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SHORT COMMUNICATION

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Preparation of anti-canine interleukin-31 receptor alpha polyclonal antibody and evaluation of its therapeutic effect in canine atopic dermatitis

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Abstract

Canine atopic dermatitis (CAD) is a prevalent genetically susceptible inflammatory and pruritic allergic skin condition affecting not only the health of dogs but also the quality of life of their owners. Interleukin-31 (IL-31) and interleukin-31 receptor alpha (IL-31RA) are essential for the development of pruritus in primates and mice. Hence, it is expected that inhibiting IL-31RA will be an effective approach to alleviate pruritus. The purpose of the study was to produce anti-canine IL-31RA polyclonal antibodies (anti-IL-31RA pAbs) and evaluate their efficacy in inhibiting house dust mite (HDM)-evoked pruritic responses. Dogs were immunized with antigens formed by IL-31RA recombinant short peptides coupled to BSA to produce anti-IL-31RA pAbs. The CAD model was developed by using HDM allergen stimulation, and the effects of IL-31RA pAbs on the reduction of pruritus in CAD model dogs were examined. The Canine Atopic Dermatitis Extent and Severity Index (CADESI)-4 and pruritus Visual Analog Scale (pVAS) were utilized to evaluate pruritic responses, and skin tissue samples were collected from the inguinal area for pathological assessment of skin inflammatory cell infiltration. The results showed that anti-IL-31RA pAbs with high titers (1:128,000) and specificity were effectively produced. In the CAD model group, the severity of skin damage, pruritus score, inflammatory cell infiltration and level of inflammatory factors were considerably elevated. Anti-IL-31RA pAbs relieved pruritic behavior and dermatitis in dogs compared to placebo-treated dogs. In conclusion, anti-IL-31RA pAbs effectively suppressed CAD in vivo and are anticipated to be an effective novel treatment for pruritic skin disorders such as CAD.

Keywords IL-31RA, Atopic dermatitis, Polyclonal antibody, Canine

Main text

Atopic dermatitis (AD), which is characterized by acute itching and recurring eczematous lesions, is one of the most prevalent allergic skin conditions in humans and dogs. Approximately 10% of dogs suffer from canine atopic dermatitis (CAD) (Hillier and Griffin 2001). CAD can lead to hair loss, dry skin, itchy skin and consequent skin lesions and infections caused by scratching (Bradley et al. 2016). Accompanied by itching, CAD may pose a significant threat to the well-being of dogs. CAD is a chronic and common inflammatory skin disease with complicated pathogenesis that is easily confused with other skin diseases, making it more difficult to treat (Gedon and Mueller 2018). Current pharmacological treatments for AD focus on reducing itch behavior and thus alleviating skin damage. For mild AD, emollients and low potency topical steroids can achieve satisfactory

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results. However, for moderate to severe AD, combined with the chronic and long-term nature of AD, these drugs can produce immunosuppressive activity (Chun and Lehman 2020; Frazier and Bhardwaj 2020; Patrizi et al. 2015). Long-term use of hormones may produce side effects such as excessive drinking and urination and may also affect the function of the animal liver and disrupt the endocrine system. Gadeyne investigated the efficacy of a glucocorticoid (prednisolone) and a Janus kinase inhibitor (hyoscine) in the treatment of AD. During therapy with both medications, they observed that several dogs had aberrant clinical symptoms, including vomiting, diarrhea, pyoderma, and otitis externa. Consequently, developing alternate strategies for alleviating AD is essential (Gadeyne et al. 2014).

Interactions between the nervous and immunological systems produce pruritus. T-cell secretory factors are associated with several inflammatory pruritic skin conditions, including AD (Cevikbas et al. 2014). Important to the development of AD is an imbalance in the Th1/Th2 differentiation of CD4+ T cells, which results in aberrant cytokine production (Czarnowicki et al. 2015). Interleukin-31 (IL-31), a four-helix bundle cytokine and a member of the IL-6 cytokine family, can activate the glycoprotein 130 (gp130) receptor subunit and further regulate the immunological response, cell proliferation, and inflammation (Datsi et al. 2021). IL-31, the primary component responsible for pruritus in AD, is generated preferentially but not solely by Th2 cells and plays a vital role in AD (Kuzumi et al. 2021). Transgenic mice that overexpressed IL-31 exhibited severe pruritus and skin lesions (Dillon et al. 2004). The mice that were injected intravenously with a single dose of IL-31 exhibited the most severe scratching behavior, and investigation has indicated that IL-31 generates a dose-dependent response to grasping (Arai et al. 2013). Moreover, binding of IL-31 to the receptor complex activates several signaling pathways, including multiple MAPK pathways (ERK, p38, and JNK) and JAK1/JAK2 and PI3K/AKT signaling pathways (Zhang et al. 2008). Activated pathways further release inflammatory mediators producing itching, triggering scratching and increasing skin breaking, contributing to the vicious cycle of pruritus-scratch (Koblenzer 1999).

Interleukin-31 receptor alpha (IL-31RA) is a subunit of the IL-31 receptor, and the only known ligand of IL-31RA is IL-31. In addition to epithelial cells and immunological cells, cutaneous neural cells and dorsal root ganglia (DRG) express IL-31RA (Zhang et al. 2008). By activating the heterodimeric receptor IL31RA/Oncostatin M receptor (OSMRβ), IL-31 induces neuronal activation and pruritus (Bağci and Ruzicka 2018; Raap et al. 2017). An anti-mouse IL-31RA neutralizing antibody, BM095, was produced for the treatment of the murine AD model, and a significant reduction in ear swelling and skin inflammation was observed (Kasutani et al. 2014). According to a recent study, blocking IL-31RA signaling was an effective therapeutic target for AD (Boguniewicz 2017).

Owing to the complex pathogenesis of CAD and its adverse impact on the quality of life of dogs, there is an unmet medical need for safe and effective treatments for AD. Cytopoint, an anti-canine IL-31 neutralizing monoclonal antibody produced by Zoetis, can exert a strong antipruritic effect. However, it is unknown whether the anti-canine IL-31RA antibody can effectively treat AD in dogs, and the rationale for its safety and tolerability is unknown. This study was designed to evaluate the effectiveness of anti-canine IL-31RA polyclonal antibodies (anti-IL-31RA pAbs), which we produced, in inhibiting the pruritic response in AD dogs.

Assessment of CAD model

To comprehend the pathogenesis and treatment of CAD, animal models are essential for clinical trials. AD models can be classified into three types based on the modeling approach and trigger cause, including spontaneous animal models, gene-edited animal models, and sensitizer-induced animal models (Hansson et al. 2002; Huang et al. 2003; Matsuda et al. 1997). The third sensitizer-induced model is widely applied in scientific research due to its simplicity, stability and low input cost. Most frequently, CAD is associated with allergen specific, allergen-mediated sensitization, particularly to house dust mites (HDMs). Notably, considerable experiments have confirmed that HDM causes characteristics such as natural CAD. In addition, the dogs were epidermally sensitized to replicate natural cases (Leung et al. 2003; Pierezan et al. 2016; Pucheu-Haston et al. 2008). Using the methodology of Marsella's CAD model, we screened dogs with high levels of lgE to be coated with HDM for modeling (Marsella et al. 2006). To verify the accuracy of the CAD model, the skin thickness and pruritus score were determined. As shown in Fig. 1A, profound erythema, papules, and bleeding were noted in the groin and armpit of CAD model dogs. Compared with saline-treated dogs, CAD-affected dogs scored higher on the CADESI-4 (Fig. 1B). After application of HDM at week 12, scratching behavior was observed, and the pruritic scores of CAD dogs were higher than those of saline-exposed dogs (Fig. 1C). The inguinal skin of dogs in the CAD model was substantially thicker than that in the control group (Fig. 1D). In addition, compared to those with dermatitis, no extensive inflammatory cell infiltration was observed after administration of saline (Fig. 1E). Our results showed that all dogs administered HDM developed pruritus and initial lesions consisting of erythematous macules and minuscule papules, which is similar to that observed in

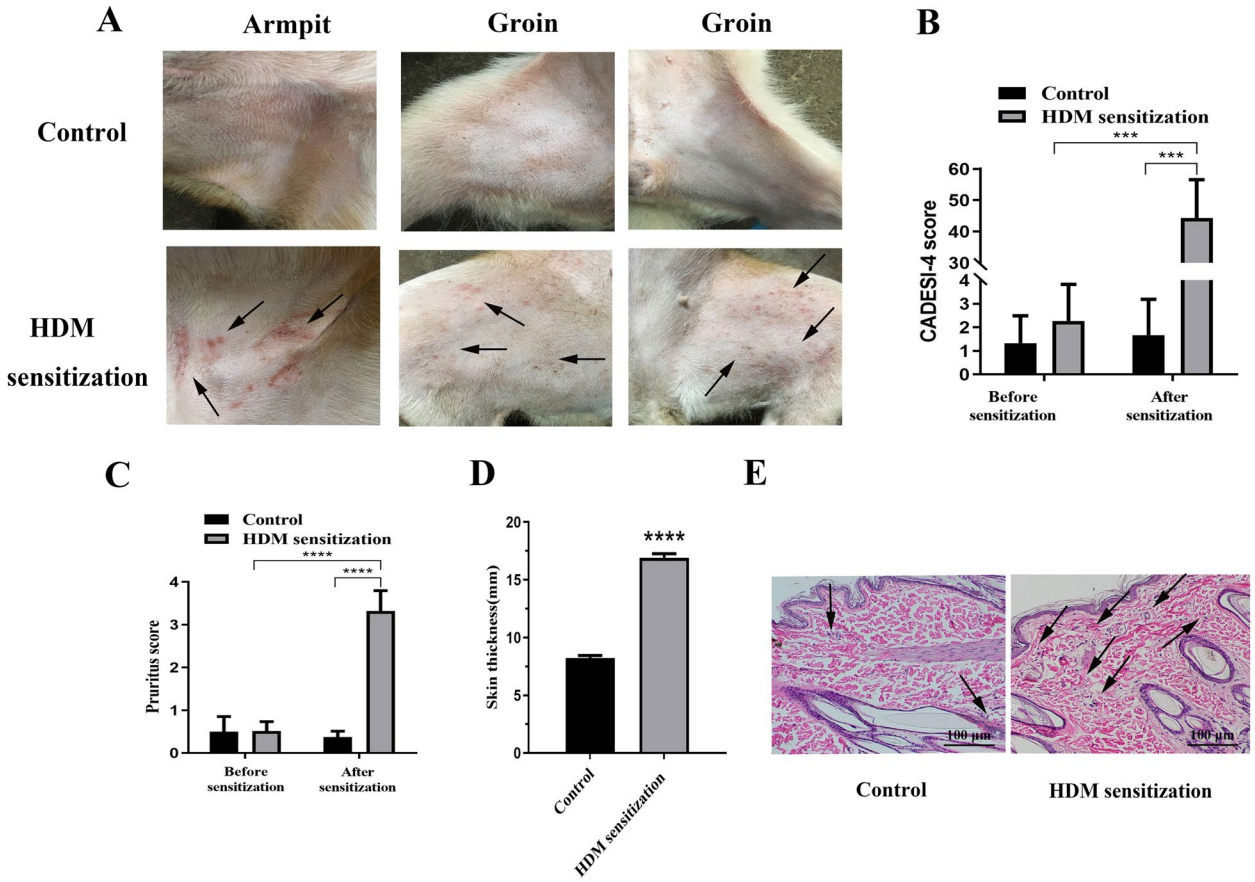

Fig. 1 Evaluation of the CAD model. **A** Characterization of skin in the CAD model induced by HDM. Black arrows refer to erythema and papules. **B** The CADESI-4 method was used to assess the severity of skin injury. *** $P < 0.001$. **C** Pruritus score was evaluated by pVAS. **** $P < 0.0001$. **D** Skin thickness of the control group and the experimental group after modeling. **** $P < 0.0001$ vs. control. **E** H&E staining of the inguinal sections was performed following the last HDM. The black arrows indicate inflammatory cells. Scale bars = 100 μm. Data are expressed as the means ± SDs

patients with AD. Additionally, inguinal skin thickness in the CAD model group increased, and the inflammation phenotype was detected in inguinal tissues. These results indicated that coating with HDM successfully induced a CAD model.

Identification and titer evaluation of anti-canine IL-31RA polyclonal antibodies

In this study, five short fragments from the anti-canine IL-31RA base sequence were chosen for synthesizing IL-31RA recombinant short peptides that were employed as antigens to immunize dogs. The specificity of the five antibodies produced was evaluated prior to assessing the efficacy of the anti-IL31RA pAbs. As depicted in Fig. 2A, the specific binding ability of five purified anti-IL31RA pAbs was confirmed in comparison to the negative antibody control. In addition, the protein level of anti-IL-31RA pAbs increased with an increasing number of immunizations (Fig. 2B). The potency of anti-IL-31RA pAbs showed that dogs immunized with IL-31RA recombinant protein generated an antibody with a titer of 1:128,000 (Fig. 2C). These outcomes demonstrated that anti-IL-31RA pAb-1~5 with high potency and specificity were produced, which can be utilized in the subsequent phase of efficacy validation.

Blockade of scratching by anti-canine IL-31RA polyclonal antibodies in CAD model dogs

In recent years, the use of AD-targeted biologics for the treatment of AD has been a promising method for advancing our understanding of treatment-resistant AD (Ratchataswan et al. 2021). IL-31 produced by CLA+ Th2 polarized T cells in response to stimulation is associated with numerous atopic disorders and is typically seen in AD skin. Increased expression levels of IL-31 significantly exacerbated inflammation and pruritus in patients with acute AD (Hawro et al. 2014). BMS-981164, a cynomolgus monkey IL-31 neutralizing antibody, significantly inhibited IL-31-induced pruritus behavior and reduced the number of scratches in experimental

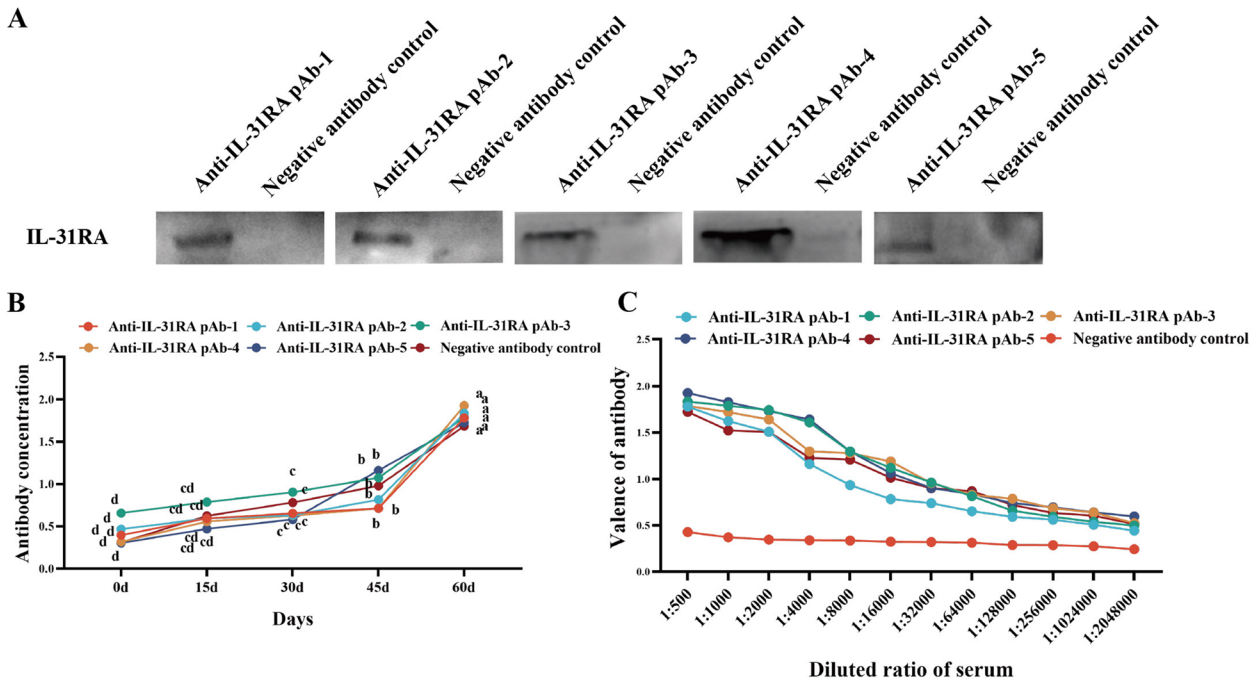

Fig. 2 Identification of anti-canine IL-31RA polyclonal antibody. **A** Specific identification of anti-IL-31RA pAbs. Anti-IL-31RA pAb 1~5 was obtained from dogs immunized with five different IL-31RA sequence short peptides coupled to BSA, while a negative antibody control was obtained from dogs immunized with intravenous BSA. **B** Indirect ELISA for anti-IL-31RA pAbs concentration after immunization of dogs with different antigens. Values with different letters (a, b, c and d) refer to the significance of differences ($P < 0.05$) at varying days of antibodies. **C** Indirect ELISA to detect the antibody titer of anti-IL-31RA pAbs

cynomolgus monkeys (Lewis et al. 2017). Our data suggested that treatment with anti-canine IL-31 polyclonal antibody (anti-IL-31 pAb) significantly attenuated HDM-evoked scratching. Of note, it was utilized as the positive antibody control to evaluate the therapeutic effects of prepared anti-IL-31RA pAbs on CAD model dogs in the present study.

Unlike other cytokines in the IL-6 cytokine family, IL-31 does not utilize gp130 as a receptor component but instead binds to IL-31RA. Similar to IL-31, IL-31RA was also upregulated in lesioned skin from the AD model (Bilsborough et al. 2006). It has been demonstrated that the inhibition of the IL-31 pathway by anti-mouse IL-31RA monoclonal antibody alleviated the pruritic/scratch cycle (Grimstad et al. 2009). Nemolizumab is an anti-IL-31RA humanized monoclonal antibody that results in a greater reduction in pruritus (Hamann and Thyssen 2018). It follows that IL-31 and IL-31RA are of crucial importance to pruritus behavior in AD. However, it remains unclear whether the pruritic symptoms of CAD can be alleviated by interfering with IL-31RA. Therefore, we hypothesized that anti-IL-31RA pAbs might relieve CAD symptoms.

To determine whether anti-IL-31RA pAbs affect the development of CAD, the therapeutic efficacy of anti-IL-31RA pAbs was investigated. Pruritus scores and CADESI-4 were reduced by injecting anti-IL-31RA pAbs, demonstrating that IL-31RA is essential for the induction of scratching. There was a statistically significant difference between the saline-treated and anti-IL-31RA pAb-1~5 groups (Fig. 3A, B). Our results revealed that polyclonal antibody-treated dogs exhibit a strong reduction in pruritus and skin damage, in line with nemolizumab. A previous study showed that AD is characterized by an increase in epidermal and dermal thickness (Lee et al. 2020). We observed attenuation of inguinal skin thickening in CAD dogs after application of anti-IL-31RA pAb-1~5. However, there was no statistically significant difference between the saline-treated and negative antibody groups (Fig. 3C). These results suggested that anti-IL-31RA pAbs effectively reduced CAD symptoms.

Anti-canine IL-31RA polyclonal antibodies inhibited the HDM-induced increase in inflammatory cytokine and Th2 cytokine levels in the skin

Because the onset of AD is likely due to persistent systemic inflammation (Fan et al. 2019), we further investigated whether anti-IL-31RA pAbs have

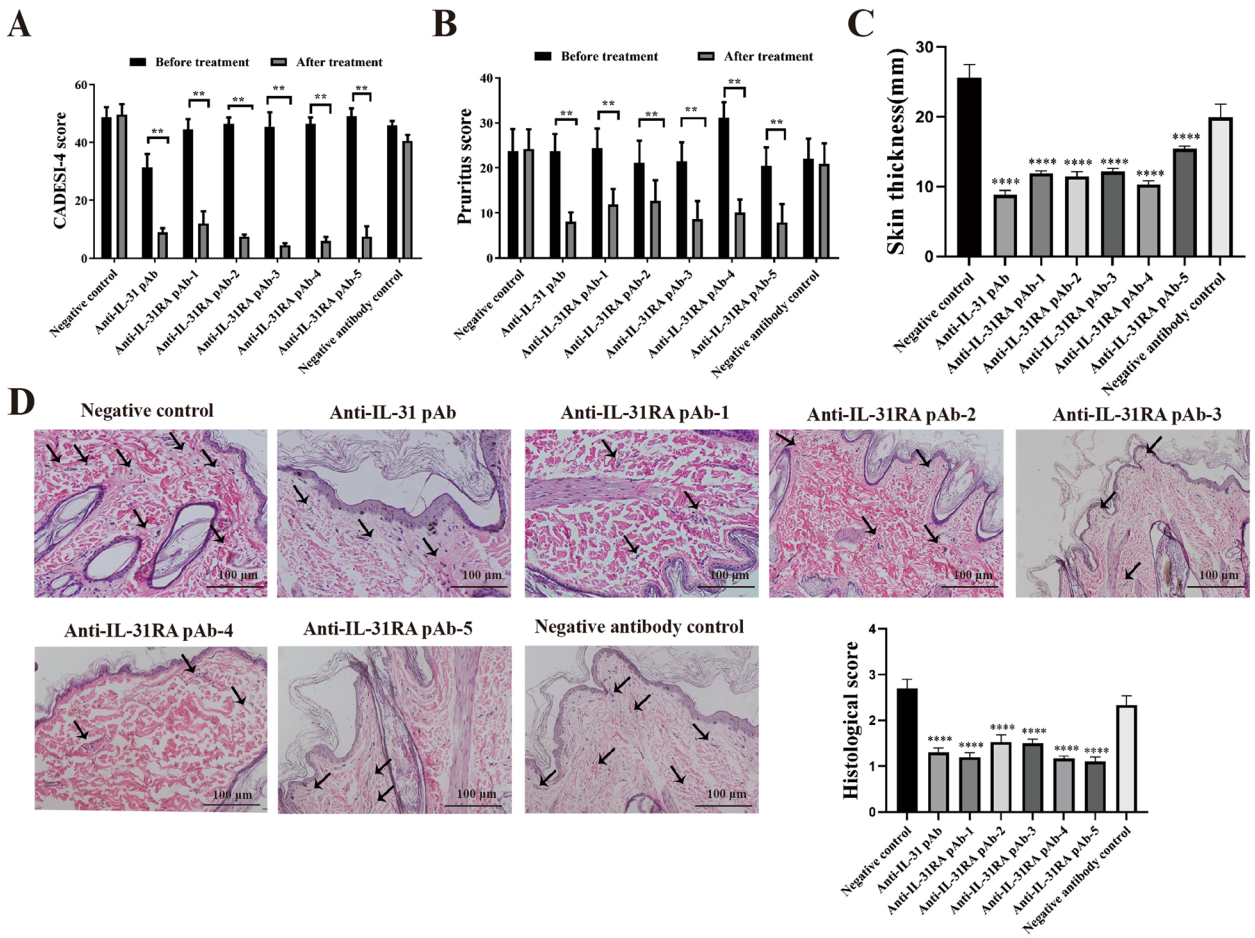

Fig. 3 Therapeutic effect of anti-IL-31RA pAbs. **A** CADESI-4 method to assess the severity of skin injury in CAD model dogs after treatment with anti-IL-31RA pAbs. ** $P < 0.01$. **B** Pruritus score from the pVAS evaluation. ** $P < 0.01$. **C** A Vernier caliper was employed to detect the inguinal thickness of the dogs. **** $P < 0.0001$ vs. negative control. **D** H&E staining of the inguinal sections was performed following the last application of the anti-IL-31RA pAbs. Scale bars = 100 μm. **** $P < 0.0001$ vs. negative control. The black arrows indicate inflammatory cells. A semiquantitative method was used to assess the severity of skin tissue inflammation. Saline treatment was the negative control group. The dogs treated with the antibody obtained by immunizing them with BSA were the negative antibody control group. Anti-IL-31 pAb was the positive antibody control. Data are expressed as the means ± SDs

anti-inflammatory properties on inguinal skin. Histological examinations of the affected skin areas were undertaken. Our data also showed that polyclonal antibodies significantly repressed inflammatory cell infiltration in skin tissue samples relative to that in the negative control. In the anti-IL-31RA pAbs-treated groups, skin tissue severity scores were considerably lower than those in the saline-treated and negative antibody-treated groups (Fig. 3D). It is evident that the therapeutic impact of our anti-IL-31RA pAb-1~5 is equal to that of the anti-IL-31 pAb (Fig. 3A-C). To verify whether the application of anti-IL-31RA pAbs has an effect on the mRNA levels of inflammatory cytokines, inguinal skin was collected, and qPCR was performed. The results showed that the mRNA levels of IL-6, IL-1β and tumor necrosis factor (TNF)-α inflammatory factors were significantly decreased in the anti-IL-31RA pAbs and anti-IL-31 pAb groups, while the negative antibody and negative control groups failed to inhibit inflammatory factor expression (Fig. 4A-C). The foregoing findings suggested that treatment with anti-IL-31RA pAbs effectively inhibited the secretion of IL-6, IL-1β and TNF-α in the skin of CAD model dogs.

Th2 cytokines, such as IL-31, IL-4, IL-5 and IL-13, play a vital role in the pathogenesis and persistent itching of AD, and their levels are elevated in acute AD lesions (Brunner et al. 2017). It has been reported that the use of dupilumab, a human IgG4 monoclonal antibody against the IL-4 receptor subunit (IL-4R), dramatically alleviated clinical severity indicators of dermatitis with a relatively

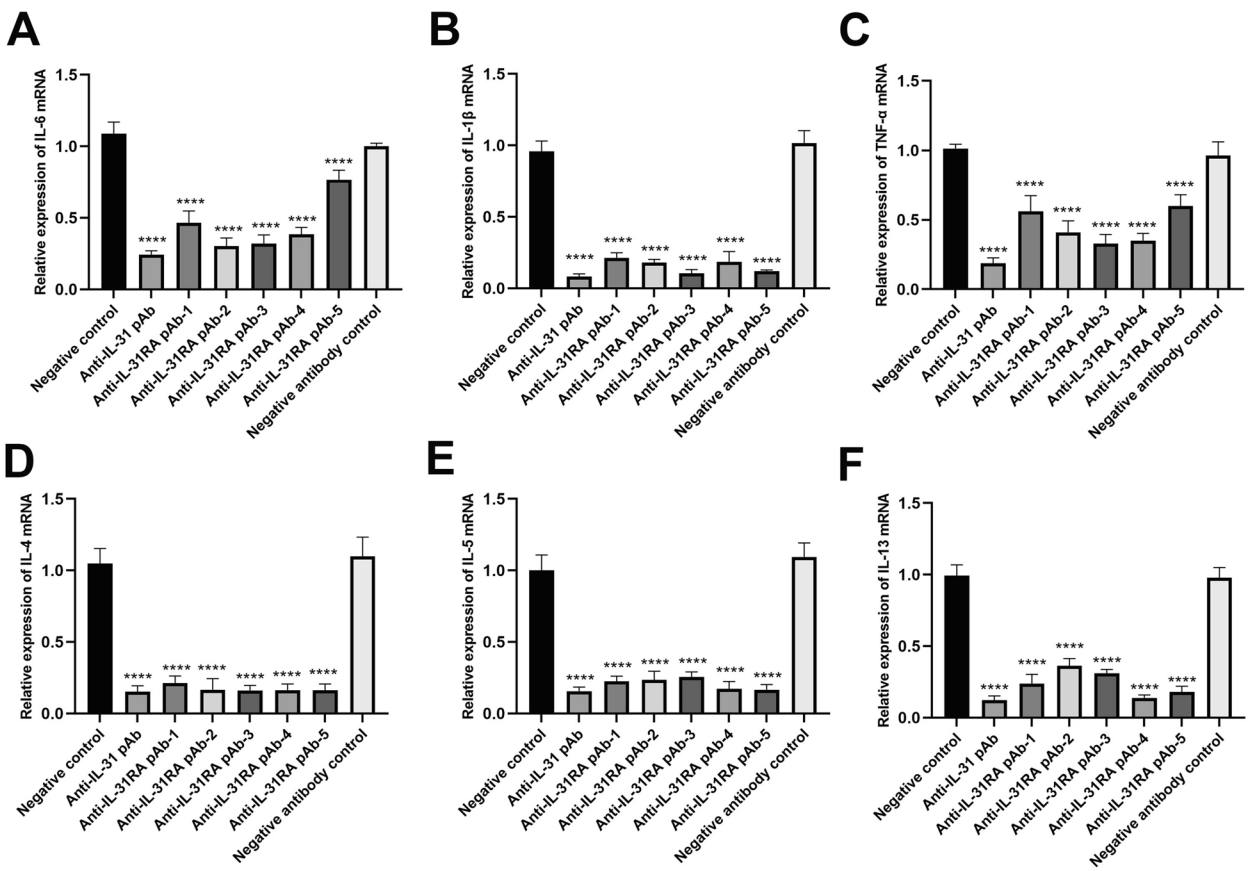

Fig. 4 Anti-IL-31RA pAbs decreased inflammatory cytokine and Th2 cytokine levels in the skin. The skin levels of inflammatory cytokines were examined with quantitative real-time PCR. **A**-**C** Effects of anti-IL-31RA pAbs on IL-6, TNF-α and IL-1β at the mRNA level in skin were determined. **D**-**E** Effects of anti-IL-31RA pAbs on IL-4, IL-5 and IL-13 at the mRNA level in skin were determined. Data are expressed as the means ± SDs. **** $P < 0.0001$ vs. negative control

favorable safety profile (Thaçi et al. 2019). This suggests that the Th2 inflammatory axis largely determines the onset of pruritus. In this study, we assessed the mRNA levels of Th2 cytokines. qPCR confirmed that the levels of IL-4, IL-5 and IL-13 were reduced in the anti-IL-31RA pAb-1~5-treated groups compared to those of the negative antibody and negative control groups (Fig. 4D-E). Furthermore, anti-IL-31 RA pAbs produced comparable inhibition of HDM-induced rise in IL-4, IL-5 and IL-13 expression as anti-IL-31 pAb. Thus, we speculated that anti-IL-31RA pAbs exerted an anti-inflammatory effect on CAD model dogs.

A limitation of this study is that we did not treat clinical dogs with AD with anti-IL-31RA pAbs; hence, the clinical applicability of the produced anti-IL-31RA pAbs requires further validation. Taken together, our findings proved that the application of HDM is effective in inducing CAD. The produced anti-IL-31RA pAbs significantly ameliorated not only the itch response but also skin inflammation and thickening, suggesting that anti-IL-31RA pAbs could be beneficial in the treatment of CAD.

Conclusion

In dogs suffering from CAD, the production of Th2 inflammatory cytokines, in particular IL-31, is a significant contributor to the development of itch and inflammation. Blocking the binding of IL-31 to its receptor IL-31RA by applying anti-canine IL-31RA pAbs is a feasible method for the treatment of CAD and lays the foundation for further research on its role in clinical treatment.

Methods

Animals

All the dogs were Miguel hounds, aged three years, and weighed 10 ± 1 kg. Thirty female high immunoglobulin E (IgE) Miguel hounds were chosen for the study and provided by the experimental animal center of Huazhong Agriculture University. The Institutional Animal Care

and Use Committee at Huazhong Agricultural University mandated that all experimental procedures be carried out in compliance with their recommendations. All of the dogs were fed the same amount of commercial dog food, had access to water on an ad libitum basis and were acclimatized to the environment before initiation of research (room temperature 18–22°C, relative humidity 60%).

Experimental design

Six healthy dogs without AD were utilized for the synthesis of polyclonal antibodies following immunization, and the remaining 24 dogs were successfully modeled to evaluate the efficacy of the acquired polyclonal antibodies (pAbs). The dogs ($n=24$) were randomly divided into four groups: saline+CAD model (negative control, $n=3$), anti-IL-31 pAb+CAD model (positive antibody control, $n=3$), control serum+CAD model (negative antibody control, $n=3$), and anti-IL-31RA pAb-1~5+CAD model (anti-IL-31RA pAbs, $n=15$). The dogs in the saline+CAD model, anti-IL-31 pAb+CAD model and control serum+CAD model groups received equal doses of saline, anti-IL-31 pAb and control serum from BSA-immunized dogs, respectively.

Establishment of a CAD model

Blood was drawn from 151 Miguel hounds (three years), and levels were determined using an IgE ELISA kit (Shanghai Renjie Biotechnology Co., Ltd.). Thereafter, 32 hounds (females, weighing 10 ± 1 kg) with high lgE levels were selected. Dust mites are the most significant environmental allergens in CAD (Marsella and Saridomichelakis 2010). Dust mite allergen skin prick tests were carried out before the application of HDM. Thirty dogs tested positive and were utilized to develop a CAD model or produce polyclonal antibodies. For the duration of the trial, HDM allergens at a concentration of 400 mg/mL and an administration volume of 0.3 mL/dog were topically applied to the skin of the groin and armpit areas of 24 dogs in the CAD model group. Throughout the study period, allergen stimulation was sustained by applying HDMs twice a week for a total of 12 weeks during that phase, and the control group only received saline.

Evaluation of pruritus score

Pruritus was assessed using a pruritus visual analog scale (pVAS) (Hill et al. 2007). Over 80 min, instances of scratching, biting, licking, chewing and rubbing signs of pruritis were photographed using cameras that were subsequently saved to a folder for observational study. The pruritus severity index was measured at four distinct time intervals in dogs prior to and after the establishment of the AD model. Likewise, measurements were performed in all dogs before and 10 h post induction of anti-IL-31RA pAbs or saline. The scores for each group were averaged after being calculated using a clear scale placed over the specified area. The researchers only rated pruritus in the groin and armpit regions of the dog, and scoring was done blindly.

Evaluation of skin damage

Skin lesions of all dogs were evaluated using a validated scoring system Canine Atopic Dermatitis Extent and Severity Index (CADESI)-4 at the same time point as the pruritus score in all dogs (Olivry et al. 2014). The four-point severity scale is rated as follows, from 0 to 3: (0) none, (1) mild, (2) moderate, and (3) severe. According to CADESI-4, the following 11 body sites were selected for this study: (1) hind feet, (2) pinna, (3) forelimbs, (4) underarms, (5) lips, (6) curved area, (7) genitals or ventral tail, (8) lateral chest or flank, (9) elbow, (10) hind limb, and (11) abdomen and/or groin area. The three components of erythema, lichenification, and alopecia were analyzed, and the severity values of each lesion site were summed to determine the overall score for each group of lesions.

Evaluation of skin thickness

The inguinal skin thickness of the dogs was measured by Vernier calipers 3 times per dog 24 h post HDM stimulation as well as 10 h post induction with polyclonal antibodies, and the average value was calculated.

Assessment of skin inflammation

Skin samples 6 mm in diameter were collected under local anesthesia with 2% lidocaine near the midpoint of the inguinal line. Harvested skin specimens were fixed in 10% paraformaldehyde (pH7.4) for 24 h, dehydrated with ethanol, and embedded in paraffin. The microtome was used for sectioning, and slides were stained with hematoxylin and eosin (H&E). The sections were sealed with neutral resin to prevent the formation of air bubbles, and they were examined using an optical microscope (Nikon ECLIPSE 80I, Japan) after they had dried at room temperature. Two investigators blindly evaluated microscopic changes using a previously reported semi-quantitative scoring system (Table 1) (Suzuki et al. 2020).

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (TaKaRa Bio, Inc., Dalian, China) according to the manufacturer's instructions. The amount of RNA was measured using a NanoDrop ND-2000 UV-Visible spectrophotometer (Fisher Scientific). Total RNA was reverse transcribed into 1000 ng of total cDNA using ABScript II RT Master Mix for qPCR (ABclonal Biotechnology, Wuhan, China). Table S1 shows the primer sequences in detail. Real-time

Table 1 Criteria for microscopic assessment of skin inflammation

Microscopic changes	Severity	Scores
Inflammatory cell infiltration	None	0
	Mild	1
	Moderate	2
	Severe	3
Epidermal erosion	None	0
	Mild	1
	Moderate	2
	Severe	3
Maximum score		6

quantitative PCR was performed using ABclonal 2X Universal SYBR Green Fast qPCR Mix on a LightCycler 96 (Roche, USA) (ABclonal Biotechnology, Wuhan, China). The ΔΔCt method and a relative expression software tool were used to examine the expression of these mRNAs. The GAPDH gene was used to standardize the mRNA levels.

Preparation of anti-canine IL-31RA polyclonal antibody

Five high-scoring fragments (CIKNYTIF, CGYNIWYF, CYNISVY, CRFRTINS, CGTKINFK) were selected from the sequences of canine IL-31RA for synthesizing IL-31RA recombinant short peptides and then coupled with vectors BSA as 1~5 antigens for immunization of dogs. Blood was collected before immunization as a control. The dogs were inoculated four times with the immunogen, each time 15 days apart. The initial utilization of multipoint subcutaneous injection, following multipoint intramuscular injection. After the last immunization, the jugular vein blood of dogs was obtained for the measurement of serum antibody titers following the last immunization. After 1 h at 37°C, the polyclonal antibody serum was precipitated at 4°C overnight and then stored at 4°C. Anti-IL-31RA pAbs were isolated by protein A column and cation exchange chromatography (Kasutani et al. 2014).

Antibody treatment methods

The anti-IL-31RA pAb group was injected intravenously with 5 mg/kg antibodies corresponding to 1~5 antigens in CAD model dogs, the sterile saline group served as the negative control group for AD model dogs receiving an intravenous saline injection, and CAD model dogs were injected intravenously with anti-IL-31 pAb as the positive antibody control group for antibody treatment.

Specific identification of polyclonal antibodies

The purified multiple antibodies were subjected to SDS–PAGE electrophoresis, and the protein bands in the gel were electrically transferred to nitrocellulose membranes, which were sealed in 5% skim milk powder for 2 h. The membrane was washed with TBST and incubated overnight at 4°C with anti-IL-31RA pAbs (1:1000). After three washes with TBS/Tween, the membrane was incubated for 2 h at room temperature with rabbit anti-canine secondary antibody. To acquire images, a gel-imaging system (Upland, California, United States) was employed, and AlphaEa-seFC (V. 4.0) was used to analyze the data.

Titer determination of polyclonal antibody

Antigens were encapsulated overnight at 4°C with 1 μg/mL encapsulation buffer. After washing the plate, an antibody was added at the following dilutions for 1 h at 37 °C: 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8000, 1:1,000, 1:16,000, 1:32,000, 1:64,000, 1:128,000, 1:256,000, 1:1024,000, and 1:2048,000. The plate was then washed and treated with rabbit anti-canine IgG at 37°C for 1 h. The monocomponent TMB substrate color development solution was added to the plate at room temperature for 10 min, and the reaction was terminated with the termination solution. Absorbance measurements at 450 nm/630 nm using an enzyme calibrator were performed.

Statistical analysis

Data collection was tested for normality using the Kolmogorov–Smirnov test (alpha = 0.05). Statistical significance was assessed by a Mann–Whitney U test for single comparisons and by a Steel–Dwass test after a Friedman test for multiple comparisons. Data are expressed as the means ± SDs. P values ≤ 0.05 were considered significant. GraphPad Prism 6 software (GraphPad Software Inc.; La Jolla, CA, USA) was used to compare all statistical data.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-023-00090-y.

Additional file 1: Table S1. Primer sequences.

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Authors' contributions

QL and YQ contributed equally to this work. YD contributed to the design of the study. QL, YQ, LY, NM and MD were responsible for collecting samples and executing animal experiments. QL drafted the manuscript. All authors approved the final version accepted for publication.

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Availability of data and materials
The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate
All experimental procedures were performed strictly in accordance with the guidelines of the Institutional Animal Care and Use Committee of Huazhong Agricultural University (ID Number: HZAUDO-2019-002).

Consent for publication
Not applicable.

Competing interests
The authors report no declarations of interest. Authors Mingxing Ding and Yi Ding were not involved in the journal's review or decisions related to this manuscript.

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