

Article

Th2 Cytokines Reshape the Transcriptome: Insights from a Canine Organoid Model of Atopic Dermatitis

Bo Chen ¹, Yuanting Zheng ², Ron Slocombe ¹ and Smitha Rose Georgy ^{1,*}

¹ Section of Anatomic Pathology, Melbourne Veterinary School, Faculty of Science, University of Melbourne, Werribee, VIC 3030, Australia; bcchen1@student.unimelb.edu.au (B.C.)

² Department of Veterinary Biosciences, Melbourne Veterinary School, Faculty of Science, The University of Melbourne, Parkville, VIC 3010, Australia

* Correspondence: s.georgy@unimelb.edu.au

Abstract

Atopic dermatitis (AD) and canine atopic dermatitis (CAD) are common allergic and pruritic skin diseases characterized by immune dysregulation and epidermal barrier dysfunction. To delineate how Th2 cytokines contribute to CAD pathogenesis, canine primary epidermal organoids (cPEOs) were established from keratinocytes, and exposure to IL-4/IL-13 induced morphologic changes characteristic of CAD. RNA sequencing analysis comparing IL-4/IL-13-treated cPEOs to untreated controls identified 224 differentially expressed genes (DEGs). Further rigorous filtering narrowed this down to 69 key DEGs, with the majority being associated with atopic dermatitis in both dogs and humans. Pathway enrichment analyses demonstrated the activation of immune and inflammatory signalling and suppression of epidermal differentiation, keratinisation, and lipid metabolism, recapitulating key features of atopic skin. Additional Th2-driven alterations included dysregulation of neuro-immune signalling, calcium homeostasis, apoptosis, extracellular matrix remodelling, and metabolic/epigenetic regulations. Together, these findings demonstrate that Th2 cytokines orchestrate multifaceted transcriptomic alterations relevant to AD/CAD. By mapping each key DEG to its known or putative role in AD/CAD, this study also provides a gene-level functional framework to inform future mechanistic studies and targeted therapeutic development. These findings also underscore the value of this model as a comparative tool for investigating both human and canine atopic dermatitis.

Keywords: canine epidermal organoids; canine atopic dermatitis; Th2 cytokines; IL-4; IL-13; RNA sequencing; molecular signature; therapeutic target

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1. Introduction

Canine atopic dermatitis (CAD) is a chronic allergic inflammatory skin disorder in which genetic susceptibility and environmental triggers interact to dysregulate immune responses and compromise epidermal barrier integrity [1]. The pathogenesis of this disease is still being actively investigated [1]. As in human atopic dermatitis (AD), two hypotheses were proposed to explain the pathogenesis, named the ‘outside-in’ hypothesis and the ‘inside-out’ hypothesis [2]. The ‘outside-in’ hypothesis suggests that impairment of the skin barrier, because of a genetic defect in skin barrier formation or changes in the environment, would lead to sensitization and subsequent development to AD [2].

Consistent with this genetic background, breed predisposition to AD is well established [3]. Conversely, the ‘inside-out’ hypothesis indicates that an intrinsic immunological imbalance predisposes individuals to atopy and that Th2 (T helper type 2)-skewed inflammation further disrupts the skin barrier [2].

The epidermal component, primarily composed of keratinocytes, plays a central role in maintaining skin barrier integrity and regulating immune responses, and its dysfunction is closely linked to various stages of AD in humans and dogs, including predisposition, onset, progression, and therapy [4,5]. Canine primary epidermal organoids (cPEOs), which are composed exclusively of keratinocytes, provide a simplified and highly controlled system for investigating the responses of this dominant epidermal cell type to the pathogenetic triggers [6]. Using this canine epidermal organoid culture system, acute CAD-like morphologic and molecular features induced by Th2 cytokines (IL-4/IL-13) were identified in our previous study, including epidermal spongiosis and impaired suprabasal differentiation in histological examination; reduced expressions of differentiated markers such as filaggrin and loricrin at the transcriptional and protein levels; and increased epidermal proliferation as evidenced by Ki67 immunostaining [6]. Th2 cytokine (IL-4/IL-13)-treated cPEOs enabled a controlled assessment of Th2 cytokine-induced effects on the epidermal barrier, allowing for an evaluation of their direct impact in a simplified organoid model. In this study, genomic profiling of this model, with and without Th2 cytokine (IL-4/IL-13) stimulation, was performed by RNA sequencing (RNA-Seq) to elucidate the transcriptional regulation of key factors involved in this induction (the ‘inside-out’ hypothesis). The goal of this study was to identify critical genes and molecular pathways associated with the IL-4/IL-13 cytokine axis in CAD pathogenesis, with the potential to reveal novel therapeutic targets and guide future treatment strategies while establishing a comparative model for investigating cross-species mechanisms shared between AD and CAD.

2. Results

2.1. RNA-seq Data Analysis

Organoids established from four different dogs were treated with and without IL-4/IL-13 cytokines for 7 days and subsequently used for RNA sequencing. The average number of sequencing reads obtained per sample was 105.59 million, with a range of 86.36 to 128.00 million reads. An average of 76.85% of the read pairs (range: 70.36–80.46%) were mapped to the canine reference genome (Supplementary File S1). A total of 36682 genes were annotated in each organoid sample. Principal component analyses (PCAs) and multi-dimensional scaling (MDS) visualized the overall gene expression per individual (Figure 1). The MDS plot illustrates the differences in gene expression profiles between cytokine-treated and control organoids, primarily along the first MDS dimension (Dim 1, 31%). In contrast, individual genetic differences, rather than variables such as age, sex, neuter status, or sample location, contributed to the distinct clustering or variation observed along the second MDS dimension (Dim 2, 24%). A visualization of the expression of all genes across the samples by the heatmap is provided in Supplementary File S2.

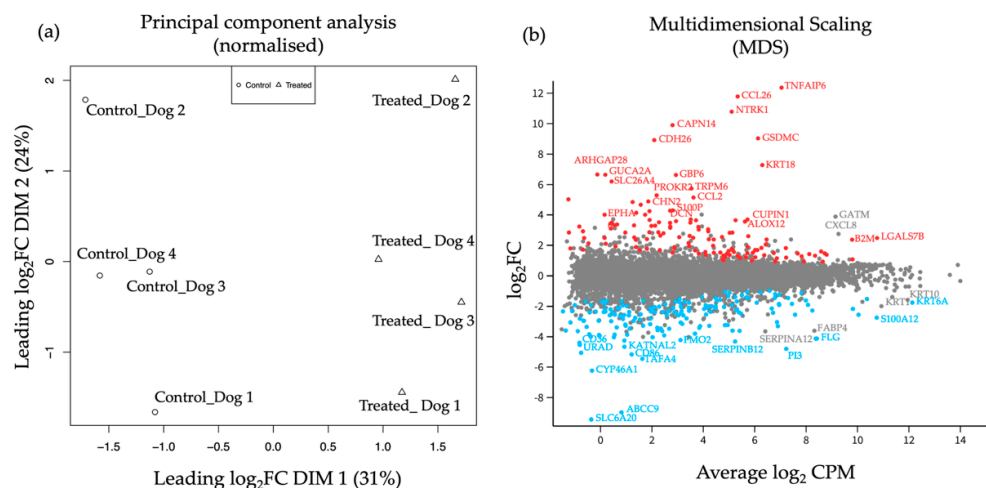


Figure 1. Principal component analysis (PCA, (a)) and multidimensional scaling (MDS, (b)) of cytokine-treated organoid samples versus controls. The PCA plot shows a clear separation between the control and treated groups. In the MDS plot, each point represents a transcript, with the x-axis indicating relative transcript abundance ($\log_2$ counts per million, $\log_2$ CPM) and the y-axis showing the $\log_2$ fold change ($\log_2$ FC) between the treated and control groups. Significantly upregulated differentially expressed genes are highlighted in red, while significantly downregulated genes are shown in blue. FC = fold change; DIM = dimension.

2.2. Differential Expression Analysis

Applying the criteria of $|\log_2\text{FC}| > 1$, $\text{FDR} \leq 0.05$ and $\log_2\text{CPM} > 1$, 224 differentially expressed genes (DEGs) were identified in cytokine-treated versus control samples. These included 107 upregulated and 117 downregulated genes, with $|\log_2\text{FC}|$ values ranging from 1.011 to 12.357 (Supplementary File S3).

A stringent set of criteria was applied to identify 76 DEGs, defined by $\text{FDR} \leq 0.01$, $|\log_2\text{FC}| > 2$, and $\log_2\text{CPM} > 2$. Among these 76 DEGs, 7 genes were poorly annotated and not well characterized in the reference genome. The remaining 69 DEGs (36 upregulated and 33 downregulated genes) were retained and designated as key DEGs in this study. To further associate the mechanisms underlying CAD, key DEGs were organized into 11 major functional categories reflecting the multi-dimensional Th2 cytokine-driven transcriptional landscape (Figure 2). The 36 upregulated genes were functionally grouped into six categories: immune activation and antigen presentation, neuro-immune, calcium homeostasis, cell death and extracellular matrix (ECM) remodelling, metabolic and epigenetic influences, and poorly defined functions. Similarly, the 33 downregulated genes were assigned to five categories: skin barrier and keratinisation function, antimicrobial defence, cell death and ECM remodelling, metabolic and epigenetic influences, and poorly defined functions. The putative function of key DEGs and its relevance in CAD/AD pathogenesis is given in Table 1.

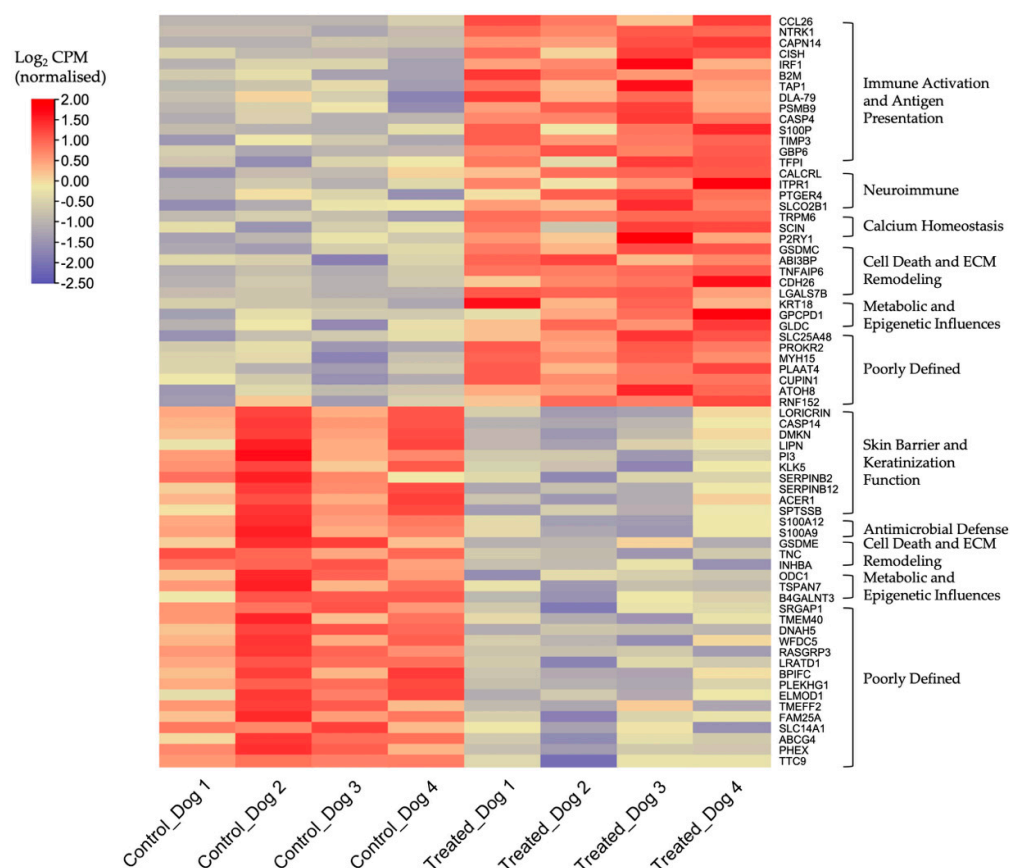


Figure 2. Heatmap of 69 identified key differentially expressed genes (DEGs): Genes are regrouped based on their reported functions or putative associations with atopic dermatitis.

Table 1. Functional summary of key differentially expressed genes (DEGs) following IL-4/IL-13 stimulation in cPEOs.

Gene	Direction log ₂ FC	Function/Pathway	Relevant further research in CAD/AD ¹	DGE in CAD? (Y/N) ²	Relevance to CAD/AD	Ref.
Immune Activation and Antigen Presentation						
<i>CCL26</i>	↑ 11.78	Eosinophil chemoattractant; Th2/JAK-STAT6 target	CAD/AD	Y	Promotes eosinophil-dominant inflammation	[7,8]
<i>NTRK1</i>	↑ 10.78	NGF receptor; neuro-immune signalling	AD	Y	Enhances CCL26 expression and sustains allergic inflammation	[7,9]
<i>CAPN14</i>	↑ 9.90	Epithelial protease induced by IL-13/JAK-STAT6	AD	Y	Implicated in eosinophilic esophagitis (EoE)	[10,11]
<i>CISH</i>	↑ 2.55	Negative regulator of JAK-STAT signalling	AD	Y	Modulates Th2 cytokine axis and eosinophilic inflammation	[12]
<i>IRF1</i>	↑ 2.83	Type I IFN transcription factor	AD	Y	Drives antigen presentation and antiviral response; prognostic biomarker of AD	[13,14]
<i>B2M</i>	↑ 2.37	MHC class I structural component	AD	Y	Supports immune activation	[15,16]
<i>TAP1</i>	↑ 2.38	MHC class I peptide transporter	AD	Y	Enhances antigen presentation	[15,17]
<i>DLA-79</i>	↑ 3.71	Non-classical MHC Ib molecule	CAD	Y	Linked to immune-mediated diseases in dogs	[18]
<i>PSMB9</i>	↑ 3.49	Immunoproteasome subunit	-	Y	Generates MHC class I peptides; reported in mild CAD	[19]

<i>CASP4</i>	↑ 3.03	Non-canonical inflammasome activator	-	Y	Mediates pyroptosis in keratinocytes	[20]
<i>S100P</i>	↑ 4.30	Calcium-binding protein	CAD/AD	Y	Reported in CAD; inconsistent in human AD	[21]
<i>TIMP3</i>	↑ 3.15	MMP inhibitor	-	Y	Regulates inflammation and ECM turnover	[22]
<i>GBP6</i>	↑ 6.63	IFN-γ inducible GTPases	-	N	Involves in tumour progression and pathogen defences	[23]
<i>TFPI</i>	↑ 2.49	Endogenous anticoagulant	-	Y	Function in keratinocytes unclear	[24]
Skin Barrier and Keratinisation Function						
<i>LOR</i>	↓ -3.31	Cornified envelope protein	CAD/AD	Y	Barrier integrity	[25]
<i>CASP14</i>	↓ -4.12	Corneocyte apoptosis enzyme	AD	Y	Terminal differentiation and desquamation	[25]
<i>DMKN</i>	↓ -2.28	Dermokine	AD	Y	Barrier maturation	[25]
<i>LIPN</i>	↓ -2.59	Lipase	AD	N	Lipid metabolism in stratum corneum	[25]
<i>PI3</i>	↓ -4.80	Protease inhibitor; Th17/Th22-linked	AD	Y	Common hub gene in AD	[26]
<i>KLK5</i>	↓ -3.60	Serine protease in stratum corneum	AD	Y	Epidermal desquamation	[27]
<i>SerpinB2/B12</i>	↓ -2.14/-4.31	Protease inhibitors in stratum corneum	AD	Y (SerpinB2)	Protect from proteolytic damage	[28]
<i>ACER1</i>	↓ -3.16	Alkaline ceramidase	AD	Y	Sphingolipid metabolism	[29,30]
<i>SPTSSB</i>	↓ -3.24	Sphingolipid biosynthesis	AD	N	Barrier lipid impairment	[31]
Neuro-immune						
<i>CALCRL</i>	↑ 3.48	CGRP receptor component	AD	Y	Hub gene; linked to neurogenic inflammation and pruritus	[32,33]
<i>ITPR1</i>	↑ 2.92	Intracellular calcium release channel	-	Y	Neurological disorders	[34]
<i>PTGER4</i>	↑ 3.52	Prostaglandin E2 receptor	AD	Y	Modulates neuro-immune crosstalk	[35–37]
<i>SLCO2B1</i>	↑ 3.23	Solute carrier transporter	-	N	Hub gene in systemic sclerosis; possible metabolic regulation	[38]
Calcium Homeostasis						
<i>TRPM6</i>	↑ 5.74	Transient Receptor Potential (TRP) family	AD	N	Regulates calcium and magnesium signals; may affect itch pathogenesis	[39]
<i>SCIN</i>	↑ 3.59	Actin-severing protein	-	N	Calcium-activated; proposed asthma biomarker	[40]
<i>P2RY1</i>	↑ 2.39	Purinergic receptor	-	Y	ADP-induced calcium mobilization	[41]
Antimicrobial Defence						
<i>S100A12/S100A9</i>	↓ -2.74/-2.17	Antimicrobial calcium-binding proteins	CAD/AD	Y	Major DEGs in CAD; temporally regulated	[42–44]
Cell Death and ECM Remodelling						
<i>GSDMC</i>	↑ 9.03	Gasdermin family protein	AD	Y	Mediates pyroptosis	[45]
<i>GSDME</i>	↓ -2.09	Gasdermin family protein	-	N, upregulated	Tumour suppressor	[46]
<i>ABI3BP</i>	↑ 2.60	Extracellular matrix-associated protein promoting cell adhesion	-	N	Associated with asthma; involved in matrix remodelling	[47]
<i>TNC</i>	↓ -2.05	ECM glycoprotein	AD	Y	Upregulated in human AD lesions; regulates adhesion and wound healing	[48]
<i>INHBA</i>	↓ -3.23	TGF-β superfamily ligand	CAD/AD	N	Modulates fibroblast activation and remodelling	[49]

<i>TNFAIP6</i>	↑ 12.36	Hyaluronan-binding glycoprotein	AD	Y	Modulates ECM stability and leukocyte migration; linked to hematopoietic malignancies in dogs	[50,51]
<i>CDH26</i>	↑ 8.92	Epithelial cadherin family adhesion molecule	AD	N	Th2-responsive; regulates epithelial remodelling	[52]
<i>LGALS7B</i>	↑ 2.48	Galectin family lectin	AD	N	Involved in Th2-biased epithelial responses and repair	[53]
Metabolic and Epigenetic Influences						
<i>KRT18</i>	↑ 7.28	Cytokeratin; stress-induced	-	N	DNA hypomethylation; linked to mammary tumours in dogs	[54,55]
<i>GPCPD1</i>	↑ 2.05	Glycerophosphodiester phosphodiesterase	-	N	Response to osmotic stress; potential barrier adaptation	[56]
<i>GLDC</i>	↑ 3.65	Glycine decarboxylase	-	Y	Reflects metabolic reprogramming in inflamed keratinocytes	[57]
<i>ODC1</i>	↓ -2.13	Ornithine decarboxylase; polyamine synthesis enzyme	-	Y	Regulates keratinocyte differentiation and antimicrobial peptide production	[58]
<i>TSPAN7</i>	↓ -3.91	Tetraspanin superfamily protein	AD	Y	Linked to cell adhesion and DNA methylation	[59]
<i>B4GALNT3</i>	↓ -2.30	Modify various kinds of glycoproteins	-	Y	involved in the clearance of N-glycoproteins; role in keratinocytes unreported	[60]

¹ Relevant research is provided in the corresponding references (Ref.). ² DEGs were compared with gene lists in prior studies [9,19,61]. ↑, upregulation; ↓, downregulation.

2.3. Pathway Analysis

Using ShinyGO 0.85.1, 103 (96%) upregulated and 104 (89%) downregulated genes were mapped to the canine ENSEMBL genome following input of the respective DEG lists. Due to differences in pathway annotation frameworks and biological relevance, interpretation was focused on Gene Ontology (GO) for Biological Process (BP), KEGG, and Reactome analyses, which together provide complementary insights into functional processes, canonical signalling pathways, and mechanistic molecular events. The top 20 pathways from upregulated GO for BP and downregulated Reactome pathways or full sets from other pathway analyses are displayed in Figure 3.

GO for BP revealed that the 101 upregulated pathways were primarily associated with immune responses, whereas 14 downregulated pathways were mainly related to epidermal development and lipid metabolism in keratinocytes (Supplementary File S4). KEGG analysis identified six upregulated pathways and one downregulated pathway, and KEGG results reflected the upregulation of mineral absorption, TRP channels of regulations, NOD-like receptor signals, and cGMP-PKG signalling pathways (Supplementary File S5). The downregulation of metabolic pathways reflects reduced metabolic activity during epidermal development. Reactome analysis revealed 20 upregulated pathways, and 25 downregulated pathways and results were largely aligned with GO for BP and KEGG findings (Supplementary File S6).

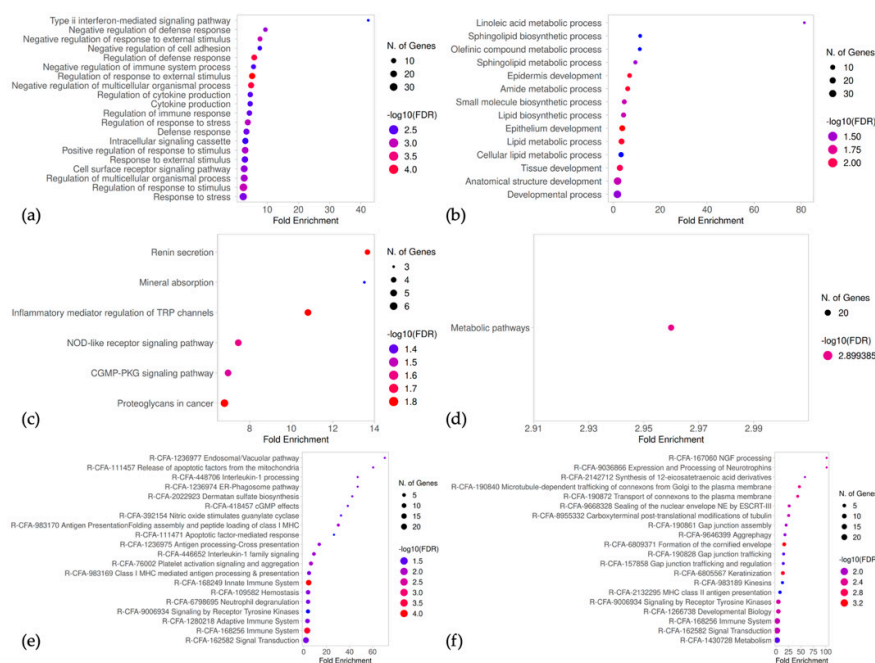


Figure 3. Biological function and pathway enrichment analyses of all significant differentially expressed genes (DEGs). Panels show enriched Gene Ontology (GO) enrichment for Biological Process (BP) (a,b), KEGG pathways (c,d), and Reactome pathways (e,f). Panels (a,c,e) represent upregulated pathways, while (b,d,f) represent downregulated pathways.

2.4. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Five upregulated DEGs (*B2M*, *CCL2*, *CCL26*, *NTRK1*, and *PTGER4*) and five downregulated DEGs (*FLG*, *LOR*, *KLK5*, *S100A12*, and *S100A9*) were selected for RT-qPCR validation. The fold changes observed by RT-qPCR were consistent with those from RNA-seq, showing significant increases or decreases in the same direction (Figure 4a). Pearson correlation analysis confirmed strong $\log_2FC$ consistency between RT-qPCR and RNA-seq results ($r = 0.99$, $p < 0.0001$), supporting the validity of the RNA-seq data (Figure 4b).

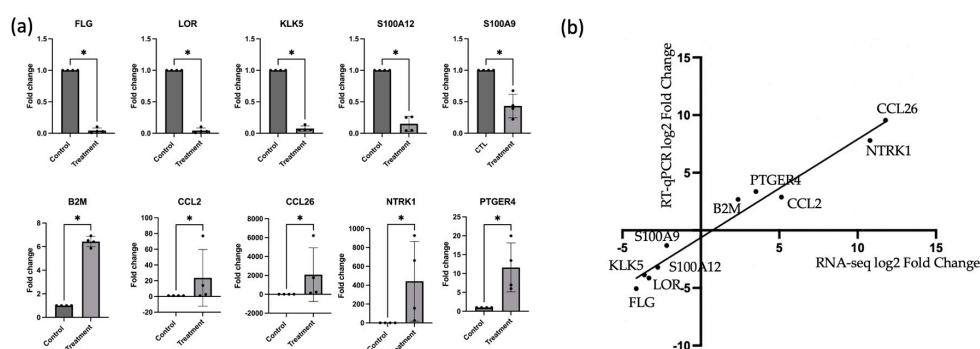


Figure 4. Validation of RNA-seq results by reverse transcription quantitative PCR (RT-qPCR). (a) Relative gene expression of ten representative differentially expressed genes (*B2M*, *CCL2*, *CCL26*, *NTRK1*, *PTGER4*, *FLG*, *LOR*, *KLK5*, *S100A12*, and *S100A9*) were quantified by RT-qPCR in the control and treatment groups. Data are presented as mean $\pm$ SEM. The data were analyzed using the nonparametric *t*-test. * $p < 0.05$. (b) Pearson correlation analysis between RNA-seq $\log_2$ -fold changes and RT-qPCR $\log_2$ -fold changes in ten genes, showing a consistent direction of regulation.

3. Discussion

This study demonstrates how IL-4 and IL-13, the signature Th2 cytokines, function as key initiators in reprogramming keratinocyte transcriptomes using canine epidermal organoids. To reduce potential breed-associated genetic bias, donor healthy skin samples were obtained from four dogs of different breeds (Table 2). Gene-level analysis enabled the interpretation of individual key DEGs in relation to their known or putative roles in AD/CAD within the Th2 axis (Figure 2 and Table 1). To characterize the functional consequences of these transcriptomic changes, three complementary enrichment analyses were employed: GO for BP for broad biological functional classification [62], KEGG for the identification of genetic processing and metabolic pathways [63], and Reactome for pathway-level insights [64], with a focus on signalling cascades predominantly curated from human systems. These results offer mechanistic insights into how Th2 cytokines orchestrate the complex and multifactorial pathology in CAD.

Table 2. Details of canine samples used for organoid culture and RNA extraction.

Animal/Organoid No.	Breed	Age	Sex/Neuter Status	Sample Location
1	Rough Collie	12y 9m	Male, Neutered	Scrotal skin
2	American Staffordshire Terrier	2y 2m	Female, Spayed	Thigh skin
3	Rhodesian Ridgeback	9y 4m	Male, Neutered	Right thoracic limb skin
4	Bernese Mountain Dog	1m	Female, Intact	Abdominal skin

3.1. Immune Activation and Antigen Presentation

Gene transcription profiles from both experimentally sensitized and naturally affected atopic dogs demonstrated activated immune and inflammatory responses including JAK/STAT and pro-inflammatory pathways [9,21,61], which aligns with the transcriptomic findings of our organoid model system.

Key genes identified in Table 1 support the involvement of canonical JAK/STAT6-dependent signalling downstream of IL-4/IL-13 stimulation. These include *CCL26*, a potent eosinophil chemoattractant, and *NTRK1*, the high-affinity receptor for NGF that reinforces neuro-immune feedback loops, both regulated by STAT6 [7]. *CAPN14*, an IL-13-inducible epithelial protease, is mediated by STAT6 and implicated in eosinophilic inflammation in mucosal tissues [10]. They may serve as a potential target for CAD therapeutic intervention. Notably, *CISH*, encoding a negative regulator within the SOCS family, was also induced. Although *CISH* limits JAK kinase activity via SH2-domain competition, its upregulation in allergic contexts has been associated with persistent eosinophilic inflammation [12]. Importantly, these gene-level associations were not captured by pathway enrichment analyses, highlighting the added value of targeted, literature-informed gene-level interpretation.

Pathway-level analyses revealed a broader immune activation landscape dominated by pro-inflammatory responses. GO for BP highlighted interferon-related and defence-response pathways, MHC class I antigen processing, and macrophage activation. KEGG analysis identified the NOD-like receptor signalling pathway, indicating engagement of innate immunity receptors. Reactome analysis revealed enrichment of IL-1 processing, MHC class I antigen processing, and additional innate immune pathways. A key gene related to immune activation and antigen presentation that was upregulated in cytokine-treated organoids was *IRF1* (Table 1). *IRF1* is a master transcription factor for interferon (IFN) responses [13] and a novel pyroptosis-related prognostic biomarker of AD [14]. *IRF1* transcriptionally regulates *TAP1*, *PSMB9*, and *B2M*, key components of the MHC class I processing machinery, thereby enhancing antigen presentation potential in keratinocytes

[15–17]. Among these genes, *PSMB9* was also identified as a DEG in a transcriptomic study using skin biopsies from atopic dogs [19]. *DLA-79*, a canine non-classical MHC class Ib gene, was also upregulated in cytokine-treated organoids compared to controls. Although its precise function remains unclear, its reported association with immune-mediated diseases and detection as a DEG in clinical atopic dogs suggests potential relevance to CAD [18,19]. The 3-fold induction of *CASP4*, a non-canonical inflammasome activator under IRF1 control [20] and additional responsive genes such as *S100P* and *TIMP3* further underscore the engagement of inflammatory stress pathways. Other transcripts involved in the inflammatory responses such as *TFPI* and *GBP6* were also detected but their specific roles in CAD remain unclear. Th2-polarized enhancement of antigen presentation in atopic skin facilitates immune activation by increasing responsiveness to allergens that penetrate through the defective skin barrier, representing an early upstream event in disease pathogenesis. Together, the findings support a keratinocyte phenotype that is both Th2-biased and stress-activated, potentially contributing to the inflammatory persistence characteristic of CAD.

3.2. Suppression of Epidermal Barrier Formation

The transcriptional suppression of skin barrier-associated pathways was equally striking. GO for BP and Reactome analyses revealed the downregulation of multiple pathways associated with keratinocyte differentiation, the formation of a cornified envelope, gap junction functions, and lipid metabolism. Key structural genes including *LOR*, *FLG*, *CASP14*, *DMKN*, and *LIPN* were suppressed with cytokine treatment, undermining cornified envelope formation, hydration, and lipid processing. *PI3*, a gene recently identified as a common hub connecting atopic dermatitis and ulcerative colitis in humans [26], was found to be one of the crucial genes associated with cornified envelope formation and was downregulated by 4.7 folds in cytokine-treated organoids. Moreover, the downregulation of *KLK5* and protease inhibitors *SerpinB2* and *SerpinB12* suggests a shift toward proteolytic imbalance, predisposing the barrier to enzymatic damage. Sphingolipid-related enzyme genes *ACER1* and *SPTSSB* were also reduced, providing a direct molecular explanation for lipid barrier impairment [29–31]. In addition, downregulation of the arachidonic acid and eicosanoid metabolism pathway, reflected by DEGs such as *ALOX12*, *ALOX12B*, and *ALOXE3*, was noted, indicating a broader disruption of epidermal lipid structures and inflammatory lipid mediators. Together, these defects point to compromised epidermal integrity and altered lipid composition, which are hallmarks of AD and CAD skin. Whether such defects represent a primary barrier abnormality or arise secondary to inflammation remains a debatable topic [2], possibly varying between individuals due to the genetically heterogeneous nature of the disease [65] and mutually exacerbating one another. However, the transcriptional patterns observed in this model suggest that at least a subset of these barrier abnormalities may be secondary to immune-mediated inflammation rather than intrinsic structural defects.

3.3. Interconnected Neuro-Immune-Calcium Triad

Neuro-immune alterations emerged as a prominent component of the Th2-driven transcriptome in our organoid model. Specifically, the upregulation of *NTRK1* and *CALCRL* points to the involvement of NGF- and CGRP-mediated pathways, which are known for enhancing pruritus and neurogenic inflammation [32,33]. Additional Th2-responsive targets, including *ITPR1*, *PTGER4*, and *SLCO2B1*, further implicate already established neuro-epidermal crosstalk in CAD pathophysiology [34,35,37,38]. IL-4/IL-13 also altered the expression of *TRPM6*, *SCIN*, and *P2RY1* genes in cPEOs involved in calcium influx [39], actin remodelling [40], and purinergic signalling [41], respectively. The enrichment of the TRP channel pathway identified by KEGG analysis suggests a close

interplay between itch signalling and calcium imbalance. Targeting TRP channel activity may represent a promising therapeutic approach for CAD. Concomitantly, genes encoding antimicrobial calcium-binding S100 proteins (*S100A12*, *S100A9*, to a lesser extent, *S100A8*, and the Th17/Th22 pathway) were downregulated, consistent with a negative regulation of the defence response revealed by the GO (BP) analysis, which may transiently diminish innate immune defences [42,43]. These findings may explain the susceptibility to microbial colonization observed in acute CAD lesions.

3.4. Alterations in Cell Death Programmes and Extracellular Matrix Remodelling

Pathways associated with cell death and extracellular matrix (ECM) remodelling were significantly altered. KEGG enrichment for apoptosis was supported by gene-level changes, including the upregulation of *GSDMC* and downregulation of *GSDME*, indicating a selective activation of inflammatory cell death mechanisms. Pyroptosis has been implicated in the pathogenesis of AD, characterized by an increased expression of *GSDMC* and *GSDMD* [66]. ECM remodelling genes *ABI3BP*, *TNC*, and *INHBA* were induced, consistent with chronic inflammation-associated fibrosis and altered keratinocyte–matrix interactions [47–49]. *TNFAIP6*, a hyaluronan-binding protein with roles in inflammation, ECM stabilization, and cell migration, was also upregulated, aligning with its reported associations with immune dysregulation in dogs and poor prognostic outcomes in human cancers [50]. *CDH26* and *LGALS7*, both associated with Th2-biased inflammation, may further modulate immune cell adhesion and epithelial remodelling [52,53]. These shifts collectively support the notion that chronic Th2 signalling reshape the epidermal microenvironment toward a pro-fibrotic, remodelling-prone state.

3.5. Metabolic and Epigenetic Reprogramming

Pathway enrichments for metabolic processes, combined with the upregulation of *GLDC* and downregulation of *ODC1* and *B4GALNT3*, suggest altered metabolic states under Th2 bias [57,58,60]. Changes in *KRT18* and *TSPAN7*, genes previously linked to methylation dynamics, may reflect concurrent regulatory adaptation [54,55,59], while *GPCPD1* upregulation is consistent with the activation of cellular stress-response pathways [56]. Although these findings are not sufficient to infer causality, they may contribute to disease chronicity and variability in therapeutic response and motivate future work connecting metabolic and epigenetic programmes to functional outcomes.

3.6. Implications for Future Therapeutic Targeting

Several significantly upregulated DEGs, including *CAPN14*, *NTRK1*, *TAP1*, *S100P*, *PTGER4*, and *PSMB9*, represent promising therapeutic targets due to their direct links to the pathogenic processes described above. For example, *CAPN14* is known to be IL-13-responsive and is implicated in eosinophilic esophagitis, where it drives epithelial changes and reduces barrier proteins such as *DSG1* [67]. *CAPN14* may play a similar role in dogs, potentially contributing to barrier impairment in CAD, making it a promising therapeutic target. Pharmacological inhibition of these proteins or blockade of their associated signalling pathways may attenuate aberrant immune activation or neuro-immune crosstalk that contribute to pruritus.

3.7. Limitations

While these transcriptomic findings offer a detailed mechanistic framework for Th2 cytokine-driven epidermal pathology, certain limitations should be acknowledged. Pathway analyses, especially Reactome, are primarily curated using human gene information, and gene annotations and functional interpretations are not fully conserved

between humans and dogs. RNA-seq captures steady-state mRNA abundance and does not directly reflect protein levels, post-translational modifications, or enzyme activity. Many of the pathways, such as JAK/STAT signalling, calcium homeostasis, and cell death, are regulated at the level of phosphorylation, proteolysis, and dynamic protein–protein interactions that cannot be inferred solely from transcriptional data. Similarly, lipid metabolic dysregulation, neuropeptide activity, and antimicrobial function depend on metabolite flux and protein function rather than transcript abundance. Future studies integrating phosphoproteomics, targeted proteomics, and metabolomics, alongside functional assays in cPEOs and in vivo animal studies, will be essential to validate and refine the mechanistic links proposed here.

Additionally, this keratinocyte-exclusive organoid system preserves key cues of cytokine–keratinocyte crosstalk, allowing us to interrogate epithelial responses in a controlled context. However, its epithelial-only nature also imposes limitations: the model cannot recapitulate the full complexity of canine atopic dermatitis, which involves dynamic, stage-dependent interactions among dermal fibroblasts, immune cells, and a broader network of cytokines. For example, the absence of immune cell populations such as eosinophils and T cells limits the ability to model the full inflammatory cascade characteristic of chronic disease. Moreover, IL-4/IL-13 stimulation primarily reflects an acute Th2-skewed environment, whereas chronic CAD is driven by additional cytokine pathways and may exhibit a distinct transcriptomic signature. Finally, the organoids used here were generated from healthy canine skin rather than atopic skin. In this study, individual fold changes in some genes (such as *CCL2*, *CCL26*, and *NTRK1*) measured by RT-qPCR and RNA seq consistently displayed significant inter-individual variability, reflecting biological heterogeneity in epithelial responsiveness to Th2 cytokine stimulation rather than technical inconsistency. Accordingly, organoids derived from atopic dogs, which may harbour intrinsic genetic or immunologic alterations, could respond differently and represent an important avenue for future investigation. Nevertheless, because clinical atopic skin samples are rarely biopsied and are therefore difficult to obtain, this organoid-based approach provides a reproducible, ethical, and cost-effective platform for investigating the genetic mechanisms underlying this disease.

4. Materials and Methods

4.1. Organoid Culture and Cytokine Treatment

Isolation of canine primary keratinocytes from the skin of dogs (Table 2) and subsequent organoid culture were conducted as previously described [6]. Keratinocytes were cultured in Matrigel with organoid culture medium supplemented with/without cytokines (30 ng/mL IL-4 and 30 ng/mL IL-13). The day of seeding was designated as day 0. The culture medium containing fresh cytokines was replenished on days 3 and 6, and the cultures were maintained until day 8.

4.2. RNA Extraction, Library Preparation, and RNA Sequencing

After the termination of organoid culture, samples were lysed, and total RNA was extracted using either the RNeasy PowerLyzer Tissue & Cells Kit (QIAGEN, Hilden, Germany) or TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturers' protocols. RNA purity and integrity were assessed with a NanoPhotometer N120 (IMPLEN, Munich, Germany), with all samples showing A260/280 ratios within the optimal range of 1.80 to 2.20. RNA quality was further evaluated using the RNA ScreenTape 4150 system (Agilent Technologies, Santa Clara, CA, USA), yielding RNA Integrity Numbers (RINs) of ≥ 9.6 .

Library preparation was performed using the RiboZero Plus kit (Illumina, San Diego, CA, USA) for total RNA. Quantification was carried out with the Illumina Quantification Kit (Illumina, San Diego, CA, USA) via qPCR, which confirmed successful adaptor ligation. These results, along with fragment size information obtained from post-PCR TapeStation analysis, were used to accurately determine library molarity for sequencing. RNA sequencing was conducted as 150 bp paired-end reads on a NovaSeq X Plus platform at the Australian Genome Research Facility (AGRF) in Melbourne, Australia.

4.3. RNA-seq Data Analyses

The primary analysis involved demultiplexing and quality control (QC). The data were then processed through an RNA-seq expression analysis workflow, which included trimming, alignment, transcript assembly, feature quantification, and differential expression analysis. The cleaned sequence reads were then mapped to the dog reference genome, ROS_Cfam_1.0 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_014441545.1/ (accessed on 28 April 2025), and gene-level counts were obtained by the STAR aligner (v2.3.5a). Further analysis was carried out using the edgeR package [68] through the Degust platform [69]. Counts per million were calculated for each gene to standardize for differences in library size, and filtering was carried out to retain genes with a baseline expression level of at least 1.0 CPM in 3 or more samples.

4.4. Identification of DEGs

Differential expression analysis between the cytokine-treated and control organoids was conducted using edgeR package (version 4.0.9). The outputs included an MDS plot and a differential expression table (Supplementary File S3) containing the Gene ID from the reference genome annotation, a $\log_2$ average fold change ($\log_2FC$) in expression between comparison groups, the $\log_2$ average counts per million ($\log_2CPM$) across all samples, the likelihood ratio statistic for each gene, and the *p*-values and FDR.

4.5. Pathway Enrichment Analysis

Biological function and pathway enrichment analyses of all DEGs were conducted in the ShinyGO 0.85.1 database (<https://bioinformatics.sdstate.edu/go/> (accessed on 26 November 2025) [70], with a false discovery rate (FDR) cutoff of 0.05 applied for significance. Enriched pathways were subsequently sorted by fold enrichment. GO, KEGG, and Reactome pathway analysis were conducted using the dog reference genome ROS_Cfam_1.0 (taxonomy ID: 9615, source: ENSEMBL) in the ShinyGO 0.85.1 database [70]. Among the three GO categories, only the Biological Process (BP) terms were used for data presentation, as they were the most relevant and interpretable for this study.

4.6. Real-Time RT-qPCR

The extracted RNA from the same samples was reverse transcribed using GoScript™ Reverse Transcriptase Mix, Oligo(dT) (Promega), and was used for first-strand complementary DNA (cDNA) synthesis as per the manufacturer's instructions. Quantitative real-time PCR for the selected gene and calculations of cycle threshold (Ct) values were performed using Rotor-Gene Q (Qiagen) with a system using GoTaq® Flexi DNA polymerase (Promega, USA) according to the manufacturer's protocol. The primers used are listed in Supplementary File S7 online. All primers were validated by generating standard curves with an R value >99% and $0.9 \leq \text{efficiency} \leq 1.1$. As a stably expressed gene in canine skin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the reference gene. The mRNA expression levels were normalized to GAPDH expression based on the ΔCt method and calculated based on fold gene change ($2^{-\Delta\Delta Ct}$) [6]. The experiment was performed in duplicate, while differences between two Ct values

in the same repetition should be less than 0.5. The average fold change in gene expression ($2^{-\Delta\Delta Ct}$) was calculated, and a nonparametric Kolmogorov–Smirnov test was applied to compare fold changes in each treatment group with the control. Pearson correlation analysis was performed between log₂ fold change values obtained from RT-qPCR and RNA-seq. Statistical analysis was conducted using GraphPad Prism (Version 10.2.0) software.

5. Conclusions

The integration of gene-level analyses with a literature-based functional review relevant to AD/CAD, together with pathway analyses using differentially expressed transcriptomes, reveals that Th2 cytokines act as master initiators driving multifaceted transcriptomic alterations in cPEOs. This in vitro canine organoid model successfully recapitulated key molecular features of AD/CAD, including immune activation and antigen presentation, dysregulation of neuro-immune crosstalk, cell death, ECM remodelling, formation of the skin barrier, and antimicrobial defences (Table 1). Many differentially expressed genes which represent downstream effects of Th2 cytokine-induced alterations are insufficiently explored in CAD and may represent candidate targets for further investigation, such as *CAPN14*, *NTRK1*, and *TAP1*.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052211/s1>, Supplementary File S1: Summary of RNA-Seq read mapping and feature assignment in control and treatment samples; Supplementary File S2: Heatmap of all expressed genes. Supplementary File S3: Detailed results of differential expression; Supplementary Files S4–S6: Detailed results of GO (BP), KEGG, and Reactome pathway analyses, respectively; Supplementary File S7: RT-qPCR primers.

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Institutional Review Board Statement: The collection and utilization of skin tissue samples were approved by the Animal Ethics Committee (AEC) of the institute (Ethics ID #22006) on 25 May 2022, in accordance with ethical standards and guidelines of the University of Melbourne. For our study, we exclusively used the primary cells isolated from the surgery scavenged tissue; this study involved in vitro research using animal-derived materials, and no live animal was involved.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

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interpretations were developed by the authors. The authors take full responsibility for the content of the published article.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

RNA-seq	RNA sequencing
CPM	Counts per million
FC	Fold change
DEG(s)	Differentially expressed gene(s)
FDR	False discovery rate
GO	Gene Ontology
BP	Biological Process
PCA	Principal component analysis
MDS	Multidimensional scaling
RT-qPCR	Reverse transcription quantitative PCR
Ct	Cycle threshold
AD	Atopic dermatitis
CAD	Canine atopic dermatitis
cPEO(s)	Canine primary epidermal organoid(s)
ECM	Extracellular matrix
NGF	Nerve growth factor
DMEM	Dulbecco's Modified Eagle Medium
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

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