Identification of Pro-Fibrotic Macrophage Populations by Single-Cell Transcriptomic Analysis in West Highland White Terriers Affected With Canine Idiopathic Pulmonary Fibrosis

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Canine idiopathic pulmonary fibrosis (CIPF) affects old dogs from the West Highland white terrier (WHWT) breed and mimics idiopathic pulmonary fibrosis (IPF) in human. The disease results from deposition of fibrotic tissue in the lung parenchyma causing respiratory failure. Recent studies in IPF using single-cell RNA sequencing (scRNA-seq) revealed the presence of profibrotic macrophage populations in the lung, which could be targeted for therapeutic purpose. In dogs, scRNA-seq was recently validated for the detection of cell populations in bronchoalveolar lavage fluid (BALF) from healthy dogs. Here we used the scRNA-seq to characterize disease-related heterogeneity within cell populations of macrophages/monocytes (Ma/Mo) in the BALF from five WHWTs affected with CIPF in comparison with three healthy WHWTs. Gene set enrichment analysis was also used to assess pro-fibrotic capacities of Ma/Mo populations. Five clusters of Ma/Mo were identified. Gene set enrichment analyses revealed the presence of pro-fibrotic monocytes in higher proportion in CIPF WHWTs than in healthy WHWTs. In addition, monocye-derived macrophages enriched in pro-fibrotic genes in CIPF compared with healthy WHWTs were also identified. These results suggest the implication of Ma/Mo clusters in CIPF processes, although, further research is needed to understand their role in disease pathogenesis. Overexpressed molecules associated with pulmonary fibrosis processes were also identified that could be used as biomarkers and/or therapeutic targets in the future.

Keywords: macrophages, canine idiopathic pulmonary fibrosis, dog, single-cell RNA-seq methods, bronchoalveolar lavage fluid, lung
INTRODUCTION

Canine idiopathic pulmonary fibrosis (CIPF) is defined as a progressive and abnormal accumulation of collagen in the lung parenchyma that threatens alveolar gas exchange and reduces lung compliance causing cough, exercise intolerance, and, finally, respiratory failure and death (1, 2). The disease affects predominantly middle-aged to old dogs from the West Highland white terrier (WHWT) breed (1, 2). Although the cause of CIPF is not identified, a genetic etiology is suspected as it affects mainly one breed. Confirmation of the diagnostic remains challenging due to absence of available diagnostic biomarkers and necessity to exclude other diseases and comorbidities. It currently relies on either thoracic high-resolution computed tomography (HRCT) or histopathology of the lung tissue or both. Despite a lot of researches on CIPF, the pathophysiology remains unclear and no curative treatment are available (1, 2).

CIPF shares several clinical findings with human idiopathic pulmonary fibrosis (IPF). However, thoracic HRCT and histopathology show features associated with both human IPF and non-specific interstitial pneumonia demonstrating that CIPF and IPF are not strictly identical (1, 2). In spite of those differences, studying CIPF in WHWTs is worth to better understand IPF. Indeed, dogs, like human, are subjected to various environmental stresses which can have an impact on lung cells especially alveolar macrophages (AMs) (3). Moreover, CIPF is a disease that develops spontaneously in WHWTs (1, 2). Those characteristics make the dog a much more interesting model compared to the mouse experimental models. In human IPF and IPF mouse models, recent studies used single-cell mRNA sequencing (scRNA-seq) to detect altered cell populations compared with healthy conditions through an unbiased approach (4–13). Indeed, the technique allows high-throughput and high-resolution analysis of thousands of cells at the same time without requiring prior knowledge of cell markers to determine cell heterogeneity (14–16). With this method, a profibrotic role of specific macrophage and monocyte populations has been described in IPF patients and IPF mouse models (4, 6, 7, 12, 17). An increased number of macrophages and proliferating myeloid cells was found in bleomycin-induced lung fibrosis mouse models, in the beginning of lung fibrosis development, before fibroblastic infiltration (6). Specific monocyte and macrophage clusters were identified in fibrosis conditions (4, 5, 7, 12). AMs from IPF patients were enriched in functions involved in fibrotic processes including “extra-cellular matrix organization” and “regulation of cell migration” for example (5). Pro-fibroptic macrophage but also monocyte clusters that expressed genes able to drive fibroblasts’ proliferation were localized in areas of fibrosis (4, 12). All these findings indicate that targeting specific macrophage and monocyte clusters could be potentially useful for the prevention and the therapy of lung fibrosis (17).

Recently, cells of the bronchoalveolar lavage fluid (BALF) of healthy dogs have been characterized by scRNA-seq, providing a comprehensive single-cell expression profiling of the canine BALF cells in healthy conditions (18). Fourteen distinct cell populations were identified including AMs (three clusters), macrophages/monocytes (Ma/Mo) (one cluster), CD8+ T cells, CD8+CD4− T cells, B cells, neutrophils, mature and immature dendritic cells (DCs), ciliated and non-ciliated epithelial cells, mast cells, and cells in division (18).

The objective of this study was to characterize, using scRNA-seq, disease-related heterogeneity within Ma/Mo populations in the BALF from WHWTs affected with CIPF compared with healthy WHWTs.

MATERIALS AND METHODS

Dog Population

The scRNA-seq analysis was performed on BALF obtained from WHWTs affected with CIPF and healthy WHWTs. Dogs were prospectively recruited between March and October 2018 at the veterinary clinic of the University of Liège (Liège, Belgium) according to a protocol approved by the ethical committee of the University of Liège (approval no. 1435). All dogs were privately owned, and samples were obtained with owners’ written consent.

The healthy or CIPF status of the dogs was confirmed according to a previously described approach (19) based on history, physical examination, complete blood work, 6-min walked distance (6MWD), thoracic HRCT, bronchoscopy, and analysis of the BALF (including macroscopic evaluation and total (TCC) and differential (DCC) cell count). WHWTs under treatment including antimicrobials drugs and corticoids were excluded from the study.

BALF Collection

BALF was obtained using the same protocol as previously described (18). Briefly, under general anesthesia, a bronchoscope (FUJINON® Paediatric Video-Bronchoscope EB-530S) was inserted into the lower airways of the dogs. Three to 4 ml/kg of sterile saline solution was instilled in the airways through the bronchoscope channel and directly reaspirated. A part of the crude BALF was used for TCC and DCC obtained using respectively a hemocytometer and a cytospin preparation. The rest of the collected fluid was then directly transferred on ice to the GIGA laboratory of cellular and molecular immunology (Liège, Belgium).

Single-Cell RNA Sequencing

ScRNA-seq was performed as already described (18, 20). Briefly, BALFs were processed to obtain a final cell suspension containing between 500 and 1,000 cells/µl suspended in
phosphate-buffered saline solution (Gibco<sup>TM</sup> 1x DPBS, Cat.14190-169) containing 0.04% (w/v) bovine serum albumin. Cell viability assessed by Trypan blue staining was considered as acceptable above 80%. Details about BALF volume, final cell concentration and cell viability for each sample can be found in Supplementary Table 1.

For each sample, approximately 3,500 cells (Supplementary Table 1) were loaded into the Chromium™ Controller (10x Genomics, Pleasanton, CA, USA) and then partitioned into nano liter scale vessels containing 10x barcoded beads from Chromium<sup>TM</sup> Single Cell 3’ Gel Bead kit v2 (10x Genomics, Pleasanton, CA, USA) according to manufacturer’s instructions. Reverse transcription of mRNAs took place into vesicles on a Veriti<sup>®</sup> 96-Well Thermal Cycler (ThermoFisher Scientific, Merelbeke, Belgium) after cell lysis and capture of polyadenylated mRNAs.

Emulsion breakage, cDNA amplification, and libraries construction were performed using Chromium™ Single Cell 3’ Reagent kit v2 (10x Genomics, Pleasanton, CA, USA) according to manufacturer’s instructions as already described (18, 20). Libraries were assessed for quality (2100 Bioanalyzer Instrument; Agilent, Santa Clara, CA, USA) and were then partitioned into NextSeq500 instrument (Illumina, San Diego, CA, USA).

Initial data pre-processing was performed using the Cell Ranger software (v1.2.0) (10x Genomics, Pleasanton, CA, USA). Reads were mapped to dog genome (CanFam3.1, GenBank assembly accession: GCA_000002285.2). The genes not well annotated were further blasted on the Ensembl genome browser (v99.31) (21) for dog species.

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Further data analyses were performed using R package Seurat (version 3.1.2) (22) after the selection of the cells with a minimum of 100 and a maximum of 2,500 unique genes mapped, the selection of the genes found in at least three different cells and the normalization of the expression values to 10,000 transcripts per cell. scRNA-seq data coming from each dog were then merged for the next analyses which were done by following Stuart et al. (2019) instructions (22). Pre-ranked gene set enrichment analyses (GSEAs) were performed using GSEA-P software (v4.0.3) (23). The enrichment score was determined using weighted Kolmogorov–Smirnov-like statistic with false discovery rate (FDR) correction for multiple testing (23). A FDR cutoff of 25%> was considered as appropriate (23). GSEAs were computed between either the Gene Ontology (GO) Biological Process gene sets (v7.1) (23), or the Hallmark gene sets (v7.1) (23) or the Comparative Toxicogenomics Database Pulmonary fibrosis gene set (24). Differentially expressed genes (DEGs) in different conditions were obtained using the “FindMarkers” command in Seurat (22). Differential gene expressions were measured using non-parametric Wilcoxon rank sum tests adjusted for multiple testing with Bonferroni correction. Only DEGs with an average log2 fold change (avg_logFC) >0.25 and an adjusted P-value <0.05 were retained.

**Statistical Analyses**

A P-value lower than 0.05 was considered as significant. Details about statistical analyses for scRNA-seq data and GSEAs can be found in the section above. Statistics used for the comparison of the WHWTs groups are reported in Tables 1, 3, and 4.

**RESULTS**

**Study Population**

BALF samples were obtained from three healthy WHWTs and five WHWTs affected with CIPF. Characteristics of the dogs included in the study are reported in Table 1. No significant differences in age, gender, and weight were reported between the groups (Table 1).

CIPF diagnosis was confirmed in all CIPF WHWTs by thoracic HRCT which revealed extensive ground-glass opacity in all dogs. Other HRCT findings included a combination of mosaic pattern, bronchial wall thickening, parenchymal and subpleural bands, bronchomalacia and bronchiectasis. Among WHWTs affected with CIPF, 3/5 (60%) had an history of both exercise intolerance and cough and 2/5 (40%) only exhibited exercise intolerance. Crackles were heard on lung auscultation in all dogs. Three dogs (60%) had a restrictive dyspnea. Among them, two also exhibited cyanosis. The 6MWD covered by each CIPF WHWT is reported in Table 1. No significant differences in age, gender, and weight were reported between the groups (Table 1).

**TABLE 1** Characteristics of the West Highland white terriers either healthy or affected with canine idiopathic pulmonary fibrosis included in the study.

| Healthy WHWTs (n = 3) | WHWTs affected with CIPF (n = 5) | P-value |
|----------------------|---------------------------------|---------|
| Age, y               | 8.2 (5.4–8.7)                  | 10.8 (10.2–12.7) | 0.14<sup>a</sup> |
| Gender, M/F          | 2/1                            | 1/4      | 0.46<sup>a</sup> |
| Weight, kg           | 8.4 (8.4–8.9)                  | 9.5 (9.1–9.8)  | 0.14<sup>a</sup> |
| 6MWD, m              | 506.1 (478.9–515.0)            | 356.4 (356.1–366.3) | 0.04<sup>a</sup> |
| BALF analysis        |                                 |          |
| TCC, cells/μl        | 760 (685–770)                  | 2,620 (2,500–3,285) | 0.04<sup>a</sup> |
| Macrophages, %       | 78 (76.5–84.5)                 | 71 (84–82) | 0.39<sup>a</sup> |
| Neutrophils, %       | 3 (2.5–3.5)                    | 10 (9–21) | 0.04<sup>a</sup> |
| Lymphocytes, %       | 11 (7–16)                      | 7 (7–16) | 0.93<sup>a</sup> |
| Eosinophils, %       | 1 (1–4)                        | 2 (1–2) | 0.93<sup>a</sup> |
| Mast cells, %        | 0                              | 0        | / |
| Epithelial cells, %  | 1 (0.5–1.5)                    | 1 (0–1) | 0.46<sup>a</sup> |

Continuous data are not normally distributed according to the Shapiro-Wilk test and are then expressed in median and interquartile range. Groups were compared using either Mann-Whitney tests (*) or Chi-squared tests (**). WHWTs, West Highland white terriers; CIPF, canine idiopathic pulmonary fibrosis; M, male; F, female; 6MWD, 6-min walked distance; BALF, bronchoalveolar lavage fluid; TCC, total cell count.
Moreover, the distance was significantly reduced in CIPF compared with healthy WHWTs (Table 1). At echocardiography, signs of secondary pulmonary arterial hypertension were present in all CIPF dogs. Changes in BALF cells analysis were consistent with non-specific chronic lung inflammation (Table 1).

Among control WHWTs included in the study, all were clinically healthy and did not have any signs or findings indicating pulmonary disease. Echocardiography excluded the presence of cardiac disease in all of them. Thoracic HRCT did not reveal significant abnormalities. BALF cells analysis was unremarkable (Table 1).

**ScRNA-Seq Identifies Multiple Cell Populations in the Dog BALF**

Droplet-based scRNA-seq analysis of BALF cells was performed with a median read depth of ~43,000 reads per cell. In total, 19,255 cells (6,703 from healthy and 12,552 from diseased dogs) coding for 11,722 unique genes were included in the final analysis. The median detected genes per cell was 788 (interquartile range 399–1,191 genes/cells, Table 2). Individual metrics about mapping and cells are displayed in Table 2, the individual distribution for transcripts and genes counts is illustrated in Supplementary Figures 1A and B, respectively.

Cells from all samples were combined and aligned to account for sample variations among dogs using Seurat package in R (version 3.1.2) (22). They were then clustered and visualized using t-distributed stochastic neighbor embedding (t-SNE) plot with a resolution set at 0.3 and a number of dimensions to use set using t-SNE plot (version 3.1.2) (22). They were then clustered and visualized for sample variations among dogs using Seurat package in R.

Population in the Dog BALF illustrated in individual distribution for transcripts and genes counts is with a resolution set at 0.3 and a number of dimensions to use set using t-SNE plot (version 3.1.2) (22). They were then clustered and visualized for sample variations among dogs using Seurat package in R metrics about mapping and cells are displayed in Supplementary Figures 1A and B, respectively.

**Comparison Between Ma/Mo Clusters**

After Ma/Mo isolation from other cell populations, we repeated clustering on those cells to better characterize changes associated with CIPF. It resulted in the identification of five transcriptionally distinct Ma/Mo clusters (M0, M1, M2, M3, and M4) (Figure 2A). Average expression of all the genes expressed by each Ma/Mo cluster can be found in Supplementary Table 3. Relative contributions of each Ma/Mo cluster into each group of dogs either healthy or diseased are displayed in Figures 2B, C. Cells repartition between healthy and diseased WHWTs was similar into each cluster except in the cluster M2 which contained more cells in WHWTs affected with CIPF (Figures 2B, C and Table 4). We then estimated differential genes expression between each cluster of Ma/Mo and performed GSEA to better characterize Ma/Mo clusters independently of the disease status of the dogs. All DEGs identified in each cluster compared to others are displayed in Supplementary Table 4.

Results of the enrichment analyses performed by mapping DEGs identified in each cluster compared to others, to Hallmark gene sets or GO Biological Process gene sets are provided in Supplementary Table 5.

Resident AMs were identified based on MARCO expression (Figure 3), a class A scavenger receptor (5, 12, 18, 25, 26) and corresponded to cells in clusters M0 and M3 (Figure 2A, Supplementary Tables 3 and 4). They represented the cluster are provided in Supplementary Table 2. Principal markers used to identify cell populations can be found in Figure 1D. Each cell population included cells coming from healthy and diseased dogs (Figure 1C and Table 3). No significant differences were reported in relative proportions of the different cell types between healthy WHWTs and WHWTs affected with CIPF, except for mature DCs (Table 3).

**ScRNA-Seq Analysis Reveals Fibrosis-Associated Transcriptomic Changes in Ma/Mo Clusters**

### Table 2 | Metrics about mapping and characteristics of the detected cells in each bronchoalveolar lavage fluid specimen.

| Sample ID | Diagnosis | Number of cells passing quality control | Reads mapped confidently to genome, % | Reads mapped confidently to transcriptome, % | Median genes/cell (range) | Median UMIs/cell (range) | Total genes detected |
|-----------|-----------|----------------------------------------|---------------------------------------|--------------------------------------------|--------------------------|--------------------------|----------------------|
| WHWT 1    | Healthy   | 3,060                                   | 67                                    | 24.4                                       | 741 (445–1,184)           | 1,711 (899–3,351)        | 12,354               |
| WHWT 2    | Healthy   | 2,345                                   | 67.2                                  | 24.7                                       | 1,091 (585–1,448)         | 2,809 (1,173–4,398)      | 12,988               |
| WHWT 3    | Healthy   | 1,298                                   | 59.8                                  | 23.9                                       | 834 (376–1,046)           | 1,889 (678–2,671)        | 10,839               |
| CIPF 1    | CIPF      | 2,551                                   | 69.2                                  | 24.6                                       | 1,147 (827–1,348)         | 2,834 (1,857–3,740)      | 12,988               |
| CIPF 2    | CIPF      | 2,696                                   | 67.3                                  | 23.4                                       | 618 (219–1,226)           | 1,362 (354–3,390)        | 12,478               |
| CIPF 3    | CIPF      | 2,564                                   | 74.8                                  | 30.4                                       | 503 (355–969)             | 960 (601–2,247)          | 11,819               |
| CIPF 4    | CIPF      | 2,556                                   | 71.5                                  | 28.1                                       | 867 (411–1,090)           | 1,939 (708–2,754)        | 11,722               |
| CIPF 5    | CIPF      | 2,196                                   | 73.1                                  | 27.5                                       | 453 (393–722)             | 833 (656–1,622)          | 11,921               |

Data were generated after passing quality control including the exclusion of cells with <100 and >2,500 genes. Only genes present in more than three cells were kept. Reads mapped confidently to genome is the number of reads that mapped only to the genome. Reads mapped confidently to transcriptome are the fraction of the reads mapped to a unique gene in the transcriptome and are considered for UMIs counting. Median genes per cell correspond to the median number of genes with at least one UMI count. Total genes detected is the detected number of genes with at least one UMI count in any cell. ID, identity; UMI, unique molecular identifier; WHWT, West Highland white terrier; CIPF, canine idiopathic pulmonary fibrosis.
FIGURE 1 | Single-cell RNA sequencing analysis identifies multiple cell populations in the canine bronchoalveolar lavage fluids (BALFs). The scRNA-seq analysis was performed on a single-cell suspension generated from eight BALFs obtained from three healthy West Highland white terriers (WHWTs) and five WHWTs affected with canine idiopathic pulmonary fibrosis (CIPF). Cells were visualized using t-distributed stochastic neighbor embedding (t-SNE) plots. (A) Cell clusters identified. (B) Cell populations identified. (C) Cells are colored according to the status of dogs either healthy or affected with CIPF. (D) Expression of differentially expressed genes representative of each cell population. Ma/Mo, macrophages/monocytes; DC, dendritic cell; MRC1, macrophage mannose receptor; MARCO, macrophage receptor with collagenous structure; CD163, scavenger receptor cysteine-rich type 1 protein M130; CD3E, T-cell surface glycoprotein CD3 epsilon chain; CD8b, T-cell surface glycoprotein CD8 beta chain; CCR7, C-C chemokine receptor type 7; CD83, CD83 molecule; SELL, selectin; TOP2A, DNA topoisomerase II alpha; FCRLA, Fc receptor like A; KRT19, cytokeratin 19; MS4A2, membrane spanning 4-domains A2.
majority of the cells composing Ma/Mo population (Figures 2A, C and Table 4). Cells in these clusters were enriched in biological processes relevant to AMs including "Hallmark reactive oxygen species pathways" for M0 cells and "GO adaptive immune response," "GO antigen processing and presentation of peptide or polysaccharide antigen via MHC class II," "GO activation and regulation of immune response," and "GO pattern recognition receptor signaling pathway" for M3 cells (Supplementary Table 5). Cells in cluster M1 were considered as monocyte-derived macrophages as they expressed markers from both macrophages, including MARCO, PPARG (encoding peroxisome proliferator activated receptor gamma), CD68, MRC1 (encoding macrophage mannose receptor, CD206), MSL1 (encoding macrophage scavenger receptor 1, CD204) and CD16 (5, 27–31), and monocytes, including CD11c (encoding integrin subunit alpha X, ITGAX), CD16, CD49d (encoding integrin subunit alpha 4, ITGA4), CD49e (encoding integrin subunit alpha 5, ITGAS) and CX3CR1 (encoding fractalkine receptor) (Figure 3, Supplementary Tables 3 and 4) (29, 30, 32, 33, 35), but they also overexpressed CD3 genes compared with other clusters (Figure 3 and Supplementary Tables 3 and 4) which are known to be T-lymphocyte markers (36). Enriched processes associated with M4 cluster were mainly focused on inflammatory response (Supplementary Table 5).

For each cluster independently of the animal status (healthy or affected with CIPF), we also performed a GSEA to determine whether overexpressed genes in each cluster, in comparison with other clusters, could be associated with signatures of pulmonary fibrosis using the Comparative Toxicogenomics Database Pulmonary fibrosis gene set (24). Only cells in cluster M1 and M2 showed significant enrichment for pulmonary fibrosis with a receptor subunit beta, CD131), CD11c, CD11b (encoding integrin subunit alpha M, ITGAM), CD49d, CD49e, and CX3CR1 (Figure 3, Supplementary Tables 3 and 4) (29, 30, 32–34). Cells in cluster M1 were enriched in functions associated with the immune response activation including "Hallmark inflammatory response," "GO interferon gamma production," and "GO leukocyte cell-cell adhesion," while cells in cluster M2 were more involved in "GO leukocyte migration" and "GO cell motility" (Supplementary Table 5), functions essential when monocytes are recruited from blood into tissues. M4 cells also expressed macrophages and monocytes markers including notably MHC-II markers, CD63, CD68, CD16, and CD49d (12, 30, 32, 33, 35), but they also overexpressed CD3 genes compared

| TABLE 3 | Relative cells repartition between healthy and CIPF WHWTs in each cell population. |

|          | Healthy WHWTs | CIPF WHWTs | P-value |
|----------|---------------|------------|---------|
| Ma/Mo    | 69.5 ± 4.7    | 52.7 ± 26.3| 0.332   |
| CD8+ T cells | 10.9 ± 10.0   | 17.6 ± 15.4| 0.533   |
| CD8 CD4 T cells | 7.1 ± 1.1    | 14.7 ± 9.0 | 0.210   |
| Immature DC | 4.6 ± 3.1    | 3.7 ± 1.6  | 0.866   |
| Neutrophils | 1.6 ± 2.1     | 4.5 ± 6.7  | 0.498   |
| Cycling cells | 2.2 ± 0.5     | 1.8 ± 0.7  | 0.456   |
| B cells    | 1.5 ± 0.6     | 2.1 ± 1.4  | 0.567   |
| Mature DC  | 0.4 ± 0.1     | 2.1 ± 1.0  | 0.041   |
| Epithelial cells | 1.0 ± 0.6 | 0.6 ± 0.2  | 0.160   |
| Mast cells | 1.2 ± 1.8     | 0.3 ± 0.3  | 0.270   |

Relative cell proportion were compared between healthy West Highland white terriers (WHWTs) and WHWTs affected with canine idiopathic pulmonary fibrosis (CIPF) using t-tests after verification of the distribution normality using Shapiro-Wilk tests. Data are expressed in mean percentage ± standard deviation.

**TABLE 4 | Relative cells repartition between healthy and CIPF WHWTs in each Ma/Mo cluster.**

|          | Healthy WHWTs | CIPF WHWTs | P-value |
|----------|---------------|------------|---------|
| M0       | 73.6 ± 4.1    | 67.2 ± 10.0| 0.342   |
| M1       | 18.8 ± 4.1    | 14.2 ± 6.6 | 0.329   |
| M2       | 2.9 ± 0.2     | 13.5 ± 4.7 | 0.009   |
| M3       | 3.2 ± 1.1     | 4.4 ± 1.8  | 0.356   |
| M4       | 1.4 ± 1.0     | 0.7 ± 0.4  | 0.185   |

Relative cell proportion in each macrophages/monocytes (Ma/Mo) cluster were compared between healthy West Highland White terriers (WHWTs) and WHWTs affected with canine idiopathic pulmonary fibrosis (CIPF) using t-tests after verification of the distribution normality using Shapiro-Wilk tests. Data are expressed in mean percentage ± standard deviation.

**FIGURE 2 | Macrophages/monocytes (Ma/Mo) clusters identified.** Cells identified as Ma/Mo after the annotation of scRNA-Seq data obtained from three healthy West Highland white terriers (WHWTs) and five WHWTs affected with canine idiopathic pulmonary fibrosis (CIPF) were selected and then clustered allowing the identification of five distinct clusters. (A) Clusters identified. Cells were visualized using a t-distributed stochastic neighbor embedding (t-SNE) plot. (B) t-SNE plot of Ma/Mo colored according to the disease status of the WHWTs either healthy or affected with CIPF. (C) Bar plot of the relative proportion in each disease status of each Ma/Mo cluster.
normalized enrichment score (NES) of 1.87 and 1.85, respectively (FDR q-value = 0.007 and 0.002) (Figures 4A, B). Differentially overexpressed genes identified in relation with pulmonary fibrosis included SFTPC (encoding surfactant protein C), CCL5 (encoding C-C motif chemokine ligand 5), FN1 (encoding fibronectin 1), CXCL8 (encoding C-X-C motif chemokine ligand 8), ATP11A (encoding ATPase phospholipid transporting 11A) and SPP1 (encoding osteopontin) in cluster M1 and CCL2 (encoding C-C motif chemokine ligand 2), SPP1, FN1, CCL3 (encoding C-C motif chemokine ligand 3), TIMP1 (encoding metalloproteinase inhibitor 1), IL1RN (encoding interleukin 1 receptor antagonist), CXCL8 and CCL4 (encoding C-C motif chemokine ligand 4) in cluster M2 (Figure 4C). M0 cells were negatively enriched for pulmonary fibrosis with a NES of −2.04 (FDR q-value = 0.002). The other clusters were not significantly associated with pulmonary fibrosis processes (FDR q-value = 0.145 and 0.289 for cells of cluster M3 and M4, respectively).

Comparison Between Animal Status

Differential gene expression between cells from healthy WHWTs and WHWTs affected with CIPF in each Ma/Mo cluster was also assessed (Supplementary Table 6) and was essentially found for cells in cluster M1. DEGs in cluster M1 between CIPF and healthy WHWTs were mapped to the Comparative Toxicogenomics Database Pulmonary fibrosis gene set to assess pulmonary fibrosis signatures. NES in pulmonary fibrosis processes was at 2.01 (FDR q-value = 0.008) (Figure 5A). Genes involved in pulmonary fibrosis processes found to be upregulated in CIPF compared with healthy WHWTs in cluster M1 included FN1, SPP1, CXCL8, and PLAU (encoding plasminogen activator urokinase) (Figures 5D–G). The differential expression between healthy and CIPF WHWTs of those molecules in all Ma/Mo clusters is illustrated in Supplementary Figure 2. Moreover, in cluster M1, enrichment analysis with Hallmark gene sets indicated that cells from CIPF WHWTs were enriched for processes known to be associated with fibrosis including “epithelial mesenchymal transition (EMT)” (Figure 5B) and “angiogenesis” (Figure 5C) (NES of 1.86 and 1.88; FDR q-value = 0.039 and 0.068, respectively). Genes associated with these two gene sets and overexpressed in CIPF dogs included VIM (encoding vimentin), FN1, SPP1, THY1 (encoding Thy-1 cell surface antigen, CD90) for “EMT” gene set and SPP1, VCAN (encoding large fibroblast proteoglycan) and S100A4 (encoding S100 calcium binding protein A4) for “angiogenesis” gene set (Supplementary Table 6).
DISCUSSION

In this study, we analyzed Ma/Mo clusters in the BALF from healthy WHWTs compared with WHWTs affected with CIPF. Five Ma/Mo clusters were identified. Among them, we described a cluster of monocytes present in larger proportion in CIPF WHWTs than in healthy WHWTs. Expression of cells in this cluster was enriched for pulmonary fibrosis processes and eight genes associated with fibrosis were overexpressed in this cluster including CCL2, SPP1, FN1, CCL3, TIMP1, IL1RN, CXCL8, and CCL4. We also identified a cluster of monocyte-derived macrophages enriched for inflammatory and pulmonary fibrosis processes in which the gene expression differed between CIPF and healthy WHWTs with an enrichment for pulmonary fibrosis but also EMT and angiogenesis processes. We identified four overexpressed genes associated with pulmonary fibrosis processes in CIPF compared with healthy dogs in this cluster including FN1, SPP1, CXCL8, and PLAU.

In this study, similar cell populations and clusters were identified compared with previously published data on scRNA-seq analysis in BALFs from healthy dogs and included Ma/Mo, T cells either CD8+ or CD8−CD4−, DCs either mature or immature, neutrophils, B cells, epithelial cells, mast cells, and cycling cells (18). We were not able to differentiate between ciliated and non-ciliated epithelial cells which can be due to either the low proportion or the absence of ciliated epithelial cells in our samples (37) as rare cell populations may be missed using scRNA-seq (15). As already reported (18), eosinophils were not identified using scRNA-seq.

FIGURE 4 | Enrichment in pulmonary fibrosis processes in M1 and M2 macrophages/monocytes clusters compared to others. (A, B) Gene set enrichment analyses between Comparative Toxicogenomics Database Pulmonary Fibrosis gene set and differentially expressed genes in M1 and M2 clusters, respectively, compared to others. (C) Dot plot showing the expression of genes involved in pulmonary fibrosis processes found to be upregulated in cluster M1 and M2 compared to others. Dot size represents the percentage of cells expressing the genes, while the dot color represents the average expression of the indicated genes.
probably secondary to their RNase content conducting to the degradation of mRNAs in those cells (38).

In healthy conditions, lung macrophages are known to be extremely heterogeneous and play a crucial role in the regulation of the homeostasis of the lung. In addition to their immune defense function, they also exerted an indispensable role in organ development, maintenance of homeostasis and repair (3, 17). In the lung, the majority of the macrophages are AMs which are resident and self-renewing macrophages (3). They have been identified in this study by their expression of MARCO and corresponded to cells of cluster M0 and M3 (3, 18, 26). In inflammatory conditions, the lung is rapidly infiltrated by recruited monocytes which gradually differentiate into monocyte-derived macrophages and then AMs (3). Here, we observed a higher proportion of monocytes (cells from cluster M2) in CIPF dogs that are probably recruited secondary to lung fibrosis in higher proportion than in healthy dogs. This increased number of macrophages and myeloid cells was also reported as an early event in bleomycin-induced lung fibrosis mouse model (6). As M2 cluster cells were enriched in pulmonary fibrosis processes, we suggest that their increased proportion in CIPF condition could participate to the onset and/or to the perpetuation of the fibrosis process in WHWTs.

The Ma/Mo involved in pro-fibrotic processes in this study can be considered as immature macrophages as they were identified as either monocyte-derived macrophages (cluster M1) or monocytes (cluster M2). Recently, transcriptomic profiling of macrophages collected over the time course of bleomycin induced fibrosis showed that during monocyte maturation, genes linked to fibrosis are most highly expressed during their differentiation and progressively downregulated with the maturation of the cells into AMs (39). This is in line with...
with results obtained in this study and suggests that recently recruited macrophages (clusters M1 and M2) have greater fibrotic capacity than mature AMs (clusters M0 and M3). Targeting those particularly pro-fibrotic recruited immature macrophage clusters could be a potential novel strategy for the prevention and the therapy of CIPF.

DEGs between healthy and CIPF WHWTs were essentially found in the M1 cluster. Moreover, M1 cells in CIPF dogs were enriched for EMT, angiogenesis and pulmonary fibrosis processes. EMT is considered as one of the phenomena by which collagen-producing fibroblasts and myofibroblasts accumulate, creating a pro-fibrotic environment (40). Indeed, epithelial cells differentiate to acquire features of mesenchymal cells including invasion, migration, and production of extracellular matrix (40). Altered EMT process is the most widely accepted pathogenetic mechanism in IPF patient (40) and could also participate in the development of CIPF as suggested by this study. Angiogenesis is another well-known mechanism involved in IPF, which is targeted by Nintedanib, an anti-angiogenesis molecule used in human for its properties against the vascular endothelial growth factor (VEGF) pathway (41). Involvement of angiogenesis in CIPF has only been assessed through the measurement of VEGF concentration in serum without results (42). To the authors’ best knowledge, none of the molecules identified in the present study and linked to angiogenesis has been studied in CIPF.

Among genes found to be associated with pulmonary fibrosis processes, only CCL2 and CXCL8 have already been associated with CIPF (2). Indeed, it has been shown that mRNA expression of CXCL8 and CCL2 was increased in CIPF lungs compared with controls (2). Moreover, CCL2 and CXCL8 chemokine concentrations were increased in CIPF WHWTs compared with healthy WHWTs in both serum and BALF and only in BALF respectively (2). The osteopontin (SPP1 gene) is a glycoprotein secreted by numerous cell types including macrophages which has been proved to be closely related to IPF (7, 43–46). Indeed, high level of expression and increased BALF protein concentration have been reported in IPF mouse models but also in IPF patients (45). Such findings suggest that osteopontin could be used as a potential biomarker and a therapeutic target for treating fibrotic lung diseases (43). The fibronectin 1 (FN1 gene) is a mediator of cell matrix adhesions. It promotes myofibroblast differentiation and is found in abundance in the lungs of IPF patients (47). CCL3 and CCL4, also known as macrophage inflammatory protein 1-alpha and beta, are chemoattractant cytokines (48–50) suspected to play a role in sustaining inflammation and the chronic course of IPF by recruiting inflammatory cells such as neutrophils (49–51). Their expression in CIPF dogs could be related to the higher rate of neutrophils found in the BALF of CIPF compared to healthy dogs. The tissue inhibitor of metalloproteinase (TIMP-1 gene) probably contributes, through its control of matrix metalloproteinase catalytic activity, to provide a non-degrading fibrillar collagen microenvironment in IPF patient as well as in IPF mouse model (52, 53). It has also a potential value as biomarker in patients with IPF (54). The interleukin-1 receptor antagonist (encoding IL-1RA) is a cytokine produced by alternatively activated AMs. The protein level was increased in IPF patients compared with healthy volunteers (55, 56) and in patients with acute exacerbation of IPF compared with stable IPF patients suggesting that this protein could be of interest as diagnostic and prognostic marker (56). The role of the plasminogen activator urokinase (PLAU gene) in pulmonary fibrosis is not clear. The protein level has been showed to be low in BALF of IPF patient (57, 58) and the molecule was showed to be protective against fibrosis development in IPF mouse model (59). Recently, the protective role of the plasminogen activator was controverted as its presence was associated with increased plasmin formation which in turn activates structural and inflammatory cells driven fibrosis (57). PLAU overexpression in this study indicates that fibrinolytic processes are present in CIPF dogs. Whether it is protective or not remains unclear. Further studies are needed to better assess the potential role of all these molecules in CIPF pathogenesis and their utility as biomarkers of disease progression and as potential therapeutic target.

The present study had some limitations. First, the analysis of scRNA-seq data remains limited by the poor annotation of canine genomic dataset highlighting the need for further studies to optimize the use of this technique in healthy and diseased dogs. Indeed, the percentage of reads mapped confidently to the transcriptome had to be from at least 30%¹, which is not the case in this study. Secondly, our study involved a relatively low number of dogs either healthy or affected with CIPF. Indeed, even if the transcriptomic profiling costs are falling, the use of the scRNA-seq remains currently quite expensive. However, even with this small number of subjects and with the lack of annotation of the canine genome, we were able to identify the different cell populations, their genes expression and their DEGs in CIPF condition. We were also able to detect the genes already identified as involved in CIPF such as CXCL8 and CCL2. Finally, it should be noted that in some Ma/Mo clusters, DEGs included markers normally expressed by other cell types, mainly in M4 cluster which expressed Ma/Mo and T cells markers. This likely results from contamination from ambient RNA released during BALF processing. This contamination is a known limitation that can occur in scRNA-seq experiments (60, 61). Another explanation would be that these cells are in fact doublets. Doublets are a known confounding factor in scRNA-seq analysis (62) that can be reduced by decreasing cell number introduced in the Chromium™ Controller (63) and by filtering out cells with a really high gene count (62) as it was done in this study.

**CONCLUSION**

Using scRNA-seq in BALF specimens from healthy WHWTs and WHWTs affected with CIPF, we were able to reveal the presence of pro-fibrotic monocytes, more abundant in CIPF than

¹https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/troubleshooting#alerts (accessed March 16, 2020).
in healthy WHWTs, reflecting the inflammation that occurs in fibrotic lung. The presence of those monocytes enriched with pro-fibrotic genes probably participates to the onset and/or the perpetuation of CIPF in WHWTs. Moreover, monocyte-derived macrophages enriched in pro-fibrotic genes in CIPF compared with healthy WHWTs were also identified. This cluster was also enriched with EMT and angiogenesis processes, which are known to play an important role in IPF.

The results of that study offer promise for the better understanding of the role of macrophages in CIPF pathogenesis and the identification of new biomarkers and therapeutic targets to better diagnose, follow and treat the disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/arrayexpress/, E-MTAB-9623.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethical committee of the University of Liège (approval no. 1435). All dogs were privately owned. Written informed consent was obtained from the owners for the participation of their animals in this study.

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AUTHOR CONTRIBUTIONS

AF, CC, and FB designed the study. AF and CC recruited the dogs and collected samples. A-CM performed and interpreted echocardiographies. GB performed and interpreted thoracic scans. A-CT performed anesthesia of the dogs. AF, DP, and LF processed and acquired the data from samples. AF and DP analyzed the data. FB, CD, and TM provided their expertise in lung immune cells and helped for data interpretation. AF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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**Conflict 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.

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