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Transcriptomic analysis reveals distinct effects of cigarette smoke on murine airspace and bone-marrow derived macrophages

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Abstract

Background Chronic obstructive pulmonary disease (COPD) is an inflammatory airway disease characterized by emphysema and chronic bronchitis and a leading cause of mortality worldwide. COPD is commonly associated with several comorbid diseases which contribute to exacerbated patient outcomes. Cigarette smoke (CS) is the most prominent risk factor for COPD development and progression and is known to be detrimental to numerous effector functions of lung resident immune cells, including phagocytosis and cytokine production. However, how CS mediates the various pathologies distant from the lung in COPD, and whether CS has a similar biological effect on systemic immune cells remains unknown.

Methods C57BL/6 mice were exposed to 8 weeks of CS as an experimental model of COPD. Bone marrow cells were isolated from both CS-exposed and room air (RA) control mice and differentiated to bone marrow-derived macrophages (BMDMs). Airspace macrophages (AMs) were isolated from the same CS-exposed and RA mice and bulk RNA-Seq performed. The functional role of differentially expressed genes was assessed through gene ontology analyses. Ingenuity Pathway Analysis was used to determine the activation states of canonical pathways and upstream regulators enriched in differentially expressed genes in both cell types, and to compare the differences between the two cell types.

Results CS induced transcriptomic changes in BMDMs, including an upregulation of genes in sirtuin signalling and oxidative phosphorylation pathways and a downregulation of genes involved in histone and lysine methylation. In contrast, CS induced decreased expression of genes involved in pathogen response, phagosome formation, and immune cell trafficking in AMs. Little overlap was observed in differentially expressed protein-coding genes in BMDMs compared to AMs and their associated pathways, highlighting the distinct effects of CS on immune cells in different compartments.

Conclusions CS exposure can induce transcriptomic remodelling in BMDMs which is distinct to that of AMs. Our study highlights the ability of CS exposure to affect immune cell populations distal to the lung and warrants further investigation into the functional effects of these changes and the ensuing role in driving multimorbid disease.

Keywords Macrophage, COPD, Cigarette smoke, Transcriptome

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Introduction

Chronic obstructive pulmonary disease (COPD) is a debilitating inflammatory airway disease and a leading cause of mortality globally. COPD is more than just a respiratory disease and is often associated with multiple comorbidities with overlapping risk factors ("multimorbidity"), which can interact in such a way to exacerbate clinical outcomes [1–5]. Inhalation of cigarette smoke (CS) is the primary risk factor for COPD, but how CS brings about the extrapulmonary manifestations of COPD remains unclear [6–12].

The biological effect of CS on immune cell populations, in particular macrophages, may be a mechanism by which smoke exerts its persistent systemic effects. CS elicits an increase in the number of macrophages in the airspaces and parenchyma of the lung as well as in other organs [12–16]. These macrophages, termed airspace macrophages (AMs), are a combination of resident and recruited monocyte-derived (MDM) populations that are functionally and metabolically impaired in COPD and directly contribute to the development of emphysema [17–23]. MDMs recruited to the lung upon smoke have enhanced chemotaxis, are more pro-inflammatory and may over time assume the identity of AMs through signals provided by the local lung niche [22, 24–26]. Whether MDMs are "primed" or transcriptionally altered by smoke prior to their recruitment to the lung is unclear [27, 28]. Peripheral blood mononuclear cells (PBMCs) from smokers and individuals with COPD are also dysfunctional and more pro-inflammatory than those from healthy controls [23, 29]. Yet, there is little overlap transcriptomically between lung macrophage populations and PBMCs from the same individuals and little overlap in inflammatory markers between the blood and the sputum of individuals with COPD, suggesting that systemic inflammation in COPD may occur in parallel to, and be distinct from, local lung inflammation [30–33]. We hypothesized that, prior to their entry into the lung, there is a compartment-specific effect of CS on the bone marrow and subsequent MDMs that primes these macrophages and directs them toward an altered inflammatory state. To test this hypothesis, we investigated the transcriptomic differences in bone-marrow derived macrophages (BMDMs) and AMs isolated from murine smoke-exposed models of COPD. We show that the transcriptome of distant macrophage populations is dysregulated by cigarette smoke, an effect which is distinct from that on airspace macrophages. Our results highlight the dysregulation of the innate immune response systemically in COPD and warrant further investigation into the role of CS-induced macrophage dysfunction in mediating pathologies distal from the lung.

Methods

Mice and cigarette smoke exposure model

C57BL/6 J wild type mice (Stock No. 000664) were purchased from The Jackson Laboratory and were maintained under specific pathogen-free conditions at Weill Cornell Medicine. Sex and age-matched mice aged between 6 and 12 weeks of age were exposed to whole-body CS using the Teague Enterprises TE-10 inhalation exposure apparatus and 3R4F cigarettes acquired from the University of Kentucky Centre for Tobacco Reference Products. Mice were exposed to CS (average total particulate matter (TPM) of 150 mg/m^3) for a minimum of 2 h per day for 5 days a week, for an 8-week period.

AM and BMDM isolation

Mice were euthanised via CO_2 asphyxiation following the CS exposure regimen. Following tracheostomy and intubation with a 20G×1″ catheter (SR-OX2025CA; Terumo), mice lungs were lavaged using 0.5 ml increments of ice-cold PBS (10,010–023; Life Technologies) supplemented with 0.5 mM EDTA (351–027-061; Quality Biological), to a total of 4 ml. Cell pellets were collected through centrifugation at 500×*g* for 5 min at 4 ˚C and treated with 1 ml of RBC Lysis Buffer (R7757, Sigma-Aldrich). Following 3 min incubation, cells were centrifuged at 500×*g* for 3 min at 4 °C, resuspended in 0.5 ml PBS, and 20 µl removed for cell counting using a hemocytometer. The majority of lavaged cells were macrophages, with less than 5% of the population being neutrophils or lymphocytes.

BMDM isolation and treatment

Bone marrow cells were isolated from the murine femur and tibia through a 25 G needle with a 12 ml syringe filled with complete DMEM attached (11,995; Life Technologies). Cell aggregates were removed using an 18 G needle and 70 µM cell strainer, after which the single cell suspension was centrifuged at 1200 rpm for 10 min. Cells were plated in complete DMEM supplemented with 10% L929 media for 8 days.

RNA isolation and sequencing

Bulk RNA-Seq was performed in AMs and BMDMs isolated from WT and CS mice as detailed above. Total RNA was extracted using the Qiagen RNEasy Micro Kit (74,004) as per manufacturer's instructions, and 1 µg total RNA used to generate RNA-Seq libraries using the Bioo kit. Samples were read as paired-end 150 bp reads on a High-Seq 4000 and reads aligned to the mouse genome (GRCm38/mm10) using STAR. Cut-offs of absolute fold

change > 1.5 and Benjamini–Hochberg adjusted $p < 0.05$ were used for all analyses.

Bioinformatic analyses

Differentially expressed protein coding genes (DEGs) were determined through DeSeq2 (https://github.com/lovelab/DESeq2). Volcano plots of gene expression were generated through ggVolcanoR (https://ggvolcanor.erc.monash.edu/). For gene ontology analysis, up and downregulated differentially expressed genes were inputted into ShinyGO (http://bioinformatics.sdstate.edu/go/; v.0.77). Ingenuity Pathway Analysis was used to identify canonical pathways and to predict upstream regulators.

Results

BMDMs from CS-exposed mice show transcriptomic remodelling

Infiltration of monocytes to the lung from circulation is a well-recognised hallmark of COPD progression. To investigate the transcriptome of these cells, we generated bone-marrow derived macrophages (BMDMs) from mice exposed to 8 weeks as previously described [34]. We analysed aligned bulk RNA-sequencing reads prepared from RNA isolated from these BMDMs alongside those

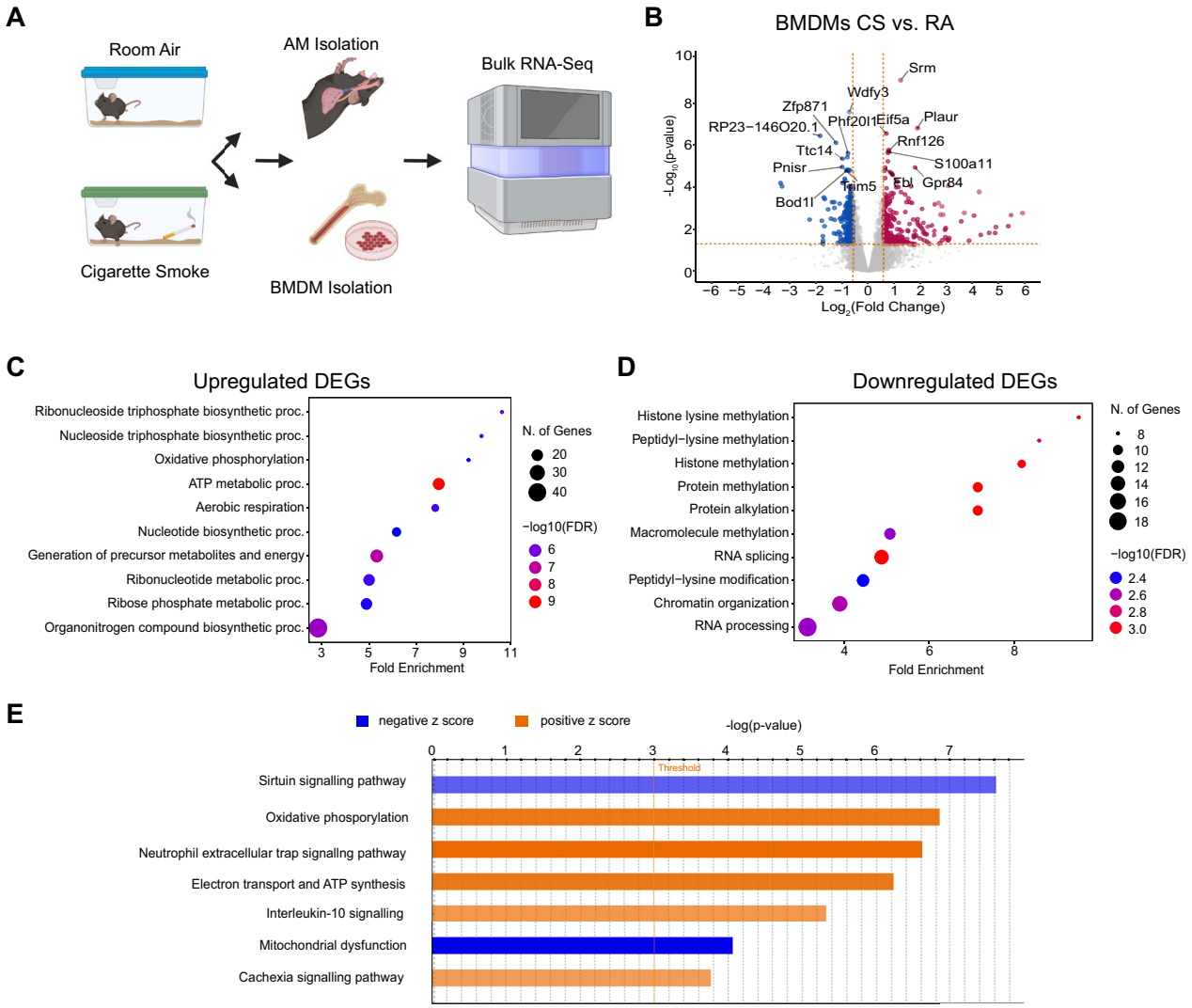

Fig. 1 CS exposure induces transcriptomic remodelling in BMDMs. **A** Schematic of experiment (created using Biorender). **B** Volcano plot of BMDM differentially expressed genes (DEGs) in CS-exposed mice (n = 3) compared to room air (RA) controls (n = 3). Biological processes associated with BMDM CS-induced upregulated (**C**) and downregulated (**D**) DEGs. **E** Canonical pathways produced from Ingenuity Pathway Analysis enriched in BMDM CS-induced DEGs and their activation z-scores (blue = inhibited, orange = activated)

generated from room air (RA) exposed controls (Fig. 1A) [35]. Following DeSeq2 analysis using cut-offs of absolute fold change > 1.5 and adjusted $p < 0.05$, our analysis identified 448 genes differentially expressed in response to CS exposure, with 190 genes downregulated and 258 genes upregulated (Fig. 1B). The most significantly upregulated gene upon CS exposure was *Srm,* encoding for the spermidine biosynthesis gene spermidine synthase; *Wdfy3,* the most significantly downregulated gene, encodes for an adaptor protein involved in selective macroautophagy and was previously identified as a regulator of macrophage efferocytosis [36]. Gene ontology (GO) analysis of upregulated genes showed enrichment for processes related to mitochondrial bioenergetics including oxidative phosphorylation (OXPHOS, GO:0006119) and ATP production (GO:0046034), while downregulated genes showed enrichment for histone (GO:0034968) and protein lysine methylation (GO:0018022) (Fig. 1C). To further elucidate the effects of CS on transcriptional pathways, we next used Ingenuity Pathway Analysis (IPA), where protein-coding genes were matched against the Ingenuity Pathway Knowledge Base to identify canonical pathways enriched in differentially expressed genes, assigning a positive z-score to pathways activated by CS exposure and a negative score to those inhibited. Sirtuin signalling was the most significantly enriched canonical pathway (absolute z score > 2, $p < 0.00001$), showing an inhibited state (Fig. 1E, Supplemental Fig. S1A). Consistent with GO analyses, the OXPHOS canonical pathway was also activated, with enrichment in genes encoding for several components of respiratory electron transport chain complexes (Fig. 1E, Supplemental Fig. S1B). Cachexia signalling, a well-documented co-morbidity of COPD, was also identified as activated (Fig. 1E). Upstream regulator analysis predicted lipopolysaccharide (LPS) as the most activated upstream regulator of CS-induced DEGs (Supplemental Fig. S1C).

CS-induced transcriptomic modelling in BMDMs is distinct to that of AMs

To compare the transcriptome of these recruited macrophages to lung resident macrophages, in parallel we isolated RNA from AMs derived from the bronchoalveolar lavage fluid of mice exposed to 8 weeks CS or RA and performed bulk RNA-Seq as before (Fig. 1A). Using the same significance cut-offs as BMDMs, DeSeq2 analysis revealed 481 genes differentially expressed in response to CS exposure, with 313 genes downregulated and 168 genes upregulated (Fig. 2A). *Vwf*, which encodes for von Willebrand factor, a platelet-binding glycoprotein involved in the regulation of hemostasis, was the most significantly upregulated gene upon CS exposure, while *H2-eb1*, which encodes for a component of the major histocompatibility type II complex, was most significantly downregulated. GO analysis of downregulated DEGs revealed an enrichment for biological processes associated with immune cell activation, including leukocyte differentiation (GO:0002521) and chemokine/cytokine response (GO:0034097, Fig. 2B). In contrast to BMDMs, IPA did not identify any activated canonical pathways, but did discover inhibited pathways reflective of a blunted macrophage response to infection including phagosome formation and pathogen-induced cytokine storm (Fig. 2C). The T1 T helper cell (Th1) pathway was also inhibited, in line with downregulated expression of major histocompatibility type II complex components (Fig. 2C). Upstream regulator analysis predicted lipopolysaccharide (LPS) as the top inhibited regulator, again in contrast to BMDMs (Supplemental Fig. S1D).

Importantly, comparing CS-induced DEGs between AMs and BMDMs, only 16 genes were commonly differentially expressed in response to CS and only 3 with the same directionality: *Psat1,* encoding for an enzyme in the serine metabolism pathway, was upregulated in both AMs and BMDMs, while *Stab1,* a transmembrane scavenger receptor, and *St8sia4,* a polysialyltransferase, were downregulated (Fig. 2D). Similarly, the only canonical pathways altered in the same direction in both cell type were pathways related to aging (sirtuin signalling and senescence) and adaptive immune system function (Th1 pathway). The largest discrepancy in activation score was pathogen-induced cytokine storm signalling, which was activated in BMDMs (z-score = 3.2) and inhibited in AMs (z score = -3.2) (Fig. 2E). Other dichotomous pathways of interest include mitochondrial dysfunction, reactive oxygen species (ROS) signalling, wound healing and tissue pathways, and a number of cancer pathways.

Discussion

Cigarette smoking is the primary risk factor for COPD, but its role in driving the pathogenesis of comorbidities is poorly understood. Here, we show that CS exposure induces the differential expression of protein-coding genes in the murine bone marrow, activating pathways associated with oxidative phosphorylation and energy production and inhibiting those associated with sirtuin signalling. These transcriptomic changes in CS-exposed BMDMs were markedly different from those observed in lung airspace macrophages, in which CS-exposure predominantly had an inhibitory effect in terms of immune signalling. Although these are not functional studies, our findings suggest that CS exposure may have systemic consequences for immune activation

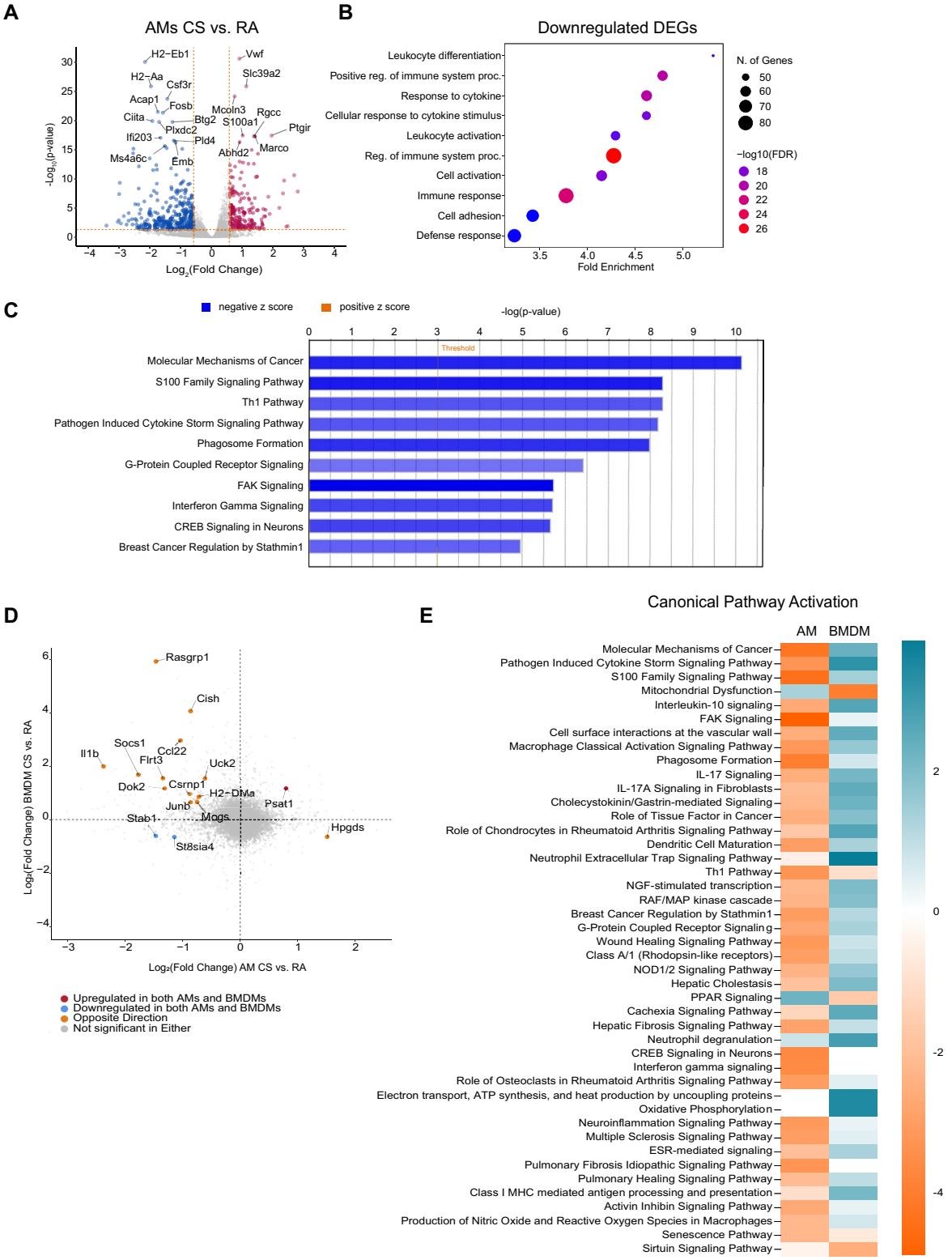

Fig. 2 CS exposure induces distinct transcriptomic remodelling in BMDMs compared to AMs. **A** Volcano plot of AM DEGs in CS-exposed mice (n = 4) compared to RA (n = 4). **B** Biological processes associated with AM CS-induced downregulated DEGs. **C** Canonical pathways produced from Ingenuity Pathway Analysis enriched in AM CS-induced DEGs and their activation z-scores. **D** Comparison of Log2 fold change values in BMDMs and AMs from CS exposed mice compared to RA, with significant DEGs common to both cell types highlighted. **E** Heat map comparing activation z-score of canonical pathways induced by CS in BMDMs and AMs

and pathogen response, which in the context of COPD multimorbidity may play a role in contributing to extrapulmonary disease.

Our data highlight the differential effects of in vivo CS exposure on macrophages from the airspaces and the bone marrow, with bulk RNA-seq results showing few overlapping gene expression changes and in some cases, diametrically opposite responses. Specifically, while CS exposure predominantly had an inhibitory effect in AMs in terms of immune signalling, CS upregulated pathways associated with mitochondrial bioenergetics and energy production in BMDMs. Aging and adaptiveimmune signalling pathways were common to both cell types, suggesting a role for a loss in sirtuin signalling and a dysfunctional interaction between CS-exposed macrophages and the adaptive immune system. These findings align with previous studies that have shown that sirtuin-1 protein levels are reduced in COPD lung tissue and macrophages, with our data suggesting recruited macrophages may also contribute to this burden [37]. While others have shown that AMs from individuals with COPD have reduced antigen presenting capabilities [38, 39], little is known about COPD MDMs and antigen presentation.

Metabolically, we and others have shown that AMs and MDMs from individuals with COPD have impaired mitochondrial respiration, with some evidence that AMs from smokers have heighted maximal respiration [23, 40]. As our 8 week CS exposure induces inflammation and mucociliary impairment but not the emphysema typical of late-stage COPD, our results may indicate that an upregulation of OXPHOS by systemic macrophages precedes these defects on mitochondrial energetics, perhaps through the increased production of reactive oxygen species associated with increased mitochondrial respiratory complex activity. While our analyses only show association, further studies are needed to determine the functional impact of these changes.

Despite the differentiation of BMDMs in cell culture media for 8 days without CS exposure, there were still 448 DEGs between BMDMs from RA and CS-exposed mice. This number is comparable to the DEGs in AMs isolated immediately after CS exposure, suggesting a memory like, long-lasting effect that is likely reflective of epigenetic changes. This is supported by the identification of several biological processes related to histone methylation in GO analysis of downregulated genes in BMDMs from CS-exposed mice, in agreement with previous studies including a demonstration of a role for the histone methyltransferase *PRMT7* in COPD monocyte migration and tissue damage in both patient and murine monocytes [41–43]. This "memory" component of CS exposure may be driven by histone modifications by metabolites produced through the several altered metabolic pathways our analyses identified in BMDMs and could potentially explain the observation of persistent inflammation and immune dysfunction in smokers long after smoking cessation [11, 44–46]; however, whether this process occurs in the bone marrow or arises systemically remains unclear. We have previously demonstrated that CS alters erythropoietic activity and iron in the bone marrow of CS exposed mice, hinting at the possibility that CS may impact myeloid progenitors as well [34]. Administration of healthy bone marrow cells to a rodent model of experimental COPD reverses emphysema, highlighting the therapeutic potential of restoring bone marrow function in COPD [47].

The notion that CS can affect the bone marrow similarly in human COPD has wide-reaching consequences. Over 75% of COPD patients present with two or more chronic diseases, which are often treated and managed in isolation rather than as part of a multimorbid condition. Diseases associated with multimorbidity can exacerbate the pathology and progression of others, but a shared mechanism remains unclear [48, 49]. Our data show that cigarette smoke, a risk factor for numerous lung, cardiovascular and metabolic diseases, can affect macrophages derived from myeloid precursors in the bone marrow, with pathway analysis also identifying cachexia, a common comorbidity in COPD patients. Multimorbidity, especially its mechanistic aspects, is understudied in COPD, and more studies are needed to elucidate the connection between extrapulmonary pathologies and CS-affected systemic immune cell populations. [39, 50].

Our study has several limitations. Although AMs comprise 93% of immune cells in the BALF at homeostasis, our isolation of RNA directly from BAL cells without purification may result in contamination from other immune populations recruited during CS-induced inflammation [51]. MDMs recruited to the lung during such inflammation are functionally distinct to lung-resident AMs and can uniquely orchestrate inflammation and repair [52, 53]. As transcriptomic differences between these lung macrophage populations have been characterised extensively in experimental and human COPD through single-cell RNA-seq previously [20], we instead chose to investigate CS-induced transcriptomic changes in BMDMs as a model of a systemic macrophage prior to any influence from the local lung niche, with this distinction evidenced by the lack of overlap observed between DEGs in AMs and BMDMs [54]. Further investigating the differential contributions of the local tissue niche compared to ontogeny on macrophage identity in CS exposure through genetic lineage tracing experiments

would prove useful in further interrogating CS-driven systemic priming. The effects of culture conditions on BMDM transcriptomic changes are unknown, as is how CS exposure affects the transcriptome and epigenome of bone marrow progenitors: this would elucidate how CS-induced transcriptomic changes may manifest in other myeloid-derived immune populations in COPD, such as neutrophils, in comparison to our macrophage data [55].

In summary, we show that CS transcriptomically remodels BMDMs distinct to that of tissue resident AMs, inducing expression of genes associated with mitochondrial metabolism distinct to AMs, but mutually altering aging and adaptive immune system-related pathways in both cell types. Our data highlight the ability of CS to alter immune cell populations outside of the lung and warrants further functional studies of this effect on driving both COPD progression as well as the development of multimorbidity associated with COPD.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-024-02939-3.

Additional file 1.

Additional file 2.

Author contributions

S.C. and S.M.C. designed the research study. W.Z.Z., M.M.S., E.K.R., E.P., and K.K. conducted the research experiments. L.F., W.Z.Z., M.M.S., E.K.R., and S.M.C. analysed the data. L.F., W.Z.Z., and S.M.C. wrote the manuscript. M.M.S. and S.C. contributed to the manuscript.

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Availability of data and materials

RNA-seq data were submitted to the National Centre for Biotechnology Information Gene Expression Omnibus database (NCBI GEO) under accession numbers GSE184571 (BMDM data) and GSE275328 (AM data).

Declarations

Ethics approval and consent to participate

All animal protocols were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine and were carried out in accordance with the relevant ethical regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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