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Veterinary Immunology and Immunopathology xxx (xxxx) xxx-xxx

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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# Food allergen-specific sublingual immunotherapy modulates peripheral T cell responses of dogs with adverse food reactions

Elisa Maina , Bert Devriendt , Eric Cox *

*Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium*

ARTICLE INFO

ABSTRACT

Food allergen-specific sublingual immunotherapy (FA-SLIT) is a novel, safe and effective approach in dogs with adverse food reactions (AFR) to reduce their clinical symptoms. However, little is known about the specific immune components which mediate this reduction in clinical symptoms. In humans, regulatory T cells seem to play an important role in this desensitisation process. Here, we investigated changes in peripheral T cell responses of dogs with AFR upon FA-SLIT. Five dogs received a dose escalation of FA-SLIT over a six-month period. An oral food challenge was performed at the beginning and end of the study to assess the efficacy of the FA-SLIT. Using *in vitro* allergen-recall assays, we assessed the proliferation of T cell subsets before and at the end of the treatment. FA-SLIT significantly increased the percentage of proliferating CD4-CD8- double-negative (DN) T cells, while the percentage of allergen-specific CD4-CD8+ and CD4+CD8+ double-positive (DP) T cells decreased upon treatment. These findings indicate that sublingual immunotherapy in dogs activates DN T cells, which might be important for the desensitisation of dogs with adverse food reactions. However, further research is needed to corroborate these findings and to further elucidate the mechanism of action of FA-SLIT in dogs with AFR.

## 1. Introduction

Currently, the only known treatment for adverse food reactions (AFR) is avoidance of offending allergens and administration of anti-inflammatory medications on accidental exposure. Given the prevalence of AFR and the significant impairment in the quality of life for both dogs and owners, the development of a safe and efficacious therapy targeting AFR is highly desirable. Sublingual immunotherapy (SLIT) is a novel approach used in the treatment of people with food allergy by inducing desensitisation and eventually tolerance (Kim et al., 2011). With SLIT, small amounts of allergen extract are delivered sublingually as drops or tablets. Although the exact mechanisms of desensitisation with allergen-specific immunotherapy are still not fully understood, it is known that skewing of T-cell responses from allergen-specific effector T cells toward regulatory T cells (Tregs) is an essential event in the development of normal immune responses to allergens and is correlated with successful allergen-specific immunotherapy (ASIT) in humans (Akdis et al., 1998). There are numerous studies on immunotherapy for food allergy in humans, but studies in veterinary medicine on the efficacy of immunotherapy in the context of allergies and the immune components involved are lacking. We have recently reported that FA-SLIT had a favourable safety profile and was associated with decreased pruritus and clinical signs in dogs with AFR (Maina et al., 2016; Maina and Cox, 2016). Moreover, we showed that FA-SLIT triggered IL-10 secretion upon stimulation of PBMCs with allergens (Maina et al., 2017). In the present study, we aimed to investigate if these clinical improvements were accompanied by changes in the allergic immune response and more specific if FA-SLIT might modulate the response of specific T cell subsets upon stimulation with allergens.

## 2. Materials and methods

### 2.1. Animals and sampling

Client-owned dogs were enrolled as reported previously (Maina and Cox, 2016). Briefly, dogs with proven AFR and no other concurrent allergic conditions were randomized to receive the treatment (T group) with FA-SLIT (n = 5). During the study dogs were fed a restrictive diet. Clinical signs were provoked by feeding the diet con-

* Corresponding author.
*Email address:* eric.cox@ugent.be (E. Cox)

https://doi.org/10.1016/j.vetimm.2019.05.003
Received 21 March 2019; Received in revised form 27 May 2019; Accepted 28 May 2019
Available online xxx
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taining the suspected allergen before and at the end of the study. The severity of clinical signs was assessed with a pruritus Visual Analog Scale (pVAS) and the fourth iteration of the Canine Atopic Dermatitis Extent and Severity Index (CADESI-04) (Hill et al., 2007; Olivry et al., 2014). The treatment was continued daily for at least six months with dose escalations every two weeks. Peripheral blood samples were collected by cephalic or jugular venipuncture during the two provocative food exposure phases (before and after treatment). Sera were frozen at -20 °C until tested.

### 2.2. IgE ELISA

Serum food allergen-specific IgE concentrations were evaluated by ELISA. Allergen extracts (pork, chicken, beef, cow's milk, fish, rice, wheat and corn) were coated on 96-well plates (Nunc MaxiSorp®) overnight at 4 °C at 0.05 mg/mL in bicarbonate buffer. Upon blocking with PBS + 0.5% gelatin (from cold water fish skin; Sigma-Aldrich, Steinheim, Germany), the wells were incubated with two-fold dilutions of the serum samples in 0.05% Tween®20 in PBS (PBST) and incubated for 1 h at RT. Upon washing with PBST, the wells were incubated with a canine IgE-specific polyclonal antibody (horseradish peroxidase-labelled, Novus Biologicals; Cambridge, UK, NB7346) for 1 h at RT. Following PBST washes, ABTS solution (Roche Diagnostics; Mannheim, Germany) was used as substrate. Finally, the optical density (OD) was measured at 405 nm with a spectrophotometer (Tecan Group Ltd; Mannedorf, Switzerland).

### 2.3. Lymphocyte isolation

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 Netherlands), 100 µg/ml streptomycin/100 U/ml penicillin (P/S), 0.8% trisodium 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 $5 \times 10^6$ cells/mL.

### 2.4. Lymphocyte stimulation and flow cytometry staining

To detect proliferating lymphocytes, cells were labelled with CellTrace Violet (CellTrace™ 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 $5 \times 10^6$ 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 and corn at concentrations of 25 µg/mL; Greer laboratories; Lenoir, NC, USA), concanavalin A (10 µg/mL; Amersham Pharmacia Biotech; Freiburg, Germany) or medium.

The PBMCs were then incubated at 37 °C in a 5% CO2 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). After doublet and dead cell exclusion with the live/dead marker 7-aminoactinomycin D (7-AAD, Life technologies), the CD3 + T cell subpopulations were analysed using a BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) and BD FACSDiva software (BD Biosciences). The gating strategy is shown in Fig. 1.

### 2.5. Statistical analyses

For between-group comparisons, the Kruskal–Wallis test was employed, whereas for paired analysis of parameters before and after intervention within groups the Wilcoxon signed-rank test was used. Correlation between T cell subpopulations and clinical scores were analysed with a Spearman correlation test (q). Statistical analyses were conducted with SPSS Statistics 24 software (IBM; Armonk, NY, USA). A p-value $< 0.05$ was considered significant.

## 3. Results and discussion

To assess if FA-SLIT affects the function of canine T cell subpopulations, peripheral blood lymphocytes of dogs in the treatment group were stimulated with food allergens in a recall assay and the resulting phenotype of the CD3 + T cell subpopulations was investigated by flow cytometry. The percentage of the CD4-CD8 + and CD4 + CD8 + T cells significantly decreased after the treatment ($p = 0.007$ and $p = 0.004$, respectively), while the percentage of the CD4-CD8- T cell subset increased ($p = 0.009$) (Fig. 2A). As shown in Fig. 2B, proliferating CD4-CD8- double-negative (DN) T cells were significantly increased after the treatment ($p = 0.001$), while the percentage of proliferating CD4-CD8 + and CD4 + CD8 + double-positive (DP) T cells was significantly decreased after the treatment ($p = 0.002$ and $p = 0.019$, respectively). There was no difference in the percentage of proliferating CD4 + CD8- T cells. The DN T cell subset was reported to have an inflammatory function, being involved in the development of autoimmune diseases and infections, as well as a suppressive function, preventing allograft rejection, graft-versus-host disease, and autoimmune diabetes (Chen et al., 2003b; Cowley et al., 2010; Hillhouse et al., 2010; Marlies et al., 2007; Reinhardt and Melms, 2000; Young KJ et al., 2003). Given our results, we speculate that this DN T cell subset activated by sublingual immunotherapy comprises regulatory T cells (Tregs). DN Tregs are unique antigen-specific regulatory cells able to suppress CD8 + and CD4 + effector T cell responses and control the function of B cells, NK cells and dendritic cells via multiple mechanisms, including the secretion of IFN-γ and IL-10 (Chen et al, 2003a; Chen et al., 2005, 2007; Ford et al., 2002; Gao et al., 2011; He et al., 2007; Hillhouse et al., 2010; Ma et al., 2007). The role of this T cell subset in tolerance induction has been investigated only in one study, which demonstrated that DN T cells mediate the suppressive effects of immunotherapy by inhibiting IgE production and attenuating clinical signs in a mouse model of type I allergy (Raker et al., 2015). In our study, the increased percentage of DN Treg was not followed by significant changes in allergen-specific IgE concentration (Table 1), but it was concurrent with a significant decrease of the CD8 + T cell percentage, implying that in dogs tolerance induction by DN Tregs may

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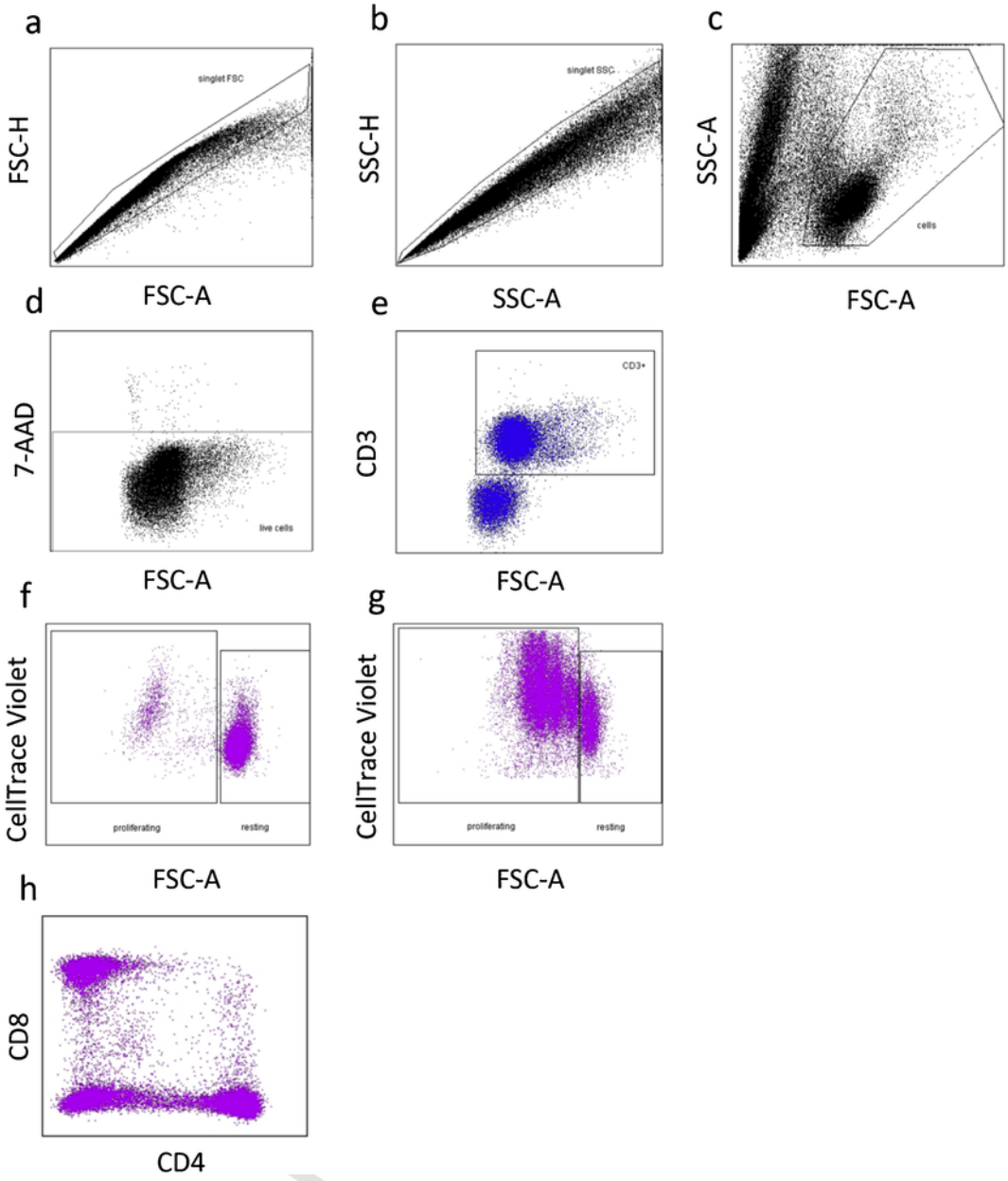


**Fig. 1.** Gating strategy to identify proliferating T cell subsets.a,b) Doublet cells were discriminated in forward scatter area (FSC)-A/FSC-H and side scatter area (SSC)-A/SSC-H dot plots. c) Based on their size and granularity, the lymphocytes within the singlet cell population were gated in a FSC/SSC dot plot. d) Living lymphocytes were gated based on a 7-AAD staining. e) Within the living lymphocyte gate, CD3 + T cells were selected. f,g) Based on CellTrace Violet, proliferating cells were identified. h) within proliferating and resting T cells, four subsets were identified based on CD4 and CD8 expression.

follow a different mechanism. Future research should include additional markers to distinguish this regulatory T cell subpopulation from other CD4-CD8- T cells.

Interestingly, also the CD4 + CD8 + DP T cells were affected by the treatment. These CD4 + CD8 + DP T cells have been described in other species, including pigs and humans. In swine, DP T cells are a multifunctional memory T cell subset involved in protection against viral infections (Gerner et al., 2015; Okutani et al., 2018), while in humans this T cell subset is associated with chronic diseases, including atopic dermatitis (Bang et al., 2001; Overgaard et al., 2015). Only a few reports describe these CD4 + CD8 + DP T cells in dogs and they seem to have an activated phenotype (Bismarck et al., 2012, 2014; Hoshino et al., 2008; Kato et al., 2007; McGill et al., 2018), but their function remains largely unclear. Recently, canine CD4 + CD8 + DP T cells were shown to expand upon infection with *Ehrlichia chaffeensis*, an obligate intracellular bacterium causing chronic infections in dogs (McGill et al., 2018). In our study, dogs with AFR had a higher baseline value of DP T cells than that reported in healthy dogs (25% vs 2.4%, resp.) and this baseline value decreased after the treatment, suggesting that DP T cells may have a role in the pathogenesis of AFR. This hypothesis is supported by a recent report demonstrating that the absolute number of DP T cells in lungs and mediastinal lymph nodes increased after immunization with OVA in mice with OVA-induced allergic asthma compared to healthy subjects. This suggests that the recruitment of DP T cells to sites of allergen-induced inflammation may be involved in the pathogenesis of allergic asthma (Zuśka-Prot et al., 2016). Together, these previous and our findings seem to indicate that CD4 + CD8 + DP T cells play a central role in chronic diseases in multiple species.

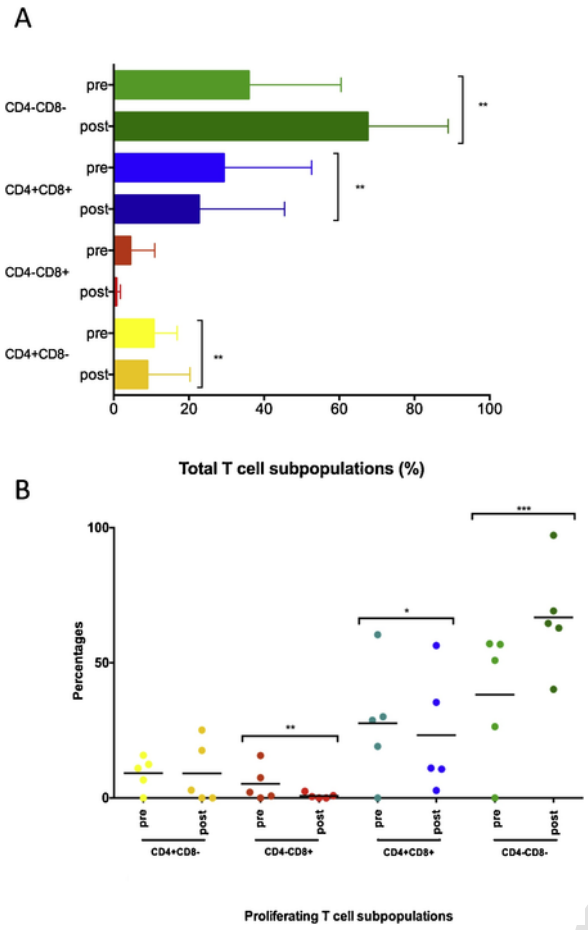


**Fig. 2.** Enumeration of the percentages of the (A) total and (B) proliferating CD3 + T cell subpopulations before and after the treatment in allergen-restimulated PBMCs of dogs in the treatment group (cases 4, 6, 8, 9 and 11). Since the responses were the same irrespective of the allergen, for dogs allergic to more than one food component only the average of all culprit allergens is displayed (for dog 4 the average of the values for Pk,R,W,B,C, and Mi is displayed; for dog 6 the average of the values for C and Fi; for dogs 8 and 9, allergic to only one food component [C]; and for dog 11 the average of the values for C,B, and W is displayed). Abbreviations: B, beef; C, chicken; Fi, fish; Mi, cow milk; Pk, pork; R, rice; W, wheat. The horizontal lines correspond to the median value. Statistical analysis: Wilcoxon signed-rank test: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

**Table 1**
Allergen-specific serum IgE levels were measured before (pre) and after (post) FA-SLIT in dogs included in the treatment group. The serum IgE levels are presented as OD values.

| Dog | allergens | allergen-specific IgE | |
|---|---|---|---|
| | | pre | post |
| 4 | pork | 0.181 | 0.184 |
| | rice | 0.244 | 0.272 |
| | wheat | 0.284 | 0.327 |
| | beef | 0.177 | 0.179 |
| | chicken | 0.195 | 0.242 |
| | milk | 0.257 | 0.217 |
| 6 | chicken | 0.445 | 0.429 |
| | fish | 0.222 | 0.270 |
| 8 | chicken | 0.170 | 0.243 |
| 9 | chicken | 0.227 | 0.163 |
| 11 | chicken | 0.382 | 0.363 |
| | wheat | 0.241 | 0.443 |
| | beef | 0.227 | 0.233 |

Despite these changes, the percentages of the total and proliferating T cell subpopulations and their changes after therapy were not correlated with changes in clinical scores (pVAS and CADESI; Maina and Cox, 2016). The most plausible explanation could be a lack of statistical power. To show this correlation between changes in T cell populations and changes in clinical scores, more animals should have been included in the study. Nevertheless, FA-SLIT appears to modulate the immune system, resulting in a functional switch from an activated T cell profile to a regulatory one, suggesting tolerance induction. We are the first to report that CD4 + CD8 + DP T cells are increased in dogs with adverse food reactions and that food-specific immunotherapy seems to decrease this subset and increase the percentage of DN T cells. However, future studies should be performed to better elucidate the role of the DP and DN T cell subsets in the pathogenesis of AFR as well as the mechanisms of FA-SLIT.

## Uncited reference

Ma et al. (2008).

## Acknowledgements

This research was funded by a grant of the special research fund of Ghent University. BD holds a postdoctoral grant of the Research Foundation Flanders (F.W.O.-Vlaanderen).

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