ABSTRACT

TAMAMOTO-MOCHIZUKI, CHIE. Investigating the Role of Interleukin-31 in an Experimental Model of Acute Canine Atopic Dermatitis (Under the direction of Dr. Thierry Olivry and Dr. Scott Laster)

Atopic dermatitis (AD) is a most common, chronically relapsing, allergic skin disease of humans and dogs. Recent studies have revealed that interleukin 31 (IL-31) represents one of the critical cytokines in human and canine AD, however, there are occasional failures of disease control with IL-31 inhibition, indicating that further researched are needed.

In our first experiment (Chapter 2), we tested if the proactive administration of the anti-canine IL-31 monoclonal antibody (mAb), lokivetmab (LKV), would prevent or delay flares of canine AD in experimental and clinical settings. Using our canine AD model, one injection of LKV prevented nearly all expected allergen-induced pruritus manifestations but not skin lesion development. In dogs with spontaneous AD, 28% of dogs did not exhibit an AD flare for at least one year while receiving LKV-monotherapy. On the other hand, the median time-to-flare after starting LKV-proactive therapy was only 63 days. This observation led to the hypothesis that IL-31 has a minor role in skin lesion development in contrast to its central role in atopic itch, which might explain the insufficient effect of current strategies for inhibiting IL-31 to delay AD flares in some dogs.

To have a better understanding of the role of IL-31 in the development of canine AD skin lesions, we assessed the chronology of the expression of IL-31 and determined the identity of cells producing IL-31 in experimental acute AD skin lesions of dogs (Chapter 3). We collected skin samples from four house-dust-mite (HDM)-experimentally-sensitized atopic dogs at 0, 24, 48 and 96 hours after allergen provocation. IL-31 single-staining immunofluorescence (IF), as well as IL-31/CD3 and IL-31/CD4 double-staining IF were performed. In three of four dogs, the highest numbers of IL-31-positive cells occurred at 24 or 48 hours, and it started to decrease at 96 hours. The majority of IL-31-positive cells co-expressed CD3 (range:91-100%) and CD4 (range:63-100%), indicating that they were helper T cells. Unexpectedly, sebaceous glands were strongly immunolabeled with IL-31; however, we could not detect *IL31* mRNA in any sebaceous glands using the RNAscope method, thus could not yet confirm these cells as representing another novel cellular source of IL-31 (Chapter 4). These findings suggested an early and

transient production of IL-31 by Th2 cells, supporting the concept of using IL-31 inhibiting LKV as a proactive therapy to prevent AD flares.

These observations led to the hypothesis that other cytokines, independent of IL-31, are responsible for developing acute atopic inflammation and contribute to the treatment failures in IL-31-inhibiting therapy. In the final set of our studies (Chapter 5), we compared the cytokine transcriptome profiling in acute AD skin, with or without IL-31 inhibition by LKV. Skin samples were obtained from six HDM-experimentally-sensitized atopic dogs at 0, 6, 12, 24, 48 and 96 hours after allergen provocation. The results revealed no significant difference in the mRNA expression of major cytokines between with or without IL-31 inhibition. We also found that *IL33*, *IL13*, *CCL17* and *IL9*, and potentially *IL6* and *CCL22,* were still significantly upregulated despite a previous IL-31 inhibition, suggesting that these cytokines could be valuable targets for inhibition to supplement LKV therapy.

Altogether, our data suggest the early and transient production of IL-31 in canine AD skin, supporting the concept of using IL-31-inhibiting mAbs as a proactive therapy in canine AD. The insufficient effect of IL-31-blockage with LKV to delay AD relapses in some clinical cases could be caused by acute flares of AD skin inflammation induced by IL-31-independent cytokines, such as IL-33, IL-13, CCL17, IL-9, IL-6 and CCL22. The inhibition of these cytokines for the prevention of canine AD flares is deserving of further studies.

Investigating the Role of Interleukin-31 in an Experimental Model of Acute Canine Atopic Dermatitis

by
Chie Tamamoto-Mochizuki

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APPROVED BY:

Dr. Thierry Olivry Committee Co-Chair	Dr. Laster Scott Committee Co-Chair
Dr. Petra Bizikova	Dr. Glenn Cruse
Dr. Santosh Mishra	Dr. Andrea Gonzales External Member

DEDICATION

I wish to dedicate this dissertation to my husband Hiroyuki Mochizuki, my family in Japan and the best mentor in my life, Dr. Thierry Olivry, for their continued support during this research.

BIOGRAPHY

After graduating from the Veterinary School of Obihiro University of Agriculture and Veterinary Medicine in Japan in 2007, Dr. Tamamoto-Mochizuki worked as a general practitioner at a private small animal clinic for a year. To receive an advanced specialty veterinary training, she underwent an internal medicine internship for two years, followed by a dermatology and oncology residency for two years at the University of Tokyo in Japan (supervisor: Prof. Hajime Tsujimoto).

Because Dr. Tamamoto-Mochizuki developed a special interest in dermatology and decided to devote her veterinary career to this field, she had further clinical training at North Carolina State University as an alternative-route resident with the Asian College of Veterinary Dermatology (AiCVD) from 2014 to 2017. She became board-certified by the AiCVD in 2018.

To strengthen her knowledge in research, and to obtain further training for her future carrier as a clinician-scientist, Dr. Tamamoto-Mochizuki began her Ph.D. in Comparative Biomedical Sciences with a concentration in cell biology (major) and molecular biotechnology (minor) at NC State University in 2017 (supervisor: Prof. Thierry Olivry). Her research focused on the role of IL-31 in the pathogenesis of acute atopic dermatitis in the dog.

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LIST OF ABBREVIATIONS

aa	amino acid
AD	Atopic dermatitis
CADESI-03	Canine Atopic Dermatitis Extent and Severity Index-03
CCL	C-C motif chemokine ligand
CCR	C-C chemokine receptor
CGRP	Calcitonin gene-related peptide
CXCL	C-X-C motif chemokine ligand
DEG	Differentially expressed gene
Df	*Dermatophagoides farinae*
DPM	Duration of pruritus manifestations
DRG	Dorsal root ganglia
EASI	Eczema Area Severity Index
EASI-50	EASI score improvement of 50 percent relative to baseline
ELISA	Enzyme-linked immunosorbent assay
FC	Fold change
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
HCA	Hydrocortisone aceponate
HDM	House dust mite
IENF	Intraepidermal nerve fiber
IF	Immunofluorescence
IL	Interleukin
IL-13Rα2	IL-13 receptor subunit alpha 2
IL-31	Interleukin-31
IL-31RA	Interleukin-31 receptor subunit α
IL-4Rα	IL-4 receptor subunit α
ILC2	Type 2 innate lymphoid cell
ISH	In situ hybridization
JAK	Janus kinase

KLK	Kallikrein
LKV	Lokivetmab
mAb	Monoclonal antibody
MBA	Maltese–beagle atopic
MDC	Macrophage-derived chemokine
Mrgpr	Mas-related G protein-coupled receptor
NK-1R	Neurokinin 1 receptor
NGF	Nerve growth factor
NTC	No-treatment control
OSMRβ	Oncostatin M receptor subunit β
PAR	Proteinase-activated receptor
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
RefSeq	Reference sequence
RPKM	Reads per kilobase of exon per million mapped reads
RNA-seq	RNA sequencing
SiMoA	Single-molecule array technology
SLS	Skin lesion score
SP	Substance P
SPF	Specific pathogen-free
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokine
Th	T helper
Th1	T helper type 1
$T_{1/2}$	Elimination half-life
Th2	T helper type 2
Th9	T helper type 9
Th17	T helper type 17
Th22	T helper type 22
Treg	Regulatory T cells
T_{max}	Maximum drug concentration-time

TPM	Transcripts per million
TRPA1	Transient receptor potential cation channel subfamily A member 1
TRPV1	Transient receptor potential cation channel subfamily V member 1
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
TTF	Time-to-flare

CHAPTER 1

THE ROLE OF INTERLEUKIN-31 IN ATOPIC DERMATITIS

1.1 Atopic dermatitis

Atopic dermatitis (AD) is an inflammatory, chronically relapsing, allergic skin disease with severe pruritus that has pronounced detrimental effects on the quality of life of the patients in both humans and dogs (Figure 1).[1,2] The pathogenesis of AD is complex and multifactorial: genetic factors, an impairment of the function of the skin barrier, and many immunologic aberrations have been described in human and canine AD.[1,3–5]

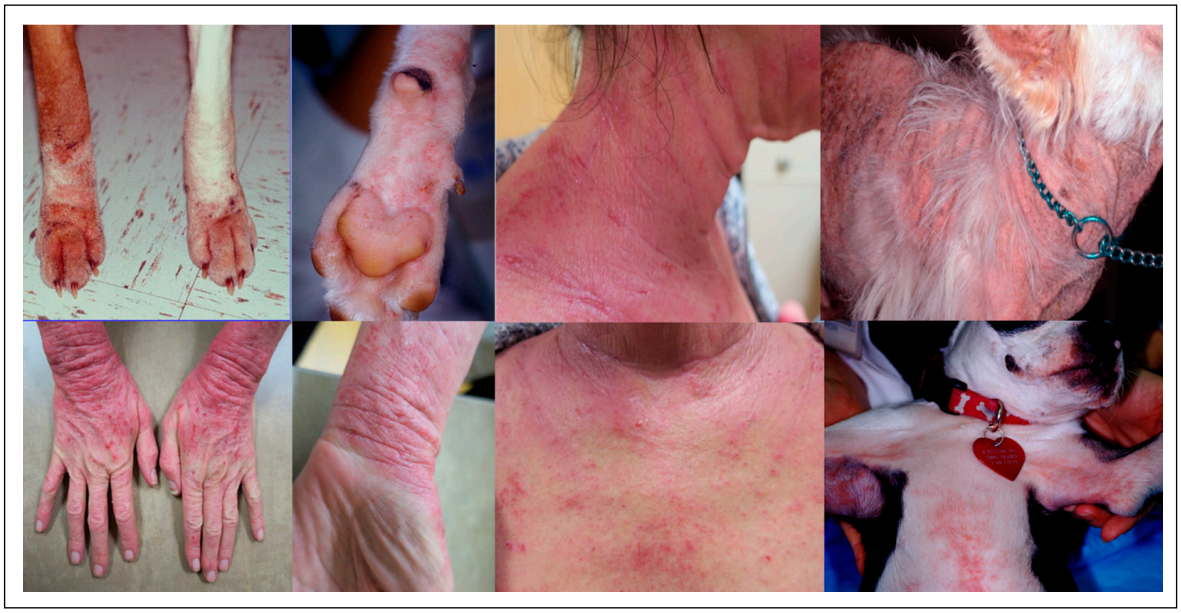

Figure 1. Clinical signs of AD in humans and dogs. (adapted from reference 2)
Because of the similarities in the appearance of clinical symptoms (pruritus and erythema) and the distribution of skin lesions (i.e., extremities, neck, chest), canine AD is considered a true homolog of its human counterpart.

The incidence of AD in humans has increased two-to-three folds in industrialized nations during the last 50 years, with currently approximately 15 to 20% of children and 1 to 3% of adults being affected worldwide.[6] Similarly, the incidence of AD in dogs seems to be increasing. The earliest study in the 1970s reported the prevalence of canine AD in a university clinic to be as low as 3.3% of dermatology cases;[7] it seems to have now increased to 78% of patients in some veterinary dermatology referral centers.[8]

Although human AD can occur at any age, its incidence peaks in infancy, with approximately 45% of all cases beginning within the first six months of life, 60% during the first years, and 80-90% by an individual's fifth birthday.[9] In canine AD, as in humans, the patients usually present their first clinical signs in their early lives; the age of onset is less than three years old for about 70% of the cases with a mean age of 2.2 years old.[10] Due to its genetic association, unfortunately, there is no "cure" per se for AD. Atopic dermatitis could resolve by the time a child reaches adulthood, but 10 to 30% of human patients continue to exhibit symptoms of the disease throughout their lives;[6] such "growing out of AD" has not been described in canine patients. As a result of their persistent disease, lifelong management is required for many patients, and there is always a need to develop novel and more effective treatments to manage AD in both humans and dogs.

1.2 Atopic itch

An intense itch is the hallmark of AD, and the itch is often the first clinical symptom at its onset, even before the first erythematous lesions appear on the skin.[10,11] Atopic itch can be divided into two categories: acute and chronic.[1,12] Atopic patients often experience intermitted AD flares accompanied by an acute itch, and the prolonged and recurrent nature of the disease leads to a more persistent chronic itch.[1]

Itch in AD results from orchestrated interactions between cutaneous sensory nerves, keratinocytes and immune cells. They communicate with each other through the release and reception of various mediators. Herein, we will explain how they interact in more detail, and we will focus on the emerging role of IL-31—one of the key cytokines that mediates this pruritogenic network—in atopic itch.

1.2.1 Nerves in atopic itch

Atopic itch occurs when sensory nerves are exposed to exogenous and endogenous stimuli (pruritogens), including allergens, amines, neuropeptides, proteases and various cytokines.[13–16] The neuronal transmission of itch can be divided into two distinctive pathways: histaminergic or non-histaminergic. While the former is involved in acute itch, the latter underlies chronic itch.[14–17] In the peripheral nervous system, the itch sensation is initiated after the binding of pruritogens to a subset of afferent somatosensory neurons that innervate the skin.[18]

These small-diameter unmyelinated sensory nerves mediating the itch sensation (the so-called C-fibers) originate from cell bodies located in the dorsal root ganglia (DRGs),[19,20] and terminate not only in the dermis but also further elongate into the epidermis (intra-epidermal nerve fibers [IENFs]).[21–26]

Exogenous pruritogens penetrate the upper layer of the epidermis because of the existing epidermal barrier dysfunction, which is believed to be the initial step in the development of human AD.[4,27] They then bind to the receptors expressed on the endings of the IENFs to directly trigger itch perception, even before atopic skin inflammation might be visible. An increase in the density of IENFs is seen in both atopic humans and dogs,[21–25] which could promote a decrease in itch's threshold; this phenomenon is named "neuronal sensitization" and is typical of atopic patients.[16,17,28] The activation of receptors expressed on sensory nerves, which are called metabotropic receptors, are coupled with voltage-gated calcium ion channels of the transient receptor potential family (e.g., TPRV1 and TRPA1). Activation of these ion channels induces calcium influx into DRGs. The marked increase in the concentration of cytosolic calcium then function as a second messenger that mediates the transmission of the depolarization signals and contributes to synaptic activities in DRGs.[29] Subsequently, the impulses ascend through the spinal cord and finally reach the brain to be processed as "itch" signal.[14,16] Although both histaminergic and non-histaminergic pathways follow the same route – i.e., starting from the peripheral nerves, DRGs, spinal cord, and then finally reaching the brain –, they appear to exist separately and independently from one another (Figure 2).[15,30,31] The separation of the transmission pathways already begins in the peripheral nerves depending on the receptors expressed on the terminates and their ligands (pruritogens). They use different areas of spinal cords and even lead to different activation patterns in the brain.[30,31]

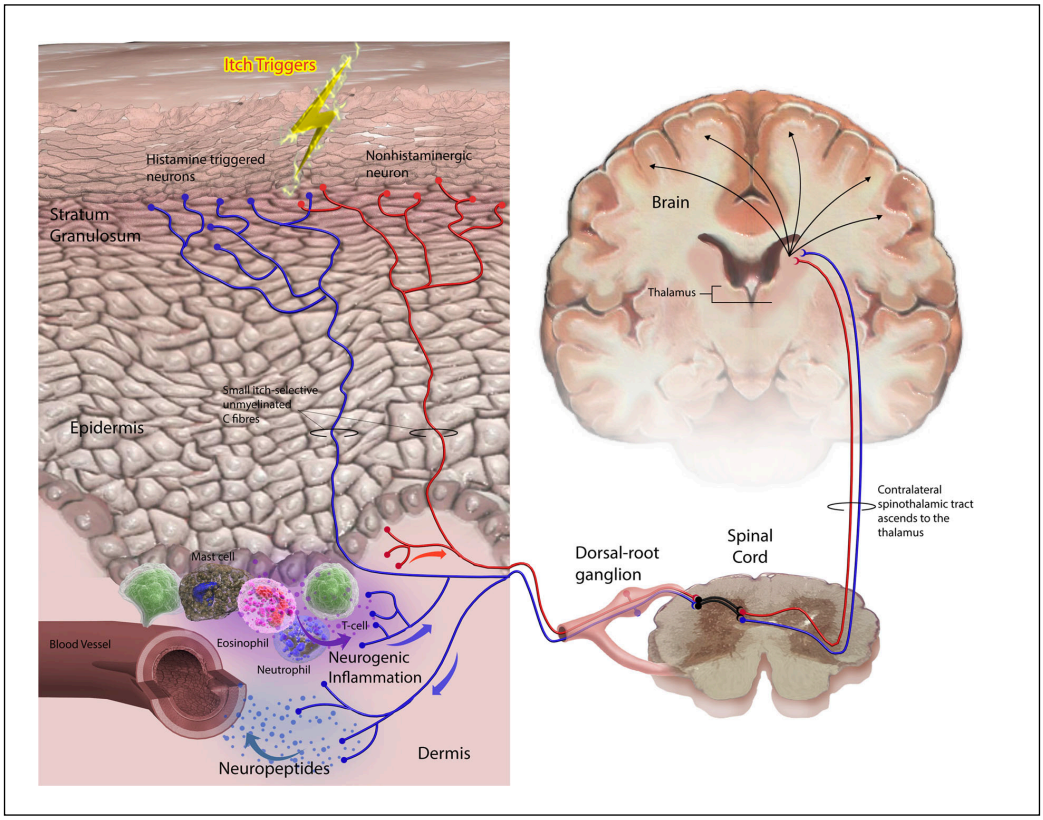

Figure 2. Histaminergic and non-histaminergic induced itch and neurogenic inflammation. (adapted from reference 15)

The neuronal pathways for histaminergic and non-histaminergic itch are independent and remain so as they travel from the periphery to the brain. The activation of the nerve fibers also leads to the axonal and antidromic release of neuropeptides, resulting in vasodilation and leukocyte-mediated inflammation (neurogenic inflammation).

1.2.2 Itch mediators in atopic itch

Histamine is the best known and the most studied pruritogenic mediator that can directly stimulate itch sensory neurons in the skin to cause acute itch.[32] In allergen challenges of sensitized atopic dogs, the itch was significantly reduced with hydroxyzine (H1 receptor antagonist), thus confirming the importance of histamine for acute atopic itch also in this species (Olivry, unpublished data). Although human patients with AD intermittently suffer from flares of acute itch due to the waxing-and-waning characteristic of this disease, chronic itch (i.e., one lasting six or more weeks) is a more defining feature of atopic itch.[11,33] In contrast to acute itch, the evidence suggests that histaminergic induced itch does not substantially contribute to the chronic itch in atopic patients. The placebo-controlled studies with a non-sedating antihistamine

(terfenadine) have failed to alleviate itch over the placebo effect in atopic humans, which supported that histamine is not the critical factor in chronic atopic itch.[34,35] Similarly, an insufficient anti-pruritic effect of H1-histamine receptor blockers has been reported in placebo-controlled studies in dogs with chronic AD.[36,37]

Chronic itch is induced by the activation of the non-histaminergic pathway by various endogenous and exogenous pruritogens. Neuropeptides (e.g., substance P, nerve growth factor), proteases (e.g., kallikreins, tryptase, exogenous proteases) and several cytokines and chemokines (e.g., thymic stromal lymphopoietin, IL-4, IL-13, IL-31) are known to be relevant to chronic atopic itch.[15,16,33]

Substance P (SP) is a neuropeptide that has been suggested to be an important itch mediator in AD.[38] It is secreted from the free nerve ending in the skin as well as from keratinocytes and various inflammatory cells.[39,40] Immunohistochemical studies have shown an increased number of substance P-positive nerve fibers in the lesional skin compared to those in the non-lesional skin of atopic patients.[41] Although the receptors of SP, neurokinin 1 receptors (NK-1Rs) and mas-related G protein-coupled receptors (Mrgprs), are both expressed on the terminates of non-histaminergic itch-mediating sensory neurons (Figure 3),[15,16] a recent mouse study has shown that only Mrgprs are responsible for SP-induced itch since SP-induced scratching behavior was dependent on Mrgprs rather than NK-1R.[42] NK-1R is also expressed on keratinocytes and immune cells, thereby, SP can induce the release of additional itch mediators from these cells.[43,44]

Nerve growth factor (NGF) is another neuropeptide that has been reported to have a role in atopic itch.[15,16,45] It is mainly produced by keratinocytes and promotes the growth of nerve endings in the skin, which is partly responsible for neuronal sensitization of itch seen in AD patients.[15,45] The number of the NGF positive keratinocytes and the intensity of its immunoreactivity were increased in the lesional skin of human and canine AD patients compared to those in the non-lesional or normal skin.[46–48] Furthermore, NGF up-regulates other neuropeptides, especially substance P, thereby further promoting itch indirectly.[49]

Various endogenous and exogenous proteases mediate non-histaminergic itch in atopic patients via binding to their receptors – proteinase-activated receptors (PARs), especially PAR2 – expressed on the itch-mediating sensory nerves in the skin (Figure 3).[13,50–52] Kallikreins (KLKs) are enzymes that regulate stratum corneum desquamation and turnover; they can activate PAR2,

resulting in the induction of itch.[15] The upregulation of KLKs is seen in the stratum corneum collected from the skin of atopic patients.[53] This increased activity of KLKs is also, in part, responsible for the barrier defects seen in the atopic skin, which further facilitate the penetration of exogenous pruritogens.[54]

Tryptase is another endogenous protease that plays an important role in atopic itch. A large amount of tryptase is released upon mast-cell degradation in the skin of AD.[55] The dermal concentration of mast cell tryptase is markedly increased in AD skin lesions compared to those in healthy controls.[56]

Finally, exogenous proteases in house dust mites – one of the most common allergens – and proteases produced by *Staphylococcus aureus* – a pathogen of one of the most common concomitant skin infection in human AD – can penetrate the compromised skin barrier of atopic patients and induce itch by directly activating itch-mediating sensory neurons via PAR2.[51,52]

Numerous cytokines and chemokines are involved in the pathogenesis of AD.[1] Among those, several cytokines are known to contribute to pruritus by directly stimulating non-histaminergic itch-mediating sensory neurons (Figure 3). Thymic stromal lymphopoietin (TSLP) is secreted primarily by epidermal keratinocytes resulting from the epidermal barrier disruption seen in atopic patients or from the mechanical damage of keratinocytes caused by scratching; it is a crucial cytokine promoting T helper type 2 (Th2) immune responses in AD.[57,58] It has been shown that TSLP directly activates sensory neurons to evoke itch through its receptor on itch-mediating sensory neurons co-expressing TRPA1.[59]

Interleukin (IL)-33 – another keratinocyte-derived alarmin over-expressed in the skin of atopic patients –[60] also has been shown to mediate the itch directly through the activation of itch sensory neurons.[61]

These alarmins activate the Th2-mediated immune response, which predominates the acute phase of AD.[1] Interleukin-4 and IL-13 – two central Th2 cytokines – have been shown to induce itch by directly activating itch-mediating sensory neurons through the IL-4 receptor subunit α (IL-4Rα), which is a shared subunit for both IL-4 and IL-13 (Figure 3).[62] A therapeutic monoclonal antibody (mAb) targeting IL-4Rα (dupilumab; Dupixent, Regeneron, Eastview/Tarrytown, NY, US) – thereby blocks the signaling from both IL-4 and IL-13 – demonstrated a 55.7% reduction in pruritus score, further implicating both cytokines as mediators in atopic itch.[63]

Lastly, another Th2 cytokine, IL-31, was found to play a key role in atopic itch.

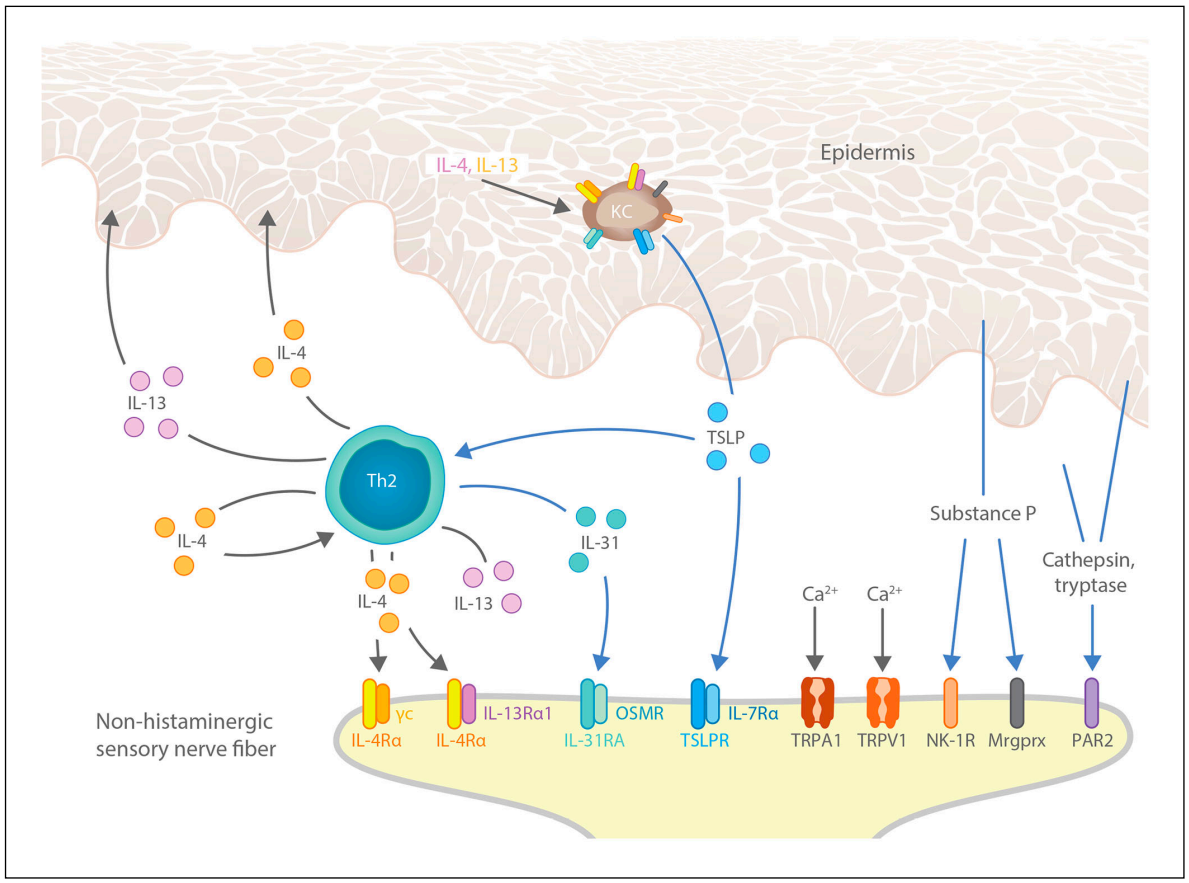

Figure 3. Crosstalk between keratinocytes, immune cells and peripheral nerves mediates atopic itch. (adapted from reference 33)

Immune cells and keratinocytes release various pruritogens in AD. They promote itch by exciting the non-histaminergic sensory nerve fibers in the skin through their receptors. Keratinocyte-derived factors can also activate immune cells, leading to further production of pruritogenic cytokines from these cells. Finally, feedback loops involving IL-4 and IL-13 contribute to itching by inducing more TSLP expression in keratinocytes.

Abbreviations: IL-13Rα1 = Interleukin 13 receptor subunit α1, IL-31RA = Interleukin 31 receptor subunit α, IL-4Rα = Interleukin 4 receptor subunit α, IL-7Rα = Interleukin 7 receptor subunit α, Mrgprx = Mas-related G protein-coupled receptors subfamily X, NK-1R = Neurokinin 1 receptor, OSMR = oncostatin M receptor, PAR2 = proteinase-associated receptor 2, TRPA1 = transient receptor potential cation channel subfamily A member 1, TRPV1 = transient receptor potential cation channel subfamily V member 1, TSLP=thymic stromal lymphopoietin, TSLPR = TSLP receptor.

1.2.3 IL-31 in atopic itch

IL-31 is a cytokine that is known to cause severe itch in humans, mice, dogs, cats and monkeys when it is either applied by skin prick test, or injected subcutaneously, intradermally or intravenously.[64–73] Because of this unique feature of this cytokine, numerous studies investigated its association with several pruritic skin diseases, especially AD.

Before the discovery of IL-31 itself, the receptor of this cytokine was first identified on CD-14-positive cells (monocytes and macrophages) in mice and humans.[74] This receptor was most homologous to the IL-6 receptor signal transducing chain, gp130, followed by the granulocyte colony stimulating factor receptor with a 25% and 24% identity, respectively.[74] Thus, it was classified as a type I cytokine, gp130-like monocytes receptor.[74] When the ligand – at that time, a chimeric molecule constructed based on the extracellular and transmembrane domains of the receptor – bound to the receptor, it induced the activation of signal transducer and activator of transcription (STAT)-3 and STAT-5.[74] Therefore, it was initially reported that this receptor and its ligand would be a novel cytokine involved in the development and the regulation of monocytes and macrophages.[74]

Two years after discovering the receptor, the same research group identified "the ligand" – IL-31 – in activated T cells; it was preferentially, but not exclusively, produced by Th2 cells.[65] Mouse and human *IL31* cDNA consist of an open reading frame encoding a 164 amino acid (aa) precursor and a predicted 141 aa mature polypeptide containing four α-helices.[65] At the aa level, the mature mouse IL-31 protein shares 31% identity with its human counterpart.[65] However, there is no cross-species activity, meaning mouse IL-31 does not bind the human IL-31 receptor and vice versa.[75] Later studies also identified *IL31* cDNA in dogs. It contains a 480-bp coding region and a predicted 159 aa sequence. The canine IL-31 aa sequence has a 28% and 54% identity to mouse and human *IL31*, respectively.[69,76] Due to this relatively low homology, it is also expected that there is no interspecies activity between these three species.

Dillon's study also uncovered a unique feature of this cytokine. Mice that received IL-31 protein via the subcutaneous insertion of a mini-osmotic pump developed severe itch followed by multiple self-induced patches of alopecia.[65] Furthermore, transgenic mice overexpressing IL-31 developed severe pruritus followed by alopecia and skin lesions that were hallmarks of AD.[65] Consistent with this finding, IL-31-receptor-deficient mice did not develop pruritus or alopecia in response to mouse IL-31.[65] Both IL-31-overexpressing and IL-31-receptor-deficient transgenic

mice did not show overt changes in their thymus or bone marrow, indicating that IL-31 actually was not important for hematopoiesis or the activation state of these cells, in contrary to their initial hypothesis.[65] Thus, ensuing studies by other researchers focused on investigating the association of IL-31 in pruritic skin diseases, such as AD.

NC/Nga mice represent one of the mouse models of AD, as they develop dermatitis and scratching behaviors when they are raised under conventional conditions.[77,78] These mice do not show any of these clinical signs when they are raised under specific pathogen-free (SPF) conditions, however.[77,78] *IL31* mRNA was expressed exclusively in the skin of NC/Nga mice raised under conventional conditions, but not in those of mice under SPF condition, and there was a good correlation between the intensity of itch and the expression level of *IL31* mRNA.[79,80] This scratching behavior was reduced by an anti-mouse IL-31 mAb, which further confirmed the importance of IL-31 in atopic itch.[68]

Later reports in humans also supported this new link between IL-31 and itch in AD. Numerous studies demonstrated significantly-elevated serum IL-31 levels in patients with AD compared to healthy controls.[81–86] It has also been reported that *IL31* mRNA is significantly over-expressed in the skin of atopic patients compared to that of nonpruritic psoriatic or healthy individuals.[66,82,87,88] Some of these studies also investigated the correlation between the serum IL-31 levels and the intensity of itch in atopic patients; however, all, except one study, failed to show such a positive correlation.[82,89–93] In two human clinical trials, the serum IL-31 levels were also compared before and after treatment to evaluate further the importance of IL-31 in the pruritogenesis of AD. While a significant reduction of itch was seen after treatment in both studies, a significant reduction of serum IL-31 level was only seen in one report in which the patients were treated with epinastine, an H1-histamine receptor antagonist known to inhibit the development of Th2 immune responses.[93,94] One of the likely reasons for this dissociation between the serum IL-31 levels and the intensity of atopic itch is poor correlation between cytokine levels in blood circulation and those in the skin. In a study of humans with chronic AD in which authors calculated the correlations between the cytokine levels in the interstitial fluid of lesional skin and the corresponding plasma samples, no correlation was found for most cytokines measured in the study, including IL-31.[95] Another study showed a moderate correlation between the serum IL-31 levels and *IL31* mRNA expression in the skin of atopic humans, but more than

half of these patients (7/13) had either normal levels of serum IL-31 or undetectable levels on *IL31* mRNA in the skin; therefore, the actual correlation seen remains questionable.[82]

Because of the increasing evidence supporting the pivotal role of IL-31 in atopic itch, this cytokine rapidly became a promising therapeutic target. In 2012, a phase I clinical study of a humanized mAb targeting human IL-31 (BMS-981164) was started in healthy subjects and patients with AD. Although it was completed in 2015, results have not been released yet (NCT01614756). Another therapeutic humanized mAb, nemolizumab, targets the human IL-31 receptor to block IL-31 signaling. Phase 2 clinical trials of nemolizumab revealed its anti-pruritic benefit in humans with moderate-to-severe AD, which further demonstrated the importance of this cytokine in atopic itch.[96–99] Recently, a phase 3 clinical trial enrolling AD patients with an inadequate response to conventional standard treatment with medium-potency topical glucocorticoids or topical calcineurin inhibitors reported that an adjunctive nemolizumab therapy showed a greater reduction in itch in those patients compared to that in the placebo controls.[100] In veterinary medicine, the treatment with a caninized anti-canine IL-31 mAb (lokivetmab, Cytopoint, Zoetis) also leads to a significant reduction of itch in atopic dogs, thereby indicating that IL-31 also plays an important role in the pathogenesis of canine AD.[101–103]

As mentioned above, there is abundant evidence to support that IL-31 is an important cytokine for atopic itch. Several studies have investigated the mechanisms by which IL-31 induces itch in AD. Increased total and allergen-specific serum IgE levels are one of the characteristics of many human patients with AD; such IgE later induce the release of various pruritogens from immune cells such as mast cells, which is one of the key pruritogenic mechanisms in AD.[1,104] On the other hand, transgenic mice overexpressing IL-31 lack such an increase in serum IgE levels.[65] Furthermore, mast cell-deficient mice treated with IL-31 still developed severe pruritus and alopecia, indicating that IgE and mast cells are not directly responsible for the itch response caused by IL-31.[65]

The first step to elucidate the role of this cytokine was to identify the target cells expressing its receptor. The IL-31 receptor is a heterodimer consisting of the gp130-like receptor chain – the IL-31 receptor subunit α (IL-31RA) – and the oncostatin M subunit β (OSMRβ) (Figure 4).[65,74,105,106] The interaction of the OSMRβ with Il-31RA is required for signal transduction, and each alone cannot transduce a signal.[84] Several isoforms of *IL31RA* have been described to date in humans (*GPL560*, *GPL610*, *GPL626*, *GPL745*, *CRL3*, *GLM-R* and

IL31RAv1-v4), which encode either long or short isoforms in the intracellular domain.[107] While both forms can activate the downstream signaling transducing molecules,[108] the short isoform (GPL560; the smallest isoform that has no transmembrane or intracellular domain) has been shown to have an antagonistic or lower activity compared with that of the long isoform (GPL745).[109] However, it has to be noted that it is presently unclear whether these different splice variants are expressed and to what extent they are relative to the full-length protein has not been clarified. Similarly, there are currently five *IL31RA* mRNA splice variants in dogs, encoding two protein isoforms: the full-length isoform X1 and the truncated isoform X2. The *IL31RA* isoform X2 lacks the N-terminal signal peptide and parts of the cytokine binding domain; therefore, it may not bind to IL-31.[110] Although transcription of *IL31RA* X2 (truncated isoform) was significantly higher than that of *IL31RA* X1 in both atopic and healthy dogs, the difference in the function of these isoform remains to be confirmed.[110]

Upon binding to its receptor, the intracellular signaling of IL-31 is mediated by the activation of three signal transduction pathways: the Janus kinase (JAK) 1/2-STAT 3 (also to less extent STAT1 and STAT5) pathway, the phosphatidylinositol-3 kinase (PI3K)-protein kinase B (AKT), and the mitogen-activated protein kinase (MAPK; extracellular-signal-regulated kinase [ERK], mitogen-activated protein kinase p38 and c-Jun N-terminal kinases) pathways.[20,111–113] The recruitment of JAKs results in the subsequential phosphorylation of STATs and the stimulation of the PI3K/AKT pathway.[20,111–113] In contrast to the IL-31RA, the OSMRβ activates the MAPK pathway.[112] These control downstream effectors of IL-31 signaling and are most likely essential for controlling target gene expression. IL-31 has been shown to activate these pathways in different primary cells and cell lines in different species, such as STAT1/3/5 in human keratinocytes and STAT1 and ERK1/2 in DH82 canine histiocytic cell lines.[65,69] Whether all pathways are activated in all cells that express the IL-31 receptor, or whether there is any tissue specificity in signal transduction awaits further investigation.

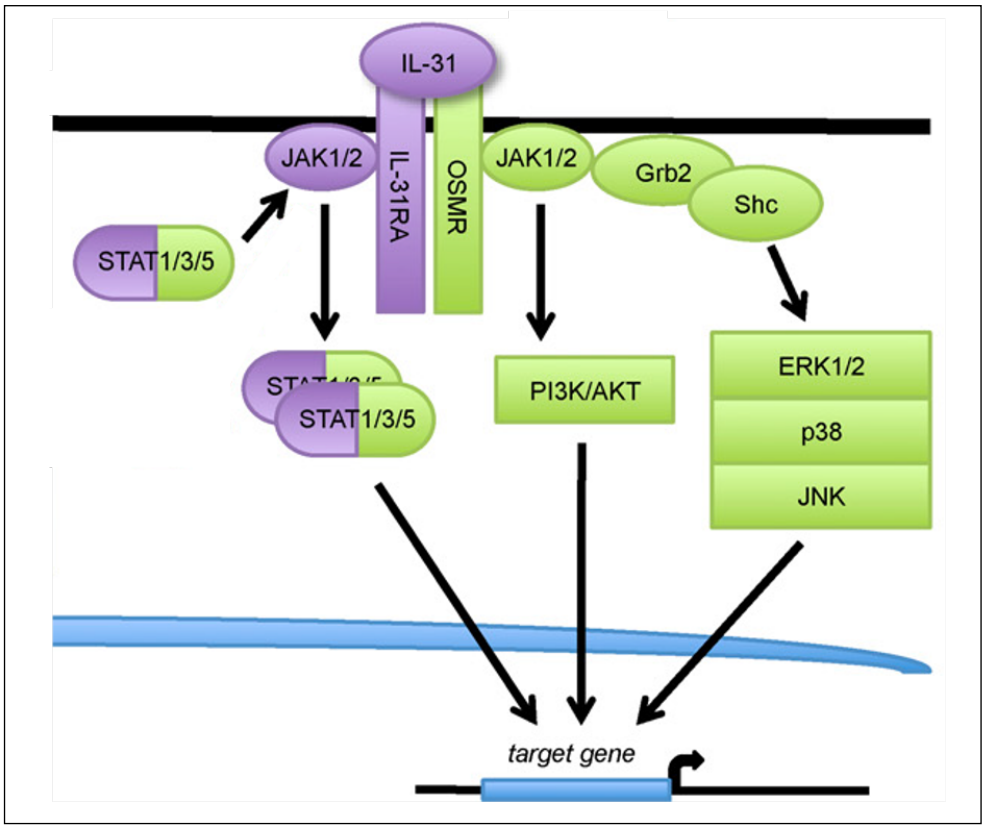

Figure 4. The IL-31 and IL-31 receptor complex and the signal cascade. (adapted from reference 112 with modification)

Upon binding to its heterodimer receptor composed of the IL-31RA and OSMRβ, IL-31 activates JAK/STAT, PI3K/AKT and MAPK (EKR1/2, p38 and JUN) signaling pathways.

Abbreviations: AKT = protein kinase B, ERK1/2 = extracellular-signal-regulated kinase 1/2, Grb2 = growth factor receptor-bound protein 2, IL-31 = interleukin 31, IL-31RA = interleukin 31 receptor subunit α, JAK1/2 = Janus kinase 1/2, JNK = c-Jun N-terminal kinases, OSMR = oncostatin M receptor subunit β, p38 = mitogen-activated protein kinase p38. PI3K = phosphatidylinositol-3 kinase, Shc = src homology 2 domain-containing transforming protein, STAT1/3/5 = signal transducing activator transporter 1/3/5.

Since the OSMRβ is shared with at least another cytokine (oncostatin M) and is thus not specific for IL-31, the expression of IL-31RA is used to identify the cells targeted by IL-31 specifically. An analysis of the tissue distribution of IL-31RA revealed that the highest *IL31RA* mRNA level among 63 different human tissue specimens examined was in DRGs — the cell bodies of cutaneous sensory neurons.[88] Later studies consistently described the expression of IL-31RA on DRGs in humans, mice and also dogs. The expression of IL-31RA also has been

demonstrated in the dermal nerve fibers of human atopic patients.[66,88,114–121] Consequently, it has been suggested that IL-31 directly binds to cutaneous sensory nerve fibers and then transmits the itch perception to the brain. This direct effect of IL-31 on sensory nerves is supported by the finding that an injection of IL-31 induces a relatively acute itch that occurs within a couple of minutes, or up to 30 minutes, in mice, monkeys and dogs.[69,72,116] On the other hand, skin prick tests with IL-31 in healthy and atopic humans induce, not an immediate, but relatively late itch responses that occur 142 minutes on average after the IL-31 challenge.[64] This delayed IL-31-induced itch in humans suggests that there are other itch pathways indirectly stimulated by IL-31, such as the release of secondary pruritogens downstream of the IL-31 signaling pathway.

The activation of neurons by IL-31, as with other pruritogens, leads to a marked increase in cytosolic calcium concentration, which then functions as a second messenger that mediates the transmission of the depolarization signals and contributes to synaptic activities.[29] Human and mouse studies have shown that IL-31 induced calcium influx into DRGs in vitro, which confirmed that IL-31 has the capability of directly activating these neurons.[66,115,121] It has been reported that the distribution of IL-31RA completely overlapped with that of the transient receptor potential cation channel vanilloid subtype 1 (TRPV1) in mouse DRGs.[66] Furthermore, 67% and 91% of IL-31–responsive cells in mouse DRGs were activated by capsaicin (TRPV1 agonist) and AITC (TRPA1 agonist), respectively, which indicates that most of these TRP channels are functional.[66] The co-expression of IL-31RA and TRPV1 on DRGs has also been reported in dogs at the mRNA and protein level.[118,119] Similarly, a recent canine study has shown that all DRG neurons that responded to recombinant canine IL-31 were within the capsaicin-responsive subset, further supporting the co-expression of these receptors (IL-31RA and TRPV1).[121] Therefore, TRPV1 and TRPA1 are considered to be responsible for this calcium influx upon IL-31 stimulation. Consistent with these observations, there is a significant reduction of IL-31-mediated itch in TRPV1- or TRPA1-deficient mice.[66]

Interestingly, the transgenic IL-31-overexpression and subcutaneously-delivered IL-31 were shown to induce an increase in the density (both elongation and branching) of cutaneous nerve fibers in the lesional skin in mice; this promotion of neuronal growth by IL-31 was selectively seen in small-diameter neurons with features of itch-mediating sensory C-fibers.[122] This increase in the density of cutaneous nerve fibers induced by IL-31 could be another

contributing factor to the neuronal sensitization of itch that results in the "sensitive skin" seen in atopic patients.[16,17,28]

1.3 Atopic skin inflammation

Skin inflammation is another central characteristic of AD.[1,10] As with atopic itch, the skin inflammation of AD can also be classified into acute and chronic stages based on clinical presentation and histopathological changes. In human and canine AD, acute skin lesions are characterized by erythema with edema, serous exudate and excoriations; in contrast, chronic skin lesions are less erythematous, dry (xerosis) and lichenified (thick).[1,123] Although the transition from acute to chronic phases is a continuous event, many studies have shown distinctive cytokine profiles in each phase. In humans, and to some degree in dogs, the currently accepted model proposes a predominant Th2 cytokine milieu in the initiation of acute AD skin lesions and a mixed T helper type 1 (Th1) and Th2 pattern in chronic skin lesions.[1,5,62] In the next section, we will reflect on the pathomechanism of atopic skin inflammation, including how itch can contribute to it, and the current conflicting findings of the role of IL-31 in skin inflammation in AD.

1.3.1 "Itch-scratch-itch" cycle and itch inflammation

Itch leads to scratching; scratching further damages the skin barrier function and thus enhances allergen penetration, leading to an exacerbated response to these exogenous allergens.[1,16] This mechanical stimulation by scratching also directly induces the release of various pro-inflammatory cytokines from keratinocytes.[1] Some of these keratinocyte-derived mediators — such as IL-33, TSLP and KLKs — can directly stimulate itch-mediating sensory neurons via their receptors expressed on free nerve endings (i.e., IENFs).[1] These mediators also activate Th2 immune cells to induce further the release of other pruritogens, such as IL-4 and IL-13, as explained earlier in this chapter.[1] These lead to the initiation of another itch signaling and perpetuates the desire to scratch; this vicious cycle is called the "itch-scratch-itch" cycle in AD (Figure 5).[124]

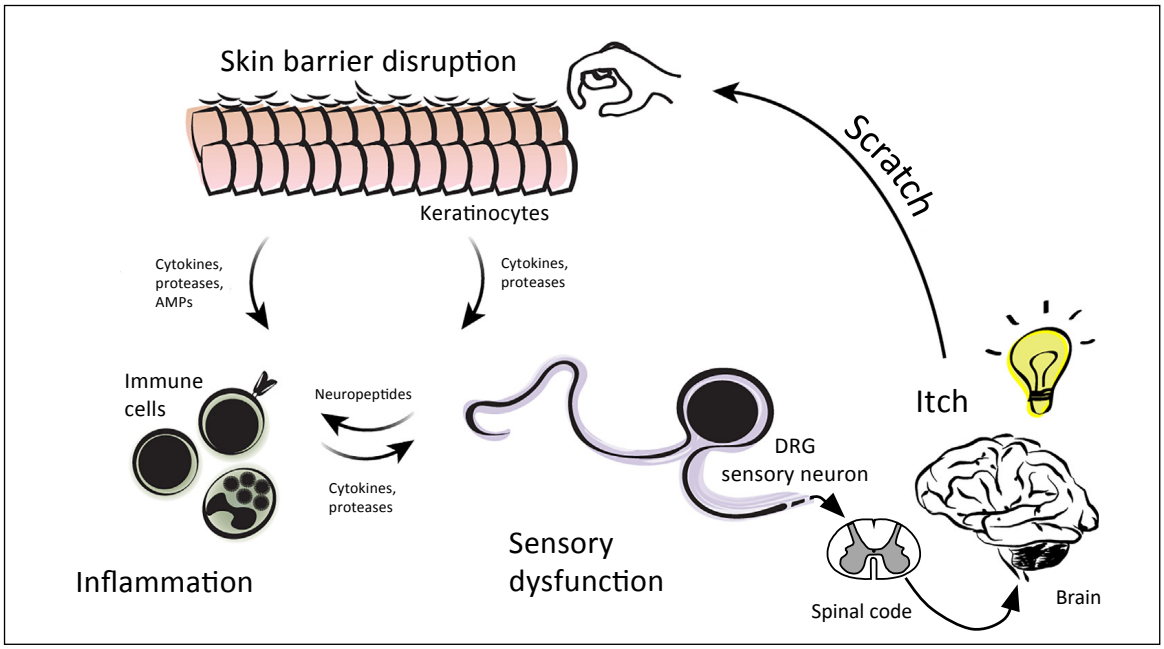

Figure 5. The itch-scratch-itch cycle. (adapted from reference 124 with some modification)
Scratching behaviors cause mechanical damage to keratinocytes, leading to the release of keratinocyte-derived pruritogens (cytokines, proteases) that directly activate itch-sensory neurons. Keratinocytes also release mediators (cytokines, proteases and AMP) that can stimulate immune cells, resulting in the release of immune-cell-derived pruritogens to mediate itch. The excitation of sensory neurons leads to the antidromic release of neuropeptides, which triggers the development of neurogenic inflammation by additional recruitment and activation of inflammatory cells.
Abbreviations: AMP = antimicrobial peptide, DRG = dorsal root ganglion.

As mentioned above, keratinocyte-derived mediators not only directly initiate the itch sensation but also directly or indirectly induce skin inflammation. For example, IL-25, IL-33 and TSLP are essential initiators of the Th2 inflammation responsible for the acute phase of AD.[1,125] Scratch injuries to epidermal keratinocytes stimulate the release of chemokine (C-C motif) ligand 20 (CCL20), which contributes to the recruitment of T helper (Th) 17 and Th22 cells.[126] The IL-17A and IL-22 that are produced by these cells are also proposed to be involved in the generation of skin inflammation in both acute and chronic phases in AD.[127,128] The effect of this itch-scratch behavior in skin inflammation was evident in a mouse contact hypersensitivity model where the severity of dermatitis was reduced by half by simply avoiding mechanical scratching by protective shields.[129]

1.3.2 Inflammatory mediators in atopic skin inflammation

Histamine is predominantly released from mast cells and basophils and plays a central role in the induction of acute atopic skin inflammation – erythema and edema – by dilating the superficial capillaries and by activating and recruiting other immune cells.[1,32,130] However, the overall contribution of histamine to the development of AD lesions appears small, as there is no high-level evidence of the benefit in controlling AD symptoms – both itch and skin inflammation – by antihistamines that inhibit the type 1 and 4 histamine receptors.[32,131]

In addition to transmitting itch perception, the excitation of itch-mediating skin sensory neurons leads to the axonal and antidromic skin release of neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), which have been implicated, to varying degrees, in the promotion of vasodilation and leukocyte-mediated inflammation (Figure 2, 5).[15,124] For example, substance P can stimulate mast cell degranulation and mediator release, including histamine. The intradermal injection of substance P and a substance P-releasing agent (mustard oil) evoked dose-dependent wheal, flare and itch reactions in both AD patients and controls.[132] Such type of inflammation is called neurogenic inflammation, and it can potentiate chronic skin inflammation in AD.[15,124]

Cytokines and chemokines are believed to be the central regulators of atopic skin inflammation (Figure 6). The latest comprehensive evaluations of the cytokine profile in acute and chronic skin lesions in atopic humans showed that the changes accompanying the transition from non-lesional to acute to chronic inflammation in AD are quantitative rather than qualitative.[133,134] An epidermal barrier dysfunction is consistently observed in the skin of atopic patients and is known as one of the critical components of the development of AD.[1,62] Keratinocytes in an epidermis stressed with exogenous and endogenous stimuli (e.g., stress from the irritation from exogenous allergens and irritants, mechanical scratching injury, infection, inflammatory cytokines and proteases) release epidermal alarmins, including IL-1β, IL-25, IL-33, TSLP, thymus and activation regulated chemokine (TARC or CCL17), and macrophage-derived chemokine (MDC or CCL22); these mediators drive the activation of skin-resident type 2 innate lymphoid cells (ILC2s) and memory Th2 cells.[1,62,135] ILC2s produce IL-5 and IL-13 to activate eosinophils and Th2 cells, respectively, thus promoting the Th2-skewed cytokine milieu of early AD.[1,62,136] This acute phase of atopic skin inflammation is characterized by profound increases of Th2 (IL-4, IL-5, IL-10, IL-13, IL-31) and Th22 (IL-22) cytokines; among these

cytokines, IL-4 and IL-13 have been demonstrated to play a crucial role in AD pathogenesis, as shown by the clinical benefit seen in human patients treated with dupilumab, a monoclonal antibody that inhibits their shared receptor.[1,62,133,136] Inflammatory dendritic epidermal cells (IDECs) and dermal dendritic cells take up exogenous allergens and present them to T cells, and then activated Th2 cells release IL-4 and IL-13, which promote IgE class switching in B cells and the production of antigen-specific IgE. Langerhans cells bearing such IgE take up allergens and further fuel the Th2 immune response.[1,62,136]

The chronic phase of atopic skin inflammation is characterized by a mixed Th1, Th2 and Th22 pattern (Figure 6).[1,62,136] Th2 and Th22 responses are intensified in chronic AD lesions, along with the parallel activation of the Th1 axis (IFN-γ, CXCL9, CXCL10, CXCL11) rather than a ''switch'' to a Th1-only milieu.[133,134,136] Th17-associated proteins (IL-17A, peptidase inhibitor 3, and CCL20) are consistently up-regulated in human patients with both acute and chronic skin inflammation compared to those with normal skin. However, the role of Th17 cell-mediated response in AD is still unclear; this is supported by the lack of response of patients to IL-17-inhibiting therapeutic monoclonal antibodies like secukinumab.[137]

As explained earlier, many mediators have dual roles in AD – as pruritogens and inflammatory mediators; this might not be the case for IL-31, however.

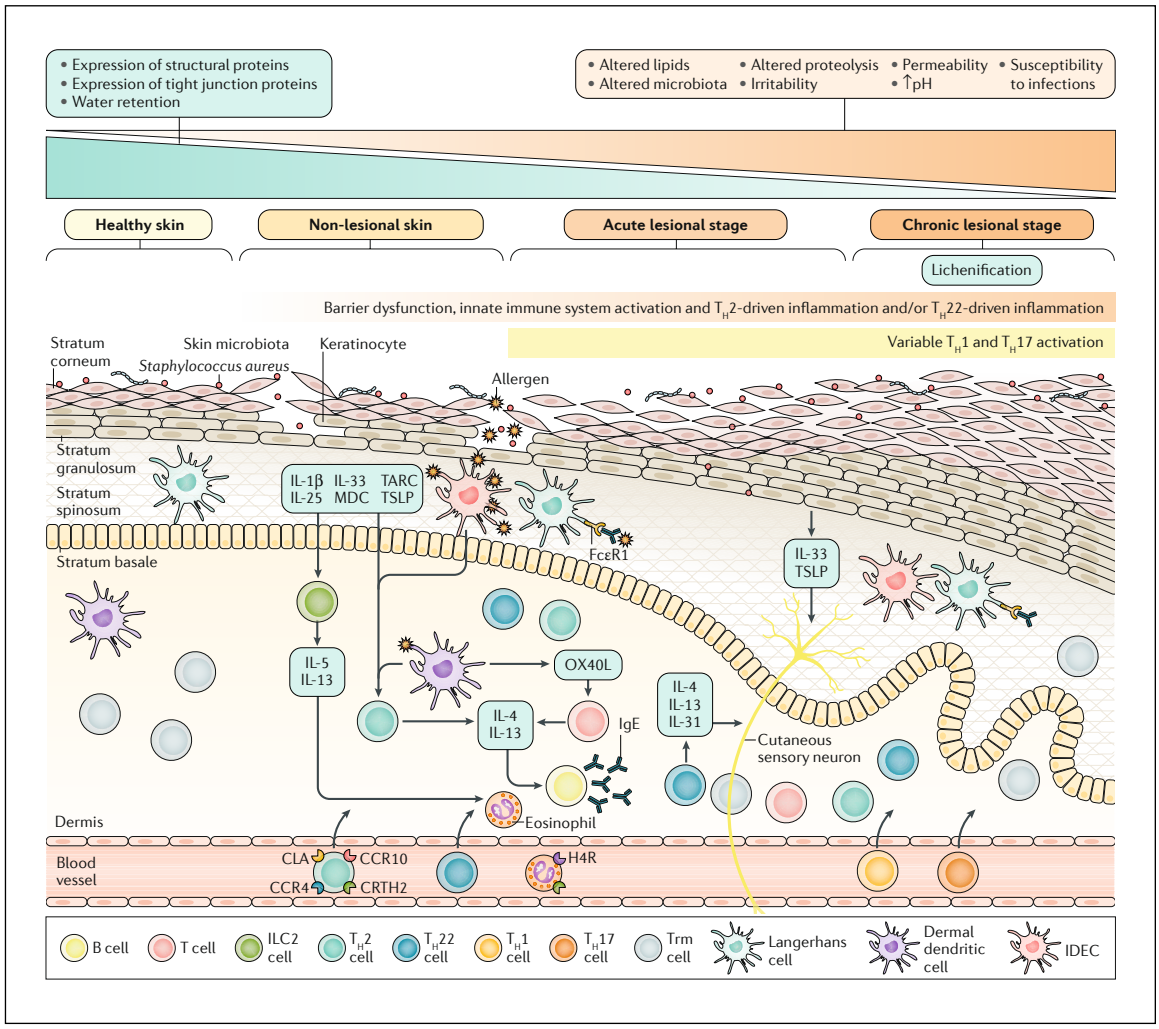

Figure 6. Stage-based pathogenesis and cytokine milieu of AD. (adapted from reference 62)
Clinically unaffected, non-lesional skin in AD patients consistently has an epidermal barrier dysfunction, which induces the expression of epidermal alarmins (IL-1β, IL-25, IL-33, TSLP, TARC, MDC); these mediators activate skin-resident ILC2s and Th2-mediated immune responses, which predominate in the acute phase of atopic skin inflammation. With increasing chronicity, there is a progressive increase of Th1 immune response, leading to the development of mixed Th1, Th2 and Th22 cytokine milieu in the chronic phase of atopic skin inflammation.
Abbreviation: CCR = CC chemokine receptor, CLA = cutaneous lymphocyte-associated antigen, CRTH2 = chemoattractant receptor-homologous molecule expressed on Th2 cell, FcεR1 = high-affinity immunoglobulin ε receptor, H4R = histamine H4 receptor, IDEC = inflammatory dendritic epidermal cell, IL = interleukin, ILC = innate lymphoid cell, OX40L=OX40 ligand, TARC = thymus and activation-regulated chemokine, T_H = T-helper cell, Trm = tissue-resident memory T cell, TSLP = thymic stromal lymphopoietin.

1.3.3 IL-31 in atopic skin inflammation

Despite the clear correlation of IL-31 and itch in AD, the role of IL-31 in atopic skin inflammation is still controversial. Various immune cells — such as macrophages, monocytes, mast cells, eosinophils and basophils — have been shown to express the IL-31 receptor.[65,74,138–142] The expression of *IL31RA* mRNA in a monocytic cell line is also reported in dogs.[143] All of these cells play essential roles in the development of atopic skin inflammation.[1] As a result, it has been suggested that IL-31 could directly recruit these cells to the lesional AD skin to induce inflammation. Although the expression of *IL31RA* mRNA was found to be elevated in human AD skin samples compared to that in healthy controls,[144] there is insufficient evidence to support that IL-31RA-expressing immune cells are actually recruited to the skin during atopic inflammation. One study showed the perivascular infiltration of $CD68^+$ (macrophage cell marker) and IL-$31RA^+$-double-positive cells in atopic skin lesions,[138] but another study could not detect any IL-31RA-positive infiltrating cells in the skin of atopic humans.[117]

IL-31-overexpressing transgenic mice develop skin inflammation that closely resembles that lesional skin from patients with AD, but none of the IL-31 protein–injected mice developed skin lesions, except for self-induced alopecia.[65] This suggests that the development of skin lesions arose merely from the excessive scratching behavior over time and that it was not the direct effect of IL-31. In a mouse model of fluorescein isothiocyanate- or 2,4-dinitrofluorobenzene-induced allergic contact dermatitis, while IL-31-deficient transgenic mice showed a reduced allergic itch compared to that of wild-type congeners, both mice showed a normal migration and maturation of skin dendritic cells and the induction of hapten-specific T cells followed by local skin inflammation.[145] Similarly, the “proactive” treatment of NC/Nga atopic mice with an anti-IL-31 mAb reduced the scratching behavior, but it did not improve clinical dermatitis scores or microscopic changes in skin biopsies compared to those of albumin control-injected mice.[68] In this study of atopic mice, the mean dermatitis score continued to increase despite repeated treatment with the anti-IL-31 mAb every fifth day for seven weeks, thereby corroborating the relative lack of importance of IL-31 in atopic skin inflammation.[68] In acute experimental canine AD skin lesions (see Appendix 1 in this chapter for more detailed characteristics and pitfalls of this canine AD model), IL-31 was uniquely responsible for the acute atopic itch because a single caninized anti-canine-IL-31 lokivetmab injection prevented most of the house dust mite (HDM)-induced pruritus manifestations. By contrast, blocking this

cytokine alone did not prevent the development of acute erythematous lesions (see Chapter 2 for more details). These findings further suggest that IL-31 is responsible for the itch but not for the induction of local skin inflammation.

Numerous studies in human AD investigated the correlation between the serum IL-31 levels and the severity of skin lesions to evaluate the importance of this cytokine in atopic skin inflammation. Although some of these studies reported a positive correlation, others showed none.[81–83,89–92,146,147] Similarly, the serum IL-31 levels in atopic dogs have shown an inconsistent correlation with disease severity, but this might have resulted from the use of assays not validated for the measurement of canine IL-31.[148,149] A study in human AD reported that subjectively-scored macroscopic and microscopic skin lesions were significantly higher — i.e., skin lesions were significantly worse — in patients who had IL-31-positive cells in their skin samples compared to those of patients who did not have such IL-31-positive cells.[117] Therefore, the inconsistent correlation between serum IL-31 levels and the severity of the skin lesions might be due to the poor correlation that exists between cytokine levels in the peripheral circulation and those in the skin. Nevertheless, another study failed to detect the correlation between the severity of AD and the expression levels of *IL31* mRNA in the lesional skin of patients.[87]

Furthermore, in phase II trials, nemolizumab — a humanized anti-IL-31RA mAb — showed at best a modest effect on AD skin lesions compared to the significant improvement in itch in those patients, further suggesting that IL-31 does not have a significant role in atopic skin inflammation.[96,97,99,113] Similarly, in dogs, the caninized anti-canine-IL-31 lokivetmab appears to have a rapid and potent anti-pruritic effect but a slower and lower anti-inflammatory potency on AD skin lesions. A blinded, randomized, placebo-controlled clinical trial with client-owned dogs with spontaneous chronic AD reported that the percentage of dogs categorized as normal–very mild (<3.5 of maximum 10 cm) for Owner Pruritus VAS scores for at least one time point post-treatment were greater for the lokivetmab-treated (85.1%) than that for the placebo-treated group (30.6%). On the other hand, there were no differences between treatment groups for the percentage of dogs categorized as normal (≤15 of a possible score of 1240) for Canine Atopic Dermatitis Extent and Severity Index-03 (CADESI-03) scores post-treatment during the 56-day study period.[101] Another double-blinded, ciclosporin (one of the standards of care for canine AD)-controlled clinical study showed a significantly rapid reduction of pruritus in the lokivetmab-treated group compared to that of the ciclosporin group. In contrast, the significant

difference in CADESI-03 score between the treatment groups was not seen until Day 84, which indicates that IL-31 might not play a significant role in the acute phase of atopic skin inflammation.[102]

Since the first discovery of IL-31RA on CD-14-positive cells (monocytes and macrophages) in 2002,[74] various other immune cells, such as dendritic cells, mast cells, eosinophils and basophils, have been reported to express the IL-31 receptors in humans.[112,150] The expression of *IL31RA* mRNA has also been demonstrated in the canine macrophage-monocyte cell line (DH82).[143] Therefore, IL-31 should have the capability to recruit these immune cells to the atopic skin lesions and fuel the atopic skin inflammation. Furthermore, these immune cells (are known to produce IL-31, leading to the autoactivation of these cells. For example, IL-31 significantly induces the release of pro-inflammatory cytokines (IL-1β and IL-6) and AD-related chemokines (CXCL1, CXCL8, CCL2 and CCL18) from peripheral blood eosinophils collected from healthy human volunteers.[140] Interestingly, such induction was further enhanced when eosinophils were co-cultured with keratinocytes.[140] These findings suggested a potential role of IL-31 in recruiting inflammatory cells and skin inflammation through the eosinophils-keratinocytes system.[140] Human peripheral blood eosinophils collected from AD patients displayed an increased release of IL-31 after 24 hours in culture compared with eosinophils of non-atopic patients.[151] The upregulation of IL-31 in eosinophils was stimulated when they were cultivated with Th2 cytokines, such as IL-4, IL-5, IL-13, and even with IL-31, indicating that there is an autoactivation of IL-31 on eosinophils.[151] However, the presence of these IL-31RA-expressing immune cells in the atopic skin lesions is inconsistent. One study in humans has shown that perivascular infiltrating of IL-31RA and CD68 (a macrophage marker) double-positive cells in the skin of atopic patients.[138] Interestingly, these IL-31RA-positive cells were negative for CD3 (a T cell marker) or tryptase (a mast cell marker).[138] On the other hand, another study could not demonstrate the infiltration of cells expressing IL-31 receptors in the skin of atopic patients.[117] Further studies are needed to attest the direct effect of IL-31 in recruiting immune cells and the consequent skin inflammation in atopic skin.

There is increasing evidence that IL-31 might also exert its effect via an indirect mechanism. Keratinocytes are another cell type that expresses IL-31 receptors.[65,117,119,138,140,152–155] Upon binding to its receptor on keratinocytes, IL-31 activates JAK-STAT signal transduction pathway and induces the release of various pro-inflammatory mediators and chemokines — such

as IL-6, MCP-1 (CCL2), TARC (CCL17) and MDC (CCL22) — from primary keratinocytes collected from both atopic and healthy individuals in humans.[65,140,153,154] As a result, IL-31 might recruit various immune cells and induce skin inflammation indirectly. Consistent with this finding, the skin prick tests with IL-31 induce long-lasting erythema in healthy and atopic humans.[64]

In summary, the late onset of IL-31-induced response further supports the notion that IL-31 likely exerts its pro-inflammatory effect indirectly via keratinocytes and secondary mediators, rather than directly stimulating and recruiting dermal inflammatory cells.

1.4 Conclusion

Itch and skin inflammation are the most critical and dual hallmarks of AD. They result from orchestrated interactions between nerves in the skin, keratinocytes and immune cells, and numerous mediators mediate their inter-communication. IL-31 is one of these emerging mediators that has key roles in the pathogenesis of AD. Although previous studies in humans, mice and dogs have revealed the importance of IL-31 in directly inducing atopic itch and, likely indirectly, stimulate atopic inflammation, more studies are needed to optimize the current IL-31-targeted therapy in human and dogs with AD.

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1.6 Appendix Characteristics of our canine AD model

The NCSU inbred colony of Maltese–beagle atopic (MBA) dogs is known to spontaneously exhibit a high IgE response to food allergens and to develop AD signs upon challenge with such allergens. These dogs are also easily experimentally-sensitized to *Dermatophagoides farinae* (Df) HDM allergen at a young age, and they reproducibly produce IgE against Df and develop atopic skin lesions after epicutaneous HDM provocations.[156,157] Dogs in this colony are maintained with restricted dietary management and in a controlled housing environment, preventing the development of spontaneous AD flares to both food and environmental allergens. Once these previously-HDM-sensitized MBA dogs are challenged with HDM, itch manifestations and macroscopic skin lesions (erythema, edema, pustule/papule, excoriation) start to appear early as 24 hours after the provocation before spontaneously subsiding within a week after challenge.[158-160] Microscopic atopic skin lesions (dermal inflammation, acanthosis) can continue even after these clinical signs disappear (see Chapter 5)[160]; however, the expression of AD-related cytokines does not last longer than 96 hours after a single HDM provocation.[159] Therefore, this canine AD model appears to only represent the induction of the earliest stages of acute flares of AD.

Due to relatively rapid spontaneous recovery from AD flares after the HDM challenge, this model does not represent the fully-developed or chronic AD skin lesions that are present in dogs with spontaneous AD. Nevertheless, dogs with spontaneous AD also experience intermittent acute AD flares, especially when the doses of anti-allergic drugs are reduced. Therefore, this model does have value and is best used to study the pathogenesis of acute AD flares. Finally, results obtained with this canine acute AD model need to be followed by clinical studies with client-owned dogs with spontaneous chronic AD so as to detect the changes present in chronic AD.

CHAPTER 2

PROACTIVE MAINTENANCE THERAPY OF CANINE ATOPIC DERMATITIS WITH THE ANTI-IL-31 LOKIVETMAB. CAN A MONOCLONAL ANTIBODY BLOCKING A SINGLE CYTOKINE PREVENT ALLERGY FLARES?

Chie Tamamoto-Mochizuki*, Judy S. Paps* and Thierry Olivry*†

* Department of Clinical Sciences, College of Veterinary Medicine, NC State University, 1060 William Moore Drive, Raleigh, NC 27607, USA

† Comparative Medicine Institute, NC State University, Raleigh, NC 27606, USA

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2.1 Introduction

Atopic dermatitis (AD) is a common, often chronic, pruritic allergic skin disease that can affect humans and dogs; it is characterized by a waxing-and-waning course with frequent flares of inflammation and pruritus.[1,2] In humans, current treatment guidelines recommend the implementation of topical anti-inflammatory therapy during active flares of AD before tapering or discontinuing the topicals once signs and symptoms abate.[3] In the last decade, this traditional "late reactive therapy" to treat existing skin lesions was complemented by a "proactive therapy" that follows the remission of skin lesions to prevent their relapse.[3,4] During proactive therapy, one keeps treating previously affected areas with topical glucocorticoids or calcineurin inhibitors, on two consecutive days each week, whether or not lesions are visible on these areas.[5]

The main strategy behind this proactive therapy is to continue dampening the residual subclinical cutaneous inflammation that persists after reactive therapy in order to prevent or delay subsequent flares of skin lesions. In human patients with moderate-to-severe AD, the proactive therapy with topical glucocorticoids or calcineurin inhibitors lowers the risk of recurrence of signs, extends the time to such relapse, and – especially in severe cases – also can lower the cost of treatment.[5]

A similar proactive treatment strategy has been proposed for the treatment of canine AD. Proactive long-term weekend therapy with a hydrocortisone aceponate (HCA) spray (Cortavance, Virbac; Carros, France) extended almost four-fold the median time-to-flare (TTF) in dogs with AD compared to a placebo spray; it was generally well-tolerated.[6] Because a lasting remission between flares of canine AD is often difficult to achieve, there would be value in exploring additional proactive strategies to prevent disease exacerbations.

Lokivetmab (Cytopoint, Zoetis; Parsippany, NJ, USA) is the first FDA-approved therapeutic monoclonal antibody (mAb) for use in veterinary dermatology. It is an almost-fully caninized mAb that specifically binds to and neutralizes the pruritogenic cytokine IL-31. Since its launch in the USA in 2016, two clinical trials have reported its benefit in client-owned atopic dogs with medium-to-high positive response rates without major adverse effects.[7–9] Lokivetmab appears to have a rapid and potent anti-pruritic effect, but a lower anti-inflammatory effect on AD skin lesions.[7,9]

In an experimental canine model of acute AD and its itch,[10] we have reported the unexpected, early, and very high expression of *IL31* mRNA after an epicutaneous allergen challenge in house dust mite (HDM)-sensitized dogs.[11] This rapid and robust expression of *IL31* mRNA after allergen challenge prompted us to evaluate the ability of lokivetmab to prevent allergy flares in dogs. We hypothesized that the proactive use of this mAb would inhibit IL-31 soon after its secretion, thereby preventing the development of IL-31-induced itch and the inflammatory cascade occurring downstream from this cytokine.

This article consists of the reports of two distinct studies. We first evaluated the validity of anti-IL-31 proactive therapy in our experimental model of acute canine AD.[10] Then, in an open trial we tested whether proactive therapy with lokivetmab would prevent flares of AD in client-owned atopic dogs whose signs were controlled with standard-of-care reactive therapy.

2.2 Methods and materials

Phase 1: experimental acute canine AD model

This study was approved by the institutional animal care and use committee. We used four Maltese-beagle atopic dogs that had been sensitized to the HDM *Dermatophagoides farinae*, as described previously (see Chapter 1 Appendix for more details about this model).[12] There were three males and one female ranging from 3- to 8-year-old. All dogs were injected once subcutaneously with the lokivetmab at an average dosage of 2.75 mg/kg (range: 2.5–3.0 mg/kg); this dosage, higher than that approved in the USA, was selected in an attempt to not miss the detection of any preventive effect on lesions or itch. Seven days after the mAb injection, the dogs were challenged epicutaneously with 25 mg of lyophilized HDM (Greer Laboratories; Lenoir, NC, USA) in 1 mL of mineral oil. We applied this suspension to an area of 200 cm^2 on the previously clipped right side of the abdomen, as reported previously.[10] Starting 24 h before HDM challenge and continuing for 24 h after it, dogs were video-monitored and the duration of pruritus manifestations (DPM), such as scratching, biting, licking or chewing on the right abdomen, was assessed as reported previously.[10] The DPM after the challenge was subtracted from the value for 24 h before it to give the DPM associated with the sole allergen provocation (i.e., the "post-minus-pre-" number of seconds in the first 24 h after challenge).

Immediately before and 24 h post-HDM challenge, erythematous macules, edema, papules/pustules, and excoriations were scored as 0 (absent), 1 (faint, mild), 2 (moderate) or 3 (strong, severe). The grades for each lesion were added to yield a skin lesion score (SLS) with an achievable maximum of 12.[12] To serve as a "no intervention" (i.e. untreated) control, we used the median DPM and SLS values obtained during all HDM challenges in the preceding three years with the same dogs. Statistical analyses were not performed due to the small number of dogs enrolled in this phase.

Phase 2: a prospective study of client-owned dogs with AD

Study design

This study was a prospective, uncontrolled, open-label trial with client-owned atopic dogs treated proactively with monthly-to-bimonthly lokivetmab injections. All dogs with AD that received the first lokivetmab injection in our dermatology service between 1 February 2016 and 30 March 2017 were eligible to enter the study. Owners consented to the injections of

lokivetmab per standard recommendations and to the need of reporting any flare needing anti-allergic treatment escalation.

Case selection

Inclusion criteria

For a dog to be selected for this study, the following three criteria had to be met:

1. It suffered from AD diagnosed from a compatible history and the fulfillment of five published criteria.[13] All skin diseases potentially resembling AD had to be ruled out, when relevant, according to diagnostic and treatment standards. We did not exclude dogs with food-induced AD if they remained on the diets known not to cause a flare of AD; and
2. It had had at least one flare of AD in the preceding six months; and
3. At the time of the first lokivetmab injection, AD was considered to be controlled with any combination of the standard of care anti-allergic medications (i.e., topical or oral glucocorticoids, ciclosporin, oclacitinib or oral antihistamines). We defined "controlled AD" as one with no or mild pruritus (subjective or Pruritus Visual Analog Scale ≤two of 10 evaluated by the owner), with no or mild cutaneous erythema and no or only focal skin or ear canal infections.

Exclusion criteria

The exclusion criteria for any dog were:

1. It had superficial pyoderma or yeast infection requiring oral antimicrobial medications at the time of the first lokivetmab injection; or
2. Any of the anti-allergic medications defined above were administered for more than four weeks after the first lokivetmab injection; or
3. Allergen-specific immunotherapy had been initiated less than 12 months before the first lokivetmab injection; or
4. Lokivetmab was injected less often than every four weeks if given at a 1 mg/kg dosage or every eight weeks for a 2 mg/kg dosage.

Treatment protocol and follow-ups

We used the commercially available lokivetmab in single 1 mL-vials containing either

10, 20, 30 or 40 mg (Cytopoint, Zoetis). Lokivetmab injections were repeated every four weeks for the 1 mg/kg dosage (EU dosage),[9] or four to eight weeks for 2 mg/kg (US dosage)[7]; each of the two dosages was selected by the clinician and owner after considering the cost and expected interval between injections. All of the enrolled dogs were followed prospectively until the pre-defined study end-point (see next section). Topical antiseptics, shampoos without glucocorticoids, or otic medications (even with a glucocorticoid) could be used, as needed, during the follow-up period.

End-point of the study

Each dog was followed until a flare of AD occurred, or one year after the last patient was enrolled (30 March 2018), whichever came first. In this study, we defined a flare of AD as an episode resulting in the need for an escalation of anti-allergic treatment (other than lokivetmab) or for seeking additional (veterinary) medical advice, as proposed previously for humans with AD.[14,15]

Outcome measures

The primary outcome measure was the TTF of AD, defined as the time (in days) elapsed between the flare and the last day of administration of any of the previously given anti-allergic medications. The Kaplan–Meier method was used to estimate the median and distribution of TTF of AD. The secondary outcome measures were the percentages of dogs not having had a flare of AD at three, six, nine and 12 months after the first lokivetmab injection.

2.4 Results

Phase 1: experimental acute canine AD model

The results of this experiment are presented in Table 1 and Figure 1. Without any intervention, the application of HDM onto the skin of these sensitized dogs led to a marked increase in pruritus manifestation duration (a median change of over 2,000%) and mild-to-moderate skin lesions with a median SLS of 4 (Table 1).

When these dogs were proactively treated with a single injection of lokivetmab, one week before the challenge, and compared with their expected "no intervention" scores, there was a reduction in both pre-challenge DPMs in the 24 h before the allergenic challenge

("prechallenge") and in those during the 24 h that followed it ("post-challenge"'; Table 1). Compared to results without intervention, the median change in DPM due to the lokivetmab proactive therapy was –90% (range: –81 to –93%; Figure 1, left panel).

By contrast with the substantial effect on preventing the development of pruritus manifestations, there was little apparent benefit of the lokivetmab proactive therapy to prevent the induction of skin lesions (Table 1, Figure 1, right panel). The median change in SLS due to lokivetmab proactive therapy was 0% (range: –75% to +50%).

Table 1. Comparison of duration pruritus manifestations and skin lesion scores with or without lokivetmab proactive therapy.

	No Intervention			
	Pre-challenge	Post-challenge	Post-minus-pre	Post-vs-pre (%)
Duration of pruritic manifestations (s/24h)	107 (52-178)	2374 (2173-2580)	2283 (2090-2402)	2115 (1349-4500)
Skin Lesion Scores	0 (0-0)	4 (2-4)	4 (2-4)	Not calculated
	Lokivetmab pretreatment			
	Pre-challenge	Post-challenge	Post-minus-pre	Post-vs-pre (%)
Duration of pruritic manifestations (s/24h)	35 (20-67)	210 (205-470)	185 (165-403)	601 (413-950)
Skin Lesion Scores	0 (0-0)	3 (1-4)	3 (1-4)	Not calculated

data presented as median (range)

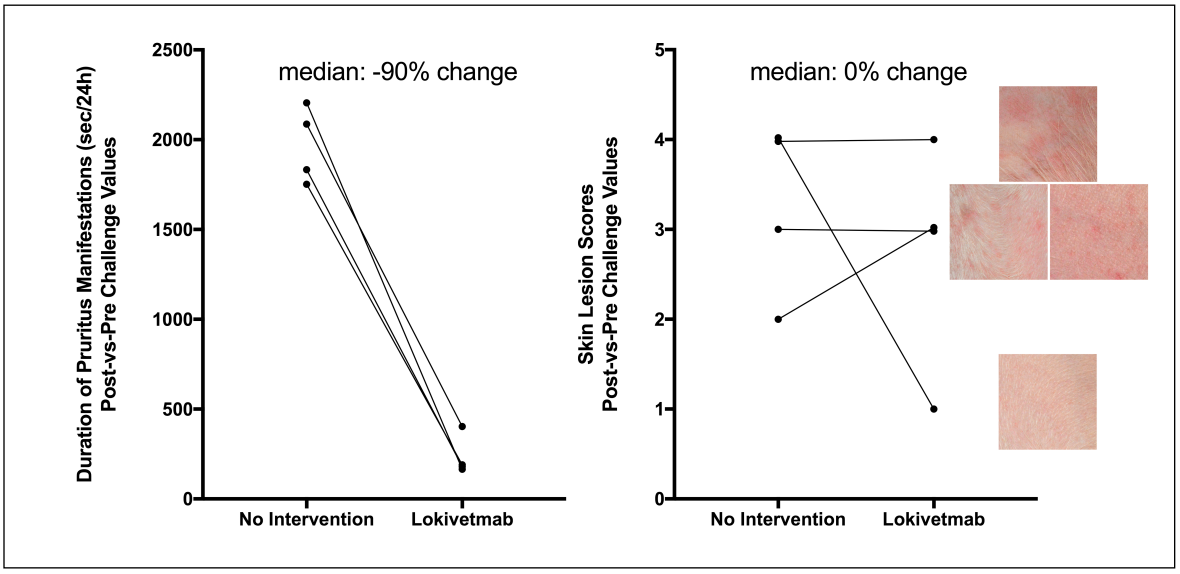

Figure 1. Comparison of duration pruritus manifestations and skin lesion scores with or without lokivetmab proactive therapy.

While the lokivetmab abolished most of the pruritus manifestations, erythematous lesions still developed within 24 h of an epicutaneous allergen challenge.

Phase 2: a prospective study with client-owned dogs with AD

In total, 103 dogs with AD received the first lokivetmab injection at our institution during the recruitment period. Of these 103 dogs, 58 did not meet inclusion criteria, 21 were removed

based on one or more exclusion criteria and we excluded three others from analysis due to different reasons (Figure 2). Therefore, 21 enrolled dogs were followed for up to one year of lokivetmab proactive therapy.

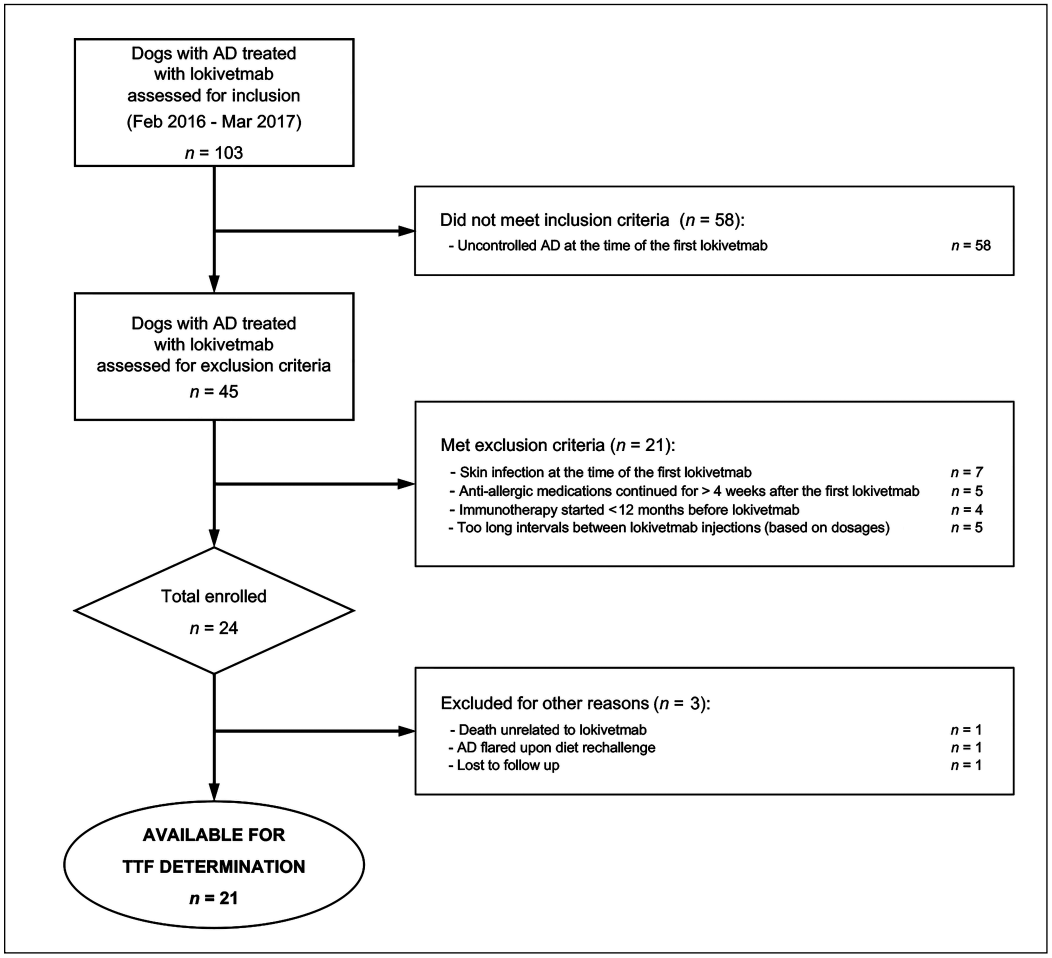

Figure 2. Flow-chart of lokivetmab proactive therapy in client-owned dogs with AD.
Abbreviation: TTF = time-to-flare

Two dogs (cases 18 and 20) stopped receiving additional lokivetmab injections in spite of not having had a flare of AD during the follow-up period; these dogs were censored (i.e., they were not analyzed further as data were incomplete or not informative) from the study on the day of the last lokivetmab injection, but they remained included in the data analysis (Appendix Table S1). Three dogs (cases 5, 6 and 15) did not have a flare of AD by the end of the study; they were included in data analysis with an end-of-study censoring.

Altogether, the earliest TTF of AD after discontinuation of anti-allergic medications was three days. By contrast, one of the censored dogs (case 5) did not have a flare of AD until the end of the study with a TTF of AD longer than 718 days (Appendix Table S1). The Kaplan–Meier curve, designed to reflect the percentage of dogs not having a flare of AD over time, yielded a median TTF of AD of 63 days for these 21 dogs (Figure 3).

The observed percentages of dogs not having had a flare of AD at three, six, nine and 12 months was 43% (9 dogs), 33% (7), 19% (4) and 19% (4), respectively (Appendix Table S1). Taking into account the censored dogs in the Kaplan–Meier analysis, the percentages of dogs not having had a flare of AD at these time points were, respectively, 43%, 33%, 28% and 28% (Figure 3).

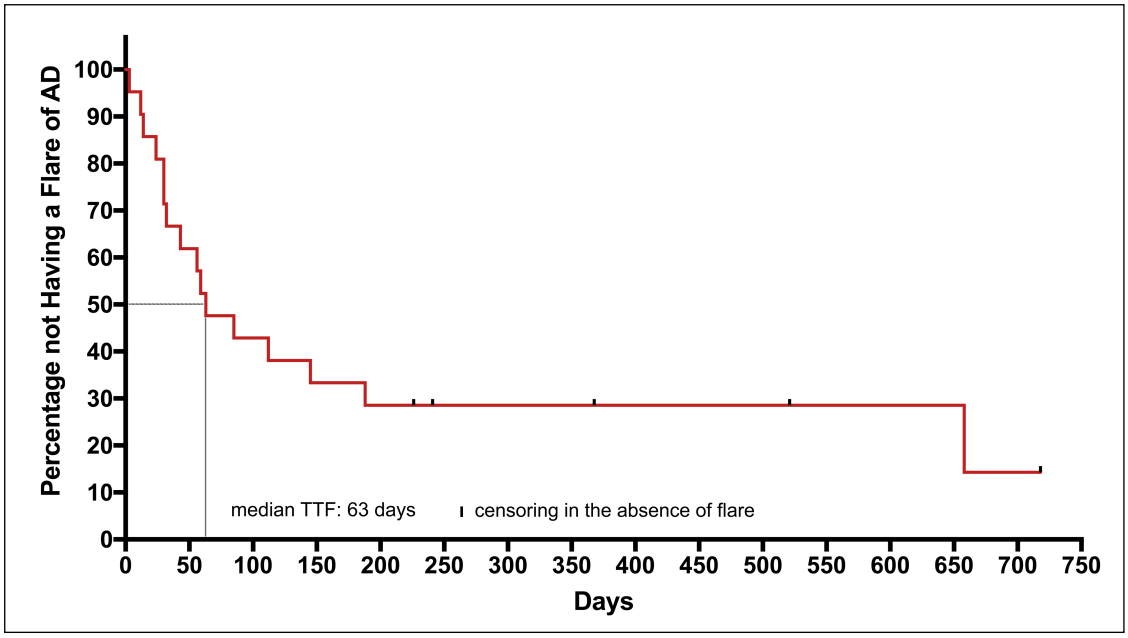

Figure 3. Probability of flares of AD in client-owned dogs receiving lokivetmab proactive therapy.
Abbreviation: TTF = time-to-flare

2.5 Discussion

In this study, we tested the ability of the anti-IL-31 lokivetmab to prevent the development of clinical signs in an experimental model of acute AD skin lesions in dogs and a prospective follow-up of dogs treated proactively with this mAb.

In our acute canine AD experimental model, we had shown previously that the *IL31*-encoding gene was rapidly transcribed after the allergen challenge and that this cytokine gene

was the most highly upregulated among those coding for the known protein pruritogens examined.[11] In the current study, we confirmed that IL-31 was uniquely responsible for the acute atopic itch, because a single lokivetmab injection prevented most of the HDM-induced pruritus manifestations. By contrast, blocking this cytokine alone did not prevent the development of acute erythematous lesions. Because skin prick tests with IL-31 induced long-lasting erythema in healthy and atopic humans,[16] we had expected that inhibiting this cytokine would have a higher effect to prevent the visible allergen-induced acute skin inflammation. Nevertheless, our results suggest a minimal role for this cytokine, or mediators derived from it, in the generation of early AD skin lesions that follow an epicutaneous allergen challenge. This observation of skin inflammation development not blocked by lokivetmab is noteworthy. Indeed, if it were not treated immediately, and were it to occur over a large area of skin, one could assume that it might evolve into a *bona fide* disease flare involving numerous inflammatory mediators and cells, epidermal hyperplasia and an inflammation-induced stratum corneum barrier defect with resulting skin surface microbial dysbiosis, thus needing a multimodal therapy and/or broadly targeting antiallergic drugs.

Similarly to our results, the "proactive" treatment of NC/Nga atopic mice with an anti-IL-31 mAb reduced the scratching behavior but did not improve clinical dermatitis scores or microscopic changes in skin biopsies compared to those of albumin control-injected mice.[17] In that study of atopic mice, the mean dermatitis score continued to increase despite repeated treatment with the anti-IL-31 mAb every fifth day for seven weeks, thereby corroborating the relative lack of importance of IL-31 in atopic skin inflammation.[17] Finally, our results mirror those seen when inducing allergic contact dermatitis in *IL31*-deficient (*Il31*$^{-/-}$) mice.[18] In this model, as in ours, whereas the allergic pruritus was reduced after hapten application, the local skin inflammation was not.[18]

The results seen with proactive lokivetmab therapy match those obtained after the "reactive" treatment of dogs or humans with active AD with – respectively – lokivetmab or the anti-IL-31 receptor nemolizumab. Both of these mAbs lead to a lower reduction of skin lesion than pruritus scores, thereby confirming the minor role of IL-31 in atopic inflammation compared to allergic itch in both species.[7,9,19]

In the second phase of our study, the prospective follow-up of client-owned dogs, half of the dogs treated proactively with monthly to bimonthly lokivetmab injections had a flare of AD

requiring treatment escalation within the first two months after discontinuing all antiallergic drugs. Even though a potential limitation of this study is that it was not controlled with a placebo — a possible unethical intervention in this situation where dogs had a controlled disease when they were selected — we chose to compare our TTFs with those generated in two previously published trials that have evaluated AD flares after standard-of-care anti-allergic drug discontinuation. In the first study,[20] 100 dogs were treated for four months with tapering doses of either oral ciclosporin (Elanco; Greenfield, IN, USA) or methylprednisolone (Oro-Medrol, Zoetis). The median TTFs after ciclosporin and methylprednisolone discontinuation, estimated from the published Kaplan–Meier figure, were 44 and 26 days, respectively.[20] In the second study,[6] 41 dogs were treated with an HCA spray (Cortavance, Virbac), and then, once signs were in remission or comparable to those of mild AD, they were treated with weekend applications of proactive HCA or placebo. The median TTFs after placebo and HCA proactive therapy were 33 and 112 days, respectively.[6] Although these results cannot be compared directly because of differences in canine populations and study designs, the TTF that we observed after lokivetmab proactive therapy seems to be about twice that seen without any treatment, but only half that obtained with twice-weekly proactive topical glucocorticoid therapy.[6,20] Furthermore, we compared our post-lokivetmab Kaplan–Meier TTF curve with those of the HCA proactive study using a partial dataset provided by the authors of that study[6]; the TTFs after lokivetmab therapy were significantly longer than those after placebo but not after the HCA (data not shown). We suspect that, compared to the topical HCA, the lower median TTF seen after lokivetmab is likely due to both a broader anti-allergic effect of the glucocorticoid and a limited role of IL-31 in the inflammation of atopic skin.

In humans with AD, there is no clear consensus for how long patients need to be treated with anti-allergic drugs (i.e., during induction therapy until the control of not only macroscopic lesions but also subclinical inflammation) before moving onto a phase of proactive maintenance therapy.[5] An "induction of remission" phase that is too short likely leads to a failure of managing AD.[5] In our study, we defined dogs with "controlled AD" as those having no or mild pruritus, no or mild erythema and no or focal skin or ear infection; such dogs were then selected to be treated proactively with lokivetmab maintenance therapy. The rapid flares seen in some dogs after discontinuation of the anti-allergic drugs, less than two weeks in three of our dogs (Appendix Table S1), suggest that inflammation was still present at the time of standard treatment cessation

and that a longer induction of anti-allergic drugs might have been needed in these patients.

Another possibility for the relatively poor flare-preventive efficacy of proactive therapy with lokivetmab is a shift in the cytokine repertoire during the evolution of AD skin lesions. In canine as in human AD, although Th2-type cytokines are involved in acute lesions, a more complex cytokine milieu involving – at least – a Th1 response is believed to predominate during chronic stages.[21,22] Interleukin-31 being one of the Th2 cytokines,[23] it would be logical to expect that the lesion-preventive effect of lokivetmab might decrease after the acute lesions begin to mature and the cytokine repertoire shifts away from a Th2-predominant one. Indeed, during a flare of canine AD lesions induced by an allergen challenge in HDM-sensitized atopic dogs, there is a variable cytokine expression over time, even in the first two days after provocation, thereby likely diluting the importance of IL-31 as lesions progress.[11,24]

Lastly, in the current model of the cytokine cascade characteristic of AD inflammation, IL-31 is secreted downstream from several other cytokine groups, including, for example, the keratinocyte-derived cytokines IL-33, thymic stromal lymphopoietin (TSLP), IL-25 and IL-17C, and the inflammatory cell-secreted Th2 cytokines IL-4, IL-5 and IL-13.[22,25,26] As a result, even if lokivetmab were to block the action of IL-31 completely, there would be ample opportunity for inflammation to develop due to the action of the many mediators secreted upstream of, or concurrently with, IL-31.

In spite of the flare of AD seen in half of the case study dogs in the first two months of proactive lokivetmab monotherapy, over a quarter of followed cases did not exhibit a disease flare for more than one year. Although this percentage does not seem high, because AD is a multifactorial disease that requires a multi-faceted treatment approach,[27] the long-term remission seen in some dogs after targeting a single cytokine suggests that IL-31 is critically involved in the pathogenesis of clinical signs of AD in these cases.

Finally, we would be remiss in not mentioning that, in humans, different phenotypes of AD have been reported to occur in different ethnic groups. For example, Asian patients with AD have increased epidermal hyperplasia and parakeratosis and a higher Th17 activation compared to the European American atopics.[28] Similarly, the early onset pediatric human AD also exhibits a robust Th17 response.[29] The reported variation of clinical phenotypes among atopic dogs of different breeds[30] could be interpreted as different genetically influenced pathomechanisms being present in canine as in human AD. Such variation in breed-associated pathogenesis — and

perhaps even one related to the age at the time of treatment — could potentially lead to heterogeneity in the efficacy of lokivetmab proactive therapy. As we only had a small number of dogs of different breeds, a breed- or age-specific response to lokivetmab prevention therapy could not be evaluated.

2.5 Conclusion

We showed herein that proactive maintenance therapy of canine AD with lokivetmab injections led to a long-lasting remission of clinical signs in some cases, but more than two-thirds of treated dogs eventually flared within two months after discontinuation of other anti-allergic drugs. These results suggest the existence of heterogeneity in lesion development mechanism in dogs as in humans with AD (that is the existence of "different endotypes"), and that factors other than IL-31 are involved in AD skin lesion development in most dogs with AD. Nevertheless, additional studies are needed to elucidate the specific roles of IL-31 in canine and human AD, and to better select patients that would benefit the most from proactive therapy with anti-IL-31 therapy.

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2.7 Appendix

Appendix Table S1. Patient signalment and individual time-to-flare data.

Case No.	Breed	Sex	Age at the first lokivetmab (yrs)	Body Weight (kg)	Lokivetmab dosage (mg/kg)		Concurrent medications at the time of the first lokivetmab injection	TTF of AD (days)	No AD flare at 3 months	No AD flare at 6 months	No AD flare at 9 months	No AD flare at 12 months
					first	last						
1	Lhasa Apso	FS	12	10.0	2.0	2.1	None	30				
2	Labrador Retriever	MC	7	45.3	1.8	1.8	Oclacitinib (7)	63				
3	Vizsla	FS	7	20.2	2.0	1.9	Oclacitinib (5)	43				
4	English Springer Spaniel	FS	5	16.0	2.5	2.5	Cyclosporine/Ketoconazole and Temaril-P (7)	85				
5	Labradoodle	MC	7	22.6	1.8	1.8	None	718*	x	x	x	x
6	Terrier	FS	9	9.7	2.1	1.9	None	658	x	x	x	x
7	Papillon	FS	6	13.7	2.2	2.3	None	56				
8	Havanese	FS	6	9.0	2.2	2.3	Prednisolone (5)	32				
9	American Cocker Spaniel	FS	5	12.7	2.4	2.4	Prednisolone (6)	14				
10	Mix Breed	FS	11	22.2	1.4	1.6	None	112	x			
11	Lhasa Apso	FS	13	4.3	2.3	2.3	Oclacitinib (3)	24				
12	Dalmatian	MC	3	23.3	1.3	1.3	Cyclosporine/Ketoconazole (14), Temaril-P (3)	3				
13	Shih Tzu	MC	8	8.0	1.3	1.3	None	12				
14	Terrier	MC	8	33.3	1.2	1.8	None	59				
15	Hound	MC	5	27.6	2.2	2.4	Cyclosporine/Ketoconazole (4)	521*	x	x	x	x
16	Shih Tzu	MC	4	6.4	3.1	3.2	Temaril-P (6)	30				
17	Golden Retriever	MC	5	37.3	2.4	2.3	Oclacitinib (3)	188	x	x		
18	Border Terrier	FS	7	9.8	2.0	2.2	None	226*	x	x		
19	Labrador Retriever	FS	5	29.6	2.0	2.0	Oclacitinib (7)	145	x			
20	Pug	MC	11	12.5	2.4	2.5	None	241*	x	x		
21	German Shepherd	FS	3	33.6	1.8	1.7	None	365*	x	x	x	x
		MC 9	Median 7	Median 16.0			( ): # of days when the medication was continued after the first lokivetmab injection	Median 63	*n* = 9 (43%)	*n* = 7 (33%)	*n* = 4 (19%)	*n* = 4 (19%)
		FS 12	Min 3	Min 4.3				Min 3				
			Max 13	Max 45.3				Max 718*				
								*day when censored				

CHAPTER 3

IL-31 AND IL-31 RECEPTOR EXPRESSION IN ACUTE EXPERIMENTAL CANINE ATOPIC DERMATITIS SKIN LESIONS

Chie Tamamoto-Mochizuki*, Judy S. Paps* and Thierry Olivry*†

* Department of Clinical Sciences, College of Veterinary Medicine, NC State University, 1060 William Moore Drive, Raleigh, NC 27607, USA

† Comparative Medicine Institute, NC State University, Raleigh, NC 27606, USA

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3.1 Introduction

In 2004, interleukin 31 (IL-31) was first identified in mice as a cytokine causing severe pruritus followed by skin lesions that closely resembled those of atopic dermatitis (AD).[1] This cytokine took the center stage in veterinary dermatology since lokivetmab (Cytopoint; Zoetis, Parsippany, NJ, USA), a caninized therapeutic monoclonal antibody (mAb) that targets and neutralizes canine IL-31, was launched in 2016 for the treatment of canine AD. Lokivetmab has a very rapid and potent antipruritic effect, is effective in up to 88% of atopic dogs, and prevents atopic flares for more than a year in 28% of the cases;[2–5] these observations highlight the important role of IL-31 in the pathogenesis of canine AD.

Although IL-31-blockade therapy is more advanced in veterinary than human medicine,

the studies of IL-31 in dogs with AD are few compared to those in humans. There are only four papers and two conference presentations that report the serum IL-31 levels in dogs, with results similar to those seen in humans. The serum IL-31 level in atopic dogs is increased compared to that in healthy dogs,[6,7] but there is an inconsistent correlation with the severity of AD skin lesions.[8,9] These results indicate that we still do not fully understand the precise role of IL-31 in AD, and that more research studies are needed to elucidate the IL-31 pathway in atopic skin. At this time, such studies are still very limited in dogs. Two papers demonstrated that T cells, likely type 2 helper T (Th2) cells, produced IL-31, however, these studies were performed in stimulated peripheral blood cells.[10,11] Another study using canine atopic skin samples reported the production of IL-31 by lymphocytes and plasma cells, however, the specificity of the primary antibody used to detect canine IL-31 was not validated.[12] Similarly, only two poster presentations confirmed the expression of the IL-31 receptor alpha chain (*IL31RA*) on canine keratinocytes, but detailed results have not been published yet.[13,14]

As is well-known in both humans and dogs, the cytokine milieu changes during the evolution of atopic skin lesions.[15,16] Thus, exploring the dynamics of IL-31 secretion in canine atopic skin lesions would permit to determine the optimal timing for the maximum effect of IL-31 targeted therapy. To the best of our knowledge, no studies have characterized the chronological change of IL-31 production at the protein level in atopic skin, be it in humans or dogs.

Therefore, our objectives were: 1) to assess the chronology of IL-31 expression in experimental canine acute atopic skin lesions, 2) to compare it with the serum IL-31 levels and the severity of macroscopic skin lesions, and 3) to determine the identity of IL-31- and IL-31RA-positive cells. Such a study would enable a better understanding of the precise signaling cascade dynamics of IL-31 secretion in acute canine AD, which will help optimize IL-31-targeted therapy.

3.2 Materials and methods

Ethics

All procedures were approved beforehand by our university's Institutional Animal Care and Use Committee (IACUC ID #17-005-O).

Animals and sample size estimation

In this experiment, we used our atopic dog model, an inbred line of laboratory Maltese-beagle dogs that were sensitized to the *Dermatophagoides farinae* house dust mite (HDM), as described previously (see Chapter 1 Appendix for more details about this model).[17]

We determined that at least four dogs would need to be included for this experiment to have a greater than 80% power to find results significant at the *p*-value at 0.05 (one-sided) using our previously published data.[18] There were two males and two females ranging in age from 7 to 10 years.

Experimental design and sample collection

Atopic skin lesions were induced by the epicutaneous application of 20 μL of HDM slurry (25 mg of lyophilized HDM [Greer Laboratories, Lenoir, NC] in 1 mL of mineral oil), as described previously.[19] A droplet of suspension was applied to three areas of the previously-clipped lateral flanks of each dog. Blood and skin samples were collected at baseline (0 hour), and 24, 48 and 96 hours after the HDM provocation. One sample was collected at each time point using an 8-mm punch from one of the HDM application sites.

Skin lesion score

Immediately before (0 hour), 24, 48 and 96 hours post-HDM provocation, erythematous macules, edema, and papules/pustules were scored as 0 (absent), 1 (faint, mild), 2 (moderate) or 3 (strong, severe) at the site of HDM application to evaluate skin lesions directly caused by atopic inflammation. The grades for each lesion were added to yield a skin lesion score with an achievable maximum of 9.

Serum IL-31 level

The serum IL-31 level was measured using a single-molecule array technology (SiMoA; Quanterix, Lexington, MA, USA), also called digital ELISA. This assay enabled to measure the target protein with a limit that is 1000-fold lower than that of conventional techniques.[20] Collected sera were immediately frozen at −80°C and sent to Zoetis where the canine IL-31 SiMoA system was available.

Immunofluorescence

Sample preparation

Collected skin samples were snap frozen and cryosectioned at 5-µm thick. Formalin fixation was not performed to prevent the loss of immunoreactivity.

Stimulated canine peripheral blood mononuclear cells (PBMCs) and neuron-rich canine nasal planum skin were used as positive controls for IL-31 and IL-31RA IF, respectively. The detailed procedures to prepare the positive controls are described in Appendix S1.

IL-31 IF single staining

A two-step indirect IF was performed to detect IL-31 using a mouse anti-canine IL-31 monoclonal antibody (Zoetis). The IL-31 IF on stimulated canine PBMCs (positive control) showed a strong cytoplasmic signal (Figure 1a); The marked reduction of IL-31 staining after pre-incubation of the primary antibody with recombinant canine IL-31 (Zoetis) supported the specificity of the anti-IL-31 primary antibody used in this study (Figure 1b). For the detailed protocol, see Appendix S1.

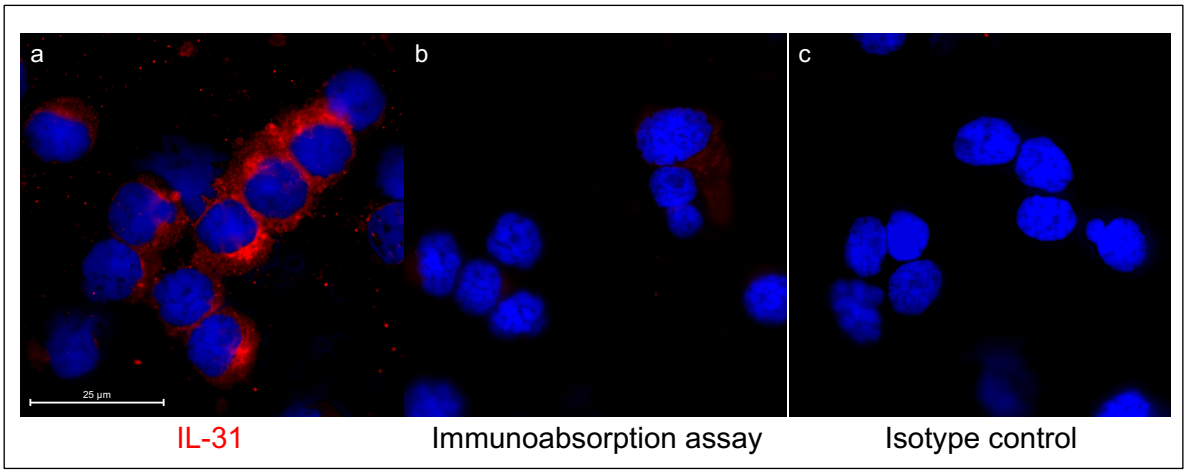

Figure 1. IL-31 single staining IF in stimulated canine PBMCs (positive control).

(a) The IL-31 IF using stimulated canine PBMCs (positive control) showed a strong cytoplasmic signal (red). (b) The marked reduction of this positivity by pre-incubating the primary antibody with recombinant canine IL-31 (immunoabsorption assay) confirmed the specificity of this primary antibody used in this study. (c) The IF using appropriate isotype control did not show any signals.

IL-31 IF double staining

To identify the nature of IL-31-positive cells, we double-stained all sections for IL-31 and

CD3 as well as IL-31 and CD4. For the detailed protocols, see Appendix S1.

IL-31RA IF single staining

A two-step indirect IF was used to identify the IL-31RA-positive cells using a goat anti-human IL-31RA polyclonal antibody (AF2769, R&D Systems, Minneapolis, MN, USA), which had been used in a previous study in dogs.[14] For the detailed protocol, see Appendix S1.

IL-31RA IF double staining

To identify the nature of the IL-31RA-positive linear structures observed in the dermis, we performed a double staining for both IL-31RA and β3-tubulin, a neuronal marker. Thicker-cut sections (10-μm thick) were used to track the linear structures more continuously. The three-dimensional image reconstructed from z-stack images of a confocal microscope showed IL-31RA and β3-tubulin double-positive linear structures in the upper dermis of canine nasal planum skin (positive control), which validated this assay for the detection of IL-31RA-immunoreactivity on dermal nerve fibers in dogs (Figure 2). For the detailed protocol, see Appendix S1.

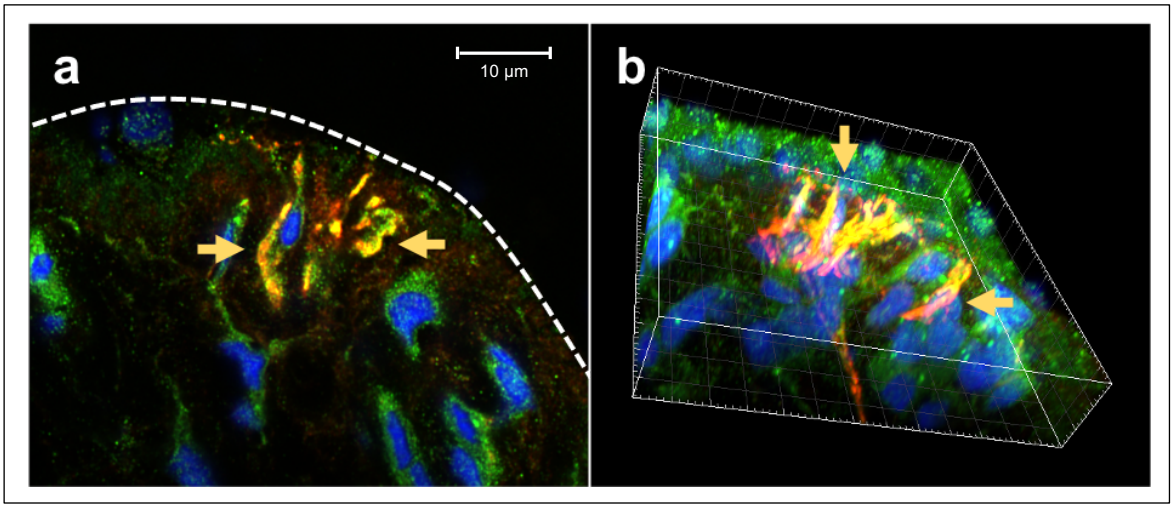

Figure 2. IL-31RA and β3-tubulin double staining IF in nasal planum of a normal dog (positive control).
The 2D (a) and 3D (b) images of IL-31RA and β3-tubulin double staining IF reconstructed from z-stack images of a confocal microscope showed IL-31RA and β3-tubulin double-positive linear structures (orange arrow) at the upper dermis of canine nasal planum (positive control). The white dashed-dotted line indicates epidermal-dermal border line.

Evaluation

IL-31 expression score

The entire section of each sample was scanned using a 40x magnification (see Appendix S1 for the detailed settings). The IL-31-positive round or dendritic cells were subjectively scored for their distribution (0 = none; 1 = one focal area; 2 = multifocal areas; 3 = diffuse) and cell number (0 = none; 1 = low; 2 = medium; 3 = high) to yield an IL-31 expression score (distribution x cell number scores with a maximum of 9).

Percentages of IL-31$^+$CD3$^+$ or IL-31$^+$CD4$^+$ double-positive cells

Digital images of the three areas with the highest apparent density of inflammatory cells of each section were acquired at a 40x magnification (see Appendix S1 for the detailed settings). The total number of epidermal and dermal infiltrating round and dendritic cells with single- or double-positive immunoreactivity was enumerated using ImageJ manual cell counting (https://imagej.nih.gov/ij/). The percentages of double-positive cells among the total number of IL-31-positive cells were then calculated.

IL-31RA-positive cells

Considering that the IL-31RA belongs to a transmembrane receptor, positive staining was considered when a cell surface staining was visualized. A positive staining for dermal nerve fibers was considered if the immunolabeling for β3-tubulin was visualized in a continuous or segmented orientation.

Statistical methods

A Friedman test (nonparametric, repeated-measure ANOVA) with Nemenyi post-hoc tests were used to compare over time the serum IL-31 levels and IL-31 expression scores. A Spearman's rank correlation test was used to investigate the correlation between the IL-31 expression score and the serum IL-31 levels or the skin lesion scores. A p-value equal to or less than 0.05 was considered significant. All analyses were performed using Microsoft Excel 16.41 (Microsoft Corporation, Redmond, WA, USA).

3.3 Results

1) Chronological change of IL-31 in acute canine atopic dermatitis

The range of the serum IL-31 level of 55 normal dogs in a previous study using the same SiMoA technique, was 0.02 to 4.1 pg/ml with a median value of 0.15.[7] All dogs in our study showed higher serum IL-31 levels than this median value, even at baseline before challenge.

The serum IL-31 levels of each dog increased throughout the study period, except for those of Dog 3, which remained unchanged (Figure 3a). On the other hand, the peaks of the skin IL-31 expression scores of each dog occurred at 24 or 48 hours after HDM provocation (Figure 3b, 4); scores started to decrease at 96 hours (Figure 3b, 4), except for, again, Dog 3 whose score continued to increase throughout the study period (Figure 3b). We did not reveal any statistically significant differences at any time points compared to the baseline value for either serum IL-31 levels or IL-31 expression scores.

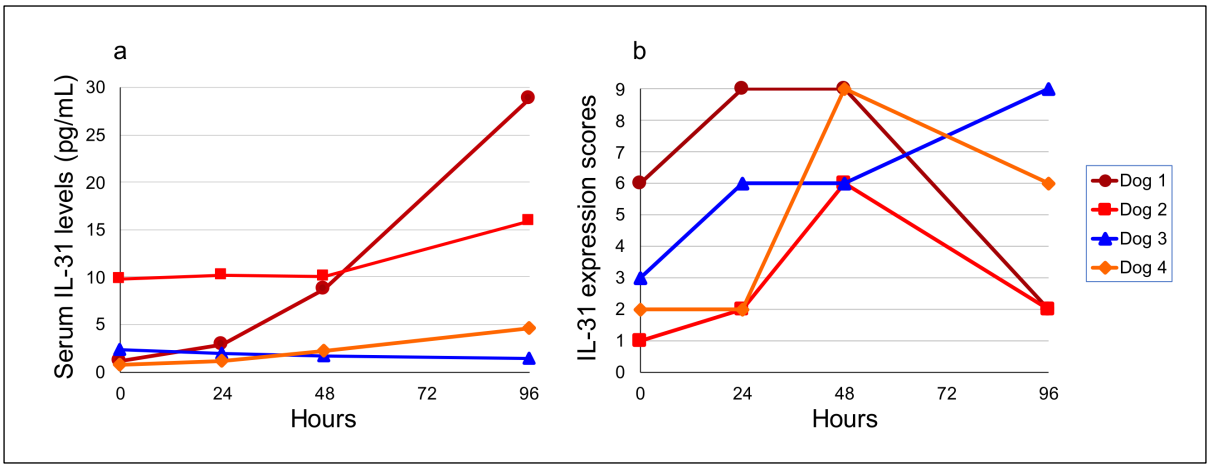

Figure 3. Chronological change in the serum IL-31 levels and the IL-31 expression scores.

(a) The serum IL-31 levels of each dog increased throughout the study period, except for Dog 3 whose serum IL-31 level was unchanged. (b) The peak IL-31 expression scores for each dog occurred at 24 or 48 hours after HDM provocation; they started to decrease at 96 hours, except for Dog 3 whose score continued to increase throughout the study period.

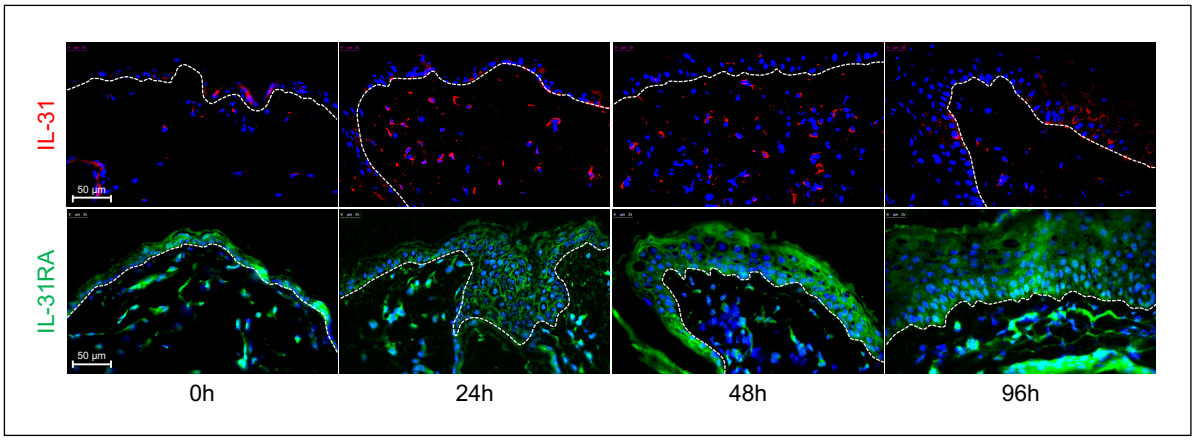

Figure 4. Chronological changes of IL-31- and IL-31RA-positive cells in the canine atopic skin.
Representative pictures of IL-31 and IL-31RA-positive cells of Dog 1 at each sampling time point (0, 24, 48 and 96 hours). Epidermal and dermal infiltrating round and dendritic cells showed a positive cytoplasmic IL-31 signal (red). The IL-31RA IF showed an epidermal intercellular pattern (green) at all time points suggesting its constitutive expression by keratinocytes. The white dotted line represents the epidermal-dermal border.

2) Correlation between the IL-31 expression scores and the serum IL-31 levels or skin lesion scores

Overall, there was only a weak negative correlation between the IL-31 expression scores and the serum IL-31 levels ($r = –0.25$, $p = 0.35$; Figure 5a). In contrast, there was a weak positive correlation between the IL-31 expression and the macroscopic skin lesion scores ($r = 0.25$, $p = 0.36$; Figure 5b).

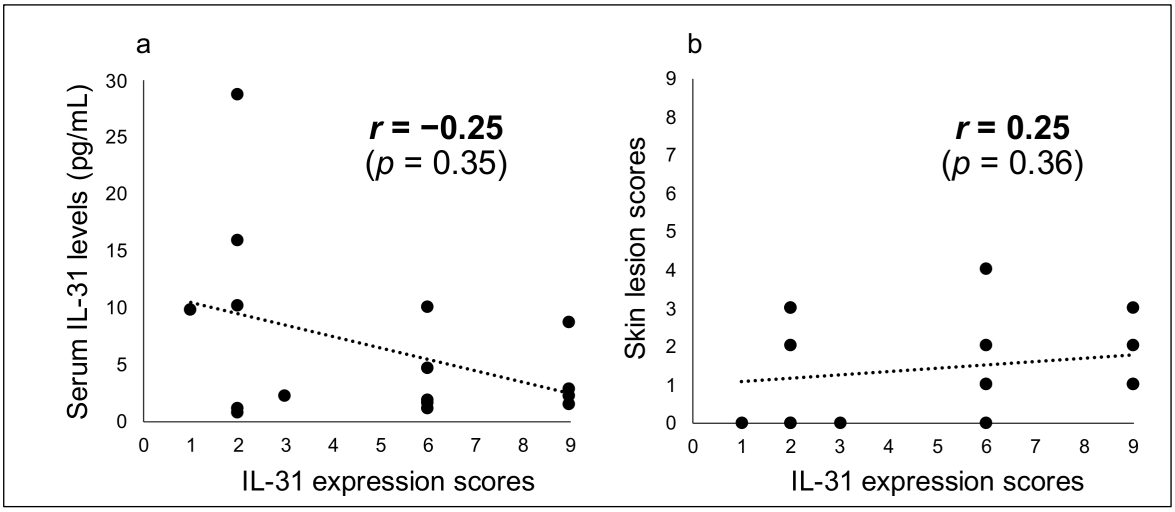

Figure 5. Correlation between the IL-31 skin expression scores and the serum IL-31 levels or the skin lesion scores.

(a) Only a weak, but a non-significant negative correlation was seen between the IL-31 expression scores and the serum IL-31 levels. (b) Similarly, only a weakly-positive but non-significant correlation was observed between the IL-31 skin expression and the macroscopic skin lesion scores.

3) Identity of IL-31- and L-31RA-positive cells

The vast majority of the round and dendritic cells were double-positive for IL-31 and CD3 (range: 91-100%, median = 98%) (Figure 6a). Similarly, most of these cells were double-positive for IL-31 and CD4 (range: 63-100%, median = 98%) (Figure 6b). Although we did not perform triple-staining, based on the percentages of these double-stainings, we concluded that most IL-31-immunolabeled cells expressed both markers, suggesting that they were Th2 cells. Unexpectedly, sebaceous glands were strongly immunolabeled for IL-31 in all dogs, even before challenge; the extinction of this positivity by immunoabsorption with IL-31 further supported the specificity of this immunostaining. (Figure 7).

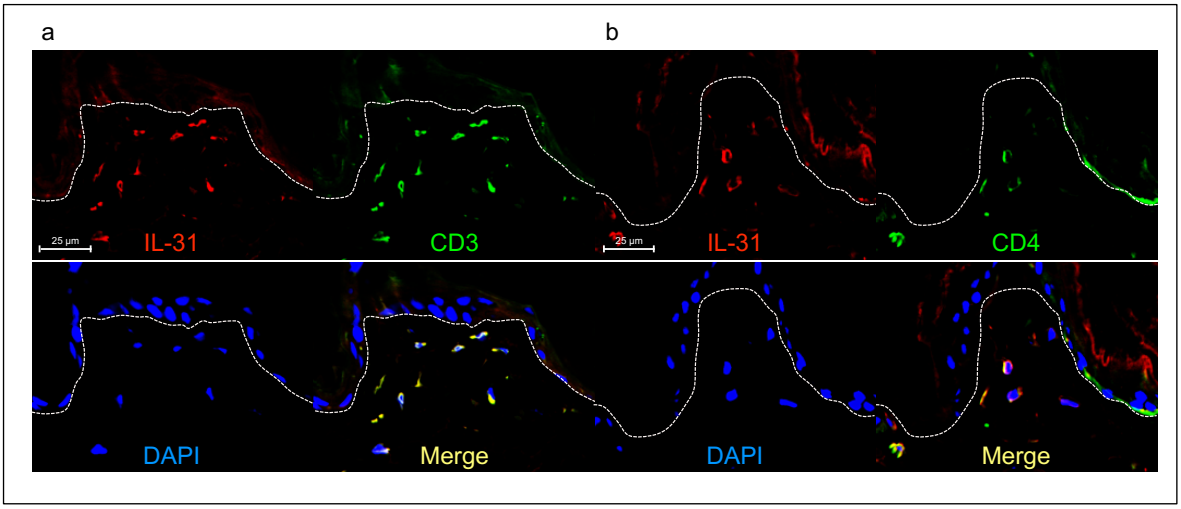

Figure 6. IL-31+CD3 and IL-31+CD4 double staining IF.

(a) The vast majority of IL-31-positive cells (red) and CD3-positive cells (green) overlapped (yellow). (b) Similarly, almost all IL-31-positive cells (red) also expressed CD4 (green). These results indicate IL-31-producing cells likely express both CD3 and CD4 cells, indicating that they represent helper T cells.

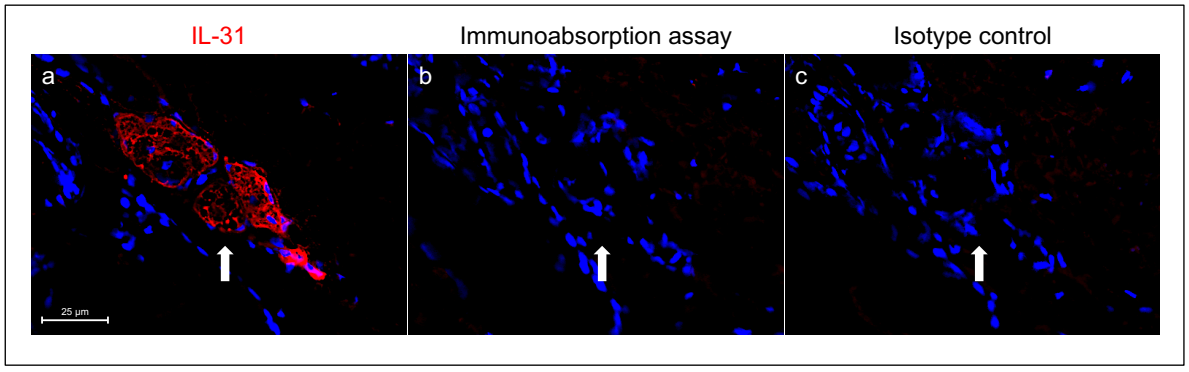

Figure 7. IL-31 single staining IF and immunoabsorption assay in sebaceous glands.

(a) Sebaceous glands strongly immunolabeled with IL-31 (red). (b) The positive signal was fully extinguished by pre-incubating the primary antibody with recombinant canine IL-31 (Immunoabsorption assay). (c) The IF using an isotype control did not show any staining. The arrow indicates the same sebaceous glands.

The IL-31RA IF single staining showed an epidermal intercellular pattern at all time points suggesting its constitutive expression by keratinocytes (Figure 4). A small proportion of the dermal β3-tubulin-positive linear structures were also immunolabeled for IL-31RA, indicating the expression of IL-31RA on some dermal nerves (Figure 8). Although a green

fluorescence was seen on some round or dendritic cells in the dermis, since they did not show a membrane pattern and a similar fluorescence was also seen with the isotype control (data not shown), we concluded these to be non-specific.

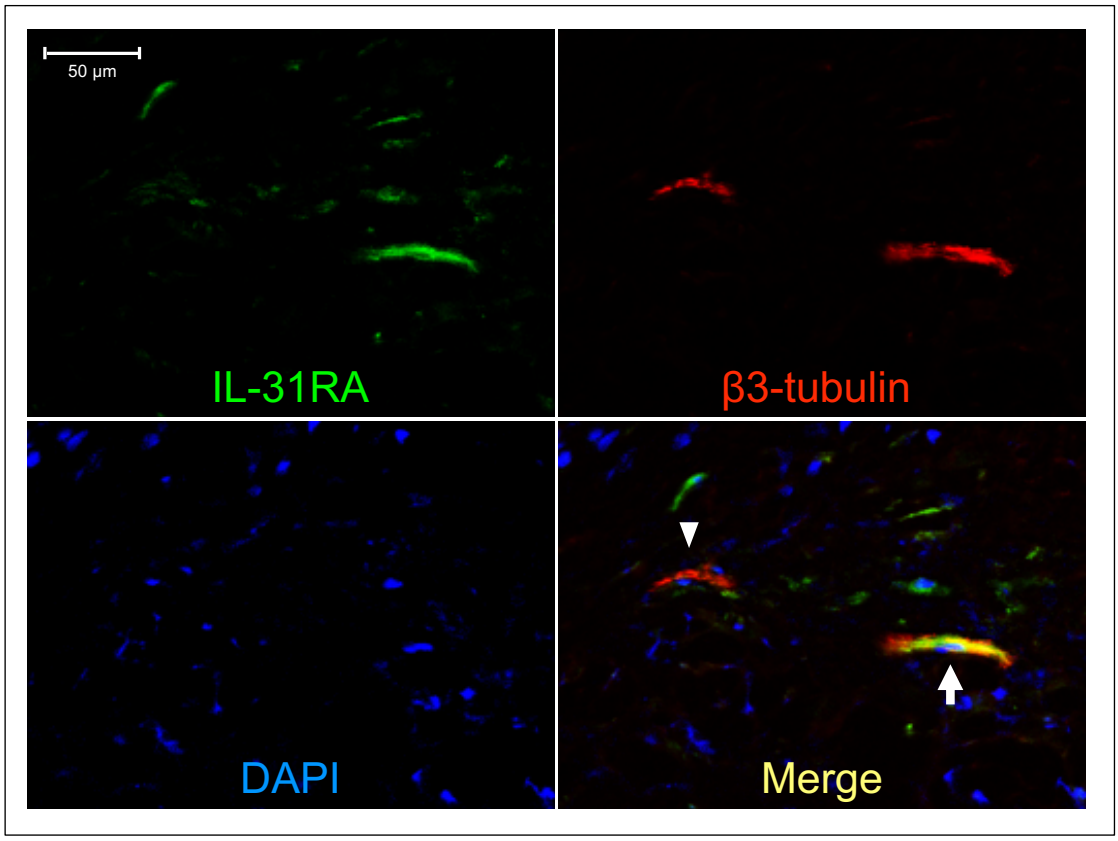

Figure 8. IL-31RA and β3-tubulin double staining IF.

A small proportion of dermal nerve fibers (red) in the dermis were also immunolabeled with IL-31RA (green). The arrowhead shows the nerve that was negative for IL-31RA, and the arrow shows the nerve that was positive for IL-31RA.

3.4 Discussion

In the present study using an experimental model of acute canine AD skin lesions, we showed the early and transient cutaneous production of IL-31 with a peak expression between 24 and 48 hours with a reduction at 96 hours after epicutaneous allergen provocation. These results indicate that IL-31 likely plays a role early on in the genesis of atopic skin lesions, which is consistent with results of a previous study investigating the mRNA expression of *IL31* and other pruritogenic mediators in the same experimental model.[18] In the previous study, a significant up-

regulation of *IL31* mRNA was detected as early as 24 hours after HDM provocation, and it continued to increase until 48 hours, the last sampling time point. For this reason, we designed our experiment to collect samples 96 hours after challenge to detect the endpoint of IL-31 production after the single allergen provocation; three out of four dogs in this study had a reduction of IL-31 expression at 96 hours post-challenge. Altogether, these results support the concept of using lokivetmab as proactive therapy in atopic dogs,[5] since the targeted cytokine of lokivetmab, IL-31, might no longer be present at high levels in the skin after atopic lesions are fully developed. We do not exclude, however, that the production of IL-31 at high levels might persist at sites other than skin lesions.

Interestingly, we did not find any significant correlation between the skin IL-31 expression scores and the serum IL-31 levels. In a study of humans with chronic AD in which authors calculated the correlations between the cytokine levels in the interstitial fluid of lesional or non-lesional skin and the corresponding plasma samples, there was a statistically-significant positive correlation only for four cytokines and chemokines (CXCL9, CXCL10, IL-1RA and IL-6) among 33 examined mediators;[21] IL-31 was also measured in that study, but a correlation with plasma levels was not found.[21] Although the reason for this finding was not explained, we suspect that it could be due to a delay in the transition from the dermal interstitium to the circulation of cytokines locally produced at the site of inflammation. It has long been known that the skin edema associated with acute inflammation leads to a lowering, rather than an increase, in the interstitial fluid pressure.[22] Thus, this lower pressure could contribute to the retention of mediators that are locally produced in the skin and prevents them from entering the peripheral circulation. In any case, this dissociation between the IL-31 expression scores and the serum IL-31 levels indicate that the latter are unlikely to be a useful biomarker for skin lesions or a valid indicator for when to select cases for IL-31-targeted therapy.

As seen in humans with AD, we did not find any significant correlation between the IL-31 skin expression and the macroscopic skin lesion scores, which indicates that IL-31 might not have a direct and/or major role in the development of early AD skin lesions. These results mirrors those of our previous study in which the development of AD skin lesions was not blocked by pretreatment with the anti-canine-IL-31 mAb.[5] On the other hand, the clinical trials of IL-31-inhibiting therapy with lokivetmab in client-owned atopic dogs have shown beneficial results on both pruritus and skin lesions. These seemingly contradictory results indicate a

complex role of IL-31 in the generation of skin lesions in atopic dogs.

In this study, we showed that Th2 cells were likely the major source of IL-31 in canine atopic skin. This observation is consistent with the previous in-vitro studies in dogs in which IL-31 was preferably produced by this cell type.[10,11] In humans, although various types of primary immune cells are known to produce IL-31 in vitro, there is still a debatable argument regarding the exact cellular source of IL-31 in atopic skin lesions. One study reported that 63% of IL-31-positive cells were Th2 cells in human AD skin lesions.[23] In contrast, another showed that most of the IL-31-immunolabeled cells stained positively for macrophage markers.[24] It is important to point out that, in the latter report, there was no overlap detected between the positive immunostains for IL-31 and CD3, a T cell marker. Since these studies used clinical skin samples, racial differences (e.g., European versus Asian) and/or the stage of AD skin lesions (i.e., acute versus chronic) might have contributed to these inconsistent results. In our study, to limit such variability of race and timing, we used an experimental canine acute AD model in which all dogs were of the same breed, lived in the same environment and had the same stage of HDM-induced AD skin lesions. Such a study should be followed by the determination of the cellular source of IL-31 production in the skin of client-owned dogs of various breeds with spontaneous AD skin lesions. Furthermore, there were still a few IL-31-immunolabeled mononuclear cells that we did not characterize in this study. These cells could be either macrophages, dendritic cells or mast cells, as these were also reported to produce IL-31 in humans.[1,24–27] Nevertheless, considering that a very small proportion of these cells (compared to Th2 cells), the significance of IL-31 production by these cells in this acute model of canine AD may be minimal.

The most unique finding of our study was a possible production of IL-31 by sebaceous glands, which has not been reported in any other species. It has been shown that a canine sebaceous epithelial cell line expressed various cytokines and chemokines, although *IL31* mRNA was not investigated in that study.[28] Interestingly, one human study demonstrated the presence of IL-31 in the eccrine sweat glands with a corresponding high level of this cytokine in sweat samples collected from both atopic and healthy individuals.[29] However, in our study, we cannot rule out that a shared epitope between IL-31 and sebaceous glands is recognized by our IL-31 mAb, which would explain the complete extinction of the sebaceous gland staining after immunoabsorption with IL-31. Further studies are underway to confirm the production of IL-31 by sebaceous glands, using in situ hybridization.

We showed that dermal nerve fibers and keratinocytes stained positively for the IL-31RA in canine atopic skin, which is consistent with the results of similar human studies.[23,24] In humans, there is increasing evidence that IL-31 exerts its effects via the direct and indirect activation of sensory neurons and keratinocytes. It has been shown that IL-31 induces the activation of dorsal root ganglia (DRGs) neurons, the cell body of cutaneous sensory neurons, and the release of neuropeptides from DRGs; these neuropeptides not only transmit itch perception to the brain but also induce the release of proinflammatory cytokines from skin keratinocytes and dendritic cells in vitro.[23,30,31] Upon binding to its receptor on keratinocytes, IL-31 also directedly induces the production of various proinflammatory cytokines from primary keratinocytes collected from both atopic and healthy humans.[1,32–34] The presence of IL-31RA on dermal nerve fibers and keratinocytes in canine skin suggests that similar activation mechanisms of IL-31 likely exist in canine AD.

3.5 Conclusions

In conclusion, we showed, in experimental acute canine AD skin lesions, the early and transient secretion of IL-31 by helper T cells — likely Th2 cells — that does not parallel the serum IL-31 levels or the macroscopic skin lesion scores. These results support the concept of using IL-31-blocking therapy as a proactive rather than reactive therapy and the minimal relevance of measuring the serum IL-31 levels prior to selecting dogs for such therapy. The detection of IL-31RA in keratinocytes and peripheral nerve fibers in canine atopic skin confirms the likely importance of IL-31 as a pro-inflammatory and pruritogenic cytokine that activates these cells in atopic dogs.

3.6 References

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3.7 Appendix

Appendix S1. Supplementary Materials and Methods.

Preparation of stimulated canine PBMCs (IL-31 IF positive control)

Stimulated canine peripheral blood mononuclear cells (PBMCs) were used as a positive control for IL-31 IF because activated T cells have been identified as a possible cellular source of IL-31 in dogs, at mRNA as well as protein levels.[10,11] A fresh whole blood sample was collected into an EDTA tube from our atopic dog model that was re-challenge by epicutaneous application of HDM allergen four days before the blood collection to induce the production of Th2 cytokines. Peripheral blood mononuclear cells (PBMCs) were collected using Lymphoprep density gradient centrifugation per manufacture's protocol. Briefly, the blood sample was diluted 1:1 with PBS and overlayed onto Leucosep (Greiner Bio-One, Kremsmuenster, Austria) containing 15 mL of Lymphoprep (Alere Technologies, Oslo, Norway). The sample was then spun at 800 x *g* for 30 minutes at room temperature with no brake. The PBMCs layer was then carefully collected with a sterile 18G catheter tube into a sterile conical centrifuge tube. The transferred PBMCs were washed three times with PBS. Isolated PBMCs were then cultured in RPMI-1640 (Corning, Corning, NY, USA) supplemented with 2% Glutamax (Thermo Fisher Scientific, Waltham, MA, USA), 10% FBS (GenClone, San Diego, CA, USA), and 1% penicillin/streptomycin solution (Corning) with the presence of cell activation cocktail of 50 ng/mL phorbol 12-myristate 13-acetate (Sigma–Aldrich, St. Louis, MO, USA) plus 2 μM ionomycin (Sigma–Aldrich) that were reported to stimulate IL-31 production from canine PBMCs,[11] for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 in the air. The protein transport inhibitor cocktail reagent composed of brefeldin A plus monensin (2 μL/mL; eBioscience, San Diego, CA, USA) was added to prevent protein secretion for the last 4 hours of culture. After removing the cell culture media, the stimulated PBMC suspension was resuspended at about 3.5 x 10^5 cell/mL in PBS. The slides of stimulated PBMCs were prepared using a Cytospin 4 cytocentrifuge (Thermo Fisher Scientific) at 800 *rpm* for 5 minutes. The slide was placed in 0.2% Tween 20 solution for 15 minutes to permeabilize the cells immediately before the IL-31 IF.

Preparation of canine nasal planum skin (IL-31RA IF positive control)

Canine nasal planum skin was collected post-mortem from a healthy dog and used as a

positive control for the IL-31RA IF. The nasal planum was selected because of its thick epidermis and its existing high density of intraepidermal nerve fibers, a type of neuronal endings known to express IL-31RA in humans.[24,31,32] The tissue was processed and cryosectioned in the same way as other experimental samples.

IL-31 IF single staining

After blocking with 10 μg/mL canine Fc receptor binding inhibitor polyclonal antibody (Invitrogen, Waltham, MA, USA) for one hour at room temperature, the sections were incubated with a mouse monoclonal IgG1 anti-canine IL-31 primary antibody (1 μg/mL; Zoetis, Parsippany, NJ, USA) or a mouse monoclonal IgG1 antibody as isotype control (1 μg/mL; MAB002, R&D Systems, Minneapolis, MN, USA) overnight at 4°C, followed by a Cy3-conjugated rabbit polyclonal anti-mouse IgG secondary antibody (5 μg/mL; AP160C, MilliporeSigma, St. Louis, MO, USA) for 6 hours at 4°C. Finally, the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in a mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA).

IL-31 immunoabsorption assay

To confirm the specificity of the IL-31 IF signals, the absorption control was prepared as followed: a mouse monoclonal IgG1 anti-canine IL-31 primary antibody (1 μg/mL; Zoetis) was pre-incubated with recombinant canine IL-31 (10 μg/mL; Zoetis) for one hour at room temperature, followed by centrifugation at 15,000 x *g* for 10 minutes at 4°C. Immunofluorescence was performed as described earlier except the primary antibody was replaced by this supernatant.

IL-31 IF double staining

For IL-31 and CD3 double staining, IF was performed as described earlier in the IL-31 IF single staining section except the primary antibody was replaced by a cocktail of a mouse monoclonal IgG1 anti-canine IL-31 primary antibody (1 μg/mL; Zoetis) and goat polyclonal anti-human CD3ε primary antibody (1 μg/mL; sc-1127, Santa Cruz Biotechnology, Dallas, TX, USA), or a cocktail of a mouse monoclonal IgG1 antibody (1 μg/mL; MAB002, R&D Systems) and goat polyclonal IgG antibody (1 μg/mL; AB-108-C, R&D Systems) as an isotype control.

For IL-31 and CD4 double staining, IF was performed using a cocktail of a mouse monoclonal IgG1 anti-canine IL-31 primary antibody (1 μg/mL; Zoetis) and a goat polyclonal anti-canine CD4 primary antibody (5 μg/mL; AF2410, R&D Systems), or a cocktail of isotype controls. The primary antibodies were visualized with a cocktail of a Cy3-conjugated rabbit polyclonal anti-mouse IgG secondary antibody (5 μg/mL; AP160C, MilliporeSigma) and an Alexa Fluor 488-conjugated rabbit polyclonal anti-goat IgG secondary antibody (5 μg/mL; A-11078, Invitrogen).

IL-31RA IF single staining

After blocking with 2.5% normal rabbit serum (191357, MP Biomedicals, Santa Ana, CA, USA) for 30 minutes at room temperature, the sections were incubated with a goat polyclonal IgG anti-human IL-31RA primary antibody (5 μg/mL; AF2769, R&D Systems) or a goat polyclonal IgG antibody as an isotype control (5 μg/mL; AB-108-C, R&D Systems) overnight at 4°C, followed by an Alexa Fluor 488-conjugated rabbit polyclonal anti-goat IgG secondary antibody (5 μg/mL; A-11078, Invitrogen) for 6 hours at room temperature. The sections were counterstained with DAPI in the mounting medium (Vectashield).

IL-31RA IF double staining

Double staining IF was performed as described earlier in the IL-31RA IF single staining section except the primary antibody was replaced by a cocktail of a goat polyclonal IgG anti-human IL-31RA primary antibody (5 μg/mL; AF2769, R&D Systems) and a mouse monoclonal IgG2a anti-human β3-tubulin primary antibody (1 μg/mL; LS-B5227, Lifespan Biosciences, Seattle, WA, USA), or a cocktail of a goat polyclonal IgG antibody (5 μg/mL; AB-108-C, R&D Systems) and a mouse monoclonal IgG2a antibody (1 μg/mL; MAB003, R&D Systems) as an isotype control. The primary antibodies were visualized with an Alexa Fluor 488-conjugated rabbit polyclonal anti-goat IgG secondary antibody (5 μg/mL; A-11078, Invitrogen) and a Cy3-conjugated rabbit polyclonal anti-mouse IgG secondary antibody (5 μg/mL; AP160C, MilliporeSigma), respectively.

Microscopic imaging

Each section was examined using a Leica DM5000 B upright fluorescence microscope with a 40x objective (Leica Microsystems, Wetzlar, Germany). The center wavelength/full

bandwidth of excitation (Ex) and emission (Em) filters were as follows: Cy3 (red), Ex = 555/25 nm and Em = 605/52 nm; Alexa Flour 488 (green), Ex = 470/40 nm and Em =525/50 nm; and DAPI (blue), Ex = 350/50 nm and Em = 460/50 nm.

CHAPTER 4

INVESTIGATION OF MESSENGER RNA EXPRESSION OF *IL31* IN SEBACEOUS GLANDS OF ATOPIC AND HEALTHY CANINE SKIN

Chie Tamamoto-Mochizuki* and Thierry Olivry*†

* Department of Clinical Sciences, College of Veterinary Medicine, NC State University, 1060 William Moore Drive, Raleigh, NC 27607, USA

† Comparative Medicine Institute, NC State University, Raleigh, NC 27606, USA

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4.1 Introduction

Interleukin 31 (IL-31) is a pruritogenic cytokine known to play a pivotal role in pruritic skin diseases, especially atopic dermatitis (AD) in humans and dogs.[1–3] Numerous human studies have demonstrated elevated serum IL-31 levels and *IL31* mRNA expression in the skin of atopic patients compared to those of healthy controls;[4,5] similar findings have also been reported in canine patients.[6,7] Our previous study in a canine model of acute AD skin lesions showed the highest up-regulation of mRNA encoding *IL31*, among that coding for other pruritogenic cytokines and mediators examined in the study, as early as 24 hours after an allergen challenge. These results indicated that IL-31 was the earliest and a major player in the pruritogenic cytokine cascade of acute canine AD.[8]

Several studies have been done to identify the cellular source of IL-31 in AD skin lesions. In humans, IL-31 is known to be expressed by various immune cells, such as T cells (both $CD4^+$ T cell and $CD8^+$ T cells), mast cells, monocytes, macrophages, dendritic cells, eosinophils and basophils;[9,10] it can be also produced by keratinocytes and dermal fibroblasts *in vitro*.[9] An increased mRNA expression of *IL31* was detected when peripheral blood mononuclear cells (PBMCs) of healthy dogs were stimulated by a T cell mitogen, indicating T cells as a source of IL-31.[11] A further study demonstrated that the production of IL-31 was induced in canine Th2-polarized PBMCs (characterized by an increased production of the Th2 representative cytokine, IL-4) *in vitro*, suggesting that Th2 cells were likely the primary source of IL-31 after exposure to allergens.[12]

In our previous study (see Chapter 3 above), we performed immunofluorescence (IF) using an anti-canine-IL-31 monoclonal antibody (mAb) to uncover the cellular source of cutaneous IL-31 in our canine acute AD model. As expected from the previous study with canine PBMCs, helper T cells — likely Th2 — were found to be the primary source of IL-31 in the skin of atopic dogs. At the same time, and rather unexpectedly, we discovered that sebaceous glands were also strongly immunolabeled for IL-31; the extinction of this positivity by pre-incubating the antibodies with recombinant canine IL-31 further supported sebaceous glands as being another potential source of IL-31 production (Figure 1). Although such an ectopic production of IL-31 has been reported in the eccrine gland (i.e., sweat glands) apparatus in both atopic and healthy humans,[13] we could not find any report about the production of IL-31 by sebaceous glands in any other species.

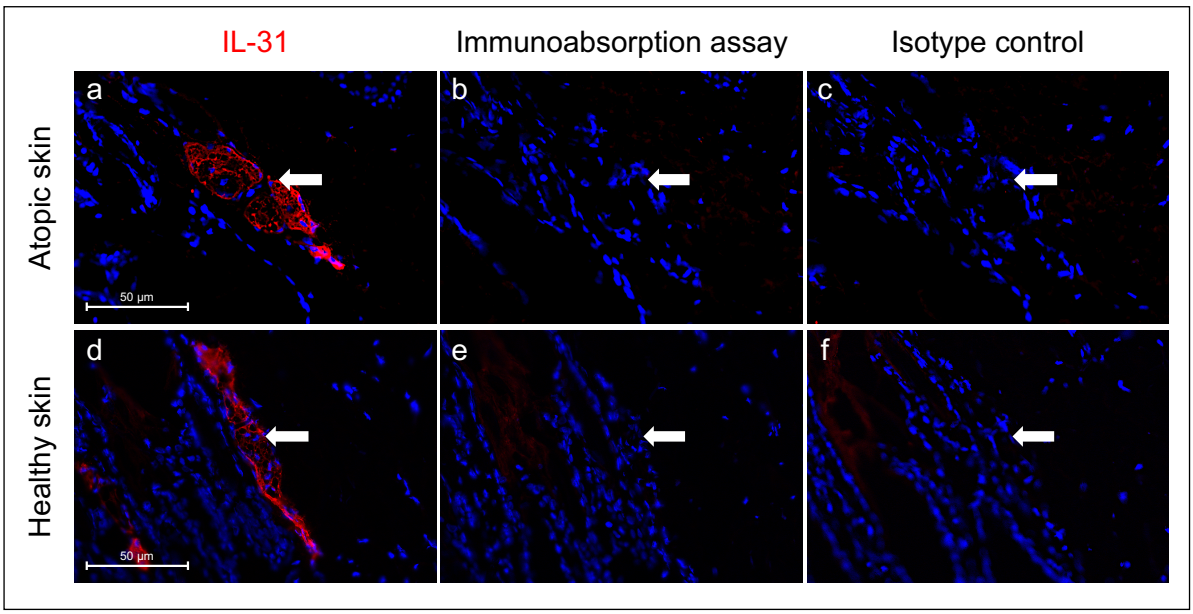

Figure 1. IL-31 IF in sebaceous glands of the skin of atopic and healthy dogs.
Sebaceous glands are strongly immunolabeled for IL-31 in the skin of both atopic and healthy dogs (a,d); the extinction of the positivity with immunoabsorption assay (b,e) and negative staining with isotype control (c,f) confirmed its specificity. Arrows indicate the location of sebaceous glands.

As IL-31-immunolabeled sebaceous glands were seen in both atopic and healthy dogs in our study (Figure 1), this source, alone, is unlikely to contribute to the development of AD skin lesions. It is well-known that an epidermal barrier dysfunction, which has been demonstrated in atopic dogs and humans but not in healthy individuals, increases epidermal penetration.[14,15] Consequently, it is logical to hypothesize that sebum IL-31 only would penetrate the impaired epidermal barrier of atopic—but not normal—dogs to stimulate upper epidermal keratinocytes and intraepidermal sensory nerves. This potential outside-to-inside effect of IL-31 might represent a novel insight into the IL-31 signaling pathway in AD.

Although our previous study demonstrated the immunoreactivity of sebaceous glands for IL-31, we believe that it is still insufficient to fully establish its production by sebocytes, as we cannot exclude a shared epitope between IL-31 and these cells. Therefore, in this study, we aimed to demonstrate the expression of *IL31* mRNA in cutaneous sebaceous glands in atopic and healthy dogs.

4.2 Methods and materials

Collection and preparation of skin samples

To ensure a better RNA quality, we used frozen tissue samples for the detection of IL-31 mRNA expression. We used skin samples from our mite-sensitized atopic dogs and from a healthy dog that had shown a positive IL-31 immunoreactivity in the sebaceous glands in our previous study (Chapter 3). The skin samples were stored at –80°C and cryosectioned at 5-µm thick one day before in situ hybridization (ISH).

IL31 RNAscope (RNA in situ hybridization)

We performed a colorimetric ISH using the RNAscope method (Advanced Cell Diagnostics, Newark, CA, USA) to locate the expression of *IL31* mRNA in sebaceous glands. We used the RNAscope 2.5 HD detection kit (Advanced Cell Diagnostics; catalog #322350) according to the manufacturer's protocol. Briefly, the section was first fixed in 10% neutral buffered formalin for 1.5 hours at 4°C, followed by serial dehydration in 50%, 70% and 100% ethanol for 5 minutes each. The endogenous peroxidase was quenched by incubating the section in 3% hydrogen peroxide solution for 10 minutes, and protease treatment was performed for 30 minutes at room temperature. The probe targeting canine *IL31* mRNA (*Cl-IL31*) (Advanced Cell Diagnostics; catalog #483891) was then hybridized for 2 hours at 40°C. RNAscope probes targeting the canine ubiquitin C mRNA (*Cl-UBC*) (Advanced Cell Diagnostics; catalog #409851) and bacterial dihydrodipicolinate reductase mRNA (*dapB*) (Advanced Cell Diagnostics; catalog #310043) were also used as positive and negative control probes, respectively. After serial RNAscope amplification, the signals were visualized using the Fast Red chromogen. Finally, the sections were counterstained with Mayer's hematoxylin (Volu-Sol Inc, Salt Lake City, UT, USA; catalog #VMH-032) for 5 minutes and washed in running water for 10 minutes.

Stimulated canine PBMCs were prepared as described earlier in Chapter 3 with some modification. Isolated PBMCs were co-incubated with T cell activators for 6 hours instead of 24 hours to yield the maximum *IL31* mRNA expression, as reported in a previous study,[11] and the protein transport inhibitor was omitted. Cytospin slides were prepared as described earlier in Chapter 3 and used as positive controls for *IL31* mRNA detection.

4.3 Results

A positive hybridization with the probe for *IL31* mRNA was seen in 3% of stimulated canine PBMCs, although 42% of PBMCs prepared from the same blood sample had exhibited a positive IL-31-immunoreactivity (Figure 2). Even though there were abundant positive signals with the positive control probe (*Cl-UBC*) on sebaceous glands in the skin of both atopic and healthy dogs, *IL31* mRNA was not detected in any sebaceous glands of either sample type (Figure 3). Also, *IL31* mRNA signals were only seen in a few mononuclear cells in dermis, which showed abundant IL-31-immunoreactivity in our previous study (see Chapter 3). To verify these results, we also performed *IL31* RNAscope using the Fluorescent Multiplex Detection kit (Advanced Cell Diagnostics; catalog #323110) on both fresh and formalin-fixed paraffin-embedded (FFPE) samples, which also did not show any *IL31* mRNA signals on sebaceous glands in any samples (Figure 4) and only a few positive mononuclear cells in dermis.

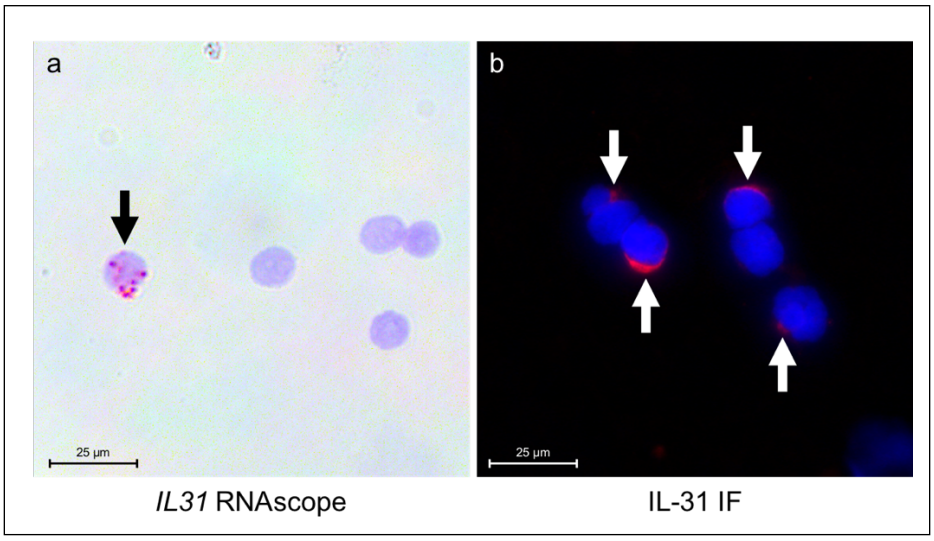

Figure 2. IL31 RNAscope and IL-31 IF on stimulated canine PBMCs.

A black arrow indicates *IL31* mRNA signals seen in one PBMC (a). IL-31 IF was also performed on the same PMBC samples as described earlier in Chapter 3, which demonstrates a positive IL-31-immunoreactivity in the cytoplasm (white arrows: b).

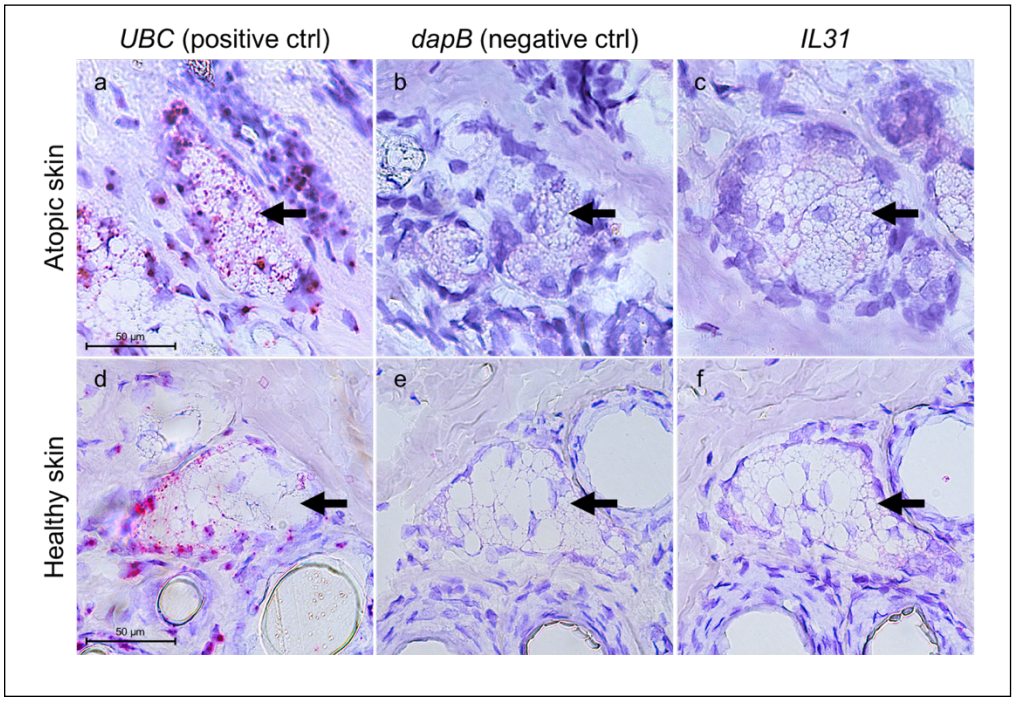

Figure 3. IL31 colorimetric RNAscope on sebaceous glands in fresh-frozen skin samples of atopic and healthy dogs.

Although the abundant signals (red dots) with the positive control probe (*UBC*) were present on sebaceous glands in the skin of both atopic and healthy dogs (a,d), *IL31* mRNA was not detected in any sebaceous glands of either skin sample (c,f). Arrows indicate the location of sebaceous glands.

Abbreviation: ctrl = control.

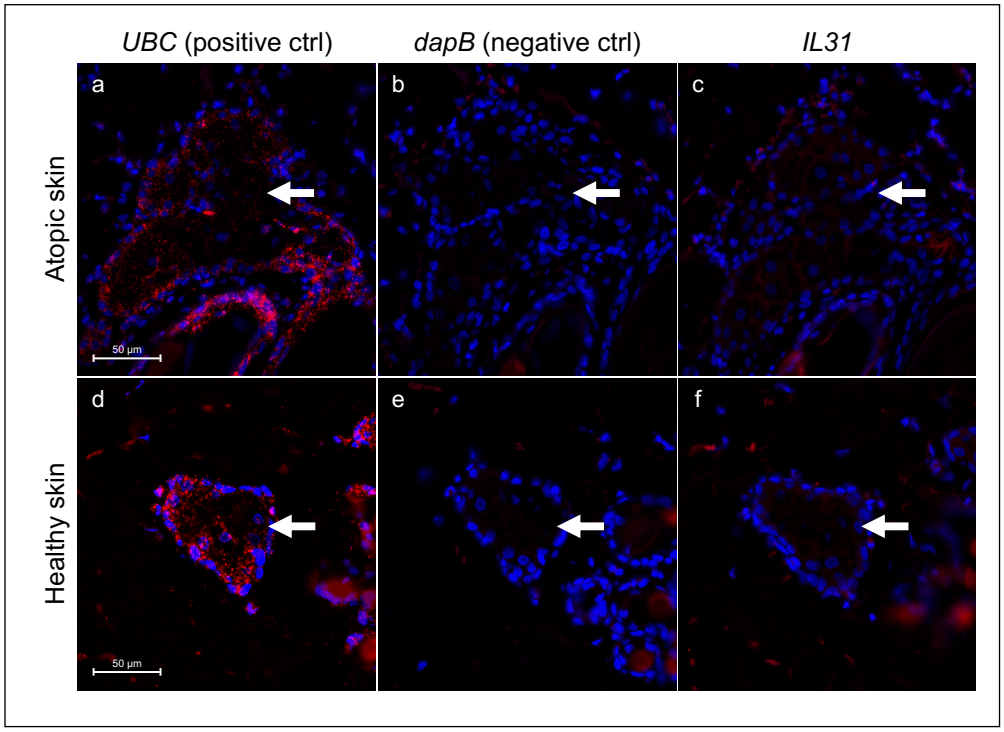

Figure 4. IL31 fluorescent RNAscope on sebaceous glands in FFPE skin samples of atopic and healthy dogs.
Similarly to the results of the colorimetric RNAscope, *IL31* mRNA was not detected in any sebaceous glands on either atopic and healthy skin samples (c,f) despite the abundant signals (red dots) with the positive probe (a,d). Arrows indicate the location of sebaceous glands.
Abbreviation: ctrl = control.

4.4 Discussion

The RNAscope used in this study is a very specific and sensitive RNA ISH assay based on gene-specific double-Z probe pairs that prevents the cross-hybridization to unintended targets with a sequential hybridization of amplifiers providing robust signal amplification (Figure 5). Although our previous study showed the presence of a strong IL-31 immunoreactivity in sebaceous glands, we could not confirm any *IL31* mRNA expression in sebocytes of atopic and healthy canine skin in this study.

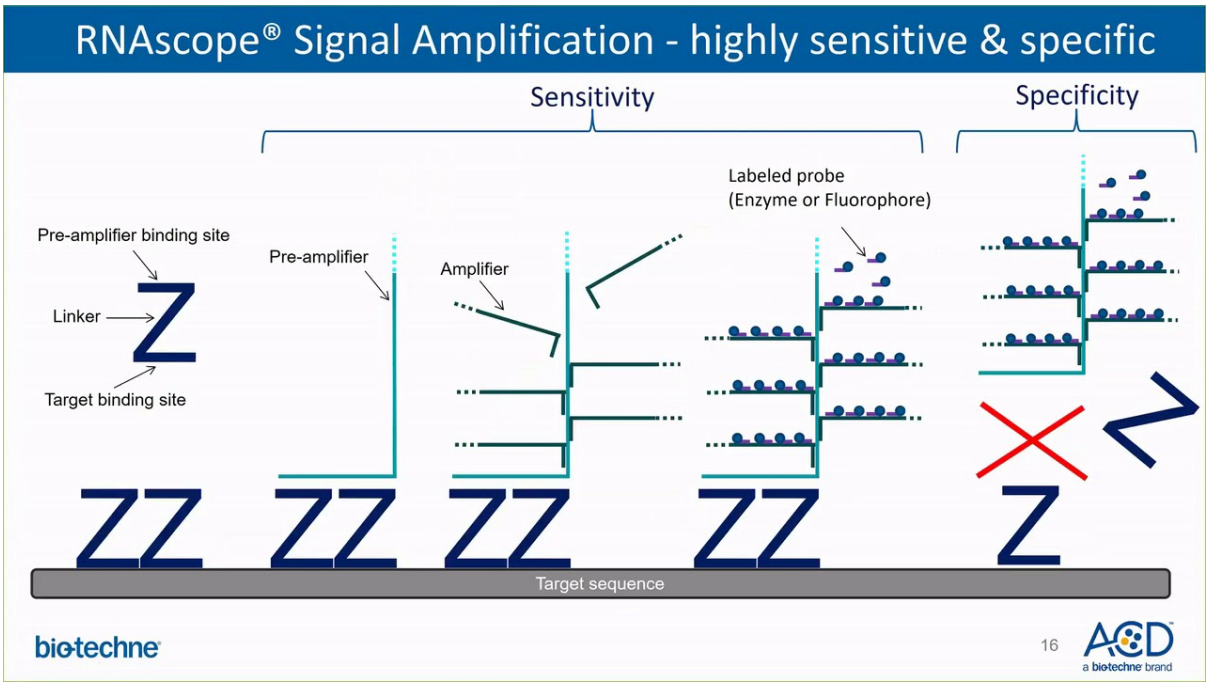

Figure 5. The principle of RNAscope. (adapted from ACDbio website: https://acdbio.com/)
Gene-specific double-Z probe pairs that enable the elimination of cross-hybridization to unintended targets (high specificity) with a sequential hybridization of amplifiers providing robust signal amplification (high sensitivity).

There are several possibilities for this discrepancy in IL-31 detection. One could first hypothesize that the mRNA could have been degraded, since the samples were already collected and had been stored at −80°C for months. However, the abundant positive signals seen with the positive control probes (*UBC*) indicated that the mRNA quality in these specimens was more than adequate. Furthermore, we also repeated this *IL31* RNAscope on freshly collected healthy canine skin samples, but *IL31* mRNA was not detected on sebaceous glands either (data not shown).

In addition to producing sebum, which is the primary function of sebaceous glands, sebocytes are known to express several ectopeptidases that are involved in the degradation of cytokines.[16] It is therefore theoretically conceivable that IL-31 was merely present in the sebaceous glands as part of its degradation process. However, currently, the decaying pathway of IL-31 is unknown, and there is no evidence to support the presence of such degradation enzymes of IL-31 in sebaceous glands.

One could hypothesize also that the mRNA expression of *IL31* is very transient so that we could not detect it with our method. Consistent with this hypothesis, *IL31* mRNA signals were only seen in a few mononuclear cells in the dermis of samples that had abundant IL-31-immunoreactivity in our previous study. Similarly, the canine PBMCs we used as positive controls for *IL31* mRNA detection showed only a 3 % positivity rate, although 42% of these cells had IL-31-immunoreactivity using IF. After stimulation, the peak mRNA expression of genes happens long before the peak expression of the encoding protein.[17] We used PBMCs that were co-cultured with T cell mitogens for 6 hours because this time point had been reported to be the peak expression of *IL31* mRNA, but the actual peak of transcription could have been earlier than 6 hours and very transient, since *IL31* mRNA had not been measured before this time point in the study.[11] The IL-31-immunolabeled sebaceous glands seen in atopic dogs before HDM allergen exposure as well as in healthy dogs, would imply a constitutive expression of IL-31 by sebaceous glands. Such a constitutive expression would mean that there is a constant transcription of *IL31* mRNA, which we should have detected.

We have tried multiple different staining conditions, such as different protease treatment conditions, more intense RNAscope signal amplification, and longer incubation time with the chromogen to optimize the RNAscope ISH assay. These modifications increased the non-specific staining with the negative control probes, but we still did not detect the *IL31* mRNA in sebaceous glands (data not shown). Furthermore, the abundant positive signals with the positive control probes indicated that the staining protocol itself was already optimized. Nevertheless, we still cannot exclude the possibility that the commercially-available probes designed to target canine *IL31* used in our study might not have been sensitive enough to detect a very low mRNA expression. Unfortunately, our request to make a new custom-made *IL31* probe was declined by the company. Therefore, other approaches to investigate the IL-31 in sebaceous glands at mRNA and protein levels will need to be considered.

The technique to establish a canine sebaceous epithelial cell line has been recently reported. Real-time PCR of this sebocyte cell line revealed the expression of various cytokines and chemokines, including *CCL2*, *CCL20*, *CXCL10*, *TNFα*, *IL1α*, *IL1β* and *IL8* following the treatment with lipopolysaccharide, an endotoxin derived from Gram-negative bacteria.[18] Further studies measuring the expression of *IL31* mRNA in such cell line, or the detection of IL-31

protein in the culture medium of these cells, are warranted to further investigate sebaceous glands as a possible cellular source of IL-31 in canine skin.

At this time, we think it is most likely that there might be a shared epitope(s) between sebaceous glands and IL-31 that is/are recognized by the IL-31 mAb used in our study; this would then explain the complete extinction after immunoabsorption with IL-31. It is a well-known issue in immunostaining that many monoclonal and polyclonal antibodies show cross-reactivity with other closely-related or non-related proteins.[19] For example, three different commercial polyclonal antibodies against the transient receptor potential channel-vanilloid subfamily member 1 (TRPV1) also recognize the urothelium of bladder tissues obtained from both wild-type and TRPV1$^{-/-}$ knock-out mice.[20] This finding indicates that these antibodies had led to a non-specific immunoreactivity, likely due to shared epitope between TRPV1 and urothelial cells. However, it is technically challenging to generate *IL31*$^{-/-}$ knock-out dogs to investigate such shared epitopes potentially detected by our IL-31 mAb.

4.5 Conclusion

In this study, we could not demonstrate the expression of *IL31* mRNA on sebaceous glands in canine skin with the RNAscope ISH method. Further studies with different approaches are needed to unequivocally confirm the secretion of IL-31 in the sebaceous glands, as the immunoreactivity for this cytokine seen in our previous study would seem to imply.

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CHAPTER 5

CYTOKINE TRANSCRIPTOME PROFILING IN ACUTE EXPERIMENTAL CANINE ATOPIC DERMATITIS SKIN LESIONS AFTER IL-31 INHIBITION — *WHICH CYTOKINES COULD BE THE NEXT THERAPEUTIC TARGETS IN CANINE AD?* —

Chie Tamamoto-Mochizuki*, Natalie Crawford‡, Andrea Gonzales‡ and Thierry Olivry*†

* Department of Clinical Sciences, College of Veterinary Medicine, NC State University, 1060 William Moore Drive, Raleigh, NC 27607, USA

‡ Veterinary Medicine Research & Development, Zoetis Inc., 333 Portage St, Kalamazoo, MI 49007, USA

† Comparative Medicine Institute, NC State University, Raleigh, NC 27606, USA

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Previous publication: None (will be submitted to Veterinary Dermatology)

5.1 Introduction

Atopic dermatitis (AD) is a common, often chronic, allergic skin disease of humans and dogs associated with mild-to-severe pruritus and characteristic inflammatory skin lesions.[1,2] Interleukin (IL)-31, a pruritogen, is known to represent one of the key cytokines of human and

canine AD.[3–5] A significant reduction of atopic itch has been seen following injections of therapeutic monoclonal antibodies (mAbs) targeting the IL-31 pathway, such as nemolizumab (an anti-human-IL-31 receptor α subunit mAb) and lokivetmab (LKV, an anti-canine IL-31 mAb, Cytopoint; Zoetis, Parsippany, NJ, USA), thus confirming that IL-31 plays a pivotal role in the pathogenesis of AD in both species.[6–13] In addition to the potent and rapid anti-pruritic effect of LKV, two randomized clinical studies have reported that about half of atopic dogs treated with LKV exhibited a 50% or greater reduction—or even a 'remission'—of their skin lesions compared to those at baseline.[10,11] Considering that AD is a highly-complex and multi-factorial disease, keeping AD under control by blocking just a single cytokine confirms the unique relevance of IL-31 as a valid therapeutic target for AD.

Despite the documented treatment successes in clinical trials, there is increasing evidence that IL-31 is not required for the induction of acute allergic skin inflammation, and there are also occasional failures of disease control, especially of skin lesion, when using IL-31-inhibiting mAbs in clinical practice. In our prospective study enrolling client-owned dogs with AD, we observed that half of dogs treated proactively with LKV monotherapy eventually needed additional anti-allergic medications after about two months, indicating that there are other essential cytokines that contribute to AD flares independent of IL-31 (see Chapter 2 above). In support of this observation, there is increasing evidence that IL-31 is not required for the induction of acute allergic skin inflammation. Indeed, the study of allergic contact dermatitis induction in *IL31*-deficient mice revealed that the local skin inflammation still developed after hapten application while pruritus was markedly diminished.[14] Such observations are consistent with what was seen in NC/NgA atopic mice in which an anti-mouse-IL-31 mAb had little impact on house dust mite (HDM)-associated skin lesions despite the mAb significantly reducing pruritus manifestations.[15] Similar findings were seen in canine AD. For example, in our acute canine AD model, we showed that the pretreatment with LKV did not prevent the development of skin lesions after an exposure to the HDM allergens despite almost completely inhibiting pruritus manifestations (see Chapter 2 above). Finally, our recent study failed to detect a correlation between the severity of macroscopic inflammatory skin lesions and the amount of infiltrating IL-31-positive cells in experimental acute atopic skin lesions in dogs, further supporting the minimal role of locally-released cutaneous IL-31 in the development of allergic skin inflammation (see Chapter 3 above). Altogether, these findings raised the following

question: if IL-31 is not required for the induction of acute atopic skin inflammation, which cytokines are still released in the presence of IL-31-inhibiting therapy?

Our objectives were thus to uncover IL-31-independent cytokines that are crucial for the development of acute atopic inflammation using a comprehensive evaluation of the lesional skin transcriptome in experimental canine acute AD. Such a study should enable a better understanding of the pathobiological mechanism of acute canine AD skin lesions (i.e., AD flares), and it should help identify additional therapeutic target(s) to supplement the use of LKV.

5.2 Methods and materials

Ethics

All procedures were approved beforehand by our university's Institutional Animal Care and Use Committee (IACUC ID #17-005-O and #19-825-O).

Animals and sample size estimation

In this experiment, we used our atopic dog model, an inbred line of laboratory Maltese-beagle dogs sensitized to the *Dermatophagoides farinae* HDM, as described previously (see Chapter 1 Appendix for more details about this model).[16]

We determined that at least four dogs would need to be included for this experiment to have a greater than 80% power to find results significant at the *p*-value at 0.05 (one-sided) using our previously published data.[17] Therefore, we included six dogs to avoid underpowering our study; there were five males and one female ranging in age from 3 to 11 years.

Experimental design and sample collection

The study was designed as a randomized, no-treatment-controlled, cross-over study with a 28-week washout period (Figure 1; note: this unusually-long wash-out occurred because of the COVID-19-associated restrictions in laboratory animal usage). We compared the cytokine profiling, with or without the LKV-induced inhibition of IL-31, to uncover IL-31-independent inflammatory pathway(s) in acute atopic skin lesions. In Phase 1, using coin-flipping, dogs were randomly assigned to one of two pretreatments; LKV (IL-31-blocking group: LKV) or none (no-treatment control group: NTC). Dogs in the IL-31-blocking group received a single injection of LKV, at 2 mg/kg subcutaneously, 10 days before the epicutaneous HDM allergen provocation.

This timing of the LKV pretreatment was decided based on its maximum drug concentration-time (T_{max}) of 10 days.[10] After a washout period of 28 weeks, dogs were switched to the other pretreatment (Phase 2; Figure 1). This long washout was largely sufficient to have a complete elimination of LKV from the circulation based on its elimination half-life ($T_{1/2}$) of 16 days.[10]

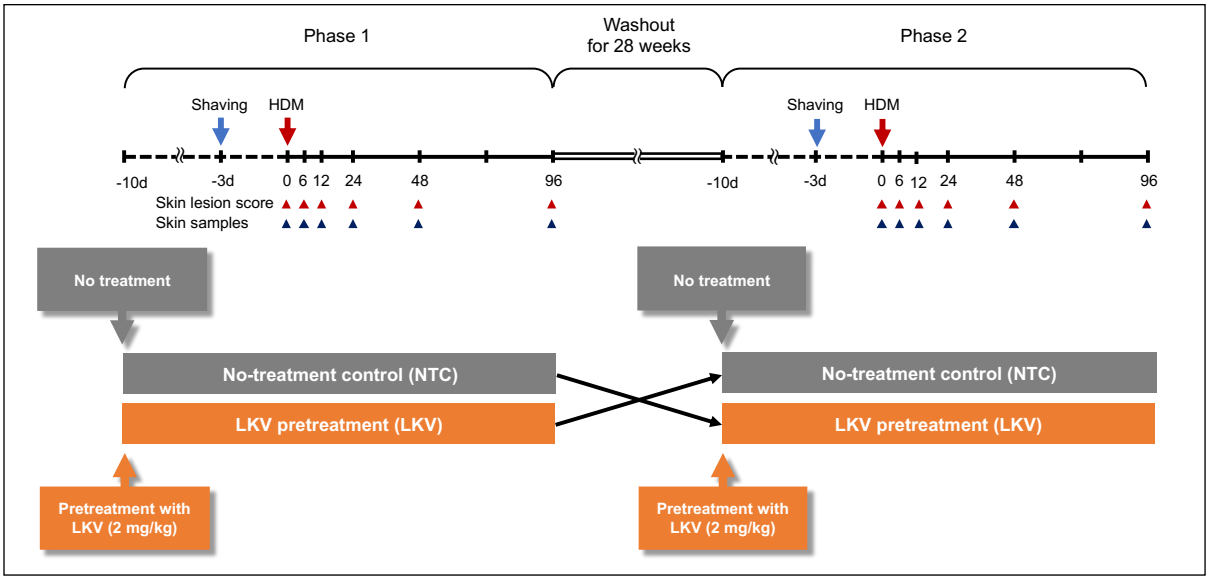


Figure 1. The study schedule.

The study was designed as a randomized, no-treatment-controlled, cross-over study with a 28-week washout period. In the second phase, dogs in each group were switched to the other pretreatment.

Abbreviation: HDM = house dust mite, LKV = lokivetmab, NTC = no-treatment control.

On Day 0 of each phase, atopic skin lesions were induced by the epicutaneous application of 20 µL of HDM slurry (25 mg of lyophilized HDM [Greer Laboratories, Lenoir, NC] in 1 mL of mineral oil), as described previously.[16] A droplet of suspension was applied to five previously-clipped areas on the lateral flanks of each dog. Skin samples were collected from every dog at baseline (0 hour) and 6, 12, 24, 48 and 96 hours after the HDM provocation; one sample was collected at each time point using an 8-mm dermal punch from one of the HDM application sites.

Macroscopic skin lesion score

Immediately before (0 hour), 6, 12, 24, 48 and 96 hours post-HDM provocation, erythematous macules, edema, papules/pustules and excoriations were scored as 0 (absent), 1 (faint, mild), 2 (moderate) or 3 (strong, severe) at the site of HDM application by a guest-

clinician who was blinded to the study protocol. The grades for each lesion were added to yield a macroscopic skin lesion score with an achievable maximum of 12, as described previously.[18]

Microscopic skin lesion score

One half of each collected skin sample was snap-frozen and cryo-sectioned at 5-μm thick, and the sections were stained with hematoxylin and eosin. The entire section of each sample was scanned using a 40x magnification (Olympus BH-2 microscope; Olympus, Shinjuku, Tokyo, Japan). The number of infiltrating cells in dermis (dermal inflammation score) was subjectively scored as 0 (none), 1 (low), 2 (medium) or 3 (high). The thickness of the epidermis (acanthosis, hyperplasia score) was also scored as 0 (normal), 1 (mild), 2 (moderate) or 3 (high). The grades for each lesion were added to yield a microscopic skin lesion score with an achievable maximum score of 6.

RNA sequencing

The second half of each collected skin sample was immediately placed of in RNAlater (Qiagen, Hilden, North Rhine-Westphalia, Germany) and frozen at −80°C. After samples from both phases were collected, they were sent to Zoetis for RNA extraction, RNA purity/integrity assessment and RNA sequencing (RNA-seq). The total RNA was isolated from each skin sample using RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacture's protocol. The RNA purity was assessed by NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) with a mean A260/A280 ratio of 2.1 (range 1.87 - 2.15; A260/A280 ratio of greater than 2.0 is considered pure RNA). The RNA integrity was assessed using the TapeStation system (Agilent, Santa Clara, CA, USA), which revealed a mean RNA integrity number of 9.1 (range 6.7 - 9.6; 10 is the highest quality and 1 is completely degraded RNA). Following RNA isolation and quality assurance, RNA libraries were prepared using the Illumina Standard TruSeq mRNA protocol and sequenced on the Illumina NextSeq550B (Illumina, San Diego, CA, USA) with 1 x 75 bp single-end reads according to the manufacture's protocol.

RNA-seq data analysis

We performed the RNA-seq data analyses and principal component analysis (PCA) using the CLC Genomic Workbench version 21.0.3 (Qiagen) with default parameters. Illumina

standard primers were trimmed, and the quality of the data was assessed. Reads were mapped to the canine reference genome (Ensembl CanFam3.1), and the number of reads for each gene was counted. A trimmed mean of M-values was used to normalize the RNA-seq data. The expression value was calculated in the transcripts per million (TPM). We used a TPM threshold of equal or greater than 1 for actively-transcribed genes.[19]

Differential expression analysis

We conducted the differential expression analysis between the two pretreatment groups (LKV vs. NTC) at each sampling time point, or between each sampling time points and baseline (0 hr), using the Differential Expression for RNA-Seq tool of the CLC Genomic Workbench version 21.0.3 (Qiagen), while controlling for the effect of individual variability. For establishing significance, we selected an absolute fold change (FC) greater than two, a false discovery rate (FDR) of equal or less than 0.05, and a maximum group mean of reads per kilobase of exon per million mapped reads (RPKM) greater than 1.

Statistical methods

A Wilcoxon signed-rank test (nonparametric, paired) was used to compare macroscopic and microscopic skin lesion scores between IL-31-blocking LKV and NTC groups at each time point. A Friedman test (nonparametric, paired, repeated-measurement) with a Conover post-hoc test was used to compare over time the macroscopic and microscopic skin lesion scores. A *p*-value equal to or less than 0.05 was considered significant. All analyses were performed using Microsoft Excel 16.41 (Microsoft Corporation, Redmond, WA, USA).

5.3 Results

1) Macroscopic skin lesion score

There was no significant difference in macroscopic skin lesion scores between the LKV and NTC pretreatment groups at any time point ($p = 0.47$ to 0.83). Although the scores remained relatively low in all dogs at all time points, small peaks of median macroscopic skin lesion scores were seen at 24 and 96 hours after HDM allergen challenge in both groups. Furthermore, significant differences in values were not seen over time within each group (LKV $p = 0.13$; NTC $p = 0.49$). (Figure 2).

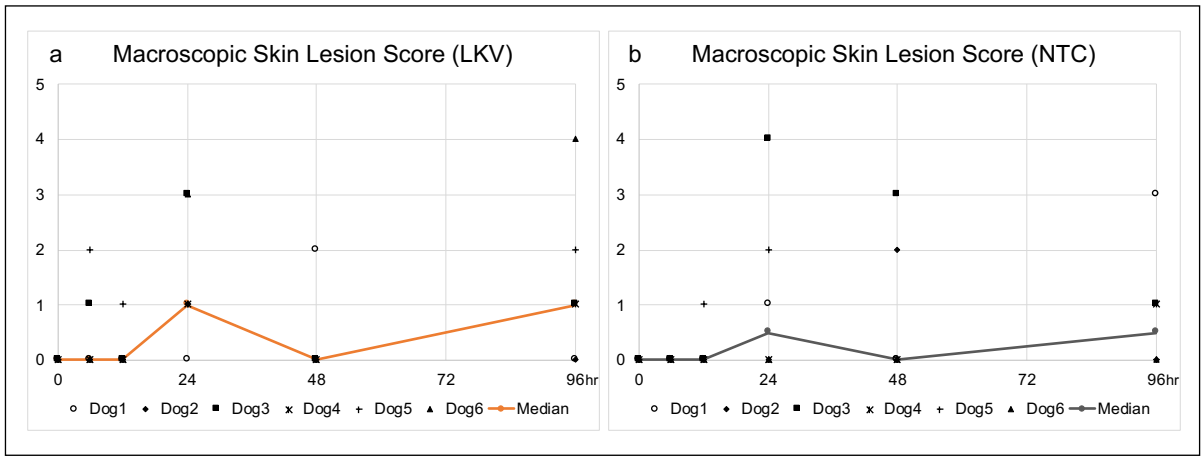


Figure 2. Macroscopic skin lesion scores in LKV and NTC.

There was no significant difference in macroscopic skin lesion scores between LKV (a) and NTC (b) at any time point ($p = 0.47$ to 0.83).

Abbreviation: LKV = lokivetmab, NTC = no-treatment control.

2) Microscopic skin lesion score

Similarly to visible skin lesions, the microscopic skin lesion scores were not significantly different between the LKV and NTC groups at each time points ($p = 0.27$ to 0.68), thus indicating that the IL-31 inhibition by LKV did not result in a change in the severity of skin inflammation (Figure 3). The median microscopic skin lesion scores increased over time, and significant increases in the scores compared to those at baseline (0 hr) were seen after 12 and 48 hours in LKV and NTC, respectively (LKV $p = 0.027$ [12 hr], $p = 0.0012$ [24 hr], $p = 0.0044$ [48 hr], $p < 0.001$ [96 hr]; NTC $p = 0.002$ [48 hr], $p < 0.001$ [96 hr]) (Figure 3).

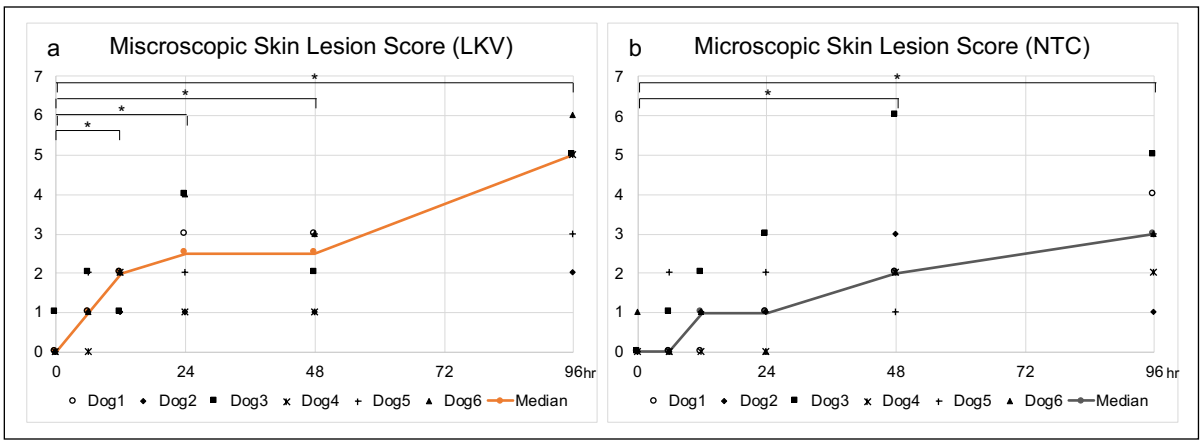


Figure 3. Microscopic skin lesion scores in LKV and NTC.

There was no significant difference in microscopic skin lesion scores between LKV (a) and NTC (b) at any time point ($p = 0.27$ to 0.68). The median microscopic skin lesion scores increased over time in both groups. Asterisks indicate a significant difference ($p < 0.05$) compared to the baseline (0 hr)

Abbreviation: LKV = lokivetmab, NTC = no-treatment control.

3) RNA-seq data analysis

RNA-seq

We were able to identify 16,491 genes in each sample.

Principal component analysis

Using PCA, we detected two outlier samples: Dog 4 in the LKV pretreatment group at 0 and 24 hours (Figure 4a,b). Because this study was a cross-over study with continuous multiple samplings, we removed all of the sample data of Dog 4 from subsequent analyses. The PCA of all other samples did not show any clear separation between the LKV and the NTC pretreatment groups, indicating that the LKV pretreatment had not resulted in a major change in gene expression (Figure 4a). In contrast, there was a clustering between the gene expression of early (0-12 hours) and late time points (24-96 hours), which suggested that the HDM challenges had triggered an apparent change in gene expression likely associated with an induction of atopic skin inflammation (Figure 4b).

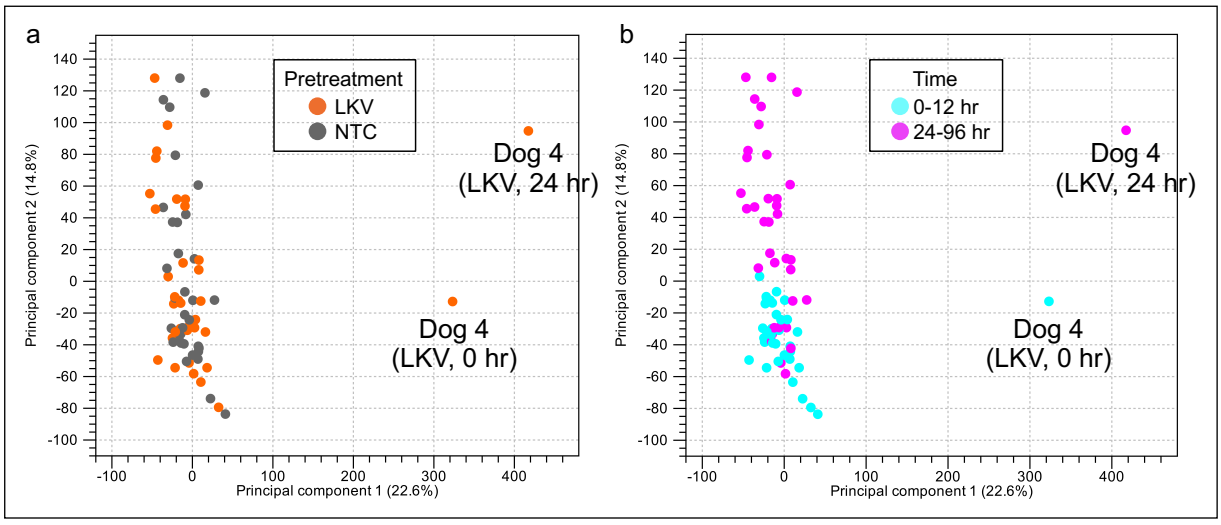


Figure 4. Principal component analysis based on pretreatments (LKV vs. NTC) and time (0-12 hr vs. 24-96 hr). Type of pretreatment (LKV vs. NTC) did not clearly separate gene expression (a). In contrast, there was a clustering between early (0-12 hours) and late time points (24-96 hours) (b).

Abbreviation: LKV = lokivetmab, NTC = no-treatment control.

Differential expression analysis

Using our selected criteria for differentially expressed genes (DEGs), we identified 211 DEGs (196 down-regulated and 15 up-regulated genes) in the LKV pretreatment samples compared to untreated (NTC) controls (Figure 5). The highest number of down-regulated DEGs in the LKV group was seen at 48 hours (Figure 5), which was consistent with the highest median *IL31* mRNA expression of all samples at 48 hours; this observation supported these changes in gene expression being likely associated with IL-31 inhibition by the LKV. Among these DEGs, only one gene (up-regulated [*IL36G*]) at 0 hour and four genes (one up-regulated [*IL37*] and three down-regulated [*CXCL8*, *IL9* and *CCL17*]) at 48 hours encoded cytokines (Figure 6), which suggested that the LKV pretreatment did not majorly affect cytokine-gene transcription. This observation could be interpreted as IL-31 acting independently, or downstream, of other pro-allergic cytokines produced during the induction of acute canine AD skin lesions.

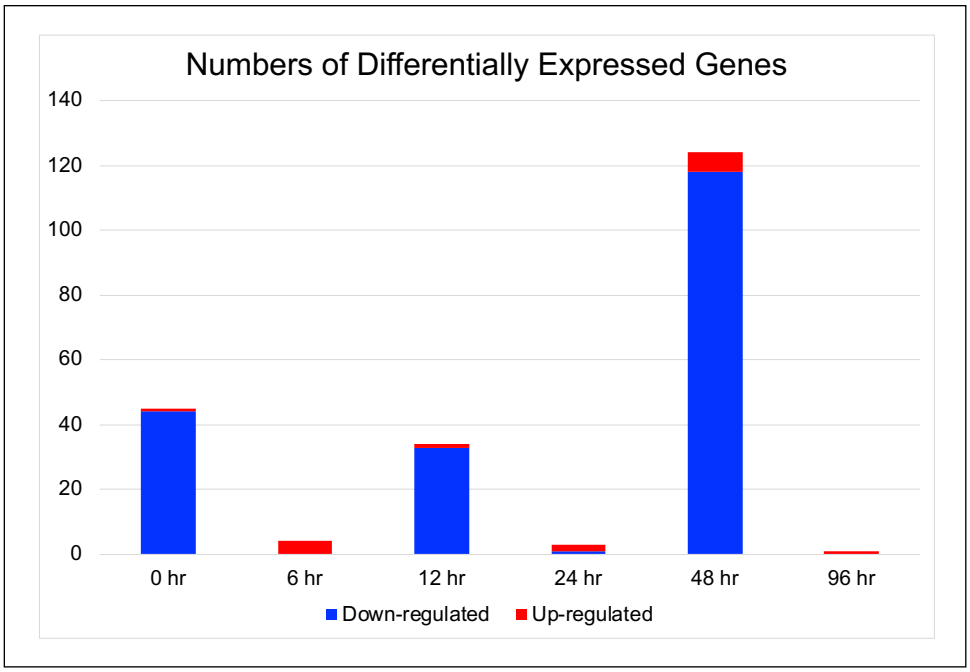


Figure 5. Numbers of differentially expressed genes (LKV/NTC) at each time point.

The highest number of down-regulated DEGs in the LKV group compared to NTC controls was seen at 48 hours.

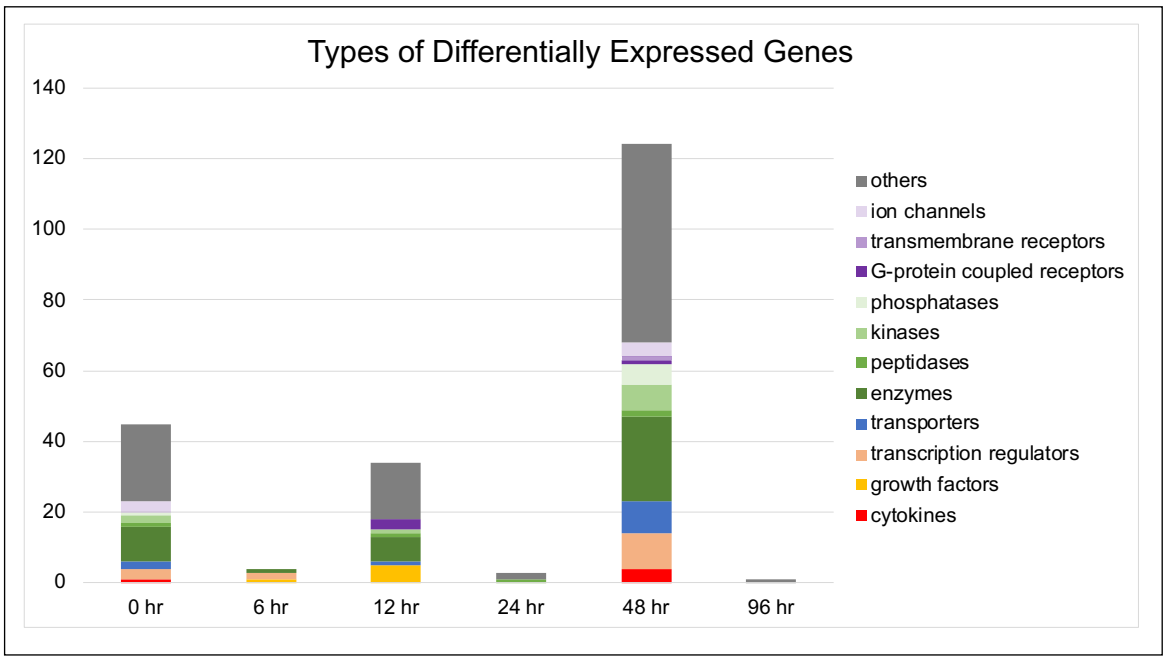


Figure 6. Types of differentially expressed genes (LKV/NTC) at each time point.

DEGs encoding cytokines were only seen one and four at 0 and 48 hours, respectively, indicating that IL-31 inhibition had a no-to-little effect on the transcription of other cytokines.

Comparison of cytokine-encoding gene expression in LKV and NTC groups

We examined fold changes of key proinflammatory, T helper type 1 (Th1), type 2 (Th2), type 9 (Th9), type 17 (Th17), type 22 (Th2) and regulatory T cells (Treg) cytokines and chemokines in the LKV pretreatment group compared to those in the NTC pretreatment group at each sampling time point. As was expected from our previous analysis of DEGs, there were no consistent significant fold changes in any cytokines and chemokines (Table 1). The chronological changes of median TPMs for these cytokines and chemokines also did not reveal any apparent difference between pretreatment groups (Table 2). These results further supported that these cytokines were likely independent—or upstream—of any IL-31-dependent pathway. About a half of these genes showed a TPM equal or greater than 1 at most-or-all sampling time points, which indicated that they were likely actively transcribed even in the LKV pretreatment group (Table 2).[19]

Table 1. Fold changes of cytokine-encoding gene expression in the LKV against NTC groups.

The numbers in each column denote the fold changes of each gene in the LKV against NTC groups at each time point. None of the cytokines and chemokines showed consistent significant fold changes during the study period. The bolded-white lettering denotes down-regulated genes with statistically significant differences at |FC| > 2, FDR ≦ 0.05 and RPKM ≧ 1 in the LKV compare to those in the NTC group at the same time point.

Abbreviation: FC = fold change, FDR = false discovery rate, LKV = lokivetmab, NaN = not a number (transcripts with zero expression across all samples in either or both groups, therefore the fold changes were not calculated), NTC = no-treatment control, RPKM = reads per kilobase of exon per million mapped reads.

			LKV/NTC					
			hr					
	Gene codes	Coding Protein	0	6	12	24	48	96
Proinflammatory cytokines	*IL1A*	Interleukin 1α	-1.0	-1.1	1.1	-1.0	-1.0	1.0
	IL1B	Interleukin 1β	-1.1	3.1	-1.2	1.3	-1.1	2.4
	IL6	Interleukin 6	-1.1	6.0	1.3	-1.4	-1.4	1.4
	IL25	Interleukin 25	1.2	1.3	-1.1	1.2	-1.8	-1.5
	IL33	Interleukin 33	-1.0	1.4	1.6	1.1	-1.4	1.1
	TNF	Tumor necrosis factor	1.8	-1.1	-1.2	-1.0	-1.5	1.1
Th1 cytokines	*IL2*	Interleukin 2	2.1	-1.2	-2.1	1.3	-4.8	1.0
	IL12A	Interleukin 12A	-1.0	1.4	1.4	-1.0	1.2	-1.1
	IL12B	Interleukin 12B	-1.8	-1.2	1.4	-2.1	1.2	-1.0
	IL18	Interleukin 18	1.3	-1.1	1.3	1.4	-1.5	1.1
	IL27	Interleukin 27	3.9	1.2	1.1	1.4	-4.0	1.4
	IFNG	Interferon γ	2.7	1.1	1.4	1.3	-1.8	-1.4
	CXCL10	C-X-C motif chemokine ligand 10	2.4	-1.2	1.1	1.3	-1.5	1.0
	CXCL11	C-X-C motif chemokine ligand 11	3.1	NaN	NaN	NaN	NaN	NaN
Th2 cytokines	*IL4*	Interleukin 4	NaN	-1.4	6.2	-1.1	-2.0	2.0
	IL5	Interleukin 5	-1.5	1.2	1.8	2.8	-1.1	1.3
	IL13	Interleukin 13	1.2	-1.4	-1.7	2.1	-2.5	1.1
	IL31	Interleukin 31	-4.3	NaN	-2.9	1.5	-2.4	1.9
	CCL17	C-C motif chemokine ligand 17	1.0	3.1	1.0	2.1	**-5.8**	-1.4
	CCL22	C-C motif chemokine ligand 22	1.4	1.3	-1.3	1.6	-1.3	-1.7
	TSLP	Thymic stromal lymphopoietin	-1.4	-1.4	-1.0	-1.2	-1.1	1.3
Th9 cytokines	*IL9*	Interleukin 9	NaN	-2.6	2.0	2.6	**-5.2**	1.3
Th17 cytokines	*IL17A*	Interleukin 17A	NaN	NaN	NaN	-14.0	-8.5	-1.3
	IL17B	Interleukin 17B	-1.0	-1.1	1.1	1.3	-1.4	-1.0
	IL17C	Interleukin 17C	-5.9	1.0	15.1	1.7	-3.6	-1.4
	IL17F	Interleukin 17F	NaN	NaN	-2.7	2.4	-7.7	5.1
	IL21	Interleukin 21	-1.6	7.3	4.6	2.3	-4.1	-1.6
	IL23A	Interleukin 23A	1.4	1.1	1.1	-1.5	-1.3	1.4
Th22 cytokines	*IL22*	Interleukin 22	NaN	NaN	7.4	1.2	-2.9	5.8
Treg cytokines	*IL10*	Interleukin 10	-1.2	1.0	1.1	1.1	-1.1	1.1
	TGFB1	Transforming growth factor β1	1.0	1.1	-1.1	-1.0	-1.2	-1.1

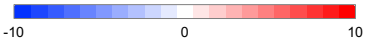
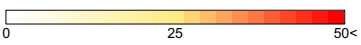


Table 2. Comparison of cytokine-encoding gene expression between LKV and NTC groups.

The numbers in each column denote the median TPMs for each gene at each time point. Comparing the median TPMs of major cytokines and chemokine genes did not reveal any apparent difference between LKV and NTC groups. The bolded-blue lettering denotes down-regulated genes with statistically significant differences at |FC| > 2, FDR ≦ 0.05 and RPKM ≧ 1 in the LKV compare to those in the NTC group at the same time point; these down-regulations, being seen at a single time point in spite of a rising expression in other samples suggest a technical error.

Abbreviation: FC = fold change, FDR = false discovery rate, LKV = lokivetmab, NTC = no-treatment control, RPKM = reads per kilobase of exon per million mapped reads, TPM = transcripts per million.

			LKV						NTC					
			hr						hr					
	Gene codes	Coding Protein	0	6	12	24	48	96	0	6	12	24	48	96
Proinflammatory cytokines	*IL1A*	Interleukin 1α	10.1	12.9	13.2	12.1	10.7	10.4	10.8	13.0	13.4	14.0	12.1	14.7
	IL1B	Interleukin 1β	6.2	8.8	7.8	7.9	6.9	6.6	7.1	6.9	7.8	9.8	8.9	9.5
	IL6	Interleukin 6	0.0	0.6	0.6	4.0	5.4	1.7	0.0	0.1	0.5	2.4	3.1	0.6
	IL25	Interleukin 25	0.5	1.3	1.2	0.8	0.4	0.5	0.6	1.1	0.9	0.8	0.9	0.9
	IL33	Interleukin 33	14.6	16.5	19.9	38.6	36.3	40.8	11.9	15.7	16.5	29.0	49.0	32.0
	TNF	Tumor necrosis factor	3.8	3.2	3.2	4.9	5.3	4.8	1.9	3.9	3.7	5.4	7.4	4.5
Th1 cytokines	*IL2*	Interleukin 2	0.0	0.0	0.2	0.3	0.2	0.2	0.0	0.0	0.3	0.1	1.0	0.3
	IL12A	Interleukin 12A	1.2	2.1	2.0	1.9	1.9	1.3	1.0	1.0	1.5	1.8	1.5	1.4
	IL12B	Interleukin 12B	0.3	0.3	0.4	0.2	0.6	0.2	0.5	0.4	0.6	0.6	0.4	0.2
	IL18	Interleukin 18	58.5	46.3	68.6	107.0	84.9	99.3	42.4	58.7	55.3	61.6	117.1	123.1
	IL27	Interleukin 27	0.0	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.2
	IFNG	Interferon γ	0.2	0.1	0.2	0.3	0.1	0.0	0.0	0.1	0.1	0.1	0.2	0.1
	CXCL10	C-X-C motif chemokine ligand 10	5.3	14.7	17.6	34.2	9.2	9.9	2.5	15.4	15.8	22.7	21.3	11.5
	CXCL11	C-X-C motif chemokine ligand 11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Th2 cytokines	*IL4*	Interleukin 4	0.0	0.0	0.0	0.3	0.2	0.0	0.0	0.0	0.0	0.4	1.1	0.2
	IL5	Interleukin 5	0.8	0.6	0.8	1.7	0.9	0.9	0.9	0.6	0.2	0.5	0.9	0.5
	IL13	Interleukin 13	0.3	0.3	0.4	8.7	5.3	7.6	0.2	0.6	1.4	2.5	9.1	4.3
	IL31	Interleukin 31	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.9	0.0
	CCL17	C-C motif chemokine ligand 17	0.5	0.3	0.8	8.7	**17.0**	68.7	0.5	0.3	2.1	5.5	91.3	56.1
	CCL22	C-C motif chemokine ligand 22	11.1	16.2	11.1	21.4	23.6	27.6	7.4	15.2	17.7	15.9	30.0	36.5
	TSLP	Thymic stromal lymphopoietin	1.0	0.3	1.8	0.6	0.6	0.3	1.1	0.5	1.3	0.5	0.5	0.3
Th9 cytokines	*IL9*	Interleukin 9	0.0	0.0	0.0	11.5	**0.8**	7.3	0.0	0.0	0.0	4.8	13.5	2.9
Th17 cytokines	*IL17A*	Interleukin 17A	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	IL17B	Interleukin 17B	6.2	5.4	5.8	10.7	7.4	8.6	6.9	6.0	5.5	6.3	9.9	8.0
	IL17C	Interleukin 17C	0.0	0.0	0.4	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2
	IL17F	Interleukin 17F	0.0	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0	0.1	0.4	0.0
	IL21	Interleukin 21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	IL23A	Interleukin 23A	2.8	2.2	2.2	2.5	3.4	1.8	2.1	2.0	2.3	2.6	4.2	2.1
Th22 cytokines	*IL22*	Interleukin 22	0.0	0.0	0.0	0.6	0.0	0.1	0.0	0.0	0.0	0.2	0.4	0.0
Treg cytokines	*IL10*	Interleukin 10	4.8	4.8	5.8	7.8	5.1	10.9	6.2	5.5	4.9	6.4	5.6	14.7
	TGFB1	Transforming growth factor β1	22.7	24.8	24.6	30.7	25.8	28.0	20.1	24.8	27.2	28.9	29.5	31.2



Identification of possible cytokine targets to supplement LKV treatment

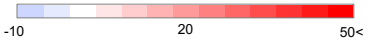
To further identify IL-31-independent cytokines whose inhibition could complement LKV therapy for the prevention of canine AD flares, we performed a differential expression analysis to assess chronological changes in the expression of these cytokine-encoding genes in the LKV pretreatment group. This analysis revealed that *IL33*, *IL13*, *CCL17* and *IL9* were statistically significantly up-regulated after 24 hours compared to the expression at baseline (0 hour) (Table 3). *IL6* and *CCL22* were also up-regulated after 24 hours, but they lost statistical significance at 96 hours (Table 3). *IL1B* at 24 hours, *IL18* and *IL10* at 96 hours were also up-regulated compared to the baseline's expression, but we did not consider them as valuable targets because they were seen to be upregulated at single time point (Table 3). There was a trend for the increased expression of *IL4*, *IL17C*, *IL17F* and *IL22* after 12-24 hours; however, none of them showed a statistically significant difference compared to the baseline's expression (Table 3). Furthermore, TPMs of these genes were less than 1, indicating that they were not actively expressed (Table 2). Therefore, we did not consider them as potential targets of interest. Altogether, we concluded that IL-33, IL-13, C-C motif chemokine ligand (CCL)17, and IL-9, and potentially IL-6 and CCL22, could be valuable targets whose inhibition could help prevent AD relapses in atopic dogs receiving LKV treatment.

Table 3. Fold changes of cytokines and chemokines at each sampling time point compared to the baseline (0 hour) in LKV pretreatment group.

The numbers in each column denote the fold changes of gene expression compared to the baseline (0 hour). Bold lettering denotes genes with statistically significant differences at |FC| > 2, FDR ≦ 0.05 and RPKM ≧ 1 at each time point compare to those at the baseline (0 hour).

Abbreviation: FC = fold change, FDR = false discovery rate, LKV = lokivetmab, RPKM = reads per kilobase of exon per million mapped reads.

			LKV					
			hr					
	Gene codes	Coding Protein	0	6	12	24	48	96
Proinflammatory cytokines	*IL1A*	Interleukin 1α		1.3	1.4	1.5	1.2	1.6
	IL1B	Interleukin 1β		2.4	1.1	**6.6**	3.1	4.4
	IL6	Interleukin 6		33.2	13.3	**122**	**64.5**	35.8
	IL25	Interleukin 25		1.6	1.4	1.2	1.2	1.2
	IL33	Interleukin 33		1.4	1.7	**2.7**	**2.5**	**2.8**
	TNF	Tumor Necrosis Factor		1.0	-1.0	1.7	1.4	1.5
Th1 cytokines	*IL2*	Interleukin 2		2.1	1.5	6.3	2.5	3.6
	IL12A	Interleukin 12 A		1.5	1.8	1.8	1.7	1.1
	IL12B	Interleukin 12 B		1.6	2.1	-1.1	1.4	-1.3
	IL18	Interleukin 18		-1.1	1.1	2.0	1.5	**2.2**
	IL27	Interleukin 27		1.7	1.6	3.5	-1.0	3.5
	IFNG	Interferon γ		-1.6	-1.1	1.3	-1.5	-3.0
	CXCL10	C-X-C motif chemokine ligand 10		-2.2	-1.0	1.1	-1.4	-1.8
	CXCL11	C-X-C motif chemokine ligand 11		-2.7	-2.7	-2.7	-2.7	-2.5
Th2 cytokines	*IL4*	Interleukin 4		4.5	6.1	48.4	18.2	2.0
	IL5	Interleukin 5		-1.2	-1.1	3.2	1.4	1.2
	IL13	Interleukin 13		2.1	2.6	**21.0**	**10.0**	**13.1**
	IL31	Interleukin 31		1.1	1.1	23.6	13.8	13.7
	CCL17	C-C motif chemokine ligand 17		1.3	2.9	**30.5**	**14.5**	**51.0**
	CCL22	C-C motif chemokine ligand 22		1.4	1.0	**2.1**	**2.5**	1.8
	TSLP	Thymic stromal lymphopoietin		-1.2	1.3	-1.4	-1.4	-1.5
Th9 cytokines	*IL9*	Interleukin 9		1.1	7.9	**508**	**143**	**271**
Th17 cytokines	*IL17A*	Interleukin 17A		1.0	-1.0	2.7	-1.0	14.2
	IL17B	Interleukin 17B		-1.2	-1.1	1.4	1.0	1.2
	IL17C	Interleukin 17C		3.5	34.8	27.7	2.4	36.0
	IL17F	Interleukin 17F		1.0	1.0	80.2	16.1	64.3
	IL21	Interleukin 21		2.6	1.6	8.0	-1.1	2.9
	IL23A	Interleukin 23A		-1.2	-1.1	-1.0	1.3	1.6
Th22 cytokines	*IL22*	Interleukin 22		1.1	6.6	44.3	19.1	28.8
Treg cytokines	*IL10*	Interleukin 10		1.1	1.1	1.5	1.0	**2.4**
	TGFB1	Transforming growth factor β1		1.2	1.1	1.4	1.2	1.5



Comparison of skin barrier-encoding gene expression in LKV and NTC groups

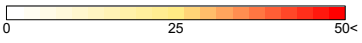
The expression of some genes associated with physical (e.g., filaggrin, desmoglein-1, desmocollin-1 and corneodesmosin) and antimicrobial skin barrier (β-defensins and S100 Ca^{2+}-binding proteins) are known to be affected by IL-31, *in vitro*, in humans.[20,21] We thus compared the expression patterns of physical and antimicrobial skin barrier-associated genes in LKV and NTC groups to evaluate the effect of IL-31 inhibition in the canine species. Since filaggrin-encoding genes were not in the canine reference genome and we could not map the reads, the gene expression of molecules and enzymes known to be involved in filaggrin metabolism were assessed to evaluate the production of filaggrin. We did not reveal any apparent difference in the transcription of these genes between groups, except for the down-regulation of *S100A9* and *S100A12* at a single time point, indicating a no-to-little effect of IL-31 inhibition to prevent acute canine AD skin barrier dysfunction (Table 4).

Table 4. Comparison of physical and antimicrobial skin barrier-encoding gene expression between LKV and NTC groups.

The numbers in each column denote the median TPMs for each gene at each time point. Comparing median TPM of physical and antimicrobial skin barrier-encoding genes did not reveal an apparent difference between LKV and NTC groups. Blue and red bold lettering denotes down-regulated and up-regulated genes, respectively, with statistically significant differences at $|FC| > 2$, FDR $\leqq 0.05$ and RPKM $\geqq 1$ in the LKV compare to those in NTC group at the same time point.

Abbreviation: FC = fold change, FDR = false discovery rate, LKV = lokivetmab, NTC = no-treatment control, RPKM = reads per kilobase of exon per million mapped reads, TPM = transcripts per million.

			LKV						NTC					
			hr						hr					
	Gene codes	Coding protein	0	6	12	24	48	96	0	6	12	24	48	96
Filaggrin metabolism	*FURIN*	Furin	162	169	158	179	164	196	157	159	156	163	158	199
	PRSS8	Protease 8/Prostasin	62	75	68	51	49	43	63	66	65	64	44	65
	KLK5	Kallikrein 5	73	82	92	57	80	67	82	83	84	71	54	68
	ST14	Matriptase	92	94	101	84	80	73	98	97	109	99	83	83
	CAPN1	Calpain 1	230	236	237	225	230	249	227	235	231	213	214	237
	ASPRV1	SASPase	206	214	233	138	117	107	182	222	194	164	102	224
	CASP14	Caspase-14	54	59	84	70	89	130	48	55	62	58	57	116
	BLMH	Beomycin hydrolase	125	130	155	94	101	83	124	139	144	104	85	130
	SPINK5	Serine peptidase inhibitor Kazal 5	1570	1549	1914	1333	1544	1575	1302	1717	1529	1371	1426	1753
Cornified envelope	*EVPL*	Envoplakin	490	441	525	337	370	370	465	529	490	390	360	414
	IVL	Involucrin	595	716	674	513	620	622	655	598	702	566	600	678
	PPL	Periplakin	827	802	881	547	616	631	776	839	769	588	548	727
	TGM1	Transglutaminase 1	159	177	188	133	131	147	142	177	167	128	116	161
	TGM3	Transglutaminase 3	153	151	165	130	203	208	120	140	113	111	146	192
	TGM5	Transglutaminase 5	55	63	71	43	44	53	44	58	49	45	39	70
Tight junctions	*CLDN1*	Claudin 1	392	375	377	352	368	295	394	393	368	359	356	307
	CLDN4	Claudin 4	58	66	57	42	48	38	57	59	65	54	40	43
	CLDN7	Claudin 7	7	8	8	8	10	9	7	8	7	8	8	12
	OCLN	Occludin	60	55	52	38	50	42	51	59	55	47	44	47
	F11R	Junctional adhesion molecule A	143	125	126	95	106	100	141	116	149	117	100	103
	TJP1	Zonula occluden 1	185	170	172	158	176	154	168	154	172	143	166	157
Keratines	*KRT10*	Keratin 10	9368	9095	13275	9769	9195	9402	6351	9628	8050	8319	6631	9727
	KRT14	Keratin 14	10045	10592	10341	9310	9884	8844	9130	10352	10828	9629	8407	8554
Corneodesmosomes	*DSG1*	Desmoglein 1	1239	1069	1104	979	1165	1098	993	1014	959	887	934	1210
	DSC1	Desmocollin 1	1649	1458	1407	1113	1381	1373	1319	1408	1232	1155	1254	1632
	JUP	Plakoglobin	1705	1760	1878	1371	1448	1534	1590	1759	1741	1479	1250	1475
	PKP1	Plakophilin 1	1512	1660	1794	1295	1434	1258	1329	1523	1559	1330	1143	1310
	PKP2	Plakophilin 2	16	18	14	13	15	14	15	15	17	13	11	14
	PKP3	Plakophilin 3	369	400	412	302	339	305	396	387	422	355	265	303
	CDSN	Corneodesmosin	652	717	726	538	594	495	544	657	651	504	417	686
Desquamation	*KLK5*	Kallikrein 5	73	82	92	57	80	67	82	83	84	71	54	68
	KLK8	Kallikrein 8	365	381	386	328	380	364	311	333	354	379	341	326
	KLK14	Kallikrein 14	1	0	1	1	1	1	1	0	1	1	1	1
	SPINK5	Serine peptidase inhibitor Kazal 5	1	0	1	1	1	1	1	0	1	1	1	1
Antimicrobial peptides	*DEFB1*	Beta-defensin 1	9	9	11	9	9	12	6	8	7	7	7	7
	cBD103	Beta-defensin 103	53	41	45	60	50	70	45	50	42	55	61	50
	CAMP	Cathelicidin antimicrobial peptide	0	0	0	0	0	0	0	0	0	0	0	0
	S100A8	Calgranulin A	525	489	484	1142	575	1373	527	475	523	554	901	800
	S100A9	Calgranulin B	5	9	**12**	218	**26**	345	4	4	5	21	144	100
	S100A12	Calgranulin C	8	16	24	391	**73**	644	6	6	9	34	290	144



5.4 Discussion

In the present study, using an experimental canine acute AD model, we investigated the effect of IL-31 inhibition on the development of AD skin lesions and on the lesional skin transcriptome at the induction of acute atopic inflammation. With this methodology, we aimed to uncover IL-31-independent cytokines that could be the additional therapeutic target(s) to supplement the use of LKV to prevent acute AD flares in dogs.

As was expected from the result of our previous study in which the pretreatment with LKV did not prevent the development of skin lesions after exposure to the HDM allergens (see Chapter 2 above), we did not observe any significant difference in macroscopic and microscopic skin lesion scores between the LKV and NTC pretreatment groups at any sampling time points. This finding further supported that IL-31 is not essential for the induction of acute allergic skin inflammation, which could explain an insufficient effect of LKV proactive therapy on preventing AD flares in some dogs (see Chapter 2 above).

Several studies in human allergic diseases have reported the direct interaction of IL-31 and other cytokines and chemokines. IL-31 significantly induced the release of proinflammatory cytokines (IL-1β and IL-6) and AD-related chemokines (C-X-C motif chemokine ligand [CXCL]1, CXCL8, CCL2, and CCL18) from eosinophils, especially when they were co-cultured with keratinocytes.[22] Human monocyte-derived dendritic cells released several proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, CXCL8, CCL2 CCL5 and CCL22, after IL-31 stimulation.[23] IL-31 also increased the gene expression of several cytokines and chemokines including *CXCL1*, *CCL1*, *CCL4*, *CCL17*, *CCL19*, *CCL22*, *CCL23*, *IL20* and *IL24* from normal human epidermal keratinocytes.[3,20] As the result of these studies, we inferred that IL-31 inhibition by LKV pretreatment would down-regulate the transcription of these cytokines and chemokines. In the present study, however, we only identified five cytokine-encoding genes (two up-regulated genes [*IL36G* at 0 hour and *IL37* at 48 hours] and three down-regulated genes [*CXCL8*, *IL9* and *CCL17* at 48 hours]) in the LKV pretreatment samples compared to untreated (NTC) controls among 211 DEGs. Since the significant difference of these gene expressions was only detected at a single sampling time point, we could not confirm that it was associated with a direct effect of IL-31 inhibition, and we thus suspect that these are aberrant methodology-related results. Instead, this observation could be interpreted as IL-31 acts independently, or downstream, of other cytokines during the induction of acute canine AD skin lesions,

highlighting that IL-31 inhibition would not prevent the development of acute atopic inflammation by these other cytokines. This finding is consistent with the result of the human AD skin transcriptome study in which IL-31 did not show a correlation with other cytokine genes that were dysregulated in acute lesional AD skin.[24]

Based on their active gene expression levels and significant up-regulation compared to the expression at baseline (0 hour), we identified IL-33, IL-13, CCL17, and IL-9, and potentially IL-6 and CCL22 as valuable targets whose inhibition could help prevent AD relapses in atopic dogs receiving LKV treatment. These cytokines and chemokines are produced at a very early stage of induction of acute flares of AD, likely even before Th2 cells produce IL-31 (Figure 7)[25] Such proposed "cytokine cascade" is consistent with our finding that IL-31 inhibition did not affect the transcription of these cytokines since they are likely upstream from the IL-31 pathway.

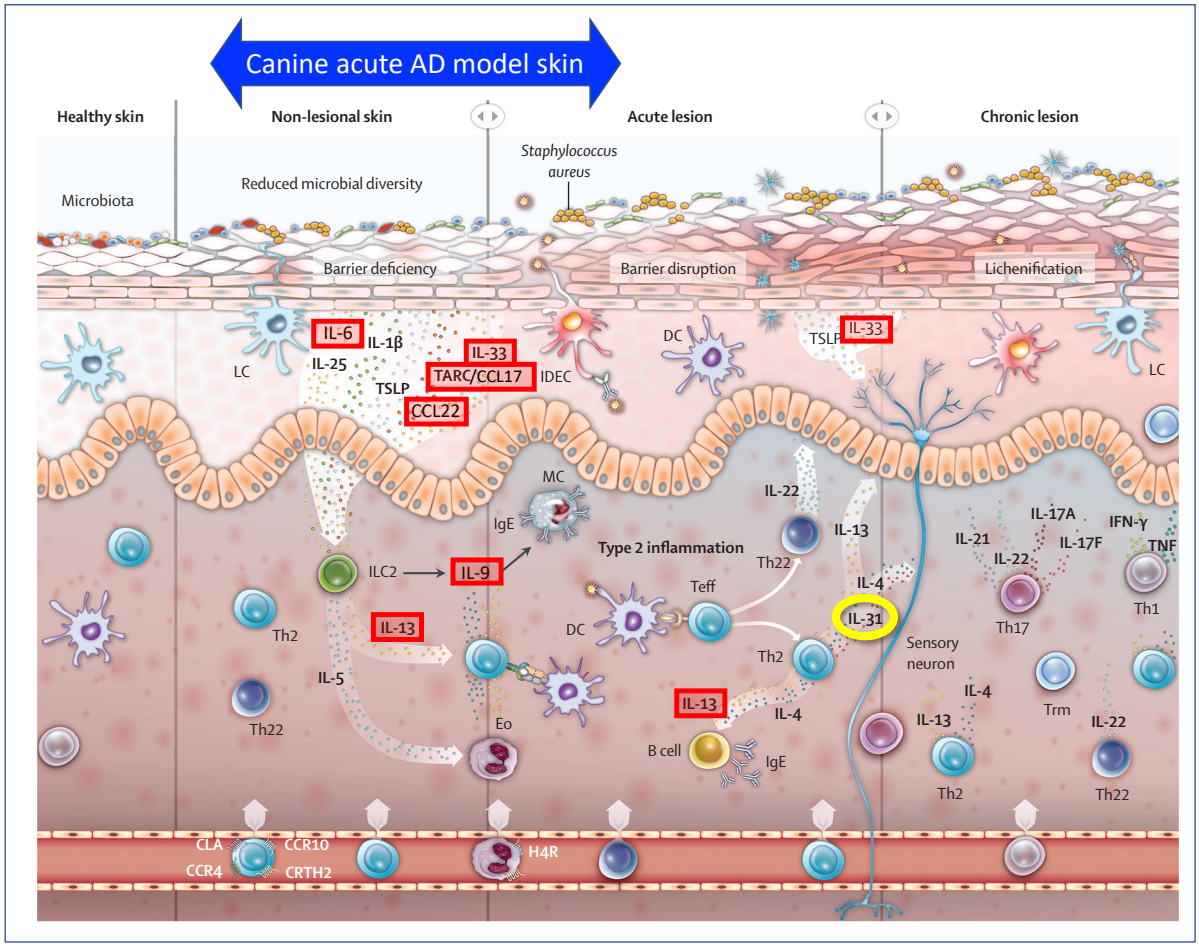


Figure 7. Potential target cytokines to prevent AD relapses under LKV treatment. (figure adapted from reference 24 with some modification)

All cytokines we identified as valuable targets (IL-6, IL-33, CCL17, CCL22, IL-13 and IL-9; highlighted with red boxes) to supplement LKV therapy are released upstream of IL-31 production (highlighted with a yellow circle) during induction of acute AD skin lesions. In this canine AD model, skin lesions only represent a very early stage of acute AD (see blue two-directional arrow).

Abbreviation: CCR = CC chemokine receptor, CLA = cutaneous lymphocyte-associated antigen, CRTH2 = chemoattractant receptor-homologous molecule expressed on Th2 cell, DC = dendritic cell, Eo = eosinophil, H4R = histamine H4 receptor, IDEC = inflammatory dendritic epidermal cell, IFN = interferon, IL = interleukin, ILC = innate lymphoid cell, LC = lymphoid cell. MC = mast cell, TARC = thymus and activation-regulated chemokine (CCL17), Teff = effector T cell, Tem = effector memory T cell, Th = T-helper cell, TNF = tumor necrosis factor, Trm = tissue-resident memory T cell, TSLP = thymic stromal lymphopoietin.

All cytokines we identified as valuable targets in this study (IL-33, IL-13, CCL17, IL-9, IL-6 and CCL22) have already been investigated as therapeutic targets in human allergic

diseases. IL-33, an alarmin cytokine that is released from various cells, including damaged keratinocytes in response to stress conditions, has emerged as a key candidate to control allergic disorders including AD.[26,27] IL-33 activates eosinophils and basophils, triggers mast cell degranulation, and promotes the development of Th2 immune response.[26,27] A phase 2a clinical trial in adult human patients with moderate-to-severe AD revealed a rapid response to a single dose of humanized anti-IL-33 mAb (etokimab), and all patients reached Eczema Area Severity Index (EASI) score improvement of 50 percent relative to baseline (EASI-50) within 57 days.[28] However, the following phase 2b, randomized, double-blinded, placebo-controlled, multi-dose study with approximately 300 moderate-to-severe AD patients failed to demonstrate a statistically-significant difference between any etokimab dose and placebo in providing an improvement in EASI score by week 16 (NCT03533751). Considering that IL-33 plays its central role in the earliest induction of AD skin inflammation, one could infer that this treatment failure might have been due to inappropriate treatment strategies of etokimab. Indeed, it might have been conceptually better to administer the etokimab at a time of disease stability to prevent further flares (i.e., "proactive" therapy). Consistent with this hypothesis, a single dosage of etokimab pretreatment significantly increased the threshold of reactivity after oral peanut challenge compared to that of placebo in human patients with peanut allergy.[29] These observations in human AD support that IL-33 could also be a promising target in canine AD when used proactively and not reactively (meaning after lesions have developed fully).

IL-13 is regarded as a primary disease-inducing effector cytokine in allergic diseases, as it contributes to IgE production and the differentiation of T cells toward the Th2 subtype.[25] A recent human study performing RNA sequencing and *in situ* hybridization on subacute-to-chronic AD skin demonstrated that *IL13* had the best ability to differentially label AD from normal or psoriasis skins among other ten cytokines examined in the study (*IL4*, *IL12B*, *IL17A*, *IL17F*, *IL22*, *IL23A*, *IL31*, *TNF*, *NOS2* and *IFNG*).[30] IL-13, therefore, is considered an important and relevant therapeutic target in asthma and AD in humans. Dupilumab, a fully human mAb that blocks IL-4 and IL-13 by blocking access to their shared IL-4 receptor α, is the first biological agent approved for AD treatment in humans.[31] Two phase 3 studies enrolled moderate-to-severe AD patients revealed that dupilumab significantly improved the signs and symptoms of AD as compared with placebo.[32] Lebrikizumab and tralokinumab are both humanized anti-IL-13 mAbs that have also been tested for human AD in advanced studies with favorable outcomes.[33,34]

A recent phase 2b randomized clinical trial of lebrikizumab monotherapy in adults with moderate-to-severe AD revealed that lebrikizumab provided a rapid and potent effect, with itch relief observed by Day 2, and a statistically-significant improvement in EASI compared to that in placebo controls at 16 weeks.[35] Similarly, a phase 3 clinical trial with tralokinumab monotherapy in human patients with AD who had an inadequate response to conventional topical corticosteroids demonstrated significantly higher treatment success rates in patients who received tralokinumab compared to those treated with placebo.[36] These findings in human AD further support the hypothesis that IL-13 could be a valid target also in canine AD. In fact, the addition of a recombinant protein including the canine Fc-IgG coupled to the IL-13 receptor subunit alpha 2 (IL-13Rα2), a nonfunctional decoy receptor counteracting IL-13 signaling, has already been shown to inhibit the production of allergen-specific-IgE from canine peripheral blood mononuclear cells (PBMCs) isolated from dogs with flea allergy.[37] This early report, more than 20 years ago, confirms the validity of inhibiting IL-13 in canine allergic diseases, like in humans with similar afflictions.

Beyond IL-33 and IL-13, there are few studies that have reported the effect of therapeutic mAbs targeting our other candidate cytokines. Mogamulizumab that inhibits C-C chemokine receptor (CCR)4, the receptor for CCL17 and CCL22, is approved for the treatment of cutaneous T cell lymphomas and asthma in humans;[38] there are, however, no data available for its effect on AD. A small molecule CCR4-specific antagonist (compound 22) was tested in an ovalbumin-sensitized, dibutyl phthalate-induced murine AD model, which demonstrated the inhibition of Th2 cell infiltration and amelioration of AD-like skin lesions.[39] Another potent inhibitor of human and canine CCR4 (AZ445) has been tested in another HDM-sensitized atopic dog model. In contrast to the apparent reduction of clinical scores observed with oral prednisolone (positive control), the CCR4 inhibitor only showed partial improvement (30-49%) of clinical signs in a subset of these dogs (5/13),[40] indicating an insufficient effect on CCR4-inhibiting therapy in canine AD. Further studies should verify these unexpected results in light of CCL17 being the most promising and prominent biomarker of disease activity in human and canine AD.[41,42]

A phase 2a clinical trial enrolling asthma patients to evaluate the safety and efficacy of enokizumab (MEDI-528), a humanized mAb against IL-9, failed to demonstrate any significant clinical improvement compared to that in placebo controls.[43] Its potential effect on AD has not been reported yet.

Lastly, tocilizumab, an anti-IL-6 receptor mAb, was used in three patients affected with severe AD that had been refractory to conventional treatments, including topical steroids, topical calcineurin inhibitors, oral ciclosporin and phototherapy.[44] Pruritus subsided within six weeks, and EASI was significantly reduced after three months of treatment in all patients.[44] Although the translatability potential of this report was limited by the small number of patients and the open design without a control group, these early results suggested the IL-6 receptor—and thus likely IL6—to be promising therapeutic targets for human AD. Further studies are needed to investigate the effect of inhibiting these cytokines or their receptors to prevent human and canine AD flares.

In vitro studies in humans have shown that IL-31 down-regulated the expression of some proteins associated with the physical skin barrier, such as filaggrin, filaggrin-processing enzymes, desmogleins, desmocollins and corneodesmosin, in a dose-depending manner.[20,21] Furthermore, a significant reduction of transepidermal water loss has been reported in dogs with AD after LKV treatment compared to that at baseline, suggesting that IL-31 inhibition might help restore a functional skin barrier.[45] Interestingly, treatment with dupilumab in human AD has also reported a strong effect on the normalization of gene expression of skin barrier function-related proteins such as loricrin, claudin 8, filaggrin, keratin 1, keratin 10, and elongation of very long chain fatty acids protein 3.[24] In contrast, the gene expression levels of Th2 cytokines such as *IL4*, *IL5*, *IL13* and *IL31* actually increased despite the clinical improvement seen in AD skin lesions in those patients after three months of dupilumab injections.[24] These findings could suggest that restoring epidermal barrier function might be more important than regulating immune response for successful treatment of AD. In this study, however, we did not detect any significant difference in the transcription of these physical skin-barrier-associated genes between LKV and NTC pretreatment groups, indicating a lack of effect of IL-31 inhibition to prevent acute canine AD skin barrier dysfunction.

There were several limitations to this study. The first limitation was the small sample size. Although we determined the number of dogs based on the sample size calculation estimated from our previous study, we observed more individual variability than expected in this experiment. Therefore, we might have missed herein some significant differences in gene expression. This experimental dog model was an inbred line of laboratory Maltese-beagle dogs whose earliest ancestors about 25 years ago were diagnosed as having AD. Due to the unique

characteristics of this colony, we only had eight descendants at the time of our study and could not easily have a larger sample size.

The second limitation of this study was a lower expression of *IL31* mRNA detected by RNA-Seq in these samples. This was in marked contrast with an earlier publication using dogs from this colony, which reported *IL31* to be the highest up-regulated gene after HDM application.[17] In this study, the *IL31* mRNA expression increased about 24 times at 24 hours compared to that at baseline (0 hour), but the median TPM indicated that it was not actively expressed throughout the study period. If IL-31 was not actively produced in the skin of our dogs in this study, we would not be able to appropriately compare the transcriptome profiling with or without IL-31 inhibition by LKV. However, we still believe that IL-31 was produced in the skin of our dogs and that the LKV pretreatment inhibited the IL-31 pathway since the highest number of DEGs in the LKV group was seen at 48 hours, which was consistent with the highest median *IL31* mRNA expression of all samples (combining both LKV and NTC groups). There were several possibilities for the low expression of *IL31* mRNA in this study. One possibility was a very transient expression followed by the fast degradation of *IL31* mRNA, which would have prevented its detection despite multiple sampling times. This possible transient expression and fast degradation of canine *IL31* mRNA was also suspected in our previous study in which we found a low percentage of *IL31* mRNA positivity in contrast to the relatively high percentage of IL-31 immunoreactivity in the same canine PBMC sample (see Chapter 4 above). Furthermore, another possibility for our low detection was an insufficient accuracy of the *IL31* sequence in the canine reference genome. The reference sequence (RefSeq) status of canine *IL31* is currently "provisional" in contrast to the "validated" status in human and mouse *IL31*. Thus, it was possible that some reads were not mapped to the *IL31* sequence resulting in the underestimation of its expression. Ideally, other methods, for example, qPCR for *IL31* mRNA expression and western blotting or immunostaining for IL-31 protein production in the skin samples, will be performed to better characterize the transcription and translation of *IL31* in our samples.

Finally, the skin samples usually collected in this canine AD model only represent the induction of the earliest stages of acute flares of AD (Figure 7). As a result, we could not assess the role of IL-31 and the complete cytokine milieu that would exist in fully-developed or chronic AD skin lesions. Unfortunately, dogs with AD are usually presented to their veterinarians when they have experienced clinical signs for days to weeks after their onset, markedly later than what

this experimental AD skin model represents. Therefore, as the cytokine milieu is known to change over time as AD skin lesions progress, the targets to supplement LKV treatment, which we identified herein, might no longer play a central role in the skin of those patients with chronic AD. Still, our model mirrored the intermittent acute AD relapses that patients experience during the waxing-and-waning characteristic evolution of this disease. Therefore, targeting one or more of our proposed cytokines proactively (i.e., before lesions develop) rather than reactively (i.e., after they have fully developed) should help to prevent AD flares and, at least theoretically, inhibit or delay the initiation of the cytokine cascade and inflammation that is known to occasionally arise when treating with uniquely targeted therapies such as LKV. Furthermore, it is interesting to note that *IL31*-deficient mice exposed to chronic topical HDM showed significantly increased rather than decreased numbers of dermal-infiltrating IFNγ, IL-4, and IL-13-producing cells compared to those in wild type mice, indicating that IL-31 actually might function as a negative regulator of Th1 and Th2 inflammation in chronic AD.[46] Therefore, this also could explain some AD flares while the patients continued on LKV injections.

5.5 Conclusion

In conclusion, in the experimental acute AD skin lesions of dogs, we demonstrated that the IL-31 pathway appears to be independent, or downstream, of major relevant cytokine groups (Proinflammatory, Th1, Th2, Th9, Th17 and Th22). We identified IL-33, IL-13, CCL17 and IL-9, and potentially IL-6 and CCL22, possibly responsible for acute flares of AD skin inflammation when receiving IL-31-blocking treatment with LKV. The inhibition of these cytokines or their receptors—especially in a proactive manner—to help prevent canine AD flares is deserving of further study.

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CHAPTER 6

CONCLUSIONS AND FEATURE DIRECTIONS

In humans and dogs, atopic dermatitis (AD) is the most common, currently incurable, chronic allergic skin disease that has a debilitating effect on patients and their "families" worldwide. Among the various endogenous and exogenous pruritogens known to be relevant to AD, interleukin-31 (IL-31) appears to play one of the most critical roles. Targeting the IL-31 or IL-31 receptor with monoclonal antibodies (mAbs) has shown favorable outcomes in AD human and canine patients. (reviewed in Chapter 1). However, there are also occasional failures of disease control with IL-31 inhibition, indicating that there are other mediators, beyond IL-31, that are released in acute AD flares. The overall objectives of our study were to identify additional therapeutic targets to optimize the strategy of IL-31-blockage therapy.

Our first study that tested the ability of IL-31-inhibition to prevent acute AD flares with the proactive administration of the anti-canine IL-31 mAb, lokivetmab (LKV), revealed an insufficient effect of LKV in preventing skin lesion development in our experimental canine AD model and flares of AD in some dogs with spontaneous AD, all of this despite having a remarkably strong preventive effect on atopic itch. This observation led to the hypothesis that IL-31 only has a minor role in skin lesion development in contrast to its central role in atopic itch, which might explain the insufficient effect of our strategies for inhibiting IL-31 to delay disease flares in some dogs with spontaneous AD. Leaving atopic inflammation flaring without control eventually will rapidly lead to the release of mediators different from IL-31 that will no longer be inhibited by LKV. Furthermore, the tendency for cells to mutually activate each other and for some cytokines (e.g., TSLP-periostin and IL-31) to self-and-mutually amplify their secretion highlights the critical need for early control of cutaneous atopic inflammation.

To have a better understanding of the role of IL-31 in the development of canine AD skin lesions and to optimize the strategy in IL-31-blocking therapy, we then assessed the chronology of the expression of IL-31 and determined the identity of cells producing IL-31 in acute AD skin lesions of dogs (see Chapter 3). In our experimental canine acute AD model, we demonstrated the early and transient production of IL-31 by Th2 cells in acute lesional AD skin, supporting the concept of using IL-31 inhibiting LKV as a proactive therapy to prevent the development of

acute AD skin lesions or itch, as we had already shown in the first study. Unexpectedly, sebaceous glands were strongly immunolabeled with IL-31; however, we could not detect *IL31* mRNA in any sebaceous glands using the RNAScope method, and, thus could not yet confirm these cells as representing another novel cellular source of IL-31 (see Chapter 4).

An insufficient effect of IL-31 inhibition on preventing skin lesion development in our experimental canine AD model and on delaying AD flares in some dogs with spontaneous AD suggested that other cytokines, independent or upstream of IL-31, could be responsible for the development of acute atopic inflammation and could contribute to the treatment failures after IL-31-inhibiting therapy. Our final set of studies compared the cytokine transcriptome profiling in acute lesional AD skin, with or without IL-31 inhibition by LKV. Our results suggested that some important cytokines (i.e., pro-inflammatory, Th1, Th2, Th9, Th17, Th22, and Treg cytokines) acted independently or upstream of the IL-31-dependent pathway in the induction of acute AD skin inflammation. Therefore, these IL-31-independent cytokines, particularly IL-6, IL-33, IL-13, CCL17, CCL22 and IL-9 could be valuable targets for inhibition to supplement LKV therapy.

The research performed as part of this doctoral degree prompted future studies on testing the effect of inhibiting the candidate cytokines mentioned above for the proactive treatment in canine AD, that is, to prevent the apparition of flares. Among these cytokines, there is strong evidence in human AD that therapeutic mAbs targeting IL-33 and IL-13 show favorable outcomes in human AD, with dupilumab (an anti-IL-4 and IL-13 receptor mAb) being the first biologic medication approved for the treatment in human AD.[1,2] Considering that human and canine AD share many characteristics, these cytokines are likely to be the next very promising targets to treat or prevent canine AD. In recent years, mAb engineering has dramatically evolved and became more assessable.[3] Our subsequent interest would be generating therapeutic mAbs targeting canine IL-33 and IL-13 to test their ability in preventing skin lesion development in an experimental canine AD model followed by clinical trials with dogs with spontaneous AD. One could, of course, envision the creation of dual- or bi-targeted mAbs, having each Fab chain targeting two atopic cytokines, one for itch (obviously IL-31) and one for inflammation (IL-13 or IL-33).

One of the questions that remains to be answered is whether there are any other cellular sources of IL-31 in dogs, particularly sebaceous glands, since it might provide new insights and

additional treatment strategies, such as more aggressive topical therapy targeting IL-31 in the sebum, in dogs with AD. We are considering testing other approaches to investigate the possible IL-31 production by sebaceous glands. For example, we will continue to try to detect *IL31* mRNA or protein using a sebaceous gland cell line, which we attempted with the culture of primary sebaceous gland cells but failed due to bacterial contamination likely coming from the hair follicles (data not shown). Another approach would be to detect IL-31 protein in the skin surface lipid-containing sebum by chromatography and mass spectrometry or SiMoA assay. It is needless to say that we will also test other in situ hybridization variants using different probes for *IL31* different from those used in the RNAscope.

Last but not least, we want to emphasize again that our experimental canine AD model only represents the induction of the acute phase of AD (that is, the first days of an AD flare) and thus cannot be translatable to the immunological changes that occur in, and for the treatment of dogs with chronic AD. Unfortunately, there is no established canine model that fully and reliably represents chronic canine AD. Therefore, further studies are warranted to investigate the role of IL-31 and cytokine milieu in the skin of client-owned dogs with chronic AD, even though there would be more individual variabilities compared to an experimental setting. Nevertheless, such studies might reveal different roles of IL-31 and other therapeutic targets in canine AD.

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