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# Gene expression analysis of Canine Demodicosis; A milieu promoting immune tolerance

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ABSTRACT

Canine demodicosis is a common skin disease seen in companion animal practice that results from an overpopulation of the commensal *Demodex* mite species. Common predisposing factors to the development of canine demodicosis include immunosuppressive diseases, such as neoplasia and hypothyroidism, and administration of immunosuppressive therapies, such as corticosteroids. Despite this, the pathogenesis of development of canine demodicosis remains unclear. Previous studies have implicated a role for increased expression of toll like receptor 2 (TLR2), increased production of interleukin (IL)-10) and T cell exhaustion. Here, we investigate gene expression of formalin fixed paraffin embedded skin samples from twelve cases of canine demodicosis in comparison to twelve healthy controls, using a 770 gene panel (NanoString Canine IO Panel). Results show an increase in the T cell population, specifically Th1 and Treg cells in dogs with demodicosis. In addition, while there is an upregulation of immunosuppressive cytokines such as IL-10 and IL-13, there is also an upregulation of immune check point molecules including PD-1/PD-L1 and CTLA-4. These findings suggest that *Demodex* spp. mites are modulating the host immune system to their advantage through upregulation of several immune tolerance promoting pathways.

## 1. Introduction

Canine demodicosis is a common skin disease of dogs that results from the overpopulation of *Demodex* mite species in pilosebaceous units (Mueller et al., 2020). Mites of the genus *Demodex* spp., Order Trombidiformes, family Demodecidae are commensal parasites found in hair follicles and sebaceous glands of most animal species (Lowenstein et al., 2005; Schonfelder et al., 2010; Miller and Campbell K, 2012; Nashat et al., 2017). In dogs, there are currently three recognised species of *Demodex* spp. mites: *Demodex canis*, *Demodex injai* and *Demodex cornei* (Sivajothi et al., 2015; Ordeix et al., 2009). *Demodex cornei* is, however, considered a variant of *Demodex canis* (De Rojas et al., 2012). There are many known predisposing factors that contribute to the overpopulation of *Demodex* spp. mites and the development of canine demodicosis. These include genetic predisposition with many reported familial cases and breed overrepresentations (Kelly et al., 2022b). Immunological disorders such as atopic dermatitis and inflammatory bowel disease together with endocrine disease, neoplasia and immunosuppressive therapies are also known as predisposing factors for disease development (Mueller et al., 2012; Bowden et al., 2018; O'neill et al., 2020; Foley et al., 2021; Kelly et al., 2022b). However, while these predisposing factors may be aiding the development of canine demodicosis, when treated solely with ectoparasitic drugs most canine patients with demodicosis recover and have a low recurrence rate which suggests that the disease is not only occurring because of an underlying systemic immunosuppressive disease/therapy or genetic predisposition (Bowden et al., 2018; Charach, 2018; Mueller et al., 2012). In these later cases, it is likely that *Demodex* mites may be modulating the immune system to facilitate their overpopulation.

To date, the proposed mechanisms for the development of canine demodicosis have focused on T cell exhaustion and inflammation-modulating cytokines. Dogs with demodicosis have been shown to

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exhibit a cytokine phenotype characterized by low production of stimulatory cytokines, such as interleukin (IL)− 2 and IL-21, high levels of suppressive or immune-modulating cytokines, such as IL-10 and transforming growth factor β (TGFβ), together with low numbers of circulating CD4 + lymphocytes, all of which suggest T cell exhaustion (Felix et al., 2013; Ferrer et al., 2014; Gasparetto et al., 2018; Lemarié and Horohov, 1996; Martinez-Subiela et al., 2014; Oliveira et al., 2015; Singh et al., 2010; Tani et al., 2002). One pathway known to result in an increase in IL-10 expression is activation of toll like receptor 2 (TLR2). *Demodex* spp. have been shown to result in a marked increase in the expression and activity of TLR2 on keratinocytes (Kumari et al., 2018; Rivas A. et al., 2013). IL-10 has several known immune-modulating effects; (1) IL-10 inhibits Major Histocompatibility Complex (MHC) class II and co-stimulatory molecule expression on monocytes and macrophages, and limits the production of proinflammatory cytokines and chemokines; (2) IL-10 can act directly on CD4 + T cells, inhibiting proliferation and production of IL-2, IFNγ, IL-4, IL-5 and tumour necrosis factor-alpha (TNFα); (3) IL-10 can directly regulate innate and adaptive Th1 and Th2 responses by limiting T cell activation (Felix et al., 2013; Ferrer et al., 2014; Gasparetto et al., 2018; Huisinga et al., 2007; It et al., 2010; Kumari et al., 2018; Lemarié and Horohov, 1996; Rivas A. et al., 2013; Yarim et al., 2015; Singh et al., 2010).

More recently, the role of the parasympathetic nervous system in the development of canine demodicosis has been investigated (Kumari et al., 2017). Acetylcholine (ACh) plays an important role in attenuating the release of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-18, without affecting the production of IL-10. Kumari et al. (2018) showed that there is significantly increased cholinesterase activity in dogs with demodicosis suggesting an overproduction of ACh. The same authors also noted that cholinesterase activity in dogs with generalised disease was significantly higher than in dogs with localised disease. These findings suggest that a neuro-immunosuppressive pathway may also be involved in the pathogenesis of demodicosis (Kumari et al., 2017).

The aim of this study was to provide a comprehensive assessment of the expression of common proinflammatory, anti-inflammatory and immune-modulatory genes in skin biopsy samples from dogs with demodicosis; it was envisaged that such data sets may provide additional insight into the pathogenesis of the disease and/or identify new pathways amenable to the development of new targeted therapies.

## 2. Materials and methods

### 2.1. Case selection

This study was approved by the Animal Research Ethics Committee (AREC) at University College Dublin (Reference Number: AREC E 19 09 Kelly).

Twelve formalin fixed paraffin embedded (FFPE) skin samples from dogs diagnosed histologically with demodicosis were selected. All twelve dogs had skin swabs for microbiological assessment taken on the day of biopsy sampling of which culture results were reported as 'normal growth'. As outlined previously in Kelly et al. (2022), 'normal growth' is considered when culture is of normal commensal skin bacteria, which includes Gram-positive staphylococci, *Corynebacterium* spp., viridans streptococci, *Micrococcus* spp. and Gram-negative *Acinetobacter* spp., without the presence of pathogenic bacteria or overgrowth of any bacterial species (Kelly et al., 2022a). Six of the twelve samples were from dogs aged less than 18 months of age (juvenile onset demodicosis) and the remaining six were from dogs over four years of age (adult onset demodicosis). Details of signalment, microbiological culture and histological findings are available in the supplementary material (Table S1).

Twelve formalin fixed paraffin embedded skin samples from dogs that had no history, clinical signs or histological evidence of skin disease were selected as controls. Control dogs were presented for surgery either for fracture repair, orchidectomy or ovariohysterectomy; skin samples were obtained from excess skin that was taken from surgical wound margins that are routinely taken for better wound apposition. Six of these samples were from dogs aged 18 months or less and the remaining six were from dogs aged over four years. Details of signalment of the control cases are available in the supplementary material (Table S1).

### 2.2. RNA extraction

Total RNA was extracted from the twelve FFPE skin samples from dogs with demodicosis and twelve FFPE control skin samples using the RNeasy FFPE kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were treated with DNase-I (Qiagen) to remove traces of genomic DNA. The RNA samples were quantified (Nano-Drop ND-8000 spectrophotometer, Thermo Scientific, Wilmington, USA); a minimum quantity of 60 ng/μl RNA was required for analysis.

### 2.3. NanoString Canine IO panel

RNA (300 ng) in 5 μl was hybridized with gene-specific reporter and capture probes (nCounter Canine IO panel, NanoString, Seattle, USA) at 65 °C for 18 h and processed on the nCounter Prep station. Data were acquired using nCounter scanner, both systems are part of the NanoString nCounter Flex system. The Canine IO Panel, which includes 770 genes (305 cytokine- and chemokine signalling genes, 47 interferon signalling genes, 67 checkpoint signalling genes, 33 complement cascade genes, 52 immune cell surface marker genes, 74 antigen presentation and DNA damage repair genes, 94 epigenetic regulation, hypoxia, TGFβ and WNT signalling genes and 101 angiogenesis and matrix remodelling genes) was used. To characterize the inflammatory infiltrate, the NanoString Advanced Analysis package was used. This analysis utilises a summary statistic of the expression of the marker genes previously shown to be characteristic of various immune cell populations to measure their abundance with a high confidence of prediction ($p \leq 0.01$) (Danaher et al., 2017).

### 2.4. Immunohistochemistry

Antigen retrieval was performed using heat-induced epitope retrieval (HIER); sections were treated at full pressure with Access Retrieval Unit (Menarini, Florence, Italy) in EDTA (pH 8) for forkhead box P3 (FOXP3), for 90 s at 125 °C. The sections were then rinsed in Tris Tween ® buffer (Thermo Fisher Scientific, Massachusetts, US) (pH 7.5). The sections were treated for 5 min at room temperature with 3% hydrogen peroxide in phosphate buffered saline to quench endogenous peroxidase activity. After washing twice with Tris Tween ® buffer (pH 7.5), sections were incubated for 30 min at room temperature with the primary antibody anti-FoxP3 (Invitrogen, Massachusetts, US)) at dilution of 1:800, then washed with Tris Tween ® buffer (pH 7.5). Primary antibody was then labelled with biotinylated Rabbit anti-Rat IgG antibody (H+L) (Vector) for 30 min, then washed with Tris Tween ® buffer (pH 7.5). For detection of primary antibody, sections were incubated with EnVision+ System horseradish peroxidase (HRP) Labelled Polymer Anti-Rabbit Secondary Antibody (Dako, Agilent, California, US) for 30 min at room temperature and then washed with TRIS Tween buffer, followed by two 5-minute incubations with 3,3'-diaminobenzidine (DAB) substrate-chromogen (EnVision+ System, Dako). Sections where then rinsed twice with distilled water for 5 min. Tissues were counterstained using Gill's haematoxylin and mounted using DPX mounting media (Cellpath, Newtown, UK) and coverslips for long-term storage.

The slides were reviewed by a single pathologist (PK) and a scoring system adapted from a published method by Tagami et al. (2022) was applied (Table S2) (Tagami et al., 2022).

### 2.5. Statistical methods

The quality control (QC,) normalization, differential expression and pathway analysis were performed using the nSolver v4 software and nSolver advance analysis module (NanoString) according to the guidance given by manufacturers. Profiled data were pre-processed, specifically background was subtracted by using threshold counts of 20, normalization was performed with positive control and housekeeping genes and no QC flags were raised. Obtained values were Log2 transformed prior to identification of differentially expressed (DE) genes ($p < 0.05$) using heteroscedastic t tests as per manufacturer's recommendation. DEs were plotted using agglomerative clustering (Euclidean distance), fold changes and adjusted p values were reported for each DE. Results of adjusted $p$ values are denoted as follows: ns $p > 0.05$, *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Differential expression is, unless otherwise stated, written as (Log2 fold change, $p$ value) throughout the manuscript. Data is visualised using Graphpad Prism 9 for all column charts, whereas heatmaps and volcano plots are visualised using NanoString nSolver software.

## 3. Results

Of the 770 genes assessed, 441 genes were found to be statistically significantly differentially expressed ($p \leq 0.05$). Implementing a Log2 fold change cut off of +/− 1, 20 genes were significantly downregulated and 309 were found to be significantly upregulated (Figs. 1 and 2). Details of all genes significantly differentially expressed and within the Log2 fold change cut off are available in the supplementary material (Table S3).

### 3.1. Immune cells

Genes and immune cell type gene signatures for T-cell, CD8 + T-cell, Th1 cell, Th2 cell, T regulatory (Treg), Th17, exhausted CD8 + cell, B-cell, cytotoxic cell, dendritic cell, macrophages, mast cell, neutrophils were assessed.

### 3.2. T cells, CD8 + Cells, T helper cells, Th1 cells, Th2 cells, Treg, Th17 and exhausted CD8 + cells

Three genes that encode CD3 proteins (T cells) were assessed for T cell signature, and all were found to be significantly upregulated; CD3D (2.9, $p \leq 0.0001$) CD3E (2.83, $p \leq 0.0001$) and CD3G (3.12, $p \leq 0.0001$). Using the cell specific gene signature analysis, there was a significant increase in the presence of T cells compared with healthy controls ($p \leq 0.001$) (Fig. 3A). To further classify the T cell infiltrate, genes for glycoproteins CD4 or CD8 that serve as co-receptors were measured. Assessment of expression of CD4 and CD8 associated genes showed a significant upregulation of genes commonly associated with both (CD4: 4.69, $p \leq 0.0001$, CD8A: 3, $p \leq 0.0001$, CD8B:1.26, $p \leq 0.05$). However, when using the cell specific gene signature analysis, while there was a significant increase in CD4 + cells ($p \leq 0.0001$), there was no significant difference in CD8 + cells in comparison to healthy controls ($p > 0.05$) (Fig. 3B and C). The ratio of differential gene expression of CD4 + :CD8 + for all cases was 1.6. Histologically all cases had varying degrees of mural folliculitis and peri-folliculitis, however, only 7 of the 12 cases of demodicosis had furunculosis and perifollicular granulomas (Table S1). Analysis of cases grouped as those either with or without perifollicular granulomas/furunculosis compared with controls showed a CD4 + :CD8 + ratio of 1.8 for those with perifollicular granulomas vs a ratio of 1.14 for those without perifollicular granulomas. CD4 + or T helper cells can be further classified as Th1, Th2, Th17 and Treg cells. While differential expression of genes associated with Th1 and Treg cells were found to be statistically significantly upregulated (Th1: TBX21: 2.4, $p \leq 0.0001$, Treg: FOXP3, 2.5, $p \leq 0.0001$), there was downregulation of GATA3, a gene that is critical for the development and maintenance of Th2 cells (Th2: GATA3: −0.913, $p \leq 0.001$) (Fig. 3D-F). Genes associated with Th17 cells, in particular IL-17, were not differentially expressed relative to control samples. Measurement of differential expression of genes associated with T helper cell cytokines showed that IFNγ and IL-2 were not differentially expressed; however, TNFα (1.98, $p \leq 0.0001$) and IL-10 (1.81, $p \leq 0.0001$) were found to be significantly upregulated. Similarly, IL-4 and IL-5 were not differentially expressed. There was, however, significant upregulation of IL-13 (4.26,

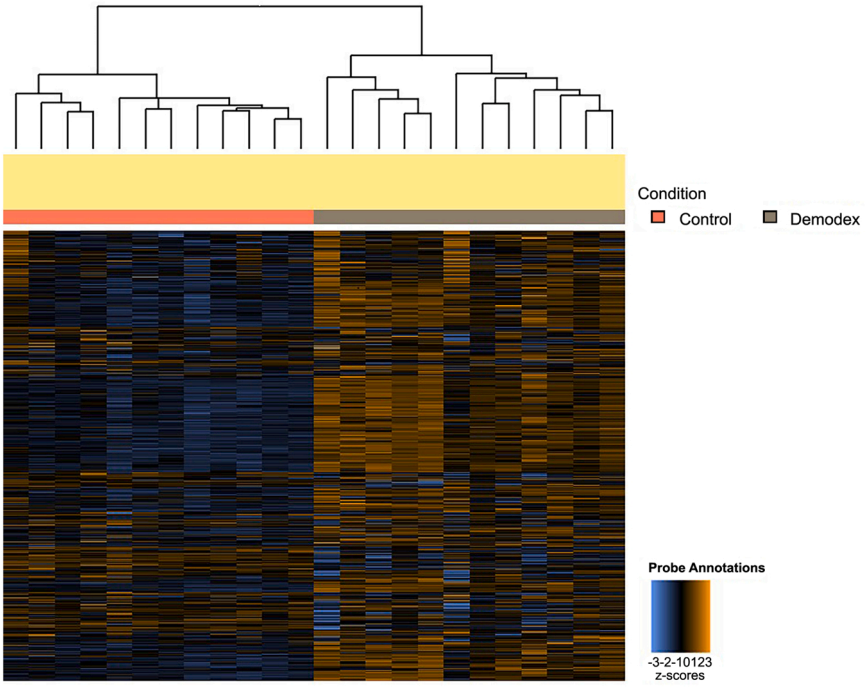


**Fig. 1.** Unsupervised hierarchical cluster heatmap of expression of all genes in all samples (n = 24). Heatmap shows clustering of gene expression in skin with demodicosis (n = 12) and control skin (n = 12).

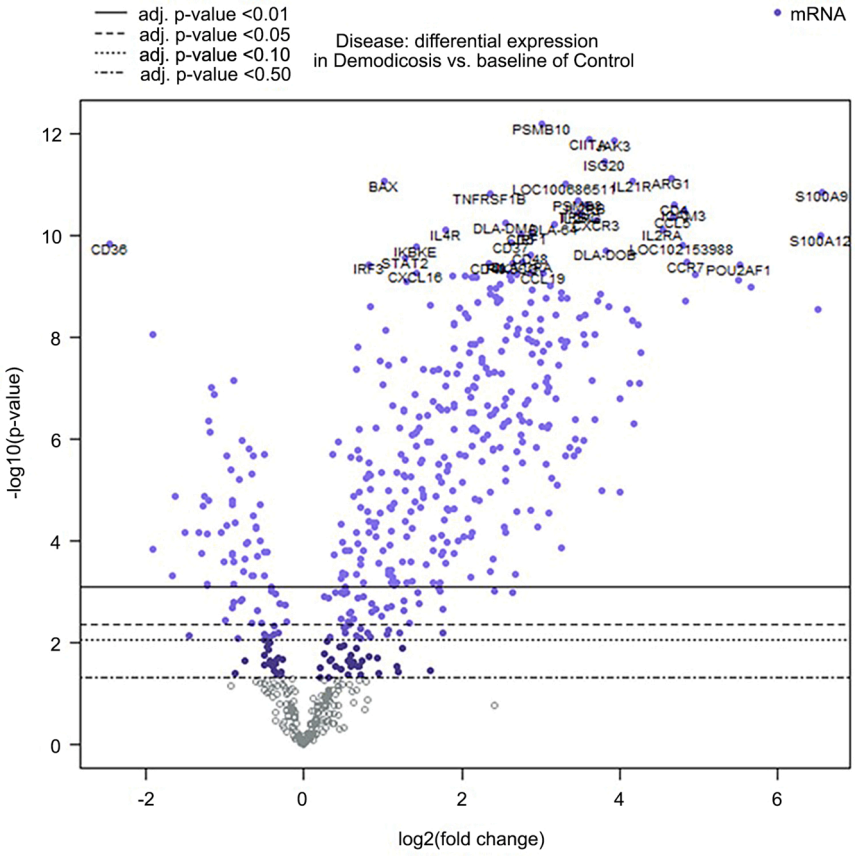


**Fig. 2.** Volcano plot of the Log2 Fold Change of all genes in canine demodicosis samples compared with the baseline of control samples. Levels of statistical significance (Adjusted p value) are also noted.

$p \leq 0.0001$). Genes commonly expressed by exhausted T cells were found to be significantly upregulated in the demodicosis group relative to controls including Lag3 (1.31, $p < 0.01$), EOMES (3.32, $p \leq 0.0001$), 2B4 (CD244: 1.24 $p \leq 0.001$), PD-1 (PDCD1: 2.59, $p \leq 0.0001$), HAVCR2 (1.37 $p \leq 0.0001$) and CTLA-4 (2.9, $p \leq 0.0001$). However, when using the cell specific gene signature analysis, there was no significant increase in the presence of exhausted T cells compared with healthy controls ($p > 0.05$).

### 3.3. B cells

Genes characteristic of B cells were measured including BLK, FCRL2, TNFRSF17 and MS4A1. All had significant upregulation; BLK (3.75, $p \leq 0.0001$), FCRL2 (3.44, $p \leq 0.0001$), TNFRSF17 (3.65, $p \leq 0.0001$) and MS4A1 (4.95, $p \leq 0.0001$). The cell specific signature for B cells was found to be significantly increased ($p \leq 0.01$) with higher infiltrates in cases of demodicosis compared with healthy control skin (Fig. 4A).

### 3.4. Cytotoxic cells

Genes characteristic of cytotoxic cells which include T cells, Natural Killer T cells and Natural Killer cells were measured. With the exception of KLRK1 and KLRD1, all were significantly upregulated as follows; GZMB (3.07, $p \leq 0.0001$), CTSW (3.02, $p \leq 0.0001$), KLRB1 (1.6, $p \leq 0.0001$), NKG7 (1.63 $p \leq 0.01$), GZMA (1.67, $p \leq 0.001$), PRF1 (1.1, $p \leq 0.01$), KLRD1 (0.906, $p \leq 0.05$), KLRK1 (0.496 $p > 0.05$). The cell specific signature for cytotoxic cells was found to be significantly increased ($p \leq 0.01$) with higher infiltrates in cases of demodicosis compared with healthy control skin (Fig. 4B).

### 3.5. Dendritic cells

Genes characteristic of dendritic cells were measured and found to be upregulated in canine demodicosis including CCL13 (1.08, $p \leq 0.01$), HSD11B1 (0.714, $p > 0.05$) and CD209 (0.984, $p \leq 0.05$). Tolerogenic DCs express the surface markers CD163, CD141, CD16 and CD14; CD14 was significantly upregulated: 2.43, $p \leq 0.0001$, whilst CD163 was not differentially expressed and neither CD16 nor CD141 were assessed. Langerhans cells are members of the DC/macrophage family. Keratinocytes express RANKL which is critical for regulating Langerhans cells which themselves express its receptor RANK. The RANKL gene was significantly upregulated in canine demodicosis, while the RANK gene showed significant differential expression but fell below the fold change cut off (RANK: 0.664, $p \leq 0.01$, RANKL: 2.11, $p \leq 0.01$). The cell specific signature for dendritic cells was found to be significantly increased ($p \leq 0.05$) with higher infiltrates in cases of demodicosis compared with healthy control skin (Fig. 4C).

### 3.6. Macrophages

While genes characteristic of macrophages were mostly statistically significantly upregulated in canine demodicosis, including CD68 (2.73, $p \leq 0.0001$), CD84 (2.13, $p \leq 0.0001$) and CD163 (not differentially expressed); the cell specific gene signature was not significant. Cytokines associated with M2 macrophages including IL-13 (4.26, $p \leq 0.0001$), IL-10 (1.81, $p \leq 0.0001$), home oxygenase-1 (HO-1), and arginase (ARG1: 4.65, $p \leq 0.0001$) were found to be significantly upregulated. Genes associated with subgroups of M2 macrophages were assessed. M2a-macrophage associated genes including CCL17 and CCL22 were found to be significantly upregulated in cases of canine demodicosis (CCL17 5.67, $p \leq 0.0001$; CCL22 2.85, $p \leq 0.0001$).

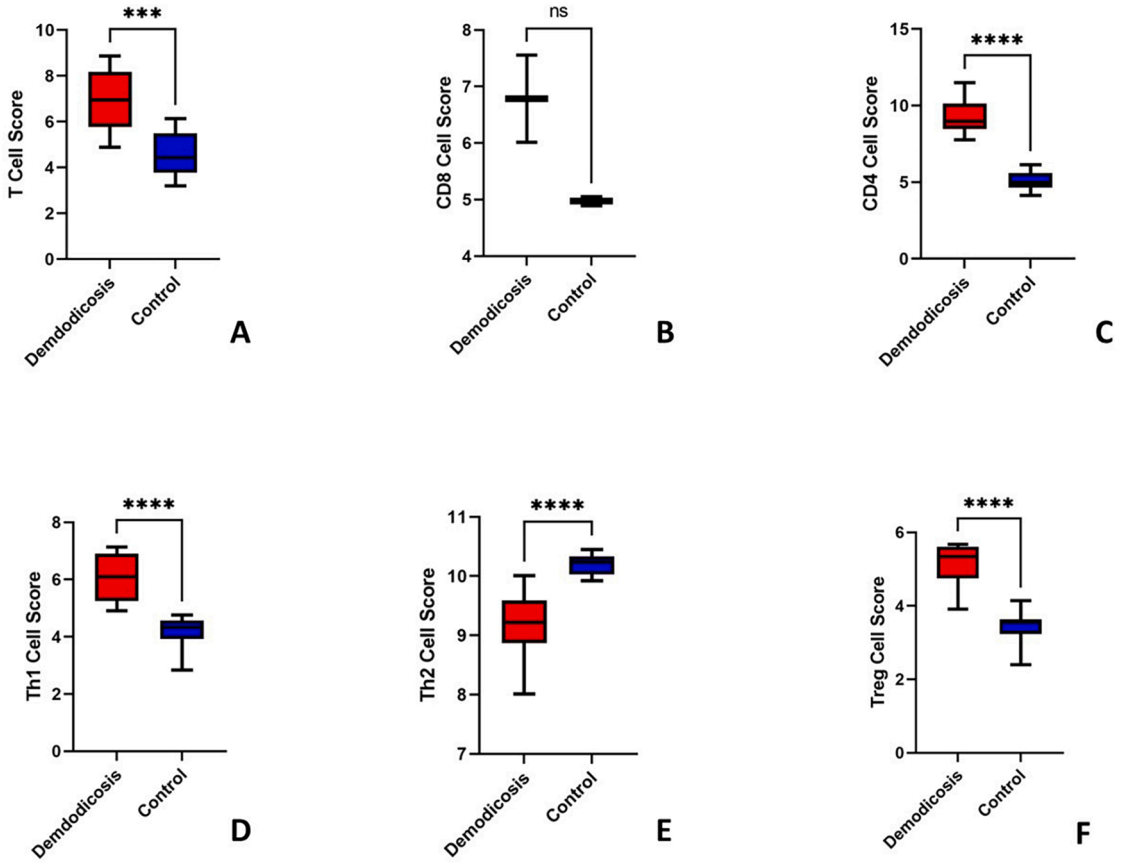


**Fig. 3.** Bar charts of cell specific gene signature scores for T cells, CD8 Cells, CD4 Cells, Th1 Cells, Th2 Cells and Treg Cells. A, T cell score, there is a significant increase in the T cell population in demodicosis cases compared with control cases ($p \leq 0.001$). B, CD8 cell score, there is no significant difference between the CD8 cell population in cases of demodicosis in comparison with control cases ($p > 0.05$). C, CD4 cell score, there is a significant increase in the CD4 cell population in demodicosis cases compared with control cases ($p \leq 0.0001$). , Th1 cell score, there is a significant increase in the Th1 cell population in demodicosis cases compared with control cases ($p \leq 0.0001$). E, Th2 cell score, there is a significant decrease in the Th2 cell population in demodicosis cases compared with control cases ($p \leq 0.0001$). F, Treg cell score, there is a significant increase in the Treg cell population in demodicosis cases compared with control cases ($p \leq 0.0001$).

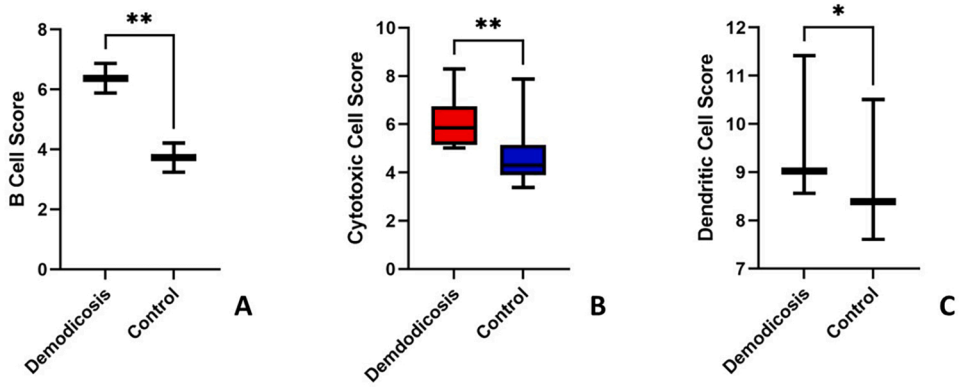


**Fig. 4.** Bar charts of cell specific gene signature scores for B cells, cytotoxic cells, and dendritic cells. A, B cell score, there is a significant increase in the B cell population in demodicosis cases compared with control cases ($p \leq 0.01$). B, Cytotoxic cell score, there is a significant increase in the cytotoxic cell population in demodicosis cases compared with control cases ($p \leq 0.01$). C, dendritic cell score, while there is a significant increase in marker genes associated with dendritic cells ($p \leq 0.05$), for a high confidence of prediction of a change in cell population, a minimum $p$ value of $p \leq 0.01$ is required. While there is a trending increase in the dendritic cell population in cases of demodicosis compared with control cases the cell score does not reach the significance level of confidence required for certainty.

Similarly, genes associated with M2b-macrophages including IL-6 and TNFα were significantly upregulated (IL-6, 2.55, $p \leq 0.0001$; TNF, 1.98, $p \leq 0.0001$). Genes associated with M2c-macrophage including IL-10 and CXCL13 were also found to be significantly upregulated (CXCL13: 2.86, $p \leq 0.0001$). The VEGFA gene that is associated with M2d-macrophage was found to be significantly upregulated (VEGFA, 1.36, $p \leq 0.0001$) as was CCL5 (4.68, $p \leq 0.0001$), CXCL10 (3.77, $p \leq 0.0001$) and CXCL16 (1.42, $p \leq 0.0001$). The results are outlined in Fig. 5.

### 3.7. Mast cells, Neutrophils

Genes characteristic of mast cells were measured including HDC (2.76, $p \leq 0.0001$), CPA3 (not differentially expressed) and MS4A2 (1.48, $p \leq 0.001$). The cell specific gene signature was not significant. Similarly, genes characteristic of neutrophils were assessed including FCAR (0.515, $p \leq 0.01$), S100A12 (6.54, $p \leq 0.0001$) and CSF3R (2.56, $p \leq 0.001$). The cell specific gene signature did not show a significant difference between cases of canine demodicosis and healthy controls.

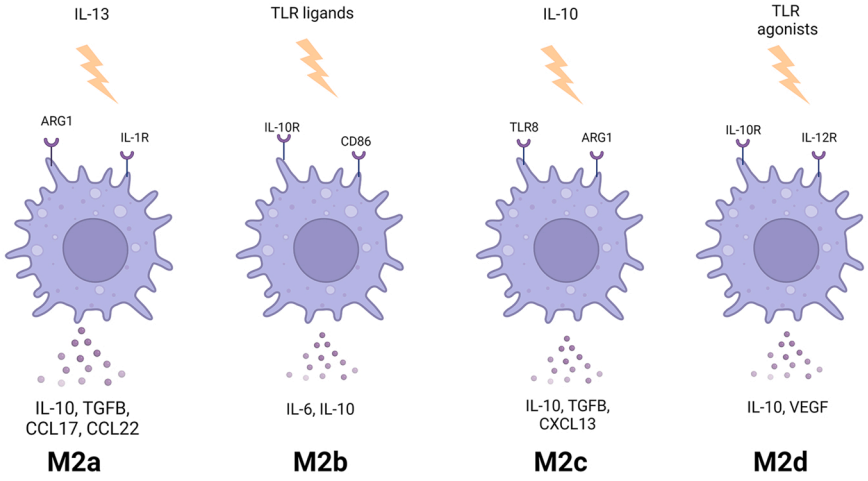


**Fig. 5.** Illustration of subtypes of M2 macrophages showing only associated genes that were statistically significantly ($p \leq 0.05$) upregulated in cases of demodicosis in comparison to control samples. M2a; induction of M2a-macrophages is triggered by IL-13 and leads to the increased expression of ARG1 and IL-1R receptors and production of IL-10, TGFβ, CCL17 and CCL22. M2b; induction of M2b-macrophages is triggered by TLR ligands, leading to an increased expression of IL-10R and CD86 and production of IL-6 and IL-10. M2c; induction of M2c-macrophages is triggered by IL-10 and leads to the increased expression of ARG1 and TLR8 receptors and production of IL-10, TGFβ and CXCL13. M2d; induction of M2d-macrophages is triggered by TLR agonists and leads to the increased expression of IL-10R and IL-12R receptors and production of IL-10 and VEGF. Illustration created with BioRender.com.

### 3.8. Myeloid derived suppressor cells (MDSC)

Myeloid-derived suppressor cells (MDSC) are pathologically activated neutrophils and monocytes with potent immunosuppressive activity. Genes used to differentiate MDSCs from classical neutrophils and monocytes include S100A9, S100A8, ARG1, NOS2, IL-10, VEGFA, CD14, TGFB1, TNF, STAT1, IRF1, IL-4R, CD84 and IL-6. S100A8 and S100A9 genes were found to be statistically significantly upregulated (S100A8: 5.51, $p \leq 0.0001$, S100A9: 6.65, $p \leq 0.0001$), as were ARG1 (4.65, $p \leq 0.0001$), IL-10 (1.81, $p \leq 0.0001$), VEGFA (1.36, $p \leq 0.0001$), CD14 (2.43, $p \leq 0.0001$), TNF (1.98, $p \leq 0.0001$), STAT1 (1.4, $p \leq 0.0001$), IRF1 (2.89, $p \leq 0.0001$), IL-4R (1.8, $p \leq 0.0001$), CD84 (2.13, $p \leq 0.0001$) and IL-6(2.55, $p \leq 0.0001$). No specific validated cell gene signature is currently available for MDSCs.

### 3.9. Pattern recognition receptors

Toll-like receptors (TLRs) are an important family of receptors that form the first line of defence system against microbes. Differential expression for genes associated with TLRs (1−10) and MyD88 were measured. The gene encoding the MyD88 adaptor protein was found to be significantly upregulated (MyD88: 1.03, $p \leq 0.0001$) together with genes for TLR1 (1.29, $p \leq 0.001$), TLR2 (1.43, $p \leq 0.0001$), TLR6 (2.57, $p \leq 0.0001$), TLR7 (1.89, $p \leq 0.0001$), TLR8 (2.3, $p \leq 0.0001$), TLR9 (1.12, $p \leq 0.001$) and TLR10 (1.82, $p \leq 0.001$) (Fig. 6). Elevated gene expression of several regulators of TLRs were found in the cases of demodicosis including ST2L (1.18, $p \leq 0.001$), SIGIRR (2.36, $p \leq 0.0001$), A20 (1.5, $p \leq 0.0001$) and BTK (2.04, $p \leq 0.0001$).

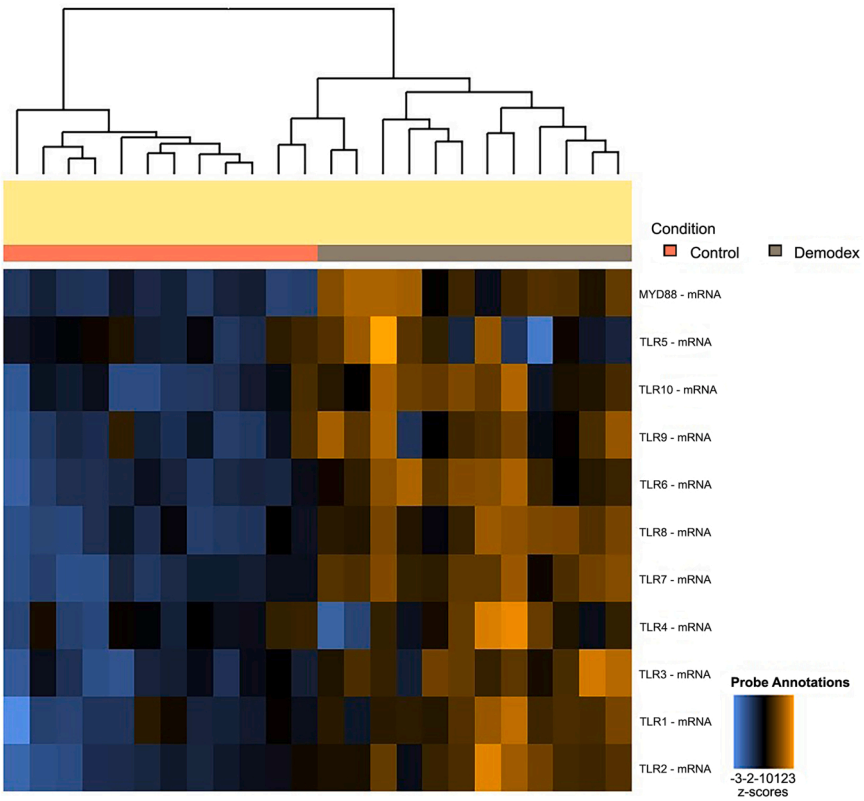


**Fig. 6.** Unsupervised hierarchical cluster heatmap of expression of TLR associated genes in all samples (n = 24). Heatmap shows clustering of TLR gene expression in skin with demodicosis (n = 12) and control skin (n = 12).

### 3.10. Tumour necrosis factor receptor superfamily

The Tumour Necrosis Factor superfamily of ligands (TNFSF) and receptors (TNFRSF) provide key communication signals between various cell types during development, maintenance, and initiation of tissue responses. An emerging feature of several of these TNFRSF members is co-signalling in Treg cells to suppress immune responses. TNFR2 was found to be significantly upregulated in dogs with demodicosis (TNFRSF1B: 2.36, $p \leq 0.0001$).

### 3.11. Senescence

Fifteen genes commonly associated with loss of cell activation or reduced proliferation (senescence) were measured, seven of which were found to have statistically significant differential expression (Fig. 7). Of these, three were within the fold change cut off including IRF5 (1.29, $p \leq 0.0001$), PLAU (1.09, $p \leq 0.001$) and SerpinB2 (−1.22, $p \leq 0.01$).

### 3.12. FOXP3 (Treg cell) Immunohistochemistry

There was a statistically significant increase in FOXP3 positive staining cells (Treg cells) within the perifollicular areas and follicular epithelium in cases of canine demodicosis compared with the control group ($p < 0.0001$) (Fig. 7). There was also a statistically significant increase in FOXP3 positive staining cells within the surrounding dermis in the demodicosis group compared with controls ($p < 0.05$) (Fig. 8).

## 4. Discussion

Canine demodicosis remains one of the most common dermatological presentations in small animal primary care practice (Mueller et al., 2020). Whilst *Demodex* spp. mites represent normal skin commensals in the dog, an overpopulation in mite numbers can result in clinical disease (Mueller et al., 2020; Foley et al., 2021). The predisposition of dogs with immunosuppressive diseases or those receiving immunosuppressive therapies to develop clinical demodicosis suggests that immune modulation plays a central role in controlling the mite population (Bowden et al., 2018; O'neill et al., 2020; Miller and Campbell K, 2012). In the current study, we report an increase in the T cell population, specifically Th1 and Treg cells in dogs with demodicosis. In addition, whilst immunosuppressive cytokines such as IL-10 and IL-13 were up-regulated, there was also an upregulation of immune check point molecules including PD-1/PD-L1 and CTLA-4. Our data sets suggest that *Demodex* spp. mites do modulate the host immune system to their advantage through upregulation of various immune tolerance promoting pathways.

A challenge often faced by researchers investigating clinical diseases is access to suitable samples for upstream analysis such as RT-PCR. Formalin fixed paraffin embedded (FFPE) tissue blocks are the most commonly available tissue specimens with many institutions, diagnostic laboratories and hospitals having large archives of stored FFPE samples. The RNA extracted from FFPE tissue is, however, most often of low quality limiting its usefulness in traditional gene expression profiling techniques. A growing platform specifically designed for low quantity and quality RNA such as that extracted from FFPE is the NanoString Technologies nCounter platform. The automated nCounter platform hybridizes fluorescent barcodes directly to specific nucleic acid sequences, allowing for the non-amplified measurement of up to 800 targets within one sample. The platform has been shown to be comparable to other technologies and offers sensitivity, technical reproducibility, and robustness for analysis of FFPE samples (Veldman-Jones et al., 2015a; Veldman-Jones et al., 2015b; Northcott et al., 2012). As this was a retrospective study FFPE tissue from canine cases that were diagnosed by histopathology with demodicosis but also had a normal skin culture on the day of biopsy and which had similar histopathological changes were selected (Table S1). The NanoString Canine IO

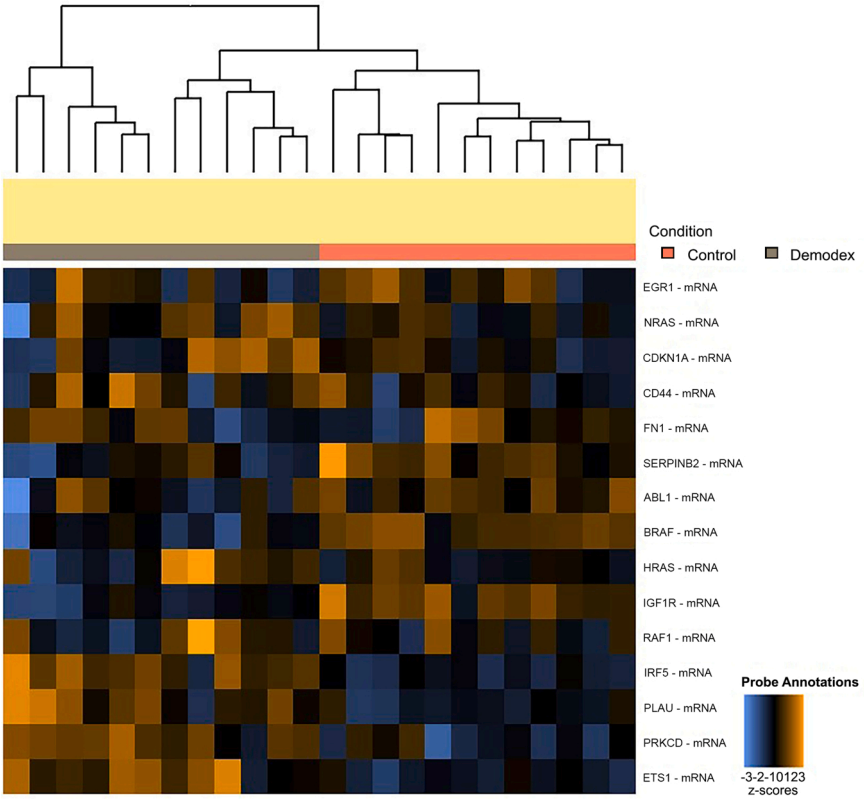


**Fig. 7.** Unsupervised hierarchical cluster heatmap of expression of cellular senescence associated genes in all samples (n = 24). Note the downregulation of BRAF in cases of demodicosis in comparison to control samples.

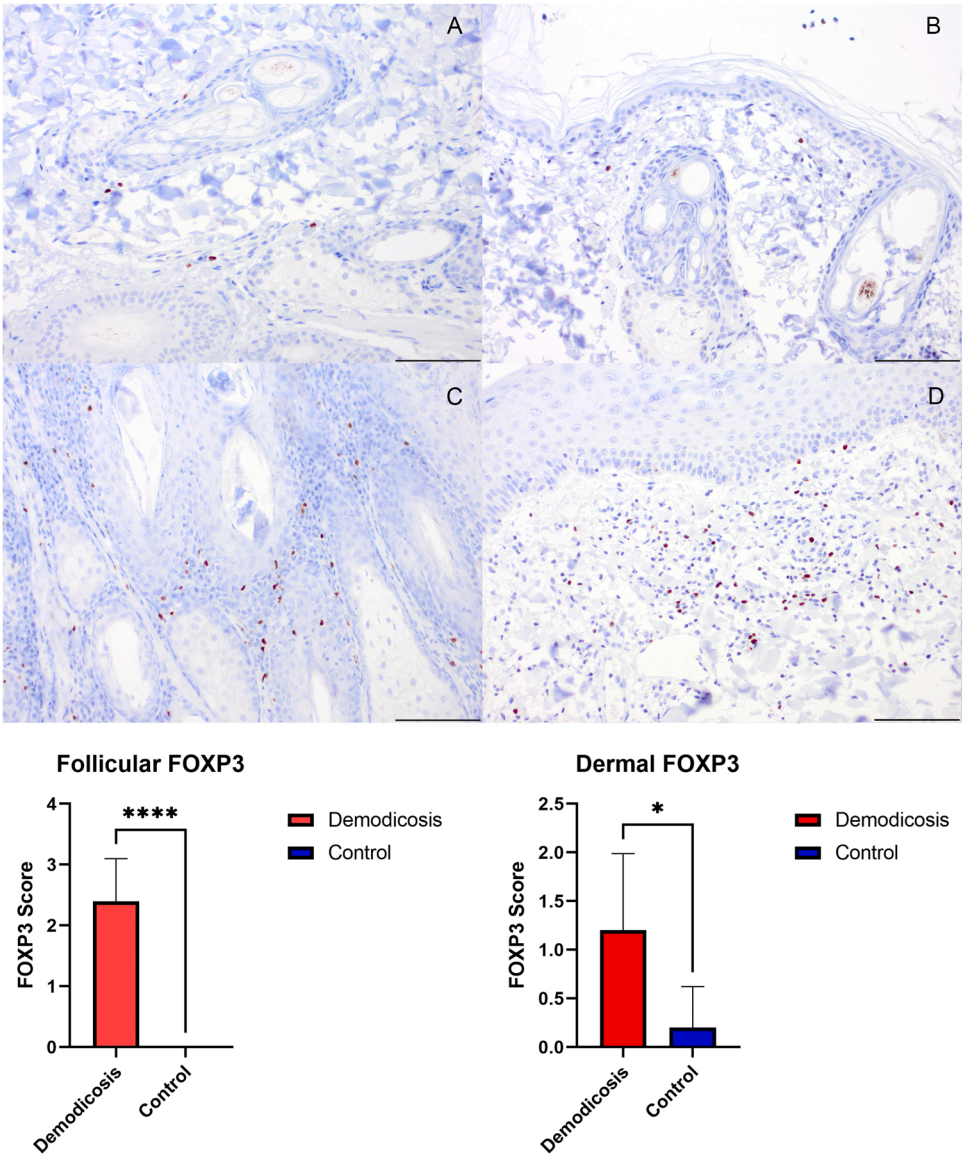


**Fig. 8.** A: Control Skin, few cells in the perifollicular areas display strong nuclear immunolabelling for FOXP3. B: Control skin, few cells within the superficial dermis display strong nuclear immunolabelling for FOXP3. C: Skin from canine demodicosis case, moderate numbers of cells in the perifollicular areas display strong nuclear immunolabelling for FOXP3. D: Skin from canine demodicosis case, moderate numbers of cells in the superficial dermis display strong nuclear immunolabelling for FOXP3. A-D: FOXP3 antibody, size bar = 10 μm. E: Bar chart of immunohistochemical score of FOXP3 cells present within and adjacent to the hair follicles in cases of demodicosis compared with controls (**** $p < 0.0001$). F: Bar chart of immunohistochemical score of FOXP3 cells present within the dermis in cases of demodicosis compared with controls (* $p < 0.05$).

Panel used here allowed for the assessment of 770 immune related genes in a single reaction for each sample (Mason et al., 2021).

Immune cell specific gene signatures were assessed using NanoString Advanced analysis which allowed for measurement of the abundance of various immune cell populations with a high confidence of prediction ($p \leq 0.01$) (Danaher et al., 2017). This showed an increase in T cells with a higher expression and level of significance of CD4 + T cells than CD8 + T cells suggesting a CD4 + T helper cell predominance in canine demodicosis compared with the control group. Immunohistochemical studies investigating the CD3 + T cell population in cases of canine demodicosis are limited (Caswell et al., 1995; Caswell et al., 1997; Day, 1997). However, these studies indicate that while there is an overall increase in CD3 + T cells, the distribution of CD4 + and CD8 + cells vary depending on the presence or absence of active mural folliculitis (Caswell et al., 1997). In one study in dogs with active mural folliculitis, which were considered as clinically active disease, CD3 + and CD8 + T cells were found infiltrating the follicular epithelium (CD4 +:CD8 + mean ratio in the epithelium was 0.032), whereas in cases without mural folliculitis, which displayed perifollicular dermatitis and or perifollicular granulomas, the perifollicular dermis contained equal numbers of CD4 + and CD8 + cells (CD4 +:CD8 + mean ratio in the epithelium was 0.9) (Caswell et al., 1997). Caswell et al. (1997) followed the histological changes during the clinical course of canine demodicosis and found that lesions without mural folliculitis were mostly found when clinical lesions were resolving (Caswell et al., 1997). The differential gene expression of CD4 to CD8 ratio for all cases in the current study was found to be 1.6; however, when the cases were analysed as those with and those without perifollicular granulomas/furunculosis compared with controls, the CD4 + :CD8 + ratio was 1.8 for those with perifollicular granulomas vs 1.14 for those without perifollicular granulomas. These findings support the previous immunohistochemical studies in that the CD4 + :CD8 + ratio increases with the presence of perifollicular granulomas (Caswell et al., 1995; Caswell et al., 1997; Day, 1997). However, in all groups, with and without perifollicular granulomas, genes associated with CD4 + cells predominated over CD8 + .

When an antigen presenting cell or dendritic cell (DC) contacts an antigen, there is activation of Naïve CD4 + T Helper cells through interaction of MHC class II and TCR molecules. Depending on the nature of the antigen, the cytokines released will preferentially promote the proliferation of Th1, Th2, Treg or Th17 helper cells. To further characterise the CD4 + population present in canine demodicosis lesions, genes associated with CD4 + T helper cell subgroups were assessed. Here we found that most genes associated with Th1 and FOXP3 + cells

(Treg cells) were significantly upregulated, whereas most genes associated with Th2 and Th17 cells were either significantly downregulated or not differentially expressed. Similarly, genes for T helper cell associated cytokines mirror this difference in Th1, Treg and Th2 cells with higher levels of Th1 and Treg cell associated cytokines in comparison to Th2 cell associated cytokines. To confirm the increase in infiltration of Treg (FOXP3 +) cells, we performed immunohistochemistry using FOXP3 antibodies on the FFPE samples. We found that Treg cells were statistically significantly increased in canine demodicosis compared with control skin. Also, the Treg cells were predominantly and more significantly distributed close to, and within, the hair follicle epithelium. Immunological studies in humans with demodicosis have shown that *Demodex* spp. mites are able to modulate the immune system with suggested methods of modulation including inhibition of the Th2 response, downregulation of T cell activation, and increased Treg cell activity (Akilov and Mumcuoglu, 2004; Liu et al., 2004; Gazi et al., 2019). As with human *Demodex* spp. mites, the gene expression profile in the current study suggests that canine mites appear to be able to modulate the T helper cell population to their advantage.

In chronic infections, T cells are exposed to persistent antigen and/or inflammatory signals (Blank et al., 2019). This persistent antigen presentation results in a reduction in T cell function, often referred to as T cell exhaustion (Blank et al., 2019; Yi et al., 2010). Several pathways have been shown to be involved in T cell exhaustion including prolonged TCR engagement and co-stimulatory and/or co-inhibitory signals, excessive levels of inflammatory cytokines (IFNs) and suppressive cytokines including IL-10 and TGFβ, as well as changes in the expression levels of chemokine receptors, adhesion molecules and nutrient receptors (Blank et al., 2019; Yi et al., 2010). Exhausted T cells are accompanied by a progressive increase in the amount and diversity of inhibitory receptors that are expressed, including PD-1, LAG3, 2B4, CD160 and TIGIT (Wherry and Kurachi, 2015). Genes commonly expressed by exhausted T cells were found to be significantly upregulated in the demodicosis group relative to controls. These findings reflect and support previous studies that indicated the presence of T cell exhaustion in the peripheral blood of dogs with demodicosis (Singh et al., 2010; IT et al., 2010; Fukata et al., 2005).

There was a significant increase in expression of B cell associated genes; further assessment of this revealed that significant expression of these genes only occurred in samples that had histological evidence of furunculosis, and perifollicular granulomas centred on fragments of mites and keratin (data not shown). In samples that had perifollicular inflammation but no evidence of follicular rupture, BLK, FCRL2 and MSFA1 genes were not differentially expressed. The gene TNFRSF17 was significantly upregulated in this latter sub-population (2.14, $p \leq 0.001$), but not to the same degree as in cases with furunculosis. It is therefore likely that significant B cell involvement in cases of demodicosis only occurs when the population of mites and the degree of inflammation is such that it results in follicular rupture. Our data suggest that B cells may therefore play a less pivotal role in the initial development of disease.

The upregulation of PD-1, PD-L1 and CTLA-4 indicates several immune checkpoint pathways by which *Demodex* spp. mites may regulate the immune system to their advantage. PD-L1 is not only expressed by DCs but has also been shown to be lowly expressed by keratinocytes (Keir et al., 2008). Activation of PD-1 and CTLA-4 results in reduced TCR signalling, reduced T cell proliferation and survival as well as increased T cell exhaustion and increased Treg cell activity, all of which would aid the evasion of the immune system by demodectic mites. Another related pathway that may be used is the induction of IDO1 in DCs by activation of CTLA-4. IDO1 is a tryptophan-catabolizing enzyme that acts as an immune checkpoint molecule that modifies inflammation. It is produced by alternatively activated macrophages (M2 macrophages) and is known to suppress T cell activity and generate Treg cells and myeloid derived suppressor cells. IDO1 results in tryptophan depletion thereby preventing T cell proliferation as well as causing the production of catabolic products (kynurenine) that are cytotoxic for T lymphocytes and NK cells (Munn et al., 1999; Lee et al., 2002; Frumento et al., 2002). IDO1 was found to be significantly upregulated in cases of demodicosis relative to control samples, suggesting a possible role in immune modulation by this parasite.

Other immune checkpoint molecules known to provide inhibitory signals to T cells were also significantly upregulated in dogs with demodicosis relative to controls; these include HVEM (TNFRS14), a member of the TNF receptor superfamily that serves as a receptor to several ligands, including BTLA and CD160, which inhibit T cell activation when bound to HVEM (Yu et al., 2019); T cell Immunoglobulin Domain and Mucin Domain 3 (TIM3), which belongs to the IgSF and regulates macrophage activation and inhibits Th1 mediated immune responses to promote tolerance (Banerjee and Kane, 2018); LAG-3 which is expressed on activated T cells, NK cells, B cells and plasmacytoid DCs and which negatively regulate proliferation, activation and homeostasis of T cells in a similar fashion to CTLA-4 and PD-1 (Graydon et al., 2021). This upregulation of a wide range of key immune checkpoint genes highlights the complexity of the immune modulation naturally occurring in canine demodicosis.

Genes associated with myeloid derived suppressor cells (MDSCs) were also found to be significantly differentially expressed. MDSCs are pathologically activated neutrophils and monocytes with potent immunosuppressive activity. Classical myeloid cell activation in response to pathogens and tissue damage is mainly driven via danger-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and TLR activation (Veglia et al., 2021). Pathological activation, however, arises from persistent stimulation such as that seen in the context of chronic infections, inflammation, cancer, autoimmune diseases or in this case, demodectic infestations (Veglia et al., 2021). MDSCs possess potent immune suppressive activities; they have been shown to downregulate type 1 interferons, inhibit T cells and T helper cells by inducing T cell exhaustion, downregulate macrophage production of IL-12, induce T reg cells and produce IL-10 and TGFβ (Ostrand-rosenberg and Sinha, 2009). Research into the role of MDSCs in canine diseases, not limited to demodicosis, is an area warranting further investigation.

Another pathway of immune regulation resulting in immune suppression is the upregulation of VEGFA. VEGFA is produced by many cell types including macrophages and keratinocytes. It is highly conserved between humans and dogs (Leis-Filho et al., 2021). Human VEGFA has been shown to inhibit DC maturation resulting in an increase in immature DCs that have an immunosuppressive function including the production of IL-10 and TGFβ. VEGFA has additionally been associated with the accumulation and proliferation of Treg cells and MDSCs (Leis-Filho et al., 2021; Bourhis et al., 2021). Of note, in human rosacea, which is often associated with an increase in the *Demodex* spp. mite population, there is increased expression of VEGF (Forton, 2020). Our study showed that VEGFA was significantly upregulated in canine demodicosis, suggesting a possible role for VEGFA in Treg and MDSC proliferation together with immunosuppressive cytokine production in this disease.

While there was upregulation of TLR2, the most commonly implicated TLR in the pathogenesis of canine demodicosis, there was also upregulation in genes for TLR1, TLR6, TLR7, TLR8, TLR9, TLR10 and adaptor protein MyD88. Heterodimers and homodimers of cell membrane TLRs result in the production of inflammatory cytokines, whereas endosome associated TLRs including TLR2, TLR4, TLR7, TLR8 and TLR9 result in the production of immunosuppressive cytokines including interferons and IL-10 (Guven-Maiorov et al., 2015). Suppression of the proinflammatory signals resulting from TLR activation is under the control of several regulators. These regulators function to prevent ligand-receptor binding, degrade the target protein and inhibit recruitment or transcription of intermediates (Yuk and Jo, 2011). One such regulator are the soluble forms of TLRs (sTLRs). They include soluble forms of TLR2 and TLR4 which function as a feedback mechanism for the inhibition of excessive TLR activation (Lebouder et al., 2003). Other regulators include Suppression of Tumorigenicity 2 L (ST2L) and Single

Immunoglobulin Interleukin-1 Receptor-Related protein (SIGIRR) which are membrane-associated TLR regulators which inhibit MyD88-dependent pathways (Brint et al., 2004). Intracellular TLR regulators which include sMyD88, A20 and small heterodimer partner (SHP, NROB2) have also been shown to negatively regulate TLR signalling (Guven-Maiorov et al., 2015). Upregulation of several regulators were found in the cases of demodicosis including ST2L, SIGIRR, A20 and BTK. These findings indicate that while TLR2 activation is playing a key role in disease development, so too are other endosome associated TLRs and TLR inhibitors. The activation of TLRs and their inhibitors in demodicosis are most likely having a cumulative effect of reducing pro-inflammatory cytokines and immune cell signalling during disease development.

Several genes associated with cellular senescence were measured. One of these genes, SerpinB2, was found to be significantly downregulated. SerpinB2 is constitutively expressed on differentiating keratinocytes and is induced during many inflammatory processes and infections and is one of the most upregulated proteins of activated monocytes/macrophages (Schroder et al., 2010). Downregulation is likely to result in reduced immune cell migration thus assisting in promoting immunosuppression in canine demodicosis. Another well-known marker of cellular senescence is BRAF, a serine/threonine-specific protein kinase, which plays a key role in cell growth. It is often mutated in cancer cells allowing for their increased proliferation. BRAF inhibitors have become a main stem treatment for several human cancers including melanoma. There have been several reports of clinical cases of demodicosis in human patients receiving BRAF inhibitors (Taritsa et al., 2022); our findings of significant downregulation of BRAF together with the increase in human cases of demodicosis on BRAF inhibitor therapy suggests that the downregulation of BRAF may be a key step in immune modulation by *Demodex* spp. mites.

The main limitation of this study was that it was a retrospective study. A prospective study following cases of canine demodicosis from presentation to the resolvement of clinical signs would show the dynamics of the immune cell population and the activation and suppression of immune modulating pathways of early to late lesions. However, this limitation is common to many clinical research projects in which repeat sampling gives rise to ethical concerns. Another important limitation, as the study was retrospective, was lesion location. Samples used in the study were from different anatomical locations. It is possible that the gene expression profile of skin from cases of canine demodicosis affecting the paws only could differ from those affecting the face only; again, a prospective study would be required to assess this.

## 5. Conclusion

The findings of this study support the hypothesis that *Demodex* spp. mites are modulating the immune system to aid their survival and proliferation. While previous studies have focused on TLRs and immune suppressive cytokines, here we show that immune checkpoint molecules, regulatory T cells and myeloid derived suppressor cells are additional potential key players in disease development. Further proteomic investigations are warranted which will help to reveal if the gene expression profiles reported here translate to a protein level.

## Animal Welfare Statement

All samples used were residual clinical samples accessed only after all diagnostic tests required by the treating veterinarian were complete. All tests were performed per the protocol approved by the Animal Research Ethics Committee, UCD (Approval ID: AREC E 19 09 Kelly).

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## CRediT authorship contribution statement

**Pamela A. Kelly, Rory Breathnach**: Funding acquisition. **Pamela A. Kelly, John Browne, Rory Breathnach**: Conceptualization. **Pamela A. Kelly**: Investigation, Writing – original draft. **Pamela A. Kelly, Jennifer S. McKay, Susan Peters**: Data curation. **Pamela A. Kelly, John Browne**: Formal analysis. **Pamela A. Kelly, John Browne, Susan Peters, Frazer Bell**: Methodology, Validation. **Pamela A. Kelly, John Browne, Susan Peters, Frazer Bell, Jennifer S. McKay, Irene Lara-Saez, Rory Breathnach**: Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109954.

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