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Changes in leukocyte counts, lymphocyte subpopulations and the mRNA expression of selected cytokines in the peripheral blood of dogs with atopic dermatitis

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ABSTRACT: Canine atopic dermatitis (CAD) is a chronic allergic disease characterised by genetic predisposition. The exact pathogenesis of the disease is still not fully understood. Blood parameters, lymphocyte subpopulations, cytokines and other molecules involved in pro- and anti-inflammatory activities, and Th1/Th2 polarisation in peripheral blood were assessed in clinically healthy dogs and dogs with AD by haematological examination, flow cytometry and qRT-PCR. The group of diseased dogs was further divided into subgroups according to the severity of clinical signs expressed as CADESI-03 (canine atopic dermatitis extent and severity index) values. The group of dogs with CAD showed mild neutrophilia without general leucocytosis, reduced numbers of B-lymphocytes ($P < 0.05$), increased relative numbers of T-lymphocytes ($P < 0.01$) decreased absolute numbers of double-positive lymphocytes ($P < 0.001$) and NK cells ($P < 0.05$) as compared with healthy controls. The expression levels of IL-10 mRNA were significantly higher ($P < 0.05$) in the group with CAD. Correlation with CADESI-03 was observed in expression levels of CCL3 ($P < 0.05$), CCL5 ($P < 0.05$) and SOCS-3 mRNAs ($P < 0.05$). In conclusion, the disease stage investigated in the present study is characterised by a chronic inflammatory response.

Keywords: chronic inflammation; B-lymphocytes; double-positive lymphocytes; stress

Canine atopic dermatitis (CAD) is a common chronic skin disease which appears to share many similarities with AD in humans (Marsella and Olivry 2003). CAD is a complex multifactorial disease which is often considered as Type I hypersensitivity. Immune dysregulation, inadequate immune response to a given allergen, epidermal barrier defects, microbial colonisation of the skin surface and environmental factors all play a role in disease pathogenesis (Scott et al. 2001).

The acute phase allergic response is characterised by Th2 lymphocytes, eosinophils and the pro-inflammatory cytokines IL-4, IL-5 and IL-13 (Thepen et al. 1996; Lee et al. 2000), whereas the chronic phase of AD is characterised by a predominance of Th1 lymphocytes, macrophages and IL-2, IL-12, IFN-γ and IL-18 cytokines (de Vries et al. 1998). In human medicine, a recent popular hypothesis has posited that the resulting type of immune response in AD depends on the ratio of IL-10- to IFN-γ- or IL-4-secreting cells (Akdis et al. 2004). A certain dysfunction in regulatory T-lymphocytes and a so-called break in tolerance to external allergens have been demonstrated in dogs with AD (Nuttall et al. 2002; Maeda et al. 2007).

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Besides lymphocytes, many other inflammatory cells play important roles in the pathogenesis of CAD: mast cells, eosinophils, neutrophils, lymphocytes, antigen-presenting dendritic cells and monocytes (Hill and Olivry 2001). However, the function of innate immune cells (NK cells and γδT-lymphocytes) and memory cells, e.g. double-positive T-lymphocytes (CD4+CD8+) is still not fully understood in connection with AD. In human medicine, a role for NK-cells and γδT-lymphocytes has been acknowledged. These cells may not only be part of the natural defence of the body, but can also affect immune response polarisation via their cytokines (TNF-α and IFN-γ). Vice versa, their function can also be influenced by Th2 cytokine responses (Kos 1998; Lahn et al. 1998; Katsuta et al. 2006).

These inflammatory cells, in conjunction with lymphocytes, are attracted towards the site of the immune response by chemokines (Homey et al. 2006) which are mostly produced by leukocytes, dendritic and dermal endothelial cells (Zlotnik and Yoshie 2000; Kaburagi et al. 2001). Besides cytokines and chemokines, the activity of cells involved in allergic responses is controlled by transcription factors that influence cytokine production at the nuclear level (Zheng et al. 1997), while suppressors of cytokine signalling (SOCS) negatively regulate cytokine signal transduction between receptors and intracellular signalling molecules (Krebs and Hilton 2000).

The aim of this study was to assess the changes not only in the previously described populations and subpopulations of peripheral blood cells, but also in the less abundant and less well studied groups of cells, in particular NK cells (CD3–CD8+), γδT-lymphocytes, double-positive (CD4+CD8+) T-lymphocytes and selected cytokines and other regulatory molecules. A group of diseased dogs was evaluated by comparison with a healthy control group and in relationship to disease severity expressed as CADESI-03 values (Olivry et al. 2007).

MATERIAL AND METHODS

Design and animals. A total of 41 dogs, patients of the Small Animal Clinic of Faculty of Veterinary Medicine, University of Veterinary and Pharmaucetical Sciences, Brno, were included in the present study with the consent of their owners. In 21 of them, CAD was previously diagnosed using the diagnostic criteria according to Favrot et al. (2010) and food allergy was excluded, using an elimination diet for eight weeks. The dogs underwent anti-parasitic treatment (selamectin, three administrations at 14-day intervals). Secondary bacterial and yeast infections were excluded in the patients and symptomatic treatment of CAD was initiated. Dogs with AD were included in the study at various stages of the disease, and glucocorticoid and antihistaminic treatments were stopped for at least eight weeks before sampling. CADESI-03 was determined in all AD dogs. For haematological and immunological examinations, 3 ml of full anticoagulated blood were used. The blood was collected from the jugular vein into tubes containing lithium heparin.

The group of dogs with AD (CAD) was divided on the basis of CADESI-03 values (Olivry et al. 2008) into three subgroups: (1) values greater than 120 (severe – SEV), (2) values of 60–120 (moderate – MOD), and (3) values of 15–59 (MILD). No animals were included in the remission stage subgroup (values lower than 15). The group of dogs with AD consisted of six non-neutered dogs, 13 intact and two sterilised bitches. A total of 11 breeds at the average age of 4.9 years (one to nine years) were included in this group.

The control group (CON) comprised 20 healthy dogs older than five years. Furthermore, these dogs were never treated for any other skin condition nor did they receive systemic or local glucocorticoid treatment. The control group comprised six non-castrated and two castrated dogs, five intact and seven sterilised bitches. A total of 11 breeds at the average age of 6.57 years (five to 12 years) were included in this group.

Haematology and flow cytometric assay. Haematological variables were measured using the BC-2800 Vet Haematology Analyser (Mindray, China). Counts of leucocyte subpopulations were performed on blood smears stained according to the Pappenheim staining method by enumeration of at least 200 cells.

Table 1. Primary antibodies

Name	Clone	Isotype	Manufacturer
CD3	CA17.2A12	IgG1	P.F.Moore, CA, USA
CD4	CA13.1E4	IgG1	P.F.Moore, CA, USA
CD8α	CA9.JD3	IgG2a	P.F.Moore, CA, USA
TCRγδ	CA20.8H1	IgG2a	P.F.Moore, CA, USA
CD21	CA2.1D6	IgG1	P.F.Moore, CA, USA
CD45	CA12.10C12	IgG1	P.F.Moore, CA, USA

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Table 2. Secondary antibodies

Name	Clone	Manufacturer
Goat Anti-Mouse Alexa Fluor 488 IgG1	A21121	Life Technologies
Goat Anti-Mouse Alexa Fluor 647 IgG2a	A21241	Life Technologies

Flow cytometric analysis of lymphocyte subpopulations was performed using a two-colour immunofluorescence method. Whole blood was washed with haemolytic buffer (8.26 g NH_4Cl, 1 g $KHCO_3$, and 0.037 g EDTA/l of distilled water) and incubated with primary antibodies (in combinations of CD3+/CD8+, CD4+/CD8+, CD21+/TCRγδ+ and CD45+ alone) (Table1). In order to prevent non-specific binding of secondary antibodies, 20 ml of goat serum was added. After rinsing with washing solution (PBS with 1.84 g/l EDTA, 1 g/l sodium azide and 4 g/l gelatine) a reaction with isotype-specific secondary antibodies labelled with two types of fluorochromes followed (Table 2). After repeated rinsing with washing solution and the addition of propidium iodide, dead cells were excluded from the measurement. Samples were measured on a flow cytometer BD LSR Fortessa and evaluated with BD FACS Diva™ software (both Becton Dickinson, CA, USA). The subsets were assessed according to our previous study (Faldyna et al. 2001).

RNA isolation and cDNA synthesis. Mononuclear cells were isolated from whole blood by gradient centrifugation using Histopaque 1077 (Sigma). In order to monitor the ability of leukocytes to respond to stimulation, 200 µl of cell suspension (2.5×10^6/ml) were stimulated in a microplate (PTT, Switzerland) with a 20 µl mixture of 0.1 µg/ml phorbol myristate acetate (PMA, Sigma) and ionomycin at a concentration of 0.4 µg/ml (Sigma). The remaining portion of cell suspension was left unstimulated. Incubation was carried out for 4 h in RPMI-1640 supplemented with 10% FBS (Gibco) at 37 °C and 5% CO_2.

Total RNA was isolated using TriReagent-RT (MRC, Cincinnati, OH, US) and then purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The amount of isolated RNA was measured using a NanoDrop 2000 (LabTech). The isolated RNA was transcribed into cDNA using M-MLV (Invitrogen, Carlsbad, US-CA) and oligo d(T).

Quantitative RT-PCR. For each reaction, 3.0 pmol of primer (Table 3) and 1.5 µl of QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) were added to 0.5 µl of cDNA in a 384-well plate. The PCR parameters were the following: initial denaturation at 95 °C/15 min, followed by 45 cycles of denaturation at 95 °C/15 s, annealing at 58 °C/30 s, extension at 72 °C/30 s, and finally melting analysis at 60–95 °C and cooling at 40 °C. The reaction was performed in a Light Cycler® 480 II (Roche, Switzerland).

HPRT was chosen as a reference (housekeeping) gene (Schlotter et al. 2009). The relative expression of each gene was normalised to expression levels of HPRT (Zelnickova et al. 2008). The resulting change in expression was calculated as a ratio of gene expression in a stimulated versus unstimulated sample, designated as the stimulation index.

Statistical analysis. The obtained data were evaluated by the unpaired Mann-Whitney *U* test using Prism (GraphPad Software). Stepwise linear regression, where the value of CADESI-03 was

Table 3. Primers used for qRT-PCR

Primer	Forward primer 5'→3'	Reverse primer 5'→3'
HPRT	CGAAGTGTTGGCTATAAACCTGACTTTGTTGG	TCAAGGGCATATCCTACAACAAACTTGTCTGGA
IFN-γ	TTGCGTGATTTTGTGTTCTTCTGGCTGT	ACGAAAAGAGACCCACCGTCCGATACA
IL-1β	CCCTGGAAATGTGAAGTGCTGCTGCC	TGCAACTGGATGCCCTCATCTACCAG
TNF-α	TTCTCCTTCCTCCTCGTCGCAGGG	TGGGGGCCGATCACTCCAAAGTG
TGF-β	CAGATCCTTGCGGAAGTCAATGT	ACACCAACTACTGCTTCAGCTCC
IL-10	TGCATGGCTCAGCACTGCTCTGTTG	AGTGGGTGCAGTCGTCCTCAAGTAGG
CCL3	TGTTGCCCAGCACCATGGAGGTC	CAGCACCAAAAGTGGAAGAGCAGGTC
CCL4	GCGCTCTCAGCACCAATGGGTTCAG	TACCACAGCTGGCTGGGAGCAGA
CCL5	GCCTCTGCCTCCCCATATGCCTCAG	GACGACTGCTGGCATGGAGCACT
CXCL10	ACCTCTCTCTAGAACTATACGCTGTACCTGTATCA	TGTGGCAATGATCTCAACATGTGGACACG
IL-8	CCAAGCTGGCTGTTGCTCTCTTGGC	CAGCTTCACAGAGAGCTGCAGAAAGGACA
SOCS3	AGAAGATCCCTCTGGTGTTGAGC	GTCTTCCGACAGAGATGCTGGAG

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given as the dependent variable, was calculated using SAS version 9.4 (Windows).

RESULTS

Blood cell populations

When compared with the healthy control group, the total number of leukocytes in AD patients was not changed. However, the percentage of neutrophils in the group of patients (70.33 ± 5.74%) was significantly increased ($P < 0.05$) at the expense of lymphocytes, the percentage (25.88 ± 5.67%) of which was significantly decreased ($P < 0.05$) compared with that of healthy controls (62.73 ± 11.6% resp. 32.55 ± 11.47%). The absolute lymphocyte count was directly proportional to CADESI-03 ($P < 0.05$) values. Differences in other blood cell populations between the groups of healthy and diseased dogs were not significant, except for the absolute monocyte count, which was significantly increased in the subgroup with a mild form of the disease (MILD) compared to the most severely affected subgroup of patients (SEV) ($P < 0.05$).

CD3+ (T-lymphocytes) versus CD21+ (B-lymphocytes)

The drop in the total absolute lymphocyte count in the group of dogs with AD in comparison with the healthy control group was mainly due to a significant decrease in B-lymphocytes ($P < 0.05$). We observed a positive correlation with the severity of CAD ($P < 0.05$). The percentage of T-lymphocytes was significantly increased ($P < 0.01$) and the percentage of B-lymphocytes was reduced ($P < 0.05$) in the group of patients as compared with the healthy controls. The proportion of T-lymphocytes to B-lymphocytes was significantly increased in the group of dogs with AD ($P < 0.05$) (Figure 1).

Subpopulations of T-lymphocytes

In dogs with CAD, the absolute counts of CD4+ (CD3+CD8–) T-cells ($P < 0.1$), CD8+ (CD3+CD4–) T-cells ($P < 0.05$) and γδT-lymphocytes ($P < 0.01$) were positively correlated with the severity of the disease. The absolute count of double-positive

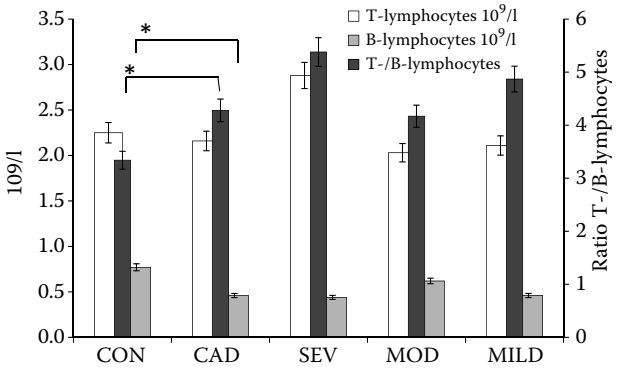

Figure 1. Absolute counts of T- and B-lymphocytes and their ratio. Absolute counts of T- and B-lymphocytes and their ratio in the control group of healthy dogs (CON), in all groups of dogs with AD (CAD) and in subgroups with severe clinical signs (SEV), with moderate clinical signs (MOD) and mild clinical signs (MILD). Columns represent the median of values. Connecting lines show significant differences between groups as determined using the Mann-Whitney *U* nonparametric test; $^{*}P < 0.05$

T-lymphocytes (CD4+CD8+) was significantly reduced ($P < 0.001$) when compared to the healthy control group. However, due to the fact that double-positive T lymphocytes comprised only a small minority of the total population (1.6 ± 0.8% of lymphocytes in healthy animals), this did not influence the total T-lymphocyte count (Figure 2).

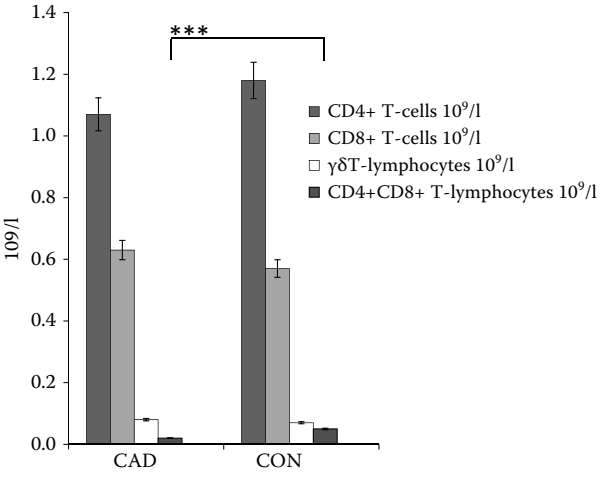

Figure 2. Subpopulations of T-lymphocytes. Subpopulations of T-lymphocytes in the control group of healthy dogs (CON) and group of dogs with AD (CAD). Columns represent the median of values. Connecting lines show significant differences between groups as determined using the Mann-Whitney *U* nonparametric test; $^{***}P < 0.005$

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CD3−CD8+ (NK-CELLS)

In the present study, CD3−CD8+ cells constituted a minor population of lymphocytes (1.7 ± 0.8% of the lymphocytes in healthy animals) but a significant decrease in both relative ($P < 0.001$) and absolute ($P < 0.05$) counts was observed in the group of dogs with AD (0.99 ± 0.65%) as compared to the healthy control group.

Cytokines and other regulatory molecules

Out of 12 cytokines and other regulatory molecules investigated in our study, significant changes were observed in mRNA expression values for IL-10, CCL3, CCL5 and SOCS-3.

The stimulation index for expression levels of IL-10 before and after stimulation was significantly higher in the group of dogs with AD as compared to the healthy control group ($P < 0.05$) (Figure 3), and an inverse correlation with CADESI-03 values ($P < 0.1$) was found. A negative correlation was also observed in the expression levels of mRNA for IL-10 after stimulation, without significant differences between the groups of healthy and diseased dogs ($P < 0.1$).

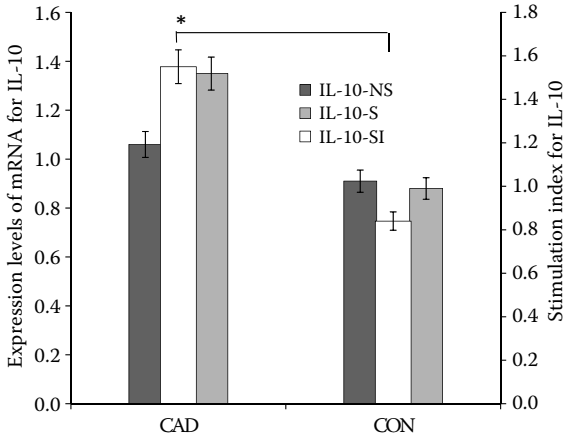

Figure 3. Relative expression levels of mRNA for IL-10 before and after stimulation and the stimulation index for IL-10. Relative expression levels of mRNA for IL-10 before (IL-10-NS) and after stimulation (IL-10-S) and stimulation index (IL-10-SI) in the control group of healthy dogs (CON) and group of dogs with AD (CAD). Columns represent the median of values. Connecting lines show significant differences between groups as determined using the Mann-Whitney *U* nonparametric test; $^{*}P < 0.05$

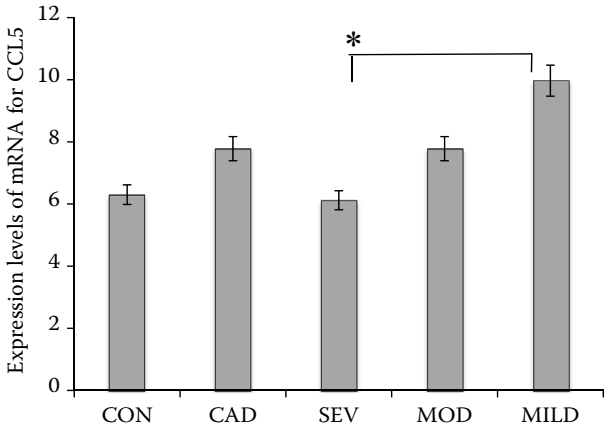

Figure 4. Relative expression levels of mRNA for CCL5 after stimulation in the control group of healthy dogs (CON), in all groups of dogs with AD (CAD) and in subgroups with severe clinical signs (SEV), with moderate clinical signs (MOD) and mild clinical signs (MILD). Columns represent the median of values. Connecting lines show significant differences between groups as determined using the Mann-Whitney *U* nonparametric test; $^{*}P < 0.05$

Expression levels of CCL3 mRNA before stimulation were directly proportional to disease severity ($P < 0.05$). In dogs with AD, CCL5 expression levels after stimulation showed a negative correlation with disease severity ($P < 0.05$). This inverse pro-

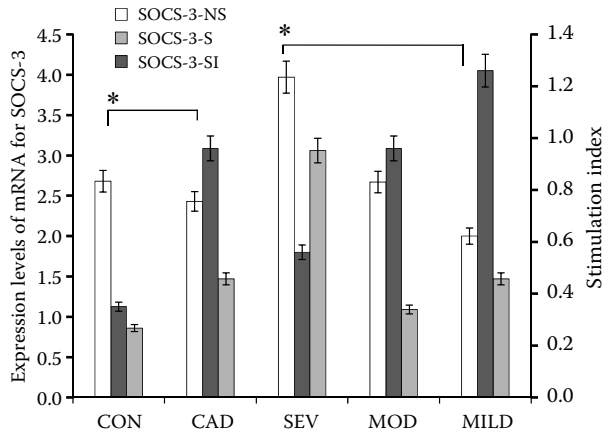

Figure 5. Relative expression levels of mRNA for SOCS-3 before (SOCS-3-NS) and after stimulation (SOCS-3-S) and stimulation index (SOCS-3-SI) in the control group of healthy dogs (CON), in all group of dogs with AD (CAD) and in subgroups with severe clinical signs (SEV), with moderate clinical signs (MOD) and mild clinical signs (MILD). Columns represent the median of values. Connecting lines show significant differences between groups as determined using the Mann-Whitney *U* nonparametric test; $^{*}P < 0.05$, $^{**}P < 0.01$

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portionality confirmed the significant decrease in the stimulated level in the subgroup of dogs with severe disease (SEV) compared to the subgroup with mild clinical signs (MILD) ($P < 0.05$) (Figure 4).

Expression levels of mRNA for SOCS-3 before stimulation were directly proportional to disease severity ($P < 0.05$), which confirmed a significant increase in expression levels in the subgroup with the highest CADESI-03 values (SEV) as compared to dogs with moderate disease ($P < 0.05$) (Figure 5). The stimulation index was significantly increased ($P < 0.01$) in the group of AD dogs as compared to the healthy control group (Figure 5).

DISCUSSION

The haematological results obtained in our study can be compared with other studies conducted thus far, which monitored changes in the peripheral blood of dogs affected with AD (Olivry et al. 1997; Griffin and DeBoer 2001; Jackson et al. 2002; Maeda et al. 2004; Maeda et al. 2007; Taszkun 2013). The reduction in the relative proportion of lymphocytes was likely a result of an increasing number of neutrophils in response to a potential secondary infection in patients with a severe form of the disease or in response to long-term exposure to stress. It was shown that stress, together with other factors such as the oestrous cycle and climate, plays a role in lowering the itch threshold and in worsening clinical manifestations without the development of secondary infection (Werfel and Kapp 1998).

The changes in the leukogram in the group in the group of dogs with CAD were characterised by a leukogram pattern typical of stress and a make-up typical of chronic inflammation (Duncan et al. 1994). The most significant changes were observed in neutrophils and lymphocytes as mentioned above. Characteristic changes for these two leukogram patterns varied, depending on whether a more important role was played by stress caused by long-term exposure to infectious pressure or by a chronic inflammatory response. When dividing groups according to CADESI-O3 values, it was evident that a gradual shift occurred from a leukogram pattern typical of a chronic inflammatory response in the subgroup with severe clinical manifestations, to a leukogram typical of stress caused by a long-term infectious pressure in the subgroup with a mild form of CAD. The conclusion that the leukogram in the subgroup of dogs with the highest CADESI-O3 values was mainly influenced by chronic inflammation and a mild form by stress was also supported by positive correlations between absolute lymphocyte counts and CADESI-O3 values. However, an unambiguous impact of stress on the course of CAD was not confirmed by the other stress indicators (concentration of cortisol etc.).

The relative increase in the number of T-lymphocytes in dogs diagnosed with AD was in accordance with the findings of other authors (Jackson et al. 2002; Taszkun 2013). The observation that the proportion of this subpopulation was increasing, while the absolute number remained at the same level, was due to the decline in other subpopulations, particularly B-lymphocytes. The direct proportion between the absolute number of T-lymphocytes and the CADESI-O3 values could, again, be explained due to chronic inflammation in patients with the severe form, where T-lymphocytes are one of the major cells involved in the response (Schlotter et al. 2011).

The number of B-lymphocytes in the peripheral blood of canine patients has been reported in some studies (Faldyna and Toman 1998; Jackson et al. 2002; Taszkun 2013). The changes reported by Jackson et al. (2002) were non-significant and Taszkun (2013) observed a higher number of these cells in dogs with AD complicated by purulent dermatitis. This decrease could be explained by the cell-mediated response in dogs with AD in our study. During the cell-mediated response, Th1-lymphocytes and macrophages predominate and this type of response has often been described in AD in association with chronic inflammation and the late-phase of an allergic response (Thepen et al. 1996; de Vries et al. 1998; Lee et al. 2000). Exposure to stress which causes more serious alterations to the percentage of B-lymphocytes than T-lymphocytes (Dhabhar et al. 1995) might play a role in the decrease of both the relative and absolute numbers of B-lymphocytes. In humans, sleep deprivation and lack of social communication have been most often described as stressful situations, whereas in dogs with AD, pruritus could play an important role. This can disrupt the regime of sleeping, feeding and also interaction with the owner (Werfel and Kapp 1998).

Subpopulations of CD4+ and CD8+ T-cells have been found to exist simultaneously in the skin and peripheral blood of patients with CAD (Olivry et

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al. 1997; Sinke et al. 1997; Maeda et al. 2004). The increase in CD4+ T-cells in dogs in association with CAD in peripheral blood has often been described (Sinke et al. 1997; Maeda et al. 2004), while the increase in the subpopulation of CD8+ T-cells was observed during secondary infections (Taszkun 2013). The relationship between CADESI-03 values and the absolute numbers of the given subpopulations could be connected with both stress and an inflammatory response in the subgroup of dogs with severe clinical signs of disease, whereas mainly stress seems to be the cause of their decline. An important role of stress in the decrease of both CD4+ and CD8+ T-cells has been previously described in both human medicine (Herbert and Cohen 1993) and animal models (Dhabhar et al. 1995). Stress may be involved not only in the development of clinical manifestations, but can also weaken the body's natural defence systems and thus facilitate the development of a secondary infection (Herbert and Cohen 1993). The CD4+ to CD8+ T-cell ratio is also important. A higher ratio in peripheral blood and skin with a predominance of CD4+ T-cells has been described in canine AD (Olivry et al. 1997; Sinke et al. 1997; Maeda et al. 2004), whereas in our study, the ratio was almost identical.

Due to the fact that the subpopulation of γδT-lymphocytes represents the transition between innate and adaptive immunity and is capable of phagocytosis and antigen presentation to other cells (Kabelitz 2011), the increase in γδT-lymphocytes in patients with severe clinical manifestations can be explained as a result of a secondary infection and the involvement of cell-mediated immunity in an allergic response. The relative increase in the number of γδT-lymphocytes observed in our study was in contradiction to the only study describing the occurrence of this subpopulation in peripheral blood in association with AD. In this study, conducted in human patients, this decrease occurred together with a decrease in NK-cells due to elevated apoptosis induced by monocytes (Katsuta et al. 2006). This difference could be explained by a different function of this subpopulation in dogs which could be controlled by other mechanisms. This subpopulation of cells deserves further study in order to reveal their exact functions.

The function of double-positive T-lymphocytes in dogs is still not completely clear, but they are assumed to be effector/memory cells responding to stimulation and which communicate via MHC-II molecules on their surface (Bismarck et al. 2012). Even though the number of double-positive T-lymphocytes in dogs is low (1.6 ± 0.8% out of all lymphocytes in healthy animals in our study), the dogs with AD had half of the above number. The decrease in double-positive T-lymphocytes could simply be due to the relative increase in other subpopulations of T-lymphocytes, whilst the decrease in absolute values could be a result of their migration to the site of the response. It remains unclear whether a chronic inflammatory response or stress predominates in patients with CAD and whether these factors could somehow affect the number, function or migration of double-positive cells.

Canine NK cells together with γδ and double-positive T-lymphocytes represent insufficiently investigated leukocyte subpopulations that are not only key players in innate immunity, but, via their cytokines (IFN-γ and TNF-α), can also affect the effector cells of acquired immunity (Akdis and Akdis 2003). Katsuta et al. (2006) described the decline of this subpopulation in parallel with a significant decrease in the expression levels of IFN-γ and TNF-α mRNA, which however, was not confirmed in our study. In human medicine, NK-cells in association with AD have been mentioned in several studies (Wehrmann et al. 1990; Bouloc et al. 2000; Katsuta et al. 2006). The percentage of NK cells did not significantly change in AD patients compared to healthy controls in the study of Bouloc et al. (2000). However, another two papers reported a significant decline in patients with AD (Wehrmann et al. 1990; Katsuta et al. 2006) which is in accordance with our study.

The role of IL-10 in AD is still not fully understood. Released from regulatory T-lymphocytes, IL-10 may serve as a suppressor of the immune response (Moore et al. 2001) and is characteristic of humoral immunity (Keppel et al. 2008; Machura et al. 2008). A reduction in its expression is associated with dysregulation of Th2-lymphocytes and the development of an allergic reaction (Maeda et al. 2007) whereas an increase is typical of humoral immunity (Keppel et al. 2008; Machura et al. 2008). Elevated levels of IL-10 mRNA expression in dogs with AD were also described by other authors (Schlotter et al. 2011; Taszkun 2013). The increase in the stimulation index for IL-10 mRNA expression levels before and after stimulation in the group of dogs with AD could be explained by the effect of humoral immunity in response to the

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stimulating agent. The inverse proportion of the stimulation indexes and IL-10 mRNA expression levels with CADESI-03 values detected in patients in our study support the theory that a Th2 immune response develops by way of a break in the natural immune tolerance attributed to Treg-lymphocyte dysfunction (Maeda et al. 2007).

Increased levels of the CCL3 chemokine in peripheral blood have only been described in humans suffering from AD (Kaburagi et al. 2001; Kakinuma et al. 2003). In our study, we did not confirm a significant increase in CCL3 mRNA expression in dogs with AD, but a direct correlation between the expression levels before stimulation and the severity of clinical signs was noted. Even though CCL3 is an important chemoattractant for B-lymphocytes (Taub et al. 1993), higher levels were found in subgroups which had a significantly lower percentage of B-lymphocytes. The given phenomenon could be explained by the fact that when CCL3 expression levels were increasing, migration of a greater number of B-lymphocytes towards the site of the inflammation might have occurred with a consequent reduction of their number in peripheral blood.

In veterinary medicine, CCL5 mRNA expression levels in peripheral blood have not yet been investigated in association with CAD. In humans suffering from AD, increased serum levels have been reported in peripheral blood (Gluck and Rogala 1999; Kaburagi et al. 2001), but they did not correlate with clinical scores (Gluck and Rogala 1999). In our study, we did not confirm these findings of increased levels and, moreover, the levels of expression after stimulation were inversely proportional to CADESI-03 values. CCL5 is one of the major chemoattractants for memory T-lymphocytes, monocytes and eosinophils (Rot et al. 1992), which could explain the significantly elevated absolute count of monocytes in the subgroup of patients with the mild clinical form of the disease. A correlation between the number of eosinophils and the levels of CCL5 mRNA was not confirmed in contrast to the results of a study of humans with AD by Kaburagi et al. (2001).

The transcription factor SOCS-3 is involved in establishing and maintaining Th2 immune responses (Krebs and Hilton 2000) and it acts to decrease the Th1-cell differentiation rate (Kashiwakura et al. 1999). Our study did not confirm the findings of Arakawa et al. (2004), who observed elevated expression of SOCS-3 in AD patients but did not find a correlation with the severity of clinical symptoms. However, a direct proportionality between unstimulated levels and clinical features was found, which corresponds to the Th2-dominated response in allergy.

This study has broadened our knowledge of the significance of cells, cytokines and other regulatory molecules with previously described functions in the pathogenesis of CAD and has suggested possible roles for the hitherto poorly characterised subsets (NK-cells, CD4+CD8+ and γδT-lymphocytes). Further, based on our results, we also suggest stress to be an important factor influencing the immune response. Some of the results obtained in our study can only be compared with studies in human medicine, and therefore, further study is required in order to draw clear conclusions on the importance of particular subpopulations and cytokines in canine atopic dermatitis.

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