Altered plasma cytokines in dogs with atopic dermatitis

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Background – Canine (*Canis lupus familiaris*) atopic dermatitis (AD) shares similar clinical signs to human AD. The abnormal immune response of AD is orchestrated by T lymphocytes, and may include variable involvement of cytokines, regulatory T (Treg) cells, eosinophils, mast cells and other immune components. Helper T (Th)2 cytokines often predominate initially, followed by Th1 cytokines in more chronic phases.

Hypothesis/Objectives – Pro-inflammatory and Treg cytokines have been shown to play a role in human AD, yet their importance is not clear in canine AD. Hence, this study aimed to measure the concentrations of cytokines/chemokines not traditionally associated with Th1/Th2 response.

Animals – Canine AD patients (n = 27), compared to control dogs (n = 11).

Methods and materials – A total of 19 plasma cytokines were assayed using canine specific multiplex immunoassays.

Results – The plasma concentrations of CXC Motif Chemokine Ligand 8 (CXCL8), interleukin (IL)-7 and IL-15 cytokines were elevated in canine AD patients, compared to control dogs. In addition, stem-cell factor (SCF) concentrations were reduced in the plasma of canine AD patients compared to control dogs. Distinct cytokine profiles were found in dogs belonging to the Staffordshire breeds, a group with increased risk of AD. In particular, granulocyte-macrophage colony-stimulating factor (GM-CSF) had significantly elevated concentrations.

Conclusions and clinical relevance – Some of the plasma cytokine alterations in canine AD described here, particularly of IL-7, have not been reported previously. Monitoring these distinctive cytokine alterations could be useful for diagnosis and monitoring of canine AD in dogs.

Introduction

Atopic diseases are hypersensitivity responses that include allergic asthma, allergic rhinitis and atopic dermatitis (AD). The pathogenesis of AD appears most complex, potentially involving variable contributions from skin barrier defects, environmental allergens and immune system dysregulation.

Attempting to better characterise and clarify the role of immune dysfunction, former research explored cytokine alterations in AD (briefly summarised below). In human AD, the focus was initially on the potential role of helper T (Th)1 and Th2 cytokines.[1,2] Elevated mRNA expression levels of interleukin (IL-4) and IL-5 in skin lesions,[3–5] and IL-13 in pCLA+ T cells[6] are documented in humans with AD. Correlation between elevated serum and plasma IL-31 and severity of AD, and between IL-31 levels in skin

lesions and concomitant pruritus is reported.[7,8] A model of disease pathogenesis arising from dysregulation of Th2 cytokines has been proposed (see review).[9] Subsequent analysis of a broader range of cytokines has led to a more refined model in which Th2 cytokine responses predominate in the initial phase of AD, followed by Th1 cytokines in the chronic phase, with elevated interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα) and IL-2, and reduced IL-12 associated with disease.[9,10] Some studies support suppression of regulatory T (Treg) function associated with the impaired skin barrier and AD, reflected by elevated IL-10.[11] Th17 and Th22 cytokines have more recently been implicated,[11] with initial Th17 (IL-17) secretion in the acute phase of AD, shifting to Th22 involvement (IL-22) in the chronic stage of the disease.[11–14] Some evidence for the role of chemokine and chemokine receptors in human AD also has been documented. This showed that CCL2 (MCP-1), a CC chemokine ligand (CCL), is expressed by keratinocytes and recruits dendritic cells, while CXCL8 (IL-8), a CXC chemokine ligand (CXCL), is secreted during the chronic phase of AD (see review).[15]

Naturally occurring AD in dogs (*Canis lupus familiaris*) (OMIA ID: 000269-9615)[16] provides a useful model of human AD due to comparable clinical signs, environmental triggers and immunological changes.[5,17] Alterations to Th1 and Th2 cytokines in canine AD (cAD) (see review)[18] are similar to those reported in human AD. However, the investigation of Treg cytokines, and especially Th17 and

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Th22 cytokines in cAD remains limited.[19,20] The majority of studies have focused on analysis of individual cytokine gene expression profiles from skin lesions,[4,5,21–24] and to a much lesser extent, reflected mRNA expression and/or changes in disease states in circulating leukocytes.[25,26] Elevated concentrations of serum IL-31, secreted by activated Th2 cells, have been reported in dogs with AD[27] and IL-31 confirmed to produce pruritus in a majority of dogs with AD.[28] Plasma concentrations of IL-13 and TNFα were increased, IL-10 was decreased in both plasma[29,30] and serum of AD dogs,[30] and tumour growth factor (TGF) β results were inconclusive when comparing these two studies. In experimental cAD, exposure to allergen resulted in increased mRNA expression of IL-4, IL-10, IL-13 and TGFβ in circulating mononuclear cells.[26] Likewise, mRNA expression from Th1-cytokines and Th2-cytokines were increased in the skin of dogs with AD, along with relative decreases in TGFβ and IL-12p40.[4,5,9,20–22] By contrast, no cytokine or chemokine mRNA was differentially expressed in peripheral blood from dogs with spontaneous AD.[29] One study investigating the phenotypes of Th17 and Th22 cells, reported IL-22 production in skin lesions from atopic dogs, but no detectable IL-17.[19] Collectively, the significance of these findings and the potential role of cytokines other than Th1 and Th2 cytokines to the pathogenesis of cAD, along with any value for diagnosis and/or monitoring response to treatment, remain to be explored.

A comprehensive analysis of cytokines not traditionally associated with human AD facilitated a more detailed understanding of the complexity of responses in AD and highlighted the potential of using circulating cytokines as biomarkers for AD.[31–36] However, there are few comparable studies of cAD. While the knowledge on the role of circulating cytokines that are not directly associated with Th1 or Th2 responses is limited in dogs, we hypothesised that they may play a role in cAD pathogenesis, as they do in humans. The availability of multiplex immunoassays, a modified enzyme-linked immunosorbent assay (ELISA) method which allows cost-effective simultaneous measure of multiple cytokine/chemokine levels in one sample well, recently has been utilised to study several other inflammatory canine diseases.[37] To reveal the potential role of circulating cytokines not traditionally associated with cAD, in this study we aimed to measure a broad panel of plasma cytokine and chemokine concentrations in dogs with AD, using canine-specific multiplex immunoassays.

Methods and materials

Dog population

Blood samples were collected from dogs diagnosed with AD by a referral clinician at the University of Sydney Veterinary Teaching Hospital. Inclusion and exclusion criteria for this study are previously described.[38] Treatments that may alter immune responses, such as glucocorticoid therapy or immunosuppressive agents (e.g. ciclosporin), were ceased three to six weeks before sampling as recommended.[39,40] Signalment and clinical data recorded for each dog included breed, sex, age of onset, date when sample taken, exclusion of other relevant potential diagnoses and intradermal allergen test (IDT) results. Data on breed (purebred or cross-bred) also was recorded, based on the patient details in the computerised file. In general, a dog was considered "purebreed" if it qualified for a pedigree certificate that confirmed it as a purebred dog, while the other dogs were considered "cross-bred".

The control samples were collected from healthy dogs that were free from AD, and had no history of skin disease or apparent skin lesions during physical examination at the day the blood sample was taken. As onset of clinical signs before three years of age is correlated with AD,[41,42] inclusion in this group was limited to >6-year-old dogs. All samples were recruited with a written informed owner consent and approved by the University of Sydney Animal Ethics Committee (AEC protocol 4949).

Plasma sample collection and preparation

Blood samples were collected from dogs before an IDT or serum immunoglobulin (Ig)E blood test. Blood samples were separated by gradient centrifugation immediately, or otherwise either stored in 4°C or shipped on ice, and then separated within 4 h of collection. Plasma samples were stored at –80°C before analysis. Before assay, plasma samples were thawed on ice and diluted with either a water-soluble protease inhibitor cocktail (Sigma-Aldrich; St Louis, MO, USA) or proprietary diluents according to the manufacturer's instructions. Plasma samples for the single-plex TGFβ immunoassay were acid-treated, then neutralised, clarified by centrifugation (20,000*g*; 10 min), and filtered using spin filters (0.22 µ; Sigma-Aldrich). The filtrates were stored short-term at –20°C before assay.

Cytokine and chemokine immunoassays

Three immunoassays were used to measure a total of 19 cytokines and chemokines. Duplicate samples (75 µL per replicate) were used for each dog. A panel of 14 cytokines and chemokines were measured using a single canine multiplex immuno-assay (Millipore Milliplex MAP canine cytokine/chemokine assay, Abacus ALS; Meadowbrook, QLD, Australia). This assay measured CCL2 (MCP-1), CXCL8 (IL-8), CXCL10 (IP-10), GM-CSF, IFNγ, IL-2, IL-4, IL-6, IL-7, IL-10, IL-15, IL-18, CXCL1 (KC) and TNFα. A panel of four additional cytokines – beta nerve growth factor (β-NGF), IL-12p40, stem cell factor (SCF), and vascular endothelial growth factor A (VEGF-A) – were measured using a second canine multiplex immuno-assay and TGF beta 1 (TGFβ) was measured using a canine single-plex immunoassay (Procarta Immunoassays, Thermo-Fisher Scientific; North Ryde, NSW, Australia).

The multiplex immunoassays use microbeads, each labelled with a different dye and coated with a different anticytokine antibody. After incubation with the sample, the microbeads are scanned with a laser, which determines both the bead colour and signal intensity. In this way, a large number of different cytokines can be measured simultaneously on a single sample well. All samples underwent semi-automated analysis (Bio-Plex Luminex 100 IS system, Bio-Rad; Gladesville, NSW, Australia). Cytokine and chemokine concentrations were extracted from standard curve plots using the five parameter logistic (5-PL) nonlinear regression curve-fitting model (Luminex 100 IS 2.3 software; Luminex Corporation; Austin, TX, USA). Multiplex immunoassay concentrations were processed using the Luminex console services provided by the core facility (Australian Proteome Analysis Facility Ltd, Sydney, Australia).

Cytokine and chemokine concentrations from dogs with AD were compared to those of the healthy control dogs. A comparison of cAD immunophenotypes within the atopic patient groups also was performed, including sex (males versus females), pedigree dogs versus cross-bred dogs, age of onset and age at sample collection date. Further, the results of the dogs with weak and moderate responses (score 1 or 2 of 4) were compared to those of the atopic dogs with strong IDT response (score 3 or 4).[43]

Statistical analysis

All statistical analysis was performed using Wilcoxon–Mann–Whitney U-test (two sample nonparametric tests) (Genstat, VSN International Ltd; Hemel Hempstead, UK). The accepted minimal power to conduct a comparison using the U-test in a small sample population in Genstat is eight. Values out of range below the standard curve

were assigned a nominal value of 0.01. A *P*-value threshold of 0.05 was considered significant.

Results

Characteristics of cAD cases

Clinical characteristics of the cAD cases and the controls are detailed in Table 1. There were 20 pedigree dogs (74%) and seven cross-bred dogs (26%) with AD, and eight pedigree (73%) and three cross-bred (27%) control dogs. Females were over-represented in both the control dogs (two males, nine females) and the dogs with AD (11 males, 16 females). Most affected dogs (58%) were from breeds that have been reported previously to have a high risk of developing cAD (five Staffordshire terrier and three Staffordshire terrier crosses; two Labrador retrievers; two Boxer crosses; one French bulldog; one West Highland white terrier; and one great Dane).[38] The mean age of onset was 20 months; less than three years for 22 cases, with the remainder between three and six years. Approximately half of the dogs (56%) with AD had strong responses on IDT (i.e. score of 3 or 4).

Plasma cytokine concentrations

Of the 19 cytokines and chemokines measured, nine median concentrations were higher in the plasma of dogs with AD compared to controls, yet only three of these

Table 1. Clinical characteristics of the canine atopic dermatitis (AD) cases and the control dogs

	AD cases (%)	Controls
Dogs in study [n (%)][†]		
Females	16 (59)	9 (81)
Males	11 (41)	2 (19)
All	27	11
Age [mean m (range)]	50 (10–95)	86 (74–110)
Age of onset [n (%)]		
<6m	8 (30)	
6m to <1y	6 (22)	
1y to <3y	8 (30)	
3 to 6y	5 (18)	
All [n (mean m, range)]	27 (20, 2–72)	
IDT response [(n (%)]		
Strong reactions	15 (55.6)	
Moderate reactions	10 (37.0)	
Weak reaction	1 (3.7)	
Serum IgE test[‡]	1 (3.7)	
AFR status [n (%)]		
Concurrent AD and AFR	1 (3.7)	
Exclusion of AFR[§]	18 (66.7)	
Elimination dietary trial not completed[¶]	8 (29.6)	
FBH status [n (%)]		
Concurrent AD and FBH	1 (3.7)	

m, month; y, year; AFR, adverse food reaction; FBH, flea bite hypersensitivity; IDT, intradermal testing; IGE, immunoglobulin E.
[†]Only 26 dogs were used for the Millipore cytokine/chemokine immunoassay and all 27 dogs participated in the Procarta immunoassays.
[‡]Allercept, HESKA, Gribbles Veterinary, VIC, Australia.
[§]Elimination dietary trials to exclude AFR (a minimum six week trial).38
[¶]All of these dogs had distinctly seasonal signs for >12 months, excluding the possibility of AFR as the sole diagnosis.

were statistically significant, including IL-7 ($P < 0.01$), IL-15 ($P = 0.05$) and CXCL8 ($P < 0.01$) (Table 2, Figure 1). While the values measured for IL-7 in 10 of the 11 controls were below the detectable range, half of the AD cases (13 of 26) had detectable levels (range 2.26 to 7,310 pg/mL), with six samples of >50 pg/mL. Nineteen of the AD and control samples had detectable values for IL-15, and all had detectable values for CXCL8. However, the median values of IL-15 (30.5 pg/mL; range <1.02 to 16,811 pg/mL) and CXCL8 (785 pg/mL; range 52.6 to 6,710 pg/mL) were five-fold higher in the AD cases when compared to the control dogs (IL-15: 6.4 pg/mL; range <1.02 to 129 pg/mL; CXCL8: 161 pg/mL; range 96.0 to 2,254 pg/mL) (Table 2, Figure 1). GM-CSF concentrations were not significantly different between AD dogs and controls ($P = 0.06$), although there was a significant difference when considering only the Staffordshire terrier subgroup (see a comparison of cytokine alterations in dogs that belong to the Staffordshire breeds section below).

Comparison of a subset of atopic dogs aged 6–10 years old at the sample collection date (n = 9) with control dogs in the same age group (n = 11) revealed similar differences in cytokine concentrations (IL-7: $P < 0.01$, IL-15: $P < 0.05$ and CXCL8: $P = 0.001$).

Stem cell factor median concentrations were significantly lower ($P = 0.03$) in cAD (30.5 pg/mL; range <1.22 to 284 pg/mL) compared to the control group (41.2 pg/mL; range 22.9 to 92.3 pg/mL), while β-NGF showed a non-significant ($P = 0.06$) decrease in canine AD (0.54 pg/mL; range 0.28 to 1.59 pg/mL) compared to controls (0.57 pg/mL; range 0.43 to 1.13 pg/mL) (Table 2, Figure 1).

A comparison of cytokines within dogs with AD

Cytokine concentrations in atopic pedigree dogs (n = 20) were compared with those of crossbred dogs with AD (n = 7). IL-7 was the only cytokine to be significantly elevated ($P = 0.03$) in the pedigree dogs with AD (7.08 pg/mL; range < 2.26 to 7,310 pg/mL) when compared to atopic cross-bred dogs (<2.26 pg/mL; range <2.26 to 73.9 pg/mL). In fact, while 68% of the pedigree dogs with AD had measurable IL-7 concentrations (n = 13), only one cross-bred dog with AD had detectable IL-7 (73.9 pg/mL). Other differences were found for IL-12p40 ($P < 0.01$), β-NGF ($P < 0.01$), TGFβ ($P < 0.01$) and SCF ($P = 0.04$) in the plasma of pedigree dogs with AD, where the median value of all four cytokines was lower in the cross-bred dogs compared to the pedigree dogs.

A comparison of cytokine alterations in dogs that belong to the Staffordshire breeds

Within the AD group, there were eight atopic dogs that belonged to the Staffordshire terrier-related breeds. IL-7 ($P = 0.01$), CXCL8 ($P = 0.02$), GM-CSF ($P = 0.05$) and IL-15 ($P = 0.03$) were significantly elevated in plasma of these Staffordshire terrier-related dogs when compared to the control dogs (n = 11) (Tables 2 and 3, Figure 2). The results from the AD cases that belonged to the Staffordshire dog breeds were compared to the rest of the cAD cases (Table 3). While the median values of IFNγ and TNFα in these two groups of atopic dogs were similar, both cytokines were found to be significantly elevated

Table 2. Quantification of 19 cytokines and chemokines in canine atopic dermatitis (AD) cases compared to control dogs

Cytokine/chemokine (pg/mL) [†]	Confirmed AD (n = 26/27)[‡]		Controls (n = 11)	
	Median	Range	Median	Range
CCL2 (MCP-1)	94.9	53.7–2,995	99.2	54.1–251
CXCL8 (IL-8)	785**	52.6–6,710	161	96.0–2,254
CXCL10 (IP-10)	<0.61	<0.61–45.6	<0.61	<0.61–30.4
GM-CSF	21.9	<1.13–81,051	7.34	<1.13–51.2
IFNγ	<0.93	<0.93–35.6	<0.93	all <0.93
IL-2	7.71	<0.46–14,804	2.06	<0.46–26.6
IL-4	<3.42	<3.42–244	<3.42	all <3.42
IL-6	<2.35	all <2.35	<2.35	all <2.35
IL-7	2.26**	<2.26–7,310	<2.26	all <2.26
IL-10	<3.38	<3.38–652	<3.38	all <3.38
IL-15	30.5*	<1.02–16,811	6.40	<1.02–129
IL-18	18.6	1.41–10,111	15.8	2.76–156
CXCL1	67.2	1.74–393	30.4	2.11–236
TNFα	<0.04	<0.04–0.91	<0.04	all <0.04
β-NGF	0.54	0.28–1.59	0.57	0.43–1.13
IL-12p40	118	<1.21–2,837	148	50.5–399
SCF	30.5*	<1.22–284	41.2	22.9–92.3
VEGF-A	<1.01	<1.01–772	3.10	<1.01–36.8
TGFβ	22.9	<1.35–253	11.6	<1.35–130

CCL, CC chemokine ligand; CXCL, CXC Motif Chemokine Ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; NGF, nerve growth factor; SCF, stem cell factor; VEGF, vascular endothelial growth factor; TGF, tumour growth factor.

(<) indicates that either the samples or the lowest standard measured had this value. Samples that had values lower than this were reported as out of range results.

[†]Cytokine and chemokine immunoassay concentrations were measured in picogram per millilitre (pg/mL) units.

*$P \leq 0.05$.

**$P < 0.01$.

[‡]n = 26 for the first 14 cytokines/chemokines listed in the table (using Millipore multiplex immunoassay); n = 27 for the last five cytokines/chemokines listed (using Procarta multiplex immunoassay)

(Table 3, Figure 3) in the Staffordshire dog breeds ($P = 0.04$ for both).

Cytokine concentrations and IDT

The results of the cytokine/chemokine immunoassays of the dogs with AD that had IDT test results (96% of the affected dogs in this study) were compared between the strong IDT response dogs group (n = 15) and dogs with moderate or weak responses (n = 11). The only cytokine that was significantly different between these groups was IL-10 ($P < 0.001$). However, upon closer inspection, this resulted from two dogs with AD and moderate IDT responses that had detectable IL-10 (3.38 and 651.72 pg/mL), while the remaining dogs had undetectable levels. Notably, of the 13 AD dogs with detectable IL-7 levels, six had strong IDT response and seven had a moderate response. No correlation was found between cytokines and either allergen response groups, sex or age of onset (data not shown).

Discussion

This study identified altered levels of five plasma cytokines and chemokines in dogs with cAD relative to healthy controls. The findings indicate that IL-7, IL-15 and SCF, all cytokines that are not necessarily associated with Th1 and Th2 responses, were either elevated (IL-7 and IL-15) or decreased (SCF) in affected dogs. Increased levels of CXCL8 and some evidence for elevated GM-CSF also were found. While a disturbance in the balance between Th1 and Th2 cytokines together with Th17 and Th22 imbalances are established in current models of AD pathogenesis in both dogs and human, the altered concentrations of IL-7, IL-15 and SCF in plasma identified in this study implicate roles for other immunological regulatory factors. These cytokines are unlikely to be specific indicators of AD, yet the role of these identified pleiotropic cytokines in regard to Th1/Th2/Th17/Th22 dysregulation in cAD is intriguing.

IL-7 is the key cytokine highlighted in this study. It has a role in cell proliferation, promoting B- and T-cell production, and is critical for the survival of mature T cells.[44–46] Elevated circulating IL-7 concentrations are reported and often correlated with changes to other cytokines in some human immune-related diseases.[47–49] Together with TGFβ and IL-12p40, IL-7 also was elevated in the remission phase of Crohn's disease when compared with the active disease phase.[49] A previous study[49] suggested that IL-7 suppresses Th1 cells through inhibition of Treg cells, yet does not suppress Th2 cell development. This may be relevant to the imbalance of Th1 and Th2 cells described previously in AD.[21,23,50] The crucial role of IL-7 in T-cell homeostasis, and the putative correlation between elevated IL-7 and other changes in Treg cytokines, are consistent with a model of AD pathogenesis in which IL-7 has an influential role on T-cell imbalance. While our data might indicate some association between high IL-7 levels and IDT response, the correlation between high levels of IL-7 and disease severity was beyond the
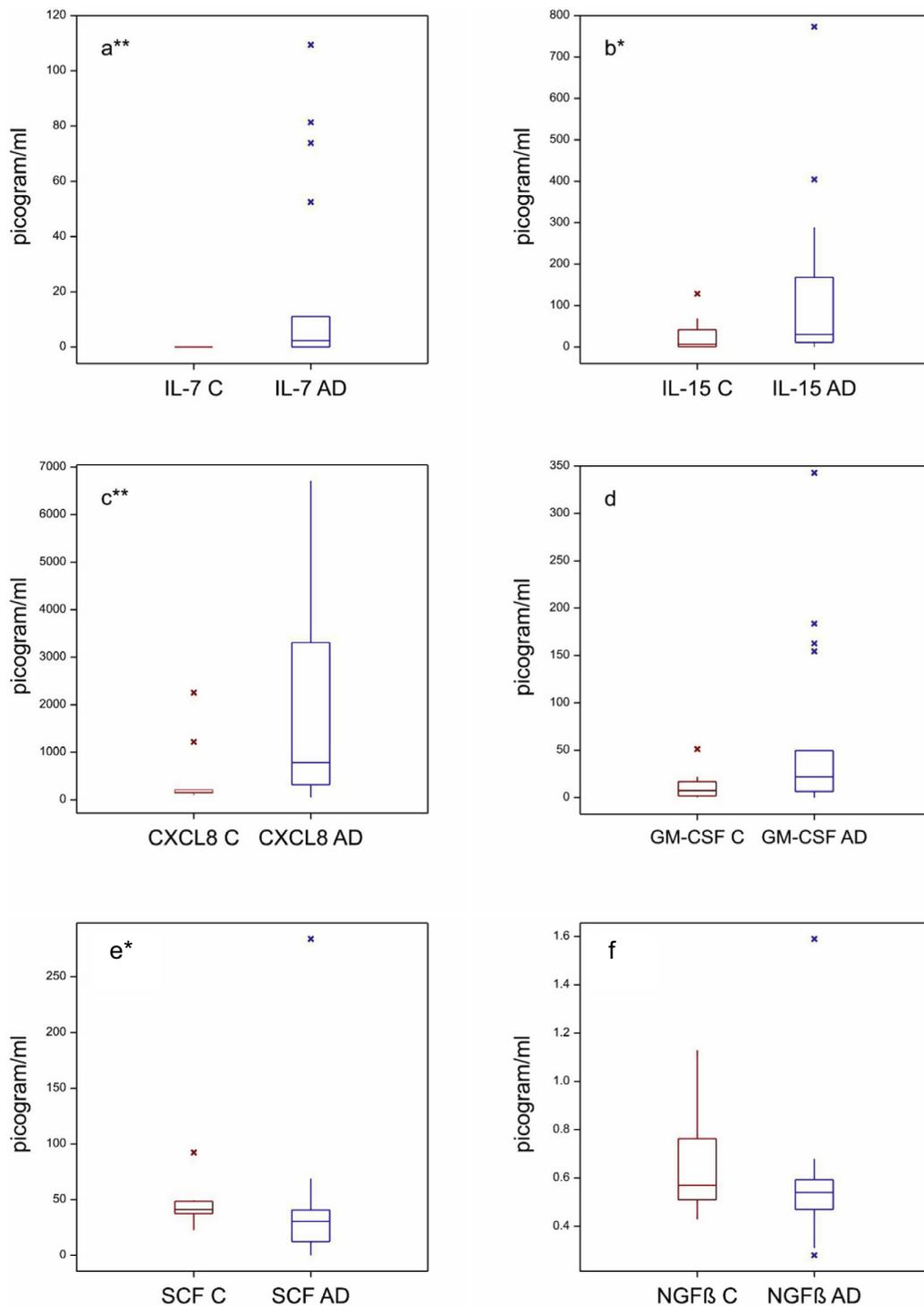
Figure 1. Cytokine and chemokine concentrations in plasma of dogs with atopic dermatitis (AD) compared to control dogs.
Box plots of plasma cytokine concentrations for normal controls (C; n = 11) and atopic dogs (AD; n = 26 or 27). (a) Interleukin (IL)-7: controls (< 2.26 pg/mL) versus AD (median 2.26 pg/mL; range <2.26 to 7,310 pg/mL) (P = 0.003). (b) IL-15: the median value for the AD cases (30.5 pg/ml) was five-fold higher than the median value for controls (6.4 pg/mL) (P = 0.052). (c) CXC motif chemokine ligand (CXCL)8/IL-8: median value for the AD cases (785 pg/mL) was five-fold higher than the median value for control dogs (161 pg/mL) (P = 0.003). (d) Granulocyte-macrophage colony-stimulating factor (GM-CSF): median value for controls (7.34 pg/mL) was three-fold lower than that of the dogs with AD (21.9 pg/mL) (P = 0.058). (e) Stem cell factor (SCF): median value of SCF for AD cases (30.5 pg/mL) was lower than the median value for the control dogs (41.2 pg/mL) (P = 0.028). (f) β-nerve growth factor (NGF): shows nonsignificant (P = 0.06) decreased concentrations in canine AD (median 0.54 pg/mL) compared to controls (median 0.57 pg/mL). *, $P \leq 0.05$. For scaling reasons, the two top values for IL-7, IL-15 and GM-CSF are not shown in these figures.

scope of this study, and will be interesting to address in future studies. From the scattered canine research using multiplex immunoassays,[37] IL-7 levels were mostly nonsignificant in a range of nine non-skin-related diseases. Cytokines in dogs with other dermatological disease need to be investigated.

Table 3. Quantification of 19 cytokine and chemokine multiplex immunoassay in Staffordshire dog breeds

Cytokine / chemokine (pg/mL)[†]	Confirmed AD (n = 8) Staffordshire dog breeds		Confirmed AD (n = 18/19)[‡] other dog breeds	
	Median	Range	Median	Range
CCL2 (MCP-1)	156	70.5–2,995	86.4	31.6–349
CXCL8 (IL-8)	13939*	119–6,710	785	52.6–3,880
CXCL10 (IP-10)	4.34	<0.61–45.6	<0.61	< 0.61–23.8
GM-CSF	33.9*	<3.22–81,051	15.6	<3.22–343
IFNγ	<0.93[$]	<0.93–35.6	<0.93	<0.93–8.86
IL-2	9.95	<0.46–14,804	7.19	<0.46–131
IL-4	<3.42	<3.42–244	<3.42	all <3.42
IL-6	<2.35	all <2.35	<2.35	all <2.35
IL-7	8.43**	<2.26–7,310	1.14	<2.26–81.4
IL-10	<3.38	<3.38–3.38	<3.38	<3.38–652
IL-15	47.6*	6.4–16,811	21.2	<1.02–773
IL-18	55.6	2.09–10,111	17.0	1.41–179
CXCL1	125	1.74–321	60.3	2.09–393
TNFα	<0.04[$]	<0.04–0.91	<0.04	<0.04–0.04
β-NGF	0.51	0.40–1.59	0.54	0.28–0.68
IL-12p40	161	36.4[c]–2,837	110	<1.21–416
SCF	27.6*	7.44–284	30.5	<1.22–51.7
VEGF-A	<1.01	<1.01–772	3.23	<1.01–10.3
TGFβ	22.0	<1.35–199	22.9	<1.35–253

CCL, CC chemokine ligand; CXCL, CXC motif chemokine ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; NGF, nerve growth factor; SCF, stem cell factor; VEGF, vascular endothelial growth factor; TGF, tumour growth factor.

< indicates that either the samples or the lowest standard measured had this value. Samples that had values lower than this were reported as out of range results.

[†]Cytokine and chemokine immunoassay concentration were measured in picogram per millilitre (pg/ml) units.

*$P \leq 0.05$.

**$P \leq 0.01$, compared to the control dogs from Table 2.

[$]$P \leq 0.05$, compared to the confirmed AD dogs belongs to other dog breeds from Table 3.

[‡]n = 18 for the first 14 cytokines/chemokines listed in the table (using Millipore multiplex immunoassay); n = 19 for the last five cytokines/chemokines listed (using Procarta multiplex immunoassay)

One of the hallmarks of atopy is allergen-induced eosinophilia and high serum IgE concentrations.[22,51–53] IL-7 influences immunoglobulin class switching in favour of IgE and IgG4.[54] Elevated IgE and IgG4 levels are part of the minor diagnostic criteria for cAD[41] and serum IgE concentrations are reduced following immunotherapy in dogs.[20] IL-7 is elevated in human psoriasis patients,[55] an immune-related skin disease in which patients have high serum IgE[56] and other atopy-like symptoms that are similar to AD.[57–60] Furthermore, IL-7 is elevated in bronchoalveolar lavage fluids after allergen challenge in human asthma patients and is correlated with increased bronchoalveolar lavage eosinophil levels.[61] Taken together, the evidence for involvement of this regulatory cytokine in skin disease which resembles the clinical signs of human atopic (i.e. psoriasis) and other human allergic diseases (e.g. asthma) supports a role for IL-7 in regulating IgE and eosinophil numbers in cAD.

The elevated plasma CXCL8 levels present in the AD dogs studied here are consistent with the observation that *IL8* is one of the most overexpressed genes in skin lesions from AD-affected animals.[62] In comparison, skin mRNA expression of *IL8* was not found to be elevated in a study of dogs from an experimental cAD colony following allergen challenge.[22] This difference may be attributed to differences between an acute phase (i.e. following allergen challenge) and the measurements made here during a chronic phase of the disease. CXCL8 and GM-CSF production by mast cells also is influenced by Thymic stromal lymphopoietin (TSLP), a cytokine that shares a receptor subunit with IL-7.[63] TSLP expression is elevated in cAD skin lesions,[64] and in one study a variant in the canine TSLP receptor was associated with cAD.[65]

Impaired Treg cell function has been proposed as a contributing factor to human atopy.[12, 31] Together with subsequent TSLP input, both IL-7 and IL-15 signals may be crucial for Treg development,[44] and the elevated IL-7 and IL-15 cytokine production found here may reflect a Treg response to allergens. However, IL-10, a key cytokine for Treg proliferation, was not elevated in the dogs with AD from the present study, and only two dogs had detectable levels, thus not supporting Treg production in AD dogs. Similar findings have been reported by others,[20,29,30] while another study found decreased serum IL-10 levels,[30] again not supporting a Treg response.

The other cytokine that showed a significant difference in plasma from dogs with cAD was SCF, which plays a crucial role in melanogenesis (see review).[66] SCF binds to the c-kit receptor,[67] which is expressed on mast cells, and effects mast cell development and differentiation. SCF in skin lesions of atopic dogs has been linked to histamine release from dermal mast cells,[68] and it is possible that lower plasma levels reflect the recruitment of SCF to the lesion.

The early onset of the disease in the set of the affected dogs examined here (mean age 20 months) aligns with
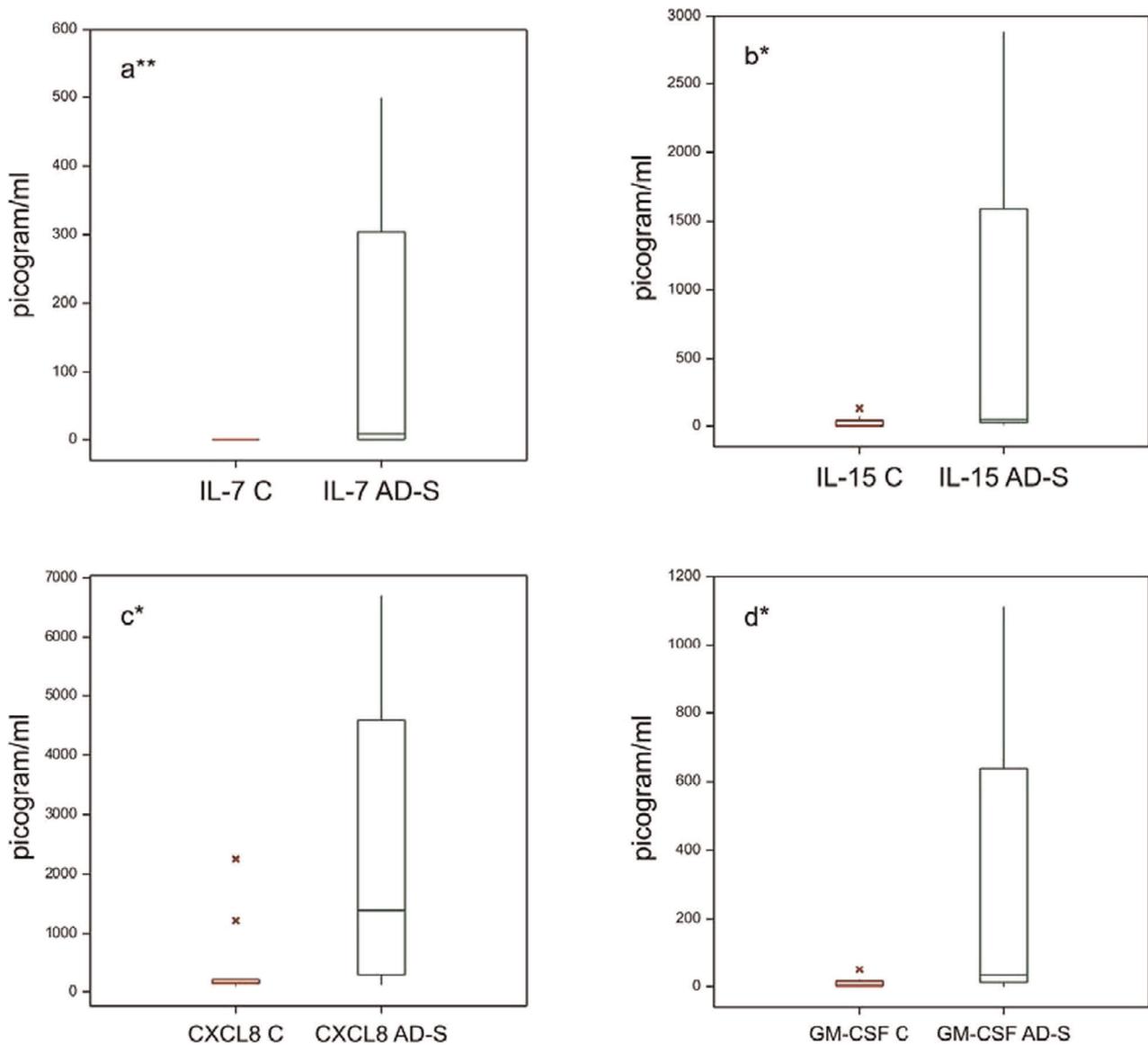
Figure 2. Cytokine concentrations in plasma of Staffordshire breeds with atopic dermatitis (AD) compared to control dogs
Box plots of plasma cytokine for normal controls (C; *n*=11) and atopic dogs belonging to the Staffordshire breed group (AD-S; n = 8). (a) Interleukin (IL)-7: controls (<2.26 pg/mL) versus Staffordshire breed dogs with AD (median 8.34 pg/mL; range <2.26 to 7,310 pg/mL) (*P* = 0.01). (b) IL-15: median value was seven-fold higher in AD cases (47.6 pg/mL) versus control dogs (6.4 pg/mL) (*P* = 0.031). (c) CXC motif chemokine ligand (CXCL)8/IL-8: median value for AD cases (1,393 pg/mL) was nine-fold higher than the median value in control dogs (161 pg/mL) (*P* = 0.016). (d) Granulocyte-macrophage colony-stimulating factor (GM-CSF): median value in controls (7.34 pg/mL) was five-fold lower than in dogs with AD (33.9 pg/mL) (*P* = 0.048). For scaling reasons, the top values for IL-7, IL-15 and GM-CSF are not shown in these figures. *, $P \leq 0.05$; **, $P \leq 0.01$.

previous reports.[9,17] Considering that often it takes time to exclude other differential diagnosis in order to confirm cAD,[38] it is likely that most cases were at the chronic phase when the blood sample was taken. This correlates with the changes in SCF and CXCL8 levels (see above in Discussion). Hence, profiling cytokine levels in early acute stages of the disease may be indicated as well. Although many of the AD dogs were younger, while all the controls were older (to rule out early cases of AD), we showed that in the subset of older dogs (aged 6–10 years) the changes in IL-7, IL-15 and CXCL8 levels are significant, compared to reported nonsignificant levels of these three cytokines when age-related changes were measured in healthy dogs,[69] ruling-out age-related changes of the cytokines.

Differences in concentrations of five cytokine and chemokines between cross-bred and pedigree affected dogs were demonstrated here (IL-7, IL-12p40, β-NGF, TGFβ and SCF). Pedigree dogs affected with AD share similar genetic backgrounds that may influence clinical presentation and/or genetic associations of cytokine concentrations,[38,65] justifying further research into breed-specific profiles of cytokines and chemokines in cAD. Dogs that belong to the Staffordshire breeds have an increased risk of developing AD.[38] In addition to elevated IL-7, IL-15 and CXCL8 concentrations in Staffordshire breed dogs with AD, we observed an elevated concentration of GM-CSF. In humans, GM-CSF may be linked to eosinophilia in allergic patients,[70] and is overproduced in keratinocytes from human AD patients.[71] In the CPEK
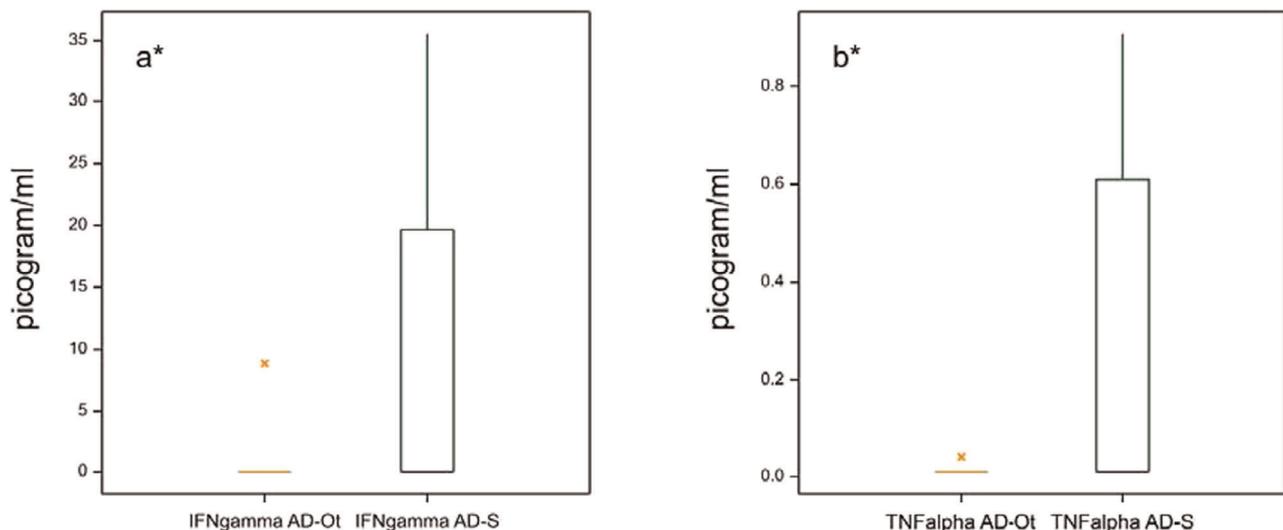
Figure 3. Interferon (IFN)γ and tumour necrosis factor (TNF)α concentrations in plasma of atopic dermatitis (AD) cases belongs to the Staffordshire breeds compared to AD cases from other breeds.
Box plot of IFNγ (a) and TNFα (b) plasma cytokine concentrations for atopic dogs from the Staffordshire breeds (AD-S; n = 8) and the AD cases from other breeds (AD-O; n = 18). (a) IFNγ: Staffordshire dogs with AD (median <0.93 pg/mL; range <0.93–35.6 pg/mL) versus dogs with AD from other breeds (median <0.93 pg/mL; range <0.93–8.86 pg/mL) (*P* = 0.043). (b) TNFα: Staffordshire dogs with AD (median <0.04 pg/mL; range <0.04–0.91 pg/mL) versus dogs with AD from other breeds (median <0.04 pg/mL; range <0.04–0.04 pg/mL) (*P* = 0.043). *, *P* ≤ 0.05.

keratinocyte cell line, a model used for cAD research, overexpression of GM-CSF was induced by allergen (see reviewed)[72] and by IL-17.[73] In addition to the aforementioned discussion on possible roles of IL-7 in AD, in the presence of GM-CSF, IL-7 supports survival of eosinophils in combination with IL-4.[61] However, IL-4 concentrations did not differ between atopic and control dogs in our study and also in others.[29,30] Another effect of IL-7 is increased secretion of GM-CSF from eosinophil granules of asthmatic patients, enhancing the allergic response in atopic diseases. Interestingly, the highest cytokine concentrations of all samples tested were detected in two dogs with AD from the Staffordshire breed group which had confirmed diagnosis of AD with either concurrent adverse food reaction (AFR) or concurrent flea bite hypersensitivity, suggesting a greater effect on cytokine levels when the immune system is combating multiple allergens. However, a breed-specific reaction also is possible.

IFNγ and TNFα were significantly elevated in the atopic Staffordshire breed group compared to the rest of the atopic dogs. Interestingly, the levels of these cytokines were not significantly different when comparing our all atopic dogs group to controls. Previous similar studies that compared atopic to control dogs were inconclusive, with increased TNFα in plasma and increased IFNγ in serum, and nonsignificant results about plasma IFNγ and serum TNFα.[29,30] Our atopic Staffordshire results may reflect a chronic phase of the disease or a breed-specific effect. It also may indicate a breed-related correlation between elevated IL-7 and IFNγ in Staffordshire breeds, in a similar way to human Vogt–Koyanagi–Harada disease, a multisystem autoimmune disease in which serum IL-7 is increased and promotes *in vivo* expression of IFNγ.[48]

The lack of directly comparable studies in the canine model limits interpretation of the present results. It also is difficult to compare direct measurement of cytokines to mRNA assays. Comparisons were further restricted by the lengthy and detailed inclusion criteria for cAD which limited the number of animals available for study. This may have been reflected in the finding that the level of some of the cytokines was different among different dog breeds. Recruitment of controls for this study was based on the rare availability of >6-year-old dogs with no historical or current skin problems. Therefore, it was not possible to breed-match healthy dogs to the affected AD cases. A follow-up study including only mastoid clade dogs may further validate our results. AD is an early onset disease and thus we could not include young controls that may develop the disease in the future. However, when we compared the results between the small (age-matched) subset of >6-year-old AD dogs (n = 9) to the controls (n = 11) there was similar pattern of significantly altered IL-7, CXCL-8 and IL-15, suggesting that age does not influence cytokine concentrations. At the time of this study, canine-specific multiplex reagent availability also was not complete. Some studies have reported varied concentrations of IL-5, IL-13, IL-22 and IL-31 in cAD,[19,22,26,27] so it will be valuable to consider measurement of an extended panel of cytokines as reagents become available.

Finally, as cytokines are produced and act locally, their plasma concentrations do not necessarily reflect their importance in the pathogenesis of the disease from the standpoint of local tissue response. Hence, while plasma cytokine concentrations may serve as an indicator to aid diagnosis, they may or may not be associated with disease severity or clinical presentation of AD. Therefore, the implication of the identified altered cytokines on disease pathogenesis, especially in relation to skin-related diseases, will need to be further explored.

Conclusion

This study identified an association between cAD and altered plasma concentrations of cytokines. Of significance were elevated plasma concentrations of CXCL8, IL-7 and IL-15, with some evidence of increased GM-CSF, especially in atopic dogs belonging to the Staffordshire breeds. The results provide potential insights into cAD pathogenesis and may provide an opportunity to explore the use of a cytokine biomarker panel to aid in the diagnosis of cAD. One model that has been suggested to account for AD pathogenesis involves dysregulation of Treg cell responses, and although this model is consistent with the elevated levels of IL-7, and with the reduced concentrations of IL-10 and TGFβ in the present study, a mechanism linking altered cytokine profiles and Treg in cAD will require further exploration. Finally, as this study identified several differences in cytokine values of Staffordshire dogs, investigations into cytokine levels in other breeds that belong to the reported susceptible dog breed clade[38] are warranted.

Acknowledgements

Author contribution

Hamutal Mazrier: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Visualization; Writing-original draft; Writing-review & editing. **Linda J Vogelnest:** Conceptualization; Investigation; Resources; Supervision; Writing-review & editing. **Rosanne M Taylor:** Conceptualization; Supervision; Writing-review & editing. **Peter Williamson:** Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Supervision; Writing-review & editing.

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Résumé

Contexte – La dermatite atopique (DA) canine (*Canis lupus familiaris*) partage des signes cliniques similaires à la DA humaine. La réponse immunitaire anormale de la DA est orchestrée par les lymphocytes T et peut inclure une implication variable des cytokines, des cellules T régulatrices (Treg), des éosinophiles, des mastocytes et d'autres composants immunitaires. Les cytokines Helper Th2 prédominent souvent au début, suivies par les cytokines Th1 dans les phases plus chroniques.

Hypothèse/Objectifs – Il a été démontré que les cytokines pro-inflammatoires et Treg jouent un rôle dans la DA humaine, mais leur importance n'est pas claire dans la DA canine. Par conséquent, cette étude visait à mesurer les concentrations de cytokines/chimiokines non traditionnellement associées à la réponse Th1/Th2.

Sujets – Chiens AD (n = 27), comparés aux chiens témoins (n = 11).

Matériels et méthodes – Au total, 19 cytokines plasmatiques ont été dosées à l'aide d'immunotests multiplex spécifiques canins.

Résultats – Les concentrations plasmatiques de CXC Motif Chemokine Ligand 8 (CXCL8), d'interleukine (IL)-7 et de cytokines IL-15 étaient élevées chez les chiens atteints de DA par rapport aux chiens témoins. De plus, les concentrations de facteur de cellules souches (SCF) ont été réduites dans le plasma des patients atteints de DA par rapport aux chiens témoins. Des profils de cytokines distincts ont été trouvés chez les chiens appartenant aux races Staffordshire, un groupe présentant un risque accru de DA. En particulier, le facteur de stimulation des colonies de granulocytes-macrophages (GM-CSF) avait des concentrations significativement élevées.

Conclusions et pertinence clinique – Certaines des altérations des cytokines plasmatiques dans la DA canine décrites ici, en particulier de l'IL-7, n'ont pas été rapportées auparavant. Le suivi de ces altérations cytokiniques distinctives pourrait être utile pour le diagnostic et le suivi de la DA canine.

Resumen

Introducción – la dermatitis atópica (AD) canina (*Canis lupus familiaris*) comparte signos clínicos similares a la AD humana. La respuesta inmune anormal de la AD está orquestada por linfocitos T y puede incluir una participación variable de citoquinas, células T reguladoras (Treg), eosinófilos, mastocitos y otros componentes inmunitarios. Las citoquinas auxiliares T (Th)2 a menudo predominan inicialmente, seguidas de las citoquinas Th1 en fases más crónicas.

Hipótesis/Objetivos – Se ha demostrado que las citoquinas proinflamatorias y Treg desempeñan un papel en la AD humana, pero su importancia no está clara en la AD canina. Por lo tanto, este estudio tuvo como objetivo medir las concentraciones de citoquinas/quimioquinas no asociadas tradicionalmente con la respuesta Th1/Th2.

Animales – pacientes caninos con AD (n = 27), en comparación con perros control (n = 11).

Métodos y materiales – se analizaron un total de 19 citoquinas del plasma utilizando inmunoensayos multiplex específicos caninos.

Resultados – las concentraciones en el plasma de la quimioquina ligando 8 del motivo de quimioquina CXC (CXCL8), interleuquinas (IL)-7 e IL-15 estaban elevadas en los pacientes caninos con AD, en comparación con los perros control. Además, las concentraciones de factor de células madre (SCF) se redujeron en el plasma de los pacientes caninos con AD en comparación con los perros control. Se encontraron perfiles de citoquinas distintos en perros pertenecientes a las razas Staffordshire, un grupo con mayor riesgo de AD. En particular, el factor estimulante de colonias de granulocitos y macrófagos (GM-CSF) tenía concentraciones significativamente elevadas.

Conclusiones y relevancia clínica – algunas de las alteraciones de las citoquinas del plasma en la AD canina descritas aquí, en particular de IL-7, no se han publicado previamente. El seguimiento de estas alteraciones distintivas de las citoquinas podría ser útil para el diagnóstico y seguimiento de la AD canina en perros.

Zusammenfassung

Hintergrund – Die atopische Dermatitis (AD) des Hundes (*Canis lupus familiaris*) zeigt ähnliche klinische Zeichen wie die AD des Menschen. Die abnormale Immunantwort der AD wird durch die T Lymphozyten verursacht und kann eine unterschiedliche Beteiligung von Zytokinen, regulatorischen T (Treg) Zellen, Eosinophilen, Mastzellen und andere Immunkomponenten zeigen. Helfer T (Th)2 Zytokine sind anfangs oft dominant, gefolgt von Th1 Zytokinen in den mehr chronischen Phasen.

Hypothese/Ziele – Es konnte gezeigt werden, dass pro-entzündliche und Treg Zytokine eine wichtige Rolle bei der AD des Menschen spielen, ihre Bedeutung bei der AD des Hundes ist jedoch unklar. Daher war das Ziel dieser Studie die Messung der Konzentrationen von Zytokinen/Chemokinen, die traditionellerweise nicht mit einer Th1/Th2 Antwort im Zusammenhang stehen.

Tiere – Canine AD Patienten (n = 27) im Vergleich zu Kontrollhunden (n = 11).

Methoden und Materialien – Insgesamt wurden 19 Zytokine mittels spezifischen caninen Multiplex Immuno-Assays im Plasma bestimmt.

Ergebnisse – Die Plasmakonzentrationen von CXC Motif Chemokin Ligand 8 (CXCL8) sowie den Zytokinen Interleukin (IL)-7 und IL-15 waren bei den Hunden mit AD im Vergleich zu den Kontrollhunden erhöht. Zusätzlich waren die Konzentrationen des Stammzellfaktors (SCF) im Plasma von Hunden mit AD im Vergleich zu den Kontrollhunden reduziert. Deutliche Zytokinprofile wurden bei Hunden, die zu den Staffordshire Rassen gehören, bei denen es sich um eine Gruppe mit einem erhöhten AD Risiko handelt, gefunden. Im Speziellen war der Granulozyten-Makrophagenkolonie-stimulierende Faktor (GM-CSF) signifikant erhöht.

Schlussfolgerungen und klinische Bedeutung – Einige der Veränderungen der Plasmazytokine, die hier bei den Hunden mit AD beschrieben wurden, im Speziellen IL-7, wurden bisher noch nicht publiziert. Eine Überwachung dieser klaren Zytokin Veränderungen könnte bei der Diagnose und beim Monitoring von Hunden mit AD nützlich sein.

要約

背景 — 犬 (Canis lupus familiaris)アトピー性皮膚炎(AD)は、ヒトのADと臨床症状が類似している。アトピー性皮膚炎の異常な免疫反応は、Tリンパ球を中心に、サイトカイン、レギュラトリーT(Treg) 細胞、好酸球、マスト細胞、その他の免疫成分が関与している可能性がある。初期にはヘルパーT(Th)2サイトカインが優勢で、より慢性的な段階ではTh1サイトカインが続くことが多い。

仮説/目的 – 炎症性サイトカインおよびTregサイトカインは、ヒトのADにおいて役割を果たしていることが示されているが、犬ADにおいてはその重要性は明らかではない。そこで本研究では、従来はTh1/Th2反応に関連していなかったサイトカイン/ケモカインの濃度を測定することを目的とした。

被検動物 – 犬AD症例(n=27)および対照犬 (n=11) を比較した。

材料と方法 　– 　計19種類の血漿サイトカインを、犬特異的マルチプレックス免疫測定法を用いて測定した。

結果 – 犬AD症例では、CXC Motif Chemokine Ligand 8(CXCL8) 、インターロイキン (IL)-7、IL-15サイトカインの血漿濃度が対照犬と比較して上昇していた。また、犬AD症例の血漿中の幹細胞因子 (SCF) 濃度は、対照犬に比べて低下していた。ADのリスクが高いとされるスタッフォードシャー種に属する犬では、独特のサイトカインプロファイルが見られた。特に、顆粒球-マクロファージコロニー刺激因子 (GM-CSF) の濃度が有意に上昇していた。

結論と臨床的妥当性 　– 　今回報告された犬ADにおける血漿中のサイトカインの変化のうち、特にIL-7の変化はこれまでに報告されていないものである。これらの特徴的なサイトカインの変化をモニタリングすることは、犬AD診断やモニタリングに有用であると考えられる。

摘要

背景 — 犬 (家犬) 特应性皮炎 (AD) 的临床症状与人类AD相似。AD的异常免疫反应由T淋巴细胞协调, 可能包括细胞因子、调节性T(Treg) 细胞、嗜酸性粒细胞、肥大细胞和其他免疫组分的不同参与。辅助性T(Th)2细胞因子最初通常占主导地位, 在更慢性阶段紧随其后的是Th1细胞因子。

假设/目标 – 促炎细胞因子和Treg细胞因子已被证明在人类AD中发挥作用, 但它们在犬AD中的重要性尚不清楚。因此, 本研究旨在测量传统上与Th1/Th2反应无关的细胞因子/趋化因子的浓度。

动物 – AD患犬(n=27)与对照犬 (n=11) 相比。

方法和材料 – 共有19种血浆细胞因子采用犬特异性多重免疫分析法进行检测。

结果 – 与对照犬相比, 犬AD患者的CXC基序趋化因子配体8(CXCL8) 、白细胞介素 (IL)-7和IL-15细胞因子的血浆浓度升高。此外, 与对照犬相比, AD患犬血浆中的干细胞因子 (SCF) 浓度降低。在斯坦德犬身上发现了不同的细胞因子谱, 斯坦福德犬罹患AD的风险增加。尤其是粒细胞-巨噬细胞集落刺激因子 (GM-CSF) 的浓度显著升高。

结论和临床相关性 – 本文描述的犬AD中的一些血浆细胞因子改变, 特别是IL-7的改变, 以前没有报道过。监测这些独特的细胞因子变化可能有助于诊断和监测AD患犬。

Resumo

Contexto – A dermatite atópica (DA) canina (*Canis lupus familiaris*) apresenta sinais clínicos similares à DA humana. A resposta imune anormal da DA é orquestrada por linfócitos T, e pode incluir o envolvimento variável de citocinas, células T regulatórias (Treg), eosinófilos, mastócitos e outros componentes imunes. As citocinas derivadas de linfócitos T *helper* (Th)2 geralmente predominam na fase inicial, seguidas pelas citocinas Th1 na fase mais crônica.

Hipótese/Objetivos – Citocinas pró-inflamatórias e citocinas Treg tem demonstrado funções na DA humana, mas a sua importância ainda não é clara na DA canina. Desta forma, este estudo teve o objetivo de mensurar as concentrações de citocinas/quimiocinas não tradicionalmente associadas às respostas Th1/Th2.

Animais – pacientes com DA canina (n = 27), comparados a cães controles (n = 11).

Materiais e métodos – Um total de 19 citocinas plasmáticas foram avaliadas utilizando imunoensaios multiplex específicos para cães.

Resultados – As concentrações plasmáticas de *CXC Motif Chemokine Ligand 8* (CXCL8), interleucina (IL)-7 e IL-5 estavam aumentaas nos cães com DA, comparado aos cães controle. Além disto, as concentrações do fator de célula tronco (SCF) estavam reduzidas no plasma dos pacientes com DA canina comparada aos cães controle. Foram encontrados perfis de citocinas distintos nos cães pertencentes às raças Staffordshire, um grupo com maior risco de DA. Em particular, o fator estimulador de colônias de macrófagos-granulócitos (GM-CSF) apresentou concentrações significativamente elevadas.

Conclusões e importância clínica – Algumas das alterações nas citocinas plasmáticas na DA canina descritas aqui, particularmente IL-7, não haviam sido descritas anteriormente. O monitoramento dessas alterações distintas de citocinas pode ser útil para o diagnóstico e monitoramento da DA em cães.