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Characterisation of T cell phenotypes, cytokines and transcription factors in the skin of dogs with cutaneous adverse food reactions

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ABSTRACT

The immunopathogenesis of cutaneous adverse food reactions (CAFRs) in dogs is unknown. Since the clinical manifestations in the skin are like those found in canine atopic dermatitis (AD), this study investigated the similarity in T cell phenotypes and gene expression of cytokines and transcription factors in CAFRs. In addition, the influence of an elimination diet on these parameters was tested.

In the skin of canine CAFRs, a predominant presence of $CD8^+$ T cells and increased expression of the *IL-4*, *IL-13*, *Foxp3* and *SOCS-3* genes were observed. *IFN-γ* gene expression was increased in lesional compared to non-lesional skin. The predominance of $CD8^+$ T cells indicates that the immunopathogenesis of CAFRs is different from that of canine AD. The elimination diet relieved clinical signs, but did not influence T cell phenotypes or expression of the cytokine and transcription factor genes in the skin of dogs with CAFRs, indicating a continuously pre-activated immune status in dogs sensitised to food constituents.

Introduction

Skin responses to dietary components in dogs are generally referred to as cutaneous adverse food reactions (CAFRs), because the underlying immunological response patterns are unknown (Hillier and Griffin, 2001). Although food allergen exposure occurs first in the digestive tract, only 20–30% of CAFR dogs have gastrointestinal signs (Hillier and Griffin, 2001) whereas the majority of the dogs only show cutaneous symptoms.

Signs of CAFRs can occur at the same predilection sites and may be clinically indistinguishable from atopic dermatitis (AD). In the most detailed retrospective study, 63% of dogs diagnosed with food hypersensitivities based on dietary elimination and provocation tests exhibited cutaneous lesions and pruritus suggestive of AD (Chesney, 2002). However, CAFRs may also manifest as papular abdominal rash, otitis, seborrhoea or recurrent superficial pyoderma (White, 1986; Carlotti et al., 1990; Harvey, 1993; Rosser, 1993; Leistra et al., 2001).

Currently, it is unknown how exposure to food leads to adverse reactions in the skin. In contrast, cell subsets and cytokine production in the skin of dogs with AD have been investigated extensively. In AD, inflammation is characterised by an influx of $CD4^+$ and $CD8^+$ T cells in the lesional skin (Olivry et al., 1997; Sinke et al., 1997) and a mixed cytokine profile with predominant expression of *IL-6*, *TARC*, *IL-4* and *IL-13* genes in the early stage followed by *IFN-γ*, *IL-12* and *IL-18* later on (Olivry et al., 1999; Nuttall et al., 2002; Marsella et al., 2006). Whereas most dogs with AD have circulating allergen-specific IgE (DeBoer and Hillier, 2001), there is controversial evidence for a similar reaction phenomenon in dogs with spontaneous manifestations of CAFRs (Jackson et al., 2003; Pucheu-Haston et al., 2008; Puigdemont et al., 2006). Increasing our insight in the immunopathogenesis of CAFRs may facilitate the development of novel modalities to treat them.

Since the clinical manifestations of CAFRs are comparable to those of AD, we hypothesised that the cutaneous reaction pattern of dogs with CAFRs is comparable to that of dogs with AD. To test this hypothesis, the inflammatory response (T cells and expression of cytokines and transcription factors representative for Th1, Th2 and regulatory T cells; reviewed in Wilson et al. (2009); Ozdemir et al. (2009)) in the skin of dogs with CAFRs was investigated after a provocation diet with the animals' original food and a subsequent elimination diet.

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Materials and methods

Animals and experimental design

Ten client-owned CAFR dogs without intestinal symptoms (five females, five males; age 10 months–8 years, median 3.5 years) were referred to the Utrecht University Faculty of Veterinary Medicine. The group consisted of four Labrador retrievers and one of each of the following breeds: shorthaired Dachshund, a German Shepherd, English Bulldog, English Cocker spaniel, West Highland White terrier, and a mixed-bred dog. There is no known breed predisposition for CAFRs. The seven healthy control dogs (three females, four males; age 4–10 years, median 8 years) included in this study were three Beagles and four mixed-bred dogs. The study fulfilled the requirements set by the Utrecht University Animal Experiments Committee.

Prior to dietary testing, flea bite hypersensitivity was ruled out by skin testing, IgE serology and flea control measures with adulticides and insect growth inhibitors at 1-month intervals. Other parasitic disorders were excluded by routine diagnostic methods. Secondary infections (staphylococcal pyoderma, *Malassezia* spp. dermatitis) were treated, if applicable, before and throughout the trial. The CAFR dogs were included based on the following dietary testing procedure. First, a full reduction in pruritus and associated symptoms had to be achieved after an elimination diet using a novel protein (ostrich, turkey, horse or goat meat) home-cooked diet for at least 8 weeks. Second, a provocative test with the original food had to result in reappearance of the pruritus (T0). Third, after a second elimination diet the pruritus had to disappear for a second time (T1). The healthy control dogs underwent a similar dietary regimen for 8, 2 and 2 weeks, being the average periods necessary for dietary responses in CAFR dogs.

Skin samples

Skin biopsies from both lesional (LS) and non-lesional skin (NLS) were taken using a 6-mm disposable punch (Kai Industries) under general anaesthesia (medetomidine 20 μg/kg and propofol 1–3 mg/kg IV on effect) within 10 days following the onset of signs due to the dietary provocation (T0) and after the disappearance of signs resulting from the second elimination diet (T1). Since clinical signs vanished after the elimination diet (T1) and LS was no longer visible, biopsies were taken at the same locations as at T0. Skin biopsies of the healthy control dogs were collected from the front legs and the thorax. Skin samples were snap frozen in liquid nitrogen for immunohistochemistry and RNA isolation, or fixed in 4% neutral buffered formaldehyde for routine haematoxylin and eosin (HE) staining.

RNA isolation

Frozen LS and NLS biopsies were immersed in TRIzol at 4 °C (Invitrogen), cut in smaller pieces and homogenised using an Ultra-Turrax disperser (T8, IKA Labortechnik). Isolation of total RNA and removal of genomic DNA was performed as previously described (Schlotter et al., 2009) and consisted of a combination of TRIzol and RNeasy mini kit (Qiagen) procedures.

Primer design and quantitative polymerase chain reaction (Q-PCR)

Oligonucleotide primers (Eurogentec) were designed for Th1-related genes (*IL-12p35*, *IL-18*, *IFN-γ*, *STAT-4*, *SOCS-5*, *TNF-α*), Th2-related genes (*IL-4*, *GATA-3*, *STAT-6*, *SOCS-3*, *IL-13*, *TARC*) and tolerance-related genes (*IL-10*, *TGF-β*, *Foxp3*) based on the sequences described in the ensemble project[1] using the Primer-3 software. Primer sequences and optimum annealing temperatures are shown in Table 1.

cDNA synthesis and Q-PCR conditions were as previously described (Brinkhof et al., 2006) with the following modifications. The Q-PCR programme included a 5 min polymerase activation step and continued with 40 cycles consisting of a denaturing step at 95 °C for 30 s, an annealing step for 30 s and an elongation step at 72 °C for 30 s with a final extension for 2 min at 72 °C. All PCR reaction efficiencies were between 95% and 105% and product melting curves showed single products and absence of a product in the negative controls (data not shown). If the expression of a gene was below the detection limit, the lowest detectable value was used for statistical analysis. The results of each sample were normalised to the average amounts of the endogenous reference genes (*HPRT* and *RPS19*) of the same sample (Vandesompele et al., 2002; Schlotter et al., 2009): the relative gene expression. *RPS5* was used as control gene for statistical analysis.

Immunohistochemistry

Biopsies were mounted in Tissue-Tek (Sakura Finetek Europe) and 6 μm cryostat sections were placed on Superfrost Plus slides (Menzel-Glaser), dried and stored at −70 °C until use. After thawing, the tissue sections were fixed in 100% acetone for 10 min at room temperature (RT). Endogenous peroxidase was eliminated by 20 min incubation in Tris-buffered saline (TBS: 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.5) supplemented with 0.3% hydrogen peroxide, followed by washing in TBS. Sections were incubated for 25 min in blocking reagent (TBS with 10% inactivated normal dog and normal goat serum) and subsequently for 1 h at RT with primary unlabelled antibodies diluted in 1% blocking reagent (Table 2). After washing, sections were incubated for 30 min with horseradish peroxidase (HRP)-labelled antibodies (Table 2), washed and blocked for 30 min with unlabelled rabbit anti-mouse antibodies (Table 2) to cover any unbound primary antibodies.

After further washing, the sections were incubated for 45 min with the second primary unlabelled antibodies, washed and incubated for 30 min with alkaline phosphatase (AP)-labelled antibodies (Table 2). Staining was developed with 3-amino-9-ethyl-carbazole (AEC, Sigma–Aldrich) resulting in a red-brownish colour (HRP) and thereafter with Fast Blue (AP), resulting in a blue colour. Double stained cells appeared purple. Cells in the epidermis and dermis from two biopsies were counted using light microscopy. Average numbers of positive cells/mm^2 were used for statistical analysis.

Table 1
Quantitative PCR primer sequences and annealing temperature.

Gene	Primer sequences 5′–3′	Annealing temperature (°C)
	Forward (F) and Reverse (R)	
IL-4	F: ccaaagaacacaagcgataaggaa	61
	R: gtttgccatgctgctgaggtt	
IL-10	F: cccgggctgagaaccacgac	63
	R: aaatgcgctcttcacctgctccac	
TARC	F: ggagccattcctatcagcag	64.5
	R: ggtcggaacagatggacttg	
IL-12p35	F: taatggatcccaagaggcag	62.5
	R: tcaagggaggatttctgtgg	
IL-18	F: gaggatatgcccgattctga	56
	R: tccggaggactcatttctg	
IFN-γ	F: agcgcaaggcgataaatg	55.8
	R: gcggcctcgaaacagatt	
TGF-β	F: caaggatctgggctggaagtgga	65
	R: ccaggaccttgctgtactgcgtgt	
GATA-3	F: tacgtccccgaatacagctc	64
	R: actccctgccttctgtgct	
STAT-4	F: actggaagaggcgacaacag	59
	R: gccttctgagttggaacagg	
STAT-6	F: aactgcagcggctctatgtc	64
	R: catgttgcagcagaaggtgt	
SOCS-5	F: tctgccgtgcagtaatctgt	61
	R: gccttgactggttctcgttc	
TNF-α	F: ccccgggctccagaaggtg	64
	R: gcagcaggcagaagagtgtggtg	
SOCS-3	F: acaccagcctgcgcctcaagacct	63
	R: cgcctcgccgcccgtca	
IL-13	F: gaggagctggtcaacatca	59
	R: tgcagtcggagacattga	
Foxp3	F: caaatggtgtctgcaagtgg	59
	R: gtgctctgcccttctcatct	
HPRT	F: agcttgctggtgaaaaggac	56
	R: ttatagtcaagggcatatcc	
RPS19	F: ccttcctcaaaaagtctggg	61
	R: gttctcatcgtagggagcaag	
RPS5	F: tcactggtgagaaccccct	62.5
	R: cctgattcacacggcgtag	

Statistical analysis

The relative gene expression and immunohistochemistry results were analysed with the SPSS version 15 software. A linear mixed model (West et al., 2007) was done to analyze the relative gene expression as outcome variable. The relative expression of all genes together was analyzed in one model. The explanatory factors in the model are gene, LS/NLS/Control group, T0/T1 group and three two-way interactions between the three factors. The subject variable dog was used as the random factor to account for the repeated measurements within the dogs and was assumed to have a normal distribution. *RPS5* was used as the control gene. Residuals were studied to check the validity of the model. Multiple comparisons were corrected with the False Discovery Rate implying that $P \leqslant 0.05$ was considered statistically significant.

Since the results for immunohistochemistry were discontinuous values, non-parametric statistical tests were used. For the comparison between skin sections of CAFRs and control dogs, the Mann–Whitney *U* test (non-parametric analogue of independent *t* test) was used and for the comparison of skin sections between T0 and T1 and between LS and NLS skin, the Wilcoxon Signed Ranks test (non-parametric analogue of the paired *t* test) was used.

[1] See: http://www.ensembl.org/, version v.32.

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Table 2
Antibodies and concentration used for immunohistochemistry.

Antibody	Origin	Concentration
CD3 (CA17.2A12) primary antibody	Serotec Ltd.	1:200
CD4 (CA13.1E4) primary antibody	Serotec Ltd.	1:20
CD8 (CA9.JD3) primary antibody	Serotec Ltd.	1:20
γδTCR primary antibody	Monoclonal antibody, P.F. Moore, University of California	1:50
αβTCR primary antibody	Monoclonal antibody, P.F. Moore, University of California	1:100
MHC-II (CA2.1C12) primary antibody	Serotec Ltd.	1:60
CD1c primary antibody	Monoclonal antibody, P.F. Moore, University of California	1:20
Rabbit anti-mouse blocking antibody	Polyclonal antibody, Dako	1:50
Goat anti-mouse-HRP secondary antibody	Polymer antibody, Envision$^+$ system, Dako	Undiluted
Goat anti-mouse-AP secondary antibody	Polyclonal antibody, Dako	1:100

Results

Cell phenotypes

Analysis of the T cell phenotypes and cell numbers in the epidermis of LS showed significantly increased numbers of $CD8^+\gamma\delta TCR^+$ T cells ($P = 0.036$) compared with control skin during the provocation diet (Fig. 1). Moreover, the dermis contained more $CD3^+CD8^+$ ($P = 0.029$) and $CD8^+\alpha\beta TCR^+$ ($P = 0.023$) T cells in LS than in the skin of control dogs (Fig. 2). The elimination diet did not affect the T cell phenotypes or cell numbers.

The T cell numbers in LS and NLS skin were highly variable in the CAFR dogs (Figs. 1 and 2), but no effect of the elimination diet was found. In biopsies of LS skin, the epidermis showed occasional acanthosis and patchy spongiosis. Dermal lesions included a mild to moderate, superficial perivascular inflammation, predominantly characterised by a mixed infiltrate with lymphocytes and histiocytes, a variable amount of mast cells and plasma cells, and few neutrophils and eosinophils. NLS skin of CAFR dogs had a normal epidermis and a very mild, superficial, mononuclear perivascular infiltrate in the dermis. Eosinophils and mast cells were rare.

Gene expression

Foxp3 and *IL-4* were increased in LS ($P = 0.001$; $P = 0.008$) and NLS ($P = 0.002$; $P = 0.001$) and in addition *SOCS-3* ($P = 0.004$) and *IL-13* ($P = 0.002$) showed higher expression in LS compared to control skin during provocation (Fig. 3). Finally, *IFN-γ* was significantly more expressed in LS than in NLS ($P = 0.007$) during provocation. The expression of the remaining genes investigated did not differ. Expression of none of the genes was affected by the elimination diet.

Discussion

The present study is the first to investigate gene expression and cell phenotypes in the skin of CAFR dogs. We found strong indications for a CD8-dependent inflammatory response in LS skin and observed an intertwined Th1, Th2 and Treg cytokine reaction pattern in the skin. Surprisingly, none of the dogs had a significant alteration in cytokine or transcription factor gene expression or cell phenotype in the skin after the elimination diet, despite full eradication of clinical manifestations.

Cell numbers and phenotypes and gene expression levels observed in the skin did not parallel the clinical alterations resulting from dietary elimination and provocation. The absence of dietary influence may indicate that once an animal was sensitised to develop CAFRs, the skin remains in a primed immune status even after dietary elimination. This observation is supported by Olivry et al. (2007), who found in experimentally corn-hypersensitised dogs that the corn-diet did not influence $CD4^+CCR4^+$ cell numbers in blood, despite visible clinical changes. Furthermore, gene expression does not necessarily reflect biological activity of related proteins; this may also explain why mRNA expression of cytokines and transcription factors did not parallel the clinical response to the elimination diet.

T cell numbers were highly variable in LS skin and stable in control skin, indicating that such variability is specific for canine CAFRs, especially since the CAFR dogs had the same clinical starting condition and showed similar clinical responses during the dietary tests. Interestingly, the immune cell population in canine CAFRs was dominated by increased numbers of $CD3^+CD8^+$, $CD8\alpha\beta^+TCR^+$ cells in LS dermis and by increased $CD8\gamma\delta^+TCR^+$ cells in LS epidermis. In a mouse model of topical allergen-induced AD, data indicated that allergen-primed $CD8^+$ T cells are required for the development of AD-like lesions in mice (Hennino et al., 2007). In addition, under different circumstances $CD8^+$ T cells are able to express *IL-10*, *TGF-β*, *IL-4*, *IL-13*, *Foxp3*, and *IFN-γ* (Teraki et al., 2000; Berg et al., 2002; Jensen et al., 2006; Mahic et al., 2008). In canine AD an increased presence of $CD4^+$ and $CD8^+$ T cells in LS skin is reported, but the ratio of $CD4^+/CD8^+$ T cells measured in the epidermis is debated. Sinke et al. (1997) observed an increased ratio of $CD4^+/CD8^+$ T cells, whereas Olivry et al. (1997) found a preferential presence of $CD8^+$ T cells. In addition, we observed that the *IFN-γ* expression in LS skin was increased in comparison to NLS skin.

We observed an increased expression of *IL-4*, *IL-13*, *SOCS-3* and *Foxp3* genes in LS skin compared to control dogs, indicating a more Th2-skewed environment. NLS skin had increased expression of *IL-*

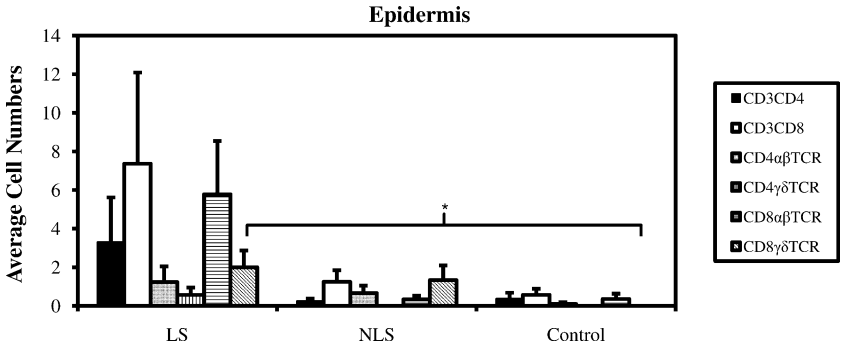

Fig. 1. Cell numbers in the epidermis of CAFR and control dogs. Each bar represents the average and SEM of the number of cells/mm length of the epidermis. $^*P \leqslant 0.05$.

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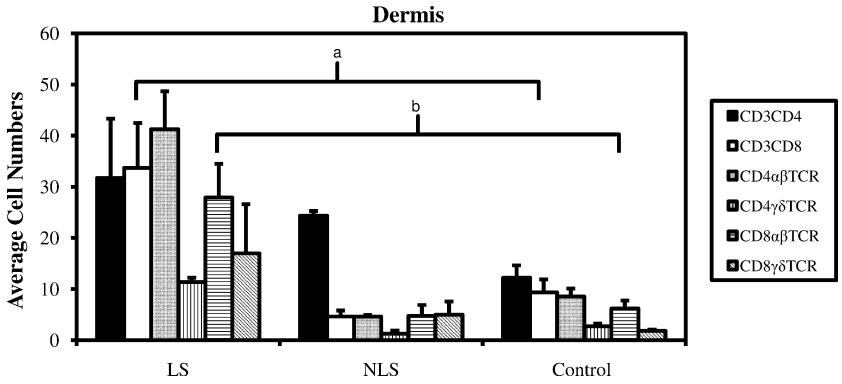

Fig. 2. Cell numbers in the dermis of CAFR and control dogs. Each bar represents the average and SEM of the number of cells/mm^2. (a) CD3$^+$CD8$^+$ LS compared to control skin ($P = 0.028$). (b) CD8$^+$αβTCR$^+$ LS compared to control skin ($P = 0.023$).

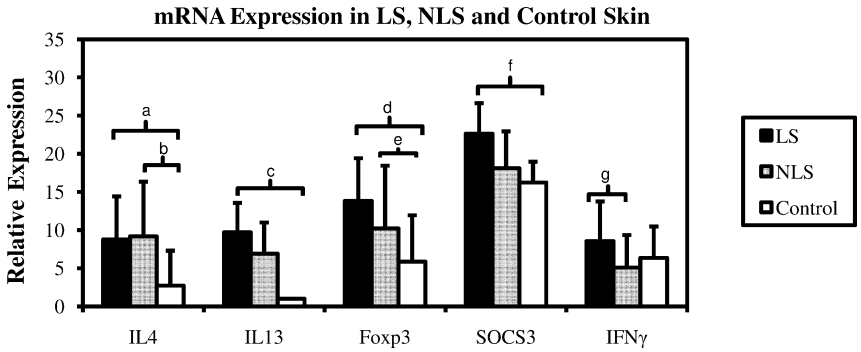

Fig. 3. mRNA expression in LS, NSL skin of CAFR dogs and canine control skin. The gene expression level is depicted as the ratio of genes expressed compared to expression of *IL-13* in control dogs, which is set to 1. Each bar represents the average and standard deviation. (a) *IL-4* LS compared to control skin ($P = 0.008$). (b) *IL-4* NLS compared to control skin ($P = 0.001$). (c) *IL-13* LS compared to control skin ($P = 0.002$). (d) *Foxp3* LS compared to control skin ($P = 0.001$). (e) *Foxp3* NLS compared to control skin ($P = 0.002$). (f) *SOCS-3* LS compared to control skin ($P = 0.004$). (g) *IFNγ* LS compared to NLS skin ($P = 0.007$).

4 and *Foxp3*, which may indicate that NLS skin is primed to become LS skin. In comparison, the cutaneous inflammation in dogs with spontaneous AD was characterised by expression of *IL-6*, *TARC*, *IL-4* and *IL-13* mRNA in the early stages, and *IFN-γ*, *IL-12* and *IL-18* in the chronic stages and no data are available on *Foxp3* expression levels (Olivry et al., 1999; Nuttall et al., 2002; Marsella et al., 2006). Regulatory CD4$^+$CD25$^+$ T cells are known to transcribe *Foxp3* and function by inhibiting the response of T cells, resulting in a tolerant environment. We suggest that despite the increased expression of *Foxp3*, they are not sufficiently effective in the LS skin, because the other cytokines and transcription factors assessed did not seem to be affected.

The CD4$^+$CD25$^+$ *Foxp3* transcribing cell population has been investigated most extensively, however, a population of CD8$^+$ T regulatory cells also exists. CD8$^+$ T regulatory cells can produce *IL-10*, *TGF-β*, *CTLA4* and *CCL4* and can express *Foxp3* as well (Cosmi et al., 2003; Jarvis et al., 2005; James and Kwok, 2007; Joosten et al., 2007; Mahic et al., 2008; Billerbeck et al., 2009). It would be interesting to investigate whether the CD8$^+$ T cells are responsible for *Foxp3* expression in CAFR skin.

Conclusions

In dogs with CAFRs, CD8$^+$ T cells are important in the initiation and maintenance of skin lesions, which is different from observations in canine AD. In addition, dietary elimination did not influence T cell profiles in the skin of dogs with CAFRs, indicating a sustained pre-activated immune status once dogs have been sensitised to food.

Conflict of interest statement

This work was financially supported by Royal Canin (Aimargues, France).

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References

Berg, R.E., Cordes, C.J., Forman, J., 2002. Contribution of CD8$^+$ T cells to innate immunity: IFN-γ secretion induced by IL-12 and IL-18. European Journal of Immunology 32, 2807–2816.

Billerbeck, E., Nakamoto, N., Seigel, B., Blum, H.E., Chang, K.M., Thimme, R., 2009. Determinants of in vitro expansion of different human virus-specific Foxp3$^+$ regulatory CD8$^+$ T cells in chronic hepatitis C virus infection. The journal of general virology 90, 1692–1701.

Brinkhof, B., Spee, B., Rothuisen, J., Penning, L.C., 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. Analytical Biochemistry 356, 36–43.

Carlotti, D.N., Remy, I., Prost, C., 1990. Food allergy in dogs and cats. A review and report of 43 cases. Veterinary Dermatology 1, 55–62.

Chesney, C.J., 2002. Food hypersensitivity in the dog: a quantitative study. The Journal of Small Animal Practice 43, 203–207.

Cosmi, L., Liotta, F., Lazzeri, E., Francalanci, M., Angeli, R., Mazzinghi, B., Santarlasci, V., Manetti, R., Vanini, V., Romagnani, P., Maggi, E., Romagnani, S., Annunziato, F., 2003. Human CD8$^+$CD25$^+$ thymocytes share phenotypic and functional features with CD4$^+$CD25$^+$ regulatory thymocytes. Blood 102, 4107–4114.

DeBoer, D.J., Hillier, A., 2001. The ACVD task force on canine atopic dermatitis (XVI): laboratory evaluation of dogs with atopic dermatitis with serum-based "allergy" tests. Veterinary Immunology and Immunopathology 81, 277–278.

Harvey, R.G., 1993. Food allergy and dietary intolerance in dogs: a report of 25 cases. Journal of Small Animal Practice 34, 175–179.

Hennino, A., Vocanson, M., Toussaint, Y., Rodet, K., Benetière, J., Schmitt, A.M., Aries, M.F., Bérard, F., Rozières, A., Nicolas, J.F., 2007. Skin-infiltrating $CD8^+$ T cells initiate atopic dermatitis lesions. Journal of Immunology 178, 5571–5577.

Hillier, A., Griffin, C.E., 2001. The ACVD task force on canine atopic dermatitis (X): is there a relationship between canine atopic dermatitis and cutaneous adverse food reactions? Veterinary Immunology and Immunopathology 81, 227–231.

Jackson, H.A., Jackson, M.W., Coblentz, L., Hammerberg, B., 2003. Evaluation of the clinical and allergen specific serum immunoglobulin E responses to oral challenge with cornstarch, corn, soy and a soy hydrolysate diet in dogs with spontaneous food allergy. Veterinary Dermatology 14, 181–187.

James, E.A., Kwok, W.W., 2007. $CD8^+$ suppressor-mediated regulation of human $CD4^+$ T cell responses to glutamic acid decarboxylase 65. European Journal of Immunology 37, 78–86.

Jarvis, L.B., Matyszak, M.K., Duggleby, R.C., Goodall, J.C., Hall, F.C., Gaston, J.S., 2005. Autoreactive human peripheral blood $CD8^+$ T cells with a regulatory phenotype and function. European Journal of Immunology 35, 2896–2908.

Jensen, J., Langkilde, A.R., Frederiksen, J.L., Sellebjerg, F., 2006. $CD8^+$ T cell activation correlates with disease activity in clinically isolated syndromes and is regulated by interferon-beta treatment. Journal of Neuroimmunology 179, 163–172.

Joosten, S.A., van Meijgaarden, K.E., Savage, N.D., de Boer, T., Triebel, F., van der Wal, A., de Heer, E., Klein, M.R., Geluk, A., Ottenhoff, T.H., 2007. Identification of a human $CD8^+$ regulatory T cell subset that mediates suppression through the chemokine CC chemokine ligand 4. Proceedings of the National Academy of Sciences U.S.A. 104, 8029–8034.

Leistra, M.G.H., Markwell, P.J., Willemse, T., 2001. Double blind evaluation of selected protein source diets in the management of dogs with adverse reactions to foods. Journal of the American Veterinary Medical Association 219, 1411–1414.

Marsella, R., Olivry, T., Maeda, S., 2006. Cellular and cytokine kinetics after epicutaneous allergen challenge (atopy patch testing) with house dust mites in high-IgE beagles. Veterinary Dermatology 17, 111–120.

Mahic, M., Henjum, K., Yaqub, S., Bjørnbeth, B.A., Torgersen, K.M., Taskén, K., Aandahl, E.M., 2008. Generation of highly suppressive adaptive CD8(+)CD25(+)FOXP3(+) regulatory T cells by continuous antigen stimulation. European Journal of Immunology 38, 640–646.

Nuttall, T.J., Knight, P.A., McAleese, S.M., Lamb, J.R., Hill, P.B., 2002. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. Clinical and Experimental Allergy 32, 789–795.

Olivry, T., Naydan, D.K., Moore, P.F., 1997. Characterization of the cutaneous inflammatory infiltrate in canine atopic dermatitis. The American Journal of Dermatopathology 19, 477–486.

Olivry, T., Dean, G.A., Tompkins, M.B., Dow, J.L., Moore, P.F., 1999. Toward a canine model of atopic dermatitis: amplification of cytokine-gene transcripts in the skin of atopic dogs. Experimental Dermatology 8, 204–211.

Olivry, T., Kurata, K., Paps, J.S., Masuda, K., 2007. A blinded randomised controlled trial evaluating the usefulness of a novel diet (aminoprotect care) in dogs with spontaneous food allergy. The Journal of Veterinary Medical Science 69, 1025–1031.

Ozdemir, C., Akdis, M., Akdis, C.A., 2009. T regulatory cells and their counterparts: masters of immune regulation. Clinical and Experimental Allergy 39, 626–639.

Pucheu-Haston, C.M., Jackson, H.A., Olivry, T., Dunston, S.M., Hammerberg, B., 2008. Epicutaneous sensitization with *Dermatophagoides farinae* induces generalised allergic dermatitis and elevated mite-specific immunoglobulin E levels in a canine model of atopic dermatitis. Clinical and Experimental Allergy 38, 667–679.

Puigdemont, A., Brazis, P., Serra, M., Fondati, A., 2006. Immunologic responses against hydrolysed soy protein in dogs with experimentally induced soy hypersensitivity. American Journal of Veterinary Research 67, 484–488.

Rosser, E.J., 1993. Diagnosis of food allergy in dogs. Journal of the Veterinary Medical Association 203, 259–262.

Schlotter, Y.M., Veenhof, E.Z., Brinkhof, B., Rutten, V.P., Spee, B., Willemse, T., Penning, L.C., 2009. A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis. Veterinary Immunology and Immunopathology 129, 115–118.

Sinke, J.D., Thepen, T., Bihari, I.C., Rutten, V.P., Willemse, T., 1997. Immunophenotyping of skin-infiltrating T-cell subsets in dogs with atopic dermatitis. Veterinary Immunology and Immunopathology 57, 13–23.

Teraki, Y., Hotta, T., Shiohara, T., 2000. Increased circulating skin-homing cutaneous lymphocyte-associated antigen (CLA)1 type 2 cytokine-producing cells, and decreased CLA1 type 1 cytokine-producing cells in atopic dermatitis. British Journal of Dermatology 143, 373–378.

Vandesompele, J., De Paepe, A., Speleman, F., 2002. Elimination of primer-dimer artefacts and genomic coamplification using a two-step SYBR green I real-time RT-PCR. Analytical Biochemistry 303, 95–98.

West, B.T., Welch, K.B., Galecki, A.T., 2007. Linear Mixed Models: A Practical Guide Using Statistical Software. Chapman & Hall/CRC, Taylor & Francis Group, Boca Raton, FL, USA. pp. 9–49 and 175–217.

White, S.D., 1986. Food hypersensitivity in 30 dogs. Journal of the American Veterinary Medical Association 188, 695–698.

Wilson, C.B., Rowell, E., Sekimata, M., 2009. Epigenetic control of T-helper-cell differentiation. Nature Reviews Immunology 9, 91–105.

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