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Quantitative analysis of $CD4^+CD25^+FoxP3^+$ regulatory T-cells in canine atopic dermatitis in Korea

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Abstract: Recently, it was suggested that $CD4^+CD25^+FoxP3^+$ Tregs (Regulatory T-cells) exist in canine skin, although their numbers were not significantly different between healthy and atopic dogs. In this study, we investigated whether Treg frequencies correlate with the clinical features of canine atopic dermatitis (cAD). The goal of this study was to compare and analyse the numbers of the circulating Tregs in atopic and healthy dogs. In the peripheral blood mononuclear cells (PBMC) of healthy dogs, Tregs defined as $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood ranged from 0.3% to 1.5%. By contrast, in atopic dogs, the same population ranged from 0.7% to 8.8%. The percentage of $CD4^+CD25^+FoxP3^+$ Tregs in gated $CD4^+$ T-cells was significantly higher in the peripheral blood of dogs with atopic dermatitis (n = 9) than in the healthy controls (n = 8). The difference in the Treg levels ($CD4^+CD25^+FoxP3^+$) (P = 0.012) between the atopic and the healthy groups was statistically significant. The circulating T-cells (phenotype $CD4^+CD25^+FoxP3^+$ and $CD4^+FoxP3^+$) were increased significantly in the atopic dogs. The proportion of $CD4^+CD25^+FoxP3^+$ Tregs of the atopic dogs decreased with advancing age. These findings suggest that changes in the Tregs may mediate the pathogenesis of CAD.

Keywords: canine; atopy; $CD4^+CD25^+FoxP3^+$

Canine atopic dermatitis (cAD) is based on a common and chronic, genetic predisposition to a relapsing inflammatory skin disease, which results from the complex interaction between environmental and genetic factors (Navarro et al. 2015). The pathogenesis of cAD is not fully understood in veterinary medicine. Compared with the traditional dogma emphasising the importance of IgE-mediated early- and late-phase hypersensitivity to air-borne allergens, evidence increasingly suggests that defects in the epidermal barrier might also contribute to the disease pathogenesis (Hauck et al. 2016). According to the current theory of cAD pathogenesis, epidermal antigen-presenting cells capture allergens via the allergen-specific IgE, and migrate to areas of dermatitis and regional lymph nodes (Olivry et al. 2005). Microbes, self-trauma and neuromediators might also contribute to the persistent inflammation in the chronic skin lesions, resulting in a continuous cycle of chemokine release (Jassies-van der Lee et al. 2014).

Recently, it has been suggested that $CD4^+CD25^+FoxP^{3+}$ Tregs exist in canine skin, although the numbers were not significantly different between healthy and atopic dogs. Using a three-colour flow cytometric method, the percentage of $CD4^+CD25^+FoxP3^+$ Tregs were specifically compared and evaluated in the peripheral blood mononuclear cells (PBMCs)

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of atopic and healthy dogs (Jassies-van der Lee et al. 2014). It was hypothesised that a statistically significant difference in the Treg frequency exists between the atopic and the healthy groups, providing new insight into the potential role of Tregs in the cAD pathogenesis (Hauck et al. 2016).

The concept of T-regulatory (Treg) cell–mediated immune suppression has been extensively explored in allergic reactions (Ling et al. 2004). Tregs are a unique T-lymphocyte subgroup, accounting up to 10% of all the clusters of differentiation (CD) (Jassies-van der Lee et al. 2014). Tregs prevent excessive immune responses through their immunoregulatory properties, and therefore, play a leading role in maintaining immunological homeostasis. Thus, an abnormal number or the function of the Tregs has been implicated in the pathogenesis of allergic diseases (Ito et al. 2009). Few studies have attempted to quantify canine Tregs and only one of them evaluated Tregs in the peripheral blood of the cAD (Keppel et al. 2008).

In this study, we investigated whether Treg frequencies correlate with the clinical features of cAD. The goal of this study was to compare and analyse the numbers of circulating Tregs in the atopic and healthy dogs.

MATERIAL AND METHODS

Animals

Dogs with cAD confirmed cytologically by both the Veterinary Medical Teaching Hospitals of Chonbuk National University and Chonnam National University, were included in this study. The atopic dogs were diagnosed according to the criteria proposed by Favrot et al. (2010), as listed in Figure 1. In this study, the atopic dogs were diagnosed based on a combination of at least five out of eight criteria. The study included eight healthy beagle dogs and nine atopic client-owned dogs older than one year of age. The control samples were obtained from healthy beagle dogs without allergic skin diseases. The exclusion criteria for the healthy controls were vaccination within the previous month, treatment with immunomodulatory agents, and any type of dermatitis or disease known to affect the immune system.

Information records included age, breed, and sex. All the atopic dogs were affected for six months prior to recruitment and were selected based on the absence of medical treatment, clinical features and fulfilment of at least five of the eight criteria. Upon enrolment, the severity of the cAD was assessed using a previously validated scoring system (Canine Atopic Dermatitis Extent and Severity Index, version 3; CADESI-03). The CADESI-03 scale was used to evaluate four different lesions at 62 body sites with a severity scale varying from 0 to 5. The severity of each lesion was classified into 4 grades (none, 0; mild, 1; moderate, 2, 3; and severe, 4, 5) (Figure 2). The proposed intervals of 62 total scores were remission, 0–15; mild AD, 16–59; moderate AD, 60–119; and severe AD, ≥ 120.25. Immunomodulatory drugs were not administered to the subjects prior to sampling the blood. None of the subjects had received allergen-specific immunotherapy.

Blood sampling

Approximately 3 ml of whole blood was collected from each control or atopic dog by jugular venepuncture and transferred into EDTA BD vacutainer tubes (Becton Dickinson and Company,

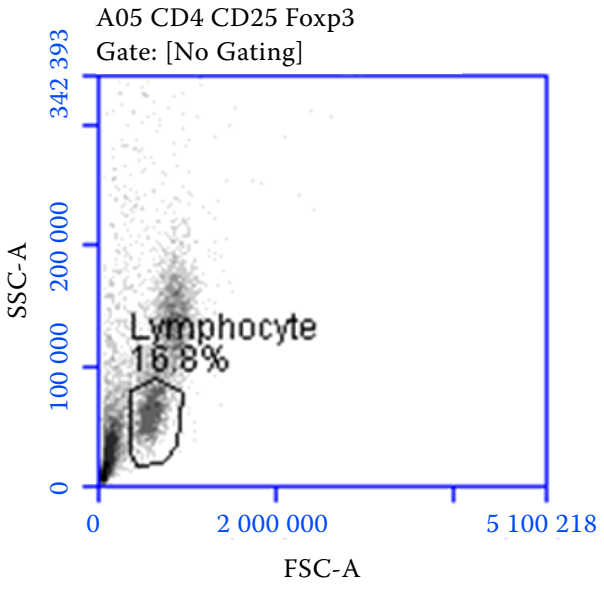

Figure 1. The peripheral blood mononuclear cells (PBMCs) of healthy and atopic dogs were stained with monoclonal antibodies against CD4, CD25 and intranuclear FoxP3. Initially, the lymphocyte population was gated based on the side scatter (SSC) and forward scatter (FSC) distribution (gate 1)

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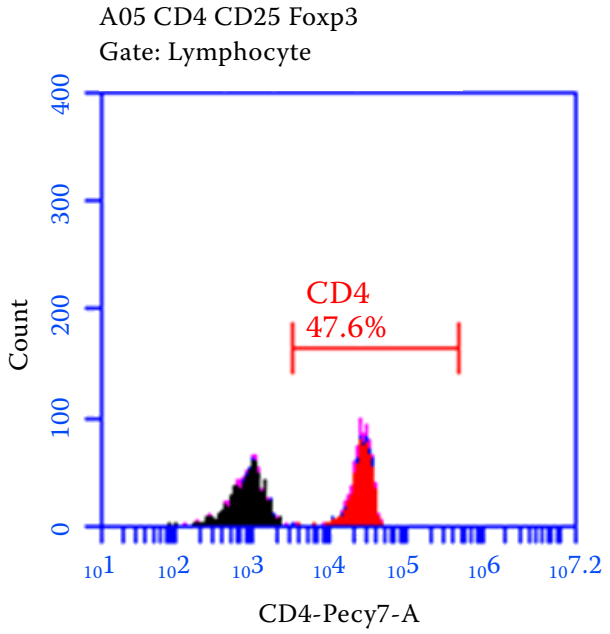

Figure 2. The CD4$^+$ cells were identified in a phycoerythrin (PE-Cyanine7) histogram (gate 2) and used in the subsequent analysis of the CD25 and FoxP3 expression (dot plot CD25 versus FoxP3). The representative dot plots from a healthy dog are shown

Franklin Lakes, NJ, USA). The blood samples were stored at room temperature (RT) and processed within 24 h after collection.

The PBMCs were isolated from the whole blood using a standard density gradient centrifugation by Histopaque-1 077 g/ml (Sigma-Aldrich, St. Louis, MO, USA) as described elsewhere. 12, 14 Anticoagulated blood was layered onto the Histopaque-1 077, followed by centrifugation (400 *g*) for 25 min at 4 °C. The layer of the PBMCs was collected from the interface, washed and re-suspended in PBS (Phosphate-Buffered Saline 1X; Welgene, Gyeongsangbuk-do, Republic of Korea).

Flow cytometric analysis of the Tregs

A typical Treg phenotype based on the simultaneous expression of CD4, CD25 and FoxP3 was analysed by flow cytometry according to protocols previously described (Biller et al. 2007; Pinheiro et al. 2011). In order to detect the cell surface (CD4, CD25) and the intranuclear (FoxP3) markers, freshly isolated PBMCs were stained with a combination of dog-specific or cross-reactive fluorochrome-conjugated monoclonal antibodies (mAbs) as follows: anti-canine CD4 phycoerythrin (PE-Cyanine7) (clone YKIX302.9; eBioscience, San Diego, CA, USA), anti-canine CD25 phycoerythrin (PE) (clone P4A10; eBioscience, San Diego, CA, USA) and cross-reactive anti-mouse/rat FoxP3 allophycocyanin (APC) (clone FJK-16s; eBioscience, San Diego, CA, USA).

Appropriate isotype controls were used for each experiment. FoxP3 staining was conducted following the manufacturer's instructions using the permeabilisation buffer and fixation/permeabilisation reagents provided (FoxP3 Staining Set; eBioscience, San Diego, CA, USA). In brief, the freshly isolated PBMCs were suspended in 100 µl of the FACS buffer (1% BSA in PBS) and incubated directly with 5 µl of both the anti-CD4 PE-Cyanine7 and anti-CD25 PE for 30 min at 4 °C in the dark. Following the surface staining, the cells were washed with PBS for 7 min at 4 °C (252 *g*). The pellet was then re-suspended in the FoxP3 fixation/permeabilization working solution and left at RT for 30 min in the dark. The cells were washed consecutively with the permeabilization buffer and probed for the intranuclear marker FoxP3 with the anti-FoxP3 APC at the concentration recommended by the manufacturer (0.5 µg per 105 to 108 cells), and allowed to interact for 30 min at RT in the dark. Subsequently, the stained cells were washed twice with the permeabilisation buffer and re-suspended in 400 µl of the FACS buffer for the flow cytometric analysis.

The flow cytometric analysis was performed using a BD Accuri C6 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) Flow Cytometer and interpreted using the corresponding software BD Accuri C6. In each sample, 10 000 events were analysed. The Tregs in the PBMCs were identified as T-cells triple-positive for CD4, CD25 and FoxP3. Initially, the fraction of the lymphocytes was selected according to the typical forward-and side-scattered properties and used for further analysis. The cells testing positive for CD4 were then gated on a PE-Cyanine7 histogram and analysed for CD25 and FoxP3. The number of Tregs was expressed as a percentage of the total CD4$^+$ T-cell population.

Data analysis

The Tregs were defined as CD4$^+$CD25$^+$FoxP3$^+$ cells for the purpose of this study. However, data involving the population of the CD4$^+$FoxP3$^+$ cells were also collected for comparison, be-

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cause the $CD4^+FoxP3^+$ phenotype has been used for the Treg characterisation in several other studies (Pinheiro et al. 2011; Gaspar et al. 2015). The correlation was determined between the two populations. The initial analyses compared the proportion of the Tregs in the atopic and healthy dogs, and correlated with the age. The correlations were also determined between the Treg proportion and the disease severity as assessed by CADESI-03.

Statistical methods

The statistical analyses were performed using the SALT 2.3 software (ISTECH Corp; Seoul, Republic of Korea). The data sets were compared using the nonparametric Mann-Whitney *U*-test for independent samples. The correlations between the quantitative variables were analysed using Pearson's *r*-test because a linear relationship between the parameters was assumed. The statistical significance level was defined as $P \leq 0.05$.

RESULTS

Animal data

The subjects included in this study were nine atopic and eight healthy dogs. The sex ratio of the atopic dogs was four females (44.4%) to five males (55.6%). The mean age was 9.22 years. Five neutered dogs were also included (55.6%). The sex ratio of the healthy dogs was 50% and the mean age was 1.5 years. The atopic and the healthy dogs used in this study were never treated. Figure 3 presents a detailed description and the clinical data for the atopic and the healthy dogs. Most subjects enrolled in the study demonstrated severe AD (CADESI-03 index ≥ 120: dogs) according to the CADESI-03 severity categories.

The proportion of the $CD4^+CD25^+FoxP3^+$ Tregs and $CD4^+FoxP3^+$ cells in the PBMCs

The proportion of the circulating Tregs was defined as a percentage of the total $CD4^+$ T-cell population. The gating strategy was utilised and the representative dot plots of the healthy and the atopic dogs are presented in Figures 1, 2, and 3.

Comparison of the $CD4^+CD25^+FoxP3^+$ Treg levels between the atopic and the healthy groups

In the PBMCs of the healthy dogs, the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood ranged from 0.3% to 1.5%. In the atopic dogs, the same population ranged from 0.7% to 8.8% (Figure 4).

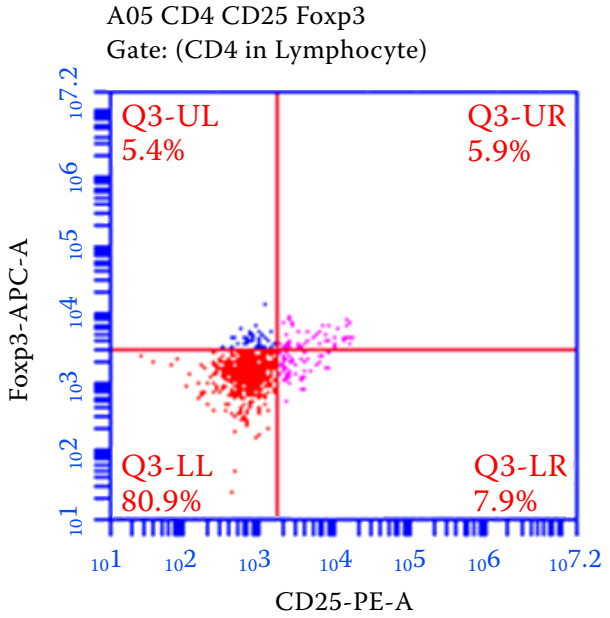

Figure 3. The numbers in the right upper quadrants represent the respective percentages of the $CD4^+CD25^+FoxP3^+$ Tregs in the gated $CD4^+$ T-cell populations

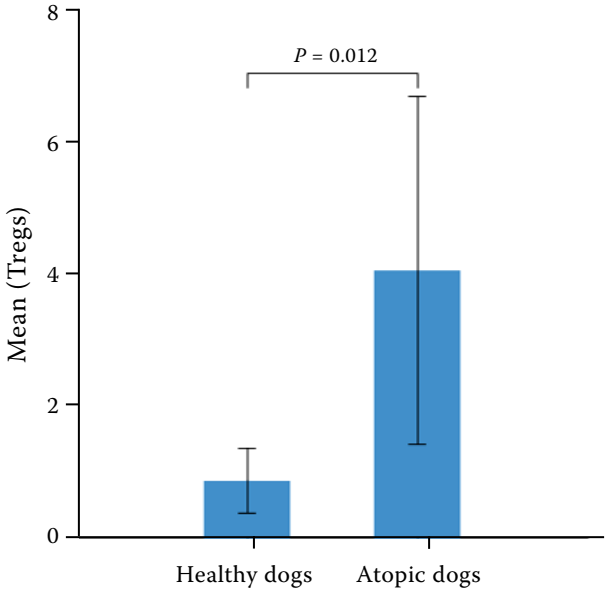

Figure 4. Comparison of the percentage of the Tregs between the atopic and healthy groups. The results are presented as plots with minimum and maximum values

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The mean Treg percentages in the atopic and the healthy groups were 4.2% and 0.912%, respectively (a mean difference of 3.288%). A non-parametric Mann-Whitney *U*-test was used to compare the population of Tregs between the atopic and the healthy groups. The percentage of $CD4^+CD25^+FoxP3^+$ Tregs in the gated $CD4^+$ T-cells was significantly higher in the peripheral blood of the dogs with atopic dermatitis (n = 9) than in the healthy controls (n = 8). The difference in the Treg ($CD4^+CD25^+FoxP3^+$) percentages (P = 0.012) (Figure 4) between the atopic and the healthy groups was statistically significant.

Comparison of the $CD4^+FoxP3^+$ cell proportions between the atopic and the healthy groups

In the PBMCs of the atopic dogs, the $CD4^+FoxP3^+$ cells in the peripheral blood ranged from 1.2% to 14%. In the healthy dogs, the same population ranged from 0.8% to 2.5%. The means of the $CD4^+FoxP3^+$ cells in the atopic (n = 9) and the healthy group (n = 8) was 10.056% and 1.762% (a mean difference of 8.293%), respectively (Figure 5). The mean difference was significantly greater in the dogs with CAD than in the healthy group (P = 0.012) (Figure 5).

Correlation of the Tregs with the clinical features of CAD

There was a negative correlation between the CAD severity (CADESI-03 scores) and the proportion of the $CD4^+CD25^+PoxP3^+$ Tregs (r = –0.757, P = 0.05) in the peripheral blood; however, no association with the $CD4^+PoxP3^+$ cells was found (Figure 6). All the atopic dogs showed a severe disease (index ≥ 120).

Correlation of the Treg proportions with the age

Within the atopic dogs, a negative correlation existed between the proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood and the age (r = –0.666, P = 0.05) (Figure 7). However, there was no statistical difference in the Treg proportions within the atopic group between the neutered dogs and the non-neutered dogs, and also no statistical difference was observed based on the sex. The atopic dogs exposed to immunomodulatory drug therapy were removed from the analysis.

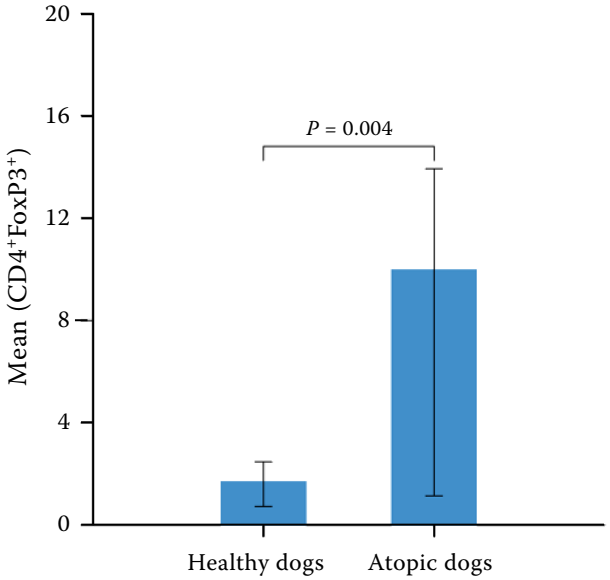

Figure 5. Comparison of the percentages of the $CD4^+FoxP3^+$ cells between the atopic and healthy dogs. The results are presented as plots with minimum and maximum values

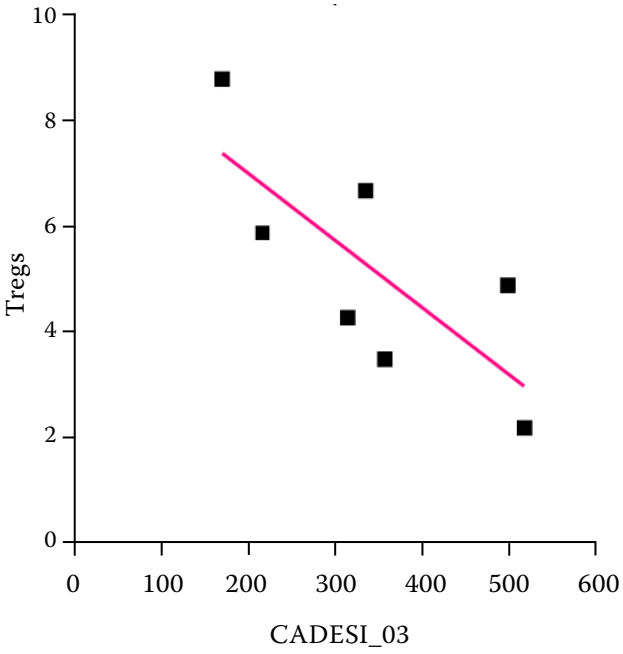

Figure 6. The correlation between the percentages of the canine regulatory T-cells (Tregs) in the atopic dogs and their disease severity (r = –0.757; P = 0.05)

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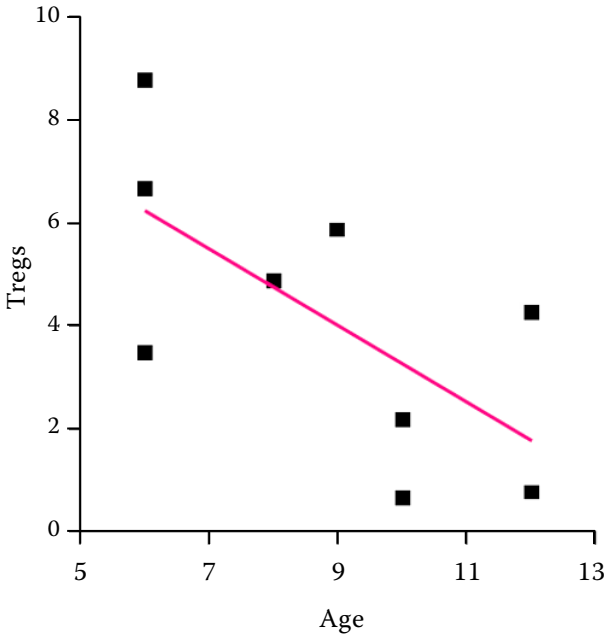

Figure 7. The correlation between the percentage of the canine regulatory T-cells (Tregs) in the atopic dogs and the age (r = –0.666; P = 0.05)

DISCUSSION

This study showed that the proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood of the dogs with cAD was significantly increased in comparison to the healthy dogs. Currently, the Tregs in dogs are generally defined by the expression of the markers CD4, CD25 and FoxP3 (Knueppel et al. 2011). Until recently, most of the previous studies of the circulating Tregs analysed the proportion of the $CD4^+FoxP3^+$ cells and the $CD4^+CD25^+$ cells in the peripheral blood of the healthy dogs. Only a few studies of the Tregs assessed the proportion of the $CD4^+CD25^+FoxP3^+$ cells in the peripheral blood of the healthy dogs (Figure 6).

Generally, the changes in the circulating Tregs of the atopic and healthy dogs at baseline can be explained in two ways. First, according to the conservative perspective, there is no difference in the circulating Tregs of the atopic and healthy dogs at the baseline. Second, according to the latest perspective, the proportion of the Tregs in the peripheral blood of the atopic dogs is increased compared with that of the healthy dogs. A recent study of atopic dogs was conducted based on the findings in humans suggesting changes in the number of circulating Tregs in AD patients compared with healthy individuals.

Hauck et. al. (2016) performed the first comparative analysis of the changes in the proportions of the $CD4^+CD25^+FoxP3^+$ Tregs and the $CD4^+FoxP3^+$ T-cells between healthy and atopic dogs. The analysis of the $CD4^+FoxP3^+$ T-cells was also performed to compare with a few previous studies. Hauck et al. (2016) compared the differences in the proportion of the $CD4^+CD25^+FoxP3^+$ Tregs between healthy and atopic dogs. The results demonstrated that the proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood of the atopic dogs (a mean of 2.1%) was significantly high compared with that of the healthy dogs (a mean 1 of %) (P = 0.002).

This study is one of the few analyses comparing the differences in the levels of the $CD4^+CD25^+FoxP3^+$ Tregs between healthy and atopic dogs. This investigation was conducted in order to examine the two perspectives about the Tregs in the peripheral blood of dogs. We found that the frequency of the population of the $CD4^+CD25^+FoxP3^+$ Tregs (a mean of 0.92%) in the healthy dogs was within the range reported in previous work studies (a mean of 0.7–1%) (Figure 2). Therefore, the results of this study involving healthy dogs are similar to those of the previous studies. However, the proportion (a mean of 4.2%) of the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood of the atopic dogs was highly increased when compared with the healthy dogs. A significant difference existed in the Treg proportions between the healthy and the atopic dogs (P = 0.012). These results are similar to those of Hauck et al. (2016).

Thus, the proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood was increased when compared with the healthy dogs. The finding suggests the need to strengthen the suppression of the immune system and promote the prevention of the pathological self-reactivity in the atopic dermatitis as in humans. Previous studies investigated the $CD4^+FoxP3^+$ T-cells in the peripheral blood of healthy dogs. The proportion (a mean of 1.76%) of the $CD4^+FoxP3^+$ T-cells in the peripheral blood of the healthy dogs detected in this study was lower than in the previous research (a mean of 1.9–4.84%). The proportion of the $CD4^+FoxP3^+$ T-cells in the peripheral blood of the atopic dogs in this experiment (a mean of 10.06%) was similar to Hauck et al.'s study (9.2%). There was a significant difference in the proportion of the $CD4^+FoxP3^+$ Tregs between the healthy and the atopic dogs (P = 0.004). Hauck et al. (2016) reported the differences in the

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$CD4^+FoxP3^+$ T-cell levels in the peripheral blood of healthy and atopic dogs. This study belongs to the few that have detected such differences.

In the case of the atopic group, approximately 28.6% of the $CD4^+FoxP3^+$ T-cells were also $CD25^+$, compared with 32.2% in the healthy group, which is similar to a previous study (Pinheiro et al. 2011). The difference between the two populations suggests that triple staining may be used to define the Tregs more precisely. Using triple staining, the $CD25^-CD4^+FoxP3^+$ population was observed, which may constitute the activated nonregulatory T-cells because both CD25 and FoxP3 are expressed in naive $CD4^+$ T-cells upon activation. However, they may represent the Tregs in an "inactive" state. It has been demonstrated that the CD25 expression on the Tregs is fully labile, and therefore, the $CD4^+CD25^-FoxP3^+$ cells may act as a peripheral reservoir of "active" $CD4^+CD25^+FoxP3^+$ Tregs upon immune activation. In addition, this study was performed to determine the correlation between the proportion of the Tregs and the disease severity.

We found that there was a statistically significant negative correlation between the proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the periphery blood and disease severity. This result was in contrast to the positive correlation demonstrated by Hauck et al. (2016) in atopic dogs. Our study was different from the above study in that the atopic dogs had only one severe category score (CADESI-03, $n = 7$), whereas in their study, the atopic dogs had several category scores (CADESI-04, remission $n = 9$, mild $n = 19$, moderate $n = 3$, and severe $n = 4$). The negative correlation may also be attributed to the differences between the dogs included in a single category of severity and those with various category scores. However, the proportion of the Tregs within the atopic dogs carrying the severe disease may show a negative correlation with the disease severity, suggesting that high levels of the Tregs may improve the disease severity.

In human studies, it was reported that the Treg numbers decline with the age and result in lifelong changes in the Treg cell subsets (Takahata et al. 2004). In a few studies mainly evaluating adult patients with AD, the circulating Tregs were increased in number compared with those of the healthy controls (Lesiak et al. 2012). This study demonstrated a significant negative correlation between the number of Tregs in the atopic dogs and the age. The proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the atopic dogs decreased with the increased age as in humans, whereas that of the $CD4^+FoxP3^+$ T-cells showed no significant association with the age. There was no significant correlation between the proportion of the $CD4^+FoxP3^+$ cells and the disease severity.

This study is a basic research effort analysing the proportion of the Tregs in the peripheral blood of atopic dogs. The study findings are as follows: The circulating T-cells (phenotypes $CD4^+CD25^+FoxP3^+$ and $CD4^+FoxP3^+$) were significantly increased in the atopic dogs. The proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the atopic dogs decreased with an increase in age. Compared with Hauck et al. (2016), the disease severity showed a negative correlation with the proportion of the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral blood. Additional studies are needed to further investigate this association. These findings suggest that changes in the Tregs mediate the pathogenesis of the cAD.

Conflict of interest

The authors declare no conflict of interest.

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