

Interleukin-31: its role in canine pruritus and naturally occurring canine atopic dermatitis

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Background – Interleukin-31 (IL-31) is a member of the gp130/interleukin-6 cytokine family that is produced by cell types such as T helper 2 lymphocytes and cutaneous lymphocyte antigen positive skin homing T cells. When overexpressed in transgenic mice, IL-31 induces severe pruritus, alopecia and skin lesions. In humans, IL-31 serum levels correlate with the severity of atopic dermatitis in adults and children.

Hypothesis/Objective – To determine the role of IL-31 in canine pruritus and naturally occurring canine atopic dermatitis (AD).

Animals – Purpose-bred beagle dogs were used for laboratory studies. Serum samples were obtained from laboratory animals, nondiseased client-owned dogs and client-owned dogs diagnosed with naturally occurring AD.

Methods – Purpose-bred beagle dogs were administered canine interleukin-31 (cIL-31) via several routes (intravenous, subcutaneous or intradermal), and pruritic behaviour was observed/quantified via video monitoring. Quantitative immunoassay techniques were employed to measure serum levels of cIL-31 in dogs.

Results – Injection of cIL-31 into laboratory beagle dogs caused transient episodes of pruritic behaviour regardless of the route of administration. When evaluated over a 2 h period, dogs receiving cIL-31 exhibited a significant increase in pruritic behaviour compared with dogs that received placebo. In addition, cIL-31 levels were detectable in 57% of dogs with naturally occurring AD (≥ 13 pg/mL) but were below limits of quantification (< 13 pg/mL) in normal, nondiseased laboratory or client-owned animals.

Conclusions – Canine IL-31 induced pruritic behaviours in dogs. Canine IL-31 was detected in the majority of dogs with naturally occurring AD, suggesting that this cytokine may play an important role in pruritic allergic skin conditions, such as atopic dermatitis, in this species.

Introduction

Canine atopic dermatitis (AD) is a genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features.¹ One clinical feature that dogs with AD commonly display is pruritus, which can have a significant impact on the quality of life for the pet as well as for the owner. However, the underlying pathways and mechanisms involved in triggering pruritic behaviours are not clear, hampering the development of effective anti-pruritic therapies.

Interleukin-31 (IL-31) is a recently identified cytokine implicated in pruritic skin conditions such as human AD. When initially characterized in transgenic mice, overexpression of IL-31 led to the development of several hallmark signs of AD, which included increased inflammatory cell infiltration into the skin, severe pruritus, alopecia and

skin lesions.² Interleukin-31 has been shown to be produced by activated T helper type 2 lymphocytes and by cutaneous lymphocyte antigen positive (CLA+) skin homing T cells from human AD patients, suggesting that these cells may represent a major source of this cytokine. Interleukin-31 has been found to be elevated preferentially in pruritic versus nonpruritic human skin conditions, and serum levels of IL-31 correlate with disease severity in human adults as well as children with AD.^{2–8}

Interleukin-31 binds to a heterodimeric receptor consisting of IL-31 receptor A and oncostatin M receptor β . Upon ligand binding to this receptor complex, signal transduction cascades such as the Janus kinase–signal transducer and activator of transcription (JAK–STAT), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways are activated.⁹ Receptors for IL-31 are found on a variety of cells, such as keratinocytes, macrophages and eosinophils, and participate in regulating immune responses in these cell types.^{9–11} Of great interest is the finding that these receptors are present on a subset of small-sized nociceptive neurons of mouse and human dorsal root ganglia,

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suggesting that this cytokine may directly activate pruritogenic signals in peripheral nerves.^{4,12}

The cloning of canine interleukin-31 (cIL-31) has been previously reported.¹³ These investigators were able to detect cIL-31 mRNA in freshly isolated canine peripheral blood mononuclear cells after concanavalin A treatment, suggesting that IL-31 may be produced by canine T cells; however, they were not able to detect cIL-31 mRNA in skin biopsy specimens from dogs diagnosed with AD, which calls into question the role of IL-31 in canine AD. To extend investigations of canine IL-31 to assessments of biological activity and protein levels in disease, the present study was conducted to evaluate the role of IL-31 in canine pruritus using purpose-bred beagle dogs and to evaluate whether IL-31 is present in the serum of animals with naturally occurring AD.

Materials and methods

Cloning and expression of cIL-31

Using total RNA isolated from canine testicular tissue and oligo-(dT)₂₀ primers, complementary DNA was synthesized with the SuperScript[®] III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Polymerase chain reactions were performed to amplify the cIL-31 gene from complementary DNA using primers TEF-1237 (5'-AGATCTGCCACCATGCTCTCCCACACAGGACCATCCAG-3') and TEF-1240 (5'-GGTACCCTACTGAGGTCCAGAGTTTAGTGAC-3'). The PCR product was cloned into pCR[®]-Blunt II-TOPO[®] according to the manufacturer's protocols (Life Technologies, Grand Island, NY, USA) and further subcloned into the expression construct pSOO524. The cIL-31 expression construct was either transiently transfected into FreeStyle[™] 293 suspension culture cells following the manufacturers' protocol (Life Technologies) or stably transfected into CHO cells using a site-specific integration system.¹⁴

Protein purification and analysis of recombinant cIL-31

Canine interleukin-31 was produced by cultured FreeStyle[™] 293 cells or CHO cells. Conditioned media from these cells was collected, dialysed with buffer (20 mmol/L Tris, pH 8.0, and 40 mmol/L NaCl) and purified by anion exchange chromatography (Q Sepharose). Protein identity was confirmed by N-terminal sequencing and by liquid chromatography-mass spectrometry (LC-MS) analysis of a tryptic digest of the protein.

Cell culture

The DH82 canine monocytic cell line (American Type Culture Collection, Manassas, VA, USA) was used to evaluate cIL-31 cytokine function. DH82 cells were plated into CoStar 96-well flat-bottomed cell culture plates (Corning, Tewksbury, MA, USA) at a density of 1×10^5 cells per well in MEM growth media (Life Technologies) containing 15% heat-inactivated fetal bovine serum, 2 mmol/L GlutaMax, 1 mmol/L sodium pyruvate, 50 µg/L gentamicin and 10 ng/mL canine interferon-γ (R&D Systems, Minneapolis, MN, USA) for 24 h at 37°C in humidified air supplemented with 5% CO₂. The following day, cells were exposed to MEM growth media without serum or interferon-γ for 2 h. Following serum deprivation, cells were treated with cIL-31 for 5 min. Cytokine treatment was terminated by removing medium and then adding AlphaScreen SureFire[™] lysis buffer (Perkin Elmer, Waltham, MA, USA) and freezing samples at -20°C.

Signal transduction pathway activation

Cell lysates were used to evaluate phosphorylation of signal transducer and activator of transcription 3 (STAT3) and extracellular signal-

regulated kinase 1/2 (ERK1/2). Activation of STAT3 was detected using the Perkin Elmer AlphaScreen SureFire[™] STAT3 p-Y705 kit, and activation of ERK1/2 was detected using the Perkin Elmer AlphaScreen SureFire[™] MAPK p-T202/Y204 kit, following the manufacturer's protocol. Specifically, 4 µL of cIL-31-treated cell lysates was sequentially incubated with streptavidin-coated donor beads bound with biotinylated capture antibody, then with protein A-coated acceptor beads bound with antibody that recognized the phosphorylation site on the target protein. Assay plates were placed on a Perkin Elmer Envision plate reader to cause excitation of the donor beads at 680 nm. Upon excitation of a donor bead, a singlet oxygen transfer occurs from the donor to an acceptor bead. Any acceptor bead in close proximity to a donor bead (due to the binding of capture and detection antibodies to the desired target protein) emits light at 520–620 nm as a result of a cascade of energy transfer triggered by the singlet oxygen. Light emission was detected by the Envision plate reader. Data were expressed as mean relative signal units, and the EC₅₀ for induction of phosphorylated STAT3 (pSTAT3) and phosphorylated MAPK (pMAPK) was determined by a nonlinear fit model in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Assessment of pruritus in animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (Pfizer Animal Health, Kalamazoo, MI, USA) and were performed in compliance with the Animal Welfare Act, Regulations, 9 CFR Parts 1, 2 and 3, and with the *Guide for the Care and Use of Laboratory Animals*, issued by the US Institute for Laboratory Animal Research Commission of Life Sciences (National Academy Press, Washington, DC, 1996).

Purpose-bred beagle dogs (Marshall BioResources, North Rose, NY, USA) were used in these experiments. Dogs were acclimated for at least 1 h to single-housed runs equipped with ceiling cameras. To evaluate the effects of cIL-31 administration via various routes on pruritic behaviour, cIL-31 (10 µg) or vehicle control [phosphate-buffered saline (PBS) containing equivalent amounts of mammalian host cell proteins to those present in the cIL-31 preparation] was administered intradermally (i.d.), subcutaneously (s.c.) or intravenously (i.v.). Pruritic behaviours (e.g. scratching, licking, chewing, scooting, head shaking and body rubbing) were monitored using video surveillance. Pruritic behaviours were measured as the time (in seconds) over a 4 h baseline period or 4 h after cIL-31 administration by one or more observers who were blinded to the treatment.

To evaluate the pruritic effects of cIL-31 in a statistically powered study, vehicle control-treated animals were compared with cIL-31-treated animals. Pruritic behaviour was evaluated for 2 h starting approximately 30 min after vehicle control or cIL-31 injection (1.75 µg/kg, i.v.) by one or more observers who were blinded to the treatment. Observed pruritic behaviour was measured using a categorical scoring system. 'Yes/no' determinations of displayed pruritic behaviour were made during consecutive, discrete 1 min intervals. The number of minutes categorized as 'yes' for displayed pruritic behaviours for an animal was then summed. The maximal achievable score for a 2 h (120 min) observation period was 120.

Canine serum samples

Blood was collected in 5 mL plastic BD Vacutainer[™] SST[™] tubes (Beckton Dickinson & Co., Franklin Lakes, NJ, USA) with owners' signed informed consent when required, allowed to clot then separated according to the manufacturer's protocol. Serum was collected from the following populations of dogs and frozen prior to measurements of serum cIL-31.

- 1 Experimentally sensitized dogs. Twenty-four purpose-bred beagle dogs (Marshall BioResources) prior to and 1 week after the last exposure to house dust mite (HDM) allergen. Animals were sensitized to *Dermatophagoides farinae* by receiving a series of three 0.5 mL injections containing 10 µg of allergen (Greer Laboratories, Inc., Lenoir, NC, USA), 2.0 mg Rehydragel

(Reheis, Inc., Berkeley Heights, NJ, USA) and 0.4 mL sterile PBS. The injections were administered 2 weeks apart. All animals were approximately 9 months of age, and 12 neutered males and 12 spayed females were evaluated.

- 2 Flea-allergic dogs. Thirty research dogs with established flea allergy (Youngs Veterinary Research Services, Turlock, CA, USA) prior to flea infestation or approximately 1 week after infestation with adult cat fleas (*Ctenocephalides felis*) began. The majority of the dogs in this colony were of mixed breed. The mean age was 10.5 years. This colony consisted of 14 intact females, two spayed females, 11 intact males and three neutered males.
- 3 Pet dogs without allergic disease. Eighty-seven client-owned dogs with subclinical periodontal disease but otherwise determined to be in good health. Samples were collected across 18 veterinary clinics in the USA to perform serum chemistries and titre assessments as part of a screening protocol for entry into a study. All owners had provided written consent for remaining serum to be used in research. No additional samples were collected for this portion of the study. Approximately 86% of the dogs were purebred and approximately 18% of the total population were retrievers [Labrador (13%) and golden (5%)]. The mean age was 3.2 years. The population consisted of 9% intact females, 47% spayed females, 13% intact males and 31% neutered males.
- 4 Pet dogs with nonseasonal atopic dermatitis. Two hundred and twenty-three client-owned animals diagnosed with chronic, non-seasonal AD of at least 1 year duration diagnosed by a board-certified dermatologist [based on modified criteria of Willemse¹⁵ and Prélard *et al.*,¹⁶ with a minimum of 'moderate itching' as assessed by the owner and a minimal skin lesion score of 25 on the Canine Atopic Dermatitis Extent and Severity Index (CADE-SI)-02]. All dogs underwent a diagnostic regimen sufficient to eliminate food allergy, flea allergy dermatitis, bacterial or fungal dermatitis, primary otitis, internal and external parasitism and metabolic disease as the cause of the pruritus. Samples were collected from 14 US specialty dermatology practices to perform serum chemistries as part of a screening protocol for entry into a study. All owners had provided written consent for remaining serum to be used in research. No additional samples were collected for this portion of the study. Approximately 75% of the dogs were purebred and approximately 25% of the total population were retrievers [Labrador (17.3%) and golden (8.2%)]. The mean age was 5.8 years. This population of dogs consisted of 3% intact females, 51% spayed females, 3% intact males and 43% neutered males.

Anti-IL-31 monoclonal antibody production

Anti-canine IL-31 monoclonal antibodies were produced at Maine Biotechnology Services (Portland, ME, USA). CF-1 mice were immunized on a biweekly schedule with cIL-31. Postimmunization, mouse sera and primary fusion products were screened for reactivity to cIL-31 by ELISA. Hybridomas were generated and subcloned by limiting dilution to ensure monoclonal cultures.

Anti-canine IL-31 hybridomas were grown in RPMI 1640 base medium supplemented with 10% ultra-low IgG fetal bovine serum, 2 mmol/L GlutaMAX, 100 U/mL penicillin, 100 µg/mL streptomycin and 55 µmol/L 2-mercaptoethanol. Antibodies were purified from the culture supernatants by protein A or protein G affinity chromatography.

Canine interleukin-31 immunoassays

A Gyrolab sandwich immunoassay was used to quantify cIL-31 levels in canine serum. Serum samples were diluted 1:2 in Rextip buffer (Gyrolab, Warren, NJ, USA) and run on Bioaffy 1000 nL CDs (Gyrolab) using the Gyrolab xP workstation. Canine interleukin-31 was captured with a biotin-labelled anti-IL-31 monoclonal antibody and detected with an Alexafluor 647-labelled anti-IL-31 monoclonal antibody. Sample concentrations of cIL-31 were extrapolated from

an eight-point standard curve with a dynamic range of 0.013–250 ng/mL using a five-parameter fit equation with Gyrolab Evaluator software. The lower limit of quantification was determined to be 13 pg/mL based on the performance of quality control standards at this concentration. Specifically, the 13 pg/mL standards gave values at least two standard deviations above background, accuracy measurements consistently within 20% of intended concentrations, and precision or the percentage coefficient of variance (%CV) within 20%.

Statistical analysis

Data generated from the evaluation of pruritic effects of cIL-31 administered i.v. in beagle dogs were analysed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). PROC MIXED for mixed linear models was used to analyse pruritic score. The model included a fixed effect of treatment and random effects for room, block within room and error. Least squares means were used as estimates of treatment means. Standard errors for treatment means were calculated at 90% confidence intervals for treatment means constructed. All tests (significance of effects and treatment comparisons) were conducted at the two-sided 10% level of significance.

Results

Identification and functional assessment of canine IL-31

Canine interleukin-31 was cloned by RT-PCR from total RNA isolated from canine testicular tissue, a tissue shown to express IL-31 mRNA by other investigators.^{2,13} The nucleotide sequence generated for cIL-31 was identical to the one independently determined and reported (GenBank AB455159).¹³ Protein produced from the generated cIL-31 mammalian expression systems was confirmed to be cIL-31 by N-terminal sequencing and tryptic mapping (see Supporting information Figure S1). To confirm biological activity of the expressed and purified protein, cIL-31 was evaluated for its ability to activate the JAK-STAT, MAPK and PI3K pathways, because these signal transduction pathways have been reported to be involved in the signalling of human and mouse IL-31.⁹ Specifically, cIL-31 treatment led to STAT3 and ERK1/2 phosphorylation in DH82 cells with EC₅₀ values of 53.2 and 84.5 ng/mL, respectively (Figure 1a,b). Phosphorylation of these proteins is indicative of JAK/STAT and MAPK pathway activation, respectively. The phosphorylation of Akt, a marker of PI3K activity, was constitutively turned on in this cell line, so induction of this pathway (PI3K/Akt) by cIL-31 could not be evaluated adequately (see Supporting information Figure S2).

Administration of cIL-31 *in vivo* to purpose-bred beagle dogs caused transient episodes of pruritic behaviour ranging from two- to 10-fold increases above baseline measurements, regardless of the route of administration (Table 1). Behaviours varied among animals. For example, some animals primarily exhibited behaviours such as scratching or head shaking (e.g. dog no. 4807448), whereas others spent most of their time licking (e.g. dog no. 4746538). One animal did not appear to respond to IL-31 injections at all (dog no. 4802098). When pruritic behaviours were displayed, they were readily seen within 4 h after cIL-31 administration and tended to return to baseline levels within 24 h (data not shown). No other obvious clinical signs were observed in the animals. Phosphate-buffered saline vehicle containing residual host cell

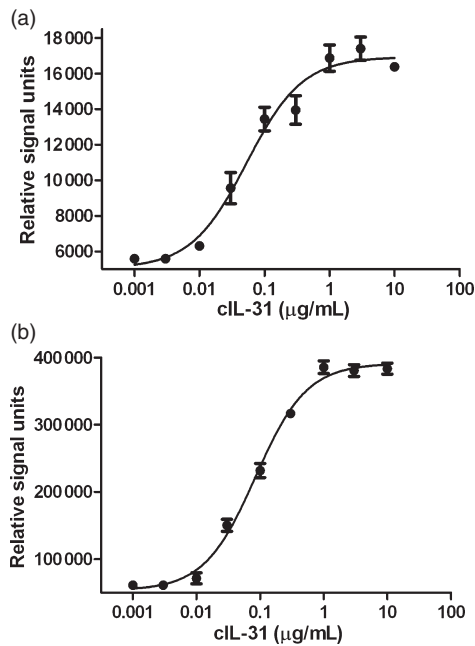


Figure 1. Canine interleukin-31 (cIL-31) induces phosphorylation of STAT3 (pSTAT3) and ERK1/2 (pERK1/2) in DH82 cells. pSTAT3 and pERK1/2 (b) were induced by cIL-31 in a dose-dependent manner. Data are expressed as mean relative signal units $\pm$ SEM ($n = 3$).

proteins at equivalent concentrations to those present in the cIL-31 preparations (mock protein) did not induce pruritic behaviours above baseline levels (e.g. dog no. 3770044). When evaluated over a 2 h period using a categorical scoring system, dogs receiving cIL-31 (i.v.) exhibited a statistically significant increase in mean pruritic score when compared with the vehicle control treatment groups (Figure 2).

Detection of cIL-31 cytokine in dogs with naturally occurring atopic dermatitis

A variety of canine populations were evaluated for the presence of cIL-31 in serum. Levels were not detectable (<13 pg/mL) in the serum from purpose-bred beagle

dogs prior to and after sensitization to HDM ($n = 24$ dogs), mixed breed dogs prior to and after flea infestation ($n = 30$ dogs) or client-owned dogs with periodontal disease but otherwise considered to be in good health, regardless of breed ($n = 87$ dogs). In the dogs with naturally occurring AD, cIL-31 was detectable (≥ 13 pg/mL) in 57% (127 of 223) of the animals, with 52% (117 of 223) of the samples showing serum cIL-31 levels between 13 and 1000 pg/mL, and 4% (10 of 223) showing levels above 1000 pg/mL (Table 2 and Supporting information Table S1).

Discussion

This report describes the generation of canine interleukin-31 protein and the biological function of this cytokine in canine systems. Canine IL-31 was found to activate the JAK-STAT pathway as well as the MAPK pathway in canine cells. Upon administration of cIL-31 to dogs, a significant increase in pruritic behaviours was observed. This study is the first to describe the biological function of IL-31 in canine models. These study results also corroborate the findings from others who have shown the same signalling cascades activated by mouse and human IL-31^{9,17} and have observed pruritic phenotypes in mice infused with or engineered to overexpress IL-31.² Interleukin-31 may therefore play a role in inducing pruritus across a variety of species.

The types of pruritic behaviours observed in dogs after IL-31 injection included scratching, licking, chewing, scooting, head shaking and body rubbing; however, not all behaviours were seen in each animal. Instead, the types of behaviours displayed by each animal varied. One animal (dog no. 4802098) displayed as much pruritic behaviour during baseline monitoring as most dogs did after IL-31 injection, and IL-31 injection in this dog did not appear to increase the amount of pruritus displayed by this animal as determined by video monitoring and quantification of time spent scratching over a 4 h observation period. This dog could have been nonresponsive to IL-31 or may have already had endogenously circulating levels

Table 1. Effects of canine interleukin-31 (cIL-31) administration via different routes on pruritic behaviour in dogs

Dog no.	Total cIL-31 dose (μ g)	Delivery route*	Observed pruritus (s) over 4 h intervals (mean $\pm$ SD)		Fold increase in pruritus after cIL-31 (versus baseline)
			Baseline ($n = 2-3$)†	After cIL-31 Delivery ($n = 1$)‡	
4340761	10	i.d.	28 $\pm$ 26	162	5.8
4807448	10	i.d.	263 $\pm$ 59	862	3.3
4746538	10	i.d.	124 $\pm$ 67	1096	8.8
4701488	10	i.d.	417 $\pm$ 80	916	2.2
3770044	0 (mock protein)	i.d.	348 $\pm$ 111	254	0
4802098	10	s.c.	988 $\pm$ 223	782	0
4814975	10	s.c.	312 $\pm$ 37	885	2.8
4477138	10	s.c.	31 $\pm$ 15	201	6.5
4711921	10	s.c.	232 $\pm$ 84	1547	6.7
4340761	10	i.v.	103 $\pm$ 125	996	9.7
4701488	10	i.v.	480 $\pm$ 235	989	2.1
4477138	10	i.v.	163 $\pm$ 123	1147	7
3770044	0 (mock protein)	i.v.	359 $\pm$ 78	137	0

Observed pruritus (in seconds) over 4 h intervals is listed for baseline observations (means $\pm$ SD) and observations taken after cIL-31 treatment.

Fold increase in pruritus from baseline is also calculated.

*i.d., intradermal injection (0.2 mL volume); s.c., subcutaneous injection (0.2 mL volume); or i.v., intravenous injection (1 mL volume).

†Replicate baseline observations were made on separate days but at the same time of day. Data represent means $\pm$ SD.

‡Administration of cIL-31 was performed on a different day from baseline observations, but at the same time of day.

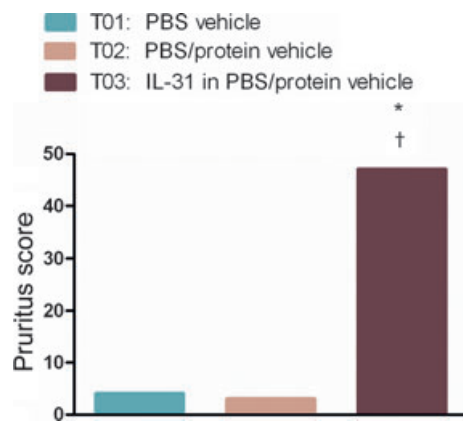


Figure 2. Canine interleukin-31 (cIL-31) administered intravenously induced pruritus in dogs. Pruritic scores from phosphate-buffered saline (PBS) vehicle-treated (T01), PBS/protein vehicle-treated (T02) and 1.75 µg/kg cIL-31 in PBS/protein vehicle-treated (T03) animals were compared. Pruritic behaviour was evaluated for 2 h starting approximately 30 min after cIL-31 injection. Mean pruritic scores (back transformed least square means) are shown. Canine IL-31 significantly induces pruritic behaviour in dogs compared with the vehicle control-treated groups (*T01 versus T03, $P = 0.0004$; †T02 versus T03, $P = 0.0003$). No significant difference was observed between T01 versus T02).

of IL-31. This animal was a neutered male 4 years of age and the heaviest dog in the study, weighing 26.8 kg. The correlation between body weight and IL-31 serum levels was not evaluated in this study but will be an important factor to consider in the future.

Evaluating the relevance of IL-31 in naturally occurring pruritic skin diseases, such as AD, was of interest in the present study, because observations from laboratory models do not always accurately reflect pathways or mediators involved in naturally occurring, chronic disease conditions. When serum samples from a variety of canine populations were analysed for IL-31 protein levels, IL-31 was detected in 57% of dogs diagnosed with naturally occurring AD. Interleukin-31 was not detected in normal

Table 2. Number of animals with detectable serum IL-31 in various canine populations

Canine population	Number of animals evaluated	Number of animals with detectable cIL-31 in serum*
Purpose-bred beagle dogs	24	0
Purpose-bred beagle dogs sensitized to house dust mite	24	0
Mixed breed dogs with no fleas	30	0
Mixed breed dogs infested with fleas	30	0
Healthy client-owned dogs of multiple breeds	87	0
Naturally occurring atopic dermatitis in client-owned dogs of multiple breeds	223	127

*Protein levels of cIL-31 were measured in canine populations using immunoassay techniques. The number of animals evaluated and the number of animals in which cIL-31 was detected (≥ 13 pg/mL) are listed for each population. A value < 13 pg/mL is below the limit of quantification.

dogs, in pruritic, flea-allergic dogs or in dogs sensitized to the HDM allergen *D. farinae*. These findings suggest that IL-31 may be dysregulated in dogs with AD and may contribute to the pathobiology of this chronic allergic skin disease. It is not surprising that IL-31 was not detected in HDM-sensitized dogs, because these dogs were not pruritic after the sensitization protocol. However, IL-31 levels were also not detected in the serum from flea-allergic dogs, even though these animals displayed pruritic behaviours. One explanation could be that this cytokine is not a key mediator of pruritus in flea allergy dermatitis. Alternatively, IL-31 could be acting locally within the skin and not readily detected in the serum. The length of flea infestation may also play a role. These animals were exposed to fleas for only 1 week prior to sampling. Interleukin-31 levels were not assessed in animals with prolonged infestation with fleas.

Although IL-31 was detected in 57% of dogs with AD, a large percentage of animals with AD (43%) did not display detectable levels of IL-31 (< 13 pg/mL). These animals may have had circulating levels of IL-31 that were below our assay limits of detection, or possibly IL-31 levels were acting locally within target tissues and not released into the circulation. Alternatively, IL-31 dysregulation may not play a significant role in the aetiology of AD in these animals with undetectable IL-31 levels. The latter interpretation is consistent with the concept that canine AD is a multifactorial disease that involves complex interactions between susceptibility genes, skin barrier dysfunction, immune dysregulation and neuroimmune interactions that collectively produce a hypersensitivity to environmental allergens and a pruritic allergic skin condition in dogs. Owing to the complexity of the disease, it is not surprising that not all dogs exhibit the same molecular or cellular changes.

Continued investigations of IL-31 in the canine AD population to determine whether serum levels correlate with a variety of parameters, such as age, breed, weight, sex and disease severity, will be important to improve our understanding of the role of IL-31 in this disease. Several investigators interested in the role of IL-31 in human AD have already extended their evaluations to the protein level and have generated data correlating serum levels of IL-31 to disease severity.^{3,7} Specifically, studies have shown IL-31 to be elevated in the serum of human AD patients compared with healthy control subjects and that serum IL-31 levels correlate with disease severity in adults as well as children with AD.

Interleukin-31 in canine AD has been a topic of interest to other groups. Mizuno *et al.*¹³ used RT-PCR techniques to evaluate IL-31 mRNA levels in a variety of canine tissues and in the skin of dogs with AD. This group was able to detect canine IL-31 mRNA in a variety of tissues; however, they were not able to detect IL-31 mRNA in the skin of dogs with naturally occurring AD ($n = 9$). They hypothesized that the biological function of IL-31 may be different in dogs versus other species or that evaluations of IL-31 protein could be more informative than looking at mRNA levels, given that mRNA levels do not always mimic changes that can be seen at the protein level. In our study, we were able to extend assessments of IL-31 to the protein level using quantitative immunoassay techniques and were able to detect elevated levels in the

serum of dogs with AD. We were also able to evaluate a large population of dogs with naturally occurring AD ($n = 224$), which may have improved our chances of detecting IL-31 alterations in the canine AD population. Future studies should extend assessments of IL-31 protein levels to the skin of dogs with AD. Investigators have begun to perform these types of assessments in human AD patients and have found that IL-31 protein is elevated in the inflammatory infiltrates of skin biopsy specimens taken from AD subjects compared with skin biopsy specimens from patients with other types of skin diseases, suggesting that IL-31 dysregulation could be unique to AD.⁸

In summary, we demonstrated that canine IL-31 injected systemically or locally can induce pruritic behaviours in dogs and that this cytokine is elevated in a significant number of dogs with AD. We believe this pathway may play a role in the pathobiology of pruritic allergic skin conditions, such as canine AD, and may represent a novel pathway for therapeutic intervention.

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References

- Halliwell R. Revised nomenclature for veterinary allergy. *Vet Immunol Immunopathol* 2006; 114: 207–208.
- Dillon SR, Sprecher C, Hammond A *et al*. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nat Immunol* 2004; 5: 752–760.
- Raap U, Wichmann K, Bruder M *et al*. Correlation of IL-31 serum levels with severity of atopic dermatitis. *J Allergy Clin Immunol* 2008; 122: 421–423.
- Sonkoly E, Muller A, Lauerma AI *et al*. IL-31: a new link between T cells and pruritus in atopic skin inflammation. *J Allergy Clin Immunol* 2006; 117: 411–417.
- Bilsborough J, Leung DY, Maurer M *et al*. IL-31 is associated with cutaneous lymphocyte antigen-positive skin homing T cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 2006; 117: 418–425.
- Kim S, Kim HJ, Yang HS *et al*. IL-31 serum protein and tissue mRNA levels in patients with atopic dermatitis. *Ann Dermatol* 2011; 23: 468–473.
- Ezzat MH, Hasan ZE, Shaheen KY. Serum measurement of interleukin-31 (IL-31) in paediatric atopic dermatitis: elevated levels correlate with severity scoring. *J Eur Acad Dermatol Venereol* 2011; 25: 334–339.
- Nobbe S, Dziunycz P, Mühleisen B *et al*. IL-31 expression by inflammatory cells is preferentially elevated in atopic dermatitis. *Acta Derm Venereol* 2012; 92: 24–28.
- Zhang Q, Putheti P, Zhou Q *et al*. Structures and biological functions of IL-31 and IL-31 receptors. *Cytokine Growth Factor Rev* 2008; 19: 347–356.
- Kasraie S, Niebuhr M, Baumert K *et al*. Functional effects of interleukin 31 in human primary keratinocytes. *Allergy* 2011; 66: 845–852.
- Kasraie S, Niebuhr M, Werfel T. Interleukin (IL)-31 induces pro-inflammatory cytokines in human monocytes and macrophages following stimulation with staphylococcal exotoxins. *Allergy* 2010; 65: 712–721.
- Bando T, Morikawa Y, Komori T *et al*. Complete overlap of interleukin-31 receptor A and oncostatin M receptor β in the adult dorsal root ganglia with distinct developmental expression patterns. *Neuroscience* 2006; 142: 1263–1271.
- Mizuno T, Kanbayashi S, Okawa T *et al*. Molecular cloning of canine interleukin-31 and its expression in various tissues. *Vet Immunol Immunopathol* 2009; 131: 140–143.
- Lindenbaum M, Perkins E, Csonka E *et al*. A mammalian artificial chromosome engineering system (ACE System) applicable to biopharmaceutical protein production, transgenesis and gene-based cell therapy. *Nucleic Acids Res* 2004; 32: e172.
- Willemse T. Atopic skin disease: a review and a reconsideration of diagnostic criteria. *J Small Anim Pract* 1986; 27: 771–778.
- Prélaud P, Guaguère E, Alhaidari Z *et al*. Reevaluation of diagnostic criteria of canine atopic dermatitis. *Rev Med Vet (Toulouse)* 1998; 149: 1057–1064.
- Diveu C, Lelievre E, Perret D *et al*. GPL, a novel cytokine receptor related to GP130 and leukemia inhibitory factor receptor. *J Biol Chem* 2003; 278: 49850–49859.

Supporting Information

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

Figure S1. Amino acid sequence of the canine IL-31 construct. The identity of purified canine IL-31 protein was confirmed using mass spectrometry to analyse a tryptic digest of the protein and also by N-terminal sequencing.

Figure S2. Detection of phospho-Akt in cIL-31-treated DH82 cells. Using western blotting techniques, phospho-Akt levels were evaluated in DH82 cells treated with varying concentrations of IL-31 (0–10 $\mu\text{g/mL}$).

Table S1. Individual cIL-31 serum levels in dogs with atopic dermatitis. Protein levels of cIL-31 were measured in serum using immunoassay techniques in client-owned animals diagnosed with atopic dermatitis. Quantitative levels of cIL-31 in serum are listed for each dog evaluated.

Résumé

Contexte – L'interleukine-31 (IL-31) est un membre de la famille des cytokines interleukine-6/gp130 qui est produit par des types cellulaires comme les lymphocytes T helper 2 et les cellules T cutanée lymphocytaire CLA positives (cutaneous lymphocyte antigen). Lorsque surexprimés chez des souris transgéniques, IL-31 provoque un prurit sévère, de l'alopecie et des lésions cutanées. Chez l'homme, les taux d'IL-31 sériques sont en corrélation avec la sévérité de la dermatite atopique chez les adultes et les enfants.

Hypothèse/Objective – Déterminer le rôle de l'IL-31 dans le prurit canin et la dermatite atopique canine naturelle (AD).

Animaux – Des chiens beagles ont été utilisés pour des études en laboratoire. Des échantillons de sérum ont été prélevés sur des animaux de laboratoire, sur des chiens sains et naturellement atopiques de cli-

ents.

Méthodes – Des beagles de laboratoire ont reçu de interleukine-31 canine (ILc-31) par plusieurs voies (intraveineuse, sous-cutanée ou intradermique) et leur prurit a été quantifié/observé par vidéo surveillance. Les techniques quantitatives d'immunomarquage ont été utilisées pour mesurer les concentrations sériques de l'ILc-31 chez les chiens.

Résultats – L'injection d'ILc-31 aux beagles de laboratoire a causé des épisodes transitoires de prurit quel que soit la voie d'administration. Lorsqu'évalué sur une période de 2 h, le prurit a augmenté de façon significative chez les chiens recevant de l'ILc-31 comparé aux chiens recevant un placebo. En outre, les niveaux de ILc-31 étaient décelables pour 57 % des chiens naturellement atopiques (≥ 13 pg/mL) mais en dessous des limites de quantification (< 13 pg/mL) chez les chiens sains de laboratoire ou appartenant à des clients.

Conclusions – L'IL-31 canine induit du prurit chez les chiens. L'IL-31 canine a été détectée dans la majorité des chiens naturellement atopiques, suggérant que cette cytokine peut jouer un rôle important dans les dermatoses prurigineuses allergiques comme la dermatite atopique chez cette espèce.

Resumen

Introducción – la interleuquina 31 (IL-31) es un miembro de la familia de citoquinas gp130/IL-6 que se producen en células tales como los linfocitos T-2 ayudantes y linfocitos T cutáneos estimulados antigénicamente con tropismo cutáneo. Cuando esta interleuquina se expresa en exceso en ratones transgénicos induce prurito severo, alopecia y lesiones de la piel. En humanos, los niveles en suero de la IL-31 están correlacionados con la severidad de la dermatitis atópica en adultos y niños.

Hipótesis/Objetivos – determinar el papel de la IL-31 en el desarrollo del prurito canino y en dermatitis atópica espontánea (AD).

Animales – Se utilizaron perros de laboratorio de raza Beagle para los estudios laboratoriales. Se obtuvieron muestras de suero de los animales de laboratorio, de animales de propietarios particulares sin enfermedad, y de animales de propietarios particulares con AD espontánea.

Métodos – se administró IL-31 a perros de laboratorio de raza Beagle por varias rutas (intravenosa, subcutánea, intradérmica) y se observó el comportamiento indicativo de prurito, cuantificándolo mediante observación en grabaciones de video. Se utilizaron técnicas cuantitativas de inmunoensayo para medir los niveles de cIL-31 en el suero de los perros.

Resultados – la inyección de IL-31 a los perros Beagle de laboratorio produjo episodios transitorios de comportamiento indicativo de prurito, independientemente de la vía de administración. Cuando se evaluaron durante un periodo de 2 horas, los perros que recibieron IL-31 presentaron un incremento perceptible de comportamiento indicativo de prurito en comparación con los animales a los que se administró placebo. Además hubo niveles detectables de cIL-31 en un 57% de perros con AD espontánea (≥ 13 pg/mL), pero estuvieron por debajo de los niveles de detección (< 13 pg/mL) en perros normales de laboratorio o sin enfermedad de propietarios particulares.

Conclusiones – la IL-31 canina induce comportamiento indicativo de prurito en perros. La IL-31 se detectó en el suero en la mayoría de los animales con AD espontánea, lo que sugiere que esta citoquina puede tener un papel importante en procesos alérgicos pruriginosos de la piel, tales como la dermatitis atópica, en esta especie.

Zusammenfassung

Hintergrund – Interleukin-31 (IL-31) ist Teil der gp130/Interleukin-6 Zytokinfamilie, welche von Zelltypen wie den T Helfer2 Lymphozyten und „skin homing“ T Zellen, die das kutane Lymphozytenantigen exprimieren, produziert werden. Wenn es in transgenen Mäusen überexprimiert wird, verursacht IL-31 hochgradigen Juckreiz, Alopezie und Hautveränderungen. Beim Menschen korrelieren die Werte von IL-31 im Serum mit dem Schweregrad der atopischen Dermatitis bei Erwachsenen und Kindern.

Hypothese/Ziele – Die Rolle von IL-31 beim Juckreiz des Hundes und bei natürlich vorkommender atopischer Dermatitis des Hundes (AD) zu bestimmen.

Tiere – Es wurden für diesen Zweck gezüchtete Beagles für die Laborstudien verwendet. Es wurden Serumproben von den Labortieren, sowie von Privathunden ohne Krankheit und von Privathunden mit der Diagnose einer natürlich auftretenden AD entnommen.

Methoden – Den für diesen Zweck gezüchteten Beagles wurde canines Interleukin-31 (cIL-31) auf unterschiedlichem Wege (intravenös, subkutan oder intradermal) verabreicht, auftretender Juckreiz wurde beobachtet bzw. quantitativ mittels Videoaufzeichnung beurteilt. Es wurden quantitative Immunassays verwendet, um die Werte von cIL-31 im Blut von Hunden zu messen.

Ergebnisse – Eine Injektion von cIL-31 bei den Beagles aus dem Labor verursachte unabhängig von der Route der Administration vorübergehende Episoden von Juckreiz. Während einer 2 stündigen Zeitspanne zeigten Hunde, die cIL-31 erhalten hatten, einen signifikanten Anstieg des Juckreizes im Vergleich zu Hunden, die ein Placebo erhalten hatten. Zusätzlich waren cIL-31 Werte bei 57% der Hunde mit natürlich auftretender AD (≥ 13 pg/mL) feststellbar, lagen aber bei normalen, bei gesunden Laborhunden oder bei privaten Tieren unter der quantitativen Nachweisgrenze (< 13 pg/mL).

Schlussfolgerungen – Canines IL-31 verursachte bei Hunden Juckreiz. Canines IL-31 wurde bei der Mehrheit der Hunde mit natürlich auftretender AD festgestellt, was darauf hinweist, dass diese Zytokine möglicherweise eine wichtige Rolle bei juckenden allergischen Hauterkrankungen, wie zum Beispiel bei der atopischen Dermatitis dieser Spezies, spielen könnten.

要約

背景–インターロイキン-31 (IL-31) はgp130/インターロイキン-6サイトカインファミリーの1つで、ヘルパーT2リンパ球とリンパ球抗原陽性の皮膚に誘導されるT細胞によって産生される。トランスジェニックマウスで、過剰発現させたとき、IL13は重度のそう痒、脱毛および皮膚症状を誘発する。ヒトではIL-31の血清レベルは成人と小児のアトピー性皮膚炎の重症度と相関する。

仮説/目的–イヌの瘙痒と自然発症性イヌアトピー性皮膚炎 (AD) でのIL-31の役割を調査する。

供与動物–目的に応じて繁殖したビーグル犬が実験に用いられた。血清サンプルは実験動物、病気のない飼い犬、自然発症性ADと診断された飼い犬から得られた。

方法–目的に応じて繁殖したビーグル犬に複数の経路 (静脈内、皮下、あるいは皮内) でイヌインターロイキン-31 (cIL-31) を投与し、ビデオでのモニターを介してそう痒の行動を観察/定量した。イヌのcIL-31の血清レベルの測定には定量的免疫測定方法を用いた。

結果–cIL-31への実験ビーグル犬の注射は投与ルートに関わらず、そう痒に関する行動を一過性に生じた。評価した2時間以上の間に、cIL-31を投与されていた犬はプラセボを投与されていた犬と比較して瘙痒に関する行動が有意に増加した。さらに、cIL-31レベルは自然発症性ADの犬の57%で検出可能であった (≥ 13 pg / mL) が、正常な病気のない実験犬あるいは飼い犬では検出値以下であった (< 13 pg / mL)。

結論–イヌ IL-31 は犬にそう痒に関する行動を誘発する。イヌ IL-31 は自然発症性 AD の犬の大多数で検出できることはこのサイトカインはこの動物種でアトピー性皮膚炎などのそう痒性アレルギー性皮膚症状へ重要な役割を果たしているかもしれない。

摘要

背景 – 白介素-31 (IL31) は糖蛋白130/白介素-6細胞因子の家族成員、由多种类型的细胞制造, 如Th-2型淋巴细胞和皮肤淋巴细胞抗原阳性的皮肤归巢T细胞。当转基因小鼠过度表达时, IL-31诱导产生严重的瘙痒、脱毛和皮肤病变。人的IL-31血清水平与成人和儿童异位性皮炎的严重程度有关。

假设/目的 – 确定IL-31在犬瘙痒和自发性犬异位性皮炎 (AD) 中的作用。

动物 – 繁殖用比格犬被用于试验研究。血清样本取自试验动物、客户饲养的健康犬和客户饲养的被诊断为自发性异位性皮炎的患犬。

方法 – 繁殖用比格犬, 通过不同途径 (静脉、皮下或皮内) 给予犬白介素-31 (cIL-31), 并通过监视器观察/量化瘙痒行为。使用定量免疫检测技术测量犬血清cIL-31水平。

结果 – 不管采取哪种给药途径, 注射cIL-31到试验比格犬都可引起瘙痒行为的短暂发作。当超过2h评估时, 接受cIL-31的犬与接受安慰剂的犬相比表现出瘙痒行为显著增加。此外, 在患有自发性异位性皮炎的犬中, 有57%可检测到cIL-31水平 (≥ 13 pg / mL), 但健康的实验动物或客户饲养的动物中低于定量极限 (< 13 pg / mL)。

结论 – 犬IL-31可诱导犬瘙痒行为。在大多数自发性异位性皮炎患犬中发现犬IL-31, 提示对于犬这个物种, 这种细胞因子在瘙痒性过敏皮肤病如异位性皮炎中发挥重要作用。

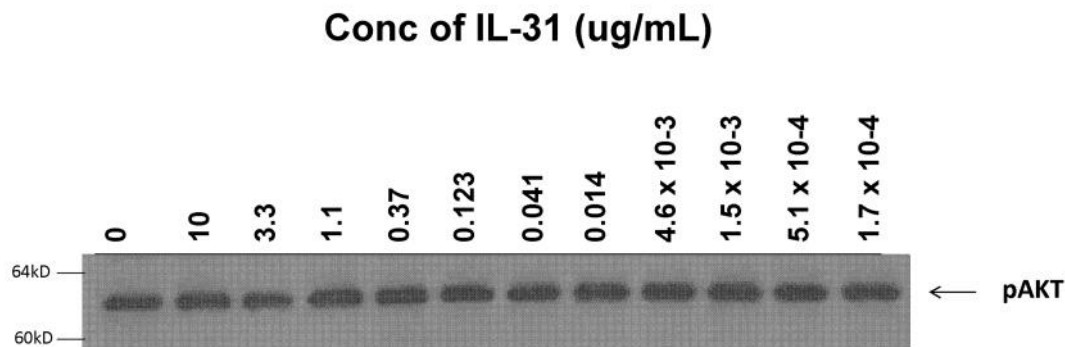
MLSHTGPSRFALFLLCSMETLLSSHMA**PT**HQLPPSDVRKIILELQPLSRGLLEDYQKK**ET**GV**PES**NR**T**
LLLCLTSDSQPPRLNSSAILPYFRAIRPLSDKNIIDKIIQLDKLK**FQ**HEPETEISVPAD**TFECK**SFILTILQQ
FSACLESVFKSLNSGPQ

Supplementary Figure 1: Amino acid sequence of the canine IL-31 construct. Bold type indicates the predicted leader sequence (removed at the time of protein secretion from mammalian cells). Highlighted portions indicate tryptic peptides identified using nanoUPLCMS. Tryptic mapping covered 69% of the protein sequence. N-terminal sequencing confirmed the start sequence of canine IL-31 to begin at AP**THQ**. This sequence is identical to the deposited sequence (GenBank BAH97742).

Methods:

The identity of purified canine IL-31 protein was confirmed using mass spectrometry to analyze a tryptic digest of the protein and also by N-terminal sequencing. For peptide mass fingerprinting, the protein sample was separated using SDS-PAGE and visualized using Simply Blue Coomassie stain (Invitrogen, Grand Island, NY, USA). The band was excised, chopped into three pieces, and washed with alternating washes of 25 mM ammonium bicarbonate (ABC) and acetonitrile (ACN). Following removal of the ACN, samples were reduced with dithiothreitol, alkylated with iodoacetamide, and washed with alternating washes of 25 mM ABC and ACN before being digested with trypsin (Promega, Madison, WI, USA; 20 µg/mL in 25 mM ABC). The digest was carried out for 16 h at 37°C before being stopped by the addition of formic acid to a final 0.3%. The acidified digest solution was pipetted away from the gel pieces and analyzed by liquid chromatography – mass spectrometry (LC-MS). Briefly, peptides were separated by reversed-phase HPLC (Waters 0.1 x 100 mm, 1.7 µm BEH column) using a 45-min, acetonitrile gradient of 0-45% in 0.1% formic acid at 1 µL/min. Mass spectrometry analysis was performed on a Waters Synapt™ High Definition MS system (Waters, Milford, MA, USA) operating in positive ion mode. The quadrupole was set for optimal transmittal of ions from m/z 50 to 2000. Accurate mass data were collected in an alternating low energy (MS, 6-eV constant collision energy) and high energy (MS^E, 15-40-eV ramp) mode of acquisition, with calibration every 30 seconds to ensure accuracy. The peak data were processed and searched using ProteinLynx Global Server™ version 2.3 (Waters), and used to query a modified, in-house Swiss-Prot database for peptide / protein identification.

For N-terminal sequencing the protein was separated by SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA USA). The band of interest was excised and analyzed by Edman degradation/sequencing. The result of the sequencing confirmed the expected N-terminal AA residues for canine IL-31 to begin at AP**THQ**.



Supplementary Figure 2: Detection of phospho-Akt in IL-31-treated DH82 cells. Using Western blotting techniques, phospho-Akt levels were evaluated in DH82 cells treated with varying concentrations of IL-31 (0-10 ug/mL). Phosphorylated levels of Akt were detectable in the DH82 cells in the absence of serum and IL-31 (first lane on the left) suggesting this protein was constitutively turned on in this cell line and not regulated by the presence or absence of serum or IL-31 cytokine. One representative western blot out of three is shown.

Methods:

The DH82 canine monocytic cell line (American Type Culture Collection, Manassas, VA, USA.) was used to evaluate cIL-31 cytokine function. DH82 cells were plated into CoStar 96-well flat bottom cell culture plates (Corning, Tewksbury, MA, USA.) at a density of 1×10^5 cells/well in MEM growth media (Life Technologies, Grand Island, NY, USA) containing 15% heat inactivated fetal bovine serum, 2 mM GlutaMax, 1 mM sodium pyruvate, 50 µg/L gentamicin, and 10 ng/mL canine IFN γ (R&D Systems, Minneapolis, MN, USA) for 24 h at 37°C, 5% CO $_2$. The following day, cells were exposed to MEM growth media without serum or IFN- γ for 2 h. Following serum deprivation, cells were treated with cIL-31 for 5 min. Cytokine treatment was terminated by removing medium then adding AlphaScreen SureFire™ lysis buffer (Perkin Elmer, Waltham, MA, USA) and freezing samples at -20°C. Protein lysates were thawed and 12 µl of lysate was combined with 4 µl of 4X protein loading buffer (Thermo Scientific, Waltham, MA, USA). Samples were boiled for 5 min and loaded onto a 4-20% Tris-Glycine gel (Life Technologies). Protein samples were then transferred to a nitrocellulose membrane, and phospho-Akt was detected by sequential incubation with an anti-p-Akt (Ser 473) rabbit polyclonal antibody (Cell Signaling Technologies, Beverly, MA, USA) and a goat anti-rabbit-HRP polyclonal antibody (BioRad, Hercules, CA USA). Bands were visualized using electrochemiluminescence (GE Healthcare, Buckinghamshire, UK).

Supplementary Table 1: Individual IL-31 serum levels in dogs with atopic dermatitis

Date Serum Sample Collected	Animal #	IL-31 serum levels (ng/mL)	Date Serum Sample Collected	Animal #	IL-31 serum levels (ng/mL)	Date Serum Sample Collected	Animal #	IL-31 serum levels (ng/mL)
14JUN10	81009	0.013	04MAY10	89001	0.083	10MAY10	79016	BLQ
09JUN10	84015	0.013	03MAY10	92002	0.085	11MAY10	79017	BLQ
30JUN10	84019	0.014	05MAY10	91002	0.094	12MAY10	79018	BLQ
27MAY10	84010	0.014	26APR10	83003	0.098	12MAY10	79019	BLQ
27MAY10	84009	0.015	23JUN10	90010	0.099	17MAY10	79021	BLQ
09JUN10	89010	0.015	20MAY10	91013	0.100	03JUN10	79022	BLQ
11MAY10	87004	0.016	06MAY10	79014	0.101	03MAY10	80001	BLQ
26APR10	83004	0.016	06MAY10	91009	0.105	10MAY10	80004	BLQ
05MAY10	84003	0.016	07MAY10	88007	0.115	10MAY10	80006	BLQ
21MAY10	83017	0.016	03JUN10	88011	0.128	10MAY10	80007	BLQ
20MAY10	90004	0.017	11MAY10	85012	0.130	10MAY10	80008	BLQ
07MAY10	83010	0.018	19MAY10	91010	0.136	24MAY10	80009	BLQ
20MAY10	90005	0.018	26JUN10	94016	0.137	01JUN10	80010	BLQ
07MAY10	88005	0.019	28MAY10	87010	0.138	01JUN10	80011	BLQ
20MAY10	87007	0.019	28MAY10	77014	0.142	14JUN10	80012	BLQ
21MAY10	94009	0.020	12MAY10	83013	0.148	21JUN10	80014	BLQ
14JUN10	80013	0.021	03MAY10	79001	0.157	03MAY10	81001	BLQ
27MAY10	90007	0.022	21JUN10	83022	0.178	03MAY10	81002	BLQ
19MAY10	85013	0.025	05MAY10	84002	0.179	06MAY10	81003	BLQ
14MAY10	94005	0.025	10JUN10	91016	0.225	17MAY10	81004	BLQ
29MAY10	94011	0.025	15JUN10	84017	0.240	20MAY10	81005	BLQ
10JUN10	90008	0.026	21MAY10	94008	0.243	10JUN10	81007	BLQ
14MAY10	94004	0.027	06MAY10	87002	0.252	01JUL10	81010	BLQ
10MAY10	92007	0.027	03MAY10	79003	0.275	26APR10	83001	BLQ
07MAY10	94002	0.028	19MAY10	89002	0.280	26APR10	83002	BLQ
14MAY10	94006	0.028	06MAY10	88004	0.306	27APR10	83005	BLQ
05JUN10	84013	0.028	07MAY10	92004	0.310	28APR10	83006	BLQ
12JUN10	94015	0.030	07JUN10	81006	0.360	30APR10	83007	BLQ
14MAY10	94003	0.031	11MAY10	87003	0.376	04MAY10	83008	BLQ
09JUN10	92012	0.031	05MAY10	92003	0.379	07MAY10	83009	BLQ
28MAY10	94010	0.031	17MAY10	90003	0.424	11MAY10	83012	BLQ
02JUN10	94012	0.031	10MAY10	83011	0.429	21MAY10	83018	BLQ
25JUN10	92013	0.032	06MAY10	91006	0.452	25MAY10	83019	BLQ
05JUN10	94014	0.032	20MAY10	91012	0.492	04JUN10	83020	BLQ
26-Jun-10	4025	0.033	10MAY10	80005	0.545	07JUN10	83021	BLQ
05MAY10	94001	0.034	10JUN10	88012	0.682	05MAY10	84001	BLQ

26MAY10	92011	0.034	14JUN10	81008	0.724	05MAY10	84004	BLQ
16JUN10	91018	0.035	06MAY10	91003	0.750	10MAY10	84006	BLQ
16JUN10	91017	0.035	07MAY10	88006	0.763	11MAY10	84007	BLQ
27MAY10	91014	0.036	14MAY10	83015	0.826	18MAY10	84008	BLQ
25MAY10	85014	0.036	03MAY10	79004	0.870	28MAY10	84011	BLQ
26-Jun-10	15009	0.037	03MAY10	80002	0.875	28MAY10	84012	BLQ
26MAY10	90006	0.038	15JUN10	87011	0.901	05JUN10	84014	BLQ
28JUN10	89013	0.038	06MAY10	91005	1.040	09JUN10	84016	BLQ
04JUN10	94013	0.041	13MAY10	79020	1.1	25JUN10	84018	BLQ
27MAY10	91015	0.043	04MAY10	88002	1.350	04MAY10	85001	BLQ
02JUN10	88010	0.045	01JUL10	90011	1.790	04MAY10	85002	BLQ
03MAY10	80003	0.047	12MAY10	83014	2.000	04MAY10	85003	BLQ
20MAY10	91011	0.048	19MAY10	77010	2.410	04MAY10	85004	BLQ
07MAY10	92005	0.050	22JUN10	85016	2.450	05MAY10	85005	BLQ
04MAY10	93001	0.050	03MAY10	87001	2.850	05MAY10	85006	BLQ
26MAY10	77013	0.051	21MAY10	83016	3.050	11MAY10	85008	BLQ
01JUL10	77016	0.052	17JUN10	88014	7.800	11MAY10	85009	BLQ
10MAY10	79015	0.0543	05MAY10	77001	BLQ	11MAY10	85010	BLQ
04MAY10	79006	0.055	06MAY10	77002	BLQ	11MAY10	85011	BLQ
12MAY10	92009	0.055	07MAY10	77003	BLQ	25MAY10	85015	BLQ
12MAY10	92008	0.058	07MAY10	77004	BLQ	17MAY10	87005	BLQ
10MAY10	84005	0.060	11MAY10	77005	BLQ	18MAY10	87006	BLQ
10MAY10	92006	0.061	12MAY10	77006	BLQ	04MAY10	88001	BLQ
14MAY10	94007	0.063	13MAY10	77007	BLQ	04MAY10	88003	BLQ
19MAY10	92010	0.064	13MAY10	77008	BLQ	11MAY10	88008	BLQ
06MAY10	91008	0.064	18MAY10	77009	BLQ	11MAY10	88009	BLQ
25JUN10	92014	0.066	19MAY10	77011	BLQ	10JUN10	88013	BLQ
03MAY10	92001	0.067	20MAY10	77012	BLQ	19MAY10	89003	BLQ
06MAY10	91004	0.068	11JUN10	77015	BLQ	19MAY10	89004	BLQ
24MAY10	87008	0.069	03MAY10	79002	BLQ	19MAY10	89005	BLQ
01JUL10	88016	0.071	04MAY10	79005	BLQ	19MAY10	89006	BLQ
01JUL10	88015	0.072	04MAY10	79007	BLQ	19MAY10	89007	BLQ
05MAY10	91001	0.073	05MAY10	79008	BLQ	02JUN10	89008	BLQ
17JUN10	90009	0.077	05MAY10	79009	BLQ	02JUN10	89009	BLQ
25MAY10	93002	0.077	05MAY10	79010	BLQ	16JUN10	89011	BLQ
11MAY10	85007	0.080	05MAY10	79011	BLQ	24JUN10	89012	BLQ
06MAY10	91007	0.081	06MAY10	79012	BLQ	30JUN10	89014	BLQ
25MAY10	87009	0.081	06MAY10	79013	BLQ	06MAY10	90001	BLQ
BLQ = Below Limits of Quantitation (<0.013 ng/mL IL-31)						12MAY10	90002	BLQ

