Lymphocyte subpopulations and Treg cells in dogs with atopic dermatitis receiving ciclosporin therapy: a prospective study

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SUMMARY

Background – Canine atopic dermatitis (CAD) is a chronic dermatological disease partly due to dysregulation of the immune system. Inappropriate activation of CD4+ lymphocytes could favour and promote the allergic response. An inadequate activation system of regulatory T cells (Tregs) is suspected to be a key immunological feature of the allergic response in atopic dogs.

Hypothesis/Objectives – To evaluate the difference in the CD4/CD8 lymphocyte ratio and the percentage of Tregs in healthy dogs, in a breed predisposed to CAD, and in dogs affected by CAD before and during therapy with ciclosporin (CsA). Additionally to assess the improvement in pruritus and skin lesions during therapy with CsA, and to compare this with CD4/CD8/Treg values.

Animals – Ten atopic dogs of different breed, sex and age, ten healthy dogs and ten English bulldogs were included.

Methods – Peripheral blood from all dogs was tested using flow cytometry to assess the CD4/CD8 ratio and percentage of Tregs. For atopic dogs, sampling was repeated after 30 and 90 days of therapy with CsA.

Results – The CD4/CD8 ratio was not significantly different between the three groups. The Treg percentage was higher, but not statistically significant, in atopic dogs compared with controls. Therapy with CsA led to clinical improvement; it was not associated with statistically significant differences in haematological variables.

Conclusion and clinical importance – This study suggests that Tregs may be involved in the pathogenesis of CAD and that ciclosporin therapy does not affect the circulating lymphocyte subpopulations

Introduction

Regulatory T cells (Tregs) are a lymphocyte subpopulation with immune modulator and anti-inflammatory activity identified in both humans and dogs. In dogs they are characterized by the CD4+ CD25high FOXP3+ phenotype.1 Tregs play an important role in the regulation of peripheral tolerance. Abnormalities of T regulatory (Treg) cell numbers or function have been implicated in several autoimmune and allergic diseases.1

Flow cytometry is a technique that allows the identification and quantification of different lymphocyte populations in a cellular suspension through the use of

monoclonal antibodies. This technique has been used to evaluate the number of Tregs compared with the total lymphocyte population, and with the CD4/CD8 (T helper to cytotoxic T lymphocyte) ratio.3,4 Alterations in the CD4/ CD8 ratio can indicate an abnormal specific immune response.4 Previous studies have demonstrated an increase in the CD4/CD8 ratio and a decrease of CD8+ circulating T cells in the blood of atopic humans.5 A reduced ratio has also been reported in the lesional skin of atopic dogs.6 To the best of the authors' knowledge, no studies have been performed on blood of atopic dogs. It could be

speculated that atopic dogs may have an increased CD4/ CD8 ratio and an alteration in the relative numbers of Tregs.

Canine atopic dermatitis (CAD) requires long-term management.7,8 Allergen specific immunotherapy (ASIT) can assist with treatment but is not always effective.7 Medical therapy to control the clinical signs of pruritus associated with CAD is often indicated.8 Ciclosporin (CsA) is an effective drug for the symptomatic therapy of CAD and is widely used.7,8 CsA has selective immune modulatory actions that reduce the proliferation of cytotoxic T lymphocytes, modulate production of antibodies from T- helper dependent B lymphocytes, and inhibit the activation of mononuclear phagocytes and of T-helper lymphocytes.7

Studies of the influence of CsA therapy on the CD4/ CD8 ratio and percentage of Tregs in human patients affected by atopic dermatitis have shown contrasting results.9–11 One study reported a decrease in the Treg percentage during CsA therapy,9 whereas others report an increase in this population.10,11 It is important to note that activated CD4+ non-Treg cells can express FOXP3

An in vitro study performed on canine blood cells showed a reduction in CD25 positivity in cells grown with CsA in comparison to a control group. However, CD25 expression alone is not unique to the Treg population; this receptor is also expressed in other classes of lymphocytes after activation.12 In dogs affected by keratoconjunctivitis sicca treated with a topical solution of 2% CsA, the number of circulating CD8 lymphocytes was lower on day 30 resulting in an inversion of the CD4/CD8 ratio at the end of the treatment.13 With respect to CAD, a preliminary study on the effects of CsA treatment on CD4/CD8 ratio in atopic dogs failed to show a statistically significant difference, but the small number of subjects included (eight dogs) may have influenced the outcome.14

We hypothesized that CsA therapy may normalize the CD4/CD8 ratio and Treg percentage in atopic dogs. The objectives of this study were: (i) to evaluate the CD4/CD8 ratio and the percentage of Treg in healthy dogs, in English bulldogs predisposed to CAD, and in dogs affected by atopic dermatitis before and during treatment with CsA, using flow cytometry; (ii) to compare pruritus and skin lesion improvement [as measured by visual analog scale (VAS)15 and CAD lesion index (CADLI),16 respectively] during therapy with CsA and the fluctuations of CD4/CD8/ Treg values during treatment; and (iii) to evaluate whether the Treg percentage could be used to monitor immunological response in atopic dogs undergoing CsA therapy.

Materials and methods

This study was designed as a prospective open controlled study. Sampling was performed with the owners' consent and according to good clinical practice (see web link).

Animals and inclusion criteria

Atopic dogs (Group A)

Ten dogs (Table 1), at least 18 months of age, with a history of at least 1 year duration of chronic, nonseasonal atopic dermatitis (AD) were selected. Dogs were included if they fulfilled the following inclusion criteria: a clinical diagnosis of nonseasonal AD according to selected criteria8 and exclusion of other allergic dermatoses based on absence of response to treatment for flea infestation (at least 8 weeks) and to an elimination diet with novel or hydrolyzed protein (at least 6 weeks); a CADLI score of at least 23 (corresponding to moderate AD);16 absence of active bacterial or yeast infections; and written informed consent by the owner. If indicated, based on clinical and cytological evidence of a microbial infection, antimicrobial treatment was administered prior to inclusion in the study (Table 2). Drug withdrawal periods before inclusion were as follows: all systemic and topical immune modulating or immunosuppressive drugs 6 months; antihistamines 14 days. Allergen specific immunotherapy was permitted if used for >12 months, the dose remained unchanged for 6 months, the clinical signs were stable and the regimen was maintained during the trial; essential fatty acids and topical therapies were permitted if in use for >8 weeks, the clinical signs were stable and the dosing regimen was maintained during the trial. Pregnant and lactating animals, or with any concurrent disease, as well as those with concomitant adverse food reaction or flea bite hypersensitivity were excluded from the study.

Control groups

Ten healthy English bulldogs, (Group B) and ten healthy dogs (Group C), without history and/or clinical signs related to CAD were selected as the control population (Table 1). The English bulldog was selected as a breed strongly predisposed to developing atopic dermatitis.16

During the study all dogs (groups A, B and C) underwent regular ecto- and endoparasite prevention and were fed a complete diet.

Experimental design

Dogs in Group A received ciclosporin (Atoplus®, Novartis Animal Health spa; Origgio (VA), Italy), 5 mg/kg once daily for 90 days. The

recommendation was to give the capsule with a meal for the first 2 weeks to minimize any gastrointestinal adverse reaction, then administer the capsules 2 h before or after food as suggested by the manufacturer.

Clinical assessments were performed on days 0, 30 and 90 by the same investigator to minimize interobserver variability. Clinical lesions were scored using the CADLI;16 pruritus was assessed using a visual analog scale (VAS).15 Briefly, owners were asked to mark the scale with a short horizontal line according to their perception of their dog's pruritus over the preceding 24 h. The pruritus score was the distance in millimetres from the bottom edge of the scale to their mark.15

Dogs were withdrawn if they required treatment with a prohibited medication (Group A), if they experienced unacceptable discomfort or when there was poor compliance. Owners of dogs with transient adverse effects (e.g. gastroenteric problems) were encouraged to continue with dosaging for a further 5 days; if the signs persisted, the dog was excluded from the study. Owners could withdraw their animals at any stage.

A peripheral blood sample was collected for haematology from all dogs on Day 0 (Group A, B and C). Additional samples were collected on daya 30 and 90 from the atopic dogs (Group A). All samples were delivered to the laboratory and analysed within 24 h. For each sample a complete blood count via an automated analyser (Sysmex XT- 2000iV, Sysmex; Kobe, Japan) was performed and the leucocyte differential was validated manually with a blood smear stained with May-Grunwald Giemsa.

A multicolour flow cytometric staining was performed as previously described including antibodies reacting with canine CD45, CD5, CD4, CD8 and CD21.17 All samples were acquired with a BD FACScalibur (Beckton Dickinson; San Jose, CA, USA) and analysed via specific software (CellQuest, Beckton Dickinson); 30,000 events were acquired for each sample. For analysis cells were gated in a morphological scattergram to exclude platelets and debris. At first an 8% ammonium chloride solution was used to lyse the erythrocytes in the samples. Thereafter, cells were resuspended in RPMI 164 medium containing 5% fetal bovine serum and 0.2% sodium azide.

Cell suspensions (50 lL), adjusted at 5 9 105 cells per tube, were incubated for 20 min at 4°C with one of the following combinations of antibodies: CD5-fitc/CD21-pe/CD45-apc or CD4-fitc/CD8-pe/CD45- apc. Finally, cells were washed twice and resuspended in phosphate- buffered saline (PBS) for the final acquisition. For each sample, the percentage of lymphoid CD4+ and CD8+ cells out of total CD45+ cells was recorded, and the CD4/CD8 ratio was calculated.

Staining for Treg cells was performed with an adaptation of a procedure using antibodies reacting with canine CD4, CD25 and FOXP3.1 Briefly, peripheral blood samples were incubated at 4°C with CD4-fitc and CD25-pe antibodies. After 20 min cells were washed and incubated for 10 min in a 1 : 4 v/v fixation/permeabilization solution (FOXP3/transcription factor staining buffer set, eBioscience; Hatfield, UK) at room temperature. Then, after two washings, cells were incubated with FOXP3-pe-cy5 antibody for 30 min at 4°C in the dark. After incubation, cells were washed twice and resuspended in PBS for the final acquisition. The percentage of Treg was calculated using a two-step procedure: first the percentage of CD4+ CD25+ cells in the lymphocyte gate was recorded (which included both Tregs and activated T-helper cells); then, the number of FOXP3+ cells within the population of CD4+ CD25+ lymphocytes was recorded (which included only Tregs).

Statistical analyses

Statistical evaluation was performed with a statistical software SPSS (v19, SPSS Inc.; Chicago, IL, USA). Once the normal distribution of data was evaluated via a Shapiro–Wilk test, the Kruskal–Wallis test was used to compare variables among the three groups. The Friedman test and Wilcoxon signed rank test were performed to compare VAS, CADLI and haematological variables at days 0, 30 and 90 among Group A dogs. Significance was set at $P \leq 0.05$.

Results

Thirty dogs entered the study. No dogs were withdrawn at any time point (Tables 1 and 2). Both pruritus and clinical lesion scores significantly improved in Group A dogs receiving CsA therapy at all time points

(Table 3). The Treg percentage decreased in Group A during CsA therapy between days 0 and 90; this was not statistically significant. No significant differences were noted in the CD4+/ CD8+ ratio between any group or time period (Table 4).

Discussion

The present study investigated the CD4/CD8 ratio and Treg percentages in dogs affected by CAD, and compared this with healthy dogs and English bulldogs (a CAD predisposed breed), and the influence of CsA therapy on these parameters. We found that the circulating CD4/ CD8 ratio was not statistically different between the different groups. Despite a previous study in humans demonstrating an increase in the CD4/CD8 ratio in the skin, as well as a decrease of CD8+ circulating T cells and resultant increase of the CD4/CD8 ratio,5 the results of this study were not entirely unexpected. Previous studies in cats have demonstrated an alteration in the normal cutaneous lymphocytic populations in allergic cases but a normal CD4/CD8 ratio in peripheral blood.18 It is speculated that the circulating and cutaneous subpopulations may have a different role in the pathogenesis of AD.

This study also found that the percentage of Tregs was higher in atopic dogs compared with all nonaffected dogs, a result that was not statistically significant. This contrasts with a previous finding that the percentage of Tregs (defined as CD4+ FOXP3+ lymphocytes) in the blood of dogs with CAD was not different from that of healthy dogs, but was significantly increased following a year of ASIT.19 However, studies in humans have shown that patients with AD have significantly increased numbers of peripheral blood Tregs20 and that the increase in Treg percentage positively correlated with the patients' clinical scoring.21

The Treg percentage appeared to decrease during CsA treatment in atopic dogs in this study, but this difference was not statistically significant. The only comparable canine study detected a reduction in CD25 positivity in blood cells grown in vitro with CsA, although this study may not have specifically identified Tregs.12 The influence of CsA therapy on Tregs in human patients affected by AD has been evaluated in many studies with conflicting results.9–11 We conclude that evaluating Tregs is probably not a useful tool to monitor the immunological response in atopic dogs undergoing CsA therapy. Furthermore, we conclude that there were no statistically significant differences in the lymphocytic subpopulations in atopic dogs after 90 days of CsA therapy. Based on these results, CsA does not appear to affect circulating lymphocyte subpopulations in treated dogs, in spite of an improvement in clinical condition.

The main limitations of this study were the small number of dogs enrolled (which may have influenced correct evaluation of lymphocytic subpopulations and their role in CAD) and that patients were at different stages of the disease and therefore a different duration of immunotherapy may have had differences in lymphocytic subpopulations. Furthermore, the atopic and control populations were only partially age and sex matched.

Further studies utilizing larger numbers of dogs are indicated to further elucidate the role of circulating lymphocyte subpopulations in dogs with CAD.

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Table 1. Summary of case details for each study group

	Breed	Age (yr)	Gender
Group A (n = 10)	German shepherd dog	6	M
	German shepherd dog	4	M
	West Highland white terrier	3	M
	Mixed breed	5	F
	German shepherd dog	5	M
	Mixed breed	4	F
	Labrador retriever	3	F
	American Staffordshire bull terrier	3	F
	Pinscher	2	F
	Beagle	4	M
Group B (n = 10)	English bulldog	0–5	5 M and 5 F
Group C (n = 10)	Pointer	5	M
	English setter	4	M
	German shepherd dog	4	M
	German shepherd dog	3	M
	Labrador retriever mix breed	5	M
	Pit-bull mix breed	5	F
	Pit-bull mix breed	3	F
	Beagle mix breed	5	F
	Shih tzu mix breed	5	F
	Shih tzu mix breed	4	F

M, male; F, female.

Table 2. Antimicrobials used in dogs in Group A, prior to study entry

Malassezia dermatitis	Pyoderma	Bacterial overgrowth	Preventive antiparasitic drugs
Itraconazole p.o. 5 mg/kg once daily	Cefalexin p.o. 30 mg/kg twice daily	Salicylic acid 2% shampoo weekly	Moxidectin spot on monthly†
Miconazole 2% solution weekly	Cefovecin s.c. 8 mg/kg once every 15 days	Chlorhexidine 3% shampoo weekly	Selamectin spot on monthly†
Chlorhexidine 0.05% solution weekly	Clindamycin 11 mg/kg twice daily	Chloroxylenol 2% shampoo weekly	Fipronil+S methoprene monthly†

p.o., per os; s.c., subcutaneous

*Based on clinical and cytological evidence of a microbial infection, 30 days of antimicrobial treatment were administered before inclusion in the study.

†Given at the manufacturer's recommended dose rate.

Table 3. Pruritus visual analog score (VAS)15 and canine atopic dermatitis lesion index (CADLI)16 values from dogs in Group A during treatment with ciclosporin

	Mean ± standard deviation	Median	Range
VAS Day 0	7.2 ± 2[*,†]	8	4–9
VAS Day 30	4.3 ± 1.4[*,‡]	4	2–7
VAS Day 90	2.2 ± 2[†,‡]	2	0–5
CADLI Day 0	96.9 ± 32.6[§,¶]	90	67–150
CADLI Day 30	60.0 ± 33.1[¶,**]	60	10–100
CADLI Day 90	42.6 ± 21.5[§,**]	40	15–75

*P = 0.003, †P = 0.003, ‡P = 0.005, §P = 0.002, ¶P = 0.003, **P = 0.005. Each symbol signifies a statistically significant difference between VAS or CADLI for each time point.

Table 4. T helper to cytotoxic T lymphocyte (CD4/CD8) ratio and regulatory T cell (Treg) percentages in dogs with atopic dermatitis (AD) undergoing treatment with ciclosporin (Group A), dogs predisposed to AD without disease (Group B) and control dogs (Group C)

	CD4/CD8 ratio Mean ± standard deviation (median; range)	Treg (%) Mean ± standard deviation (median; range)
Group A Day 0	1.98 ± 0.52 (2.02; 1.09–2.67)	48.35 ± 14.30 (48.22; 40.67–68.76)
Day 30	2.57 ± 1.96 (1.90; 0.85–6.12)	30.61 ± 14.07 (35.56; 12.03–47.05)
Day 90	2.26 ± 1.39 (1.99; 0.98–4.90)	32.74 ± 19.47 (22.64; 11.00–64.6)
Group B	2.19 ± 0.80 (2.25; 0.99–3.31)	35.22 ± 6.35 (35.31; 22.10–43.98)
Group C	2.16 ± 1.47 (1.52; 0.89–5.53)	33.15 ± 8.95 (32.3; 20.38–51.79)

No statistically significant differences were found between any group or time point.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Box-plots showing pruritus VAS15 values in 10 dogs with atopic dermatitis at inclusion (Day 0) and after 30 and 90 days of ciclosporin treatment. The line within the box represents the median of the recorded values; the top and the bottom of the box represent the 3rd and 1st quartiles, respectively; the ends of the whiskers represent the maximum and the minimum values, respectively. • = outlier.

Figure S2. Box-plots showing CADLI16 values in 10 dogs with atopic dermatitis at inclusion (Day 0) and after 30 and 90 days of ciclosporin treatment. The line within the box represents the median of the recorded values; the top and the bottom of the box represent the 3rd and 1st quartile, respectively; the ends of the whiskers represent the maximum and the minimum values, respectively.

Figure S3. Box-plots showing CD4/CD8 ratio in 10 dogs with atopic dermatitis (Group A), 10 dogs from a predisposed breed (Group B) and 10 healthy dogs (Group C). The line within the box represents the median of the recorded values; the top and the bottom of the box represent the 3rd and 1st quartile, respectively; the ends of the whiskers represent the maximum and the minimum values, respectively. • = outlier.

Figure S4. Box-plots showing Treg percentage in 10 dogs with atopic dermatitis at inclusion (Day 0) and after 30 and 90 days of ciclosporin treatment. The line within the box represents the median of the recorded values; the top and the bottom of the box represent the 3rd and 1st quartile, respectively; the ends of the whiskers represent the maximum and the minimum values, respectively.