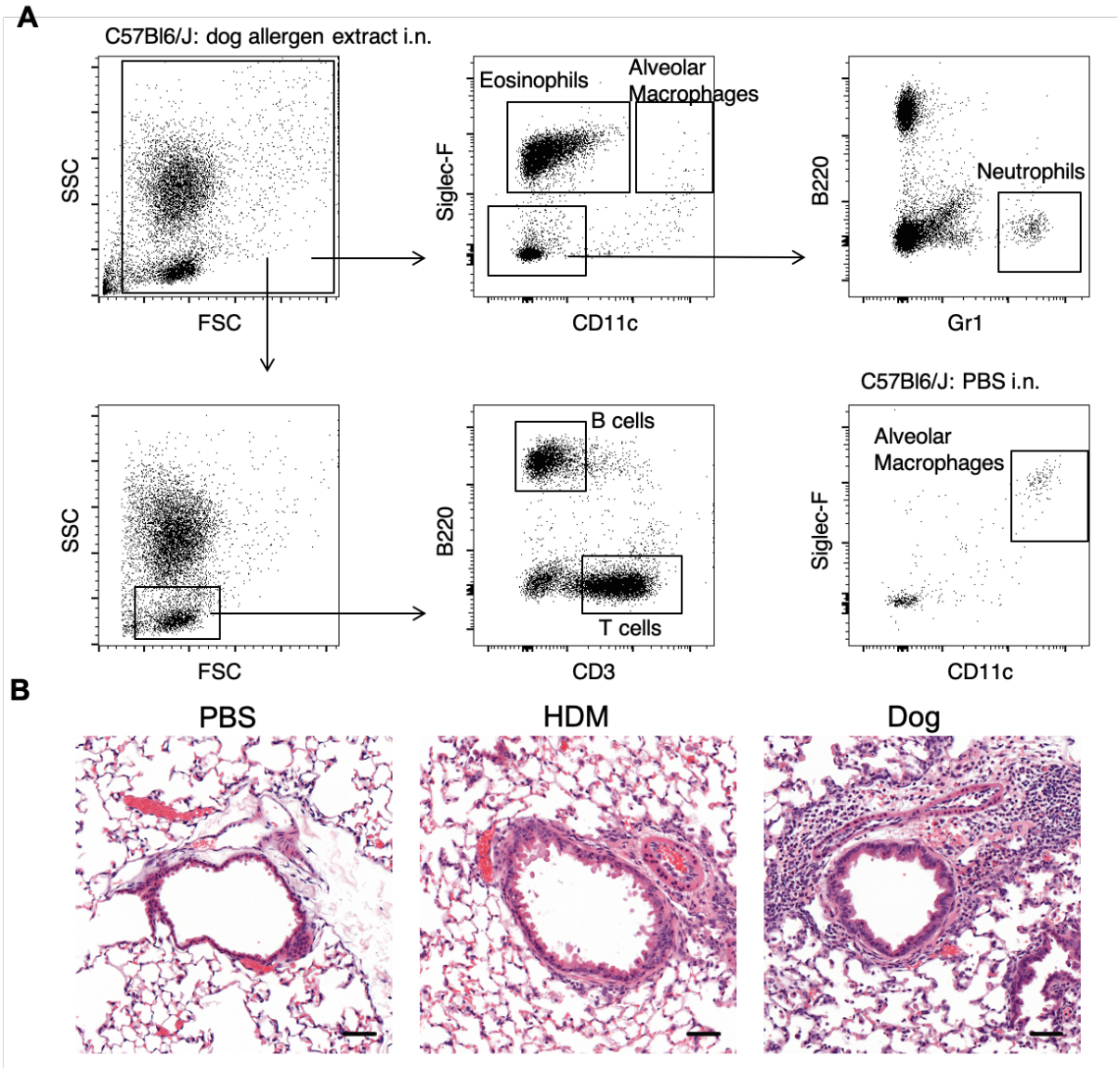

Figure S1. BAL gating strategy and H&E staining of lungs. A, Gating for Eosinophils, Macrophages, B cells and T cells from the BAL of a C57Bl6/J mouse administered dog allergen extracts. Gating for Macrophages also shown for C57Bl6/J mouse administered PBS only. B, H&E staining of lungs from mice administered PBS, HDM or Dog allergen extracts. Representative of 4 mice per group (line = 50 µm).

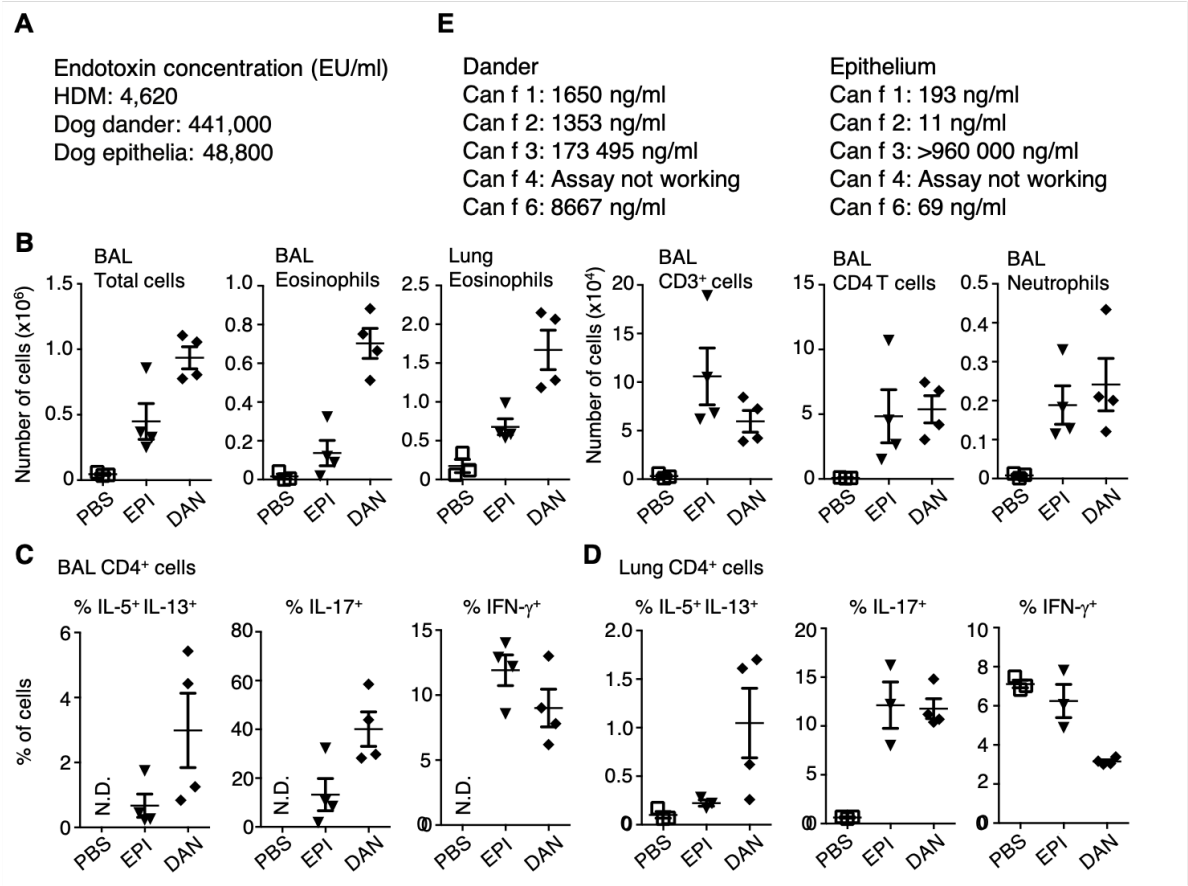

Figure S2. Characterisation of composition of allergen extracts and responses to dog dander and epithelium. A, Endotoxin in HDM, dog epithelium and dog dander extract was measured by LAL assay. B-D, Mice were sensitised and challenged with either dog dander or epithelium extracts according to the standard timeline and airway inflammation was assessed at day 15. B, total number of indicated cells is shown. C-D, graphs of the frequency of IL-5$^+$ IL-13$^+$, IL-17$^+$ and IFN-γ$^+$ cells among total effector CD4$^+$ cells from the BAL (C) and lung (D). E, Concentration of Can f 1, f 2, f 3, f 4 and f 6 were quantified from vials of dander and epithelium.

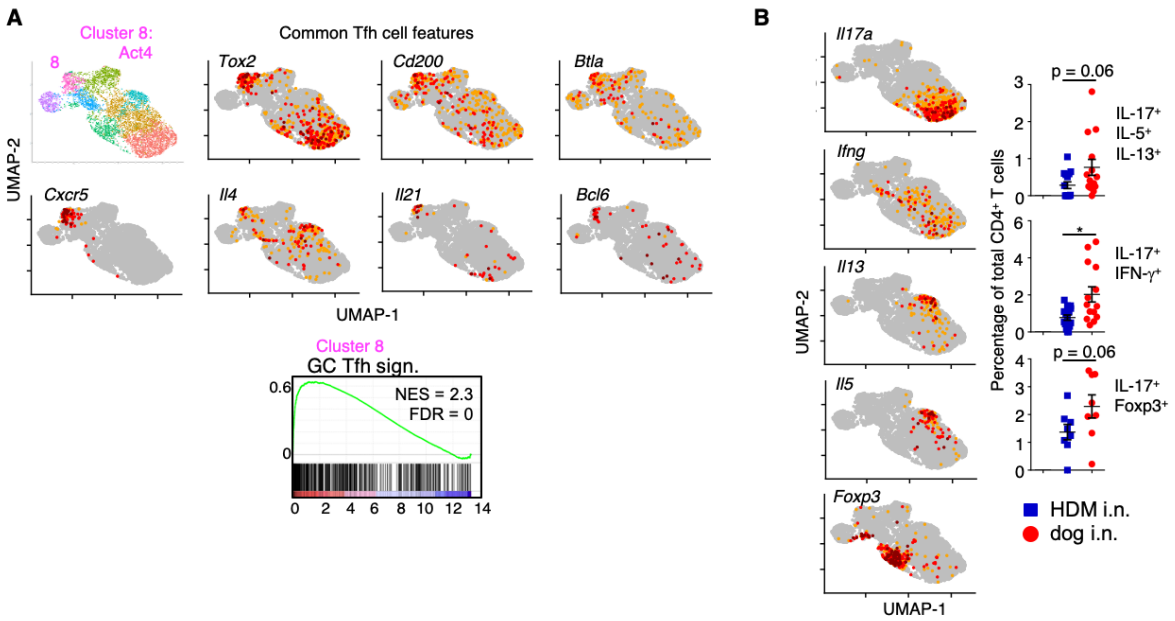

Figure S3. (A) Act4 cluster resembles Tfh cells. Feature plots of several classical Tfh cell markers are shown and gene set enrichment analysis of Act4 cluster for genes associated with germinal center (GC) Tfh cells is shown. **(B) Greater overlap in cytokine secretion in the dog allergen model compared with the HDM model.** Feature Plots of *Il17a, Ifng, Il13, Il5* and *Foxp3* mRNA. Graphs of the frequency of airway IL-17$^+$ IL-5$^+$ IL-13$^+$ (HDM blue square n = 15, dog allergen extracts red circle n =14), IL-17$^+$ IFN-γ^+ (HDM n = 15, dog allergen extracts n = 14) and IL-17$^+$ Foxp3$^+$ cells (HDM n = 8, dog allergen extracts n = 8) of total CD4 T cells.

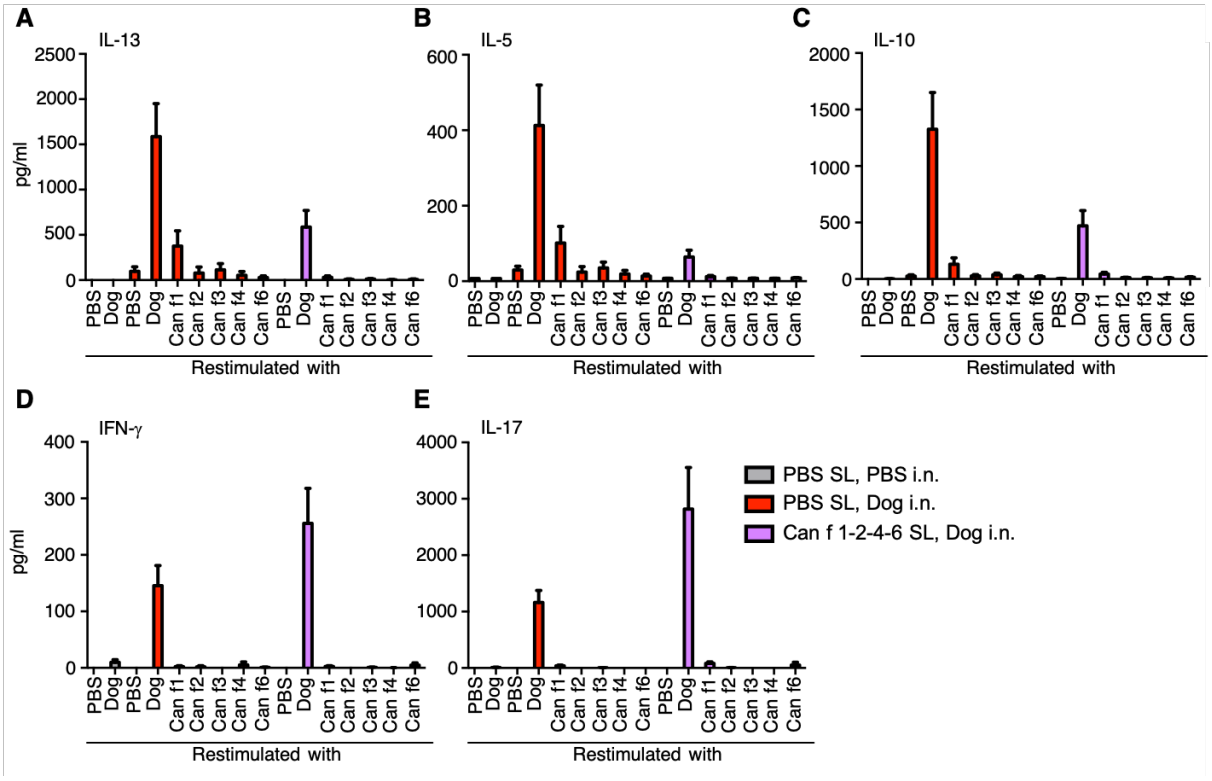

Figure S4. Reduced IL-5, IL-13 and IL-10 in lymph node restimulation cultures following SLIT, but increased production of IL-17 and IFN-γ. A-E, The concentration of the indicated cytokine in supernatant from mediastinal lymph node cell cultures after 48 hours. Cells were restimulated with PBS, whole dog allergen extracts, Can f 1, Can f 2, Can f 3, Can f 4 or Can f 6 (PBS sublingual, PBS intranasal n = 4, PBS sublingual, dog allergen extracts intranasal n = 4-5, Can f 1-2-4-6 sublingual, dog allergen extracts intranasal n = 5). This shows that Can f 1 is the dominant reactivity among these antigens, with very little cytokine production observed in response to Can f 2, f 3, f 4 or f 6.

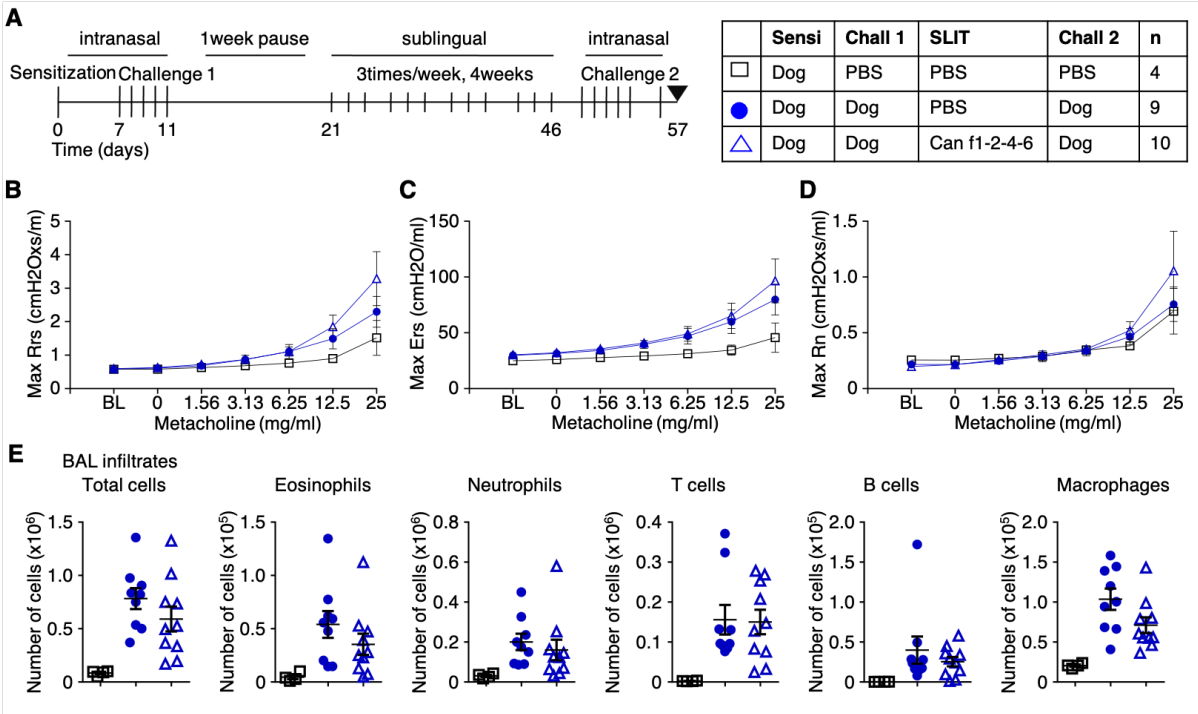

Figure S5. Sublingual immunotherapy after allergen sensitization and challenge does not reduce airway inflammation to dog allergen extracts. A, Mice were sensitized and challenged as indicated and sublingually administered either PBS or Can f 1-2-4-6 protein 3 times per week for four weeks. B, Overall resistance Rrs. C, Elastance Ers. D, Newtonian resistance Rn. E, After analysis of airway resistance on the FlexiVent apparatus, bronchoalveolar lavage was performed and total cells, eosinophils, neutrophils, T cells, B cells and alveolar macrophages were enumerated.

Table S1. List of the twenty most differentially expressed genes per cluster

Cluster1: T_H17					Cluster5: T_H2				
Gene	p_val_adj	avg_logFC	pct.1	pct.2	Gene	p_val_adj	avg_logFC	pct.1	pct.2
Ramp1	1.4484E-253	0.996931495	0.837	0.412	Il1rl1	2.65E-290	1.10670815	0.542	0.04
Il17a	6.0201E-248	1.336157352	0.343	0.023	Plac8	5.678E-234	1.573616561	0.582	0.068
Capg	2.3633E-237	0.762057865	0.964	0.674	Trav5-1	7.1197E-127	0.862550385	0.21	0.011
Tmem176b	1.7186E-203	0.889284116	0.743	0.304	Il6	1.2368E-118	0.249445037	0.119	0.001
Il17re	3.3549E-186	0.679615509	0.373	0.06	Il13	3.5885E-115	0.602540613	0.205	0.012
Tmem176a	9.5558E-172	0.838919508	0.638	0.242	Il5	2.7388E-106	0.349218644	0.171	0.008
Ckb	1.9619E-166	0.73054249	0.424	0.102	Gata3	2.99419E-97	0.960495802	0.834	0.39
Ucp2	6.1657E-158	0.610895742	0.966	0.852	Pparg	1.53255E-78	0.377919996	0.215	0.024
Thy1	3.7754E-145	0.45973996	0.988	0.933	Alox5ap	8.7852E-66	0.444471829	0.101	0.004
Sdc4	4.0658E-141	0.622580924	0.337	0.067	Icosl	7.7485E-63	0.362869471	0.168	0.018
Ccr6	3.5113E-136	0.687842872	0.489	0.161	Cd200r1	1.75284E-57	0.481422756	0.304	0.066
Aqp3	9.6798E-136	0.694340767	0.64	0.292	Zcchc10	1.10586E-51	0.469852842	0.322	0.079
Cxcr6	9.0076E-131	0.570424898	0.917	0.616	Galnt3	1.62395E-40	0.200559356	0.106	0.011
Rorc	4.5982E-127	0.597883369	0.383	0.103	Ptgir	5.51738E-40	0.196322009	0.106	0.011
Rnf208	5.0439E-122	0.47379899	0.203	0.019	Rpl37a	1.91079E-39	0.233557713	1	0.999
Smox	1.0542E-102	0.530042187	0.341	0.098	Csf1	5.66931E-38	0.224349101	0.114	0.013
Znrf1	5.5061E-100	0.444125785	0.922	0.718	Trbv1	1.76162E-37	1.074257341	0.255	0.066
Stk24	3.1567E-99	0.494039472	0.87	0.667	4932438H23Rik	4.64171E-37	0.232074722	0.119	0.015
Nfkb1	1.28089E-98	0.552529815	0.755	0.476	Cysltr1	1.2288E-36	0.397737744	0.252	0.065
S100a4	2.24431E-97	0.486389414	0.856	0.565	Aff4	1.47864E-33	0.504836098	0.599	0.299
Cluster2: Act1					Cluster6: Act3				
Gene	p_val_adj	avg_logFC	pct.1	pct.2	Gene	p_val_adj	avg_logFC	pct.1	pct.2
Trgv2	1.36505E-56	0.635658881	0.232	0.072	Ccl5	1.4707E-132	2.569577497	0.345	0.04
Ctla2a	1.03481E-45	0.369333294	0.546	0.331	Ly6c2	5.0148E-113	0.772042242	0.14	0.003
Ifngr1	8.55323E-42	0.353453648	0.801	0.659	Gzmk	2.1551E-109	0.664128534	0.128	0.002
Zfp36l2	6.54642E-41	0.351513138	0.863	0.751	Rps16	3.5686E-99	0.406644452	1	0.999
Crip1	2.16252E-33	0.301075049	0.97	0.9	Rps27	1.24944E-95	0.429058163	1	0.999
Fth1	1.9836E-31	0.156348531	0.998	0.993	Ifitm10	1.6759E-92	0.439417654	0.14	0.006
Tcrg-C2	4.59893E-26	0.307896572	0.122	0.038	Rps24	2.38976E-90	0.363231989	1	0.999
Fgl2	1.42153E-23	0.257986655	0.496	0.33	Rps5	3.85777E-78	0.377022816	0.998	0.997
H2-D1	2.20955E-22	0.147062345	0.997	0.997	Bcl2	8.45126E-73	0.916851835	0.47	0.139
Klf6	2.14744E-21	0.293761506	0.661	0.546	Gramd3	6.06901E-71	0.64837122	0.285	0.051
AW112010	2.2298E-17	0.219993514	0.841	0.769	Nkg7	9.89023E-69	1.233086422	0.335	0.074
Iqgap1	3.30848E-14	0.210154262	0.704	0.616	Rpl8	6.46264E-66	0.375423966	1	0.994
Ccr2	3.52433E-14	0.236819564	0.523	0.396	Rps21	9.65962E-63	0.326496889	1	0.996
Cd47	5.3779E-14	0.201292942	0.841	0.805	Rpl38	1.22943E-53	0.273970715	0.998	0.999
H1f0	1.23044E-13	0.276764339	0.463	0.346	Rps2	3.27035E-51	0.321631853	0.998	0.995
H2-K1	4.21668E-13	0.131093582	0.99	0.993	Rpl23	4.45864E-51	0.266852037	1	1
Sorl1	5.37471E-13	0.261458639	0.382	0.279	Rpl10	3.56718E-48	0.402847588	0.998	0.982
Pstpip1	1.35899E-10	0.202010383	0.533	0.451	Rpl36	2.52118E-47	0.2671087	1	0.998
Rad50	1.60212E-10	0.219470633	0.301	0.21	Rpl12	2.47084E-45	0.388343286	0.988	0.977
Txn1	8.23823E-10	0.207371882	0.592	0.523	Rpl9	7.56908E-45	0.256789616	1	0.995
Cluster3: Act2					Cluster7: Naive				
Gene	p_val_adj	avg_logFC	pct.1	pct.2	Gene	p_val_adj	avg_logFC	pct.1	pct.2
Klf2	2.1169E-283	1.382423752	0.839	0.225	Igfbp4	0	2.98992484	0.884	0.008
S1pr1	1.0567E-167	0.804485924	0.734	0.235	Atp1b1	0	0.876955418	0.416	0.005
Cdc25b	4.668E-144	0.805956497	0.629	0.204	Sell	0	1.300791426	0.681	0.021
Itgb1	1.2697E-114	0.865405074	0.779	0.407	Ccr7	0	1.695857015	0.835	0.044
Atp1b3	9.4889E-114	0.705375641	0.88	0.576	Treml2	9.8944E-280	0.701605848	0.368	0.011
1110034G24Rik	3.268E-111	0.76262547	0.728	0.335	Pdlim4	8.2347E-240	0.742753214	0.322	0.01
Ggt1	2.05666E-97	0.293616082	0.114	0.003	Actn1	2.0348E-191	0.918706775	0.546	0.068
Itga4	2.17882E-97	0.64935016	0.899	0.599	Dapl1	6.0141E-178	0.466650429	0.186	0.002
Il7r	1.92401E-80	0.545401957	0.946	0.723	Ly6c1	2.944E-153	0.628650206	0.203	0.006
Gm45552	2.17973E-80	0.51653011	0.322	0.078	Tbxa2r	1.034E-135	0.529784235	0.268	0.018
Rasa3	2.8378E-75	0.541882026	0.486	0.181	Tmie	2.2507E-130	0.401522925	0.192	0.007
Tmsb10	1.75544E-74	0.298981958	0.999	0.999	Gm32633	2.2319E-120	0.275361029	0.132	0.002
Tcf7	1.09978E-62	0.440011454	0.768	0.42	Gpr18	1.3992E-111	0.406887139	0.227	0.016
Rpl18	1.67053E-62	0.222332055	1	0.997	Sgk3	1.10211E-93	0.258462831	0.122	0.003
Serp2	6.69529E-62	0.290662486	0.139	0.017	Slc16a5	4.38169E-93	0.25566961	0.114	0.002
Ass1	7.37542E-62	0.605040286	0.817	0.577	Patj	1.74298E-92	0.373907875	0.165	0.009
Myo1f	6.27214E-59	0.511978384	0.497	0.207	Gm34006	7.60336E-84	0.296196239	0.138	0.006
Tdrp	1.30731E-56	0.326908012	0.202	0.042	Acvrl1	2.36314E-82	0.219711949	0.105	0.003
Ms4a4b	2.63194E-56	0.424987637	0.736	0.395	Rgmb	2.20846E-77	0.23135584	0.122	0.005
Slamf6	6.23286E-56	0.428559246	0.416	0.155	Klhdc1	6.27467E-77	0.565039153	0.332	0.06
Cluster4: Treg					Cluster8: Act4				
Gene	p_val_adj	avg_logFC	pct.1	pct.2	Gene	p_val_adj	avg_logFC	pct.1	pct.2
Foxp3	0	1.472230248	0.49	0.013	Cxcr5	2.63E-197	0.503051436	0.22	0.003
Izumo1r	1.2333E-112	0.882909837	0.43	0.088	Gm14718	5.31364E-71	0.438196782	0.176	0.014
Klrg1	5.253E-99	0.661500043	0.155	0.008	H2-Q2	4.38737E-58	0.314616629	0.118	0.007
Ncmap	1.48956E-85	0.779717916	0.476	0.142	Gltp	1.07968E-53	0.747178238	0.841	0.577
Ly6e	1.82149E-84	0.555485743	0.996	0.969	Ntrk3	9.64269E-44	0.271553383	0.108	0.008
Ly6a	6.47324E-84	1.150041131	0.801	0.468	Art2b	1.43037E-40	0.268109053	0.125	0.012
Ccr5	1.52634E-79	0.478556401	0.181	0.018	Lrig1	1.22925E-32	0.487819416	0.341	0.103
Tigit	2.22253E-72	0.764573775	0.472	0.154	St3gal1	1.4855E-31	0.471137629	0.385	0.129
Tnfrsf4	4.65643E-68	0.874916782	0.498	0.184	2310001H17Rik	7.87548E-31	0.654001253	0.584	0.278
Neb	6.20874E-65	0.441580789	0.155	0.017	Cd200	7.29834E-30	0.416110113	0.236	0.057
Il2ra	1.22809E-57	0.656292432	0.295	0.074	Fas	6.44723E-28	0.381082574	0.213	0.049
Ikzf2	3.89695E-54	0.752387032	0.351	0.108	Rnf19a	6.87615E-25	0.446719816	0.301	0.098
Snx9	4.98477E-54	0.329337339	0.157	0.021	Tspan5	1.48579E-21	0.381736784	0.253	0.079
Il10ra	1.44799E-43	0.440281687	0.267	0.074	Mrps6	1.74286E-20	0.442063842	0.375	0.155
Entpd1	1.07529E-41	0.308970293	0.161	0.029	Marcksl1	2.78378E-20	0.316829599	0.122	0.022
Srgn	1.69539E-41	0.497272865	0.958	0.884	Cd28	3.31722E-20	0.463225305	0.807	0.59
Peli1	4.20726E-41	0.584195467	0.777	0.553	Nav2	5.50853E-14	0.286378416	0.149	0.04
Gbp3	1.05822E-38	0.288115717	0.12	0.017	Npepl1	1.40651E-13	0.455470434	0.372	0.179
Arl5a	1.18645E-38	0.489408723	0.275	0.086	Stk4	2.83219E-13	0.434109	0.632	0.417
Rapsn	1.36737E-37	0.393359643	0.157	0.031	Ankrd46	3.36675E-13	0.33530938	0.25	0.098

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