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Activation-induced cell death of NK cells in canine atopic dermatitis revealed by Droplet-Sequencing data of peripheral blood mononuclear cells

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Original Article

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Abstract

Atopic dermatitis (AD) is a mutual allergic skin disease between humans and dogs manifesting varieties of similarities in their immune responses to the disease. Recently, activation-induced cell death (AICD) of NK cells was acknowledged as a novel disrupted immunoregulation in human AD. To gain insight into its plausible commotion in canine AD, NK cell expression profiles extracted from PBMC Drop-Sequencing (Drop-Seq) data of normal and AD dogs were analyzed along with human NK cell profiles of healthy and AD patients previously reported. According to the results, a total of 2,689 differentially expressed genes (DEG) between healthy and AD conditions ($P \leq 0.0001$) were presented—and some of them were associated with cell apoptosis regulation ($P \leq 0.1$). Interestingly, enhanced expressions of 13 AICD-associated genes were demonstrated in NK cells obtained from AD dogs concordant with those previously reported in AD patients. This study hereby supports the idea of AD inducing AICD of canine NK cells using a novel strategy to analyze target immune cell profiles presented in canine PBMC single-cell RNA-Seq data.

Keywords: Activation-induced cell death, NK cell, atopic dermatitis, Droplet-Sequencing, peripheral blood mononuclear cells

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Introduction

Atopic dermatitis (AD) is a common allergic skin disease among mammals. For decades, AD has been regarded as one of the most concordant disorders between humans and dogs sharing almost identical disease characteristics including its parthenogenesis, clinical signs, diagnostic criteria and also immunoregulation (Nagata and Nanko, 2010; Terada *et al*., 2011). Like its human counterpart, redundant type 2 helper T cell (Th2) cytokines—IL-4, IL-5, and IL-13 are also the hallmark of canine AD (Czarnowicki *et al*., 2015; Früh *et al*., 2020). These excessive cytokines contribute greatly to AD pathogenesis by promoting several allergic skin reactions and exacerbate the enhanced immunoglobulin E (IgE) production (Terada *et al*., 2011; Czarnowicki *et al*., 2015; Majewska *et al*., 2016; Olivry *et al*., 2016; Früh *et al*., 2020).

Continuous AD studies in humans have further suggested other immune cell types such as basophils and group 2 innate lymphoid cells (ILC2s) as the other important sources of type 2 cytokines disrupted AD immunoregulation (Roediger *et al*., 2013; Salimi *et al*., 2013). Recently, the natural killer (NK) cell has been suggested as another novel cell population in human AD development (Mack *et al*., 2020). Regarded as a major effector lymphoid cell of innate immunity, NK cells are present in several immune compartments and also in peripheral blood. Interestingly, peripheral blood NK cells have recently been suggested to regulate exacerbating Th-2 response in human AD via their IFN-γ production. The study demonstrates the reduction of peripheral NK cell population in AD patients—by which activation-induced cell death (AICD) contributes to the cell's death (Mack *et al*., 2020).

AICD refers to a process of persistent lymphocyte stimulation augmenting its apoptosis induction via precise receptor-induced caspase signaling. In the case of NK cells, this has been common via stimulation of CD2, CD16, or CD94. AICD of NK cells has been regarded as a crucial contributor aggravating several systemic autoimmune diseases (Johansson *et al*., 2005) and even hypersensitivity diseases like AD as previously mentioned (Mack *et al*., 2020). In canine AD, the reduction of NK cell pool contributing to the disease's severity was also implied by the previous report of decreasing NK cells in peripheral blood of dogs with AD (Hoskova *et al*., 2015). While absolute confirmation of AICD induction required both direct evaluation of NK cell's phenotypes and functions, the available Droplet-Sequencing (Drop-Seq) data of canine peripheral blood mononuclear cells (PBMCs) (Früh *et al*., 2020) might offer us a chance to gain insight into such knowledge in advance.

Drop-Seq is a high throughput single-cell mRNA sequencing (scRNA-Seq) technology that enables the sequencing of individual cells present in droplets. Implementing of Drop-Seq on heterogeneous cell populations like PBMCs hereby allows segregated analysis of each immune cell type including NK cell based on its specific cell marker genes (Chen *et al*., 2018; Ding *et al*., 2020). Similar to humans, several NK cell-specific markers including CD16 were also expressed in canine NK cells (Foltz *et al*., 2016; Gingrich *et al*., 2019). Since the NK cell profiles in scRNA-Seq data of human PBMC are acknowledged to be electable by expression of such marker genes (Chen *et al*., 2018; Ding *et al*., 2020), a similar concept should hereby be applicable to canine PBMC Drop-seq data (Früh *et al*., 2020).

To gain more insight into possible AICD of NK cells caused by canine AD, the canine NK cell expression profiles were retrieved from PBMC Drop-seq data of normal and AD dogs (Früh *et al*., 2020) and analyzed. Interestingly, several genes associated with AICD signaling, the AICD-associated genes were found to be enhanced in NK cells acquired from AD dogs supporting the idea of their decease in canine AD.

Materials and Methods

Programming environment and software: The open-source web application, 'Jupyter Notebook' was utilized to create and run Python and R coding. The versions of Python and R used in this study were 3.8.5 and 4.0.3, accordingly.

Sample datasets: The Drop-Seq data of PBMC isolated from normal dogs and dogs with AD was utilized for extraction of NK cell expression profiles. In this study, bulk RNA-Seq data of human NK cells isolated from normal humans and AD patients was implemented as references for parallel analysis. Both human and canine data were presented in NCBI with accession numbers GSE125916 (Mack *et al*., 2020) and GSE144730 (Früh *et al*., 2020), respectively. In detail, the canine blood samples for PBMC isolation were collected from both healthy dogs or dogs diagnosed with AD at the Cornell University Hospital for Animals Dermatology Service (CUHA Derm) and in Houston, Texas (West Highland White Terriers). Dogs with neoplasia or receiving systemic corticosteroid treatment were not included. For human PBMC isolation, the peripheral blood of healthy humans was collected from Mohs surgery patients. Meanwhile, the peripheral blood samples of AD patients were acquired from patients diagnosed with moderate-to-severe AD in the Division of Dermatology at WUSM/BJH from November 2015 to September 2018, All datasets were retrieved from the sequence read archive (SRA) database (https://www.ncbi.nlm. nih.gov/sra) with their brief descriptions provided in Table 1.

Data pre-processing: Pre-processing of human mRNA-Seq was performed utilizing methods provided in our previous study (Chokeshaiusaha *et al*., 2019). For canine Drop-Seq data pre-processing, 'FLEXBAR' software (Dodt *et al*., 2012) was applied to remove adapter sequences, trimming contaminated sequences and selecting the qualified sequences (length ≥ 30 nucleotides and mean Phred score ≥ 25). Cell barcode demultiplexing, Unique molecular identifier (UMI) collapsing, sequence alignment and quantification were utilized by the 'STARsolo-2.7.3a' package (Blibaum *et al*., 2019) based on the canine genome assembly (CanFam3.1) available in the Ensembl genome browser. Only sample cell datasets with total count read numbers more than 10^5 counts in at least 200 genes would be considered in this study. All selected

cell datasets had a Fraction of mitochondrial reads < 20% indicating that they were not in process of apoptosis. Normalization of canine pre-processed data was performed with Count Per Million (CPM) output using the 'scater' package (McCarthy *et al.*, 2016).

Canine NK cell selection: Available human-canine orthologous genes were selected by procedures described in our previous study (Chokeshaiusaha *et al.*, 2015). With scaled expressions of the selected immune cell marker genes (Table 2), hierarchical clustering—complete linkage method with Euclidean distance of canine PBMC data obtained from both healthy and AD dogs was performed. Each immune cell type was further categorized based on its major marker gene expression levels. Conventional canine NK cells, B cells, and monocytes were identified based on their remarkable CD16A, CD14, CD19 and CCR2 expressions, accordingly. T cells were selected for their consistent presence of only CD3D, CD3E, and CD3G expressions in the absence of other marker gene expressions. The leftover cells that could not meet these criteria were excluded from this study. The similarities among the selected cells were further determined by t-distributed stochastic neighbor embedding (t-SNE) using the 'calculateTSNE' function provided by the 'scater' package (McCarthy *et al.*, 2016).

Table 1 Human and canine datasets

Dataset	*Species*	*Data type*	*Description*
SRR8508517	Human	Bulk RNA-Seq	NK cells sorted from AD patient's PBMC
SRR8508520	Human	Bulk RNA-Seq	NK cells sorted from AD patient's PBMC
SRR8508523	Human	Bulk RNA-Seq	NK cells sorted from AD patient's PBMC
SRR8508524	Human	Bulk RNA-Seq	NK cells sorted from AD patient's PBMC
SRR8508518	Human	Bulk RNA-Seq	NK cells sorted from healthy person's PBMC
SRR8508519	Human	Bulk RNA-Seq	NK cells sorted from healthy person's PBMC
SRR8508521	Human	Bulk RNA-Seq	NK cells sorted from healthy person's PBMC
SRR8508522	Human	Bulk RNA-Seq	NK cells sorted from healthy person's PBMC
SRR11014432	Dog	Drop-Seq	Heathy dog's PBMC
SRR11014433	Dog	Drop-Seq	Heathy dog's PBMC
SRR11014434	Dog	Drop-Seq	Heathy dog's PBMC
SRR11014435	Dog	Drop-Seq	Heathy dog's PBMC
SRR11014436	Dog	Drop-Seq	Heathy dog's PBMC
SRR11014437	Dog	Drop-Seq	AD dog's PBMC
SRR11014438	Dog	Drop-Seq	AD dog's PBMC
SRR11014439	Dog	Drop-Seq	AD dog's PBMC
SRR11014440	Dog	Drop-Seq	AD dog's PBMC
SRR11014441	Dog	Drop-Seq	AD dog's PBMC

Table 2 Immune cell marker genes

Immune cell	*Marker gene name*
NK cell	**CD16A**
T cell	CD3D
	CD3E
	CD3G
B cell	CD19
Monocyte	CCR2

Differentially expressed gene (DEG) and candidate AICD-associated genes: DEG between healthy and AD conditions of both human and canine NK cells was acquired from the negative binomial generalized linear model (DESeq2 package) (Love *et al.*, 2014) and the Zero-inflated regression model (MAST package) (Finak *et al.*, 2015), accordingly. The differentially expressed genes acquired from comparisons in both species would be considered together. In an account of the AICD-associated genes, only genes previously reported for their upregulation in NK cells acquired from AD patients would be implemented (Mack *et al.*, 2020). Of note, only genes that were mutually upregulated/downregulated in both species would be considered in this study ($P \leq 0.0001$)

Gene-annotation enrichment analysis: Gene-annotation enrichment analysis was performed based

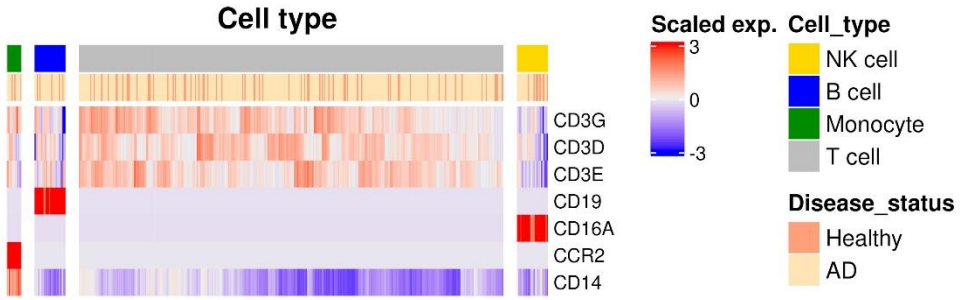
on acquired DEG using the 'goseq' package as previously described in our study (Chokeshaiusaha *et al*., 2019).

Data virtualization: Heatmaps and UpSet plots were generated by the 'ComplexHeatmap' package (Gu *et al*., 2016). The t-SNE scatter plot was drawn by the 'scatterplot3D' package (Ligges and Martin, 2003), and the word cloud of significant Gene Ontology (GO) terms obtained from enrichment analysis was plotted by the 'Gosummaries' package (Kolde and Vilo, 2015).

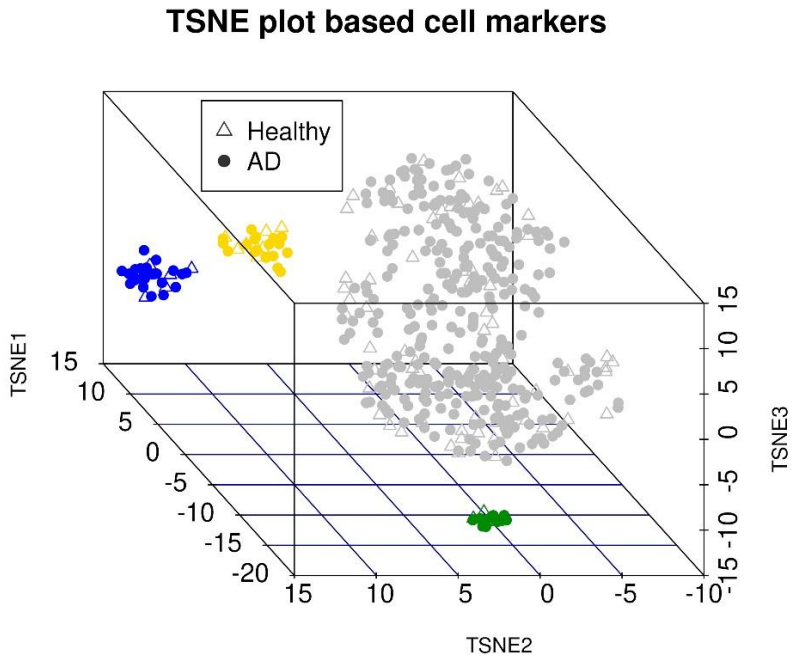
Results

Canine NK cell populations were successfully separated by clustering PBMC data based on the selected cell marker genes: Hierarchical clustering by the selected cell marker genes (Table 2) successfully revealed 4 immune cell populations—B cells, T cells, monocytes, and NK cells in canine PBMCs (Fig. 1A). For each cell type, the cells acquired from both healthy and AD dogs were homogeneously clustered together indicating that they were from the same categorized cell population. The numbers of identified T cells, B cells, monocytes, and NK cells of both healthy (Fig. 1C) and AD dogs (Fig. 1D) are demonstrated in Table 3. Scatter plot of t-SNE also manifested agreeable cell cluster results—by which clear segregation among cell types was lucidly visible with mingled cells acquired from both healthy and AD dogs (Fig. 1B).

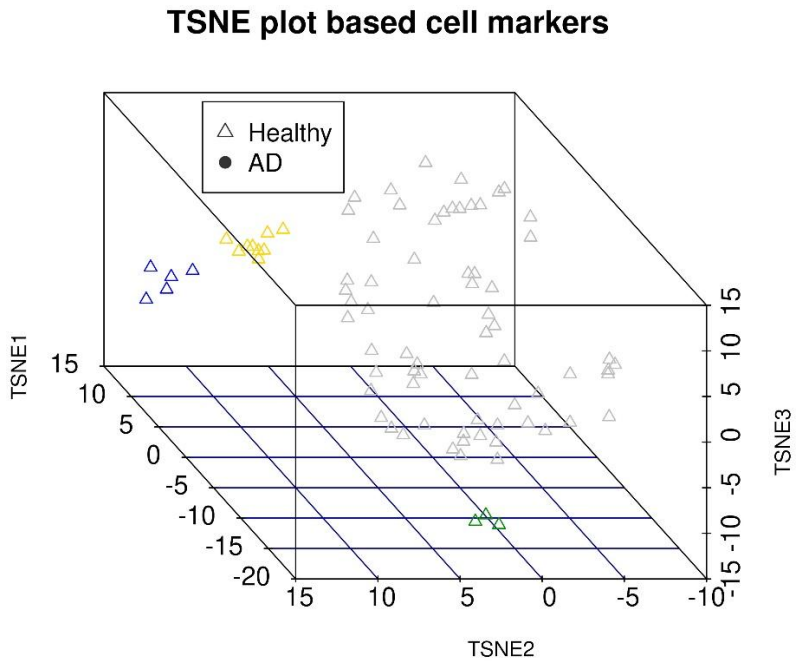
Several AICD-associated genes were upregulated in NK cells of dogs with AD: The candidate canine NK cells were further categorized into NK cells obtained from healthy and AD dogs—9 and 19 cells, respectively. According to the DEG analysis between NK cells acquired from healthy and AD conditions in both humans and dogs, 2,689 DEGs were shared between the two species. The shared DEG consumed 89% of DEG found in dogs (P≤0.01) (red bar in Fig. 2A). On account of gene-annotation enrichment results, most of these DEGs were generally associated with mRNA metabolisms of cells (Fig. 2B). However, the term "negative regulation of apoptotic process" was also presented (P≤0.1) (Fig. 2B). Concordant with this, further inspection also revealed previously reported 13 AICD-associated genes (Mack *et al*., 2020) co-upregulated in AD patients and AD dogs. These genes were alphabetically ordered as follows: BCL2L1, BID, CASP2, DFFA, FASLG, IL1R1, PARP1, PIK3CD, PPP3CA, PRKACB, TNFRSF1A, TNFSF10, and XIAP (Fig. 3).



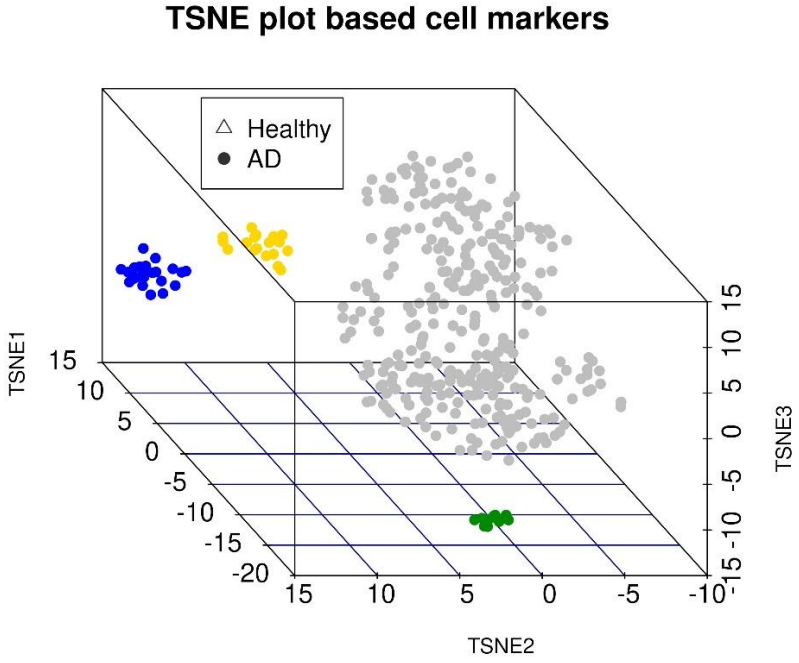
1A



1B



1C

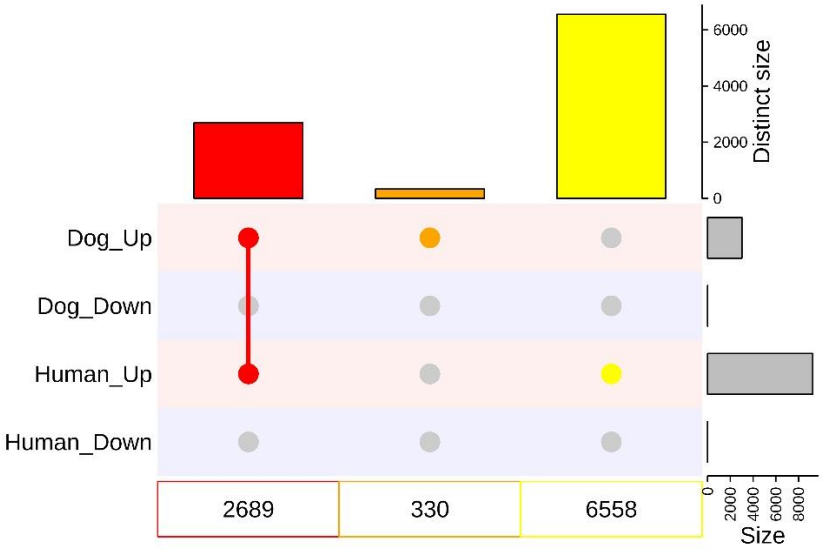


1D

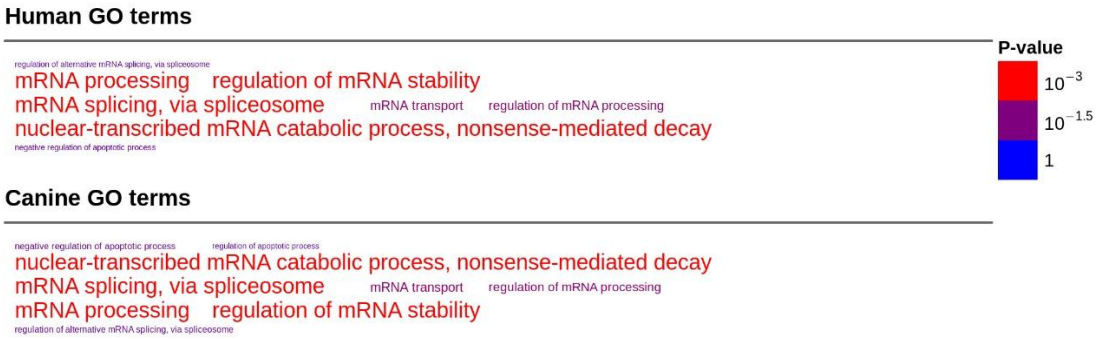
Figure 1 Categorization of cells presented in canine PBMC data. The heatmap illustrating the hierarchical clustering result of canine PBMC data acquired from both healthy and AD dogs was drawn (Figure 1A) along with its scatter plot of t-SNE (Figure 1B). For clearer manifestation, the cells acquired from healthy dogs (Figure 1C) and AD dogs (Figure 1D) were also individually demonstrated. NK cells, B cells, Monocytes and T cells were represented by the colors—yellow, blue, green, and gray as indicated in the 'Cell type' legend. The legend indicating the source of PBMC (acquired from either healthy or AD dogs) was also separately addressed in each plot.

Table 3 Numbers of the categorized cells

Immune cell	*Dog's status*	*Cell number*
NK cell	Healthy	9
	AD	19
T cell	Healthy	66
	AD	319
B cell	Healthy	5
	AD	23
Monocyte	Healthy	3
	AD	10



2A



2B

Figure 2 DEG and gene enrichment analysis. DEG between healthy and AD conditions of both human and canine NK cells was illustrated by the UpSet plot (Figure 2A). For the UpSet plot (Figure 2A), the line drawn between the 2 dots represented the intersect genes (orthologs) shared between the groups of interest. A sole dot represented the genes solely presented in the group of interest with no intersection with any other group. There were 4 groups of genes. The 'Up_Dog' and 'Down_Dog' groups represented the genes upregulated and downregulated in NK cells acquired from AD dogs compared to those of healthy ones. Similarly, the 'Up_Human' and 'Down_Human' groups represented the genes upregulated and downregulated in NK cells acquired from AD patients compared to those of healthy ones. The red bar of the plot hereby represented genes that were co-upregulated in both AD dogs and AD patients. The orange and yellow bars represented the genes uniquely upregulated in AD dogs and AD patients, respectively. The word cloud of significant Gene Ontology (GO) terms obtained from enrichment analysis using these co-upregulated genes was subsequently drawn showing terms associated with mRNA metabolism and negative regulation of apoptosis (Figure 2B).

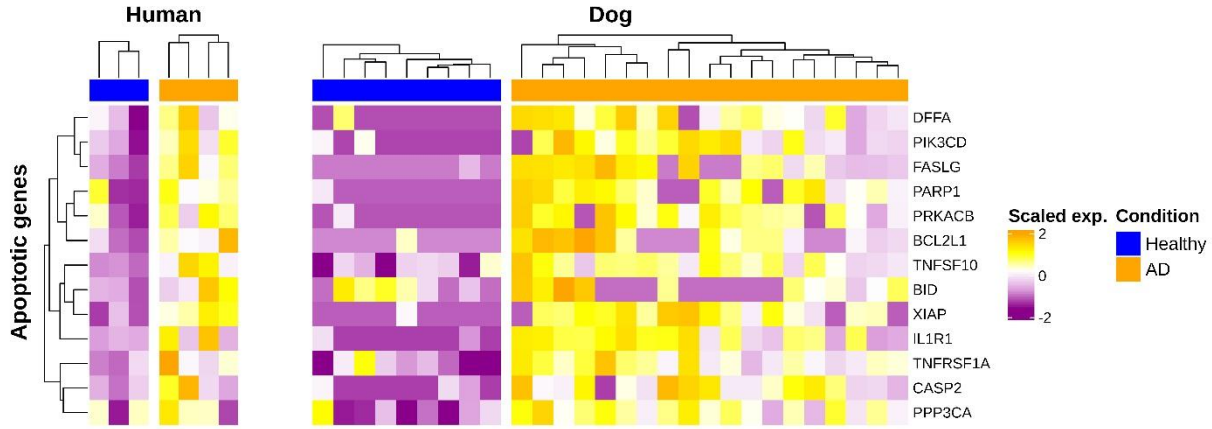


Figure 3 Heatmap of significant AICD-associated genes. The significant AICD-associated genes between healthy and AD conditions in both humans and dogs were illustrated. The heatmap was separated into two sections according to the species (Dog and Human) contributing to the NK samples. The condition legend indicated the source of NK cells either they were acquired from healthy or AD dog/human—by which the upregulation of all significant genes in AD samples was demonstrated.

Discussion

Enhanced AICD-associated gene expressions of NK cells caused by canine AD were demonstrated in the current study. While our designed procedures could successfully render the expected results, some concerns should be noted as the limitation of the process. The most crucial one should be regarded to the limited count read numbers—limited sequencing dept we found distributed among cells in canine PBMC data during pre-preprocessing (data not shown). Such limited read counts should hereby be accounted for both restrictedly available NK cell datasets and expressed genes—including the cell marker gene manifested in this study (Zhang *et al*., 2020). For instance, possible contamination of $CD16^{+}$monocyte subpopulations in the categorized NK cells should be concerned despite their clear clustering.

Successful cell type categorization of PBMC acquired from both healthy and AD dogs were demonstrated in the current study (Fig. 1). The result hereby helped to verify the same NK cell subpopulation obtained by both healthy and AD dogs applied for subsequent differential gene expression and enrichment analyses. Despite the cells of the same cell types being clustered together, the remarkable differences in frequencies of T cells, B cells, monocytes, and NK cell numbers acquired from healthy and AD dogs' PBMCs should be noted as another limitation of this study. While such results could be improved by modifying the stringency of the pre-processing procedures, such a performance should affect the subsequent differential expression and enrichment analyses of NK cells. We were hereby inclined to focus on only the well-defined and reliable NK cell populations in this study. Since previously reported AICD-associated gene expressions in human AD was focused on NK cell population with CD16 expressions (Mack *et al*., 2020), the usage of the CD16A gene as a canine NK cell marker in this study was implemented on purpose despite a few other markers being available (Foltz *et al*., 2016; Lee *et al*., 2018; Gingrich *et al*., 2019). It should however be noted that the NK cell categorization system in each mammalian species was critically complex and also depended on the cell characteristics of interest. Through this, further studies associated with the properties of canine NK cells with high CD16A expressions are required to ensure their compatibility with human $CD16^{+}$NK cells.

Most acquired DEG between healthy and AD conditions in dogs are shared with those of humans (Fig. 2A). While these DEG can be associated with a variety of NK cell metabolisms, we hereby performed enrichment analysis to narrow down the most significant cell processes—by which mRNA metabolisms and regulation of apoptosis annotations are revealed (Fig. 2B). Generally, mRNA metabolism was associated with various cell functions and processes including AICD. Concordant with this, 13 AICD-associated genes previously reported to be upregulated in NK cells of AD patients (Mack *et al*., 2020) were also noticeable in AD dogs (Fig. 3). Some of these genes—FASLG, IL-1R, TNFRSF1A, and TNFSF10 encoded protein receptors, ligands or cytokines directly aggravating AICD of NK cells (Busuttil *et al*., 2010; Zhang *et al*., 2017). The other genes encoded signaling proteins in the apoptosis pathway were BID, CASP2, DFFA, PIK3CD, PPP3CA, PRKACB and XIAP (Chantry *et al*., 1997; Brustovetsky *et al*., 2003; Wang and El-Deiry, 2003; Krumschnabel *et al*., 2009; Kumar, 2009; Hammond and Udvadia, 2010; Chen *et al*., 2013). Enhanced expression of these genes in canine AD should hereby influence the NK cell's sensitivity to death by AICD induction. While the regulatory role of canine peripheral NK cells in AD pathogenesis via its IFN-γ production was still unknown, their similar AICD fate with those in humans (Mack *et al*., 2020) implied in this study interestingly suggested the possible immunoregulatory role of canine NK cells in AD as a novel issue for the future study.

In conclusion, the current study introduced and demonstrated the procedures to extract and analyze canine NK cell expression profiles from canine PBMC Drop-Seq data of healthy and AD dogs. Concordant changes in their expression profiles with those previously reported in humans were manifested—by which AD-enhanced AICD-associated gene expressions were presented. Although this study was limited to only expression profile analysis which required further confirmation at the biological level, our findings partially implied an evolutionarily conserved role of NK cells in AD pathogenesis and regulation between humans and dogs. Of note, this study achieved introducing a novel strategy to analyze target immune cells presented in canine PBMC single-cell RNA-Seq data—from which a similar process could be applied with other immune cell types of interest in future studies.

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