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Effect of Allergen-Specific Immunotherapy on Transcriptomic Changes in Canine Atopic Dermatitis

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Abstract: Canine atopic dermatitis (cAD) is a genetic, chronic, and recurrent inflammatory and pruritic skin disorder. Allergen-specific immunotherapy (ASIT) is presently recognized as the only clinically effective disease-modifying treatment for allergies. The aim of our study was to analyze the changes in gene expression observed in the peripheral blood nuclear cells of cAD patients subjected to ASIT. Blood samples designated for transcriptomic analyses were collected from AD dogs twice, before and six months after ASIT, and also from healthy dogs. Statistical analysis revealed 521 differentially expressed transcripts, among which 241 transcripts represented genes with well-described functions. Based on the available literature, we chose nine differentially expressed genes (*RARRES2*, *DPP10*, *SLPI*, *PLSCR4*, *MMP9*, *NTSR1*, *CBD103*, *DEFB122*, and *IL36G*) which may be important in the context of the dysregulated immune response observed in cAD patients. The expressions of five out of the nine described genes (*DPP10*, *PLSCR4*, *NTSR1*, *DEFB122*, and *IL36G*) changed after the application of ASIT. The expressions of three of these genes returned to the level observed in the healthy control group. The genes listed above need further investigation to determine details of their role in the molecular mechanism of immune tolerance induction in response to allergen-specific immunotherapy.

Keywords: allergen-specific immunotherapy; canine atopic dermatitis; transcriptome; gene expression; microarrays

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1. Introduction

Canine atopic dermatitis (cAD) is a genetic, chronic, and recurrent inflammatory and pruritic skin disorder that affects 10% of the canine population. CAD is a complex and multifactorial disease related to immune dysregulation, allergic sensitization, skin barrier defects, microbial colonization, and environmental factors. The inflammatory reaction is caused by an imbalance between the T helper cells Th2 and Th1 and the predominant secretion of the interleukins IL-4, IL-13, IL-5, and IL-31. This induces the recruitment of eosinophils into the inflammatory site and the activation of B lymphocytes, which are stimulated to produce environmental allergen-specific IgE. The binding of the allergen-specific IgE to mast cells causes the degranulation of these cells. The secreted inflammatory mediators along with an insufficient response from T regulatory (Treg) suppressor cells lead to inflammation. Depending on sensitivity to the allergen, the symptoms may be seasonal (e.g., pollen) or not seasonal (e.g., mites). Approximately 80% of dogs with seasonal signs are symptomatic in spring or summer [1,2]. The treatment of atopic dermatitis (AD) is difficult and challenging due to its complex, variable, and multifactorial pathogenesis. The best treatment for AD is avoiding allergens, which is impossible in most cases. For this reason, symptomatic treatment is necessary. The treatment strategy used—either the treatment of acute flares of cAD, the treatment of chronic stages, or the prevention of

the recurrence of clinical signs—depends on the clinical condition [3]. Pharmacological treatment is based on the administration of the appropriate drugs, depending on the phase of the disease. Drug treatments may include the topical and/or oral administration of glucocorticoids, ciclosporin, oclacitinib, and lokivetmab. These medicaments treat cAD symptoms, but they can also cause side effects. According to Olivry and Banovic [3], once the patient has remained clear of clinical signs for several weeks, it is time to move to the second phase of AD treatment, described as "proactive therapy", which aims to prevent the development of flares. At this stage, allergen-specific immunotherapy (ASIT) can be used.

ASIT is presently recognized as the only clinically effective disease-modifying treatment for both human and canine allergy diseases [4,5]. The sensitization method is effective in about 50–75% of dog patients [5–7]. ASIT induces long-term clinical tolerance to allergens to which the patient was previously allergic and allows for reduced use of systemic anti-inflammatory agents, thus improving the quality of life of patients suffering from AD. Despite the positive effects of treatment, in most cases, the exact molecular mechanisms of ASIT are still not completely elucidated.

The induction of immune tolerance to an allergen through ASIT is a complex process which involves both innate and adaptive immunity. The influence of innate immunity is mainly based on the inhibition of the activation and degranulation of effector cells, such as basophils and mast cells, as well as the modulation of the action of dendritic cells and decreasing the level of type 2 innate lymphoid cells [8,9]. The latter, owing to the production of IL-5 and IL-13, are involved in allergic inflammation [9]. Changes in adaptive immunity are related to the induction of allergen-specific T regulatory (Treg) and B regulatory (Breg) cells, which produce the crucial cytokines (IL-10, TGF-β and IL-35) for suppressing allergic inflammation. The suppressive milieu causes a decline in IgE production and induces the production of IgG4 by Breg cells. The IgG4 antibodies compete with IgE for the allergen, neutralize the allergen, and block the formation of the allergen IgE complex, therefore inhibiting the degranulation of effector cells (basophils, mast cells, and eosinophils). The suppressive regulatory cells cause a decrease in the number and activity of Th2 cells, involving a shift from a Th2 to a Th1 immune response, and modulate the activity of dendritic cells [9–12].

Much more is known about the abovementioned cellular mechanisms of the response to ASIT in humans as this has been extensively studied. Much less is known in the case of cAD. There have been very few studies referring to the use of ASIT in dogs, and the results described in these reports are variable. Most of them have focused on the effectiveness of the therapy and its ability to alleviate clinical signs and prevent the progression of the disease, leaving the mechanism of the immune response poorly described. There are several reports on changes in the level of cytokines involved in the immune response or in the number of Treg cells. Often, these results are ambiguous and not easy to interpret. There are also no reports on whether and how immunotherapy affects the regulation of gene expression. Therefore, the aim of our study was to analyze the changes in the gene expression of peripheral blood nuclear cells (PBNCs) observed in cAD patients subjected to ASIT. The choice of tissue analyzed in this research was not random, but based on the fact that blood samples constitute a valuable source of information about the state of an organism and are easier to collect than skin samples. The use of the microarray technique enabled us to seek genes that are differentially expressed in healthy dogs and cAD patients before and after ASIT. The results of our study identify several new candidate gene-encoding proteins that are directly or indirectly involved in signaling pathways that are important for the induction of immune tolerance to allergens. The determination of new potential molecular markers of cAD showing differential expression in the PBNCs of dogs affected with this disease and of those subjected to ASIT may be helpful in finding new therapeutic targets in the future.

2. Results

In this study, gene expression in PBNCs was tested using a microarray technique in AD dogs subjected to ASIT for 6 months. Six out of seven dogs receiving ASIT showed a positive response to the therapy. A detailed description of the patients' responses to the ASIT and the changes in their cytokine profiles and lymphocyte subpopulations is presented in our previous publication [13]. The microarray analysis was performed to search for differentially expressed genes that can be detected in the blood samples of cAD patients before ASIT and after 6 months of treatment. In this transcriptomic analysis, differentially expressed genes in PBNCs were compered among three groups of dogs: healthy dogs—control (ctrl), dogs with AD before treatment (0), and dogs with AD after 6 months of ASIT (6 months). The following comparisons were analyzed: dogs with AD before treatment vs. healthy control dogs (0 vs. ctrl); dogs with AD after 6 months of ASIT vs. healthy control dogs (6 months vs. ctrl); cAD patients before and after 6 months of ASIT (0 vs. 6 months). Statistical analysis revealed 521 differentially expressed (DE) transcripts. Out of these probes, it was possible to identify 241 transcripts representing specific genes. The profiles of the differentially expressed genes of the three investigated groups of dogs are presented on a heatmap (Figure 1). The expressions of 405 transcripts in dogs with AD and 236 transcripts in dogs after 6 months of treatment were regulated relative to the healthy control (Figure 2). Within this group of transcripts, 141 DE genes were common in both comparisons (0 vs. ctrl, and 6 months vs. ctrl); whereas ASIT caused changes in the expression of 166 genes compared to before treatment (6 months vs. 0) (Figure 2). Table 1 presents differentially expressed genes that we found interesting in the context of canine atopic dermatitis and applied ASIT. The expressions of some of these genes have never been associated with atopic dermatitis, nor have they been analyzed in cAD. A full list of significantly differentially expressed genes is presented in the Supplementary Materials (Table S1).

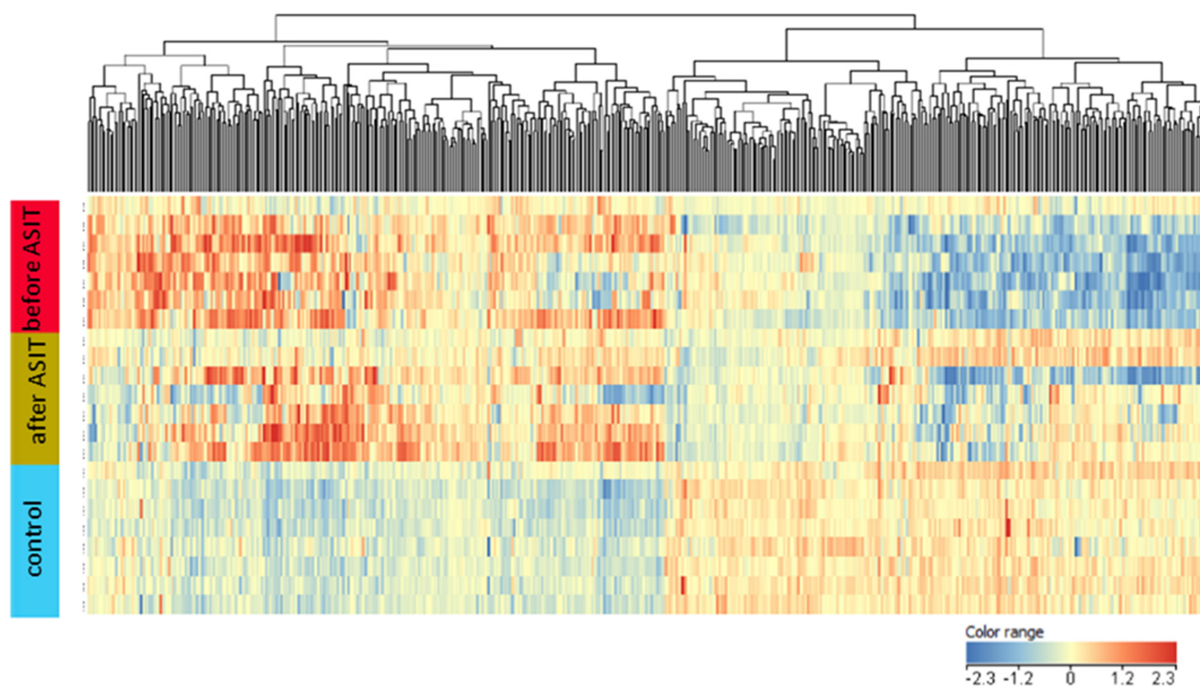

Figure 1. Heat map presenting supervised hierarchical clustering of differentially expressed genes for three investigated groups (healthy control, and cAD patients before ASIT and 6 mounts after ASIT). The distances were calculated using the Euclidean correlation metric and Ward's method. The columns show individual genes, and rows represent individual patients. Red color indicates increased gene expression, whereas blue color indicates decreased expression of genes.

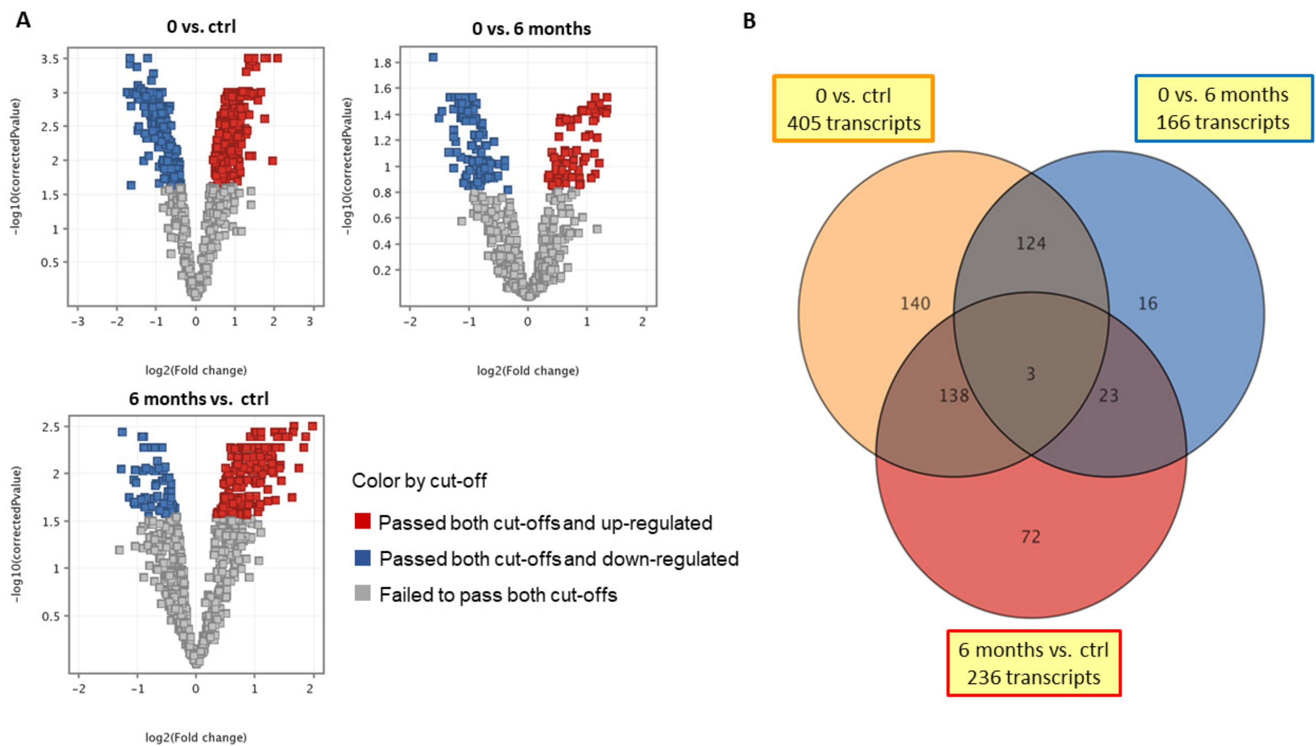

Figure 2. (**A**) Volcano plots presenting relationship between corrected p value (log10) and fold change (log2) in genes that were differentially expressed in cAD patients before therapy in comparison to healthy control group (0 vs. ctrl), in cAD patients before therapy in comparison to patients 6 months after ASIT (0 vs. 6 months), and in cAD patients 6 months after ASIT compared to healthy control group (6 months vs. ctrl). (**B**) Venn diagram based on microarray analysis showing number of differentially expressed genes in tested groups: cAD patients before therapy vs. healthy control group (0 vs. ctrl), cAD patients before therapy vs. 6 months after ASIT (0 vs. 6 months), and cAD patients 6 months after ASIT vs. healthy control group (6 months vs. ctrl). The above results were obtained using one-way ANOVA and Tukey's HSD post hoc test ($p < 0.05$) and Benjamini and Hochberg multiple testing correction (FDR < 0.05).

The microarray data were validated using real-time quantitative PCR to confirm changes in the gene expression obtained from microarray analysis. The expressions of four selected genes, *CBD103*, *IL36G*, *RARRES2*, and *SLPI*, were analyzed (Figure 3).

Table 1. The list of differentially regulated genes in: AD dogs before ASIT vs. dogs after 6 months of ASIT (0 vs. 6 months); AD dogs before ASIT vs. healthy control dogs (0 vs. ctrl); and AD dogs after 6 months of ASIT vs. healthy control dogs (6 months vs. ctrl). The differences in gene expression are presented as fold changes (FCs). The bold FC values are statistically significant (FDR < 0.05), and the "ns" symbol describes changes that are not significant.

Gene Symbol	Gene Name	GenBank Accession	FC (0 vs. 6 Months)	*p*-Values (0 vs. 6 Months)	FC (0 Months vs. Ctrl)	*p*-Values (0 Months vs. Ctrl)	FC (6 Months vs. Ctrl)	*p*-Values (6 Months vs. Ctrl)	Corrected *p*-Values (Ctrl vs. 0 Months vs. 6 Months)
RARRES2	*retinoic acid receptor responder 2*	XM_005629654	−1.39 down	ns	**−2.00 down**	**0.0015**	−1.44 down	ns	**0.0089**
DPP10	*dipeptidyl peptidase-like 10*	CX005277	**−1.90 down**	**0.0006**	**−1.69 down**	**0.0028**	1.12	ns	**0.0045**
SLPI	*secretory leukocyte peptidase inhibitor*	NM_001113171	−1.01	ns	**1.45 up**	**0.0049**	**1.46 up**	**0.00426**	**0.0081**
PLSCR4	*phospholipid scramblase 4*	XM_005634546	**2.28 up**	**0.0222**	**2.34 up**	**0.0144**	1.03	ns	**0.0192**
MMP9	*matrix metalloproteinase 9*	NM_001003219	1.08	ns	**3.36 up**	**0.0239**	**3.10 up**	**0.0357**	**0.0244**
NTSR1	*neurotensin receptor 1*	XM_543088	**2.40 up**	**0.0014**	**1.67 up**	**0.0492**	−1.44 down	ns	**0.0083**
CBD103	*beta-defensin 103*	NM_001129980	−1.60 down	ns	1.66 up	ns	**2.66 up**	**0.0240**	**0.0431**
DEFB122	*beta-defensin 122*	NM_001024641	**−2.14 down**	**0.0408**	**−2.88 down**	**0.0034**	−1.35 down	ns	**0.0123**
IL36G	*interleukin 36 gamma*	XM_005630449	**−1.71 down**	**0.0059**	**−1.99 down**	**0.0005**	−1.16	ns	**0.0045**

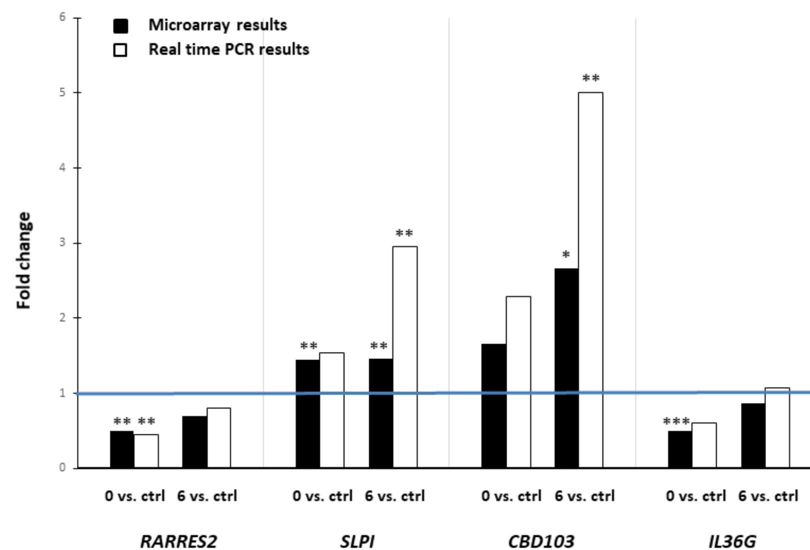

Figure 3. Expression of *RARRES2*, *SLPI*, *CBD103*, and *IL36G* genes in peripheral blood nuclear cells of cAD patients before ASIT and 6 months after ASIT and in healthy control dogs. Gene expression was analyzed using microarray and real-time PCR. Data are presented as fold change in gene expression in cAD patients before ASIT vs. healthy control dogs (0 vs. ctrl) and in cAD patients 6 months after ASIT vs. healthy control dogs (6 vs. ctrl). Bar graph presents upregulated genes with fold change values >1, and downregulated genes with fold change values <1. Statistically significant differences are depicted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Discussion

The molecular mechanisms of ASIT in cAD treatment have not been fully elucidated yet. Furthermore, existing reports describing the immune response of AD dogs subjected to ASIT often show incoherent results. For example, a study by Hou and coworkers [14] demonstrated an increase in total IgG antibodies in response to various antigens from mites after using ASIT in dogs. Research published later by DeBoer and coworkers [15] showed that after 6 months of using sublingual immunotherapy (SLIT), serum mite-specific IgE dropped, and mite-specific IgG levels increased, but were more variable over time. In addition, the changes described did not completely reflect the clinical response, and were not always associated with clinical improvement [15]. In a study by Keppel and coworkers [16], an increase in the concentration of IL-10 was reported after ASIT, but recently, another research group found no difference in the level of IL-10 between healthy and AD dogs after using different combinations of ASIT therapies [17]. Only intralymphatic immunotherapy (ILIT) caused changes in the level of IL-10 [17]. A previous study by our research group showed that only in some cAD patients after 6 months of ASIT did the level of IL-10 increase [13]. The data regarding the role of Treg suppressor cells are also inconsistent. Some studies have shown that the number of Treg cells is the same in healthy and atopic dogs, and increases after therapy [16], whereas other reports describe a higher number of Treg cells in cAD patients than in healthy dogs [18,19]. Our research [13,20] showed more Treg cells in atopic than in healthy dogs. After 3 months of ASIT, the number of Treg cells decreased, but after 6 months it increased again. In addition, our studies showed a decrease in IL-13 levels during ASIT, suggesting a shift in the immune response from Th2 to Th1. In parallel, we observed a decrease in the level of TNF-α and in the number of $CD8^{+}$ cells, which may be linked with a reduced inflammatory response [13]. It should be added that six out of seven dogs receiving ASIT showed a positive response to the therapy [13]. Therefore, in the continuation of our research, we focused on the possible use of peripheral blood nuclear cells as a source of information about the changes in gene expression that could be characteristic of cAD and could be regulated by ASIT. The results obtained demonstrated a substantial number of differentially expressed genes. When analyzing the data from the microarray experiment, we focused on several genes

that were regulated in the three compared groups of dogs (healthy control, AD dogs before ASIT, and AD dogs 6 months after ASIT) (Table 1). These genes have not been previously described in the literature as markers of atopic dermatitis, but their function seems to be directly or indirectly associated with cAD.

One of the differentially expressed genes found in our study is *retinoic acid receptor responder protein 2 (RARRES2)*, also known as *TIG-2 (tazarotene-induced gene-2)*. *RARRES2* showed significantly lower expression in AD dogs than in healthy control dogs (0 vs. ctrl), and also lower expression (although not significantly) in dogs after six months of ASIT (6 months vs. ctrl; 0 vs. 6 months). This gene encodes prochemerin, which can be converted into an active form of chemokine known as chemerin. Chemerin binds to the retinoic acid receptor, causing chemoattraction of dendritic cells and macrophages, which links innate and adaptive immunity [21,22]. *RARRES2* belongs to the group of retinoid response target genes. Several skin diseases, including AD and psoriasis, are related to alterations in retinoid metabolism/signaling [23,24]. Mihály and coworkers [24] showed that the all-trans retinoic acid (ATRA) concentration was lower in both lesional and non-lesional skin in human AD patients. The expression of the *RARRES2* gene was downregulated in AD skin in comparison with skin from healthy volunteers [20]. Although there are no available data referring to the changes in *RARRES2* expression in peripheral blood cells, or the molecular mechanism of chemerin activity in relation to AD, a protective role of the chemokine in other allergic diseases, such as allergic asthma, has been documented. A study using a BALB/c mouse model demonstrated that the administration of chemerin attenuated allergic airway inflammation, decreased the accumulation of $CD4^{+}$ T cells and eosinophils in the bronchoalveolar lavage fluid of mice with allergic asthma, and decreased the gene expression of the Th2-attracting chemokines CCL17 and CCL22, causing suppression of the airway recruitment of inflammatory $CD11c^{+}CD11b^{+}$ dendritic cells [22]. These results suggest that *RARRES2* expression is downregulated in various allergic diseases, and perhaps the use of chemerin could have a positive protective or therapeutic effect.

Another gene that showed significantly lower expression in the cAD group compared to the healthy control (0 vs. ctrl) and the treated group of dogs (0 vs. 6 months) was *DPP10 (dipeptidylpeptidase-like 10)*. The expression of the *DPP10* gene can be connected to the expression of the *SLPI (secretory leukocyte peptidase inhibitor)* gene, which showed the same level in cAD patients before and after therapy (0 vs. 6 months), and was significantly upregulated compared to the healthy control dogs (0 vs. ctrl; 6 months vs. ctrl). These genes have not been previously described in the context of AD, but some studies link the expression of *DPP10* and *SLPI* with allergy symptoms. According to Zhang and coworkers [25], point mutation in the *DPP10* gene leads to increased airway responsiveness following allergen challenge. The overexpression of *DPP10* significantly enhanced glucocorticoid receptor (GR) activation even without glucocorticoid treatment. This suggests that the DPP10 protein may influence endogenous anti-inflammatory corticosteroid production. Meanwhile, the knockdown of *DPP10* in human airway epithelial cells diminished the ability of GR to translocate to the nucleus and bind to DNA, and the overexpression of *DPP10* caused a reduction in the level of SLPI induced by IL-1β. TNF-α and IL-1β act in synergy, but also, TNF-α stimulates the secretion of IL-1β. In our studies, we did not test the level of IL-1β; however, we determined the level of TNF-α in the plasma of the investigated dogs [13]. The highest concertation of TNF-α in the plasma was noted in cAD patients before therapy. Perhaps TNF-α could have complicity in the upregulation of *SLPI* expression. The human *DPP10* gene was identified as a candidate gene for the prognosis of susceptibility to asthma, a common disease of the airways involving atopic inflammation and hyper-responsiveness to various agents [25,26]. This suggests that DPP10 may also play a protective role in cAD. On the other hand, the SLPI protein was shown to mediate the suppression of TGF-β expression and interfere with the differentiation of Treg cells [27]. Additional elastase activity is needed for the higher level of TGF-β expression in dendritic cells, which promotes an increase in the number of $CD4^{+}FOXP3^{+}$ cells [28].

Our study also determined that the *PLSCR4 (phospholipid scramblase 4)* gene showed differential expression, with the highest expression occurring in AD dogs before therapy (0 vs. ctrl; 0 vs. 6 months), whereas its expression in the healthy control dogs and in the cAD patients after ASIT was similar (6 months vs. ctrl). Py and coworkers [29] demonstrated that the phospholipid scramblase membrane proteins (PLSCR1 and PLSCR4), which largely localize in the membranes of T lymphocytes $CD4^+$, are receptors for SLPI, but also interact with the CD4 receptor. The SLPI protein can disrupt the association between PLSCR1 and CD4. SLPI binding to the endofacial domain of PLSCR1 and PLSCR4 can induce its translocation from the extracellular matrix to the cytoplasm and nuclei of monocytes, macrophages, and B and Th lymphocytes. Perhaps the increased expression of the *SLPI* gene is connected with the increased expression of the *PLSCR4* gene observed in our study.

Our transcriptomic analysis also demonstrated changes in the expression of the *MMP9* gene *(encoding matrix metalloproteinase 9)* among the groups of dogs subjected to this study. *MMP9* expression was similar in two cAD groups (before and after ASIT (0 vs. 6 months)) and significantly higher compared to the healthy control group (0 vs. ctrl; 6 months vs. ctrl). MMP-9 (also known as gelatinase B) and other metalloproteinases are able to degrade extracellular matrix (ECM), enabling tissue remodeling and cell migration. MMP-9 is one of the key enzymes in the development and course of the inflammatory reaction following allergen challenge. Several inflammatory cells secrete MMP-9, such as eosinophils, neutrophils, T cells, macrophages, and mast cells. This metalloproteinase promotes the migration and activation of immune cells by cleaving pro-inflammatory chemokines and cytokines, and therefore, contributes to the inflammatory processes [30–32]. Zhang and coworkers [33] indicated the dependence of the migration of CD4+ cells on MMP activity. The migratory capacity of Th1 cells was higher than that of Th2 cells, and there was also a difference in the levels of metalloproteinases (MMP-2 and MMP-9) secreted by the Th1 and Th2 cells. A similar effect was observed in human and murine cells [34]. Harper and coworkers [35] indicated the dominant presence of MMP-8 and MMP-9 in a mixture of MMPs detected on the skin surface in acute human AD. The presence of MMP-9 in this environment is associated with the presence of immune cells, such as eosinophils, lymphocytes, and dendritic cells, which require MMP-9 activity to penetrate the inflamed skin. The expression of the *MMP-9* gene was elevated in acute compared with chronic AD lesions. Purwar and coworkers [36] demonstrated the influence of IL-13 on MMP-9 expression in the basal keratinocyte layer of human skin biopsies. The addition of TNF-α to human peripheral blood eosinophils cultured in vitro from atopic asthmatic patients, stimulated a 95% increase in MMP-9 activity above baseline [37]. Our results showed a significant increase in the levels of IL-13 and TNF-α in the blood of cAD patients before therapy [13]. It is possible that these cytokines are among the factors contributing to the increase in *MMP9* gene expression.

Furthermore, our present study demonstrated higher expression of both *SLPI* and *MMP9* genes in AD dogs. The SLPI protein was shown to have a regulatory effect on MMP-9 quantity through transcriptional upregulation. SLPI may either directly or indirectly induce the transcription of *MMP-9*. This, coupled with SLPI extracellular interaction with plasmin, regulates MMP-9 activation and release. Thus, the net pro-invasive effect of SLPI on MMP-9 results from both increased gene transcription and protein production and secretion, tempered, in part, by SLPI's inhibition of the plasmin activity required to cleave the propeptide from MMP-9 [38]. A study by Poachanukoon and coworkers [39] showed, additionally, that active proteases present in allergen extract from house dust mites (HDM) directly activate MMP-9 by cleaving pro-MMP-9. It can be assumed that even though the activity of MMP-9 comprises SLPI's inhibition of plasmin, this metalloproteinase can be activated by HDM or other allergen proteinases in allergy patients.

Another differentially expressed gene that could potentially be important in cAD is *NTSR1 (neurotensin receptor 1*, also known as *NTR*). Significantly, the highest expression of *NTSR1* was noted in cAD patients before ASIT compared to the healthy control dogs (0 vs. ctrl) and to cAD patients after therapy (0 vs. 6 months). The lowest expression was

observed in the cAD group after therapy, but the difference was not significant (6 months vs. ctrl). The NTSR1protein induces intracellular signaling through phospholipase C and the inositol phosphate signaling pathways. It also functions through the production of cGMP, cAMP, and arachidonic acid, through the MAP kinase pathways, and through the inhibition of Akt activity [40]. Neurotensin (NTS), which is a ligand for NTSR1, functions as a neurotransmitter in the nervous system, and as a hormone in the peripheral tissues. Its action in the periphery is mediated by the G protein-coupled receptor, NTSR1 [41,42]. Aside from the role of neurotensin in the nervous system, it is also known for its pro-inflammatory role, induction of vasodilatation, vascular permeability, activation of mast cell degranulation, and enhancement of the directional migration and phagocytosis of neutrophils [43]. NTS can be involved in the pathogenesis of inflammatory skin disorders, including AD, especially those exacerbated by stress, scratching, and sweating [44], however the mechanism is still not well understood. It was shown that NTS concentration increased in rodent skin as a consequence of acute stress, and induced vascular permeability acting on mast cells through NTS receptors expressed by these cells [45,46]. Some studies indicated [47,48] that NTS also stimulated rodent mast cells to secrete histamine through NTSR. Alysandratos and coworkers [49] demonstrated a relationship between the corticotropin-releasing hormone (CRH) and neurotensin (both released in the stress response) and their receptors (CRHR-1 and NTSR) in a human culture of mast cells. CRH induced *NTS* and *NTSR1* gene expression, whereas NTS induced *CRHR1* gene expression. The authors also showed that NTS stimulated human mast cell degranulation and the release of VEGF, which contributed to skin vascular permeability, and intensified the effect of CRH on VEGF release through NTSR. An increased level of VEGF was observed in the serum of AD patients, and this growth factor can be synthesized and released by different cells (platelets, eosinophils, and mast cells) [50,51]. Studies also demonstrated that the level of NTS in the serum of AD patients was higher than in that of control patients, similarly to the expression of *NTS* gene, which was increased in the lesional skin of AD patients in comparison to controls [52]. However, there was no difference in the expression of the *NTSR1* gene in the lesional skin of AD patients compared to the control [52]. NTS and NTSR proteins were detected in the lesional skin, but not in the skin of healthy control individuals. Additionally, NTSR activation also results in the secretion of the inflammatory cytokines IL-8 and TNF-α [49]. Our previous study demonstrated an increased level of TNF-α in the plasma of cAD patients before treatment [13], and the microarray analysis revealed an elevated expression of the *NTSR1* gene in AD dogs before treatment. In the available literature, there is no information about the role of neurotensin and its receptor (NTSR1) in mast cells localized in the canine skin or in the peripheral blood, and there is no published research on the role of these proteins in the course of cAD. However, it seems that this neuropeptide and its receptor should be further investigated in the context of cAD.

CBD103 and *DEFB122* were the next interesting genes to show differences in expression. Both genes encode proteins belonging to β-defensins: *beta-defensin 103 (CBD103)* and *beta-defensin 122 (DEFB122)*. The highest expression of *CBD103* was noted in the dogs after ASIT and differed significantly compared to the expression detected in the healthy control dogs (6 months vs. ctrl). CAD patients before therapy also showed a higher expression of the *CBD103* gene compared to the healthy control dogs (0 vs. ctrl), but this difference was not statistically significant. However, the expression of *DEFB122* was significantly lower in cAD patients before therapy compared to the healthy control dogs (0 vs. ctrl) and AD dogs after ASIT (0 vs. 6 months). It seems that immunotherapy increased the expression of *CBD103*, but the mechanism of this process remains unclear. In the case of *DEFB122*, immunotherapy restored the expression of this gene to a normal level. In the cAD group, before immunotherapy, the expression of *DEFB122* was reduced. It is difficult to interpret the observed changes in the expression of these two defensins. β-defensins play a role in both innate and adaptive immunity, and are considered a bridge between the innate response and adaptive immune cell requirements [53,54]. β-defensins are cationic peptides, which show antibacterial activity. These proteins can directly kill microbial pathogens

through their interaction with anionic components in the microbial membrane [55,56]. Canine atopic dermatitis is accompanied by secondary microbial infections most often caused by *Staphylococcal pyoderma* and *Malassezia dermatitis* [1]. It is possible that abnormal levels of defensins may contribute to a defective innate immune response. Beta-defensins may show pro- and anti-inflammatory effects that depend on many factors, including the disease stage and exposure to pathogens, among others [53]. Beta-defensins can affect the chemoattraction of CD4+ memory T cells and immature dendritic cells by binding to CCR6 [57], but these proteins can also suppress the effects of TNF-α and IL-6.

Van Damme and coworkers [56] detected the expression of the *CBD103* gene in both the skin and peripheral blood mononuclear cells of all tested healthy dogs, as well as in many other tissues (duodenum, kidneys, testes, bone marrow, and lungs), whereas *DEFB122* was not detected in all dogs. *DEFB122* was expressed in over 50% of skin samples from the tested dogs, but not in the peripheral blood. In a study by Lancto and coworkers [58] the expressions of the *CBD103* and *DEFB122* genes were reported in skin samples from various body sites of healthy dogs (axilla, forehead, inner thigh, scapula, and ventral abdomen). Van Damme and coworkers [56], as well as Lancto and coworkers [58], showed decreased expression of the *CBD103* gene in the skin of AD dogs with and without lesions compared to the skin of healthy dogs. On the other hand, Leonard and coworkers [59] reported no difference. All authors showed no difference in *CBD103* expression between lesional and non-lesional skin in cAD patients. Lancto and coworkers [58] indicated a lower level of the *DEFB122* transcript in the lesional and non-lesional skin of AD dogs than in the skin of healthy dogs. The results of the present study, together with the reports of other researchers, highlight the need for further research into the role of β-defensins (CBD103 and DEFB122) in cAD.

Our analysis of the microarray results also showed a significant reduction in *IL-36γ* expression in dogs with AD before therapy compared to the healthy control dogs (0 vs. ctrl) and AD dogs after ASIT (0 vs. 6 months). IL- 36γ is one of the IL-36 isoforms belonging to the IL-1 superfamily, acting as a receptor agonist for pro-inflammatory functions. IL-36R ligands bind to the IL1RL2/IL-36R receptor and use the IL-1 receptor accessory protein (IL-1RAcP) as a co-receptor that triggers signal transduction, and activates mitogen-activated protein kinase (MAPK), and nuclear factor-kappa B (NF-kB) signaling pathway [60]. IL-36 is involved in immune cell activation, antigen presentation, and pro-inflammatory factor production [61]. The IL-36 interleukins are most active in barrier tissues, like the skin, lungs, bronchia, and intestines. Their main function is to regulate the interactions of the environment and the body, because they serve as the first line of defense against microorganisms [62]. IL-36 expression can be found in keratinocytes, B-lymphocytes, T-lymphocytes, dendritic cells, and monocytes [63–68]. In normal skin, IL-36 cytokines are expressed constitutively at low levels. Jiang and coworkers [69] determined that IL-36γ is important for the control of wound healing after skin injury. mRNA expression and the protein abundance of IL-36γ were elevated in wounded skin. However, the overexpression of IL-36γ appears to be problematic, as it may cause the loss of homeostatic balance, which leads to a pathological pro-inflammatory milieu and to disorders such as psoriasis. Many authors suggest that IL-36 may be a biomarker of psoriasis, and is also used as a therapeutic target. However, the level of *IL-36γ* expression in other inflammatory skin diseases, such as atopic dermatitis, lichen planus, contact eczema, subacute cutaneous lupus erythematosus, and mycosis fungoides, was found to be much lower than in psoriasis [67]. Genes with higher expression in atopic dermatitis are induced to a greater extent by the Th2 cytokines IL-13 and IL-4 than by the cytokines IL-17A, IL-17A/TNF, IL-36α, β, γ, and IFN-α; thus, the low expression of the *IL-36γ* gene observed in our study in the PBNCs of cAD patients is in agreement with this theory [70]. In a study by D'Erme and coworkers [71], the level of the *IL-36γ* transcript in AD skin was slightly higher than in healthy skin, but these results were not statistically significant. Our immunohistological analyses demonstrated a very low level of IL-36 protein in atopic skin. Also, other authors found no changes in *IL-36γ* expression in the eczematous skin of atopic dermatitis patients compared to

the healthy control [72]. Other studies have shown that *IL-36* expression depends on the phase and severity of the disease. Increased expression of *IL-36α*, *IL-36γ*, and *IL-36 Ra* was demonstrated in the lesional skin of AD patients compared to non-lesional skin [67,73]. Tengvall and coworkers [74] reported increased expression of *IL-36γ* in cAD skin with mild lesions versus healthy control skin. It should be emphasized that all the results described above refer to the changes in *IL-36γ* expression directly in skin samples, whereas in our study, the lower expression of the *IL-36γ* transcript was characteristic of nuclear blood cells of dogs with AD. Perhaps the deficiency of IL-36γ causes disorders in the maintenance of homeostasis, which is important in the first line of defense and wound healing. The decreased expression of the *IL-36γ* gene observed in our study in the PBNCs of cAD patients may be one of the factors contributing to the overreaction to the allergen, and should be further investigated as a potential marker in this disease.

In summary, the present study analyzed changes in the transcriptomic profiles detected in the PBNCs of dogs affected by AD in comparison to healthy dogs and in comparison to their state after 6 months of conducting ASIT. Statistical analysis revealed 521 differentially expressed transcripts, among which 241 transcripts represented genes with well-described functions. Based on the available literature, we chose nine differentially expressed genes (*RARRES2*, *DPP10*, *SLPI*, *PLSCR4*, *MMP9*, *NTSR1*, *CBD103*, *DEFB122*, and *IL36G*) that may be important in the context of dysregulated immune responses observed in cAD patients. Table 2 summarizes the functions of the genes described. Eight of these nine genes (except *CBD103*) showed different expression levels in cAD patients before therapy compared to healthy dogs. The expression of five of the nine described genes (*DPP10*, *PLSCR4*, *NTSR1*, *DEFB122*, and *IL36G*) changed after the application of allergen-specific immunotherapy, but only in the case of three genes (*DPP10*, *PLSCR4*, and *IL36G*) did the expression return to the levels observed in the healthy dogs. The expression of the *RARRES2* gene was the lowest in cAD patients prior to immunotherapy, which may be connected to the alternations in retinoid metabolism/signaling in dogs affected by AD. The increased expression of *DPP10* in dogs after 6 months of ASIT in comparison to these patients prior therapy suggests a protective role of *DPP10* in cAD, which is linked with the inhibition of the proinflammatory response through the stimulation of endogenous anti-inflammatory corticosteroid production. In addition, the decreased expression of *DPP10* may be directly connected to the increased levels of *SLPI* transcripts in AD dogs, which, in turn, results in the suppression of TGF-β secretion, causing alterations in the differentiation of Treg cells. Our study also revealed an elevated expression of the *PLSCR4* gene, encoding the phospholipid scramblase membrane proteins that are receptors for SLPI. *PLSCR4* showed the highest expression in cAD patients before therapy, whereas its expression in healthy control dogs and cAD patients after ASIT was similar. The parallel increase in the expression of the *SLPI* and *PLSCR4* transcripts suggests a common pathway of regulation of these genes. Another differentially expressed gene described in our study, *MMP9*, encodes an important matrix metalloproteinase secreted by many inflammatory cells. *MMP9* expression was increased in cAD patients compared to healthy dogs, which might have contributed to the stimulation of the inflammatory processes due to the role of MMP-9 in cleaving the pro-inflammatory chemokines and cytokines secreted by eosinophils, neutrophils, T cells, macrophages, and mast cells. The expression of *MMP9* also remained elevated in dogs subjected to ASIT, suggesting that immunotherapy does not downregulate the levels of *MMP9* transcripts. *NTSR1* was also among the detected genes showing increased expression in AD dogs before ASIT, and lower expression in healthy dogs as well as in cAD patients subjected to therapy. The increased expression of *NTSR1* may be linked with the inflammatory response induced by allergens, due to the fact that this gene encodes neurotensin receptor 1, whose activation results in the secretion of the inflammatory cytokines IL-8 and TNF-α. The genes of β-defensins *CBD103* and *DEFB122* were also among the differentially expressed transcripts detected in our microarray analysis. Beta-defensins show pro- and anti-inflammatory effects, which depend on many factors, such as the disease stage and exposure to pathogens. In our study, the highest expression

of *CBD103* was noted in dogs after ASIT and differed significantly from the expression observed in healthy dogs, whereas the expression of *DEFB122* was significantly lower in cAD patients before therapy compared to its expression in healthy dogs and in AD dogs subjected to ASIT. Taken together, this study identifies some novel directions of research on the molecular mechanisms of cAD and immunotherapy used in cAD treatment. Figure 4 illustrates the direct and indirect interactions among the proteins encoded by the differentially expressed genes and cytokines analyzed in the present study. Further research is needed to study the roles of the presented genes and proteins in immune tolerance induction in response to allergen-specific immunotherapy.

Table 2. Functions of differentially expressed genes described in this study.

Gene Symbol	Gene Name	Summary of Gene Function
RARRES2	*retinoic acid receptor responder 2*	Encodes a precursor of chemerin that acts via retinoic acid receptor (RAR), causing chemoattraction of dendritic cells and macrophages, linking innate and adaptive immunity [21,22]. *RARRES2* gene is downregulated in skin of AD patients [24].
DPP10	*dipeptidyl peptidase-like 10*	Encodes a single-pass type II membrane protein with no detectable protease activity, but with ability to bind other proteins, such as specific voltage-gated potassium channels, altering their expression and biophysical properties. Mutations in this gene have been associated with asthma [25]. Overexpression of *DPP10* enhances glucocorticoid receptor (GR) activation even without glucocorticoid treatment. DPP10 protein may influence endogenous anti-inflammatory corticosteroid production [25].
SLPI	*secretory leukocyte peptidase inhibitor*	Encodes a secreted inhibitor protecting epithelial tissues from endogenous serine proteases. SLPI protein also mediates the suppression of TGF-β expression and interferes with the differentiation of Treg cells [28]. SLPI has an affinity for leukocyte elastase and blocks its activity, which is needed for increased TGF-β expression in dendritic cells, in turn, leading to an increased number of CD4 + FOXP3+ cells [28].
PLSCR4	*phospholipid scramblase 4*	Encodes a cell membrane protein necessary for CD4 receptor binding activity in T lymphocytes [29]. SLPI binding to PLSCR4 induces its translocation from extracellular matrix to cytoplasm and nuclei of monocytes, macrophages, and B and Th lymphocytes [29].
MMP9	*matrix metalloproteinase 9*	Encodes MMP-9—one of the key metalloproteinases involved in the development and course of inflammatory reactions following allergen challenge. MMP-9 is secreted by several inflammatory cells, e.g., eosinophils, neutrophils, T cells, macrophages, and mast cells. MMP-9 promotes migration and activation of immune cells by cleaving pro-inflammatory chemokines and cytokines, therefore contributing to inflammatory processes [30–32]. Increased expression of *MMP9* gene is observed in acute compared with chronic AD skin lesions [36].
NTSR1	*neurotensin receptor 1*	Encodes a membrane protein belonging to the superfamily of G protein-coupled receptors. NTSR1 is a specific receptor for neurotensin, which, aside from its role as a neurotransmitter, is also known for its pro-inflammatory role, induction of vasodilatation, vascular permeability, activation of mast cell degranulation, and enhancement of directional migration and phagocytosis of neutrophils [43]. Neurotensin can be involved in the pathogenesis of inflammatory skin disorders, including AD, especially those exacerbated by stress, scratching, and sweating [44].
CBD103	*beta-defensin 103*	Encodes a protein belonging to defensins—a family of microbicidal and cytotoxic peptides. *CBD103* can be detected in skin and peripheral blood mononuclear cells, but shows decreased expression in cAD patients [56].
DEFB122	*beta-defensin 122*	Encodes a protein belonging to defensins—a family of microbicidal and cytotoxic peptides. Decreased expression of *DEFB122* transcript was observed in lesional and non-lesional skin of cAD patients in comparison to the skin of healthy dogs [58].

Table 2. *Cont.*

Gene Symbol	Gene Name	Summary of Gene Function
IL36G	*interleukin 36 gamma*	Encodes interleukin 36 gamma (IL-36γ) belonging to the IL-1 superfamily. IL-36 is involved in immune cell activation, antigen presentation, and pro-inflammatory factor production [61]. IL-36 cytokine expression can be found in keratinocytes, B-lymphocytes, T-lymphocytes, dendritic cells, and monocytes [63–68]. Increased expression of *IL36G* was shown in lesional skin of AD patients compared to non-lesional skin [67,73,74].

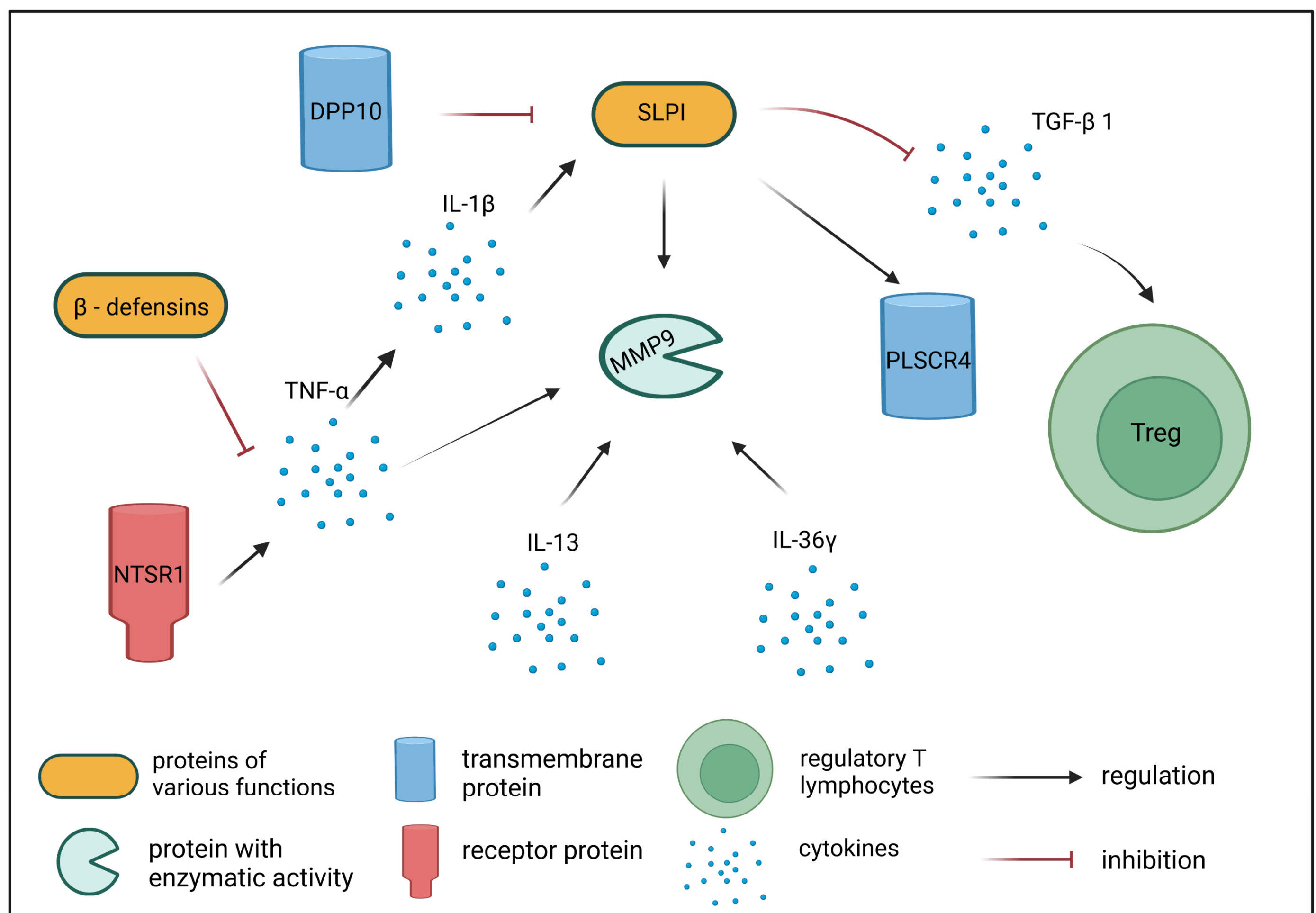

Figure 4. Scheme of potential direct and indirect interactions among proteins encoded by differentially expressed genes detected in the microarray analysis, and cytokines linked with the course of cAD and ASIT used for cAD treatment. Scheme was created using BioRender.com.

4. Materials and Methods

4.1. Animals

This study complies with national and institutional guidelines on the use of ani-mals in clinical research according to the Polish legal act concerning experiments performed on client-owned animals (Act on Experiments on Animals of 21 January 2005—Journal of Laws of Republic of Poland, abbreviated Dz.U. 2005, No. 33, item 289, as amended). Concerning experiments performed on client-owned animals. All dogs were patients of the Small Animal Clinic at the Warsaw University of Life Sciences. A high standard of care was adhered to throughout each examination. In the case of cAD patients, research was carried out as part of the routine veterinary diagnostic procedure. Dogs included in the control

group were blood donors from "Milusia" Veterinary Blood Bank that were submitted to the Small Animal Clinic for routine checkup.

Seven privately owned dogs of various breeds with cAD (five females and two males) were included in this study. The breeds were: Labrador retriever (2), Golden retriever (2), American Staffordshire terrier (2), and small Münsterlander (1). Their ages ranged from 2 years and 2 months to 6.5 years. Eight healthy dogs served as a control group (three females and five males), with their ages ranging between 3 and 8 years. The following breeds were included in the healthy control group: American Staffordshire terrier (2), Labrador retriever (2), bulldog (1), great Dane (1), Staffordshire bull terrier (1), and Weimaraner (1). Detailed information about the dogs included in this study was published in our previous research article [13].

4.2. cAD Diagnosis and Sample Collection

The diagnosis of cAD was based on compatible history and clinical signs determined using Willemse and Préalud diagnostic criteria, followed by Favrot criteria, as follows: pruritus sine materia, an indoor lifestyle, and the exclusion of other causes of pruritus ongoing for at least one year.

In all dogs with chronic pruritus, other causative factors were excluded, i.e., skin parasites (sarcoptic mange, demodectic mange, flea allergic dermatitis). Bacterial pyoderma and Malassezia dermatitis were excluded on the basis of the negative results of in vitro culture assays. The role of food antigens as a cause of the skin condition was assessed using elimination diets for 6–8 weeks. Clinical diagnosis of atopic dermatitis was confirmed via serological allergy testing (IDEXX allergic panel test) and intradermal skin testing (Artuvetrin test set, Lelystad, The Netherlands). No anti-inflammatory drugs were given for at least 3 weeks prior to the serological test and intradermal test.

All dogs classified into the investigated group had positive reactions in serological allergy testing and intradermal skin testing, which was described in our previous article [13]. Peripheral blood samples were collected just before the dogs were subjected to the intradermal skin test, and thus, at the stage when clinical signs of AD were visible, as well as after 3 months and 6 months of therapy. Hematological, morphological, and biochemical blood tests were conducted on samples from qualified patients. Each dog with AD, as well as the animals included in the healthy control group, showed morphological parameters of blood within the reference value range.

The intradermal skin test (Artuvetrin test set, Netherlands) used in the dogs that qualified for the experiment showed that the dogs were primarily sensitized to the storage mites Tyrophagus putrescentiae 86% (6 dogs), Acarus siro 86% (6 dogs), and Lepidoglyphus destructor 57% (4 dogs); the house dust mites Dermatophagoides farine 71% (5 dogs) and Dermatophagoides pteronyssimus 29% (2 dogs); tree pollen mixture 43% (2 dogs); weed pollen mixture 29% (2 dogs); and grass pollen mixture 29% (2 dogs).

4.3. Allergen-Specific Immunotherapy (ASIT)

Allergen extracts were prepared on the basis of the results of intradermal tests by the Artuvetrin Therapy company. Allergen extracts were administered subcutaneously in increasing concentrations according to the manufacturer's recommendations, as described previously [13].

The first dosage for the subcutaneous Artuvetrin Therapy started at 0.2 mL, after which it was gradually increased over longer intervals to a maximum of 1.0 mL. In the 3rd week, the dose was increased to 0.4 mL; in the 5th week, to 0.6 mL; in the 7th, to 0.8 mL; and in 10th, 13th, and 17th, to 1 mL. Then, 1 mL was administered every 4 weeks. In two patients, the time between doses was extended due to hypersensitivity reactions that followed each dose of the allergen extract.

When the 1 mL dose was reached after 12 weeks, a fixed dose of 1.0 mL was administered monthly. In some cases, this treatment schedule was too fast for the patient. If so, it was possible to deviate from the standard dosage schedule.

4.4. Gene Expression Microarray Analysis

The blood samples designated for transcriptomic analyses were collected from the dogs twice: before intradermal skin test and immunotherapy, and after six months of ASIT.

Blood samples were collected from AD and healthy dogs into RNeasy Protect Animal Blood Tubes (Qiagen, Germantown, MD, USA). Total RNA from peripheral blood nuclear cells was isolated using an RNeasy Protect Animal Blood Kit (Qiagen, USA). Additionally, contamination with DNA was eliminated via DNAse I digestion, included as an additional step of the isolation protocol. The quantity of RNA was measured using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). The analysis of final RNA quality and integrity was performed using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) and an RNA 6000 Nano Kit (Agilent, Waldbronn, Germany). To ensure optimal data quality, only RNA samples with RIN number $\geq$ 7.8 were included in the analysis.

The analysis of gene expression profiles was performed using a Canine (V2) Gene Expression Microarray, 4 $\times$ 44 K (Agilent Technologies, USA) and an Agilent Technologies Reagent Set according to the manufacturer's procedure. An RNA Spike In Kit (Agilent Technologies, USA) was used as an internal control, a Low Input Quick Amp Labeling Kit was applied to amplify and label (Cy3 or Cy5) target RNA to generate complementary RNA (cRNA) for oligo-microarrays, a Gene Expression Hybridization Kit was used for fragmentation and hybridization, and a Gene Expression Wash Buffer Kit was used for washing slides after hybridization. Acquisition and analysis of hybridization intensities were performed using an Agilent DNA Microarray Scanner G2505C. Data were extracted and backgrounds subtracted using the standard procedures included in the Agilent Feature Extraction (FE) Software version 10.7.3.1. FE was used to perform Lowess normalization.

The experiment was performed using a common reference design, in which the common reference comprised a pool of equal amounts of RNA from 13 healthy control dogs. These dogs did not take part in the experiment. The cRNA of the common reference samples was Cy3-labeled, whereas the cRNA of healthy dogs (control group of dogs taking part in experiment) and of cAD patients before ASIT (0) and after 6 months of ASIT (6 months) was labeled with Cy5. Two-color microarrays were performed, one for each patient: 7 microarrays with samples from AD dogs (0), 7 with samples from AD dogs after therapy (6 months), and 8 from healthy control dogs (ctrl). On each microarray, 825 ng of each sample of cRNA (Cy3-labeled common reference and Cy5-labeled control or patient) was hybridized.

To identify signaling pathways and gene function, the microarray data were analyzed using Pathway Studio 12 (Ariadne Genomics, Rockville, MD, USA). This database consisted of millions of individually modeled relationships between proteins, genes, complexes, cells, tissues and diseases.

4.5. Real-Time RT-PCR

To verify the microarray results, the expression of four genes (*CBD103*, *IL36G*, *RARRES2*, *SLPI*) was analyzed using real-time qPCR. The sequences of chosen genes were obtained from the Ensembl or NCBI database. Primers were designed using Primer-Blast software (NCBI database) and verified using Oligo Calc: Oligonucleotide Properties Calculator (free software available online, provided by Northwestern University) to exclude sequences showing self-complementarity. To reduce the chances of amplifying traces of genomic DNA, the primers were positioned in different exons. Reference gene *RPS19* was determined using the Genorm and NormFinder programs. All primer sequences are listed in Table 3. Total RNA was reverse transcribed to first-strand complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). All analyses were performed on individual samples of total RNA using SYBR Select Master Mix (Applied Biosystems, USA) on a Stratagene Mx3005P Quantitative PCR instrument for RT-PCR, following the manufacturer's protocol. For all genes, the annealing temperature was 58 °C. The relative expression of the target gene was quantified as the mean of tripli-

cate measurements for each biological sample. Results were calculated using the $2^{-\Delta\Delta Ct}$ method [75].

Table 3. Primer sequences for real-time qPCR verification of microarray results.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	NCBI Accession Number
CBD103	TGTGTGTCCTGCAACCTTAT	CACCGACCGCTCCTTATTC	NM 001129980.1
IL36G	ATCACTGTTCTCCCATGCAA	CCAGTATCTCCTCCTCCTTTAG	XM_005630449.2
RARRES2	GGAGACCAGTGTGGACAGA	CATTTCCGCTTCCTCCCATT	XM_022403838.1
SLPI	ATCCCGTTAATGTCTCCAACTC	AATGGCAGGTATCAGGCTTATT	NM_001113171.1
RSP19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	XM_533657.3

Primers for *SLPI* were designed based on the previously published study by Lancto and coworkers [58], and primers for *RPS19* were designed based on the previous reports by Brinkhof and coworkers [76], Schmitz and coworkers [77], and Majewska and coworkers [20].

4.6. Statistical Analysis

The statistical analysis of microarrays was performed using Gene Spring14 software (Agilent, USA). The probe sets were filtered by flags to remove poor quality probes (absent flags); additionally, filtering by error was applied, and outlier data (≥75% of coefficient of variation (CV)) were eliminated.

The statistical significance of the differences observed was evaluated using a one-way ANOVA and Tukey's HSD post hoc test ($p < 0.05$). Multiple testing correction was performed using Benjamini and Hochberg False Discovery Rate (FDR) < 0.05. Microarray data were deposited in the Gene Expression Omnibus data repository under the number GSE 168109.

The statistical analysis of the real-time qPCR results was performed using GraphPad Prism 7.05 software via one-way ANOVA, in which the mean values of $2^{-\Delta\Delta Ct}$ for samples of patients before ASIT and after 6 months of immunotherapy were compared to the healthy control samples. Statistically significant differences were defined as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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Institutional Review Board Statement: This study complies with national and institutional guidelines on the use of animals in clinical re-search according to the Polish legal act concerning experiments performed on client-owned animals (Act on Experiments on Animals of 21 January 2005—Journal of Laws of Republic of Poland, ab-breviated Dz.U. 2005, No. 33, item 289, as amended). Concerning experiments performed on client-owned animals. Since sample collection was performed as part of routine clinical diagnostics and did not require any additional procedures that would be harmful or painful for animals, this study did not require any formal ethics approval. Before enrolling a dog into this study, informed consent from its owner was obtained, and a high standard of care was adhered to throughout each examination.

Informed Consent Statement: Not applicable.

Data Availability Statement: The microarray data presented in this study are openly available in the Gene Expression Omnibus data repository under the number GSE 168109.

Conflicts of Interest: The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Veterinary Dermatology

Transcriptomic profiling of dorsal root ganglia in atopic and healthy dogs: A comparative RNA sequencing study with implications in cutaneous itch research

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Abstract

Background: Itch is a common clinical sign in skin disorders. While the neural pathways of itch transmission from the skin to the brain are well understood in rodents, the same pathways in dogs remain unclear. The knowledge gap hinders the development of effective treatments for canine itch-related disorders.

Hypothesis/Objectives: This study aimed to investigate the differential gene expression in the dorsal root ganglia (DRGs) between healthy and atopic dogs to identify specific molecules potentially involved in itch signalling and neuroinflammation in canine atopic dermatitis (cAD).

Animals: Two atopic and four healthy dogs.

Materials and Methods: DRGs were collected from atopic and healthy dogs to compare their transcriptional profiles using RNA sequencing.

Results: Principal component and heatmap analyses revealed two distinct clusters separating atopic from healthy dogs. Consistent with this observation, we identified 627 (543 upregulated and 84 downregulated) differentially expressed genes (DEGs) in atopic compared with healthy dogs. We further narrowed down our genes of interest to common DEGs in each atopic dog, which revealed 159 (132 upregulated and 27 downregulated) DEGs. Among these genes, when we focused on itch signalling–associated molecules, *P2RY12*, *IL-2RG*, *TLR1* and *POSTN* were significantly upregulated, while *MRGPRD* and *LPAR3* were both significantly downregulated in atopic dogs compared with those in healthy dogs. Pathway analysis showed a significant upregulation of CREB signalling in neurons, myelination signalling and neuroinflammation signalling pathways in atopic dogs.

Conclusions and Clinical Relevance: Our study suggested that dysregulation of neuroinflammatory pathways might play a role in the pathomechanism of cAD as in humans.

KEYWORDS
atopic dermatitis, cutaneous sensory ganglia, dog, itch signalling pathway, RNA-Seq

INTRODUCTION

Atopic dermatitis (AD) manifests as a common, often chronic, allergic skin disease in both humans and dogs, characterised by mild-to-severe pruritus and inflammatory skin lesions.[1,2] The pathogenesis of AD involves multiple factors. In 2023, the International Committee on Allergic Diseases of Animals (ICADA) revised the definition of canine AD (cAD) to reflect current knowledge. The revised definition states that cAD is a hereditary, typically pruritic and predominantly T-cell-driven inflammatory skin disease involving interplay between skin barrier abnormalities, allergen sensitisation and microbial dysbiosis.[3] This updated definition highlights the multifactorial nature of the disease, advocating for a multimodal approach

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to manage the disease's complex factors. However, a key pathogenic aspect of human AD remains unknown in cAD.

Recent studies in humans strongly support that neuroimmune pathways perpetuate atopic itch and skin inflammation, through cross-talk between the nervous system, cutaneous immune system and keratinocytes.[4,5] Pruritogens, such as allergens and cytokines, bind to receptors that are present on primary afferent C-fibre somatosensory neurons innervating the skin, initiating an atopic flare.[4,5] Activated neurons transmit signals from the skin to the brain through dorsal root ganglia (DRGs), where they are interpreted as itch.[4,5] Additionally, activated cutaneous sensory neurons release neuropeptides, such as substance P and calcitonin gene-related peptide, from the cutaneous nerve endings into the skin, inducing vasodilation, cell recruitment and the release of pro-inflammatory mediators from keratinocytes to fuel further inflammation, keratinocyte proliferation and epidermal thickening.[4,5] This cascade, termed 'neurogenic inflammation', is now recognised as a pivotal contributor to AD pathogenesis in humans.[4,5] While human and canine AD share similarities, the role of neuroimmune pathways in cAD is unexplored. Our objective was to identify key differences in gene expression in the DRGs between atopic and healthy dogs using RNA sequencing (RNA-Seq), paving the way for novel therapeutic targets.

MATERIALS AND METHODS

Ethics

Sample collection from atopic dogs was approved beforehand by our university's Institutional Animal Care and Use Committee (IACUC; ID no.: 18-130-B). Samples from healthy dogs were sourced from cadavers euthanised at the local shelters for population control purposes; thus, the IACUC approval was not deemed necessary.

Sample-size calculation

Given the lack of previous studies comparing DRG gene expression between spontaneous AD and healthy individuals in any species, we conducted a power analysis based on a previous study using an interleukin (IL)-31-induced atopic-like mouse model.[6] The analysis indicated that a minimum of three dogs per group would be required to achieve >80% power to detect the significant differences at a *p*-value of 0.05. However, as a consequence of the limited availability of the DRG samples of atopic dogs, we were unable to meet this sample size requirement.

Animals

Samples were collected from two dogs in an atopic dog model colony (one intact female and one intact male, both aged 13 years) and four healthy dogs (two females [neuter status unknown] and two intact males); all were of young-to-middle age (their exact ages were not documented) and had no gross skin issues at the time of sample collection. The atopic dog model utilised in this study was an inbred line of laboratory Maltese-beagle dogs that are known to spontaneously exhibit a high immunoglobulin (Ig)E response to food allergens and to develop cAD signs upon allergen challenge. These dogs also are easily experimentally sensitised to *Dermatophagoides farinae* (Df) house dust mite (HDM) allergen during early life stages, and reproducibly produce IgE against Df and develop atopic skin lesions following epicutaneous HDM provocations.[7,8] Dogs in this colony are maintained with restricted dietary management and reside in a controlled housing environment to prevent the development of spontaneous AD flares triggered by both food and environmental allergens. At the time of sample collection, both atopic dogs were already retired from laboratory duties yet remained in a controlled housing environment. Consequently, they had not been exposed to HDM allergens for several years (957 and 541 days for the female and male atopic dogs, respectively) and did not have active skin lesions at the time of sample collection. Hence, within the scope of this study, we designate them as atopic dogs rather than 'dogs with cAD'.

Sample collection

We collected two cervical DGRs (the cluster of the cell bodies of sensory neurons) from each atopic dog (AD1-C, AD1-C, AD2-C2 and AD2-C4) and three cervical DRGs (Dog1-C2-4, Dog2-C2-4, Dog3-C2-4 and Dog4-C2-4) pooled together as one sample from each healthy dog. All samples were stored at −80°C until further processing.

Additionally, two extra cervical DRGs from one of the atopic dogs (AD1-C#1 and AD1-C#2) and one each DRG from two additional healthy dogs (Dog5-C3 and Dog6-C3) were collected for RNA in situ hybridisation (ISH). These collected samples were embedded in an optimal cutting temperature embedding medium (OTC Compound, catalogue no.: 4585; Fisher HealthCare;) and snap-frozen on dry ice. The embedded samples were cryosectioned at 5 μm thick on the day of RNA ISH staining. All sections were arranged on the same slide to enable simultaneous staining, thereby minimising the potential for batch effects.

The signalments and locations of the samples are summarised in Table S1.

RNA isolation and RNA sequencing

Total RNA extraction from each DRG sample was conducted using an RNeasy Fibrous Tissue Mini Kit (reference no.: 74704; Qiagen) following the manufacturer's protocol. Following quality assurance and RNA library preparation, RNA-Seq was carried out using the NextSeq 500 platform (Illumina). The

detailed procedures and results for RNA extraction, RNA purity/integrity, library preparation and RNA-Seq are described in Appendix S1. The raw sequences are publicly available under study PRJNA1148493.

RNA-seq data analyses

We performed the RNA-Seq data analysis, principal component analysis (PCA) and heatmap analysis using the CLC Genomic Workbench v22 (Qiagen) with default parameters. Further elaboration on the data processing procedures is found in Appendix S1.

Differential expression analysis

Differential expression analysis between the atopic and healthy dogs (atopic versus healthy) was conducted using the Differential Expression for RNA-Seq tool within the CLC Genomic Workbench (Qiagen). Criteria for significance included an absolute fold change (FC) greater than two, a false discovery rate (FDR) of ≤0.05, and a maximum group mean of reads per kilobase of exon per million mapped reads (max RPKM) of >1. The FCs were calculated by dividing gene expressions in atopic dogs by those in healthy dogs; thus, positive numbers indicate upregulation, and negative numbers indicate downregulation in atopic compared with healthy dogs.

RNA in situ hybridisation (RNAscope)

We performed a fluorescence ISH using the RNAscope method (Advanced Cell Diagnostics) to localise the expression of *IL-33* mRNA in DRGs from both atopic and healthy dogs. The RNAscope[T] Multiplex Fluorescent V2 Assay (catalogue no.: 323110; Advanced Cell Diagnostics) was employed according to the manufacturer's protocol. Details regarding the probes used in the staining and corresponding fluorophores to visualise the signals are summarised in Table 1. A comprehensive protocol and the fluorescence microscope settings are described in Appendix S1.

Ingenuity pathway analysis

Differentially expressed genes (DEGs) exhibiting our criteria of significant difference between groups were then subjected to canonical pathway analysis (CPA) using the Ingenuity pathway analysis (IPA) program (Qiagen), aiming to predict up- or downregulated pathways in atopic compared with healthy dogs. To align with the purpose of this study, we restricted the analysis of pathways to neurotransmitters and other nervous system signalling. Statistical significance was determined by p-values ≤0.05 and absolute z-scores >2.

RESULTS

RNA-Seq data analysis

The average number of sequencing reads obtained per sample was 132.0 million, with a range of 119.4 to 152.5 million reads. An average of 90% of the read pairs (range: 88%–91%) were mapped to the canine reference genome (CanFam3.1). A total of 19,989 genes were annotated in each DRG sample. The PCA plot illustrates the differences in gene expression profiles between atopic and healthy dog DRGs (Figure 1a). Different sexes in atopic samples also showed a distinct clustering (Figure 1a). However, because two samples from each sex were obtained from the same dog, it was unclear whether the difference was caused by sex or individual variability. Likewise, visualisation of the expression of genes across the samples by the heatmap revealed two distinct hierarchical clusters separating atopic from healthy dogs (Figure 1b).

Differential expression analysis

Using our predefined criteria, we identified 627 DEGs (543 upregulated and 84 downregulated genes) in the atopic DRG samples compared with healthy dogs. Owing to significant individual differences between the two atopic dogs detected by PCA, we focused on common DEGs in both atopic dogs when each was independently compared with healthy counterparts, revealing 159 DEGs (132 upregulated

TABLE 1 RNAscope probes utilised for staining and corresponding fluorophores.

Target gene	Protein coding	Probe type	Channel no.	ACD catalogue no.	Fluorophores
IL-33	Interleukin-33	Target probe	1	484161	Opal 570 (red)
GFAP	Glial fibrillary acidic protein	Target probe	2	877971-C2	Opal 520 (green)
TUBB3	Tubulin beta 3 class III	Target probe	3	1122091-C3	Opal 690 (magenta)
Polr2a	Polymerase II polypeptide A	Positive control probe	1	323931	Opal 570 (red)
PPIB	Peptidylprolyl isomerase B	Positive control probe	2	323931	Opal 520 (green)
UBC	Ubiquitin C	Positive control probe	3	323931	Opal 690 (magenta)
dapB	Dihydrodipicolinate reductase	Negative control probe	1,2,3	321831	Opal 570, 520, 690

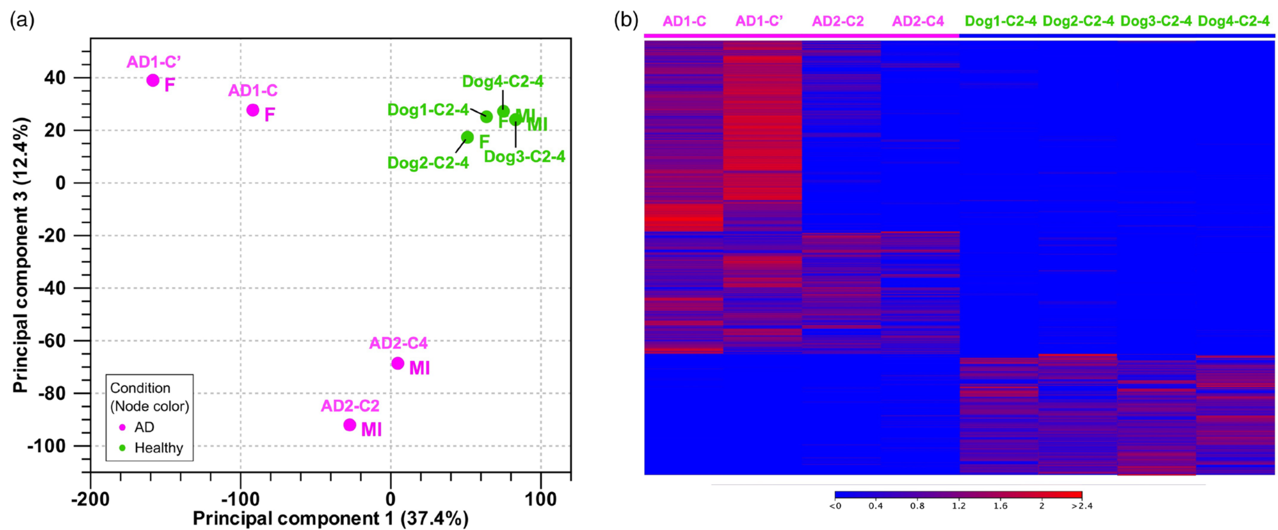

FIGURE 1 Principal component analysis (PCA) (a) and heatmap analysis (b). Both PCA (each plot represents a sample) and heatmap analysis (each column represents a sample) revealed a distinct separation between atopic and healthy dogs. Refer to Table S1 for sample IDs. F, female; MI, male intact.

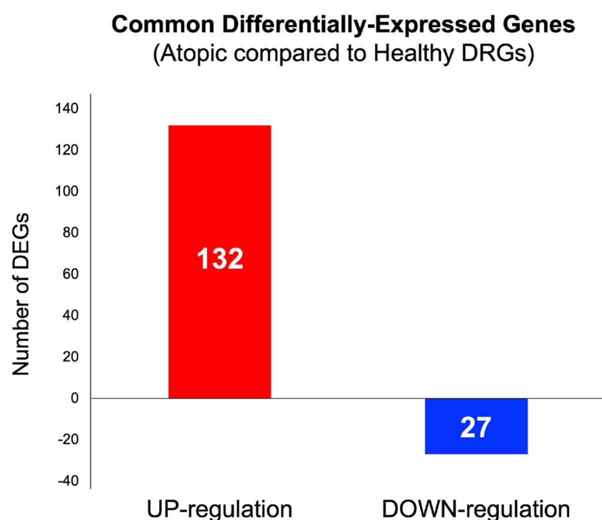

FIGURE 2 Number of common differentially expressed genes (DEGs). Our refined DEG analysis identified 159 common DEGs (132 upregulated and 27 downregulated) in atopic compared with healthy dogs. DRGs, dorsal root ganglia.

and 27 downregulated) (Figure 2; Table S2). To further investigate the mechanisms underlying itch sensation, we focused on 144 genes related to itch-associated receptors, neurotransmitters, neuropeptides and signalling molecules (Table S3).[4] Among the common DEGs, *P2RY12* (purinergic receptor P2Y12; FC=4.54, FDR=1.2E-04, max RPKM=1.37), *IL-2RG* (IL-2 receptor subunit gamma; FC=3.78, FDR=4.0E-04, max RPKM=1.47), *TLR1* (toll-like receptor 1; FC=3.32, FDR=1.2E-04, max RPKM=1.73) and *POSTN* (periostin; FC=2.22, FDR=6.7E-03, max RPKM=1.85) showed significant upregulation, while *MRGPRD* (MAS-related GPR family member D; FC=–2.39, FDR=3.5E-03, max PRKM=4.17) and *LPAR3* (lysophosphatidic acid receptor 3; FC=–2.19, FDR=7.63E-05, max RPKM=8.46) showed significant downregulation in both atopic dogs compared with those in healthy dogs (Figure 3). The remaining 138 genes showed no significant differences between atopic and healthy dogs.

RNA in situ hybridisation (RNAscope)

Our preliminary RNA-Seq analysis revealed low *IL-33* mRNA expression levels in healthy human, murine and feline DRGs, with mean transcript per million (TPM) values of 6.3, 19.6 and 5.5, respectively (data not yet published). By contrast, both healthy and atopic canine DRGs exhibited moderate expression of *IL-33*, with mean TPM values of 267.0 and 436.2, respectively. Moreover, *IL-33* expression was higher in atopic than in healthy dogs (FC=1.65, FDR=3.61E-03, max RPKM=134.45, Figure 4a), although with FC<2. A mouse study showed IL-33 protein production by satellite glial cells in DRGs, yet the cellular source of IL-33 in canine DRGs is still unknown to the best of the authors' knowledge.

RNAscope ISH showed strong *IL-33* mRNA expression in both healthy and atopic DRGs (Figure 4b). Notably, *IL-33* mRNA signals were mainly expressed on *GFAP*-positive satellite glial cells, with insufficient presence on *TUBB3*-positive cells to support the expression of *IL-33* mRNA on neurons. Owing to challenges in cell segregation, the exact count of positive cells was not feasible. By contrast, *IL-1RL1*, a subunit of the IL-33 receptor, was not detected by either RNA-Seq or RNAscope (Figure 4a,b).

Ingenuity pathway analysis

Canonical pathway analysis on neurotransmitters and other nervous system signalling unveiled significant upregulation of cAMP responsive element binding protein (CREB) signalling in neurons (z=2.50, p=6.16E-05), myelination signalling pathway (z=2.45, p=2.31E-02) and neuroinflammation signalling pathway (z=2.12,

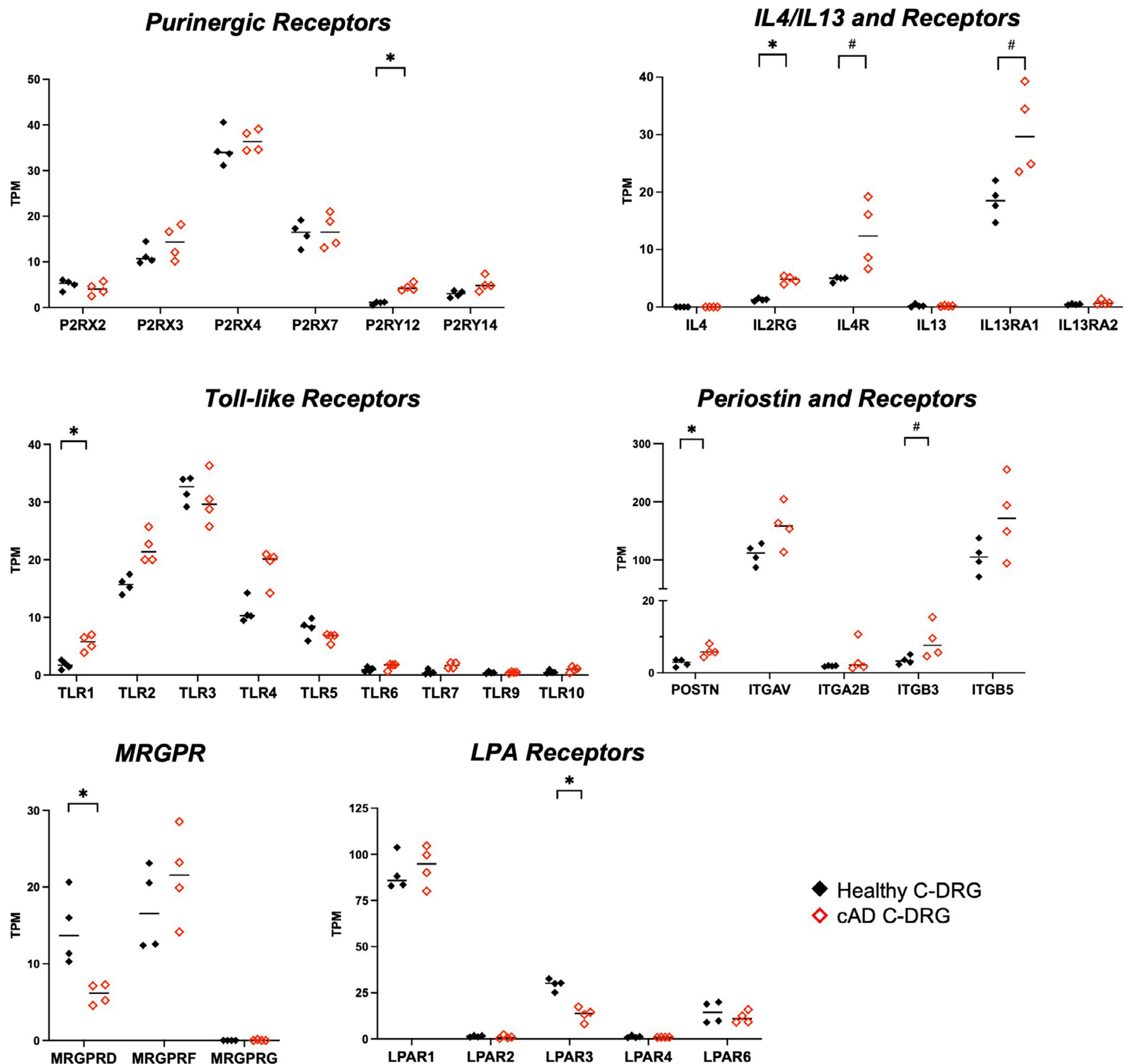

FIGURE 3 Genes showing significant differences in atopic dogs compared with healthy dogs. Each plot represents the TPM value of each sample, with lines (–) indicating the median TPM of each group. Asterisks (*) indicate common DEGs that showed statistically significant differences at |FC|>2, FDR ≦ 0.05, RPKM ≧ 1. Hashtags (#) indicate genes that did not fulfil all criteria for significant differences yet showed a tendency of difference (not common DEGs or 1.5<|FC|<2) that we still believe are interesting to point out. cAD, canine atopic dog; C-DRG, cervical dorsal root ganglia; DEG, differentially expressed gene; FC, fold change; FDR, false discovery rate; RPKM, reads per kilobase of exon per million mapped reads; TPM, transcripts per million.

p=2.98E-04; Figure 5). Table S4 presents the associated DEGs for each pathway.

DISCUSSION

Recent studies have implicated neuroimmune pathways in AD pathogenesis, yet their role in cAD is still unknown. In this study, we compared the transcriptomic profiles of DRGs between atopic and healthy dogs to identify molecules involved in cAD and neuroinflammation. Specifically, we aimed to investigate the expression of neuroimmune axis-related genes. Notably, the atopic dogs in this study did not have active skin lesions when samples were collected, ensuring that the observed differences in gene expression are not secondary to skin inflammation. We found significant differences in the expressions of *P2Y12*, *IL-2RG*, *TLR1*, *POSTN*, *MRGPRD*, *LPAR3* and, potentially *IL-33*, in atopic DRGs compared with healthy ones.

P2 purinergic receptors are activated by nucleotides, such as ATP, ADP, UTP and UDP, which are abundant in the nervous system and other tissues.[9] ATP, besides being an energy source, also functions as a pruriceptive neurotransmitter, initiating and sustaining neuronal excitability, and contributing to neuroinflammation.[10–13] In a mouse model of type 2 diabetes mellitus (DM), silencing or inhibiting *P2Y12* alleviated chronic itching and reduced reactive oxygen species, NLRP3 inflammasome, IL-1β and IL-18.[13] In our study, *P2Y12* was significantly upregulated in atopic dogs, suggesting a potential role in atopic itch. Additionally, we observed upregulation of

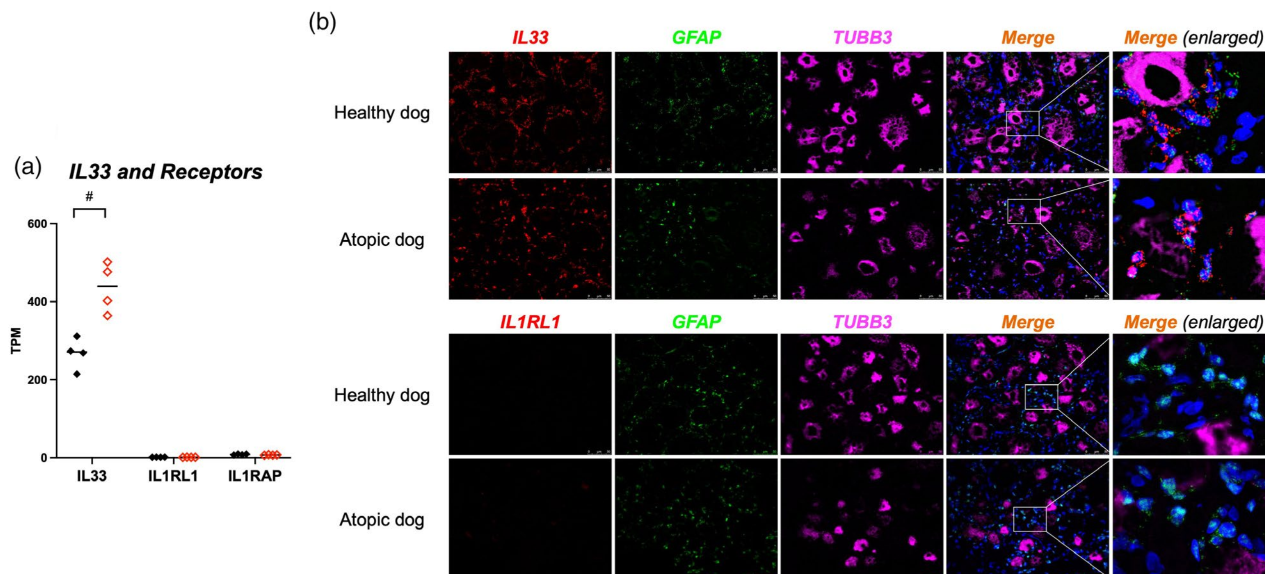

FIGURE 4 mRNA expression levels of *interleukin (IL)33* and its receptors by RNA-Seq (a) and 3-plex fluorescent RNAscope (b) in atopic and healthy dorsal root ganglia (DRGs). One each representative staining for atopic and healthy dogs showing *IL-33* signals (red) co-expressed on *GFAP* (glial cell marker, green)-positive cells but not on *TUBB3* (neuron cell marker and magenta)-positive cells. Conversely, the expression of its receptor (*IL-1RL1*, red) was not detected either by RNA-Seq or RNAscope in both atopic and healthy dogs. DAPI, 4′,6-diamidino-2-phenylindole; *GFAP*, glial fibrillary acidic protein; *TUBB3*, tubulin beta 3 class III.

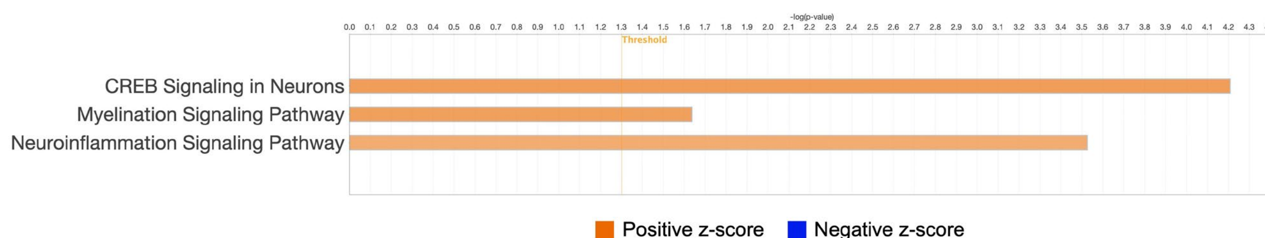

FIGURE 5 Ingenuity pathway analysis on neurotransmitters and other nervous system signalling. Based on our criteria of significant difference at $p<0.05$ ($-\log$ [p-value]>1.3) and $|z\text{-score}|>2$, we observed upregulation of CREB signalling in neurons, and the myelination signalling and neuroinflammation signalling pathways. Darker colours correlate with higher z-score. CREB, cAMP responsive element binding protein.

NLRP3 (FC=3.09, FDR=2.25E-4, max RPKM=0.996) and *IL-18* (FC=5.07, FDR=1.22E-6, max RPKM=3.50; Table S2) consistent with findings in a pruritic DM mouse model.[13] The exact role of *P2Y12* in canine AD remains unclear and needs further investigation.

IL-4 and IL-13, key T-helper 2 (Th2) cytokines, play a central role in both human and canine AD.[1,14] They induce scratching behaviour in mice and enhance neuronal responses to pruritogenic stimuli in human DRG neurons,[15,16] indicating their involvement in itch induction. However, their effects on itch in dogs are not well understood. We found significant upregulation of *IL-2RG*, a receptor subunit of IL-4 and other cytokines, in atopic DRGs. *IL-4R* (a shared receptor subunit for IL-4 and IL-13) and *IL-13RA1* (a receptor subunit for IL-13) showed elevated expression, yet did not meet our predefined criteria of significant difference, in DRGs of atopic compared with healthy dogs. Notably, our atopic dogs did not have active atopic flare-ups when samples were collected, implying that the upregulation was not the result of elevated levels of IL-4 and IL-13 in the skin. Instead, these findings suggest increased susceptibility to IL-4 and IL-13 stimuli.

Emerging evidence suggests that keratinocyte TLRs contribute to human AD by stimulating cytokine release and modulating tight junctions.[17] In mice, TLR3, TLR4 and TLR7 on DRG neurons regulate itch sensation in AD pathogenesis.[18] Here, we found significant upregulation of *TLR1* in atopic dogs, which recognises bacterial lipoprotein and glycolipids in complex with TLR2. Remarkably, a recent genome-wide association study identified *TLR1* as a candidate gene associated with cAD in Labrador retrievers.[19] Further research is needed to understand the role of TLR1 in sensory neurons in atopic dogs.

Periostin, an extracellular matrix protein, plays a pivotal role in tissue remodelling and repair.[20] Additionally, it has been implicated in chronic allergic inflammation in human AD patients and can directly activate itch sensory neurons, triggering itch behaviours across species, including dogs.[21,22] While periostin expression is elevated in the skin of dogs with cAD,[23–25] the present study is the first to demonstrate significant upregulation of *POSTN* mRNA in DRGs of atopic compared with healthy dogs. *ITGB3*, a receptor subunit, also showed upregulation in atopic DRGs, although it did not meet our criteria for significant difference owing to substantial individual difference between

the two atopic dogs. In mice, periostin is produced by satellite glial cells in DRGs and facilitates the migration of Schwann cells.[26] However, the cellular source of periostin in canine DRGs is unidentified. Further exploration is warranted to elucidate the role of periostin in DRGs in human and canine AD.

MrgprD is a G protein-coupled receptor, and nerve fibres expressing MrgprD terminate as free nerve endings in the epidermis.[27] Additionally, MrgprD is associated with histamine-independent chronic itch in humans.[28,29] We found significantly decreased *MRGPRD* expression in atopic dogs, suggesting a potential adaptive protective response against pruritogenic stimuli. Further investigation is needed to confirm this hypothesis. Notably, canine MrgprD is unresponsive to histamine, similar to humans,[30] yet its functional equivalence to human MrgprD remains uncertain owing to limited homology (76.4%).

LPARs (LPAR1-6) are a family of G protein-coupled receptors activated by lysophosphatidic acid (LPA), a bioactive phospholipid that is produced during the synthesis of cell membranes.[31] LPA has been linked to pruritus in human cholestatic patients through LPAR5 receptor activation.[32] However, pruritus associated with systemic diseases, such as cholestatic disorders, has not been reported in canine patients. Although *LPAR5* expression data were unavailable owing to its absence in the canine reference genome, we noted a significant downregulation of *LPAR3* in atopic dogs. While LPAR5 is widely acknowledged for its role in itch in humans, direct evidence of LPAR3's involvement in itch or AD remains elusive. It is interesting to note that a recent mouse study utilising single-cell RNA-Seq demonstrated that *LPAR3* and *LPAR5* were exclusively expressed on one of the three discrete populations of nonpeptidergic nociceptor DRG neurons (NP1) alongside other itch receptors, channels and neuropeptides, suggesting a potential role for LPAR3 in chronic itch, including AD.[15,33] However, it is still unclear whether this classification applies to dogs. Additionally, we lack a satisfactory explanation for the significant downregulation, instead of its upregulation, of *LPAR3* in atopic DRGs. In the aforementioned study, *MRGPRD* was likewise found to be exclusively expressed within the NP1 neuron population.[33] This decline in both *MRDPRD* and *LPAR3* expression in our atopic DRG samples might be caused by a reduction in the NP1 neuron population. Further investigation is required to validate this hypothesis and uncover its underlying cause.

IL-33 is constitutively expressed as a nuclear protein in epithelial tissues across organs, such as the lung, stomach and skin.[34] It is released extracellularly upon tissue damage, cell death or cell stress, acting as an endogenous danger signal (alarmin).[34] Accumulative evidence suggests that IL-33 is involved in human and canine AD.[35,36] However, a recent Phase 2b clinical trial in human AD using a humanised anti-human IL-33 monoclonal antibody (etokimab) failed to demonstrate treatment benefits over the placebo control group (NCT03533751), highlighting our incomplete understanding of the precise role of IL-33 in AD pathogenesis. We found a higher *IL-33* expression in atopic DRGs compared with the healthy control group, although it did not meet our significance criteria. Notably, this is the first report showing *IL-33* expression in DRGs of both healthy dogs and those with cAD. Interestingly, unlike dogs, our preliminary RNA-Seq analysis found very low *IL-33* in healthy human, murine and feline DRGs, suggesting the uniqueness of *IL-33* expression in canine DRGs. Although anti-IL-33 therapy failed in human AD, it may still be a therapeutic target in cAD, especially if targeting IL-33 in DRGs, where its role may differ from that in human AD. Furthermore, our RNAscope staining showed that *IL-33* is expressed on satellite glial cells in canine DRGs, regulating neuronal homeostasis and promoting regenerative growth in sensory neurons.[37,38] This finding aligns with a report in mouse DRGs,[39] and our study is the first to identify the cellular source of *IL-33* in canine DRGs. Although the IL-33 receptor expression has been detected in human and murine DRGs,[16,40] our RNA-Seq analysis and RNAscope staining failed to detect the IL-33 receptors mRNA expression in canine DRGs. This suggests a puzzling absence of cells capable of receiving the IL-33 signal. Further investigation is needed to determine the recipient cells of the IL-33 and to understand its role in canine DRGs.

In our study, CPA using IPA predicted the upregulation of three nerve system-related pathways. CREB signalling activation in neurons leads to various biological responses, such as neuronal excitation and proliferation. Myelin, a lipid-rich sheath enveloping long axons, is generated by Schwann cells in DRGs. The significance of upregulated CREB and myelination signalling in atopic DRGs is still unknown. Neuroinflammatory signalling plays a critical role in maintaining nervous system homeostasis, functioning in the removal of damaging agents and clearance of injured neural tissues. Although neuronal damage is unlikely in atopic dogs, upregulation of the neuroinflammatory signalling pathway may still occur as a consequence of the potential activation of neuroimmune circuits between the nervous system and the cutaneous immune system, potentially associated with these findings.

This study encountered several limitations, with the most prominent being the small sample size. Samples from atopic dogs were sourced from only two individuals, resulting in a total of four samples. Moreover, we found significant individual variability in gene expression between the two dogs, still leaving a question about whether this discrepancy stemmed from inherent individual differences or potentially from sex-specific factors. To address individual variation, we focused on identifying common DEGs. However, this approach may still lead to overestimation or underestimation of true DEGs. This experimental dog model utilised in this study was an inbred line of laboratory Maltese-beagle dogs, whose lineage traces back approximately 25 years to ancestors diagnosed with cAD. Given the unique characteristics of this colony and extremely limited opportunities for DRG collection, expanding the sample size proved challenging. Furthermore, while three DRGs were pooled for analysis in healthy dogs, two individual DRGs were collected and analysed separately for each atopic dog, and this decision to process the atopic dog samples separately

was based on the valuable nature of the samples and the need to minimise the risk of losing entire samples if processing or sequencing errors occurred. Additionally, RNA-Seq analysis does not allow for averaging TPM values of samples from the same dog as these values are calculated based on the total gene expressions of each individual sample, not the dog, which introduces pseudoreplication in the atopic group. We recognise that this decision may have impacted the statistical analysis.

Another limitation was the age range difference between atopic and healthy groups. While the exact ages of healthy dogs were not recorded, they were presumed to be adults based on their physical examination. By contrast, both atopic dogs were 13 years old and considered senior. Thus, the observed difference in gene expression in atopic dogs could potentially be attributed to age-related changes. In humans, the elderly population is more susceptible to chronic itch, which can stem from various pathophysiological mechanisms, including age-related degeneration of central or peripheral nerves.[41,42] For instance, aged mice (24 months old) were more susceptible to mechanically evoked itch compared with young mice (2 months old).[43] This increased in susceptibility is a result of the apoptosis of neuropeptide Y-producing neurons in the spinal dorsal horn, which counteract mechanical itch.[43] However, *NPY* (FC = 2.5, FDR = 0.34 and max RPKM = 0.72) expression did not differ significantly in atopic dogs. The impact of senile neuropathic change on chronic itch has yet to be investigated in companion animals. Additional transcriptome comparison using age-matched healthy dogs is warranted to mitigate potential age-related DEGs. However, despite several limitations, the use of atopic samples from dogs, combined with high throughput sequencing, provides unprecedented resources from a naturally occurring disease model, revealing molecules that may inform future research.

Finally, it is important to note that this study did not investigate gene expression differences during atopic flares, as the atopic dogs involved did not exhibit active skin lesions when samples were collected, although the microscopical skin inflammation was not examined owing to the lack of skin samples. As genes may be upregulated after initial atopic stimuli, owing to animal welfare concerns, we could not obtain DRG samples from an experimentally induced canine atopic model. Likewise, sampling from client-owned dogs with spontaneous cAD was challenging because these dogs are not typically necropsied. Although some genes may have been underestimated in this study, we believe that characterising gene expression in atopic dogs without active atopic flares can aid future research.

In conclusion, our analysis revealed distinct differential expression patterns in DRG transcriptional profiles of atopic versus healthy dogs, suggesting that neuroimmune pathways are involved in cAD, similar to human findings. Notably, genes, such as *P2Y12*, *IL-2RG*, *TLR1*, *POSTN*, *MRGPRD*, *LPAR3* and *IL-33*, warrant further investigation. These results provide a foundation for future studies to explore the functional implications of these genes in cAD potentially leading to new therapies.

AUTHOR CONTRIBUTIONS

Chie Tamamoto-Mochizuki: Conceptualization; methodology; software; data curation; investigation; validation; formal analysis; visualization; writing – original draft; writing – review and editing. **Santosh K. Mishra:** Conceptualization; methodology; validation; supervision; funding acquisition; project administration; resources; writing – review and editing.

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Self-funded.

CONFLICT OF INTEREST STATEMENT

No conflicts of interest have been declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NIH Genbank at https://www.ncbi.nlm.nih.gov/genbank/, reference number PRJNA1148493.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Zusammenfassung

Hintergrund: Juckreiz ist ein häufiges klinisches Zeichen bei Hauterkrankungen. Während die neuronalen Bahnen der Juckreiz Übertragung von der Haut ins Gehirn bei Nagern sehr gut bekannt sind, bleiben dieselben Bahnen bei Hunden unklar. Dieser Wissensmangel verhindert die Entwicklung von wirksamen Behandlungen für Erkrankungen des Hundes, die mit Juckreiz in Zusammenhang stehen.

Hypothese/Ziele: Diese Studie zielt darauf ab, unterschiedliche Genexprimierungen in den dorsalen Wurzelganglien (DRGs) zwischen gesunden und atopischen Hunden zu untersuchen, um spezifische Moleküle zu identifizieren, die beim Juckreiz Signal und bei der Neuroentzündung der caninen atopischen Dermatitis (cAD) involviert sind.
Tiere: Zwei atopische und vier gesunde Hunde.
Materialien und Methoden: DRGs wurden von atopischen und gesunden Hunden gesammelt, um ihre Transkriptionsprofile mittels RNA-Sequenzierung zu vergleichen.
Ergebnisse: Hauptkomponenten und Heatmap-Analyse zeigten zwei deutlich unterschiedliche Cluster, die atopische von gesunden Hunden trennten. Übereinstimmend mit dieser Beobachtung identifizierten wir 627 (543 hochregulierte, 84 hinunterregulierte) differenzial-exprimierte Gene (DEGs) bei atopischen im Vergleich zu gesunden Hunden. Weiters konnten wir die wichtigen Gene auf die gewöhnlichen DEGs bei jedem atopischen Hund eingrenzen, was 159 (132 hochregulierte, 27 hinunterregulierte) DEGs zeigte. Unter diesen Genen waren bei Konzentration auf Juckreiz-signalisierende Moleküle, *P2RY12*, *IL2RG*, *TLR1*und *POSTN* signifikant hochreguliert, während *MRGPRD* und *LPAR3* beide bei atopischen im Vergleich zu gesunden Hunden signifikant hinunterreguliert waren. Die Analyse dieser Bahnen zeigte eine signifikante Hochregulierung der CREB signalisierenden Neuronen, der Signalisierung der Myelinisierung und der signalisierenden Bahnen der Neuroinflammation bei atopischen Hunden.
Schlussfolgerungen und klinische Bedeutung: Unsere Studie weist darauf hin, dass eine Dysregulierung der neuroinflammatorischen Bahnen eine Rolle beim Pathomechanismus der cAD beim Menschen spielen könnte.

摘要
背景: 瘙痒是皮肤病的常见临床症状。虽然啮齿动物的瘙痒从皮肤传递到大脑的神经通路已得到充分了解，但犬的相同通路仍不清楚。知识差距阻碍了对犬瘙痒相关疾病有效治疗的开发。
假设/目标: 本研究旨在研究健康和过敏性犬的背根神经节 (DRGs) 中的差异基因表达，以确定可能参与犬过敏性皮炎 (cAD) 瘙痒信号传导和神经炎症的特定分子。
动物: 两只过敏性犬和四只健康犬。
材料和方法: 从过敏性和健康犬中收集 DRGs，使用 RNA 测序比较它们的转录谱。
结果: 主成分和热图分析揭示了将过敏性犬与健康犬区分开的两个不同簇。与此观察结果一致，我们在过敏性犬和健康犬中发现了 627 个(543 个上调，84 个下调)差异表达基因 (DEGs)。我们进一步将感兴趣的基因范围缩小到每只过敏性犬的常见DEGs，结果发现了159个(132个上调，27 个下调)DEGs。在这些基因中，当我们关注瘙痒信号相关分子时，与健康犬相比，过敏性犬的 P2RY12、IL2RG、TLR1 和 POSTN 显著上调，而 MRGPRD 和 LPAR3 均显著下调。通路分析显示，过敏性犬的神经元、髓鞘形成信号和神经炎症信号通路中的 CREB 信号显著上调。
结论和临床意义: 我们的研究表明，就像人类一样，神经炎症通路失调可能在 cAD 的发病机制中发挥作用。

Résumé
Contexte: Les démangeaisons constituent un signe clinique courant dans les affections cutanées. Alors que les voies neurales de la transmission des démangeaisons de la peau au cerveau sont bien comprises chez les rongeurs, les mêmes voies ne sont pas élucidées chez les chiens. Ce manque de connaissances entrave le développement de traitements efficaces pour les affections liées aux démangeaisonx chez le chien.
Hypothèse/Objectifs: Cette étude vise à étudier l'expression génique différentielle dans les ganglions de la racine dorsale (DRG) entre les chiens sains et les chiens atopiques afin d'identifier des molécules spécifiques potentiellement impliquées dans la signalisation des démangeaisons et la neuroinflammation dans la dermatite atopique canine (DAC).
Animaux: Deux chiens atopiques et quatre chiens sains.
Matériels et méthodes: Des DRG ont été prélevés chez des chiens atopiques et sains afin de comparer leurs profils transcriptionnels à l'aide du séquençage de l'ARN.
Résultats: Les analyses des composantes principales et des cartes thermiques ont révélé deux groupes distincts séparant les chiens atopiques des chiens sains. Conformément à cette observation, nous avons identifié 627 (543 gènes régulés à la hausse, 84 gènes régulés à la baisse) gènes différentiellement exprimés (DEG) chez les chiens atopiques par rapport aux chiens sains. Nous avons ensuite réduit le nombre de nos gènes d'intérêt aux DEG communs à chaque chien atopique, ce qui a révélé 159 DEG (132 régulés à la hausse, 27 régulés à la baisse). Parmi ces gènes, lorsque nous nous sommes concentrés sur les molécules associées à la signalisation des démangeaisons, P2RY12, IL2RG, TLR1 et POSTN étaient significativement régulés à la hausse, tandis que MRGPRD et LPAR3 étaient tous deux significativement régulés à la baisse chez les chiens atopiques par rapport aux chiens sains. L'analyse des voies a montré une augmentation significative de la signalisation CREB dans les neurones, de la signalisation de la myélinisation et des voies de signalisation de la neuroinflammation chez les chiens atopiques.
Conclusions et pertinence clinique: Notre étude suggère que la dysrégulation des voies neuroinflammatoires pourrait jouer un rôle dans le pathophysiologie de la DAC comme chez l'homme.

要約
背景: かゆみは皮膚疾患における一般的な臨床症状である。皮膚から脳へのかゆみ伝達の神経経路はげっ歯類ではよく理解されているが、イヌでは不明な点が多い。この知識のギャップが、犬のかゆみ関連疾患に対する効果的な治療法の開発を妨げている。

仮説/目的: 本研究の目的は、犬アトピー性皮膚炎(cAD)におけるかゆみシグナルと神経炎症に関与する可能性のある特定の分子を同定するために、健常犬とアトピー犬の後根神経節(DRG)における遺伝子発現の差を調べることであった。
対象動物: アトピー犬2頭および健常犬4頭。
材料と方法: アトピー犬および健常犬からDRGを採取し、RNAシーケンスを用いて転写プロファイルを比較した。
結果: 主成分分析およびヒートマップ分析により、アトピー犬と健常犬を分ける2つの異なるクラスターが明らかになった。この観察と一致して、アトピー犬では健常犬と比較して627個(543個が発現上昇、84個が発現下降)の発現差のある遺伝子(DEG)を同定した。さらに、各アトピー犬に共通するDEGに注目遺伝子を絞り込むと、159個(発現上昇132個、発現低下27個)のDEGが明らかになった。これらの遺伝子のうち、かゆみシグナル関連分子に注目すると、P2RY12、IL2RG、TLR1、POSTNが有意に発現上昇し、MRGPRDとLPAR3はともに健常犬に比べ有意に発現低下していた。パスウェイ解析の結果、アトピー犬では神経細胞におけるCREBシグナル伝達経路、髄鞘形成シグナル伝達経路、神経炎症シグナル伝達経路が有意に上昇していた。
結論と臨床的意義: 我々の研究は、神経炎症経路の調節異常が、ヒトと同様にcADの病態メカニズムに関与している可能性を示唆した。

Resumo
Contexto: O prurido é um sinal clínico comum em doenças de pele. Enquanto as vias neurais de transmissão do prurido da pele para o cérebro são bem compreendidas em roedores, as mesmas vias em cães permanecem pouco esclarecidas. Estas lacunas de conhecimento dificultam o desenvolvimento de tratamentos eficazes para doenças relacionadas pruriginosas em cães.
Hipótese/Objetivos: Este estudo visa investigar a expressão gênica diferencial nos gânglios da raiz dorsal (DRGs) entre cães saudáveis e atópicos para identificar moléculas específicas potencialmente envolvidas na sinalização do prurido e neuroinflamação na dermatite atópica canina (DAC).
Animais: Dois cães atópicos e quatro saudáveis.
Materiais e métodos: Os DRGs foram coletados de cães atópicos e saudáveis para comparar seus perfis transcricionais utilizando sequenciamento de RNA.
Resultados: As análises de componentes principais e de mapa de calor revelaram dois grupos distintos dividindo cães atópicos de saudáveis. Consistente com essa observação, identificamos 627 (543 regulados positivamente, 84 regulados negativamente) genes diferencialmente expressos (DEGs) em cães atópicos em comparação com cães saudáveis. Reduzimos ainda mais nossos genes de interesse para DEGs comuns em cada cão atópico, o que revelou 159 DEGs (132 regulados positivamente, 27 regulados negativamente). Entre esses genes, quando focamos em moléculas associadas à sinalização do prurido, *P2RY12, IL2RG, TLR1 e POSTN* foram significativamente regulados positivamente, enquanto *MRGPRD e LPAR3* foram significativamente regulados negativamente em cães atópicos em comparação com aqueles em cães saudáveis. A análise da via mostrou uma regulação positiva significativa da sinalização de CREB em neurônios, sinalização de mielinização e vias de sinalização de neuroinflamação em cães atópicos.
Conclusões e relevância clínica: Nosso estudo sugeriu que a desregulação das vias neuroinflamatórias pode desempenhar um papel no patomecanismo da DAC como em humanos.

RESUMEN
Introducción: El prurito es un signo clínico común en los trastornos de la piel. Si bien las vías neuronales de transmisión del prurito desde la piel hasta el cerebro se conocen bien en los roedores, las mismas vías en los perros siguen sin estar claras. La brecha de conocimiento obstaculiza el desarrollo de tratamientos efectivos para los trastornos relacionados con el prurito canino.
Hipótesis/Objetivos: Este estudio tiene como objetivo investigar la expresión genética diferencial en los ganglios de la raíz dorsal (DRG) entre perros sanos y atópicos para identificar moléculas específicas potencialmente involucradas en la señalización del prurito y la neuroinflamación en la dermatitis atópica canina (cAD).
Animales: Dos perros atópicos y cuatro perros sanos.
Materiales y métodos: Se recolectaron DRG de perros atópicos y sanos para comparar sus perfiles transcripcionales mediante secuenciación de ARN.
Resultados: Los análisis de componentes principales y mapas de calor revelaron dos grupos distintos que separan a los perros atópicos de los sanos. En consonancia con esta observación, identificamos 627 genes expresados de forma diferencial (DEGs) (543 sobreexpresados y 84 infraexpresados) en perros atópicos en comparación con perros sanos. Además, redujimos nuestros genes de interés a los DEGs comunes en cada perro atópico, lo que reveló 159 DEGs (132 sobreexpresados y 27 infraexpresados). Entre estos genes, cuando nos centramos en las moléculas asociadas a la señalización del picor, P2RY12, IL2RG, TLR1 y POSTN se sobreexpresaron significativamente, mientras que MRGPRD y LPAR3 se infraexpresaron significativamente en perros atópicos en comparación con los perros sanos. El análisis de las vías mostró una sobreexpresion significativa de la señalización de CREB en neuronas, la señalización de mielinización y las vías de señalización de neuroinflamación en perros atópicos.
Conclusiones y relevancia clínica: Nuestro estudio sugirió que la disregulación de las vías neuroinflamatorias podría desempeñar un papel en el patomecanismo de la cAD Al igual que en los humanos.