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Research paper

Allergen-induced production of IL-31 by canine Th2 cells and identification of immune, skin, and neuronal target cells☆

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ABSTRACT

The canine cytokine IL-31 induces pruritus in dogs and can be detected in dogs with atopic dermatitis; however very little is understood around its interactions with specific canine cells. We hypothesize that IL-31 is involved in the progression of allergic skin disease by coordinating the interaction between the immune system with skin and neuronal systems. The goal of the following work was to identify cells that produce IL-31 as well as cells that may respond to this cytokine. Peripheral blood mononuclear cells (PBMCs) were collected from naïve and house dust mite (HDM) allergen-sensitized beagle dogs and used for *ex vivo* characterization of cytokine production assessed using ELISpot and quantitative immunoassay. Sensitization to HDM allergen induced a T-helper type 2 (Th2) cell phenotype characterized by an increase in the production of IL-4 protein. Interestingly, repeated allergen challenge over time also resulted in an increase in IFN-γ. Further evaluation showed that co-stimulation of Th2 polarized cells with antigen and the bacterial component *Staphylococcus* enterotoxin B (SEB) produced higher levels of IL-31 compared to either stimulant alone. Production of IL-31 when PBMCs were stimulated by T cell mitogens suggests T cells as a source of IL-31. Quantitative real-time PCR was utilized to determine expression of the IL-31 receptor alpha chain in canine cell lines and tissue. Canine monocytic cells, keratinocytes, and dorsal root ganglia were shown to express the IL-31 receptor alpha chain mRNA. In a multifaceted disease such as canine atopic dermatitis, the combination of Th2 polarization and microbial presence may lead to IL-31 mediated effects driving inflammation and pruritus by immune cells, keratinocytes, and direct neuronal stimulation.

1. Introduction

Canine atopic dermatitis (AD) is a common allergic skin disease characterized by pruritus and inflammation. AD is genetically pre-disposed and results from dysfunction in the skin and immune system (DeBoer, 2004). Skin abnormalities in the ultrastructure, stratum corneum, and barrier proteins have been identified in dogs with clinical disease (Olivry, 2011). Imbalance in the immune response characterized by increased levels of T helper type 2 (Th2) cytokines including IL-4, IL-5, and IL-13 (Hayashiya et al., 2002; Maeda et al., 2009; Nuttall et al., 2002b; Schlotter et al., 2011) is also associated with AD. The role for the Th1 cytokine IFN-γ has been less clear although it has been detected in both lesional skin and peripheral blood mononuclear cells (PBMCs) from dogs with AD (Nuttall et al., 2002a; Stehle et al., 2010). Interaction between the skin and immune system are highlighted in studies demonstrating that canine keratinocytes can respond to both cytokine and allergen challenge with the production

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of inflammatory mediators such as GM-CSF (Kimura et al., 2012; Maeda et al., 2009).

IL-31 is a relatively novel cytokine that when over-expressed in transgenic mice leads to the recapitulation of many of the hallmark signs of AD including inflammatory infiltrate in the skin and pruritus (Dillon et al., 2004). In human AD, IL-31 levels correlate with disease severity and have been shown to be increased in both the skin and serum (Ezzat et al., 2011; Kim et al., 2011b), and reports suggest that Th2 cells and skin-homing T cells are sources of IL-31 (Bilsborough et al., 2006; Gutzmer et al., 2009). Initial efforts to clone canine IL-31 resulted in the detection of IL-31 mRNA in mitogen-activated PBMCs and various tissues (Mizuno et al., 2009). Recently canine IL-31 was shown to rapidly induce pruritus when directly administered to dogs (Gonzales et al., 2013). In addition IL-31 was detected in the serum of dogs with atopic dermatitis but not in healthy controls (Gonzales et al., 2013).

IL-31 receptors have been detected on a variety of cell types including primary human CD1c(+) cells, human monocyte-derived dentritic cells, human macrophages, keratinocytes, eosinophils, mast cells, bronchial epithelia, colonic subepithelial myofibroblasts, and human as well as rat dorsal root ganglion tissue (Cornelissen et al., 2012). Some investigators have also reported that IL-31 receptor expression is induced by the pro-inflammatory cytokine IFN-γ (Diveu et al., 2003; Heise et al., 2009; Kasraie et al., 2011), suggesting that the IL-31 cytokine and its receptor system may provide positive feedback to drive inflammation in non-immune tissues to lead to conditions like inflammatory skin disease.

The objective of the following work was to test the hypothesis that IL-31 is involved in the propagation of atopic disease in dogs *via* coordinating an interaction between the immune system, skin and neuronal systems. To address this, we investigated the polarization of immune cells before and after allergen sensitization as well as during allergen re-challenge over time and characterized IL-31 production. In order to determine cell types that may be targets of IL-31 activity, we tested monocytic cells, keratinocytes, and neuronal tissue for mRNA expression of the IL-31 receptor alpha chain.

2. Materials and methods

2.1. Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (Zoetis, Inc., Kalamazoo, MI, USA) and were performed in compliance with the Animal Welfare Act, Regulations, 9 CFR Parts 1, 2 and 3, and with the Guide for the Care and Use of Laboratory Animals Eighth Edition, issued by the US Institute for Laboratory Animal Research Commission of Life Sciences (National Academies Press, Washington, DC, 2011). Purpose-bred beagle dogs (Marshall BioResources, North Rose, NY, USA) were used for all experimental work. Samples were collected from normal or house dust mite (HDM)-sensitized dogs. In brief, HDM-sensitization consisted of a series of three injections of antigen (10 μg, Greer Laboratories, Lenoir, NC, USA) and Rehydragel (0.1 mL, Reheis Inc, Berkley Heights, NJ, USA) at two week intervals. The same allergen formulation was used for any re-exposures subsequent to the initial sensitization. Sensitization was confirmed by intradermal skin testing. Whole blood samples were collected by venopuncture. Dorsal root ganglia (DRG) tissue samples were collected from non-HDM-sensitized dogs that were euthanized for purposes unrelated to this study. Immediately post-mortem, tissue was snap-frozen and stored at −80 °C until RNA processing was completed at a later time.

2.2. Cell isolation, culture, and treatment

Fresh whole blood samples were collected into lithium heparin tubes, diluted with PBS and overlayed onto prepared AccuSpin tubes (Sigma–Aldrich, St. Louis, MO, USA) for PBMC isolation. Samples were spun at 800 × *g* for 30 min at room temperature with no brake, collected, washed twice and resuspended in culture media. Monocytic cells (DH82, ATCC, Manassas, VA, USA) were grown in MEM-based media containing 15% FBS. Canine progenitor epithelial keratinocytes (CPEKs, ZenBio, Research Triangle Park, NC, USA) were grown in Epidermal Keratinocyte-based media (ZenBio, Research Triangle Park, NC, USA) containing 10% FBS. Cytokines were purchased (canine IFN-γ, R&D Systems, Minneapolis, MN, USA) or generated by our group (Gonzales et al., 2013) (canine IL-31). For mitogen stimulation experiments, 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich, St. Louis, MO, USA) plus 2 μM ionomycine (Sigma–Aldrich, St. Louis, MO, USA), 100 ng/mL lipopolysaccharide (LPS, Sigma–Aldrich, St. Louis, MO, USA) or 5 μg/mL concanavalin A (ConA, Sigma–Aldrich, St. Louis, MO, USA) were incubated with PBMCs overnight at 37 °C. The following day supernatants were collected and assessed for cytokine production.

2.3. ELISpot

PBMCs isolated from HDM-sensitized dogs were incubated on pre-coated IFN-γ and IL-4 ELISpot plates (R&D Systems, Minneapolis, MN, USA) in AIM-V AlbuMax serum free medium (Invitrogen, Grand Island, NY, USA) together with 10 μg/mL HDM antigen (Greer Laboratories, Lenoir, NC, USA) for 24 h at 37 °C then removed by washing. Biotinylated detection antibody was added and plates incubated overnight at 4 °C. Alkaline phosphatase conjugated streptavidin was then added followed by substrate. The number of IFN-γ or IL-4 secreting cells (spots) was quantified using an AID ELISpot reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

2.4. IL-31

Isolated PBMCs were resuspended in RPMI-1640 complete media containing 10% FBS and cultured in the presence of HDM antigen at 10 μg/mL and *Staphylococcus* enterotoxin B (SEB) (Sigma–Aldrich, St. Louis, MO, USA) at 0.10 μg/mL for 96 hours at 37 °C. A GyroLab sandwich immunoassay was used to quantitate canine IL-31 levels in cell culture media supernatant as previously described (Gonzales et al., 2013). For IL-31Rα chain Q-RT-PCR, RNA

Table 1
Primer and probe sequences for of IL-31Rα and GAPDH (control).

Gene	Reagent	5′–3′ sequence
IL-31RA	Forward primer	ATGGATGCTCCTTCTACTCTGTAAACT
	Reverse primer	CAGGAAATGTTCTCAGGCTTAGC
	Probe	AGCCTGGCAGTTCT
GAPDH	Forward primer	GGCACAGTCAAGGCTGAGAAC
	Reverse primer	CCAGCATCACCCCATTTGAT
	Probe	TCCAGGAGCGAGATC

was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions from cells treated with 10 ng/mL IFN-γ (to up-regulate IL-31 receptor expression, R&D Systems Minneapolis, MN, USA) and untreated controls or from frozen dorsal root ganglia disrupted using a mortar and pestle. RNA was quantitated using a Nano Drop 8000 (Thermo Scientific, Waltham, MA, USA) and quality/integrity was assess using a 2100 Bioanalyzer (Agilent Technologies, Fort Worth, TX, USA). PCR reactions were run in a total volume of 20 μL using 500 ng of RNA with the SuperScript III Platinum One-Step (Invitrogen, Grand Island, NY, USA) system according to manufacturer's instructions. Samples were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and normalized to the housekeeping gene GAPDH (Saliaris et al., 2007). Relative quantitation was calculated by standard method. In brief, the values used to express fold increase are generated by ($2^{\hat{}}$(-treatment ΔCt – control ΔCt)) where ΔCt = Ct IL-31RA – Ct GAPDH (Table 1).

3. Results

3.1. Allergen sensitization induces a Th2 polarized phenotype

PBMCs from allergen-sensitized dogs were collected and frozen at both pre- and post-HDM sensitization for simultaneous analysis of cytokine production in an ELISpot assay using IFN-γ protein as an indicator of Th1 polarization and IL-4 protein as an indicator of Th2 polarization. The dramatic increase in HDM-induced IL-4 production (Fig. 1A and B) suggests that sensitization with HDM induces a Th2 phenotype. The alteration in polarization was very consistent (Fig. 1C and Supplementary Table 1) and repeatable in two entirely separate experiments.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.10.017.

3.2. Polarization of cells changes upon re-exposure to allergen

In order to further characterize the polarization of PBMCs collected from dogs chronically sensitized to allergen, dogs were initially sensitized to allergen with three injections two weeks apart and then re-exposed to allergen three more times at approximately 1 month intervals after the initial sensitization regime (Fig. 2, arrows indicate allergen exposure). After re-exposure, IL-4 remained elevated and IFN-γ expression increased. Dogs then remained unchallenged, and cytokine production was continually measured. As time from challenge increased, the response to allergen stimulation decreased for both IL-4 and IFN-γ (Fig. 2 days 201 and 242). As may be expected based on previous experimental data and clinical presentation, re-challenge with allergen induced increased cytokine production including IL-4 and interestingly IFN-γ as well (Fig. 2 day 291).

3.3. Th2 polarized cells produce IL-31 in response to HDM and SEB

In order to identify which cell types may be capable of IL-31 production, naïve PBMCs were treated with a variety of immune stimulating agents including LPS, ConA, and

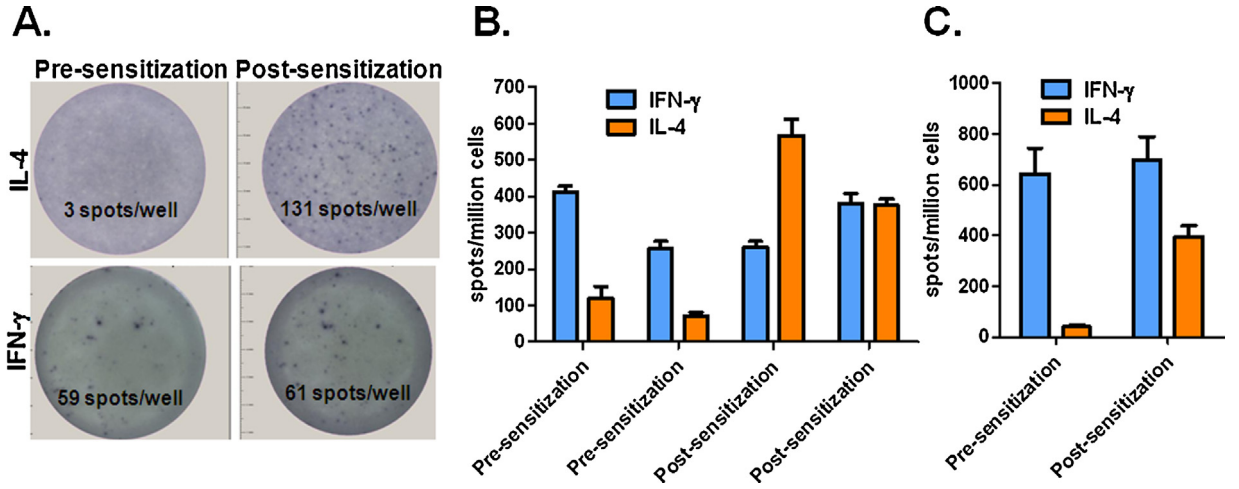

Fig. 1. ELISpot analysis of IFN-γ and IL-4 production by PBMCs isolated from dogs pre- and post-HDM allergen sensitization showing Th2-polarization. (A) Representative images from ELISpot wells from pre- (week −1) and post- (week 5) sensitization are shown. (B) Analysis at two independent times before (week −1 and day 0) and after (weeks 5 and 7) sensitization each from a single individual is shown. (C) Average cytokine production pre- (week −1) and post- (week 5) sensitization where $n = 30$.

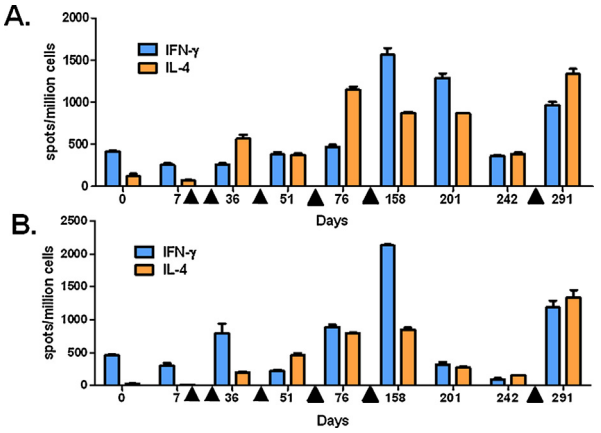

Fig. 2. Cytokine response to allergen stimulation of PBMCs over time shown in days. Pre-sensitization time points are days 0 and 7. Arrowheads indicate administration of allergen. Data from two different individuals (A and B) are shown.

PMA plus ionomycin, and cell supernatants were analyzed for IL-31. The mitogen that induced the most IL-31 production compared to any other treatment group tested was PMA plus ionomycin (Fig. 3A).

To further characterize the cellular source of IL-31, cells were collected both pre- and post-sensitization to represent a Th1 and Th2 phenotype, respectively. Since mitogen-activation of T cells can be considered artificial, we wanted to build an *ex vivo* system relevant to assessing a more natural scenario in which IL-31 may be produced. To accomplish this, we used the Th2-polarized (characterized as above by expression of the Th2 representative cytokine IL-4) HDM-specific PBMCs, and cultured them in the presence of HDM and/or *Staphylococcus* entertoxin B (SEB) and found these conditions resulted in the synergistic production of IL-31 (Fig. 3B).

3.4. Monocytic cells, keratinocytes, and neurons express IL-31Rα

Canine monocytic cells (DH82) where examined by quantitative-real time PCR (Q-RT-PCR) for expression of the IL-31Rα chain. Although the IL-31Rα mRNA was detectable in naive cells, treatment with IFN-γ resulted in a 10-fold increase in IL-31Rα message (Fig. 4A). Under resting conditions, canine epidermal keratinocytes (CPEKs) had no detectable IL-31Rα mRNA (data not shown) but interestingly activation with IFN-γ resulted in expression of IL-31Rα transcript (Fig. 4B). A relative comparison of CPEK IL-31Rα message levels indicates that under these conditions, CPEKs (activated with IFN-γ) still express approximately 3 log-fold less IL-31Rα compared to inactivated monocytic cells (Fig. 4B). Q-RT-PCR analysis of DRG tissue revealed expression of the IL-31Rα in four healthy dogs (Fig. 4B). Interestingly, the level of IL-31Rα transcript expressed by inactivated monocytic cells and DGR tissue is remarkably similar (Fig. 4B).

4. Discussion

Atopic dermatitis is a multifactorial disease with involvement of multiple tissues including a diverse range of cell types and commonly associates with bacterial infections (DeBoer, 2004). Like many allergic conditions, AD is characterized as a Th2 polarized disease (Marsella and Girolomoni, 2009). Much of the previous work to date has investigated the Th1 and Th2 hallmark cytokines including IL-4 by evaluating messenger RNA in clinical samples and in established models of canine allergy (Hayashiya et al., 2002; Marsella et al., 2005, 2006). Data from the current study is in agreement with previous reports of dogs with AD having increased IL-4 mRNA levels (Hayashiya et al., 2002; Nuttall et al., 2002b; Olivry et al., 1999) and builds on this body of work with the demonstration of cytokine protein levels increasing after sensitization to HDM, a common canine allergen (Kim et al., 2011a; Masuda et al., 2000). The Th2 polarization in this model is clear with almost no IL-4 production pre-sensitization followed by a distinct increase post-sensitization. Although a Th2 imbalance is widely associated with atopic dermatitis there have been reports of IFN-γ expression in lesional skin samples and data suggesting that IFN-γ expression may be associated with a chronic disease state (Nuttall et al., 2002a; Olivry et al., 1999), similarly to what is seen in the human disease (Hamid et al., 1994; Thepen et al., 1996). Although our data is generated using PBMCs as opposed to skin, it is consistent with previous studies demonstrating increased IFN-γ production during what may be characterized as the chronic

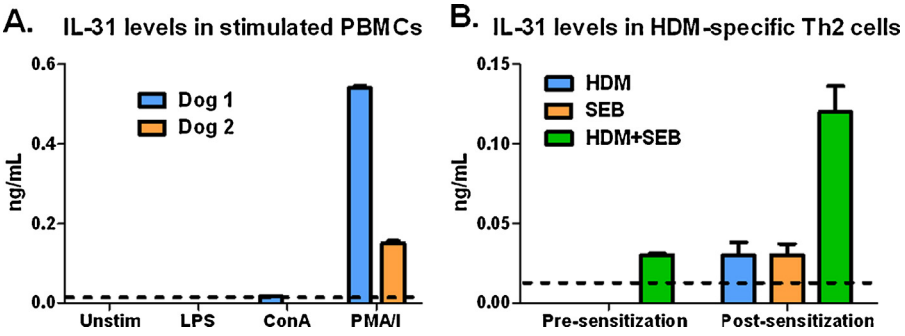

Fig. 3. Treatment of normal PBMCs with various immunostimulants induces production of IL-31. (A) Treatments show differential stimulation of IL-31 cytokine. (B) IL-31 production is associated with allergen-sensitized Th2 polarized PBMCs and is increased upon HDM and SEB treatment. Dashed line indicates assay limit of IL-31 detection.

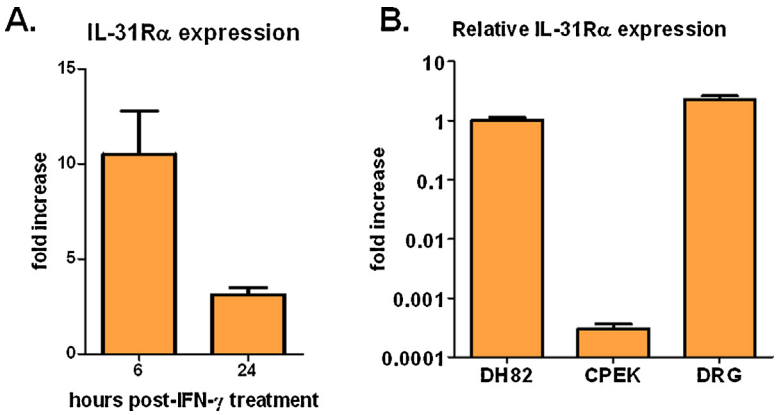

Fig. 4. Fold increase in IL-31 receptor expression as assessed by Q-RT-PCR. (A) IL-31Rα mRNA in monocytic cells (DH82) at six and 24 hours post-treatment with IFN-γ. (B) Relative expression of IL-31Rα mRNA in keratinocyte progenitor cells (CPEK) treated with IFN-γ (24 hours) and RNA isolated from dorsal root ganglia (DRG) tissue compared to untreated DH82s where $n = 3$.

phase of the disease model. These results suggest that in this model there is a clear Th2 response to allergen but also involvement of Th1 cytokines and supports the hypothesis that IFN-γ is associated with a chronic disease state in dogs.

Based on the ability of PMA to activate protein kinase C downstream of the T cell receptor (Berry et al., 1989; Chatila and Geha, 1988) and the lack of IL-31 production from the monocyte-activator LPS, the data in this study suggests that canine T cells are a source of IL-31. Under more physiologically relevant conditions using PBMCs isolated from HDM-sensitized dogs, we have seen that IL-31 is produced after exposure of Th2 polarized cells to allergen and bacterial endotoxin. The polarization status of the cells appears to be an important characteristic associated with IL-31 production as cells collected from dogs pre-sensitization have significantly reduced cytokine production compared to post-sensitization. To confirm polarized T cells as the source of IL-31, cell-type specific characterization along with isolation or intracellular labeling techniques within the polarized PBMCs cultures would be needed. Concurrent staphylococcal infections are common in dogs with atopic dermatitis (DeBoer and Marsella, 2001) and colonization with *Staphylococcus pseudintermedius* is more prevalent in dogs with AD compared to healthy controls (Fazakerley et al., 2009). *Staphylococcus aureus* has been shown to promote the production of Th2 cytokines by PBMCs from humans with AD (Dillon et al., 2004; Lin et al., 2011) including IL-31 (Niebuhr et al., 2011; Sonkoly et al., 2006). Importantly, recent studies have demonstrated that IL-31 is detected in dogs with AD but not non-diseased animals (Gonzales et al., 2013).

Based on the IFN-γ production we saw in the characterization of the HDM allergen model over time, we were interested in further investigating the involvement of this Th1 cytokine in a classically Th2 disease. The IL-31 receptor has been characterized as heterodimic consisting of the OSMR chain and the IL-31 receptor alpha chain (IL-31Rα) (Dillon et al., 2004). Studies have shown that while both receptor chains are required to induce signaling, the IL-31Rα has the predominant interaction with the cytokine (Diveu et al., 2004). Data from the current study indicate that although the production of IL-31 came from polarized cultures exhibiting a Th2-cell phenotype, the ability to respond to the cytokine, *i.e.* receptor expression, may depend at least in part on Th1 cytokine presence since IFN-γ was required to induce IL-31Rα gene expression in cells. Additionally our results showed that IFN-γ increased expression of the IL-31Rα, which is in agreement with previous findings that IFN-γ can increase expression if the IL-31 receptor in human monocytes (Diveu et al., 2003) and skin-resident cells including keratinocytes (Heise et al., 2009; Kasraie et al., 2011). Recent data from our group confirms that IFN-γ induced the presence of functional IL-31 receptor on canine monocytic cells by causing IL-31-mediated phosphorylation of STAT3 and ERK1/2 (Gonzales et al., 2013). Although receptor expression implicates both monocytes and keratinocytes as having the potential ability to respond to IL-31, additional studies are needed to confirm and identify the physiological effect.

IL-31Rα mRNA was also detected in DRG tissue. DRG are neuronal cells involved in the transition of the pruritic signal to the spinal cord (Buddenkotte and Steinhoff, 2010) and have been shown to express relatively high levels of classically hematopoietic receptors including the H4 histamine receptor (Strakhova et al., 2009) as well as the IL-31 receptor (Bando et al., 2006). These data imply that neurons may be able to directly respond to IL-31. There is evidence to support an IL-31 mediated direct effect on neuronal cells as exogenous administration of IL-31 induces pruritus in dogs within two hours (Gonzales et al., 2013). Although reports have shown upregulation of cytokines and other inflammatory mediators post-treatment with IL-31 (Cornelissen et al., 2012; Zhang et al., 2008), physiological effects seen in such a short time frame may be the result of direct neuronal action.

Taken together, these data suggest a dual-involvement of both Th1 and Th2 cytokines in responses in canine allergy. An important role for IL-31 in pruritus has been suggested in human AD (Ezzat et al., 2011; Raap et al., 2008; Sonkoly et al., 2006) and most recently characterized in canine AD (Gonzales et al., 2013). Expression of the IL-31 receptor on not only monocytic cells, but also keratinocytes

and DRG tissue suggest that this cytokine may mediate dialog between the immune system and the peripheral organ sites. The results of the current study identify stimulated Th2 polarized cells as a source of canine IL-31 and suggest disease-relevant tissues as the target. This data builds on growing understanding of the mechanism of action of IL-31 in canine AD and highlights the complexity and breadth of action that this cytokine may have in orchestrating cellular changes within the animal and inducing clinical signs associated with allergic skin disease in dogs.

Conflict of interest

All authors are employees of Zoetis Inc.

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