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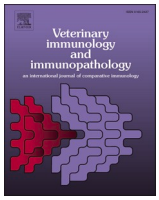

Exploring CD4 +CD8 + double-positive T cells in canine allergy and health: A pilot study

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ABSTRACT

Background: CD4 +CD8 + double-positive (DP) T cells are present in low numbers in the peripheral blood of both healthy and sick humans and dogs. In humans, these cells play cytotoxic or suppressive roles depending on the disease, but their function in dogs remains unclear.
Objectives: This study aims to investigate the presence of DP T cells in a cohort of dogs with adverse food reactions (AFR), compare their frequency among AFR, non-food-induced atopic dermatitis (NFICAD), and healthy dogs (HTY), and evaluate whether DP T cells could serve as a diagnostic tool to differentiate between AFR and NFICAD and identify the culprit allergens in AFR dogs.
Methods: Peripheral blood samples were collected from dogs with AFR, NFICAD, and healthy controls. PBMCs were isolated and analyzed by flow cytometry to assess T cell subpopulations. AFR dogs were grouped by their specific culprit allergens, and DP T cell proliferation in response to each allergen was compared across groups. An overall comparison of DP T cell proliferation was made between the three groups (AFR, NFICAD, HTY) under both stimulated and non-stimulated conditions. The mean percentage of proliferating DP T cells in healthy dogs was used as a cut-off to correlate with oral food challenge (OFC) results.
Results: DP T cells proliferated in all groups, with the greatest proliferation observed in the AFR group when stimulated with food allergens. Statistically significant differences were found between AFR and NFICAD groups, with AFR dogs showing more proliferation. The test identified the culprit allergens in 28.57 % of cases, with false positives occurring in 17.86 %.
Conclusions: DP T cells showed greater proliferation in food-allergic dogs compared to those with other allergic conditions like NFICAD. Despite these differences, overlapping results indicate that DP T cells are not a reliable screening test for distinguishing allergic from healthy dogs. While the test holds potential for identifying allergic phenotypes, it lacks sufficient diagnostic value for pinpointing specific allergens. Future studies with larger sample sizes and refined methods are needed to improve diagnostic accuracy.

1. Introduction

Adverse Food Reactions (AFR) in dogs are a significant and growing concern in veterinary medicine. AFR are immunologically mediated hypersensitivity reactions to specific food components, manifesting in gastrointestinal, dermatologic, or occasionally systemic symptoms. The prevalence of AFR has been rising, with increasing awareness among veterinarians and pet owners. However, the exact immunological mechanisms underlying AFR remain incompletely understood, making it challenging to develop effective diagnostic and therapeutic strategies. What we do know is that, similar to humans, the immune response to food allergens in dogs involves a complex interplay between various immune components. Upon exposure to the allergenic food, both innate and adaptive immune responses are activated, leading to the production of allergen-specific antibodies and the recruitment of immune cells. Despite progress in understanding, the immune mechanisms driving AFR in dogs remain incompletely elucidated, and reliable biomarkers for diagnosis and treatment are still lacking. One potential avenue for deeper investigation lies in the role of DP T cells, which are involved in immune responses across various pathological conditions.

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During thymic development, thymocytes transiently express both CD4 and CD8 co-receptors on their surface. Upon maturation, peripheral T cells lose the expression of one of the two co-receptors, differentiating into $CD4^+$ or $CD8^+$ single-positive T cells. Over 20 years ago, $CD4^+CD8^+$ double-positive (DP) T cells were identified in peripheral blood. Like their thymic progenitors, they express both CD4 and CD8 simultaneously, but, in contrast, they have a longer lifespan and can express memory markers (Bagot et al., 1998; Nascimbeni et al., 2004). While most studies suggest that peripheral DP T cells originate from mature $CD4^+$ T cells, some evidence indicates they may also derive from mature $CD8^+$ T cells. The functional role of DP T cells remains unclear, with studies presenting conflicting results (Blue et al., 1986; Flamand et al., 1998; Kitchen et al., 1998; Luhtala et al., 1997; Macchia et al., 2006; Molteni et al., 2002; Parel et al., 2007; Sullivan et al., 2001). In healthy individuals, DP T cells are typically present in low numbers (Parel et al., 2007; Blue et al., 1985; Kay et al., 1990; Colombatti et al., 1998; Patel et al., 1989). However, their frequency increases in various pathological conditions, such as viral infections (where they exhibit cytotoxic potential), autoimmune diseases (where they display suppressive functions), and neoplastic conditions (where they show both cytotoxic and suppressive cytokine profiles) (Bagot et al., 1998; Das et al., 2003; Desfrançois et al., 2010; Kitchen et al., 2005, 2004). In addition, atopic patients showed an increased frequency of DP T cells in their skin and peripheral blood as compared to healthy individuals (Bang et al., 2001).

DP T cells have also been identified in other species, including rats, mice, monkeys, swine, chickens, and dogs (Alexandre-Pires et al., 2010; Zuckermann, 1999). Notably, a study in mice found that the absolute number of DP T cells in the lungs and mediastinal lymph nodes increased following ovalbumin (OVA)-induced allergic asthma, suggesting that DP T cells may be recruited to sites of allergen-induced inflammation and contribute to the pathogenesis of allergic conditions (Zuśka-Prot et al., 2016). Despite extensive research on human DP T cells, their role in dogs remains relatively underexplored. In healthy dogs, DP T cells are present in small

percentages (~2.4 %) but can increase to 20 % following stimulation (Bismarck et al., 2012). Their proportion also rises in cases of visceral leishmaniosis and after viral or bacterial stimulation (Bismarck et al., 2012; Schütze et al., 2009). Rothe also discussed the complexity of DP T cell differentiation, further emphasizing the need for deeper investigation into their role in allergic conditions (Rothe et al., 2017). Our findings add to this complexity, demonstrating that canine DP T cells are not a homogeneous population, but rather a heterogeneous group of cells with distinct subsets. This heterogeneity may influence their functional roles in different immunological responses, including those observed in allergic diseases. Building on this, we previously observed that dogs with adverse food reactions (AFR) exhibited a higher baseline percentage of non-restimulated DP T cells (4.1 %; range: 0–19 %) compared to healthy dogs (2.4 %; range: 1.4–4.2 %) (Maina et al., 2019). Following food allergen-specific sublingual immunotherapy the frequency of DP T cells decreased (Maina et al., 2019). This unexpected finding prompted us to hypothesize that DP T cells may play a role in the pathogenesis of AFR in dogs and to explore the role of DP T cells in the context of hypersensitivity reactions, more specifically their potential role in AFR in dogs and NFICAD. It aligns with human studies, including work by von Buttlar, which showed an increased frequency of DP T cells in the skin and peripheral blood of atopic patients compared to healthy individuals (von Buttlar et al., 2015).

Therefore, the objectives of this study were to: (1) investigate the presence of DP T cells in a larger cohort of dogs with AFR, (2) compare the percentages of DP T cells among dogs with adverse food reactions (AFR), dogs with non-food-induced atopic dermatitis (NFICAD), and healthy (HTY) dogs, and (3) assess whether DP T cells can be used to develop a screening test to distinguish AFR from NFICAD.

2. Methods

2.1. Groups selection and sampling

The study was approved by the Ethical Committee of Ghent University, Belgium (EC 2013/189 for healthy dogs and EC 2013/198 for allergic dogs) and by the Deontological Committee of the Belgian government (232663/13_11_2/14). Three groups of dogs were included: healthy dogs, dogs with adverse food reactions, and dogs with non-food-induced atopic dermatitis.

Eight clinically healthy, laboratory-raised beagle dogs were included in the HTY group. These dogs were housed in a research facility at the Faculty of Veterinary Medicine, Ghent University. Clinical histories were reviewed, and thorough clinical examinations were conducted prior to inclusion to exclude allergies or other diseases. While these dogs were free of known health issues and allergies, it is important to note that their use as controls is limited to the specific context of our study.

Dogs with a history and clinical signs consistent with non-seasonal atopic dermatitis, such as pruritus and/or inflammatory skin conditions, were recruited during clinical visits conducted by the principal investigator. These dogs underwent a comprehensive diagnostic investigation, which included an assessment of ectoparasitic infestations through skin scrapings, trichography, coat brushing, and trial treatments with insecticidal and acaricidal therapies. The evaluation of microbial skin infections involved cytological examination, bacterial and fungal cultures, and trial antimicrobial treatments if necessary.

Dogs displaying allergic phenotypes and failing to respond to parasitic, bacterial, or fungal treatments were subjected to an elimination diet. Dog food for the elimination diet was selected in accordance with guidelines for managing adverse food reactions (Nuttall et al., 2019; Favrot et al., 2010; Olivry, 2010). The dogs were primarily fed a home-cooked diet consisting of novel protein and carbohydrate sources that the animal had not previously consumed, and that did not have known cross-reactivity with proteins already ingested. When a home-cooked diet was not feasible, a highly hydrolyzed diet (Royal Canin Anallergenic) was recommended. The animals were strictly fed the prescribed diet, for at least eight weeks, with no table scraps or treats allowed, to minimize potential confounding variables. For dogs that improved on the restriction diet, a provocation test was conducted. Specific food components, such as pork, chicken, beef, cow milk, fish, lamb, rice, corn, and wheat, were reintroduced one at a time over a period of 14 days (or less, if signs relapsed). These foods were selected based on the animals' previous dietary history, including those already consumed by each dog. This process aimed to identify the culprit food allergens based on a relapse of clinical signs.

Allergic dogs that did not respond to the dietary trial but had clinical signs consistent with atopic dermatitis and fulfilled diagnostic criteria for NFICAD were allocated to the NFICAD group (Nuttall et al., 2019; Favrot et al., 2010; Olivry, 2010). Dogs that improved on the restriction diet, showed cutaneous and/or gastrointestinal clinical signs during the challenge period, and then improved again after reintroduction of the restriction diet were allocated to the AFR group. At the time of inclusion, none of the dogs had received glucocorticoids, ciclosporin, oclacitinib, lokivetmab or antihistamines within the previous month.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood samples (10 mL each) were collected from dogs via the cephalic vein into heparinized tubes. Samples from dogs exhibiting clinical signs and/or pruritus were obtained during the acute phase of their condition. Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood was performed as described previously (De Bruin et al., 2007). Briefly, blood (10 mL) was incubated with 2 mL of arabic gum/carbonyl iron solution at 37 °C for one hour and gently turned every 10 min. Upon Ficoll density gradient centrifugation (600 g, 30 min, RT), the interphase was collected, diluted in an equal amount of

Alsever's solution (2.05 % D-dextrose (Serva, Heidelberg, Germany), 1 % heat inactivated fetal calf serum (hi-FCS) (Integro, Zaandam, The Nether- lands), 100 µg/mL streptomycin/100 U/mL penicillin (P/S), 0.8 % tri- sodium citrate dihydrate (Merck, Darmstadt, Germany), 0.055 % water- free citric acid (UCB Pharma, Leuven, Belgium) and 0.42 % NaCl (VWR prolabo chemicals, Leuven, Belgium) in ultra-pure water, pH 6.1) and centrifuged at 550 g for 15 min. RBC were lysed with lysis buffer (0.747 % NH4Cl (VWR) and 0.206 % Tris (VWR) in distilled water, pH 7.2) and the remaining cells were washed with PBS and finally resuspended in 1 mL PBS. The cell concentration was determined with a hemocytometer and the cell suspension was adjusted with PBS to a concentration of 5×10^6 cells/mL.

2.3. Lymphocyte stimulation and flow cytometry staining

To detect proliferating lymphocytes, cells were labelled with CellTrace Violet (CellTraceTM Violet Cell Proliferation Kit, Life technologies, Carlsbad, CA, USA) following the manufacturer's instructions. The labelled cells were resuspended in complete medium (RPMI 1640 (Life technologies, Gibco®, Ghent, Belgium) with 10 % hi-FCS, 1 % P/S, 1 % L-glutamine (Life technologies), 1 % Non-Essential Amino Acids (Life technologies,), 1 % sodium pyruvate (Life technologies), 0.5 % gentamycine (Life technologies) and 0.1 % β-mercaptoethanol (UCB Pharma)) at a concentration of 5×10^6 cells/mL and 100 µL of this cell suspension was added to a flat-bottomed 96-well plate (Greiner bio-one, Frickenhausen, Germany) (De Bruin et al., 2007). Cells were then stimulated with allergen extracts (pork, chicken, beef, cow's milk, fish, rice, wheat, corn and lamb at concentrations of 25 µg/mL; Greer laboratories; Lenoir, NC, USA), concanavalin A (10 µg/mL; Amersham Pharmacia Biotech; Freiburg, Germany) or medium. In the AFR group, the allergens tested included both the identified culprit allergens, which were based on each dog's clinical history and diagnostic evaluation, as well as additional allergens included in the panel.

The PBMCs were then incubated at 37 °C in a 5 % CO_2 humidified atmosphere for five days and subsequently collected and stained with a mixture of three fluorochrome-conjugated primary anti-dog antibodies (AbD Serotec, Oxford, UK). This mixture contains FITC-conjugated anti-dog CD3, R-Phycoerythrin-conjugated anti-dog CD4 and Alexa Fluor 647-conjugated anti-dog CD8. As a control, PBMCs were also stained with fluorochrome-conjugated isotype-matched antibodies (AbD Serotec). Staining was performed at 4°C in the dark for 30 min. Following extensive washing, 7-aminoactinomycin D (7-AAD, Sigma-Aldrich) was added to identify viable cells. $CD3^+$ T cell subpopulations were analyzed using a BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) with BD FACSDiva™ software (BD Biosciences). Doublet cells were excluded, and viable 7-AAD-stained cells were gated for CD3, CD4, and CD8 expression. For details on the gating strategy, the reader is referred to our previous work (Maina et al., 2019).

2.4. Data analysis

The final percentages of $CD4^+CD8^+$ lymphocytes were calculated by subtracting the percentage of background proliferation observed in non-restimulated cells. Statistical significance was defined as a P-value of < 0.05. For the comparison of DP T cell proliferation across the three groups (AFR, NFICAD, HTY), a Friedman test was applied, followed by Dunn's multiple comparison test for pairwise comparisons. For the comparison of DP T cell proliferation across the two groups (AFR, NFICAD), a Kruskal-Wallis test was applied, followed by Dunn's multiple comparison test for pairwise comparisons between the two groups (AFR vs. NFICAD, AFR vs. HTY, NFICAD vs. HTY). Additionally, Wilcoxon tests were used to compare the AFR group with the healthy group for each allergen. Statistical analyses were performed using GraphPad Prism version 9.

2.5. Results

A total of 44 dogs, including 28 with AFR, 8 with NFICAD, and 8 healthy (HTY), representing various breeds, were included in the study. A summary of the demographic characteristics is provided in Table 1, which indicates no statistically significant differences in age between the groups.

The mean baseline percentages of non-restimulated DP T cells (cultured in medium alone) were as follows: 3.87 % (range 0–22.03 %) for the AFR group, 1.28 % (range 0.01–3.9 %) for the NFICAD group, and 7.18 % (range 1.74–18.15 %) for the healthy group (Fig. 1A).

The mean percentage of total (proliferating and non-proliferating) $CD4^+CD8^+$ cells, stimulated with food allergens, was 0.28 % (range 0–24.93 %) in the AFR group. This wide range of responses was due to the variability in the responses across the dogs and allergens. Notably, one dog exhibited an unusually high response to fish antigens (24.93 %), which significantly contributed to the broad range. Excluding this outlier, the range of responses was more consistent, with most dogs showing DP T cell percentages within a narrower range (0–8 %). The mean percentage of total (proliferating and non-proliferating) $CD4^+CD8^+$ cells, stimulated with food allergens, was 0.15 % (range 0–1.70 %) in the NFICAD group, and 0.95 % (range 0–1.83 %) in the healthy group.

Dogs with adverse food reaction were divided based on their specific culprit allergen, and the DP T cell proliferation upon stimulation with each respective allergen was compared across the different groups. The values of DP T cells proliferating after stimulation with chicken extract in dogs allergic to chicken were compared with those from non-food induced allergic dogs and healthy dogs. This approach was repeated for each of the other allergens tested (pork, rice, wheat, beef, milk, corn,

Table 1
Descriptive data of the dogs included in the study.

Group	Breed	Sex	Age (years)	Weight
AFR	Boston terrier Cavalier King Charles spaniel Cross-bred dog (x8) Dachshund Dobermann English setter French bulldog (x4) German Shepherd (x2) Golden retriever Italian Shepherd Jack Russell Terrier Poodle (x2) Sharpei Shih tzu West Highland white terrier Yorkshire terrier	9 Females 6 Females spayed 12 Males 1 Male neutered	mean 3.40 (0.5–13)	mean 15.31 (4−42)
NFICAD	Bull terrier Dalmatian English bulldog French bulldog German Shepherd Labrador retriever (x2) Spitz	2 Females 3 Females spayed 2 Males 1 Male neutered	mean 3.50 (1−8)	mean 21.13 (6.5–35)
HTY	Beagles	4 Females 1 Females spayed 1 Males 2 Male neutered	mean 6.25 (2−10)	mean 10.54 (8.2 – 12.3)

Abbreviations: AFR, canine adverse food reactions; HTY, healthy group; and NFICAD, non-food-induced canine atopic dermatitis.

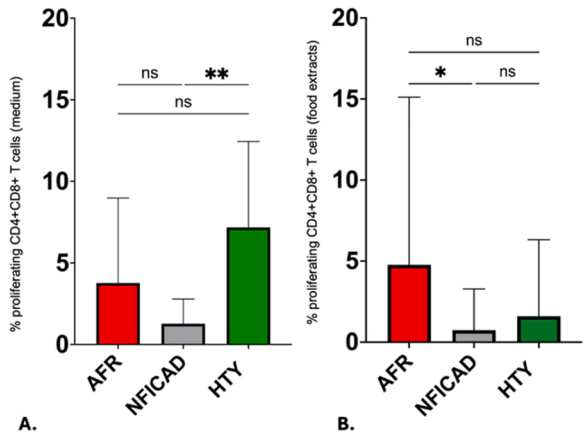

Fig. 1. Panel A: Baseline DP T cell proliferation in dogs with AFR, NFICAD, and HTY. The bar plot shows the mean and standard deviation (SD) for each group. The data indicate a significantly higher baseline DP T cell proliferation in the HTY group compared to both the AFR and NFICAD groups. Statistical significance was determined using the Kruskal-Wallis test, followed by Dunn's multiple comparison test (*P < 0.05, ns = not significant). Panel B: DP T cell proliferation in dogs with AFR, NFICAD, and HTY. Proliferation is measured after stimulation with specific food allergens. The bar graph displays the mean and standard deviation (SD) for each group. Statistical significance was determined using the Friedman test, followed by Dunn's multiple comparison test. The asterisk indicates significant differences between groups (*P < 0.05).

and fish). Although numerical differences in DP T cell proliferation were observed across the groups, a statistical analysis (Kruskal-Wallis test followed by Dunn's multiple comparison) revealed that these differences were not statistically significant (P > 0.05). Therefore, despite the observed trends in the data, there was no statistically significant difference in DP T cell proliferation between the groups for the tested allergens.

In addition to comparing the DP T cell proliferation between the groups based on specific allergens, an overall comparison of DP T cell proliferation across the three groups (AFR, NFICAD, and HTY) was conducted. This overall DP T cell proliferation differed significantly between the groups (P = 0.0008, Friedman test). A more detailed analysis showed that DP T cell proliferated more in the AFR group as compared to the NFICAD group (P = 0.0119, Friedman and Dunn's multiple comparisons), No significant differences in DP T cell proliferation were found between the other groups (AFR to HTY; P = 0.0545; NFICAD vs. HTY: P > 0.9999) (Fig. 1B.).

The mean percentage of proliferating DP T cells restimulated with food allergens in the healthy group was used as a cut-off value to assess whether the proliferating DP T cells of allergic dogs (AFR and NFICAD groups) correlated with the results of the oral food challenge (OFC). Among the 28 dogs in the AFR group, allergies to 42 different food constituents were identified. The percentage of proliferating DP T cells exceeded the cut-off value for 12 of 42 allergens (28.57 %) in 10 of 28 dogs (37.71 %) (Table 2). However, the cut-off value was also exceeded for 10 incorrect allergens in 5 of 28 dogs (17.86 %). In the NFICAD group, only 2 of 8 dogs (25 %) exceeded the cutoff value, and none of these dogs were allergic to food.

3. Discussion

In this study, we confirmed that DP T cells are indeed present in dogs with AFR. However, contrary to our expectations, when not stimulated with antigens, the mean percentage of DP T cells in healthy dogs was found to be higher than that in the AFR group. This seemingly contradictory result may be attributed to inherent biological variability, rather than differences in sample collection conditions. Although the blood from healthy lab dogs was fresher due to faster collection and processing of PBMCs, we used CellTrace Violet (CellTraceTM Violet Cell Proliferation Kit, Life Technologies) to specifically stain viable cells, excluding dead ones. This technique helped ensure that only live cells were counted, minimizing the impact of nonviable cells.

The baseline percentage of non-restimulated DP T cells should not be directly dependent on their ability to proliferate, as the variability may be influenced by multiple factors, including immune status, prior exposure to allergens, or individual dog differences. While T cells from healthy dogs can show different proliferative responses, we adjusted for potential confounding factors by subtracting the background proliferation percentage to better focus on the proliferative capacity of T cells in response to allergen stimulation. Although this method is commonly used in immunological assays to control for nonspecific or baseline proliferative activity, we acknowledge that the validity of this approach has not been fully validated in prior studies. Background proliferation could include contributions from factors unrelated to the immune response, such as nonspecific activation. By subtracting the baseline, we aimed to highlight the true proliferative responses to specific allergens.

Interestingly, when focusing on proliferating DP T cells, stimulation with food allergens resulted in higher proliferation in the AFR group than in the NFICAD group. Similarly to humans and mice, also dogs with NFICAD show an increase in DP T cells. This is not surprising since these lymphocytes show features of activated cells and both NFICAD and AFR are hypersensitivity disorders (Bang et al., 2001; Zuśka-Prot et al., 2016). Considering these observations, along with the reported reduction in DP T cells following food-allergen-specific immunotherapy in our previous study, it is tempting to speculate that DP T cells may play a role in the development of allergic diseases (Maina et al., 2019). However, further investigations are required to fully elucidate the role of DP T cells in the pathogenesis of such conditions.

Unfortunately, the overlap in proliferating DP T cell percentages between these groups prevents establishing a clear-cut threshold for differentiation. This means that the test cannot currently serve as a reliable screening tool to distinguish AFR from NFICAD. The small sample size of the NFICAD group is a limitation of our study, and as such, conclusions regarding the differences between the AFR and NFICAD groups should be made with caution. Further studies with larger cohorts would provide better insights into the immunological differences between food allergies and non-food-induced atopic dermatitis. Larger cohorts would allow for more robust conclusions and a better understanding of the role of DP T cells in these conditions. However, we believe that even with a higher number of animals, we will not be able to develop a test with sufficient sensitivity to discriminate between AFR dogs and those with NFICAD. This is because there are certainly individual-related variables and differences in the type of allergens. In this study, only certain allergens triggered DP T cell proliferation in specific dogs, and these allergens were not consistent across all animals. While we hypothesize that the identified allergens are the culprits, the diagnosis depends on changes seen during the elimination diet and provocative testing, which are often based on owner observations and can be influenced by mild or subtle reactions. This variability might also be linked to the heterogeneity of the DP T cell population, including potential subpopulations based on their expression levels of CD4 and CD8α, such as the $CD4^{dim}CD8\alpha^{bright}$, $CD4^{bright}CD8\alpha^{bright}$, and the $CD4^{bright}CD8\alpha^{dim}$ subsets (von Buttlar et al., 2015). Investigating these subsets in future studies could help clarify their role in allergic responses and improve our understanding of DP T cells in allergic conditions. To better understand the factors regulating DP T cell activation and their role in canine allergies, future research should focus on identifying the precise mechanisms driving these responses. This could involve exploring genetic predispositions, cytokine profiles, and examining the interactions between T cells and other immune cells within the affected tissues. More refined techniques, such as single-cell RNA sequencing, would allow for a deeper analysis of gene expression and cellular pathways involved in DP T cell activation. Additionally, advanced flow cytometry techniques, combined with detailed immune profiling, could

Table 2
List of dogs with AFR, NFICAD, including the culprit allergens (highlighted in bold) and all tested food allergens. The table presents the percentages of proliferating double-positive T cells for each dog and each tested food allergen. Positive results are indicated in red.

Dog with AFR	pork	rice	wheat	chicken	beef	milk	fish	corn	lamb	rabbit
1	0.0	0.0	**0.0**	0.0	-	-	-	-	-	-
2	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	1.01	-	-	-
3	0.0	0.08	0.0	**0.0**	0.0	0.07	0.0	-	-	-
4	21.3	35.16	29.52	28.2	**35.21**	35.54	30.36	-	-	-
5	0.0	0.0	0.0	**1.73**	0.0	0.0	**5.37**	-	-	-
6	3.27	0.4	1.78	**4.02**	2.8	1.8	-	-	-	-
7	0.8	0.2	0.3	**0.4**	0.7	0.5	0.5	1.5	-	-
8	-	-	-	**14.22**	-	-	-	-	-	**9.97**
9	0.2	0.5	0.8	**0.1**	0.0	0.4	1.0	0.7	-	-
10	0.1	0.0	0.0	0.0	0.0	0.0	**0.3**	0.5	0.1	-
11	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	**0.1**	-
12	**9.83**	0.41	0.4	3.53	6.8	4.76	0.0	4.25	-	-
13	**3.65**	0.0	1.91	**5.2**	2.91	2.25	1.22	1.31	-	-
14	**5.92**	2.93	1.27	0.32	2.73	1.88	**7.28**	7.32	-	-
15	**4.45**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-
16	**1.91**	1.05	1.39	1.62	1.42	1.33	**3.48**	**2.92**	-	-
17	0.4	**1.1**	0.0	0.0	0.0	0.0	0.0	0.0	-	-
18	**5.3**	0.0	0.5	0.6	0.0	0.3	0.0	0.0	-	-
19	**0**	0	0	0	**0**	0	0	0	-	-
20	1.2	0.0	0.0	0.0	0.0	**10.9**	0.0	0.0	-	-
21	**0.0**	0.17	-	**0.0**	0.0	-	0.0	-	-	-
22	0.33	**0.05**	0.0	**1.46**	**0.19**	4.44	-	-	-	-
23	0.1	0.0	0.0	0.0	0.0	0.0	**0.3**	0.5	0.1	-
24	1.2	0.1	0.2	**0.0**	0.1	0.1	0.2	1.0	-	-
25	3.41	1.97	3.52	0.0	**5.48**	0.08	0.0	1.53	-	-
26	0.86	0.0	0.0	**5.88**	0.0	1.43	24.92	-	-	-
27	2.42	**0.68**	0.0	**8.74**	0.59	3.69	23.13	-	-	-
28	0	0	**0**	**0**	**0**	0	0	-	-	-

Dog with NFICAD	pork	rice	wheat	chicken	beef	milk	fish	corn	lamb	
1	**0.0**	0.7	1.0	-	0.0	0.0	0.0	-	-	
2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	-	
3	0.0	14.43	0.0	0.0	0.0	0.0	0.0	-	-	
4	0.04	0.0	0.0	0.0	3.13	1.84	11.6	-	-	
5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-	
6	0.0	0.4	0.2	0.0	0.0	0.0	0.1	0.0	0.0	
7	0.0	0.2	0.0	0.1	0.1	0.0	0.0	0.0	-	
8	0.0	4.7	0.5	2.6	2.9	3.0	0.6	0.4	-	

provide insights into the functional state of these cells and their interactions with other immune populations. In parallel, combining DP T cell analysis with cytokine profiling would allow us not only to assess the frequency and proliferation of DP T cells but also to evaluate the specific cytokines they produce in response to stimulation. This would offer a more comprehensive view of the immune responses associated with AFR and NFICAD, potentially identifying biomarkers that could more effectively differentiate between these conditions. Moreover, using flow cytometry panels that assess a broader range of T cell subsets such as memory T cells or regulatory T cells could reveal further immune dynamics that may help distinguish between AFR and NFICAD by identifying specific T cell populations that are more prevalent or more active in one condition compared to the other.

Another objective of the study was to determine whether this test could be used to identify the food allergens responsible for food allergies in dogs. Unfortunately, the high number of false-positive results within the AFR group limits the use of DP T cells as a diagnostic tool for identifying the culprit food allergens. The observed inconsistency in DP T cell proliferation across dogs, irrespective of the food allergen used for stimulation, suggests that other factors, such as individual immune status, may influence these responses. This variability could reflect differences in immune activation or chronic immune responses, highlighting the importance of standardizing sample collection and analysis to minimize heterogeneity. To improve the reliability of such tests, it would be beneficial to select dogs with similar allergic manifestations (e.g., clinical signs, type of allergen, duration, severity, etc.) to better control confounding variables. Additionally, performing a Lymphocyte Stimulation Test (LST) in both AFR and NFICAD groups, using both food and aeroallergens, would help determine whether NFICAD dogs exhibit greater proliferation when stimulated with aeroallergens compared to those with AFR.

4. Conclusions

Regarding our initial objectives, we have confirmed that DP T cells are present in food-allergic dogs, and their proliferation, although variable, seems to be more pronounced in these dogs. However, we also found that the proliferation of these cells is not exclusive to food allergies but is also observed in dogs with other allergic conditions, such as non-seasonal atopic dermatitis, albeit to a lesser extent.

As for the second objective, we demonstrated that this test is not effective for distinguishing specific types of allergies. While it may help to identify allergic phenotypes, it currently lacks sufficient diagnostic value. Lastly, we aimed to identify the culprit allergens, but unfortunately, the test did not achieve this goal.

These findings underscore the need for more refined study designs, incorporating advanced techniques to better understand the cellular subtypes involved and how they vary according to the patient and the allergen.

CRediT authorship contribution statement

Maina Elisa: Writing – review & editing, Writing – original draft, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Devriendt Bert:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Cox Eric:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare no conflicts of interest related to the content of this study. This research was conducted in adherence to the highest ethical standards, and all funding sources are acknowledged in the manuscript. The authors confirm that there are no financial, personal, or professional relationships that could be perceived to influence the outcomes or interpretation of this research.

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