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The Fight for Iron at Mitochondria: Implications for Respiratory Infection and Disease

Thesis submitted to the University of Dublin in fulfilment of the degree
Doctor in Philosophy

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Declarations

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, apart from collaborative work which is explicitly highlighted in-text and detailed below:

Fig. 2.2: Gating strategy was designed by Dr. Anna Ledwith of the Sheedy lab, TCD.

Fig. 2.3 Gating strategy was designed by Cian Horneck Johnston of the Sheedy lab, TCD.

Fig. 3.6: Deconvoluted data was provided by Dr. Min Hyung Ryu and Prof. Craig Hersh of the Channing Institute, Harvard Medical School, Boston, which I analysed and graphed.

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Fig. 3.18-19: I performed the CS exposure model with Sonia Gesmundo of the Cloonan lab, and euthanised mice and isolated tissue. Staining, acquisition and analysis of samples for flow cytometry was performed in collaboration with Dr. Anna Ledwith and Cian Horneck Johnston in the Sheedy Lab, TCD.

Fig. 6.3-4: I administered DFP with Ruairaidhrí Jordan of the Cloonan lab.

Fig. 6.13-14: I performed CS exposure model with Sonia Gesmundo of the Cloonan lab.

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Lynne Faherty, 31/03/2025

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Summary

Biological systems have co-opted iron to exploit its electron donor capabilities, fuelling essential enzymatic reactions. Mitochondria are the primary sites of iron storage and utilisation in mammalian cells, generating heme and iron-sulfur clusters which fuel macrophage immunometabolism. As such, mitochondrial iron must be exquisitely regulated to supply enough iron for anabolic processes whilst curtailing oxidative damage driven by excessive overload. Numerous pathogens hijack host mitochondria for essential nutrients, but whether changes in mitochondrial iron levels dictate macrophage function in response to infection is unknown. Mitochondrial iron overload has been implicated in the lung disease chronic obstructive pulmonary disease (COPD). Acute respiratory infections, like with *Streptococcus pneumoniae*, are a significant cause of mortality in COPD, yet whether mitochondrial iron is increased in lung alveolar macrophages (AMs), and how in turn this would alter outcomes of the host-pathogen fight for iron, is unclear. We hypothesised that excess AM mitochondrial iron is a bacterial target for iron piracy which in turn renders individuals with COPD more susceptible to infection.

In this thesis, this hypothesis was tested through *in silico* data mining approaches and experimentally through *in vitro* models of live infection (*S. pneumoniae*), genetic and experimental murine models, and human AMs isolated via bronchoscopy and pharmacologically manipulated *ex vivo*. Together, these approaches revealed alterations in AM mitochondrial iron in human COPD and experimental murine models, and in *S. pneumoniae* infection. Murine AMs subjected to experimental COPD may have higher total, mitochondrial and heme iron, with elevated expression of ferritin and the transferrin receptor. These “COPD” macrophages show an increased bacterial burden and blunted pro-inflammatory cytokine secretion following *S. pneumoniae* infection, confirmed in human AMs isolated from smokers. Subcellular compartment-specific iron loading and depletion suggested this increased bacterial burden may associate with mitochondrial iron loading in murine and human AMs. A novel murine model of genetic mitoferrin 2 deficiency specific to AMs exhibited decreased inflammatory cell infiltration following *S. pneumoniae* infection, associated with decreased weight loss and bacterial dissemination.

This work represents the most in-depth characterisation of mitochondrial iron in the macrophage response to infection to date, including the generation of the first transgenic strain of mice with targeted loss of a mitochondrial iron importer in an immune cell population. Our results suggest mitochondrial iron as a candidate mediator of nutritional immunity in the lung and present mitochondrial iron chelation as a potentially druggable target in both COPD and other diseases characterised by recurrent acute infections.

Lay summary

The cells in our body are made from biological building blocks. Some of these parts we can make ourselves mainly through mitochondria, factories for generating these blocks inside cells. Other components, like iron, we need to take in through our diet to store inside mitochondria. Our body will use this stored iron in small amounts for processes essential to life, such as the generation of energy from inhaled oxygen and the production of new cells to replace those that are damaged. As iron is so important, our body has intricate means of hiding it away from bacteria that invade our body and who also need this iron to survive. Alveolar macrophages, immune cells that patrol our lungs, are particularly important players in limiting the iron bacteria need to acquire from us to cause serious lung infections, like pneumonia. In chronic obstructive pulmonary disease (COPD), the fourth most common cause of death in Ireland which is often driven by cigarette smoking, alveolar macrophages are 'clogged' with iron and cannot store it correctly in mitochondria, which are leaky and porous. As individuals with COPD are prone to serious, recurring lung infections which often require them to be admitted to hospital, we wondered if the reason why infections are more common in these patients is because bacteria can take iron more readily from these defective alveolar macrophages.

To answer this question, we studied iron in different types of alveolar macrophages, including from individuals undergoing bronchoscopy in Tallaght University Hospital to diagnose disease. We have shown that alveolar macrophages have higher amounts of iron in smoking, particularly in mitochondria, and that if we take this excess iron away using a targeted drug, we can improve the ability of these cells to control the bacteria that cause pneumonia. We developed a strain of laboratory mice that lack the genes necessary to bring iron into mitochondria in their alveolar macrophages and showed that these mice have a better immune response to infection and less bacteria in their lungs.

No treatments currently exist that can directly cure a person of COPD, and factors like increased pollution in urban areas and the rising popularity of e-cigarettes mean that new types of therapies are urgently needed. Patients who develop a lung infection are prescribed antibiotics, but at that point are quite sick and have developed a serious infection that can leave lasting scars in their lungs and which antibiotics might not be able to stop. In this work, we have identified a new way to hinder bacterial infections in COPD patients through drugs which take iron away from the mitochondria of alveolar macrophages. Targeting iron like this will take away the fuel needed by bacteria before patients even develop an infection, limiting any further damage to their lungs and directly improving their quality of life.

List of abbreviations

5-ALA	5-aminoleuvinic acid
ABCB	ATP Binding Cassette Subfamily B Member
ACO1	Aconitase 1
ACO2	Aconitase 2
ALAS	5-aminoleuvinate synthase
AM	Alveolar macrophage
ANOVA	Analysis of variance
AT1	Alveolar epithelial type I cell
AT2	Alveolar epithelial type II cell
ATP	Adenosine triphosphate
BACH	BTB domain and CNC homologue 1
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid assay
BMDM	Bone marrow-derived macrophage
BNIP3	Bcl-2 interacting protein 3
CCR2	C-C chemokine receptor type 2
CFU	Colony forming units
CISD	CDGSH iron sulfur domain
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CO₂	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
CSE	Cigarette smoke extract
DAMP	Damage associated molecular pattern
DEG	Differentially expressed gene
DFO	Deferoxamine
DFP	Deferiprone
DMSO	Dimethyl sulfoxide
DMT1	Divalent metal transporter 1
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain

FAC	Ferric ammonium citrate
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDR	False discovery rate
Fe	Iron
FECH	Ferrochelataase
FeL	Ferrous lactate
FeS	Iron sulfur
FLAM	Fetal liver-derived alveolar macrophage
FLVCR1	Feline leukaemia virus receptor 1
FPN	Ferroportin
FTH1	Ferritin heavy chain 1
FTL	Ferritin light chain
FXN	Frataxin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GF-AAS	Graphite furnace atomic absorption spectrometry
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte myeloid progenitor
GO	Gene ontology analysis
HRI	Heme responsive inhibitor
HSC	Hematopoietic stem cell
iBMDM	immortalised bone marrow derived macrophages
ICP-MS	Inductively coupled plasma mass spectrometry
IFN	Interferon
IL-10	Interleukin-10
IL-6	Interleukin-6
IM	Interstitial macrophage
IMM	Inner mitochondrial membrane
IPA	Ingenuity pathway analysis
IRE	Iron response element
IRP	Iron regulatory protein
ISCU	Iron-sulfur cluster assembly enzyme
KDM	Lysine demethylase
KO	Knockout
LDH	Lactate dehydrogenase

Ly6C	Lymphocyte antigen 6 family member C1
MALDI-ToF	Matrix-assisted laser desorption/ionization-time of flight
MARCO	Macrophage receptor with collagenous structure
M-CSF	Macrophage colony stimulating factor
MCU	Mitochondrial calcium uniporter
MFI	Mean fluorescence intensity
MFRN1	Mitoferrin 1
MFRN2	Mitoferrin 2
MHC	Major histocompatibility complex
MLE12	Mouse lung epithelial-12 cells
MPP	Multipotent progenitor
MRC-1	Mannose receptor C-type 1
mtDNA	mitochondrial DNA
mTOR	Mammalian target of rapamycin
mtROS	mitochondrial reactive oxygen species
NCOA4	Nuclear receptor co-activator 4
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMMIX	N-methyl-mesoporphyrin IX
NRAMP1	Natural resistance-associated macrophage protein 1
NRF2	Nuclear factor erythroid 2-related factor 2
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCBP	Poly(RC) binding protein
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RA	Room air
RBC	Red blood cell
RIP-seq	RNA immunoprecipitation sequencing
ROS	Reactive oxygen species
RT-qPCR	Real-time quantitative qPCR
<i>S. pn</i>	<i>Streptococcus pneumoniae</i>
SA	Succinylacetone
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	Standard error of the mean
STING	Stimulator of interferon genes
TBST	Tris-buffered saline tween
TCA	Tricarboxylic acid
TET	Ten-eleven translocase
TFRC	Transferrin receptor
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF-α	Tumour necrosing factor α
ToF-SIMS	Time of flight secondary iron mass spectrometry
WT	Wild type
ZIP	ZRT/IRT-like protein

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Publications

Healy C, Munoz-Wolf N, Strydom J, **Faherty L**, Williams NC, Kenny S, Donnelly SC, Cloonan SM. Nutritional immunity: the impact of metals on lung immune cells and the airway microbiome during chronic respiratory disease. *Respir Res.* 2021 Apr 29;22(1):133. doi: 10.1186/s12931-021-01722-y.

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Chapter 1: Introduction

Iron is an abundant, bioavailable micronutrient which is essential to almost all forms of life. Eukaryotes and prokaryotes alike have co-opted iron for its use as an enzymatic co-factor, leveraging its readiness to donate and accept an electron to switch between its Fe^{2+} and Fe^{3+} valencies. This same property which is exploited by cells can also be detrimental if iron is not properly handled, owing to cytotoxic free radical production through the Fenton reaction. As such, systemic and cellular iron homeostasis is tightly orchestrated and sensitive to intrinsic and extrinsic cues. Mitochondria, double membraned organelles present in virtually all eukaryotic cells, are the primary consumers of intracellular iron. Drawing on iron stored in the mitochondrial matrix or imported from the cytosol, they produce the co-factors iron-sulfur (FeS) clusters and heme which facilitate respiration, DNA synthesis and cell division among myriad other processes. Appropriate cellular iron handling is paramount in immune cells not only in potentiating its toxicity, but also in preventing iron theft from invading bacteria, hijacked to fuel their own proliferation. Despite the central role of mitochondria in immune cell effector function, particularly macrophages, how they may govern 'nutritional immunity' upon infection is not understood, particularly in disease conditions. This introduction will summarise our knowledge surrounding factors governing iron transport and utilisation at a systemic, cellular and mitochondrial level, and the pathological effects of any disruption to these axes. The unique functional, metabolic and iron-handling properties of alveolar macrophages (AMs) will be discussed, with particular attention paid to their role as enforcers of nutritional immunity during respiratory infections. Finally, the pathogenic role of defective mitochondrial and iron metabolism in the lung disease chronic obstructive pulmonary disease (COPD) will be discussed, and the evidence for how these pathologies may drive failures in the AM nutritional immune response to benefit pathogens.

1.1 Iron

1.1.1 Systemic iron homeostasis

In eukaryotes, dietary iron is absorbed in the duodenum, where non-heme Fe^{3+} iron is reduced by local ferric reductases, transported across the enterocytic membrane and complexed to the serum iron carrying protein transferrin (1). Iron is most abundant systemically in heme bound to the oxygen-carrying proteins hemoglobin (erythrocytes) and myoglobin (muscle) but is essential for many other homeostatic functions at tissue and cellular levels. Erythropoiesis is the most iron-demanding process systemically and

accounts for an estimated 75% of systemic iron, with any excess iron stored in the liver (2). No regulated process for the excretion or loss of iron is defined in mammals, and iron is strictly recycled to ensure blood plasma iron levels remain stable to facilitate delivery to tissues. This plasma iron availability is regulated by hepcidin, a small peptide hormone produced primarily in the liver. Hepcidin decreases extracellular iron levels, primarily through binding and degrading the cellular iron exporter ferroportin (FPN) to inhibit further efflux of iron to plasma (3). Hepcidin can also bind FPN to sterically occlude iron export, which may be a faster means of blocking cellular iron export than endocytic degradation (4). Impeding FPN also prevents efflux of iron to plasma from duodenal enterocytes, directly reducing levels of dietary iron absorbed. Cues which promote hepcidin secretion include bone morphogenic protein 6 (BMP6) signalling, interleukin-6 (IL-6), and the direct sensing of extracellular transferrin-bound iron by liver hepatocytes. The hormone erythroferrone is secreted by erythroblasts to inhibit hepcidin production, increasing plasma iron availability for erythropoiesis (1). Together, these processes act in concert to ensure adequate iron availability at a tissue level to meet cellular metabolic requirements.

1.1.2 Cellular iron homeostasis

Cells predominantly import iron through the transferrin receptor (TFRC), with transferrin-bound iron the predominant form of iron extracellularly under physiological conditions (**Fig. 1.1**). Endosomal acidification upon TFRC binding liberates Fe^{3+} transferrin bound iron to Fe^{2+} for cellular uptake. Fe^{2+} iron can be imported by divalent metal transporter 1 (DMT1) (5), with additional iron import also facilitated through the zinc uptake receptors ZRT/IRT-like protein 8 and 14 (ZIP8 and ZIP14) (6). Free heme can be acquired through SLC48A1, or through CD91 and CD163 when complexed in the hemoglobin derivatives hemopexin and haptoglobin, respectively (7, 8). Other more cell-specific means of iron import include receptor-mediated endocytosis of extracellular ferritin, and macrophage-specific recycling of iron from damaged or senescent erythrocytes through erythrophagocytosis.

Upon import, iron is chaperoned to the cytosolic labile iron pool, where it is used metabolically or stored in ferritin. Labile iron is thought to be chaperoned by the poly(RC) binding proteins 1 and 2 (PBCP1/2) to the ferritin nanocage, a heteropolymer composed of heavy (FTH1) and light (FTL)-chain subunits. The ferroxidase activity of FTH1 mineralises this iron for inert storage in inactive FTL. Each ferritin nanocage can store up to 4500 iron atoms, with this storage capacity determined by the ratio of FTH1 to FTL (9). This ratio varies between cell types and tissues to reflect inherent iron needs. In iron-deplete conditions, iron can be liberated from ferritin through a specific form of autophagy

termed ferritinophagy. FTH1 is marked for lysosomal degradation through complexation with nuclear receptor coactivator 4 (NCOA4), and iron liberated for metabolic use (10).

A limited pool of cytosolic labile iron exists at steady-state, which can be incorporated into cytosolic enzymes which utilise elemental Fe^{2+} as a cofactor, like prolyl hydroxylases in the hypoxia signalling pathway (11) or chromatin-remodelling ten-eleven translocase (TET) enzymes (12). Chaperone proteins, like glutathione and the aforementioned PCBP1 and 2 are thought to transport iron from the labile iron pool to storage or to metabolic utilisation (1). Ferroptosis, an iron-dependent form of regulated non-apoptotic cell death driven by lipid peroxidation, is promoted by genetic loss of PCBP1 (13), illustrating the importance of these chaperones in limiting toxicity from Fe^{2+} derived free radicals.

Elemental Fe^{2+} is exported from cells through the transmembrane protein ferroportin (SLC40A1, FPN), which is thought to alternate between its inward and outward-facing conformations to move iron across the plasma membrane to the extracellular space where it is complexed to transferrin (14). This iron appears to be delivered to FPN bound

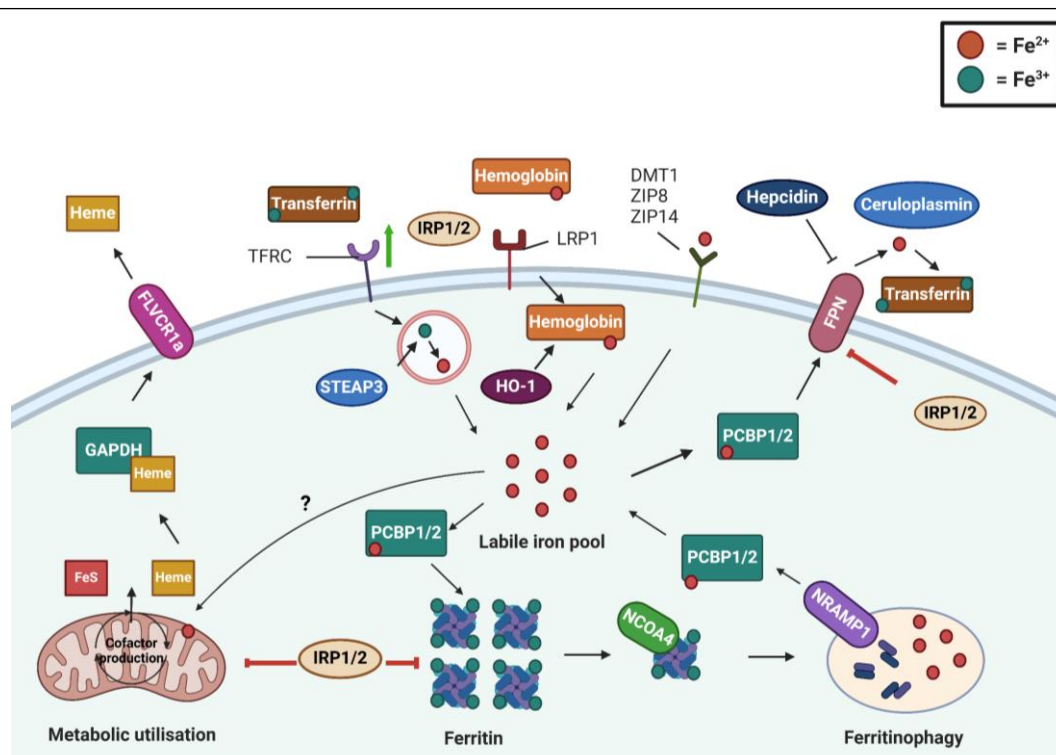


Fig. 1.1: Cellular iron metabolism. Free Fe^{2+} iron can be imported into the cell from the extracellular space through the plasma membrane divalent metal transporters DMT1, ZIP8 and ZIP14. Fe^{3+} iron carried in transferrin is internalised via TFR1, endosomally degraded and reduced to Fe^{2+} by STEAP3. Hemoglobin is internalised via LRP1 and degraded by HMOX1 to liberate Fe^{2+} . This iron is then incorporated into ferritin for storage, which can be recycled via NCOA4-mediated ferritinophagy, transported to mitochondria for metabolic usage or chaperoned to FPN by PCBP1/2 for export and complexation to transferrin. In iron deplete conditions IRP1 and 2 act post-transcriptionally to promote iron import and minimise iron loss.

to glutathione by PCBP2 (15). Heme is thought to be exported through feline leukaemia virus subgroup C receptor-1 (FLVCR1), but its specificity has been questioned through recent identification of FLVCR1 as a choline importer (16).

The cytosolic iron regulatory proteins 1 and 2 (IRP1 and IRP2) post-transcriptionally control iron import, storage and export to increase or decrease intracellular iron ability dependent on need. In iron-deplete conditions, IRP1/2 bind conserved hairpin loop structures termed iron response elements (IRE) in the untranslated regions of mRNA transcripts with a role in iron metabolism. IRP1/2 can occlude mRNA translation through 5' UTR binding to decrease iron storage and export (ferritin, FPN) and stabilise transcripts through 3' UTR binding to promote iron uptake (transferrin, DMT1) (17). Other IRE-containing targets to indirectly reduce intracellular iron usage by restricting iron-demanding processes include mitochondrial aconitase (ACO2) and hypoxia-inducible factor 2 alpha (HIF2 α) (1). IRP1 senses intracellular iron levels through 4 bound iron-sulfur (FeS) clusters: under iron replete conditions, IRP1 acts as an aconitase, but loss of FeS clusters as a result of cellular iron deficiency facilitates its IRE binding and post-transcriptional regulation (18, 19). Notably, other proteins involved in iron trafficking and metabolism can be themselves reliant on iron for function, such as NCOA4 and the mitochondrial glutathione importer SLC25A39. A role for heme-regulated inhibitor (HRI) in regulating the unfolded protein response in immune cells was recently defined, expanding on its function in regulating heme synthesis in erythroid cells (20): whether HRI maintains this ability to regulate heme synthesis in non-erythroid cells is not known. Heme availability also regulates intracellular iron levels through binding and degrading the transcription factor BTB domain and CNC homologue 1 (BACH1), facilitating nuclear factor erythroid 2-related factor 2 (NRF2) upregulation of FPN expression to increase iron export (1). Combined, this results in an intricate network of transcriptional and chemical regulation of iron trafficking to allow cells to efficiently remodel iron metabolism in response to intrinsic and extrinsic cues.

1.1.3 Mitochondrial iron homeostasis

Mitochondria constitute the primary iron storage and utilization sites in eukaryotes and can account for up to 50% of cellular iron stores (**Fig. 1.2**) (21). The precise mechanism for delivery of ferrous iron to the mitochondria is unknown, with two methods proposed. The first is the delivery of cytosolic labile iron to the outer mitochondrial membrane (OMM) by an unidentified protein (22), with glutathione, PCBP1 and GAPDH all suggested chaperones (1). The second is the direct endosomal delivery of transferrin-bound iron, referred to as the 'kiss and run' hypothesis (23).

The presence of voltage-dependent ion channels (VDACs) in the outer mitochondrial membrane (OMM) makes it extremely permeable to small ions and is hypothesised to allow the diffuse transport of iron. In contrast, the tight diffusion barrier of the inner mitochondrial membrane (IMM) requires hydrophobic carrier proteins for ion transport. **Mitoferrin 1** and **mitoferrin 2 (MFRN1 and MFRN2)** are the **only known mitochondrial iron importers**, originally characterised through their yeast orthologues (24). The exact molecular details surrounding iron transport by the mitoferrins remain unclear, although the two appear to possess complementary functionalities. Expression patterns suggest that MFRN1 is the predominant mitochondrial iron transporter in erythroid cells, with MFRN2 preferred in non-erythroid cells (25). In non-erythroid cells silenced for MFRN1, ectopic expression of MFRN2 restored heme synthesis levels to baseline, with the ectopic expression of MFRN2 on a MFRN1-silenced background yielding similar

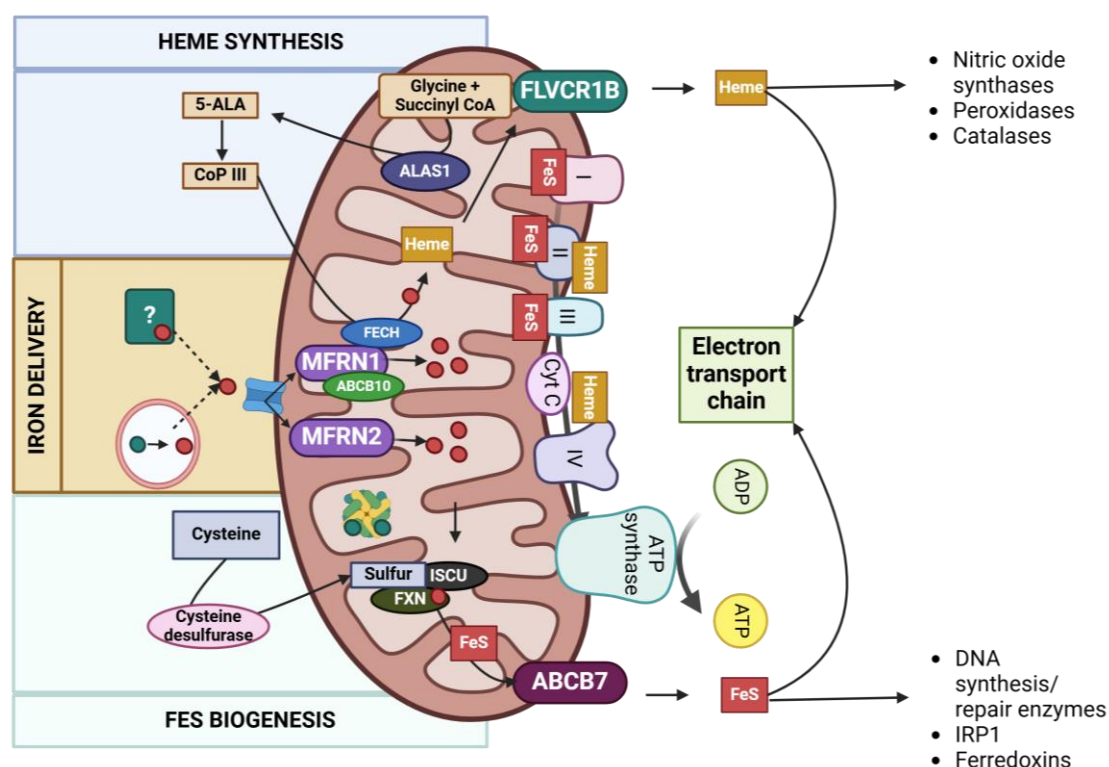


Fig. 1.2: Mitochondrial iron metabolism. Iron is thought to pass through the OMM via VDAC porins following delivery via the endosome or via a chaperone protein. Iron is imported across the IMM to the mitochondrial matrix via MFRN1 and 2, where it can be stored inertly in mitochondrial ferritin or utilised in heme or FeS cluster synthesis. Heme synthesis commences with the condensation of glycine and succinyl CoA by ALAS1 to yield 5-ALA, which is exported to the cytosol and further enzymatically converted to CoP III. CoP III re-enters the mitochondrion, where the insertion of a single iron atom into the centre of its porphyrin ring is catalysed by FECH to form heme. Alternatively, iron can be loaded to an assembly complex containing FXN and ISCU which uses cytosolically derived cysteine to form iron-sulfur clusters. Heme and iron sulfur clusters are exported to the cytosol by FLVCR1b and ABCB7 respectively, where they participate in a multitude of metabolic processes as enzymatic co-factors.

results. Conversely, MFRN2 could not rescue heme production in MFRN1-silenced erythroid cells due to insufficient accumulation on mitochondria, with MFRN1 observed to have an extended half-life (26). MFRN1 is bound and stabilised in erythroid cells by ATP Binding Cassette Subfamily B Member 10 (ABCB10), which likely extends its half-life during erythroid cell maturation and differentiation to meet demand for heme production in complex with ferrochelatase (27, 28).

A mitochondrially-localised homologue of FTH1, termed mitochondrial ferritin (FTMT), is thought to facilitate limited iron storage in the mitochondrial matrix. FTMT maintains the ferroxidase activity of FTH1, but the *Ftmt* gene does not have an IRE, suggesting its function is somewhat independent of iron (29). Expression is protective against iron overload associated oxidative damage, but at steady state expression does not appear to correlate with tissue mitochondrial iron levels, raising the possibility that its role lies in iron detoxification rather than storage. Overexpression of FTMT depletes cytosolic iron levels to drive an increase in the mitochondrial matrix (30), indicating potential cross-talk with MFRN1 and 2-mediated iron import. The iron demand of FeS biogenesis and heme synthesis means that all iron imported to the mitochondrion likely exits incorporated into one of these two co-factors. Export of free iron has only been reported through ATP Binding Cassette Subfamily B Member 8 but exclusively in conditions of cardiotoxicity (31).

1.1.3.1 FeS cluster synthesis

FeS clusters are prosthetic groups complexing iron with bridging sulfides, typically ligated to proteins at cysteine residues. FeS cluster biogenesis is a highly conserved process, with participating factors primarily identified and studied in *S. cerevisiae*. Biogenesis relies on a series of assembly steps utilising scaffold proteins both inside and outside of mitochondria. Nascent cluster assembly takes place on a heterologous complex, in which cysteine desulfurases donate sulfur to a complex including the scaffolds iron sulfur cluster assembly protein (ISCU) and frataxin. FeS clusters, or iron-free intermediates, are exported through ATP Binding Cassette Subfamily B Member 7 (ABCB7), where they are loaded to the cytosolic CIA machinery for their incorporation into extramitochondrial proteins (32).

The structural versatility of FeS clusters is conducive to their multifaceted functions as cofactors across cellular processes, housed in proteins encoded by an estimated 1% of the human genome. FeS clusters can exist in forms with up to 16 iron atoms bound, and can even house other metal ions (33). The pervasiveness of FeS clusters in metabolic pathways has led to theories proposing the origin of life on iron-sulfide minerals (34). FeS

clusters catalyse electron transport bound to electron transport chain (ETC) complexes I, II and III, with the respiratory complexes estimated to account for 17% of cellular iron (35). Multiple steps of DNA synthesis rely on FeS-mediated catalysis, including unwinding of the double-helix by helicases, purine nucleotide synthesis and *de novo* DNA polymerisation. The final step of mitochondrial heme synthesis, where iron is inserted into the protoporphyrin IX ring (detailed below), is catalysed by an FeS cluster in ferrochelatase. As outputs of mitochondrial iron metabolism FeS clusters therefore govern multiple essential cellular processes, which extend to normal mitochondrial function and iron usage itself.

1.1.3.2 Heme synthesis

While most extensively studied in an erythroid context, heme is an essential cofactor present in all mammalian cells. Heme synthesis begins in the mitochondrion with the condensation of glycine and succinyl-CoA by 5-aminolevulinase synthase (ALAS) to yield 5-aminolevulinic acid. Two isoforms of ALAS exist to control the rate of heme synthesis in erythroid and non-erythroid tissues: ALAS1, which is ubiquitously expressed, and ALAS2, which has been considered as an erythroid tissue-specific isoform. 5-aminolevulinic acid is exported to the cytosol through an as-of-yet undefined mechanism, where sequential enzymatic conversions yield coproporphyrinogen III which is imported back to the mitochondrial matrix via benzodiazepine receptors. Further oxidation steps yield the tetrapyrrole ring protoporphyrin IX, in which ferrochelatase catalyses the insertion of an Fe²⁺ atom to ultimately form heme. Heme is exported from mitochondria through FLVCR1b, encoded by an alternative isoform of FLVCR1 (36). Key families of hemoproteins include cytochromes, cyclooxygenases and peroxidases (37). Autoregulatory mechanisms control the rate of heme synthesis in non-erythroid cells, with the binding of heme to a short heme-regulatory motif in the ALAS1 protein product preventing its import into the mitochondrion to initiate heme synthesis (38). Furthermore, heme synthesis itself can be regulated by FeS cluster availability, with the catalytic activity of ferrochelatase facilitated by an FeS cluster.

1.1.3.3. Iron and mitochondrial function

As both producers and consumers of catalytic iron complexes, mitochondrial function is intimately linked to normal iron homeostasis. Mitochondrial integrity relies on cytosolic iron metabolism, illustrated through the dependence of mitochondrial DNA (mtDNA) maintenance on iron (39, 40). Similarly, mutations in mtDNA cause lethal macrocytic anaemia (41). Cytosolic iron availability for mitochondrial import is essential for mitochondrial health: chemical and genetic impairment of ferritinophagy reduces

mitochondrial membrane potential and respiratory complex assembly (42), and genetic loss of the iron chaperone PCBP1 reduces mitochondrial oxygen consumption (43). Increases in mitochondrial iron import promote membrane depolarisation and oxidative damage (44). Genetic and chemical iron depletion induces mitophagy, through mechanisms reported to be both dependent and independent to the PINK1/Parkin system (45). In iron-limiting conditions, mitophagy increases overall lifespan in the lower order model organisms *Candida glabrata* and *Caenorhabditis elegans* (46), suggesting this may be a protective stratagem for cells to repurpose mitochondrial iron stores. Frataxin loss promotes mitophagy in *Drosophila* (45), implicating mitochondrial iron itself in mitophagy regulation. Cellular metabolism can also influence mitochondrial iron dynamics, with loss of acetyl CoA production through deletion of pantothenate kinase 2 leading to mitochondrial iron accumulation in astrocytes (47).

To conserve intracellular iron usage in iron-limiting conditions, iron-regulatory proteins appear to disproportionately target mitochondrial iron-consuming proteins and processes. In yeast, the transcription factor *Cth1* is indispensable for survival during pharmacological iron deprivation and degrades mRNAs encoding for components of the ETC and heme synthesis pathways to conserve iron (48). The mammalian *Cht1* orthologue, tristetraprolin, is negatively regulated by mammalian target of rapamycin (mTOR), further entangling central metabolism and mitochondrial iron dynamics (49). Under iron starvation in a human bronchial epithelial cell line, IRP2 associates with mRNAs encoding for mitochondrially functioning proteins, most of which have no defined role in iron homeostasis or usage (50).

Mitochondrial iron dynamics reciprocally influence cellular health. Mitochondrial iron depletion stabilises DAP3 Binding Cell Death Enhancer 1 (DELE1) on the OMM, where its ensuing interaction with HRI triggers the integrated stress response (51) and can induce mitophagy (52). Through FeS cluster production, mitochondrial iron decreases glutathione import from the cytosol to maintain oxidative balance through FeS-mediated occlusion of the mitochondrial glutathione importer SLC25A39 (53). Genetic perturbations in components of the mitochondrial FeS cluster biogenesis machinery can cause neurodegenerative and muscular disorders associated with losses in respiratory complex function (21). Normal mitochondrial iron dynamics are therefore reliant on, and reciprocally influence, cellular metabolism and iron handling.

1.2 Alveolar macrophages

Macrophages are paramount in the maintenance organismal health by engulfing foreign bacteria and antigens (phagocytosis) and senescent and apoptotic cells (efferocytosis). Macrophages at mucosal sites like the lung face the additional oxidative challenges presented by exogenous iron exposure. This creates an environment of fluctuating increases and decreases in extracellular iron and necessitates a rapid remodelling of cellular iron metabolism, which, as discussed, centres on mitochondria.

1.2.1 AMs and the alveolar microenvironment

Alveolar macrophages (AM) are the predominant immune cell in the lung. As the first line of defence against pathogens, pollutants and irritants in the estimated 10,000 litres of air inhaled per day, AMs are highly plastic and pivotal in surveillance and lung defence (54) (**Fig. 1.3**). Through interactions with integrins presented by the alveolar epithelium, AMs can crawl across the luminal surface of alveoli and migrate from one alveolus to the next through the pores of Kohn (55). AMs are naturally quiescent, and their unique positioning between inhaled air and the pulmonary vasculature necessitates a rapid and efficient inflammatory response to neutralise invading pathogens while limiting

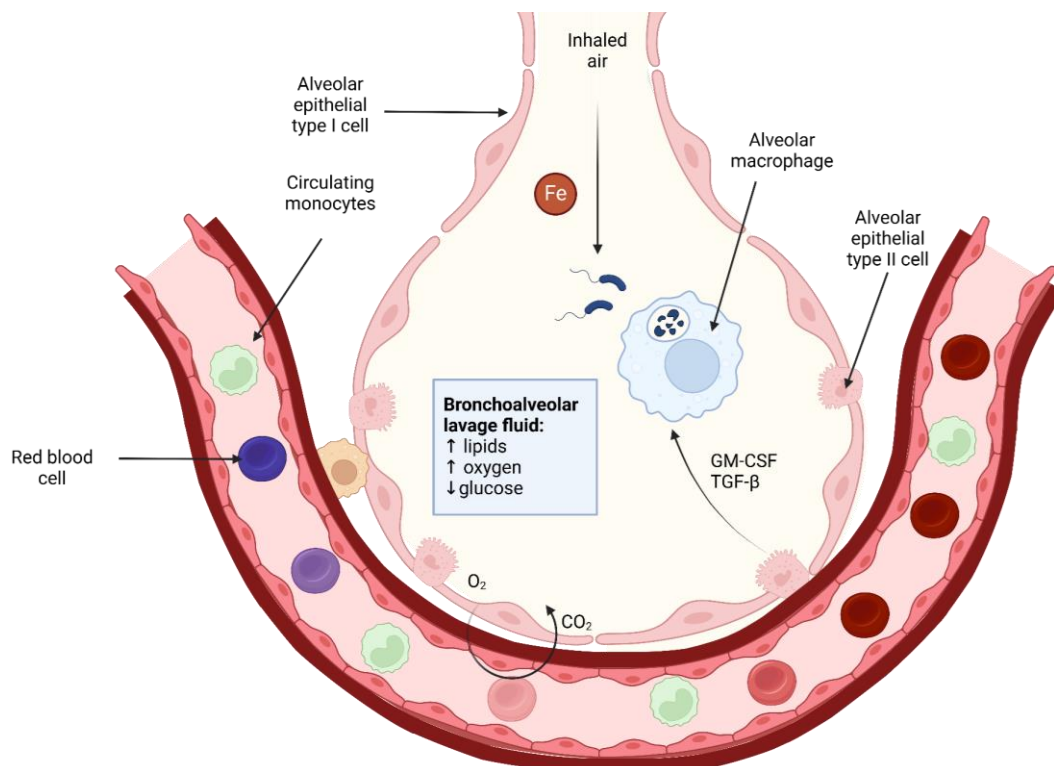


Fig. 1.3: AMs and the alveolar microenvironment. AMs reside in the alveolar space, where they are functionally informed by the unique metabolic composition of bronchoalveolar lavage fluid and by secreted cytokines from AT2 cells. AMs process air to mitigate the deleterious effects of potentially damaging inhalants such as iron and pathogens, maintaining gas exchange at the alveolar epithelium and preventing excessive recruitment of monocytes from circulation.

excessive tissue damage to protect alveolar integrity and gas exchange (56). These distinct immune properties relative to other macrophage populations are reflective of their unique ontogeny. Circulating progenitors from the yolk sac (fetal liver) travel to the pre-natal lung, where granulocyte macrophage colony stimulating factor (GM-CSF) produced by the alveolar epithelium dictates the reprogramming of these fetal monocytes to become AMs (57). At homeostasis, irradiated rats depleted of hematopoietic stem cells maintain AMs in the lung, as do mice deficient in macrophage colony stimulating factor (M-CSF), a key cytokine in the differentiation of myeloid precursors towards macrophages (58). Of note, a small population of macrophages exists in the lung interstitial spaces (interstitial macrophages, IMs). IMs are outnumbered ten-to-one by AMs and are significantly less studied in comparison, primarily attributed to the need for enzymatic tissue dissociation to isolate IMs and the lack cell-type specific surface markers identified for flow cytometric analysis (54, 59). In contrast to AMs, IMs are present in other organs and appear to maintain similar transcriptional states regardless of their tissue localisation, suggesting they may be less honed to the alveolar niche (60).

Key to dictating and maintaining AM function is their communication with the local alveolar niche, and chiefly the alveolar epithelium. The alveolar lining is predominantly composed of alveolar epithelial type I cells (AT1), which are highly specialised for gaseous exchange. The remaining 5% of the alveolar wall is comprised of alveolar epithelial type 2 cells (AT2). AT2 have stem-like capacities and can both self-renew and transdifferentiate to AT1 to regenerate damaged alveoli. AT2 also secrete surfactant, a detergent composed of 90% phospholipids and 10% specialised surfactant proteins that decrease surface tension at the air-liquid interface to facilitate normal breathing (61). AMs are critical in surfactant turnover to maintain appropriate alveolar surface tension and prevent collapse, and AM functional impairment leads to surfactant accumulation and the development of pulmonary alveolar proteinosis (62). Mice with a constitutive deficiency in GM-CSF or the GM-CSF receptor do not have AMs (54, 63). GM-CSF signalling triggers a transcriptional program regulated by peroxisome proliferator-activated receptor gamma (PPAR- γ), involving the expression of several lipid importers and catabolic enzymes important in surfactant recycling (63). Transforming growth factor beta (TGF- β) is also critical for AM development, and can be secreted in its latent form by alveolar epithelial cells and AMs themselves, where it is activated through binding the $\alpha_v\beta_6$ integrin on the alveolar epithelium (64, 65). For AMs, this interaction with the epithelium is crucial in maintaining their immunosuppressive properties, and when lost leads to a hyper-inflammatory phenotype upon toll-like receptor (TLR) stimulation (65). AMs also function in the clearance of apoptotic epithelial cells and other debris through efferocytosis to resolve tissue injury

and inflammation. The cumulative effect of these local environmental factors and pressures imposes a distinct set of functional properties on AMs, and removal of AMs to *ex vivo* culture diminishes lipid metabolism, adhesion marker expression and TGF- β signalling (66).

Developments in single-cell RNA sequencing technologies in the past decade have re-characterised AMs as a heterogeneous population of cells, with subsets identified based on distinct transcriptomic profiles governing processes like cholesterol biosynthesis, chemokine secretion and interferon (IFN) signalling (54). Furthermore, sterile and infectious insults that perturb normal lung homeostasis drive the recruitment of circulating immune cells to the airspaces, expanding this heterogeneity further. This recruitment is driven by chemoattractants secreted by AMs in response to danger or pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) (67). Acute inflammation typically associates with the recruitment of myeloid cells like neutrophils and monocytes, which under chronic inflammatory conditions can be joined by lymphocytes (68). AMs can express both major histocompatibility (MHC) I and II complex components, but their communication with the adaptive immune system in both health and disease is poorly understood.

Intricate fate-mapping models have delineated the unique contributions of monocyte-derived macrophages during inflammation and their ability to persist and integrate into the AM pool (69, 70). After the resolution of inflammation, these recruited monocyte-derived macrophages persist in the lung and for the most part assume the transcriptomic and metabolic profile of fetal-liver derived AMs as directed by the alveolar niche (71). In mice, AMs of monocytic origin can still be distinguished through low Siglec-F and macrophage receptor with collagenous structure (MARCO) expression (54). In disease, monocyte-derived AMs can remain phenotypically distinct from fetal liver-derived AMs, with reports this persistence can have beneficial consequences in tissue repair (70), but also detrimental consequences in perpetuating lung inflammation (72). The AM pool in naïve mice shows little contribution from monocyte-derived AMs, but human sex-mismatched lung transplant studies in individuals with a history of chronic lung disease revealed the AM pool was primarily comprised of recipient monocyte-derived AMs, rather than donor fetal liver-derived AMs (73). Inhalation of ambient particulate matter and endotoxins means humans are faced with an 'exposome' that likely requires a continuous level of AM replenishment from circulating monocytes (74), which is not a factor in the controlled laboratory animal environment.

1.2.2 AM metabolism

The advent of immunometabolism has repositioned mitochondria as crucial signalling hubs with remarkable plasticity to dictate macrophage effector functions in response to insult (75). Briefly, macrophages primarily utilise glycolysis or oxidative phosphorylation (OXPHOS) for adenosine triphosphate (ATP) generation from glucose derivatives. Glycolytic metabolism is less efficient in terms of output, yielding 2 ATP molecules to the 36 produced through OXPHOS, but is a faster form of energy production. Metabolic intermediates and by-products of cellular energetics can act as signalling molecules, which are now appreciated to influence immune cell effector functions beyond their roles in ATP production. Macrophages activated *in vitro* with signals associated with bacterial infection, like lipopolysaccharide (LPS) or IFN- γ , preferentially downregulate OXPHOS and switch to glycolysis for ATP generation (76). Proper mitochondrial function is essential to this metabolic repurposing, which in mice is inhibited in the absence of mitochondrial nitric oxide production (77), reactive oxygen species signalling or sufficient mitochondrial copy number (78). The critical importance of energy production in lung homeostasis is underscored by the presence of a lung-specific isoform of cytochrome c oxidase, the terminal enzyme in the electron transport chain - this increases its oxygen binding capacity compared to other tissues for maximal ATP production (79). Aside from energetic functions, reactive oxygen species (ROS) produced as byproducts of OXPHOS are key second messengers and are also harnessed by lung immune cells for bactericidal functions, like oxidative burst and phagocytosis (80).

In vivo, macrophages are exposed to diverse, concurrent stimuli. Endogenous host factors like tissue mechanical pressures, nutrient availability and the local microbiota can combine to distinctly impact macrophage metabolic needs (81). For AMs, these factors are layered with the exogenous influences of transient oxygen concentrations and ambient pollutant inhalation. Combined, these exogenous and endogenous pressures manifest in a unique AM metabolic program. Glucose levels are estimated to be 12 times lower in the airways than in blood (82), and resultingly AMs display little capacity for glycolysis at baseline. Instead, through the combinatorial effects of their exposure to lipid-rich surfactant and high oxygen concentrations, AMs readily employ OXPHOS to produce ATP, which is fuelled by the end products of fatty acid oxidation (FAO). Macrophage TLR activation typically leads to a shift towards glycolysis for ATP production, with the ensuing breaks in the tricarboxylic acid (TCA) cycle leading to the accumulation of metabolites with functions in inflammatory signalling like succinate, fumarate and citrate (83). AMs have higher glycolytic reserves compared to MDMs (84), but AMs do not show this same shift upon stimulation with LPS (85), and appear to engage glycolysis in response to a limited number

of cues like IFN- γ (86) and house dust mite (87). Monocyte-derived AMs recruited to the lung during inflammation can retain distinct metabolic properties compared to resident AMs: recruited monocytes show elevated arginine metabolism upon helminth infection (88) and diminished FAO during influenza infection (89). Interestingly, both studies note chromatin remodelling in monocyte-derived AMs after the resolution of infection compared to fetal liver-derived AMs. The dynamic deposition and removal of acetylation groups on histone lysine residues determines the accessibility of DNA polymerase at specific promoter regions, which controls the expression and silencing of inflammation-related genes (90). Acetyl-CoA is the dominant carbon source for histone acetylation, meaning the extent of its production and metabolic consumption dictates acetyl group availability for chromatin remodelling, a phenomenon which may be at play in monocyte-derived AMs. Mitochondrial metabolism is therefore intricately linked to AM function and provides the necessary energy and plasticity for AMs to preserve alveolar integrity against inhaled threats.

1.2.3 AMs, iron and nutritional immunity

Macrophages are essential for normal iron homeostasis. Macrophages supply, sequester and recycle iron systemically, primarily through erythrophagocytosis, to ensure adequate iron availability for tissues (91). Macrophages are the third most important reservoir of iron after erythrocytes and the liver and can uptake and export iron as required to meet local tissue demands (92). In line with this, AMs are the primary iron-handling cell in the lung, and their intracellular iron stores account for approximately 80% of total BALF iron content (93). As described, the acquisition of dietary iron by the host is essential for normal cellular energetics, important for AMs in homeostatic processes like OXPHOS and fatty acid oxidation. The necessity of iron for survival extends to invading pathogens, with virtually all successful pathogens having evolved sophisticated mechanisms for iron acquisition from the host to fuel their own virulence. To limit bioavailable iron (and other trace elements) to invading pathogens, the host sequesters iron both intracellularly and in the extracellular space in large macromolecules through processes largely mediated by macrophages, strategies collectively referred to as nutritional immunity (**Fig. 1.3**) (94). AMs are uniquely positioned as both the main iron-positive cell in the lung and the guardians of the alveolar microenvironment against invading pathogens. Up to 25 μg of iron is inhaled per day – this means that any failures in iron detoxification, storage and metabolism in AMs will impair not only their own effector functions but also their ability to limit pathogen outgrowth through iron sequestration (95, 96). A functional nutritional immune response in AMs is therefore paramount to lung health.

Selective evolutionary pressures driven by the host nutritional immune response has led to the reciprocal development of pathogenic mechanisms to acquire both elemental and complexed host iron. Viruses can invade cells via TFRG, but their specific means of acquiring host iron are not understood (97). The most intensively studied pathogenic mechanism of iron acquisition is the bacterial siderophore system, where bacteria secrete low molecular weight proteins into the extracellular space to trap iron (primarily Fe^{3+}). These iron laden siderophores are then internalised by bacteria, and iron liberated by reductases for use in bacterial proliferation or effector functions. The presence of siderophore-encoding genes can predict intra-species virulence (98) and are often a requirement for bacterial biofilm formation, where secreted bacterial polysaccharides facilitate epithelial adherence and tissue persistence (99). Notably, siderophores appear to have a higher affinity for iron than host-iron binding proteins. Certain bacteria have further evolved to secrete chemically modified siderophores resistant to host binding via lipocalin-2, such as the glycosylated siderophores secreted by *Salmonella* species (100) or the sterically hindered yersiniabactin used by *Klebsiella pneumoniae* (101).

While siderophores are the most studied bacterial iron uptake mechanism, some of the most successful pathogens in humans, including *Streptococcus pneumoniae*, *Haemophilus influenza* and *Legionella* sp. cannot produce siderophores. Other iron acquisition methods can be employed to fuel bacterial virulence, which can be complementary to siderophore production in relevant bacteria. Intracellular bacteria can acquire iron intracellularly through diverse mechanisms including the manipulation of the phagosomal iron exporter natural resistance-associated macrophage protein 1 (NRAMP1) (102), the induction of ferritinophagy (103), or direct uptake of Fe^{2+} both from the labile iron pool or bacterially-reduced from Fe^{3+} (94). Intracellular bacteria can also manipulate extracellular iron dynamics. The intracellular *Salmonella* induces IL-6 signalling to stimulate hepcidin-mediated degradation of FPN, preventing host intracellular iron loss, and can also promote macrophage erythrophagocytosis (104). Other, more atypical iron sources can also be targeted - recently characterised hemophores can be secreted by gram-negative bacteria (and select fungal pathogens) to extract heme from heme-bound proteins (105, 106). Heme is essential for *Staphylococcus aureus* and *Pseudomonas aeruginosa* survival in the presence of the host iron-chelating molecule calprotectin (107). *S. aureus* utilises fermentative metabolism in iron limiting conditions to produce lactate, which can dissociate iron bound to transferrin by decreasing pH (108). Agonistic interactions between invading bacteria and commensals of the microbiota at mucosal sites like the lung can also promote virulence. Bacteria that cannot produce endogenous siderophores can often hijack those secreted by commensals for iron acquisition, like the

Escherichia coli siderophore enterobactin (109). Interkingdom interactions also provide bacteria with iron liberated from viral infections to mutually benefit both pathogens. For example, prior infection with respiratory syncytial virus induces release of transferrin-containing extracellular vesicles from the alveolar epithelium, which transfer iron to *P. aeruginosa* biofilms to further their growth (110). These mechanisms, in isolation or in tandem, threaten organismal health and must be efficiently negated by the host.

The host responds to infection with an intricate network of stratagems that can both limit iron and depend on iron, and which vary depending on the intracellular or extracellular nature of the infectious agent (**Fig. 1.4**). Systemically, serum hepcidin is elevated in infection to decrease extracellular iron, which can be induced in the liver by IL-6. AMs are unique among other systemic macrophage populations in their production and secretion of endogenous hepcidin, which is essential for their bactericidal function and can promote the intracellular sequestration of iron through FPN degradation to limit extracellular availability (111, 112). AM production of ROS and nitric oxide (NO), indispensable for bacterial killing, relies on the use of heme as a cofactor (113). Alongside lung epithelial

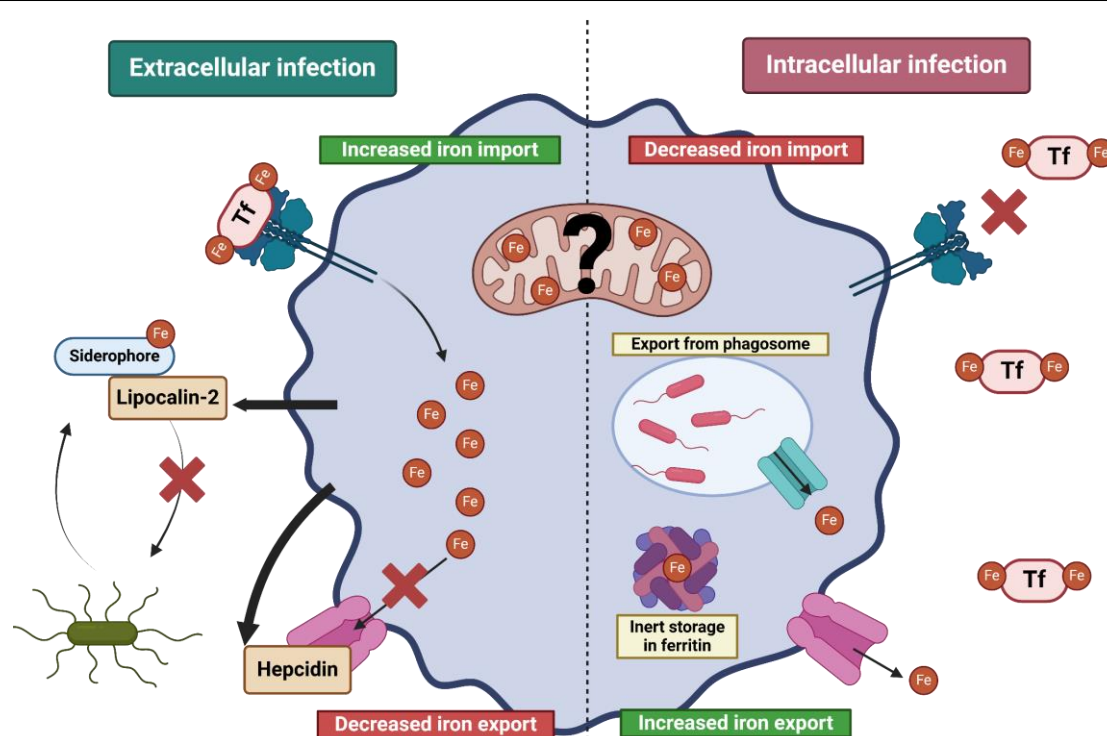


Fig. 1.4: AM nutritional immunity. Upon infection, AMs dynamically remodel their iron metabolism to deprive invading pathogens of bioavailable iron in a compartment-specific manner. During extracellular infection, AMs increase uptake of transferrin (Tf) bound iron through TRF and decrease FPN-mediated iron export through production of endogenous hepcidin. Secreted lipocalin-2 binds iron-laden siderophores to prevent re-uptake by bacteria. During intracellular infection, iron import is decreased and export increased. Intracellular iron is stored inertly in ferritin, and iron is exported from the phagosome through NRAMP1 to create an iron-starved environment for phagosomally-replicating bacteria. How mitochondrial iron dynamics change in response to intracellular or extracellular infection is unknown.

cells and neutrophils, AMs secrete lipocalin-2 in response to TLR stimulation, which binds bacterial iron acquisition molecules. Iron-laden lipocalin-2 can be internalised by mammalian cells through SLC22A17 (114). Upon infection with an intracellular bacterium, AMs recruit NRAMP1 to the phagosomal membrane to deprive bacteria of the necessary iron for their replication (115). An intracellular variant of lipocalin-2 can also restrict phagosomal iron availability to *Mycobacterium tuberculosis* (116). In AMs, these mechanisms can combine to dynamically manipulate the compartmentalisation of iron depending on intracellular or extracellular pathogen lifestyle. Plasma iron bioavailability is reduced during infection through complexation in soluble iron-binding proteins like transferrin, lactoferrin and calprotectin, but how BALF concentrations of these mediators change in acute infection is less characterised.

The importance of winning this 'arms race' against pathogens for scarce amounts of iron has rendered correct iron metabolism indispensable to the immune response. Consequently, this appears to have driven an evolutionary intertwining of iron and immune cell function. Perhaps most exemplary of this, evolutionary analysis of the transferrin gene across humans and non-human primates identified the C-terminal of transferrin as a site of accelerated evolution, and accumulated point mutations inhibit the binding of transferrin by *Nisseria gonorrhoeae* and *Haemophilus influenzae* (117). Patients and murine models carrying missense mutations in the TFRC gene associated with an approximate 50% reduction in cellular iron uptake exhibit severe immunodeficiency, affecting the development and function of both innate and adaptive immune cells. This partial loss in transferrin-bound iron import associates with neutropenia, impaired memory B cell antigen presentation and reduced T cell proliferation upon stimulation, rescued by supplementation with iron citrate (118). Neutrophils rely on the oxidative capacities of the heme-containing myeloperoxidase for extracellular trap formation and oxidative burst (119). Transcriptional factors regulated by iron availability can also target cytokines. In macrophages, tristetraprolin can bind 3' AU rich regions of chemokine (*Ccr1*, *Ccr2*) and cytokine (*Tnfa*, *Ptsg2*) mRNAs (120). Heme-regulated BACH2, expressed in AMs among a limited number of cell types, can similarly regulate expression of IL-4 and IL-33 (121). More directly, PCBP1 post-transcriptionally regulates T-cell production of IL-2 and GM-CSF (122). The iron-targeting lipocalin-2 has pleiotropic functions and is induced during inflammation by nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling regardless of sterile or infectious etiology (123). Conversely, IFN- γ , a master regulator of the inflammatory response, also has iron-targeting functions and limits TFRC expression in macrophages to prevent intracellular bacterial survival (124). Cellular immune receptors can also recognise and mount a response to iron – TLR4 binds heme,

which increases cellular ROS and pro-inflammatory cytokine production (125) and can activate the inflammasome (126).

How pre-existing alterations in AM iron can affect the immune response is less clear. Historically, iron loading has been associated with a pro-inflammatory phenotype typical of an M1-type macrophage and iron deprivation with M2-type macrophage anti-inflammatory properties. But, like the M1-2 dichotomy itself, this has proven in recent years to be a reductive model. IFN- γ signalling in primary human monocytes was enhanced in iron deplete conditions and blunted with iron excess (127). While the functional impacts of iron overload or depletion on host AM function are incompletely researched, numerous examples exist in the literature detailing the heightened bacterial virulence in response to host iron levels. Intracellular iron accumulation in BMDMs promotes *Mycobacterium avium* survival (128), and pharmacological iron depletion inhibits *M. tuberculosis* and *Ehrlichia chaffeensis* replication in macrophages. Failures in cellular iron metabolism appear to directly impede the effector functions of immune cells in the nutritional immune response. This may result in a scenario where cells are not only unable to properly harness iron for their own effector functions but are themselves leaving this excess iron prone to bacterial siphoning.

Specifically, mitochondrial iron metabolism is crucial in dictating cellular energetics through supply of FeS clusters and heme. Any fluctuation in mitochondrial iron levels would likely have detrimental consequences for macrophage effector functions, including those bacteriostatic. However, this phenomenon is poorly studied, and to date only one study investigating the effects of mitochondrial iron loss through the chelator deferiprone (DFP) on the macrophage metabolome has been reported (129). Independently to iron, pre-existing mitochondrial dysfunction associates with an elevated risk of infection. Individuals with Barth syndrome carrying a loss-of-function mutation in the *TAFazzin* gene show a loss in cardiolipin synthesis, a phospholipid exclusive to the IMM, and frequently suffer from recurrent bacterial infections (130). Primary mitochondrial disease, involving point mutations in mtDNA, confers heightened susceptibility to respiratory tract infection (131). How failures in mitochondrial function can affect the AM nutritional immune response remains an open area of research.

1.3 COPD

Correct mitochondrial and iron metabolism are both essential for AM function. These two axes converge to facilitate metabolic remodelling upon infection, allowing AMs to starve invading pathogens of iron and ensure efficient killing to preserve alveolar integrity and prevent systemic infection. Failures in the ability of the host to sequester iron

from pathogens could cause an accumulation of iron which can induce oxidative damage and provide bacteria with improved access to iron. How endogenous, cell intrinsic failures in iron handling and sequestration associated with disease could create an iron-replete environment to fuel bacterial proliferation is remarkably understudied. As a disease where defective iron handling appears characteristic of both disease development and progression, COPD presents a unique opportunity to study the fight for iron between host and pathogen.

1.3.1 Disease epidemiology and molecular pathology

COPD is a highly debilitating, inflammatory lung disease. Disease pathology is underpinned by chronic bronchitis, emphysema (alveolar tissue destruction) and remodelling of the small airways. COPD affects an estimated 300 million people globally and is the fourth leading cause of death worldwide, disproportionately affecting low and middle-income countries. Disease progression is classified by Global Initiative in COPD (GOLD) guidelines, with four stages (I-IV) defining disease severity (132). COPD is commonly underdiagnosed, with an estimated two thirds of individuals with persistent airflow limitation not diagnosed (133). Cigarette smoking was identified as the primary risk factor for COPD over 50 years ago and is the subject of extensive research, which ultimately has not yielded any therapeutic advances capable of stopping, or reversing, disease progression. Treatments like glucocorticoids and bronchodilators manage symptoms but fail to augment disease outcomes. Any available biologics for COPD treatment target type II inflammation only, meaning they are only effective in a small subset of patients (134). Successful treatment is limited by a dearth of knowledge regarding specific disease endotypes and a reliance on spirometric measurement of lung function decline to diagnose patients, by which a degree of irreversible damage has already occurred. Further complicating the ability to diagnose early-stage COPD is the heterogeneity of disease origins. The view of COPD as a self-inflicted disease solely attributed to cigarette smoking is outdated, and 25-40% of COPD patients have no smoking history. Disease in these patients is attributed to both genetic (*SERPINA1*, *FAM13A*, *IREB2*) and environmental (particulate matter-driven urban pollution, wood smoke, second-hand tobacco smoke exposure) factors, often interacting across all stages of life making it difficult to attribute disease to a single root cause (132, 135). Factors like continuing rapid urbanisation, an aging population and the rising popularity of e-cigarettes and other tobacco replacement products will converge in coming years to further increase disease burden (136). This presents an urgent need to deepen our understanding of the molecular mechanisms driving disease to inform therapeutic design and meet demand.

Lung function decline in COPD patients is non-linear, and is accelerated by exacerbations, acute worsening of disease symptoms that result in hospitalisation (137). An estimated 80% of exacerbations are infectious in origin, caused by viruses or bacteria (predominantly *Streptococcus*, *Moraxella* and *Haemophilus* species) (138, 139). Despite the inflammatory nature of COPD, individual response to infection is inefficient and can lead to pathological tissue damage without effectively controlling the infectious agent. There is currently limited mechanistic insight into why individuals with COPD are more susceptible to frequent respiratory tract infections and why they are at an elevated risk of morbidity and mortality from infection compared to the general population. However, the prevalence of exacerbations does not appear to change with disease etiology (137), making the treatment and prevention of infectious exacerbations a universal target in COPD progression.

An impairment in inflammatory responses is a molecular hallmark of COPD progression. Several pro-inflammatory cytokines (IL-6, IL-8, TNF- α) and chemokines (CCL2/3, CXCL8) are elevated in COPD BALF (140, 141). Defective ciliary beating on epithelial cells impairs mucociliary clearance, and pathogenic cycles of cell death in the alveolar epithelium and subsequent hyper-replication of remaining cells leads to irreversible airway remodelling and air trapping. Impaired tissue repair associates with defective lung fibroblast function, which elevate pro-fibrotic markers and appear senescent. Elastase and matrix metalloproteins secreted by recruited immune cells (142) further compound tissue destruction.

There is an up to 20-fold increase in macrophages in the COPD lung (**Fig. 1.5**) (143). The self-proliferative capacity of lung-resident AMs increases in cigarette smoke (CS) exposure (143), but these macrophages are predominantly monocyte-derived (144). Paradoxically, despite the overall increase in BALF inflammatory mediators, COPD AMs show blunted pro-inflammatory responses. *Ex vivo*, AMs from COPD patients, smokers or animal models show reduced phagocytosis and decreased pro-inflammatory cytokine production (112, 145). Decreased expression of pro-inflammatory cytokines and chemokines in AMs isolated from smokers is worsened in COPD, with the opposite trend observed for genes associated with tissue remodelling (146). AMs both from smokers and smoke-exposed *in vitro* show dysfunctional autophagy and an accumulation of protein aggregates and autophagic vesicles (147). AM efferocytosis is also defective in smoking and COPD, and inefficient clearance of apoptotic epithelial cells (148) and neutrophils (149) can promote secondary necrosis and perpetuate lung damage. These failures in AM homeostatic functions in COPD appear to be accompanied by a direct role for AMs in lung tissue destruction in COPD. Rats treated with anti-monocyte/macrophage antibodies prior

to CS exposure were protected from emphysema, which was not observed upon blocking of neutrophil function (150). Further, AMs localise to emphysematous sites in the lung, and increases in infiltrating monocytes inversely correlate with lung function (151). Monocyte-derived AMs can uniquely orchestrate tissue destruction in experimental COPD (143), but how functional AM defects in COPD may differ with macrophage ontogeny is not known.

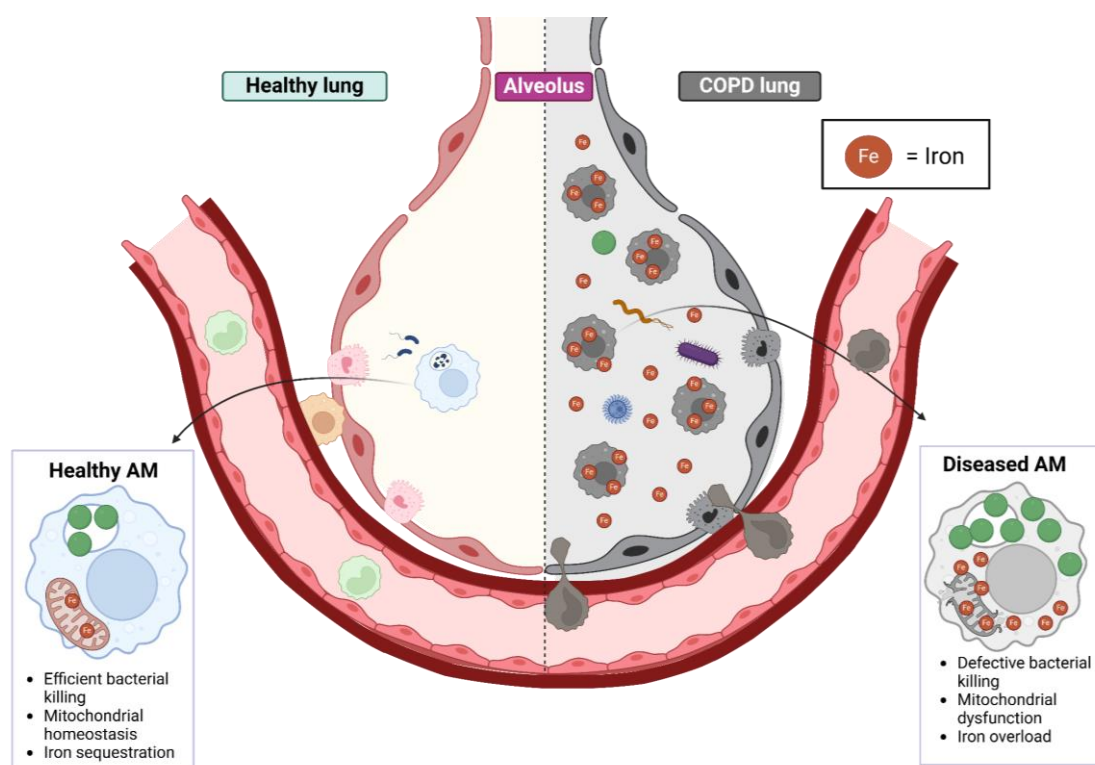


Fig. 1.5: AMs and the COPD microenvironment. In the healthy lung, AMs patrol alveoli to effectively neutralise any threats posed by inhaled pathogens or pollutants. In COPD, increased numbers of AMs are detected in the alveolar space: these AMs appear dysfunctional and are unable to mount a defence against invading bacteria. This defective function associates with an increase in intracellular and extracellular iron, alongside AM mitochondrial dysfunction.

1.3.2 Alveolar macrophage mitochondria and COPD

As discussed, mitochondria are essential for cellular bioenergetics and redox metabolism, and mitochondrial dysfunction holds severe consequences for health. Widespread changes in mitochondrial morphology and biochemistry are present across structural and immune cells in the COPD lung. Soluble mediators arise from failures in the mitochondrial antioxidant response, like mtROS, or mitochondrial damage, like mtDNA or cytochrome c leakage (152). These factors can directly contribute to tissue destruction both directly through oxidative damage and indirectly through secondary cell death and ensuing inflammation. Reduced mitochondrial membrane potential is observed in whole

lung tissue in experimental COPD (50), and COPD patients show elevated mtDNA compared to healthy control in both plasma (153) and, in specific endotypes, urine (154).

AM effector functions like phagocytosis and efferocytosis are indispensable to the maintenance of the alveolar microenvironment and are reliant on proper mitochondrial function. While little is known about quality control mechanisms in COPD AMs, failures in AM bioenergetics have been the subject of intense research. Regarding OXPHOS, basal respiration is unchanged in AMs from non-smokers, healthy smokers and COPD: however, AMs from smokers show increased maximal respiration and spare respiratory capacity, both of which are decreased in COPD AMs and accompanied by increased proton leak (155). AMs from smokers have higher levels of glycolysis compared to reduced baseline glycolysis in COPD AMs, suggesting an inability in COPD for AMs to switch to glycolysis to compensate for loss of ATP production through OXPHOS (155). Despite elevations in basal glycolysis, AMs from smokers stimulated with irradiated *M. tuberculosis* showed reduced levels of the glycolytic end product lactate compared to non-smokers, suggesting this increase in baseline glycolysis is ultimately ineffective in the AM immunometabolic response to infection (84). This depleted oxidative and glycolytic metabolism extends to MDMs generated from COPD patients (156) or treated with cigarette smoke extract (CSE), a liquid preparation of cigarette smoke for use *in vitro* (84). COPD AMs also show differential expression of genes associated with mitochondrial bioenergetics and FAO (155), but the functional effects of these changes have not been characterised. Aside from energetic changes, COPD AMs and MDMs show unregulated mitochondrial ROS production, implicated in defective phagocytosis (145). Importantly, a small molecule activator of NRF2, a transcriptional regulator of the antioxidant response, restored COPD AM phagocytosis and efferocytosis to the level of healthy AMs (157).

1.3.3 AM iron and COPD

A genome-wide association study comparing COPD patients to smoking controls associated single nucleotide polymorphisms in *IREB2*, encoding for the IRP2 protein, with disease (158). The *IREB2* gene is part of a genetic locus on chromosome 15q25 previously implicated COPD risk, which interestingly also encompasses genes encoding for components of the nicotinic acetylcholine receptor. IRP2 mRNA and protein levels are elevated in lung tissue in human and experimental COPD (50). Historically, exposure to exogenous iron associates with COPD development independently to smoking, further highlighting the intrinsic and extrinsic roles iron can play in disease. Ferrous metallurgy workers are at increased susceptibility to disease (159, 160), and mice housed near metal mines develop airway inflammation, emphysema and iron deposition in AMs (161). Lung iron accumulation is a phenomenon noted in COPD patients for decades, with stark iron

deposition observed upon Perls' Prussian Blue staining, which detects Fe^{3+} thought to be complexed in ferritin-lysosomal complexes (hemosiderin) (162) (**Fig. 1.6**). Total iron and iron-containing proteins including ferritin, transferrin, lactoferrin and lipocalin-2 are all elevated in COPD BALF and lung tissue irrespective of smoking status (163).

AMs are the primary iron-positive cell in COPD lung tissue. Time of flight secondary ion mass spectrometry (ToF-SIMS) of lung tissue from GOLD stage IV COPD revealed a stark localisation of iron to lung macrophages relative to other lung cell types and niches (164). Iron deposits in AMs from human (165) and experimental COPD (112) are present upon Perls' Prussian Blue staining (**Fig. 1.6B**). AMs in smoking show increased ferritin, both intracellular and secreted (166). The rate of ferritin secretion from these AMs *ex vivo* is significantly higher than lactate dehydrogenase, a surrogate marker of cell death, suggesting this increase in intracellular iron is not cytotoxic (166). Increased ferritin in AMs from smokers with chronic airflow obstruction skews towards increased FTL relative to FTH1, indicative of higher storage in FTL relative to detoxification by FTH1 (167). ToF-SIMS analysis suggests only a subpopulation of AMs appear iron-loaded in COPD, but importantly the proportion of iron-positive macrophages increases with disease stage (164, 165). A subset of AMs expressing metallothioneins and HO-1 were identified in GOLD III and IV stage COPD (144), but whether these AMs overlap with those chemically confirmed to have increased iron remains to be proven.

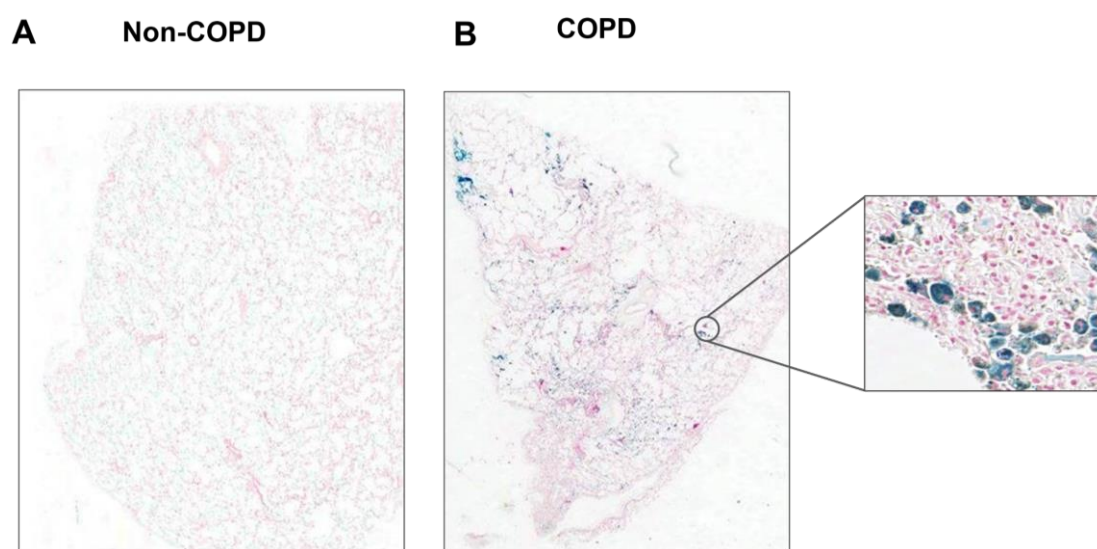


Fig. 1.6: Iron deposition in COPD. Perls' Prussian Blue staining for iron in (A) non-COPD and (B) GOLD stage IV COPD human lung tissue sections. Images provided courtesy of Dr. Henric Olsson.

The source and chemical nature of this iron remains elusive. A single cigarette contains 0.3 μg of iron, but individuals living in urban areas inhale up to 25 μg of iron per day suggesting that in this context, cigarette smoke-inhaled iron is negligible. Cigarette

smoke condensate and unburned tobacco even appear capable of themselves binding iron (168). Removal of iron from CSE had no effect on observed changes in AM iron gene expression, so the contribution of iron directly supplied by cigarettes to this phenotype appears to be minimal (112). Increases in plasma iron concentration associate with increased AM iron levels, suggesting AMs can utilise iron supplied by the pulmonary vasculature in the absence of haemorrhage (169). AM uptake of the heme degradation byproduct haptoglobin appears to be enhanced in COPD, with higher surface expression of the haptoglobin receptor CD163 in COPD AMs (170). Despite reports of increased FPN in AMs from experimental COPD (112), evidence suggests the flux of this iron through cells is impaired. Smokers and individuals with chronic obstructive bronchitis showed reduced clearance of inhaled ferromagnetic microparticles compared to healthy individuals (171). AMs appear unable to digest and liberate iron from intracellular deposits, and smokers and COPD patients show decreased bioavailable iron in exhaled breath condensates compared to non-smoking controls (172), with impaired pharmacological iron chelation in COPD AMs also noted (173). Cumulatively, these observations suggest this iron is 'stuck' in cells, a phenomenon which cannot be addressed by normal iron export or regulated cell death programs. This may arise from iron becoming sequestered in a chemically inaccessible form, or from failures in iron sensing mechanisms. Irrespective of the overall intracellular increase, if this iron is unusable, cells will ultimately lack the necessary bioavailable iron to meet their metabolic needs. COPD AMs show elevated TFRC protein levels (165), perhaps to counter this pseudo-iron deficiency through increasing iron uptake.

Converging evidence suggests that this iron overload in COPD AMs may be mitochondrially localised. Experimental validation of a role for IRP2 in COPD pathogenesis revealed a mitochondrially-targeted mechanism, where IRP2 pathogenically increased mitochondrial iron levels, leading to mitochondrial dysfunction and experimental COPD. Mice exhibited a time-dependent increase in whole lung iron levels with CS exposure, and increased baseline mitochondrial iron through genetic frataxin deficiency had impaired mucociliary clearance independent of CS exposure (50). Importantly, therapeutic administration of DFP, a mitochondrially-active chelator, was protective against CS-induced inflammatory cell infiltration to the airspaces and improved mucociliary clearance (50). IRP2 deficiency is protective against restrictive banding-induced pulmonary inflammation, suggesting a broader pathogenic role of IRP2, and potentially mitochondrial iron dynamics, in respiratory disease beyond COPD (174). These studies were performed in mice with global IRP2 deficiency, so the relevance of mitochondrial iron loading to individual cell types in the lung remains underexplored. However, ToF-SIMS analysis of

COPD AMs noted that iron deposition appeared punctate, contained in subcellular structures suggested to be mitochondria (164). Alterations in mitochondrial FeS and heme output in smoking or COPD have not been studied, but as previous evidence suggests this iron is not bioavailable to cells so co-factor production is likely impaired. The heavy reliance of AMs on both iron and mitochondrial metabolism to fulfil their homeostatic functions and protect the airways from infection positions means any failures in these processes would be particularly detrimental. Considering the increase in lung mitochondrial iron observed in experimental COPD, and the damage to mitochondrial metabolic processes in AMs, the pathological increase in AM iron appears likely to be mitochondrial. Determining the presence of mitochondrial iron increases in COPD AMs and its impact on AM function may therefore present a druggable therapeutic axis with the potential to reverse multiple molecular failures in COPD through targeting a single site.

1.3.4 Iron and infection in COPD

The mechanisms employed by the host to protect iron both intracellularly and extracellularly to evade bacterial iron uptake systems are well defined (94). How bacteria may capitalise on any failures in these host safeguards to hijack iron is surprisingly understudied. Cellular iron storage and metabolism converge at mitochondria to support the AM immune response, and several bacteria target mitochondria. If mitochondria are battlegrounds for iron, any pre-existing mitochondrial pathologies will likely favour the pathogen. In COPD, AMs are pathologically loaded with iron, with suggestions it may be mitochondrially localised (50, 164), and fail to limit pathogen outgrowth with devastating consequences for patient mortality. COPD therefore presents an unrivalled opportunity to study how failures in AM mitochondrial iron homeostasis can contribute to pathogen success, and most importantly if this mechanism can be therapeutically targeted.

1.3.4.1 *Streptococcus pneumoniae*

As mentioned, acute respiratory infections contribute significantly to mortality in COPD and are frequently causative of exacerbation events. *S. pneumoniae* is one of the most commonly cultured pathogen from the COPD lung and associates with up to 15% of all-cause exacerbations (175). Carried as a nasopharyngeal commensal in up to 10% of adults, the precise mechanisms governing its transition from an innocuous bacterium to cause invasive disease in the lower respiratory tract are unclear. Pre-existing inflammation represents the primary risk factor for invasive disease, which may explain its prevalence in the elderly and those suffering from chronic respiratory disease (176). The capacity of *S. pneumoniae* to cause invasive disease is highly strain specific, with over 100 capsular serotypes characterised thus far (177). Viral co-infection can promote aspiration to the

lungs, with virally-induced nasal inflammation leading to an increase in production of pro-inflammatory cytokines, a loss of epithelial integrity and an increased availability of nutrients (178). Pneumolysin, the primary pneumococcal virulence factor, can also promote colonisation of the lower respiratory tract through induction of an IL-10 driven inflammatory response via interaction with mannose receptor C-type 1 (MRC1) on macrophages in the upper respiratory tract (179). Indeed, murine models of infection utilising pneumolysin-deficient strains show decreased nasopharyngeal colonisation, bacteraemia and pulmonary damage, along with increased host survival (180-183).

Despite its documented reliance on iron for pathogenicity (184, 185), *S. pneumoniae* lacks the machinery necessary to synthesise or secrete endogenous siderophores (103). A variety of uptake mechanisms targeting host labile iron or heme are favoured for iron uptake instead. *S. pneumoniae* encodes for three operons expressing the iron-binding ABC transporters PiaA, PiuA and PitA on its cell surface. The natural substrate of each transporter remains to be fully defined and may vary depending on colonisation niche. All three can utilise Fe^{2+} as a substrate, with PiuA and PiaA also capable of binding hemoglobin (185, 186). Recently, proteomic studies of a knockout mutant of these ABC transporters revealed a novel iron transporter, SPD_1590 capable of binding hemin to promote virulence (187, 188). Supplementation of culture media with hemoglobin robustly increases *S. pneumoniae* growth and biofilm formation (189). These studies investigating the effects of iron sources on *S. pneumoniae* growth and virulence dynamics were all performed through *in vitro* supplementation of pneumococcal growth medium: how *S. pneumoniae* may use or exploit host iron in infection models has yet to be studied.

S. pneumoniae is unable to utilise lactoferrin or transferrin as an iron source *in vitro*, although the surface protein PspA can bind lactoferrin to inhibit the formation of the bactericidal apolactoferrin in a serotype-dependent manner (190-192). The stress hormone norepinephrine, elevated in plasma and the lung of infected patients, acts as a pseudo-catecholamine siderophore, liberating ferric iron from serum transferrin to facilitate its uptake by PiuA (193, 194). Crystallisation of PiaA also revealed the iron transporter to bind ferrichrome, a hydroxamate siderophore produced by a number of bacteria for host iron chelation (195). The capacity of *S. pneumoniae* to hijack these siderophores from the microbiota for its own iron acquisition has not yet been proven, although siderophore-deficient *P. aeruginosa* was observed to uptake exogenous hydroxamate siderophores *in vitro* to facilitate its survival in iron deplete conditions (196). Structural and biochemical analysis of PiuA revealed a catechol binding site similar to its *S. aureus* homologue SstD, suggesting *S. pneumoniae* can also utilise tetradentate siderophores from the microbiota

or host-derived catecholamines as iron sources (197). PiuA can also bind the products of enterobactin hydrolysis, although *S. pneumoniae* is unlikely to encounter the *E. coli* siderophore in the lung microenvironment (197). This highlights the ability of *S. pneumoniae* to utilize atypical iron sources derived from both the host and microbiota, a phenomenon which remains poorly explored in host infection models.

Metabolic remodelling of macrophages in response to bacterial infection relies heavily on mitochondria as hubs of multiple metabolic processes, like the production of ATP and reactive oxygen species (198). In line with this, *S. pneumoniae*, like many bacteria, has evolved means in which to target host mitochondria. *In vivo*, *S. pneumoniae* induces a loss in mitochondrial membrane potential, respiratory chain complex expression and antioxidant responses in lung macrophages, alongside release of the DAMP mtDNA (199). *In vitro* imaging assays revealed pneumolysin disrupts ATP production and mitochondrial morphology in human AT2 cells. Pneumolysin-induced release of mtDNA associates with stimulator of interferon genes (STING) signalling in lung macrophages (200-202). Pneumolysin also induces the release of microvesicles from AT2 cells, containing mitochondrial proteins which are internalized by neutrophils (203). Clearly, despite its predominantly extracellular lifestyle, *S. pneumoniae* is capable of targeting mitochondria in lung cells, although any potential interactions with mitochondrial-derived nutrients or metabolites remains to be explored.

1.3.4.2 Mitochondria as nutrient battlegrounds

Mitochondrial iron is an as-of-yet unexplored axis in the nutritional immune response. Mitochondria are the primary consumers of cellular iron and retain up to 50% of cellular iron complexed in mitochondrial ferritin and respiratory chain complexes. (204). The origins of mitochondria in eukaryotic cells are widely acknowledged to trace back to an internalised prokaryote, which ultimately developed a symbiotic relationship with its eukaryotic host. As a consequence, bacterial and eukaryotic metabolic pathways, and the metabolites consumed, are structurally and chemically near-identical (205). This shared evolutionary history seems to have equipped bacteria with the necessary knowledge to target host mitochondrial metabolism for pathogenic benefit. For example, the tuberculosis necrotising toxin depletes mitochondrial NAD⁺ to induce a non-canonical form of cellular necroptosis, to propel mycobacterial dissemination (206). mtROS trigger a phenotypic switch in internalised *Leishmania* sp. from a dormant to virulent form (207).

Further to this targeting, metabolites directly associated with mitochondria or generated as byproducts of mitochondrially-mediated pathways can be directly consumed by bacteria (**Fig. 1.7**). This concept of nutrient siphoning from mitochondria has yet to be

characterised in terms of iron, but several examples of bacteria hijacking host mitochondria for other essential nutrients are documented in the literature. This concept is elegantly detailed by the work of *Pernas et al.* examining the piracy of mitochondrial-derived fatty acids by the intracellular pathogen *Toxoplasma gondii*. Radiolabelling of host fatty acids and knockout of the ATG genes illustrated how the pathogen can co-opt host autophagy to liberate and uptake fatty acids from lipid droplets. In response, the host mounts a 'metabolic defence' against the bacterium through elongation of the mitochondria, suggesting a model where mitochondrial fusion is a host defence to increase fatty acid oxidation (208). More recently, *Rosenberg et. al.* illustrated how *Salmonella enterica* serovar Typhimurium (S. Tm) capitalises on the accumulation of succinate resulting from the glycolytic shift in activated macrophages to promote its own virulence. Radiolabelling of succinate *in vitro* revealed direct uptake of succinate by S. Tm in BMDMs, with genetic loss of the succinate transporter *dcuB* attenuating virulence. RNA-seq illustrated a transcriptomic shift towards bacterial genes promoting virulence and intracellular survival upon succinate uptake, illustrating how S. Tm can sense reprogramming of the macrophage metabolic environment and hijack its by-products to promote its own virulence (209). A similar effect is observed in the metabolic adaptation of *P. aeruginosa* to exogenous itaconate: while itaconate is initially utilised by the host as an antimicrobial agent, its accumulation imposes a selective pressure on *P. aeruginosa* leading to the accumulation of mutations promoting itaconate catabolism and biofilm formation (210, 211). *M. tuberculosis* possesses similar machinery for itaconate catabolism, which is essential for virulence (212). Clearly, a dynamic host-pathogen interplay surrounding nutrients exists at mitochondria, although this phenomenon is poorly studied *in vivo*.

While mitochondrial nutrients have a known role in bacterial pathogenicity, mitochondrial iron has been poorly studied in terms of this phenomenon. In the absence of iron, some bacteria appear capable of repurposing their iron acquisition mechanisms to target mitochondria: the *P. aeruginosa* siderophore pyoverdine induces mitophagy in *C. elegans*, which is not observed when pyoverdine is pre-incubated with iron (213). The targeting of ferritinophagy for iron uptake in human monocytes was recently described in *E. chaffeensis* infection, highlighting the capacity of bacteria to directly extract iron complexes in host structures. The bacterial virulence factor Etf-3 binds to FTL and recruits NCOA4 to initiate ferritinophagy, with the concomitant increase in the cellular labile iron pool utilized by *E. chaffeensis* upon infection (103). Importantly, *E. chaffeensis*, like *S. pneumoniae*, does not secrete siderophores for iron acquisition; it would be intriguing to speculate if this ability to liberate host iron from atypical sources to fuel bacterial virulence is something shared by *S. pneumoniae*. With both mitochondrial iron homeostasis and

mitochondrial integrity compromised in COPD, and with the significant burden of infection, it seems plausible that an invading pathogen could capitalise on this disarray to use iron for its virulence. If mitochondrial iron is indeed elevated in COPD macrophages, their positioning as host defenders may switch to prime bacterial targets.

Extracellular space

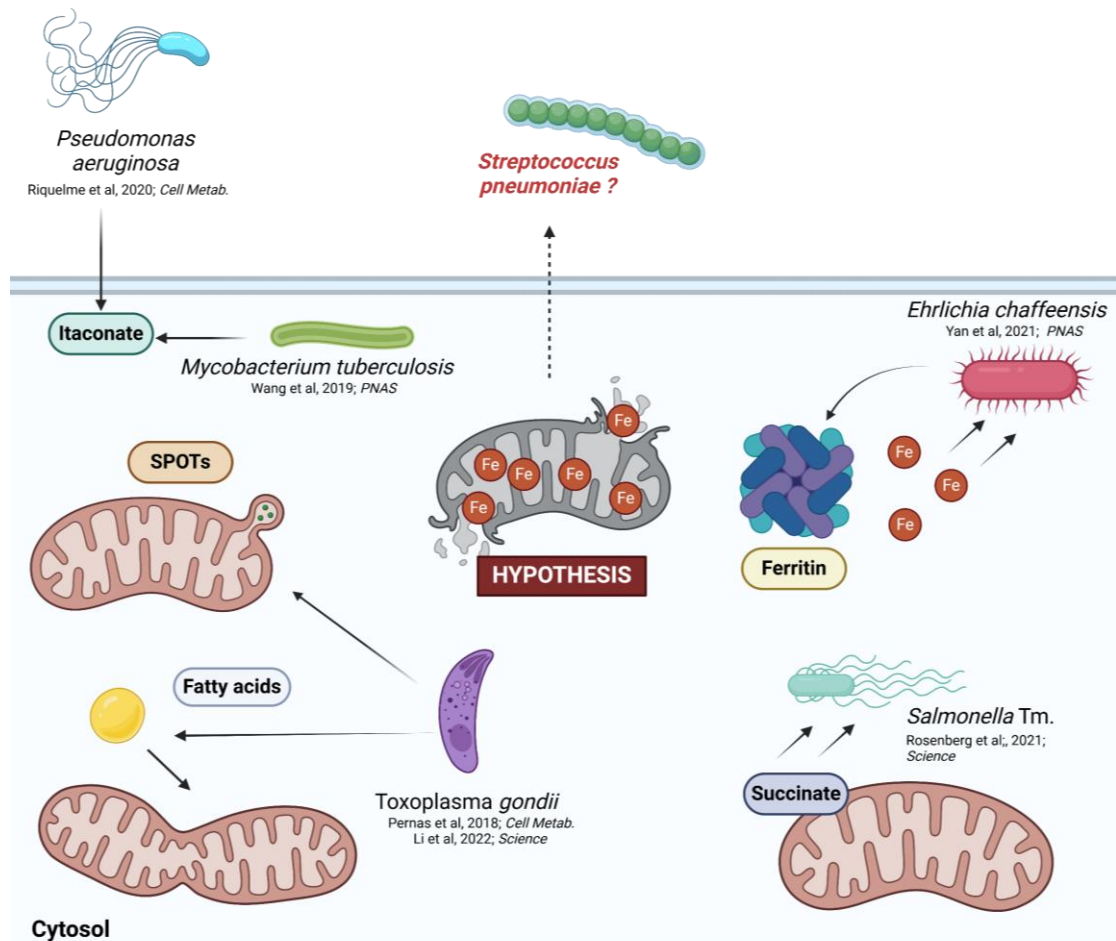


Fig. 1.7: Evidence in the literature for metabolite piracy.

1.4 Aims

Based on the above literature, we hypothesised that **alveolar macrophage mitochondrial iron dictates macrophage function in response to infection and is a target for bacterial iron piracy**. This thesis will address the presence of pathological macrophage mitochondrial iron loading in COPD, and how this confers susceptibility to microbial pathogenesis in these individuals through provision of excess iron to bacteria. With a focus on *S. pneumoniae* as an exemplar bacterium, a combination of approaches will be used, including *in silico* data mining, *in vitro* cell lines, *ex vivo* human primary AMs and transgenic and experimental murine models. This question will be addressed through the following specific aims, each of which will be detailed in a dedicated results chapter (Fig 1.8):

- Assessing the transcriptomic evidence for mitochondrial iron changes in COPD macrophages (**Chapter 3**).
- Characterising the effects of cigarette smoking on AM mitochondrial iron and *S. pneumoniae* infection *in vitro* and *ex vivo* (**Chapter 4**).
- Defining the effects of cellular and mitochondrial iron manipulation on *S. pneumoniae* virulence *in vitro* and *ex vivo* (**Chapter 5**).
- Investigating the contribution of AM mitochondrial iron in the host response to acute respiratory infection and experimental COPD through generation of a novel transgenic mouse strain with AM-targeted mitochondrial iron loss (**Chapter 6**).

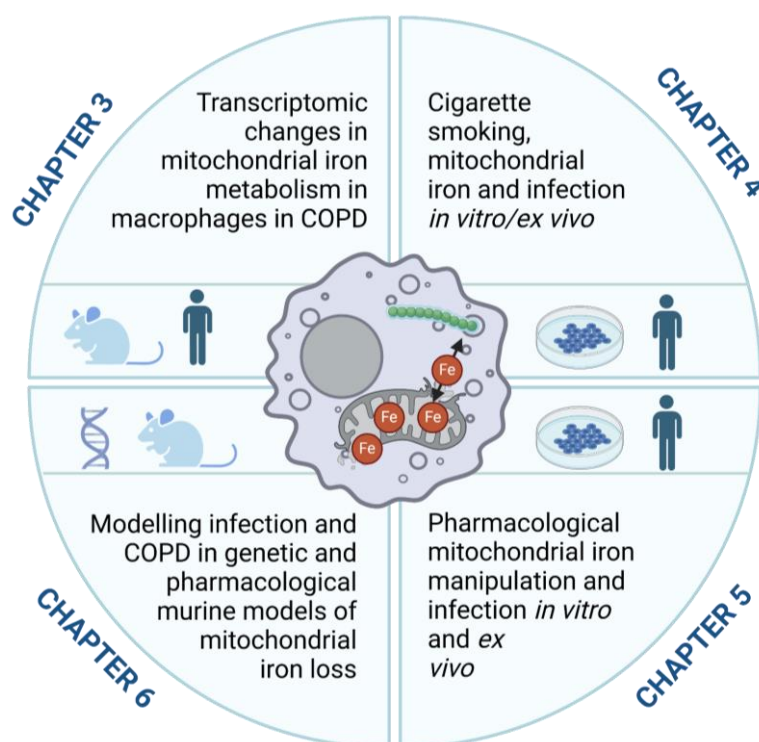


Fig 1.8: Aims of the thesis.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Buffers

Buffer name	Buffer composition
PBS (10X, diluted to 1X before use)	1.45 M NaCl, 39 mM NaH ₂ PO ₄
Sample loading buffer	0.125 M Tris pH 6.8, 10 % glycerol, 0.02 % SDS, 0.05 M DTT
RIPA buffer	10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, 140 mM NaCl
SDS-PAGE running buffer (10X, diluted to 1X before use)	25 mM Tris, 192 mM glycine 0.1 % SDS
SDS-PAGE transfer buffer (10X, diluted to 1X before use)	0.25 M Tris, 1.9 M glycine, 35 mM SDS
Tris buffered saline tween (TBST) (10X, diluted to 1X before use)	20 mM Tris, 150 mM NaCl, 0.1% Tween-20
Mitochondrial fractionation buffer	1 mM mannitol, 350 mM sucrose, 50 mM HEPES, 5 mM EGTA

Table 2.1: Composition of buffers used in this thesis.

2.1.2 Cell culture reagents

DMEM and RPMI were purchased from Gibco Biosciences. Fetal bovine serum (FBS), penicillin-streptomycin (P/S), and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Sigma Aldrich. Recombinant GM-CSF and TGF- β were purchased from Miltenyi Biotech. Insulin-transferrin-sodium selenite, transferrin, hydrocortisone, β -estradiol and HEPES were bought from Sigma Aldrich.

2.1.3 Cell lines

Fetal liver-derived alveolar-like macrophages (FLAMs) and HEK293T-GMCSF cells were a gift from Prof. Susan Carpenter, University of California, Santa Cruz, generated as described (214, 215). L929 cells were a gift from Prof. Luke O'Neill, Trinity College Dublin. Mouse lung epithelial-12 (MLE12) cells were purchased from ATCC (#CRL-2110). iBMDMs were a gift from Prof. Kate Fitzgerald, University of Massachusetts.

2.1.4 Bacterial culture

Todd Hewitt broth was purchased from BD Biosciences. Defibrinated sheep's blood and agar was acquired from Thermo Fisher. Yeast extract was purchased from VWR. Saponin was purchased from Sigma Aldrich.

2.1.5 Bacteria

Serotype 2 *Streptococcus pneumoniae*, strain D39 (216), was a gift from Prof. Ed Lavelle, Trinity College Dublin. GFP and mKate2-expressing strains were a gift from Prof. Jan-Willem Veening, University of Lausanne, Switzerland. A clinical isolate of *Streptococcus pneumoniae* was isolated from a bronchoalveolar lavage fluid sample obtained from a research bronchoscopy at Tallaght University Hospital, Dublin.

2.1.6 Mice

All mice used were maintained on a C57BL6/J background. Cd11c-Cre (*Itgax*-Cre) mice were purchased from Jackson Laboratories (#008068). *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl} mice were a gift from Prof. Diane Ward at the University of Utah.

2.1.7 Cell treatments

Ferric ammonium citrate (FAC), ferrous lactate (FeL), deferoxamine mesylate salt (DFO), 4,6-dioxoheptanoic acid (commercially sold as succinylacetone, SA) and N-methyl-mesoporphyrin IX (NMMIX) were purchased from Sigma Aldrich. DFP (DFP) was purchased from MedChem Express. DFO-naph was a gift from the Elmes lab at Maynooth University.

For cigarette smoke extract (CSE) generation, a Masterflex L/S Easy-Load 3 peristaltic pump was used to bubble smoke from 1 16RF research cigarette (University of Kentucky) with filters removed through 10 mL of cell culture media (RPMI or DMEM depending on cell type) without any additives at a rate of 150 rpm/min until ~ 10-20 mm remained. Following filter sterilization, CSE extract was stored at -80°C. CSE generated in this manner was deemed 100% strength and was used immediately after thawing for treatment.

2.1.8 RT-qPCR

Invitrogen PureLink RNA extraction kit was purchased from Thermo Fisher. High-capacity cDNA Reverse Transcription Kit, 8-strip PCR tubes and caps, 96 well qPCR plates and seals and Sybr Green PCR Master Mix were all purchased from Applied Biosciences.

2.1.7.8 Primers

Primers (**Table 2.2**) were designed in house and purchased from Eurofins at a stock concentration of 100 μ M and diluted to 10 μ M for use. All primers listed target murine gene expression.

Target	Forward primer sequence	Reverse primer sequence
<i>ActB</i>	TGT TAC CAA CTG GGA CGA CA	GGG GTG TTG AAG GTC TCA AA
<i>Flvcr1a</i>	CCG TCG CCT CGG TAT GG	CAC TAA AAC AGG TGG CAA CAA AAA
<i>Flvcr1b</i>	TCGCTTCCTATTGACAGCTATTA ACA	CAC TAA AAC AGG TGG CAA CAA AAA
<i>Fpn</i>	CCA TAG TCT CTG TCA GCC TG C T	CTT GCA GCA ACT GTG TCA CC G T
<i>Fth1</i>	GCC GAG AAA CTG ATG AAG CTG C	GCA CAC TCC ATT GCA TTC AGC C
<i>Ftl</i>	CGT CTC CTC GAG TTTCAG AAC	CTC CTG GGT TTT ACC CCA TTC
<i>Hmox1</i>	CCT CAC AGA TGG CGT CAC TT	GCT GAT CTG GGG TTT CCC TC
<i>Lcn2</i>	ATG TCA CCT CCA TCC TGG TCA G	GCC ACT TGC ACA TTG TAG CTC TG
<i>Mcu</i>	GAG CCG CAT ATT GCA GTA CGG T	AAA CAC GCC GAC TGA GTC AGA G
<i>Mtft</i>	CAA GCA CAT CAG CTC TGC AC	TCC AGG TTG ATT TGG CGG TT
<i>Ncoa4</i>	AAG AAA GTG GGA AGC CTC AG	
<i>Rps18</i>	CCC TCT ATG GGC TCG AAT TT	GGA TGT GAA GGA TGG GAA G T
<i>Slc25a28</i>	TCG TCA AGC AGA GGA TGC AGA T	GTT AAA GTG CTC TTG CAG GAA C
<i>Slc25a37</i>	TTG AAT CCA GAT CCC AAA GC	GTT TCC TTG GTG GCT GAA AA
<i>Trfc</i>	GTT TCT GCC AGC CCC TTA TTA T	GCA AGG AAA GGA TAT GCA GCA

Table 2.2: Primers used for RT-qPCR in this thesis.

2.1.9 Western blotting

Pierce BCA Protein Assay Kit was purchased from Thermo Fisher. Sodium dodecyl sulfate (SDS), ammonium persulfate (APS), Tris, HCl and sodium azide were purchased from Sigma Aldrich. N, N, N', N' Tetramethylethylenediamine (TEMED) was acquired from Millipore. 30% acrylamide (Protogel) was acquired from National Diagnostics. Spectra Multicolour Broad Range Protein Ladder was purchased from Thermo Fisher. Polyvinylidene difluoride (PVDF) membrane was acquired from Millipore. Marvel milk powder was purchased from Tesco. Bovine serum albumin (BSA) was purchased from

Sigma Aldrich. Immobilon Western Chemiluminescent HRP Substrate was bought from Thermo Fisher.

2.1.9.1 Antibodies

Primary and secondary antibodies used in western blotting are detailed below (**Table 2.3**). All antibodies were diluted in TBST with 5% w/v milk: for primary antibodies, 0.09% sodium azide was added to facilitate reuse.

Target	Category code	Manufacturer	Dilution used
Anti-rabbit	111-035-003	Jackson	1:10,000
Anti-mouse	115-035-003	Jackson	1:10,000
ACO2	ab129069	Abcam	1:1,000
β -actin	4970	Cell Signalling	1:1,000
FTH1	3998	Cell Signalling	1:1,000
NCOA4	A302-272A	ThermoFisher	1:1,000
TFRC	13113	Cell Signalling	1:1,000
TIM23	34822	Cell Signalling	1:1,000

Table 2.3: Primary and secondary antibodies used for western blotting in this thesis.

2.1.10 Flow cytometry

Stain buffer and perm/wash buffer were purchased from BD. Fluorophore-conjugated antibodies used in this study are detailed in **Table 2.4**.

Target	Fluorophore	Clone	Manufacturer
CCR2	APC	SA203G11	Biolegend
CD11b	PerCP	M1/70	Biolegend
CD127	BV711	PC61	BD
CD135	BV421	A2F10.1	Biolegend
CD150	eFluor450	mShad150	eBiosciences
CD16	AF700	3D8	Biolegend
CD3	APC-Cy7	17A2	Biolegend
CD34	FITC	RAM34	Biolegend
CD48	PerCPef710	HM48-1	Invitrogen
cKit	APC	2B8	Biolegend
H3K27Ac	AF488	-	Abcam
Ly6C	PE-CF594	HK1.4	Biolegend
Ly6G	BV785	1A8	Biolegend
NK1.1	BV570	PK136	Biolegend
Sca-1	PE-Cy7	D7	Biolegend

Table 2.4: Fluorophore-conjugated antibodies used for immunoblotting in this thesis.

2.1.11 Human bronchoalveolar lavage processing

Amphotericin B (commercially Fungizone) was acquired from Gibco. Sterile gauze was acquired from Hickey's Pharmacy. ACK lysis buffer was acquired from ThermoFisher.

2.1.12 Heme assay reagents

Oxalic acid was purchased from Sigma Aldrich.

2.1.13 Graphite furnace atomic absorption spectrometry

Nitric acid was acquired from VWR. Ultrapure water was acquired from ThermoFisher. Metal-free Eppendorf tubes were acquired from Nest Scientific USA. 0.3% $\text{Mg}(\text{NO}_3)_2$ modifier solution and 1 mg/mL Pure (Fe) Iron Standard were purchased from Perkin Elmer.

2.1.14 Alamar blue assay

Resazurin sodium salt was acquired from Sigma Aldrich. 40 mg of resazurin was dissolved in 100 mL of PBS (0.04% w/v) to make a 10X stock solution. The 10X stock was filter sterilised and stored at 4°C for short term storage, or -20°C for long term storage.

2.1.15 ELISA

DuoSet ELISA kits measuring murine IL-6, TNF-alpha and lipocalin-2 were bought from BioTechne.

2.1.16 Confocal microscopy

Hoechst and FeRhoNox (sold commercially as FerroOrange) were purchased from Merck. MtFluNox (sold commercially as Mito-FerroGreen) was acquired from Dojindo. RPMI minus phenol red was purchased from Gibco.

2.1.17 Seahorse reagents

XF RPMI base medium without phenol red, glucose, sodium pyruvate, glutamine, XF calibrant solution and MitoStress Test kit (oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), rotenone/antimycin A) were all purchased from Agilent.

2.2: Methods

2.2.1 Cell culture

The specific culture conditions of each cell type utilised in this project are detailed below. For the purposes of cells cultured for *in vitro* infection experiments with *S. pneumoniae*, penicillin/streptomycin (P/S) was absent from culture media to prevent a confounding effect on infection. Otherwise, media was supplemented with 1% P/S.

2.2.1.1 FLAMs

FLAMs were cultured in 10 cm² dishes in RPMI with Glutamax supplemented with 10% FBS, 30 ng/mL GM-CSF (recombinant protein or 16.6% HEK293T-GMCSF media) and 20 ng/mL TGF- β . Cells were detached through addition of PBS with 1.5 mM EDTA and incubation at 37°C and 5% CO₂ for 10 min. PBS was flushed over the dish surface to enhance cell yield and lifted cells centrifuged at 400 x g for 5 min. The cell pellet was resuspended in 5 mL media, with 2 mL used to continue passage of the cells and the remainder counted using a hemocytometer and plated for experiments.

2.2.1.2 HEK293T-GMCSF

HEK293T-GMCSF cells were cultured in T175 flasks in DMEM with Glutamax supplemented with 10% FBS. To prepare supernatant, cells were detached using incubation with trypsin-EDTA at 37°C and 5% CO₂ for 3 min. 2 volumes of media were added and cells centrifuged at 200 x g for 3 min. The cell pellet was resuspended in 5 mL media and cells counted using a hemocytometer. 9×10^6 cells were added to a T175 flask with a total volume of 50 mL of media. Conditioned media containing GM-CSF was collected after 3 days of culture, filtered through a .22 μ M filter and frozen at -80°C.

2.2.1.3 BMDM

Bone marrow cells were isolated from the exposed murine femur and tibia through a 25 G needle with a 10 mL syringe filled with complete DMEM (10% FBS, 1% P/S) attached. Cell aggregates were removed using a 70 μ M filter and bone marrow cells centrifuged at 400 x g for 5 min. Cells were incubated in 1 mL ACK lysis buffer on ice for 5 min to deplete red blood cells and washed once in PBS. The cell pellet was resuspended in 5 mL media and cells counted using a hemocytometer. 9×10^6 cells were added to a T175 flask with a total volume of 30 mL complete DMEM supplemented with 20% L929 M-CSF conditioned media. Progenitors were differentiated to bone-marrow derived macrophages (BMDMs) following 8 days in culture, with media changed after 4 days.

BMDMs were lifted through addition of ice-cold PBS with 0.5 mM EDTA and incubation at 4°C for 10 min. Cells were centrifuged at 1500 rpm, resuspended in 5 mL media and counted with a hemocytometer for plating in DMEM supplemented with 10% L929 media.

2.2.1.4 L929

L929 cells were cultured in T175 flasks in DMEM with Glutamax supplemented with 10% FBS. To prepare supernatant, cells were detached using incubation with trypsin-EDTA at 37°C and 5% CO₂ for 10 min. 2 volumes of media were added and cells centrifuged at 1500 pm for 5 min. The cell pellet was resuspended in 5 mL media, with 1 mL added to a T175 flask containing a total volume of 50 mL media. Conditioned media containing M-CSF was collected after 8 days of culture, filtered through a .22 µm filter and frozen at -80°C.

2.2.1.5 MLE12

MLE12 cells were cultured in T175 flasks in RPMI with Glutamax supplemented with 2% FBS, 0.01 mg/mL transferrin, 5 mg/mL insulin, 30 nM sodium selenite, 10 nM HEPES, 10 nM hydrocortisone and 10 nM β-estradiol. Cells were detached via incubation with trypsin-EDTA at 37°C and 5% CO₂ for 10 min. 2 volumes of media were added and cells centrifuged at 1500 pm for 5 min. The cell pellet was resuspended in 5 mL media and counted with a hemocytometer for plating.

2.2.1.6 iBMDM

Immortalized BMDMs (iBMDMs) were cultured in T175 flasks in DMEM with Glutamax supplemented with 10% FBS. Cells were detached through scraping into culture medium and centrifuged at 400 x g for 5 min. The cell pellet was resuspended in 5 mL media and counted with a hemocytometer for plating.

2.2.2 Bacterial culture

S. pneumoniae was cultured through inoculation of 8 mL Todd Hewitt broth supplemented with 2% yeast extract with one loopful of a stock culture (OD_{600nm} of 0.7, frozen in 20% glycerol at -80°C). The culture was incubated at 37°C in 5% CO₂ until desired growth was achieved.

2.2.3 *In vitro* infection

Mid-log phase (OD_{600nm} 0.3-0.7) cultures were centrifuged at 3000 x g for 10 min and the pellet washed once in PBS. The pellet was resuspended in 2 mL PBS and 600 μ L removed to determine OD_{600nm} . Bacteria were diluted down in antibiotic free media as appropriate to achieve the desired multiplicity of infection and added to each well of the infected groups (100 μ L in a 96 well plate, 500 μ L in a 12 well plate and 1 mL in a 6 well plate). Fresh media was added to mock infected wells containing the same volume of vehicle PBS and plates were centrifuged at 300 x g for 5 min. Cells were incubated at 37°C and 5% CO₂, with varying incubation times for specific experiments detailed in individual figure legends.

2.2.3.1 Invasion assay

After incubation of cells with bacteria (30 min for FLAMs, 4 hr for human AMs), media was removed and 100 μ L of 200 ng/mL gentamicin in cell culture media added to each well. Cells were incubated at 37°C and 5% CO₂ for 15 min and washed once in PBS. 100 μ L of 1% saponin in PBS was added to each well for 15 min at room temperature to permeabilise cells. To confirm lysis, cells were mechanically disrupted through pipetting up and down 10 times. Homogenates were serially diluted tenfold in PBS and streaked on Todd Hewitt Agar plates containing 5% v/v defibrinated sheep's blood. Plates were incubated at 37°C and 5% CO₂ overnight and CFU enumerated and extrapolated to undiluted values.

2.2.4 Mice

Mice were housed under specific pathogen-free conditions in individually ventilated cages with *ad libitum* access to food and water in the Comparative Medicine Unit, Trinity College Dublin, apart from those used in **Fig. 3.8** and **Fig 3.13-17**, which were housed in the same conditions at Weill Cornell Medicine, New York City. All mice were euthanised with increasing concentrations of CO₂ followed by cervical dislocation to confirm death.

2.2.4.1 Creation of a strain of transgenic mice with targeted deletion of MFRN1/2

The Cre-lox system was used to generate a murine model of targeted MFRN1/2 deletion in Cd11c-expressing cells. Cre recombinase is a bacterial enzyme which catalyses site-specific recombination at a recognised 34 base pair sequence termed loxP (217, 218). LoxP sequences direct the Cre protein to a specific genomic region, where its recombinase activity excises genomic DNA between two loxP sites (219). *Slc25a37^{fl/fl}*,

Slc25a28^{fl/fl} mice were bred with *Itgax*^{Cre+/-} mice, after which *Slc25a37*^{fl/WT}, *Slc25a28*^{fl/WT}/*Itgax*^{Cre+/-} mice were backcrossed with the *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl} parental line to yield *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl}/*Itgax*^{Cre+/-} progeny (Fig. 2.1). *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl}/*Itgax*^{Cre+/-} mice were bred with *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl} mice for colony maintenance. All genotyping was performed on tissue derived via ear punch using Transnetyx genotyping services.

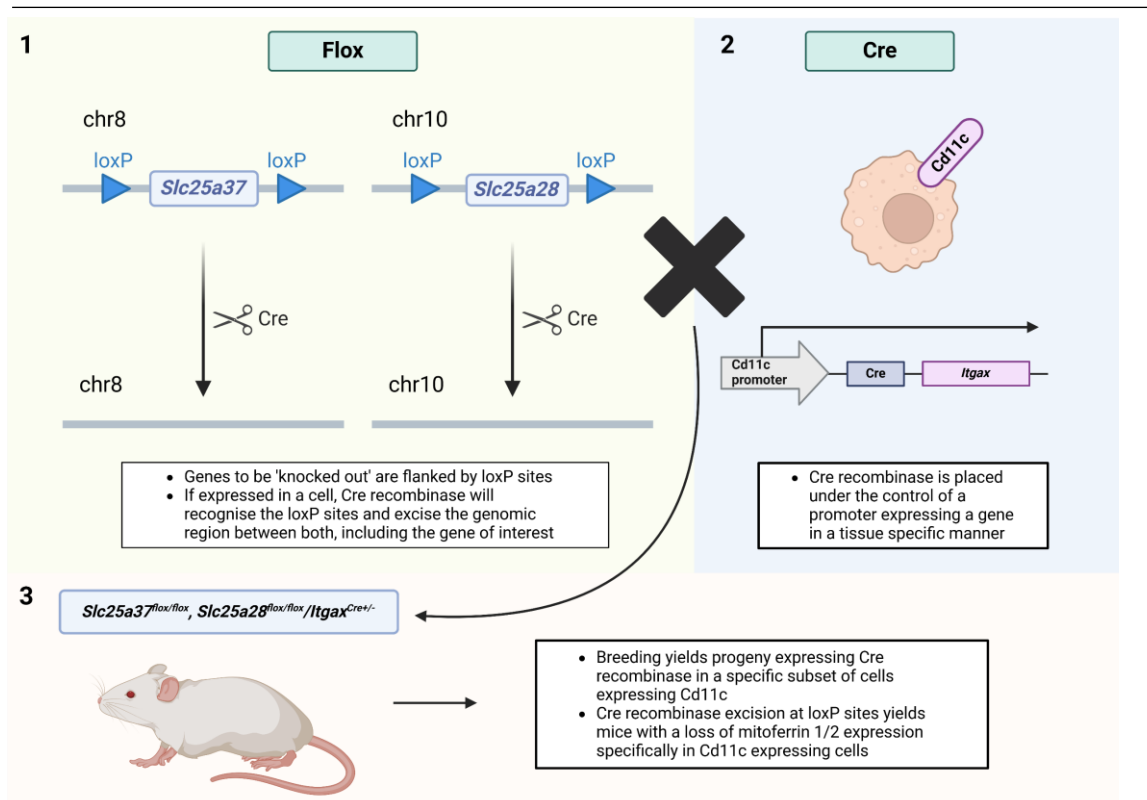


Fig. 2.1: Generation of *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl}/*Itgax*^{Cre+/-} mice using the Cre-lox system.

2.2.4.2 In vivo *S. pneumoniae* instillation

Mice were anaesthetised through subcutaneous injection of a weight-matched solution of ketamine/xylazine (80 mg/kg ketamine, 5 mg/kg xylazine), using a 25 G needle. Using a P200 pipette, 50 μ L of 5×10^6 CFU *S. pneumoniae* in PBS was continuously applied to the nasal area of the mouse, after which mice were left to recover in a 37°C chamber for 15 min and removed to their cage. Mice were monitored twice daily over the course of the experiment using a severity scoring system in line with ethical requirements.

2.2.4.3 Cigarette smoke exposure

Sex and age-matched mice aged between 6-12 weeks of age were exposed to whole-body CS using two methods:

For data presented in **Fig. 3.8** and **Fig. 3.13-17**, C57BL/6J wild type mice (Stock No. 000664) were purchased from The Jackson Laboratory. These mice were exposed to CS using the Teague Enterprises TE-10 inhalation exposure apparatus and 3R4F cigarettes acquired from the University of Kentucky Centre for Reference Products. Mice were exposed to CS (average total particulate matter of 150 mg/m³) for a minimum of 2 hr per day for 5 days a week, for an 8-week period.

For data presented in **Fig. 3.18-19** and **Chapter 6**, control and MFRN1/2^{ΔCd11c} mice were bred in house in the Comparative Medicines Unit at Trinity College Dublin. These mice were exposed to CS using the SciReq inExpose system and 1R6F cigarettes acquired from the University of Kentucky Centre for Reference Products. Mice were exposed to CS at an average of 150 mg/m³ particulate matter for 1 hr per day for 5 days a week, for a 6-week period. Mice were monitored both acutely during smoke exposure and longitudinally during the experimental regimen using a severity scoring system in line with ethical requirements.

2.2.4.4 Deferiprone administration

1 mg/mL deferiprone (DFP) was prepared using filtered H₂O and provided to mice *ad libitum* for 3 weeks. Mice were monitored weekly during the experimental regimen using a severity scoring system in line with ethical requirements, and water intake per cage calculated to ensure comparable drinking levels between experimental groups.

2.2.4.5 Bronchoalveolar lavage

Following confirmation of death, the mouse trachea was exposed, and mice were intubated with a 20 G 1 inch catheter tied in place with silk sutures. 0.7 mL of ice-cold PBS was inserted slowly into the lung using a 1 mL syringe, withdrawn and removed to a 1.5 mL Eppendorf tube. Depending on the downstream analyses, a further 2 (cell counts and cytopins) or 9 (RNA isolation) 0.7 mL increments of ice-cold PBS with 0.5 mM EDTA were instilled and retrieved from the lungs and removed to a 15 mL falcon tube. Following centrifugation at 500 x g for 5 min at 4°C, the cell-free first increment of bronchoalveolar lavage fluid removed to a fresh Eppendorf tube and frozen at -80°C with protease and phosphatase inhibitor. Cell pellets were combined in 500 µL ACK lysing buffer and incubated on ice for 5 min to deplete red blood cells. Cells were centrifuged at 6000 x g for 5 min and washed once in PBS. For BAL cell RNA isolation, the pellet was lysed immediately in RNA lysis buffer from the Qiagen RNEasy Micro Plus Kit and extracted as per manufacturer's instructions.

2.2.4.6 Cytospin preparation and differential calculations

20 µL of resuspended BAL cells was used for cell counting with a hemocytometer, representative of cells isolated within the first three 0.7 mL increments of BAL retrieved

from mice combined. Total cell number was calculated for each mouse relative to the volume of BALF retrieved. Cytospin preparations were generated by depositing suspended cells to positively charged slides using a Shandon Cytospin Preparation. Cytospins were immediately fixed using methanol (5 x 1 sec dips) and stained with eosin (8 x 1 sec dips) and methylene blue (2 x 1 sec dips). Slides were washed in H₂O to remove excess stain and dried at room temperature. For imaging, slides were mounted with a coverslip fixed with DPX mounting medium and images captured using a Leica DM 3000 LED microscope. Differential cell populations were enumerated using the Cell Counter plugin on Image J across at least 5 fields of view, with percentages of each population present calculated used to estimate the number of each population in total BAL cells.

2.2.4.7 Tissue CFU enumeration

Organs were extracted from mice, washed in 100% ethanol to deplete contaminating microbes, washed in PBS and weighed as appropriate. Organs were placed in a screwcap tube containing 1 mL PBS and 7 x 1 mm zirconium beads. Tissue was disrupted using a Bead Bug bead homogeniser at maximum speed for 2 x 1 min intervals, rested on ice in between. Homogenates were serially diluted tenfold in PBS and streaked on Todd Hewitt Agar plates containing 5% v/v defibrinated sheep's blood. Plates were incubated at 37°C and 5% CO₂ overnight and CFU enumerated and extrapolated to undiluted values.

2.2.5 RNA sequencing and bioinformatic analyses

For **Fig 3.2** AMs were adherence purified from the BALF of non-smokers, healthy smokers and COPD patients and RNA-seq performed by HG-U133 Plus 2.0 microarray as previously described (81).

For **Fig 3.8** and **Fig. 3.14-18**, RNA sequencing (RNA-Seq) was performed in AMs and BMDMs from room air and cigarette smoke-exposed mice isolated as described. Total RNA was extracted using the Qiagen RNEasy Micro Kit 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 an Illumina High-Seq 4000 and reads aligned to the mouse genome (GRCm38/mm10) using STAR. Differentially expressed protein coding genes (DEGs) were determined through DeSeq2 analysis (<https://github.com/lovelab/DESeq2>). Volcano plots of gene expression were generated through ggVolcanoR (<https://ggvolcanor.erc.monash.edu/>). Using an absolute Log₂FC cutoff of 0.58 (equivalent to 1.5 fold increase or decrease), genes with a Benjamini-Hochberg adjusted *P* value <0.05 were determined to be significantly differentially

expressed. For gene ontology analysis, up and downregulated differentially expressed genes were separately inputted into ShinyGO (<http://bioinformatics.sdstate.edu/go/v.0.77>). All gene ontology analyses used FDR <0.05 and considered the top 10 enriched pathways ranked by significance. Ingenuity Pathway Analysis (IPA) was used to identify canonical pathways in all differentially expressed genes, with significance cut-offs of absolute z score >2 and FDR <0.0001 used.

2.2.6 Flow cytometry

2.2.6.1 Bone marrow cell preparation

Bone marrow was isolated from one femur and one tibia from an individual mouse as in 2.2.1.3. Bone marrow cells were resuspended in 120 μ L PBS and incubated with 20 μ L anti-CD117 (cKit) microbeads for 25 min at 4°C shielded from light. The cell suspension was loaded into a 1 mL syringe and passed through a MACS column via a 27 G needle. Column-bound cells were washed with 1 mL PBS, the column detached from the MACS magnet and cKit⁺ cells purged from the column through flushing with 5 mL PBS. The cell suspension was centrifuged at 400 x g for 5 min to isolate cells for subsequent staining.

2.2.6.2 Blood preparation

Blood was isolated from mice via cardiac puncture and added to microtubes containing 100 μ L 0.5 M EDTA. 300 μ L blood was incubated in 1 mL ACK lysis buffer for 10 min, followed by addition of 10 mL PBS and centrifugation at 400 x g for 10 min. ACK lysis was repeated and cells collected for subsequent staining.

2.2.6.3 Cell staining and sample acquisition

For extracellular staining, cells were incubated in 20 μ L live/dead stain in blocking solution for 10 min on ice, after which 20 μ L of antibody mixture against desired surface markers was added for a further 20 min. Cells were washed and permeabilised with permeabilization buffer for 30 min, followed by 20 min incubation with intracellular antibody mixture. Data was acquired on a Cytex Aurora flow cytometer and analysed using FlowJo software, with gating strategies for bone marrow cells (**Fig. 2.2**) and blood (**Fig. 2.3**) depicted below.

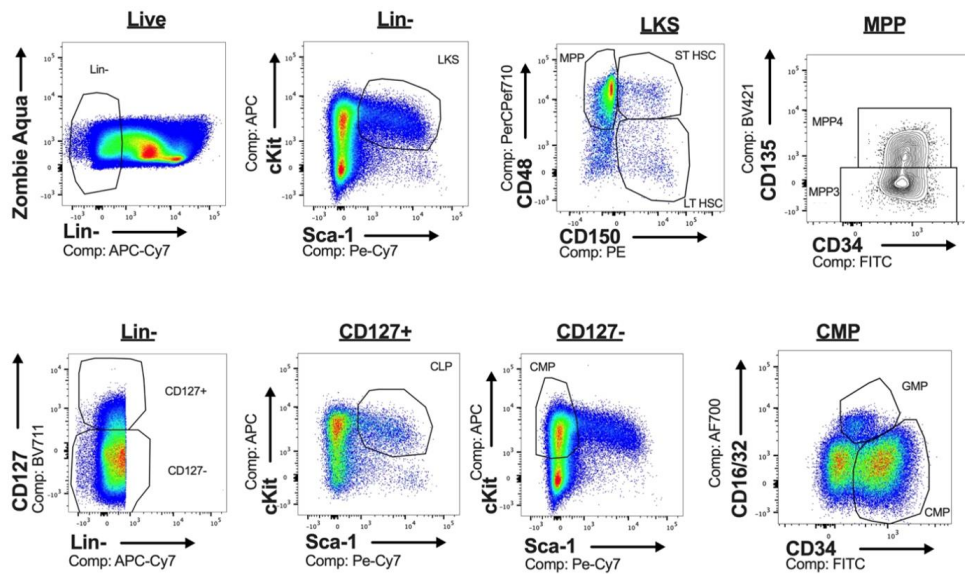


Fig. 2.2: Gating strategy for bone marrow cell profiling. Flow panel and gating strategy were designed by Dr. Anna Ledwith of the Sheedy lab, Trinity College Dublin.

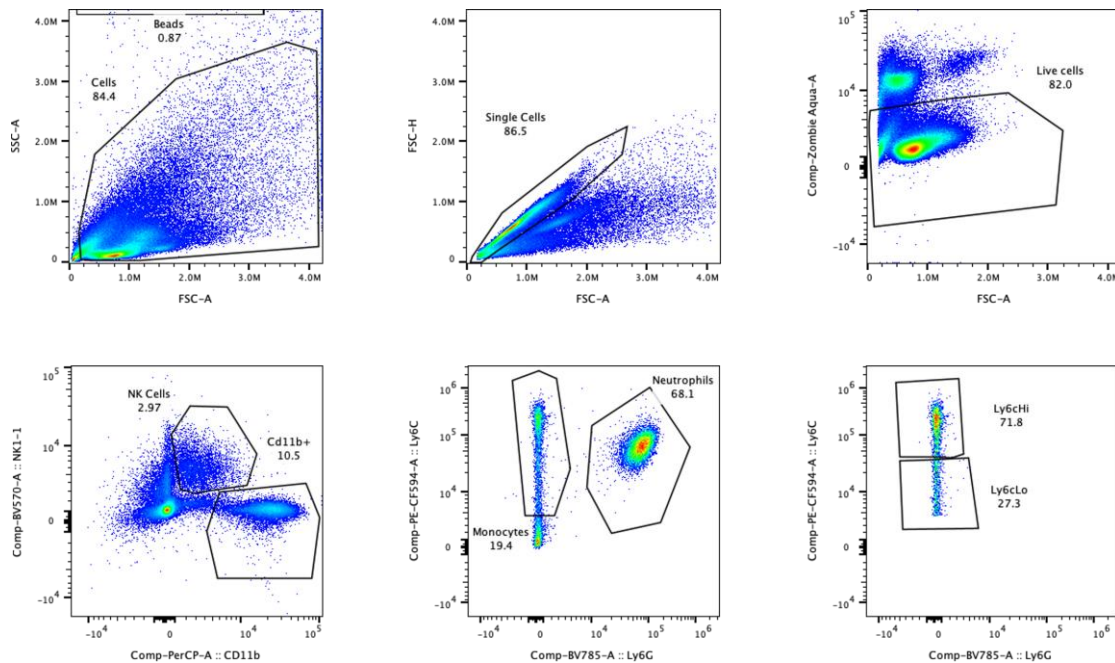


Fig. 2.3: Gating strategy for blood cell profiling. Flow panel and gating strategy were designed by Cian Horneck Johnston of the Sheedy lab, Trinity College Dublin.

2.2.7 RT-qPCR

2.2.7.1 RNA Extraction

RNA was extracted as per manufacturer's instructions and quantified using a Nanodrop 2000 spectrophotometer. RNA was stored at -80°C prior to use.

2.2.7.2 cDNA synthesis

RNA was normalized to a concentration of 50 ng/ μL and used to make complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. The reaction mix was prepared on ice and incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min. cDNA was stored at 4°C for short-term storage or -20°C for long-term storage.

2.2.7.3 Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) was performed on cDNA, using primers designed to target specific genes of interest (see **table 2.2** for primer sequences used). Master mixes were assembled for each primer and sample as per **table 2.5**, with each sample measured in triplicate. cDNA was diluted with H_2O as appropriate and added to a 96 or 384 well plate along with master mix. qPCR was performed using the Applied Biosystems StepOnePlus machine (96 well) or a QuantStudio 5 Real-Time PCR system as per manufacturer's instructions, and data analysed using the comparative Ct method.

Reagent	Volume (μL)
Sybr Green	5
dH ₂ O	0.7
10 μM forward + reverse primer mix	0.3
cDNA	4

Table 2.5: RT-qPCR reaction setup.

2.2.8 Western blotting

2.2.8.1 Cell lysis and protein extraction

Media was aspirated and adherent cells washed once in PBS. Cells were removed from plate into PBS using a cell lifter and centrifuged at $300 \times g$ for 5 min. The supernatant was discarded and the pellet resuspended in 50 μl RIPA lysis buffer. After 15 min incubation on ice, cell lysates were centrifuged for 10 min at $13000 \times g$ at 4°C and the supernatant removed to a new tube for storage at -20°C . Prior to loading, protein content

was determined via BCA assay as per manufacturer's instructions. 5X sample buffer was added to the appropriate volume of lysate, and protein was denatured at 95°C for 5 min.

2.2.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved by molecular weight using SDS-PAGE. 10 µg of each sample was loaded and ran at 60 V through the stacking gel for 30 min followed by 100 V through the 10% resolving gel for 1.5 hr (see **table 2.6** for gel compositions). A protein ladder was resolved alongside samples to extrapolate predicted band lengths.

Reagent	Resolving gel	Stacking gel
Acrylamide	10%	2%
1 M Tris, pH 6.8	-	0.06 M
1.5 M Tris, pH 8.8	0.4 M	-
SDS	0.1%	0.1%
APS	0.1%	0.1%
TEMED	0.003%	0.003%

Table 2.6: Concentrations of reagents used in stacking and resolving gels.

2.2.8.3 Electrophoretic transfer

The BioRad wet transfer system was used to transfer resolved proteins from gel to a PVDF membrane. A sponge was placed on to the bottom (cathode) side of a transfer cassette. Two pieces of filter paper (6.5 x 9.2 mm) were soaked in transfer buffer and placed on top of the sponge. The resolving gel was inverted and placed on top of the filter papers. A PVDF membrane activated in 100% methanol for 30 seconds was then placed directly on top of the gel. A further two pieces of soaked filter paper and an additional sponge were placed on top of the membrane, and air bubbles removed through gentle pressure. The cassette was then loaded into the transfer tank filled with transfer buffer and containing an ice pack, and proteins were transferred at 200 mA for 2 hr or 60 mA overnight.

2.2.8.4 Protein incubation and antibody visualization:

The PVDF membrane containing the transferred proteins was blocked in 1-5% (w/v) milk powder or BSA in TBST for 1 hr at RT. The membrane was then washed once for 10 min in TBST and incubated overnight at 4°C in various dilutions of primary antibody diluted in 3 mL of 1-5% milk or BSA. Following primary antibody incubation, the membrane was washed 3 x 10 min in TBST and incubated in varying dilutions of secondary antibody (see **Table 2.3**) in TBST or 5% milk. The membrane was then washed a further 2 times for 15 min, and protein was visualised using chemiluminescent substrate as per manufacturer's instructions on the ChemiDoc MP gel imaging system (BioRad).

2.2.9 Viability assay

Cells plated at 3×10^4 /well in a 96 well plate were treated in technical triplicate as appropriate and incubated for a defined interval. Resazurin dye was added to each well to a final concentration of 1X and cells incubated for a further 3 hr. Fluorescence was read from the bottom of the plate at 560_{ex}, 590_{em} using a BioTek Synergy H1 plate reader. Media only blank values were subtracted from each well and viability presented as a percentage relative to vehicle control-treated cells.

2.2.10 Mitoplast isolation

Media was removed from 2.5×10^7 cells seeded in a 10 cm² dish, and the cells washed gently in 5 mL ice cold mitochondrial fractionation buffer (Buffer A, see **Table 2.1** for composition). Cells were detached from the dish through gentle scraping into a further 5 mL Buffer A and centrifuged at 700 x g for 5 min at 4°C. The pellet was washed once and resuspended in 1 mL buffer A with 0.5 mM EDTA and incubated on ice to induce cell swelling. The resuspended pellet was placed in a Dounce homogeniser and mechanically homogenized 150 times. The homogenised solution was then placed in an ice-cold metal-free Eppendorf tube and centrifuged at 11000 x g for 20 min at 4°C. The supernatant containing the cytosolic fraction was removed to a separate metal-free tube and stored at -20°C and the pellet washed a further 4 times in Buffer A. The pellet was then resuspended in Buffer A with 0.5% w/v digitonin and incubated on ice for 15 min. The supernatant, containing the lysosomal fraction, was removed to a separate metal-free tube and stored at -20°C. The pellet was then washed twice in Buffer A, lysed in 50 µL RIPA and stored at -20°C for further analysis.

2.2.11 Graphite furnace atomic absorption spectrometry

2.2.11.1 Sample preparation

2.2.11.1.1 Mammalian cells

Media was aspirated and removed to a metal-free Eppendorf tube for storage at -80°C prior to digestion. Cells were scraped into 500 µl PBS, removed to a metal free Eppendorf tube and centrifuged at 400 x g for 5 min. Cell pellets were washed once in PBS and resuspended in 50 µL RIPA lysis buffer. One volume of 50% nitric acid was added to each lysate, and samples digested at 70°C for 2 hr. Following digestion, samples were centrifuged at 6000 x g for 5 min and supernatants used for iron measurements.

2.2.11.1.2 Bacterial cells

Prior to centrifugation, 20 µL of *S. pneumoniae* culture was removed, serially diluted tenfold as appropriate in PBS and plated on Todd Hewitt agar with 5% v/v defibrinated sheep's blood to determine CFU for data normalization. Cultures were centrifuged at 3000 x g for 10 min and the pellet washed once in PBS. Bacteria were then resuspended in 1 mL PBS and removed to a metal-free Eppendorf tube. Following centrifugation at 3000 x g for 10 min, the supernatant was discarded, and the bacterial pellet frozen at -20°C. To lyse cells, the pellet was resuspended in 500 µl PBS and transferred to a screw cap tube containing 200 µl glass beads. Bacteria were homogenized through bead beating using a Beadbug 3 Position Bead Homogeniser, performed 3 times at max speed for 30 sec, with samples cooled on ice between cycles. Samples were centrifuged at 8000 x g for 30 sec and supernatants removed to a new metal-free Eppendorf tube. Lysates were centrifuged for 1 hr at 4°C to remove bacterial cell wall components, and the supernatant containing the cytosolic fraction removed to a new tube. One volume of 50% nitric acid with 0.1% digitonin was added to each cytosolic fraction, and samples were digested at 70°C for 2 hr. Following digestion, samples were centrifuged at 6000 x g for 5 min and supernatants used for iron measurements.

2.2.11.1.3 Tissue

Over dry ice, approx. 10-30 mg of tissue was removed from the lower left lobe of murine lungs flash-frozen at -180°C. Tissue was blotted dry on a kim wipe, removed to a metal-free Eppendorf tube and weighed. 200 µL of undiluted nitric acid was added to the tube, which was heated at 70°C for 2 hr. The solution was cooled, one volume of ultrapure H₂O added and heated for a further 1 hr. Samples were centrifuged at 6000 x g for 5 min and supernatants used for iron measurements.

2.2.11.1.4 BALF

BALF was digested in one volume of 50% nitric acid at 70°C for two hr, centrifuged at 6000 x g for 5 min and supernatants used for iron measurements.

2.2.11.2 Iron measurement

Total iron levels were determined using the Perkin Elmer PinAAcle 900Z Atomic Absorption Spectrometer. Samples were diluted as appropriate in 0.2% nitric acid in ultrapure H₂O and iron measured in triplicate against iron standards. For *in vitro* studies, technical triplicate treatment wells were used within each individual experiment and the mean calculated for data representation.

2.2.12 Heme assay

Cells were seeded at 1×10^6 /well in a 6 well dish, treated as desired, and media aspirated. Cells were scraped into 500 μ L PBS and removed to a metal-free Eppendorf tube. Cells were centrifuged at $400 \times g$ for 5 min and a portion removed for protein measurement through BCA assay, supernatant discarded, and the pellet washed once in PBS. The pellet was resuspended in 2 M oxalic acid and split into two equal parts, with one incubated at room temperature for 30 mins and the other boiled at 95°C . Lysates were centrifuged at $8000 \times g$ and supernatants removed to a dark bottomed 96 well plate. Fluorescence at 400nm_{ex} , $620 \text{ nm}_{\text{em}}$ was read using a BioTek Synergy H1 plate reader, with total heme values calculated as 1 μM heme per 15200 absorbance units (220), after subtracting the value for the room temperature blank from that of the boiled sample.

2.2.13 Human bronchoalveolar lavage fluid processing

Bronchoalveolar lavage fluid (BALF) was retrieved via research bronchoscopy. Mucus clumps were dissociated through pipetting 8-10 times with a 25 mL stripette, and, if necessary to remove further mucus clumps, filtered through sterile gauze. BALF was centrifuged at $300 \times g$ for 10 min and 4°C and washed once in complete RPMI (10% FBS, 1% P/S, 2.5 $\mu\text{g}/\text{mL}$ Amphotericin B). If necessary, BAL cells were resuspended in 5 mL ACK lysis buffer and incubated at room temperature for 5 min to deplete red blood cells (RBCs), followed by centrifugation at $300 \times g$ for 10 min. Cells were counted using a hemocytometer and plated as appropriate in complete RPMI. Following overnight incubation at 37°C and 5% CO_2 , media containing non-adherent cells was removed, cells washed once and fresh media added to the adherent, purified AM population.

2.2.14 ELISA

DuoSet ELISA kits against murine IL-6, TNF-alpha and lipocalin-2 were used to measure cytokine levels in appropriately diluted cell supernatants as per manufacturer's instructions. 1N H_2SO_4 was added to each well to stop reactions for measurement of absorbance at 450 and 570 nm using a BioTek Synergy H1 plate reader. Values measured at 570 nm were subtracted from those at 450 nm to correct for optical impurities. Sample concentrations were extrapolated from a non-linear regression model of the sigmoidal standard curve and dilution corrected.

2.2.15 Confocal microscopy

7.5×10^5 cells were seeded in a 35 mm glass-bottomed dish and treated as desired. Media was removed and cells washed once in PBS. 1 mL RPMI (minus phenol red and any additional supplements) containing a final concentration of 1 μ M FerroOrange was added to dishes for 30 min. Cells were washed and the nuclear Hoechst dye added at a final concentration of 5 μ g/mL, and incubated for 15 min. Media was replaced with fresh phenol red-free RPMI, and cells imaged live using a Leica SP8 scanning confocal microscope. Images were processed and scale bars added using the LAX Imaging Suite software.

2.2.16 Seahorse extracellular flux analysis

Primary murine BAL cells were seeded in technical triplicate at 4×10^4 cells/per well in 100 μ L complete RPMI (10% FBS, 1% P/S) in an XFp 8-well cell culture miniplate and incubated overnight at 37°C, 5% CO₂ to facilitate AM adherence purification. The injection cartridge was placed in a utility plate containing XF calibrant solution and hydrated overnight at 37°C in the absence of CO₂. Prior to the assay, 60 μ L media was removed and 160 μ L complete Seahorse media (XF RPMI Base medium without phenol red, 10 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine) added and removed twice sequentially to wash cells. After washing, 160 μ L complete media was added to achieve a final volume of 180 μ L in each well. 180 μ L Seahorse media was added to the outermost wells as cell-free blanks. Cells were incubated at 37°C in the absence of CO₂ for a minimum of 45 min. Oligomycin, FCCP and rotenone/antimycin A were reconstituted in Seahorse media as per manufacturer's instructions and added to cartridge injection ports as per table 2.7.

Compound	Injector port	Concentration in injector port (μ L)	Volume in injector port (μ L)	Final concentration in well (μ M)
Oligomycin	A	50	20	1
FCCP	B	100	22	1
Rotenone/ antimycin A	C	25	25	.5

Table 2.7 Concentrations and injection strategy of compounds used in Mito Stress Test.

The cartridge was loaded into a Seahorse XFp Analyser for calibration, after which the cell plate was inserted. Measurements were taken at 3 x 5 min intervals at baseline

and following injection of each compound. Following completion of the assay, media was aspirated and 60 μ L RIPA added to each well and incubated on ice. Protein was measured in lysed cells by BCA to determine protein content for normalisation. Using normalised data, respiratory measurements were calculated using the Agilent Wave software.

2.2.17 Statistical methods

Statistical analysis was performed using GraphPad Prism 9 software. Data are presented as mean $\pm$ SEM of at least 3 independent experiments, unless otherwise stated in figure legend. Statistical testing was not performed in instances where only two biological replicates were carried out for an experiment, and data is instead displayed descriptively. Where a representative graph derived from one of several independent experiments is shown, the statistics displayed on the graph were calculated considering the mean values of all independent experiments. Differences were compared using unpaired or paired t-test or one- or two-way ANOVA as stated in figure legends, with differences considered significant using a 95% confidence interval at values of $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****).

Chapter 3: Characterising macrophage mitochondrial iron in smoking and COPD

3.1 Introduction

Tissue-resident macrophages, like AMs, exhibit unique gene expression patterns and metabolic features to meet the homeostatic needs of their tissue niche. As an extension of these specialised functions, tissue-resident macrophages have been termed ‘ferrostats’ due to their unique iron recycling and sequestration abilities in their local environment (92). In the lung, AMs are the primary line of defence against inhaled pathogens and irritants, uniquely positioning them at the interface between exogenous inhaled iron and endogenous iron of the pulmonary vasculature. The ever-changing terrain that AMs reside in, particularly in terms of iron availability, necessitates an efficient iron sequestration or liberation response to ensure both proper cellular function and a homeostatic alveolar microenvironment (**Fig. 1.3**). Consequently, failures in AM iron-handling mechanisms have deleterious consequences for the host (112). AMs show iron loading in both human and experimental COPD, with the number of iron-positive macrophages increasing with disease severity (112, 165, 221) alongside an increase in iron and iron-containing proteins in the extracellular milieu (163). **The nature and localisation of this intracellular iron remains unclear**, as does the mechanism driving its accumulation.

Whole lung mitochondrial iron loading is an observed pathology in experimental COPD, the chelation of which ameliorates lung injury (50). Mitochondria are the primary consumers of iron in mammalian cells, yielding FeS clusters and heme, essential enzymatic cofactors in bioenergetic pathways including the ETC and several components of the TCA cycle (21). These pathways, and the precursor metabolites and byproducts generated, govern the energetic and immunometabolic response of macrophages to infectious or sterile stimuli (222). COPD AMs have dysfunctional mitochondria, with reduced OXPHOS and compensatory glycolysis, ATP content, and dysregulated ROS production (145, 155, 156). It is unknown if mitochondrial iron loading is observed in AMs specifically in COPD, and how this would affect their metabolic and iron-handling plasticity. Whether mitochondrial iron dynamics change in human COPD is also unexplored. Considering this, we **hypothesised that AM mitochondrial iron is pathologically elevated in COPD and smoking**.

To assess this, we sought to investigate the mitochondrial iron-related transcriptome in lung macrophages across a range of disease etiologies and cellular ontogenies. Import of iron to mitochondria is mediated by the paralogous IMM proteins MFRN1 and 2, to date the sole mitochondrial inner membrane iron importers characterised in mammalian cells (1). Despite the continuous demand for the mitochondrially produced iron containing co-factors FeS clusters and heme in metabolic processes, little is known about how the expression of either of the mitoferrins can change to meet dynamic metabolic needs. Immune cells are particularly reliant on these products of mitochondrial iron metabolism for their effector functions upon activation, like oxidative burst or ROS production. However, MFRN1 and 2 remain understudied in immune cells in both health and disease.

Tissue resident AMs are fetal liver-derived and have limited capacity for self-renewal at baseline (54). When under stress, this AM pool is replenished by monocytes derived from hematopoietic multipotent progenitor cells in the bone marrow. Monocyte recruitment to the lung is chronically increased in smoking and COPD (144), however these recruited monocytes ultimately show pathological similarities to resident AMs. Driving an approximately 20-fold increase in macrophages in the COPD lung, these macrophages appear tissue-destructive (143) and are unable to defend the lung against invading bacteria (223). The mechanisms perpetuating this cycle of monocyte recruitment despite their seeming incapability in aiding tissue repair in smoking and COPD remain an open area of research, as does any pathological implications of dysregulated mitochondrial iron metabolism.

More than 70% of COPD cases in the developed world can be attributed to cigarette smoke (CS) exposure (224). Murine CS exposure is a well-established model of COPD commonly used in the literature to mimic pathological hallmarks of COPD to investigate disease mechanisms. Mice are exposed to CS generated from universally-used reference cigarettes from the University of Kentucky, which are endotoxin-free and contain a known chemical composition to ensure reproducibility across studies (225). Mice can be exposed to CS using a whole-body or nose-only approach. While a nose-only system ensures efficient inhalation and prevents non-respiratory ingestion of CS components deposited externally on mice through grooming, the level of restraint necessary inflicts stress upon mice and systems only allow for exposure of a small number of animals. As such, the whole-body exposure system is a universally used, refined method to study disease pathobiology. Impairment of mucociliary clearance emerges after approximately 4 weeks of whole-body CS exposure, where mice show an accumulation of airway mucus and chronic bronchitis (50, 226). This is accompanied by increased levels

of immune cells recruited to the airspaces and elevated BALF protein levels indicative of a loss of epithelial barrier integrity (50). Longer courses of exposure ranging from 4-6 months can induce clinical features of emphysema, with mice showing alveolar thickening and increased chord length (50). Given the difficulties in obtaining healthy human lung tissue, animal models of experimental COPD therefore provide an invaluable tool to study the mechanisms underpinning disease progression at a cellular level.

How absolute levels of mitochondrial iron can change in COPD AMs is unknown. As the low input material from humans precludes this in primary cells, an *in vitro* approach is required: however, existing models of AMs *in vitro* are limited. In contrast to circulating monocytes originating from the bone marrow, AMs are derived from the fetal yolk sac during embryogenesis and are thought to persist as a self-renewing population in the homeostatic adult lung. AMs are heavily reliant on GM-CSF and TGF- β production from the alveolar epithelium to maintain their phenotype and function (227, 228) and express a distinct complement of cell surface receptors including Cd11c, MERTK, MARCO and, in mice, Siglec-F (229). AMs are therefore ontogenically and functionally distinct from BMDMs, which are typically used as a primary model of a macrophage *in vitro*. Similarly, immortal cancer cell lines such as mouse RAW 264.7 macrophages or THP-1 monocytes will not reflect the AM phenotype. The MH-S cell line, generated through retroviral transfection of murine Balb/c AMs, fail to express Siglec-F or the GM-CSF receptor CD116 and do not recapitulate the antiviral responses of primary murine AMs (230). Using primary murine AMs for *in vitro* analyses is limited by their low abundance *in vivo* and their poor persistence over time in standard cell culture conditions. To properly interrogate AM dysfunction in COPD, novel models of AMs are needed.

3.1.1 Aims

Pathogenic lung mitochondrial iron loading can be therapeutically targeted to ameliorate murine COPD but has not been investigated at a cellular level. Macrophages appear to be the primary site for iron accumulation in the COPD lung, but whether this iron accumulates in mitochondria remains unknown. This chapter will address if mitochondrial iron loading is observed in COPD macrophages through the following specific aims (**Fig. 3.1**):

- Interrogating transcriptional evidence for mitochondrial iron loading in macrophages in human smoking and COPD.
- Investigating the transcriptomic response of murine lung resident and systemic macrophages to CS as a COPD model and delineating potential iron and mitochondrial iron-related changes.
- Characterising a recently established *in vitro* model of a murine alveolar-like macrophage cell line and the iron-associated response to cigarette smoke *in vitro*.

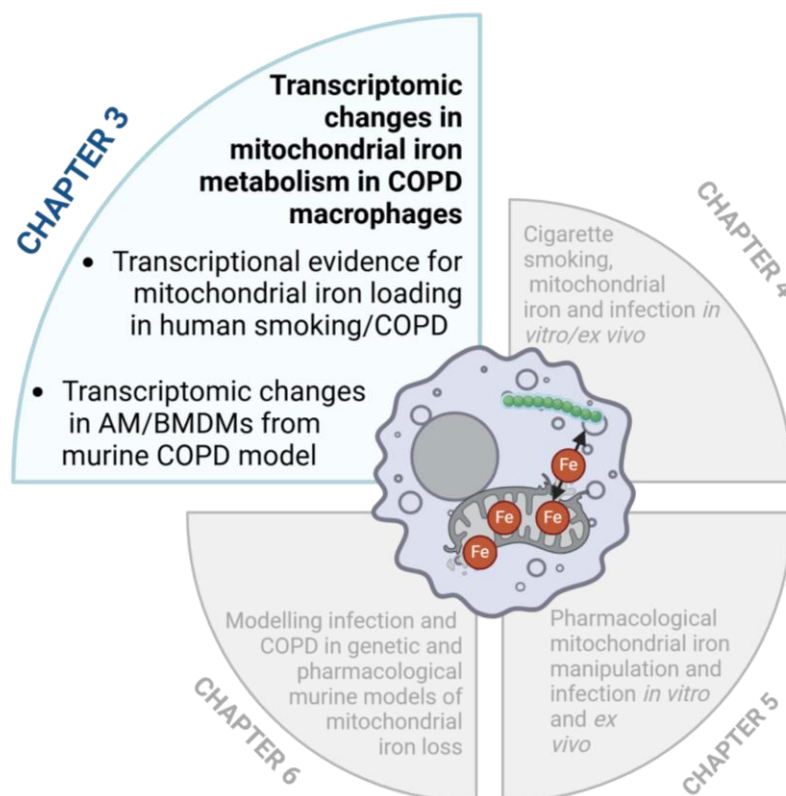


Fig. 3.1: Summary of aims of Chapter 3.

3.2 Results

3.2.1 Mitochondrial iron-related gene expression in macrophages in human smoking and COPD

To assess if mitochondrial iron metabolism is altered specifically in macrophages in COPD, we interrogated gene expression datasets, both previously published or made available to our group from collaborators (144, 146, 231-233), or generated during the course of this thesis (234) (**Table 3.1**). As expression data is often only published for genes meeting arbitrary cut-offs for significance or fold change, an unbiased approach was prohibited; instead, available data was mined manually using pre-determined gene IDs. This was performed using a curated a list of genes involved in cellular iron metabolism, obtained through combining genes listed in the EMBL Gene Ontology (GO) Biological Process pathways 'iron ion transport' (GO:0006826), 'intracellular iron ion homeostasis' (GO:0006879), 'iron ion binding' (GO:0005506) and 'ferroptosis' (GO:0097707). Genes encoding for subunits of hemoglobin were excluded from analyses: as these genes should not be expressed by non-erythroid cells, any expression values in sequencing datasets likely represents red blood cell contamination.

Dataset	Compartment analysed	Species	Cohorts	Sequencing method	Figure	Ref.
<i>Crystal et. al.</i>	Purified AMs isolated via bronchoscopy	Human	Non-smoker (n=28), healthy smoker (n=70) and COPD (n=53)	Bulk RNA-seq (microarray)	Fig. 3.2	(146) (partial)
<i>Sauler et. al.</i>	Explanted lung tissue	Human	Non-COPD (n=15), COPD (n=17)	Single-cell RNA-seq (scRNA-seq)	Fig. 3.3	(144)
<i>Huan et. al.</i>	Whole blood	Human	Non-smoker (n=4857), current smoker (n=1421)	Bulk RNA-seq (short-read)	Fig 3.4	(231)
<i>Xu et. al.</i>	Whole blood	Human	Current (n=454) and former (n=767) smoker	Bulk RNA-seq (short-read)	Fig 3.4	(232)
<i>Ryu et. al.</i>	Whole blood	Human	Current and former smoker with and without COPD (n=3618)	Bulk RNA-seq (short-read) + cellular deconvolution analysis	Fig. 3.6	(233)
<i>Faherty et. al.</i>	AMs isolated via bronchoalveolar lavage; BMDMs generated from bone marrow	Mouse	Room air (n=3-4) and smoke (n=3-4)	Bulk RNA-seq (short-read)	Fig. 3.8, 3.13-17	(234)

Table 3.1: List of RNA-seq datasets analysed in Chapter 3.

3.2.1.1 Mitochondrial iron-related gene expression in AMs in human smoking and COPD

Iron-related gene expression was first mined in RNA-seq data from bronchoalveolar lavage fluid (BALF) derived-macrophages of n=28 healthy non-smokers, n=70 smokers with preserved lung function and no COPD and n=53 individuals with COPD by microarray profiling at Weill Cornell Medical College, New York City (*Prof Ronald Crystal and Dr Robert Kaner, partially published (146)*). Using a Bonferroni age and sex-adjusted *P* value cut-off of 0.05, 25 iron-related genes were identified as significantly differentially expressed in smokers when compared to non-smokers (**Fig 3.2A**). In these 25 genes, GO analysis using the Cellular Component Ontology (235) database revealed their protein products were primarily mitochondrially localised (**Fig 3.2B**). Comparing non-smokers and COPD, 10 iron-related genes were differentially expressed, (**Fig 3.2C**), 9 of which were differentially expressed in the same directionality as smokers as in **Fig. 3.2A**. Expression of the mitochondrial iron importers *SLC25A37* (MFRN1) and *SLC25A28* (MFRN2) was significantly reduced in both smokers and subjects with COPD compared to non-smokers (all Bonferroni-adjusted $P < 0.001$, except for *SLC25A37* for which $P = 0.043$) (**Fig. 3.2D**). There were no differentially expressed iron-related genes in COPD compared to smokers.

In smoking and COPD, monocytes are continuously recruited from circulation to replenish the local AM pool. This results in distinct populations of monocyte-derived macrophages that take on varying degrees of alveolar macrophage-like traits as directed by the alveolar microenvironment and persist alongside the yolk sac-derived AMs (144). Considering this, and the significant downregulation of *SLC25A37* and *SLC25A28* observed in **Fig. 3.2D**, the publicly available COPD Cell Atlas (144) was used to analyse the expression of *SLC25A37* and *SLC25A28* at a single-cell level across lung immune cells in COPD. *SLC25A37* (MFRN1) was found to be ubiquitously expressed across lung macrophages, with a particular enrichment in monocyte-derived macrophage populations (**Fig. 3.3A, C**). *SLC25A28* (MFRN2) expression was detected ubiquitously without any strong enrichment in a particular immune cell population (**Fig. 3.3B, C**). This confirms *SLC25A37* and *SLC25A28* expression is abundant across ontogenically-distinct AM populations, with *SLC25A37* expression particularly heightened in those of monocytic origin.

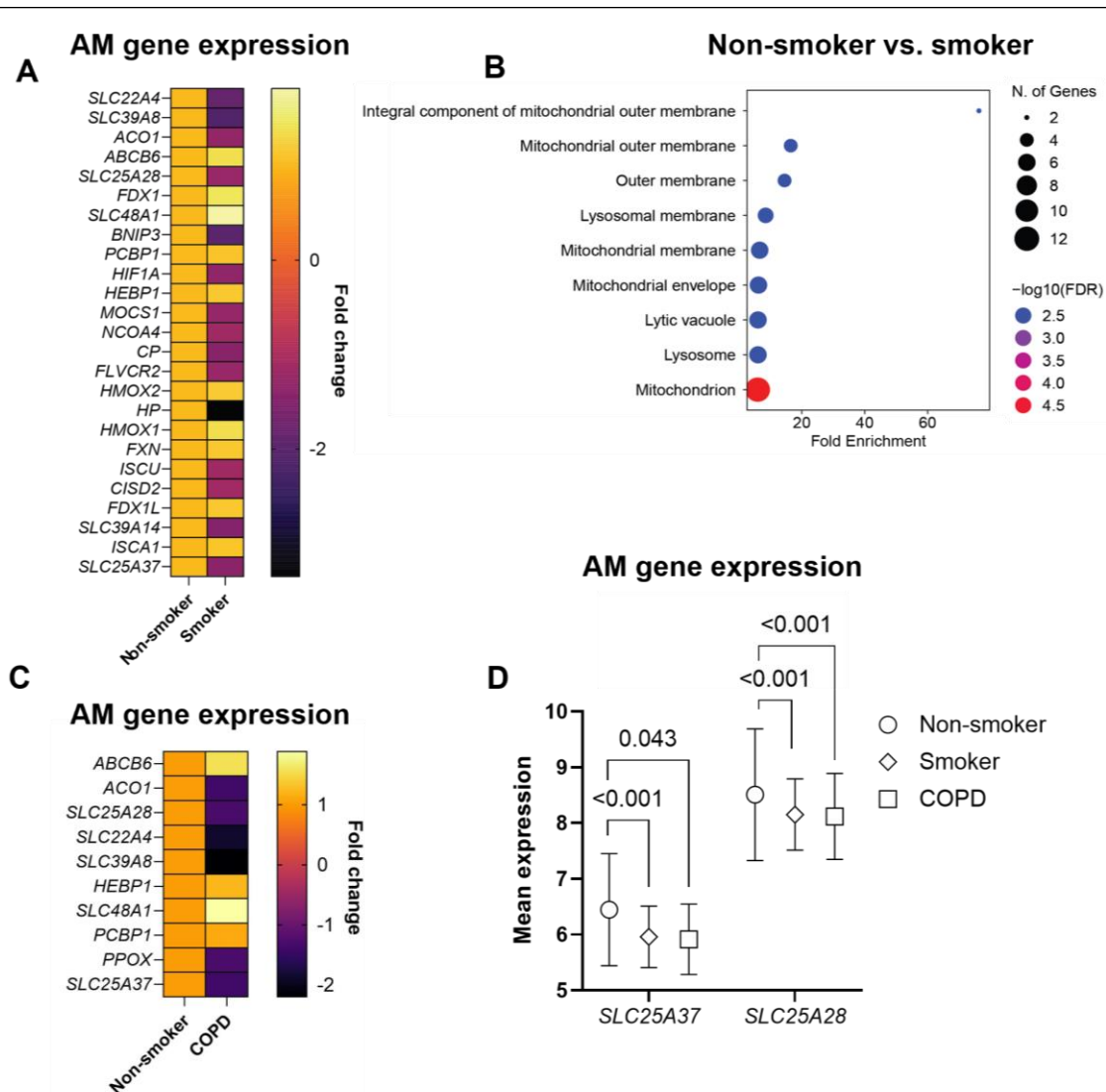


Fig 3.2: Human AMs show altered mitochondrial iron-related gene expression in smoking and COPD: AMs were isolated through research bronchoscopy from n=28 non-smokers, n=70 smokers and n=53 COPD patients and gene expression analysed by HG-U133 Plus 2.0 microarray. (A) Fold change in iron-related gene expression in smokers compared to non-smokers (Bonferroni-adj. $P < 0.05$ by t-test, heatmap ranked by significance). (B) GO Cellular Component analysis of genes significantly differentially expressed between smokers and non-smokers in (A) (FDR < 0.05). (C) Fold change in iron-related gene expression in non-smokers compared to COPD (Bonferroni-adj. $P < 0.05$, heatmap ranked by significance). (D) Mean expression values of SLC25A37 and SLC25A28 across non-smokers, smokers and COPD (Bonferroni-adj. P value by t-test, error bars representative of SD).

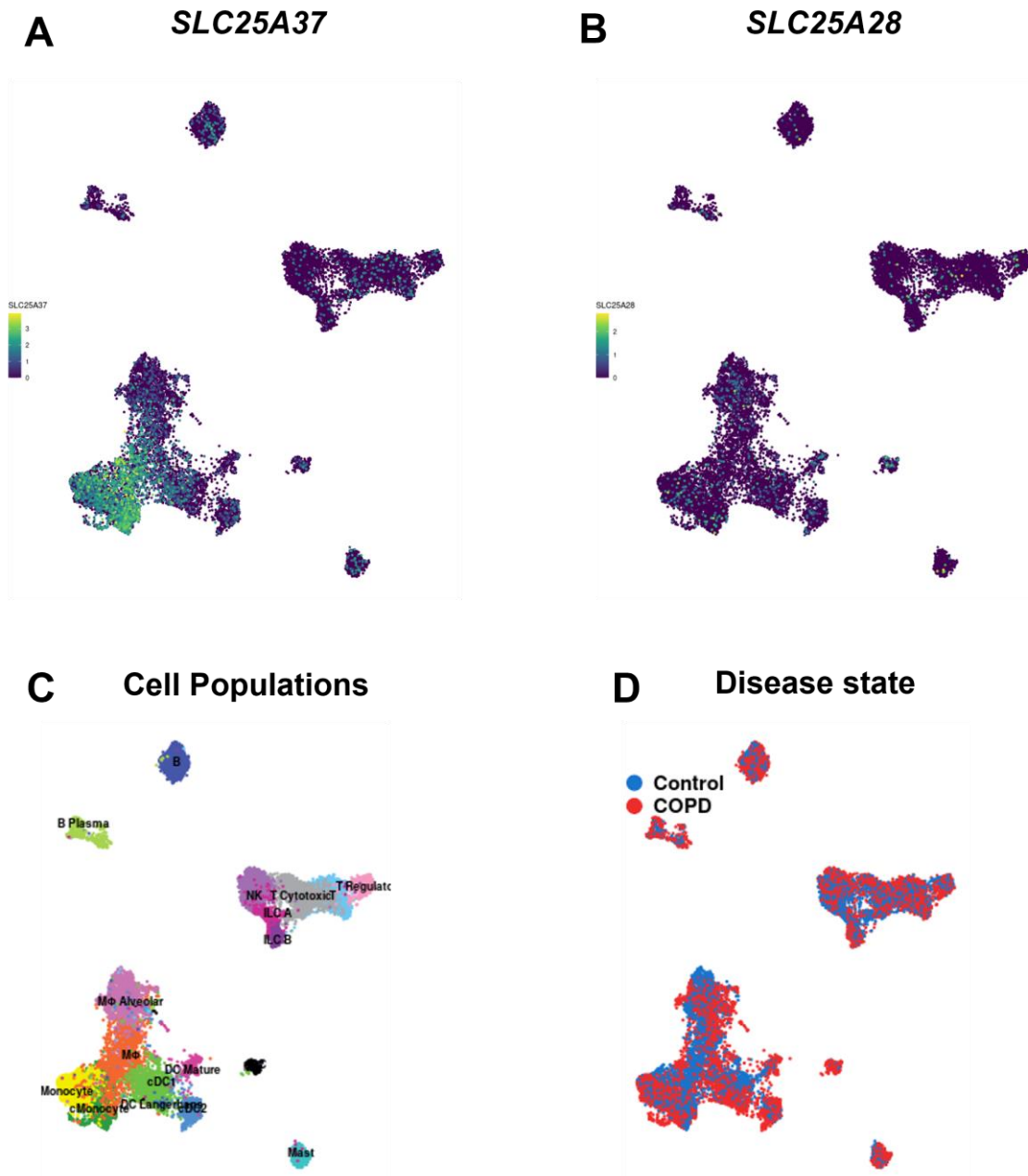


Fig. 3.3: MFRN1 expression is enriched in macrophage populations in COPD: Expression of *SLC25A37* (A) and *SLC25A28* (B) was queried across lung immune cell populations (C) present and absent in COPD and healthy controls (D). Data acquired using the COPD Cell Atlas Browser (142).

3.2.1.2 Mitochondrial iron changes in blood in ever-smokers

To expand analysis of mitochondrial iron-related gene expression across different COPD settings and compartments, peripheral blood RNA-seq data associated with varying phenotypes, endotypes and risk factors of COPD was analysed. In a meta-analysis of current smokers (n=1241) compared to non-smokers (n=4860) across six international smoking cohorts (231), 8 iron-related genes were identified as differentially expressed in whole blood, including *SLC25A37* (MFRN1) which was significantly downregulated (**Fig. 3.4A**). As a previous history of smoking increases an individual's disease risk even after smoking cessation, we next investigated if this genetic signature can persist in whole-blood RNA-seq data from ex-smokers (n=767) compared to current smokers (n=454) (232). 19 iron-related genes were significantly differentially expressed in ex-smokers, with *SLC25A37* again decreased (**Fig. 3.4B**). Intriguingly, downregulation of *SLC25A37* in both studies was accompanied by a significant downregulation of *SLC25A39* expression, a mitochondrial glutathione transporter recently identified to be co-regulated with *SLC25A37* expression in a synthetic lethality CRISPR screen (236). The most significantly downregulated gene in both datasets was *ALAS2*, an isoform of the enzyme encoding for the first step in heme synthesis previously reported to be erythroid cell-specific: this dogma has been challenged by the implication of *ALAS2* expression in MHC-II antigen presentation in blood monocytes (237).

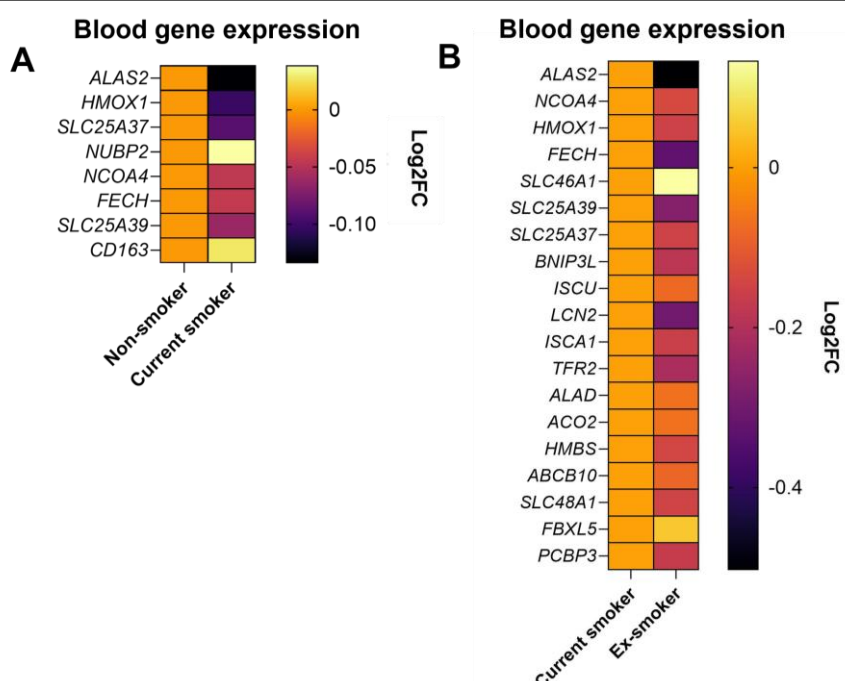


Fig 3.4: Mitochondrial iron-related gene expression is altered in blood of smokers and ex-smokers: (A) Heat map of significantly differentially expressed iron-related genes in whole blood RNA-seq (227) from non-smokers (n=1241) and current smokers (n=4860). (B) Heat map of significantly differentially expressed iron-related genes in whole blood RNA-seq (228) from current smokers (n=454) and ex-smokers (n=767). Bonferroni age and sex-corrected $P < 0.05$.

We next sought to investigate expression of *SLC25A37* and *SLC25A28* across blood immune cells to identify which cell type may drive these expression changes, in lieu of having cell-specific data available. In blood immune cell gene expression profiles generated using the Immunological Genome Project (IMMGEN) database (238), *SLC25A37* is most enriched in monocytes, showing slightly elevated expression in CD16⁺ non-classical monocytes compared to CD14⁺ classical monocytes (**Fig. 3.5A**). *SLC25A28* expression is more evenly distributed across blood leukocytes in comparison, while still expressed in both CD14⁺ and CD16⁺ monocytes albeit to a lesser extent than *SLC25A37* (**Fig. 3.5B**). These results show monocytes are the primary *SLC25A37*-expressing cells across blood leukocytes, suggesting them as a putative driver for the gene expression changes observed in the whole blood sequencing data in **Fig. 3.4**.

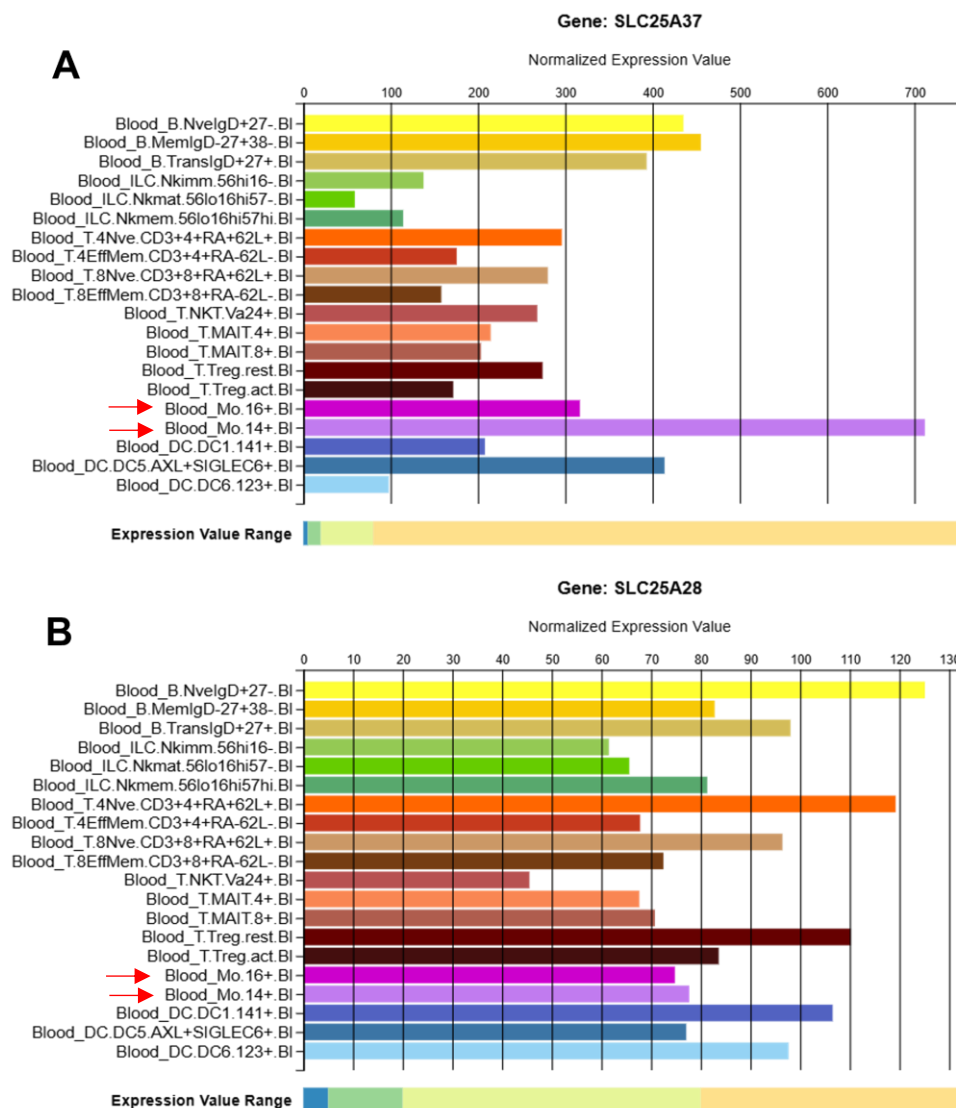


Fig. 3.5: MFRN1 gene expression is primarily localised to monocytes in blood: The Immunological Genome Project (IMMGEN) Gene Skyline RNA-seq dataset was queried for *SLC25A37* (A) and *SLC25A28* (B) expression across blood leukocyte populations. Blood monocyte populations are indicated with a red arrow.

3.2.1.3 MFRN1 expression in blood monocytes associates with exacerbation frequency:

Exacerbation events, often infectious in origin, are a large driver of morbidity and mortality in COPD, and frequent exacerbations can constitute a distinct disease endotype (137). A recent study by *Ryu et. al.* analysed whole-blood sequencing data investigating the transcriptome of COPD subjects who experience frequent, acute exacerbations in a cohort of 3618 ever-smokers (both current and former smokers), with and without COPD, enrolled in the US-based COPDGene or ECLIPSE cohorts (233). In this paper, unbiased analyses revealed higher blood leukocyte expression of *SLC25A37* (MFRN1) to be positively associated with exacerbation history as well as prospective exacerbation rate (233). To interrogate which cell type drives this enriched expression, we collaborated with Dr. Min Hyung Ryu and Prof. Craig Hersh (*The Channing Institute, Harvard Medical School*) to perform deconvolution analysis of this whole-blood RNA seq data (n=3618)*. Deconvolution of the data to yield cell type specific gene expression revealed 4 iron-related genes that associated with exacerbation rate specifically in monocytes (**Fig 3.6**): strikingly, *SLC25A37* was the most enriched across all genes (mean expression 37.57, *P* adj. 0.0055). Other iron-related genes whose expression was positively associated with exacerbation frequency in monocytes included mitochondrial FeS-binding protein CDGSH iron sulfur domain protein 2 (*CISD2*) (mean expression 1.12, *P* adj. 0.0055) and *EIF2AK1*, encoding for HRI (mean expression 0.7, *P* adj. 0.0401). The gene encoding for the transferrin receptor, the primary plasma membrane iron importer (*TFRC*) was also identified as positively enriched (mean expression 0.22, *P* adj. 0.0272).

* Deconvoluted data was provided by Dr. Min Hyung Ryu, which I analysed for iron-related gene expression and graphed.

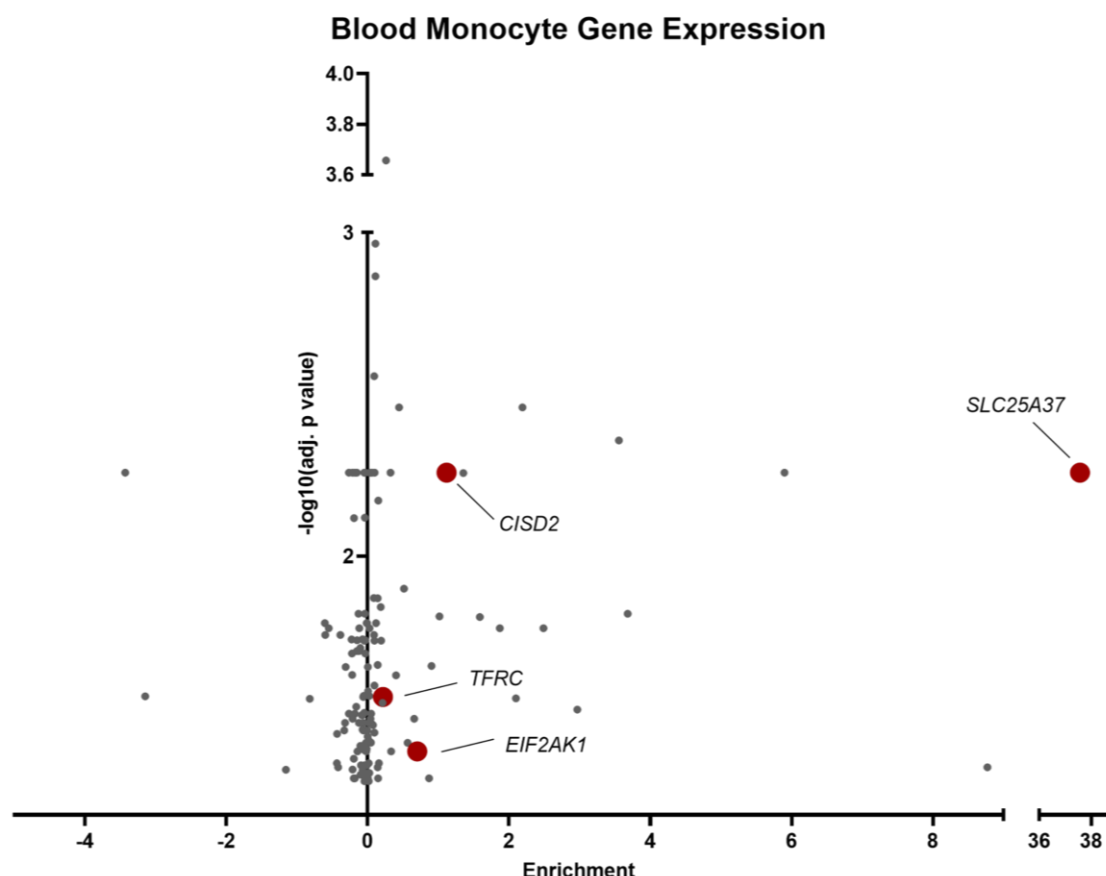


Fig 3.6: MFRN1 gene expression is associated with exacerbations in blood monocytes: Cellular deconvolution of whole-blood RNA-seq data (229) (n=3618) was performed using CIBERSORTx to yield monocyte specific gene enrichment values of significantly differentially expressed genes in monocytes associated with exacerbation rate (Bonferroni age and sex-adjusted P value <0.05). Genes related to iron metabolism are highlighted in red and labelled, with all other significantly differentially expressed genes coloured in grey.

3.2.1.4: CS AMs show altered iron-related gene expression suggestive of iron loading.

To recapitulate the above observations, the expression of iron and mitochondrial iron-related genes was assessed in an 8-week murine CS exposure model of experimental COPD*. From the same mice in tandem, AMs were isolated through bronchoalveolar lavage and bone marrow-derived macrophages (BMDMs) generated from bone marrow progenitors through culture in 10% L929 media for 7 days. The transcriptome of AMs and BMDMs was assessed through RNA-seq (**Fig 3.7**), and the expression of iron and mitochondrial iron-related genes assessed across both cell types.

* CS exposure and tissue and RNA isolation was performed by Dr. Bill Zhang, Elizabeth Perez and Kihwan Kim of Weill Cornell Medicine, New York. RNA-seq and DeSeq2 analysis was performed by Mays Mohammed Salih and Elektra Robinson of the Carpenter Lab, University of California, Santa Cruz. I analysed DeSeq2 output and created all graphs and heatmaps pertaining to this data. Parts of this data have been published in *Faherty et al., 2024; Resp. Res.* (230).

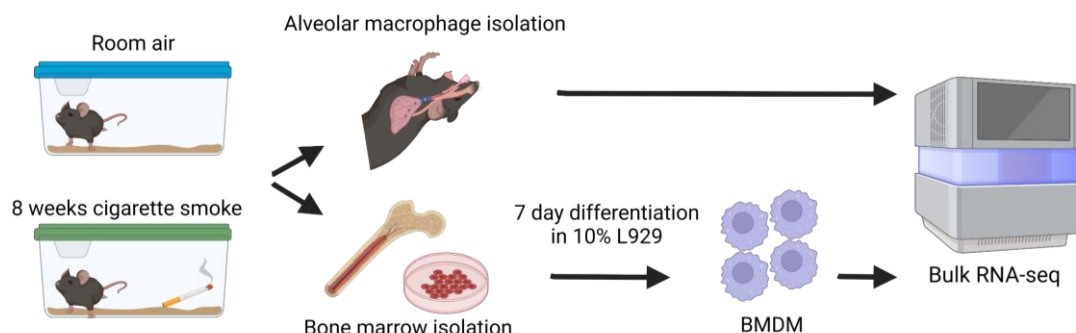


Fig 3.7: Schematic of CS exposure model and simultaneous AM/BMDM RNA-seq.

AMs isolated from CS-exposed mice showed significantly upregulated expression of *Fth1* (iron storage) and *Tfrc* (iron import), with expression of the divalent metal importer *Slc11a2* (DMT1) trending towards increase ($P = 0.12$), all suggestive of increased iron acquisition and storage (**Fig 3.8A**). Expression of *Slc25a37* and *Slc25a28* was unchanged in CS-exposed AMs ($P = 0.735$ and $P = 0.131$, respectively, **Fig 3.8B**). These findings were further probed by assessing the expression of *Slc25a37* and *Slc25a28* via RT-qPCR in bronchoalveolar lavage fluid-derived cells from mice exposed to 8 weeks of CS, where expression of both *Slc25a37* and *Slc25a28* showed no significant change as in our RNA-seq data (**Fig 3.8C-D**, $P = 0.4464$ and $P = 0.1383$ respectively).

Pertaining to BMDMs, *Ireb2* (encoding for the iron regulatory protein IRP2) and *Pcbp1* (encoding for a cytosolic iron chaperone) were significantly downregulated and upregulated respectively in response to CS exposure (**Fig 3.8E**). Contrasting AMs, *Slc25a37* expression in BMDMs trended towards decrease ($P = 0.15$) and *Slc25a28* towards increase ($P = 0.175$) (**Fig 3.8F**). Taken together, the above findings show changes in intracellular iron metabolism in both lung macrophages (AMs) and monocyte derived macrophages (BMDMs) isolated from murine CS exposure model, with gene expression changes in AMs suggesting increased intracellular iron import compared to BMDMs.

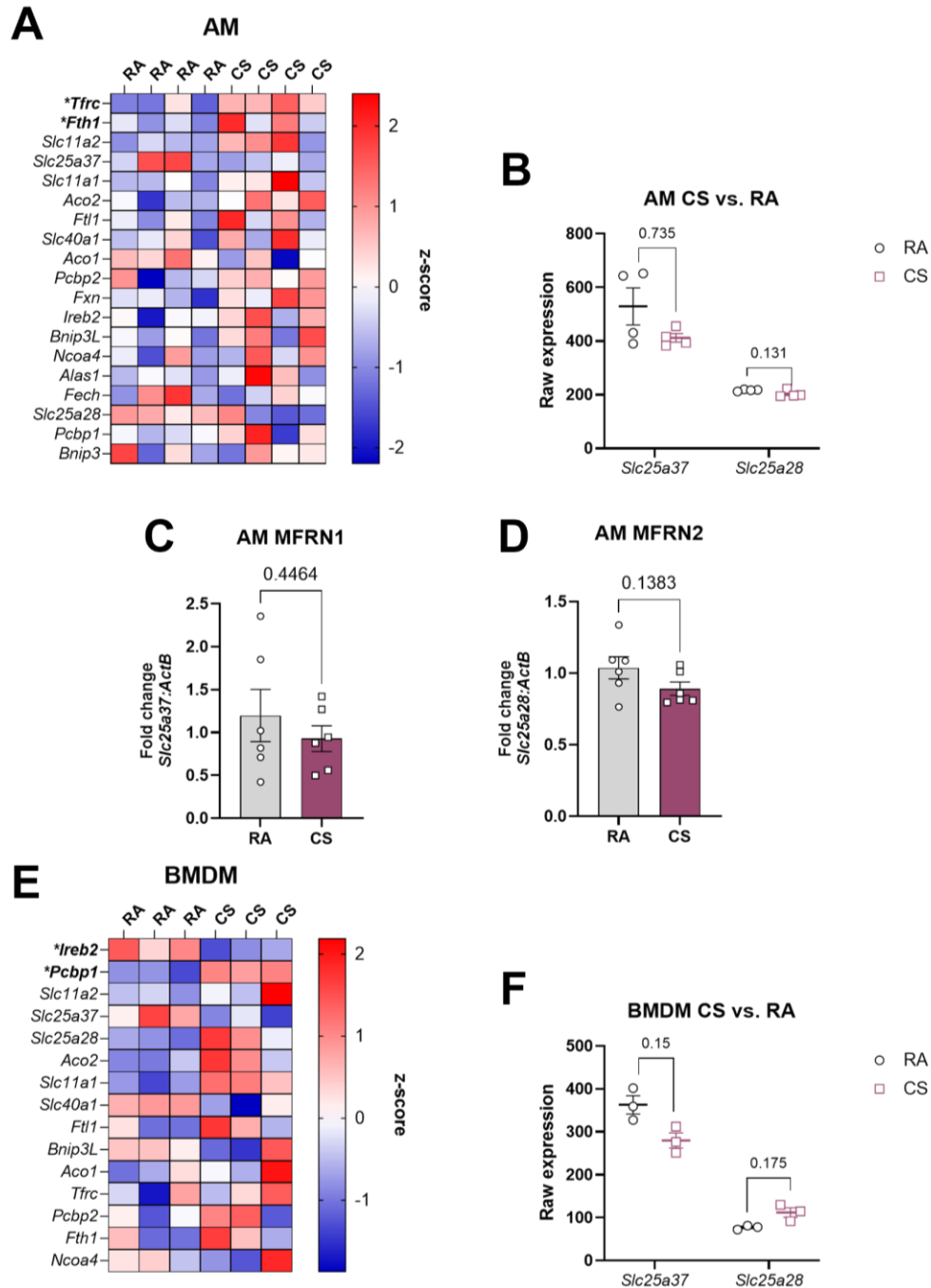


Fig. 3.8: AMs and BMDMs from CS-exposed mice show altered iron-related gene expression. (A) Heat map of iron related gene expression in AMs isolated from CS-exposed mice compared to RA assessed through RNA-seq. (n=4 in each group). (B) Mean RNA expression values of *Slc25a37* and *Slc25a28* in AMs from RA and CS mice. AMs were isolated through bronchoalveolar lavage from mice exposed to 8 weeks CS and RA controls and *Slc25a37* (C) and *Slc25a28* (D) expression measured through RT-qPCR relative to *ActB* expression as an endogenous control. Data presented as mean $\pm$ S.E.M. representative of n=2 independent experiments, n=6 mice. (E) Heat map of iron related gene expression in BMDMs generated from CS-exposed mice compared to RA assessed through RNA-seq (n=3 in each group). (F) Mean RNA expression values of *Slc25a37* and *Slc25a28* in BMDMs generated from RA and CS mice.

3.2.2 Alveolar macrophage-like cells show cellular and mitochondrial iron loading in response to CS exposure *in vitro*

Data in **Fig. 3.8** revealed AMs isolated from a murine CS exposure model of COPD show gene expression changes associated with increased iron uptake and storage, without significant change in expression of *Slc25a37* and *Slc25a28*. However, how expression of MFRN1 and 2 transcripts relates to mitochondrial iron levels is poorly understood, and whether our observed changes are reflective of actual change in cellular iron levels is also not known. The use of primary murine AMs for functional analyses is limited by the low number of AMs resident in the lung at steady state (~100,000), and there are currently no reliable commercially available AM immortalised cell lines. As evidenced in **Fig. 3.8**, CS exposure appears to drive opposing transcriptomic responses relating to iron metabolism in BMDMs compared to AMs *in vivo*, making them an unsuitable model for AMs. To better recapitulate the distinct phenotype of AMs *in vitro*, a recently published model of fetal liver-derived alveolar-like macrophages (FLAMs) was used (239). Mimicking the ontogeny of AMs *in vivo*, these cells are derived from the murine fetal liver and are differentiated to alveolar-like macrophages through GM-CSF and TGF- β for *ex vivo* expansion. As an *in vitro* model of cigarette smoke exposure, cigarette smoke extract (CSE) was generated and its ability to reduce FLAM viability confirmed through viability assay, with a decrease in viability after 18 hr treatment observed at treatment doses greater than 10% (**Fig. 3.9**).

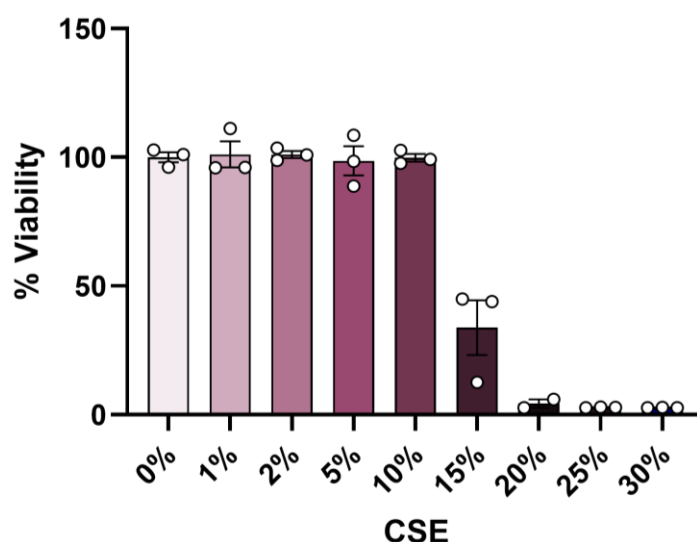


Fig 3.9: CSE induces cell death in an alveolar macrophage-like cell line: FLAMs were plated at a density of 3×10^4 cells/well. Varying concentrations of CSE (RPMI vehicle control) were added directly to each well and cells incubated for a further 18 hr, and viability assay performed as described. Data presented as a percentage relative to untreated control minus blank, representative of mean $\pm$ S.E.M. of one biological replicate with technical triplicate values pictured. CSE = cigarette smoke extract.

Having established an *in vitro* model of cigarette smoke-exposed AMs, we next sought to examine the iron status of these cells. To fully assess cellular iron storage and usage, measurements of absolute iron levels were coupled with assessments of gene and protein expression of key players in iron handling to capture the cellular 'ironome'. A 5% CSE treatment regimen for 18 hr was used to ensure no cell death was observed. To assess changes in total cellular iron levels with CSE treatment, total iron was measured in CSE-treated FLAM lysates and vehicle controls through graphite furnace-atomic absorption spectrometry (GF-AAS). At this dose and time of CSE exposure, total cellular iron trended towards increase relative to control ($P = 0.0744$) (**Fig 3.10A**). Next, the effects of CSE on heme production were assessed through heme assay, with CSE-treated cells again trending towards increase ($P = 0.0522$) (**Fig 3.10B**). Upregulation of FTH1 was confirmed at the protein level through western blot (**Fig 3.10C**). Assessing the expression of iron-related genes through qPCR revealed a significant upregulation of *Fth1*, *Ncoa4* and *Tfrc* expression (**Fig 3.10D-F**), with *Slc11a2* (DMT1) unchanged (**Fig. 3.10G**). *Lcn2* expression was downregulated, while the cellular exporters of free iron and heme, *Slc40a1* (FPN) and *Flvcr1a*, were both upregulated (**Fig 3.10G-I**).

To investigate if AMs in this *in vitro* model of COPD show altered mitochondrial iron metabolism, as suggested by earlier findings in humans (**Fig. 3.2**), we next measured mitochondrial iron. Mitoplasts (mitochondria without an outer membrane) were generated from CSE-treated FLAMs using a subcellular fractionation-based method (**Fig. 3.11A**). To eliminate any possible contamination of non-mitochondrial iron in the subcellular fraction from other organelles involved in iron metabolism and storage, such as lysosomes and peroxisomes, an additional lysis step was performed after fractionation with the detergent digitonin. This leverages the inability of digitonin to break down cardiolipin-containing membranes such as the IMM, yielding a pure population of mitochondria stripped of their OMM but with an intact inner membrane and preserved mitochondrial matrix (**Fig 3.11A**). Western blotting of mitoplasts generated in this manner and the corresponding cytosolic fraction confirmed enrichment of the IMM translocase TIM23 (240) specifically in mitoplasts (**Fig. 3.11B**). Both the cytosolic fraction and mitoplasts from CSE-treated FLAMs show a trend towards increased iron levels compared to controls ($P = 0.0879$ and $P = 0.1075$ respectively) (**Fig 3.11C-D**). Interrogation of mitochondrial iron-related genes through qPCR revealed, in contrast to mouse and human *in vivo* data, CSE significantly upregulated *Slc25a37* (MFRN1) and *Slc25a28* (MFRN2) expression (**Fig 3.11E-F**). No significant change in expression of *Mcu*, *Flvcr1b*, *Mtft* or *Bnip3* was observed (**Fig 3.11G-J**). These results show CSE can induce both cellular and mitochondrial iron loading *in vitro*.

in alveolar macrophage-like cells, which associates with increased expression of genes involved in iron uptake and storage processes.

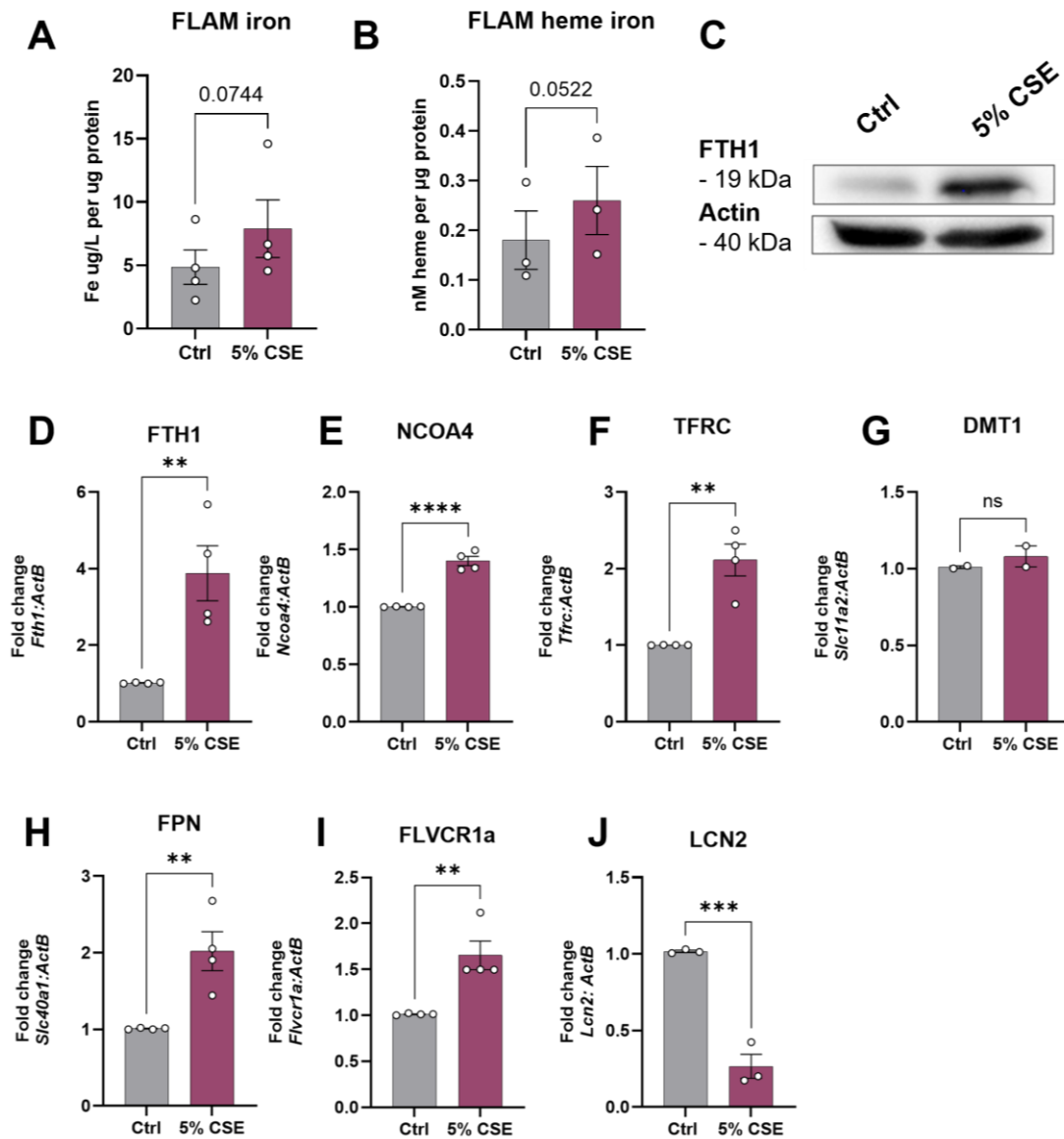


Fig 3.10: CSE may induce FLAM cellular iron loading. FLAMs were treated with CSE or vehicle control (RPMI) for 18 hr. (A) FLAMs plated in a 12 well plate were lifted through scraping, washed once, and lysed in RIPA buffer prior to digestion in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS. Each data point is representative of one biological replicate constituting the average of three technical triplicate wells of cells, normalised to protein content. (B) FLAM intracellular heme content was measured through heme assay and normalised to protein content. (C) Representative western blot of FLAM lysates treated with 5% CSE and vehicle control probed for FTH1 and β -Actin as loading control. (D-J) FLAMs plated in a 6 well plate were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. Data representative of mean $\pm$ S.E.M. of $n \geq 3$ biological replicates. All *P* value by t-test; * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $p < 0.0001$, ns = non-significant.

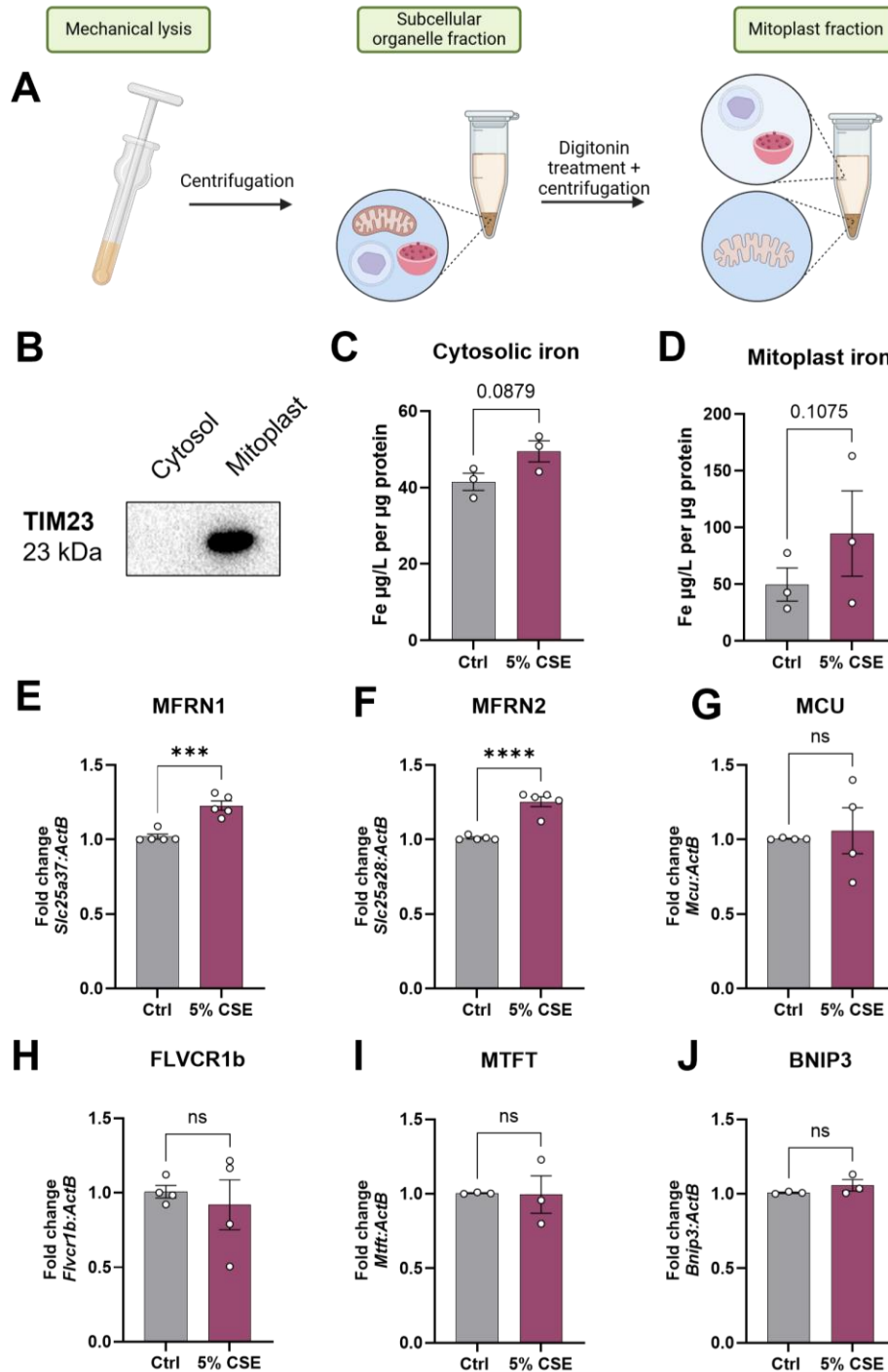


Fig 3.11: CSE may induce a degree of FLAM mitochondrial iron loading: (A) Schematic of mitoplast preparation. FLAMs plated in a 10cm² dish were lifted through scraping, centrifuged, washed and lysed mechanically. Centrifugation yielded a pellet of all subcellular organelles, which was treated with digitonin and centrifuged a yield a pure mitoplast pellet. FLAMs were treated with CSE or vehicle control (RPMI) for 18 hr. (B) Mitoplasts were lysed in RIPA lysis buffer, and, alongside the cytosolic fraction, TIM23 protein levels analysed by western blot. (C-D) Lysates were digested in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS, normalised to protein content. Data representative of n=3 biological replicates. (D-I) FLAMs plated in a 6 well plate were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. Data representative of mean $\pm$ S.E.M. of n $\geq$ 3 biological replicates. All *P* value by t-test; ***=*P*<0.001, ****=*P*<0.0001, ns = non-significant.

3.2.3 CSE does not induce iron loading in an alveolar epithelial type II cell line

While previous work from our lab has shown that removing metal ions from CSE using Chelex resin has no effect on the ability of CSE to alter the iron status of a cell (112), we sought to validate that the iron loading observed in our CSE-treated FLAM model is not an artefact of any increased iron supplemented to cells that may be present in CSE. To confirm if these findings are a generic macrophage response to CSE treatment, an immortalised BMDM cell line (iBMDMs) were treated with CSE. There was no significant change in mitoplast iron levels (**Fig. 3.12A**), and no change in *Slc25a37* (MFRN1) and *Slc25a28* (MFRN2) expression detected (**Fig. 3.12B-C**).

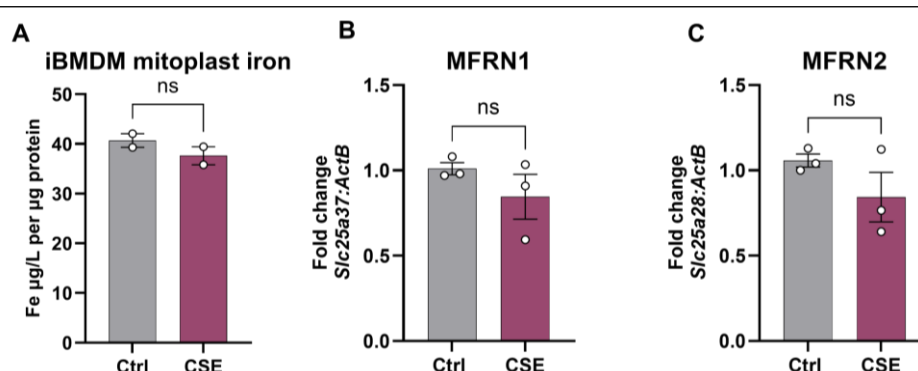


Fig. 3.12: iBMDM mitoplast iron is unchanged upon CSE treatment. iBMDMs were treated with 5% CSE or vehicle control (DMEM) for 18 hr. (A) Mitoplasts were lysed in RIPA lysis buffer, and, alongside the cytosolic fraction, were digested in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS, normalised to protein content. Data representative of n=2 biological replicates. (B-C) iBMDMs plated in a 6 well plate were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. Data representative of mean $\pm$ S.E.M. of n $\geq$ 3 biological replicates. All *P* value by t-test; ***=*P*<0.001, ****=*P*<0.0001, ns = non-significant. CSE = cigarette smoke extract.

To further validate the specificity of CSE-induced AM iron loading, an AT2 cell line, MLE-12 cells, were also exposed to CSE. AT2 cells are also heavily reliant on proper mitochondrial function for their production of pulmonary surfactant, and show a loss of function in COPD (241). Total cellular iron and heme trended toward decrease in CSE treated MLE12s, with mitochondrial iron unchanged (**Fig. 3.13A-C**). While *Fth1* gene expression increased, *Tfrc* expression was unchanged, accompanied by significantly upregulated expression of *Slc40a1* (FPN) (**Fig. 3.13D-F**). Considering mitochondrial iron-related gene expression, *Slc25a37* (MFRN1) and *Slc25a28* (MFRN2) showed no significant change, with *Mtft* expression significantly reduced (**Fig 3.13G-I**). These results suggest CS-induced iron loading may disproportionately affect AMs compared to other macrophage and cell types.

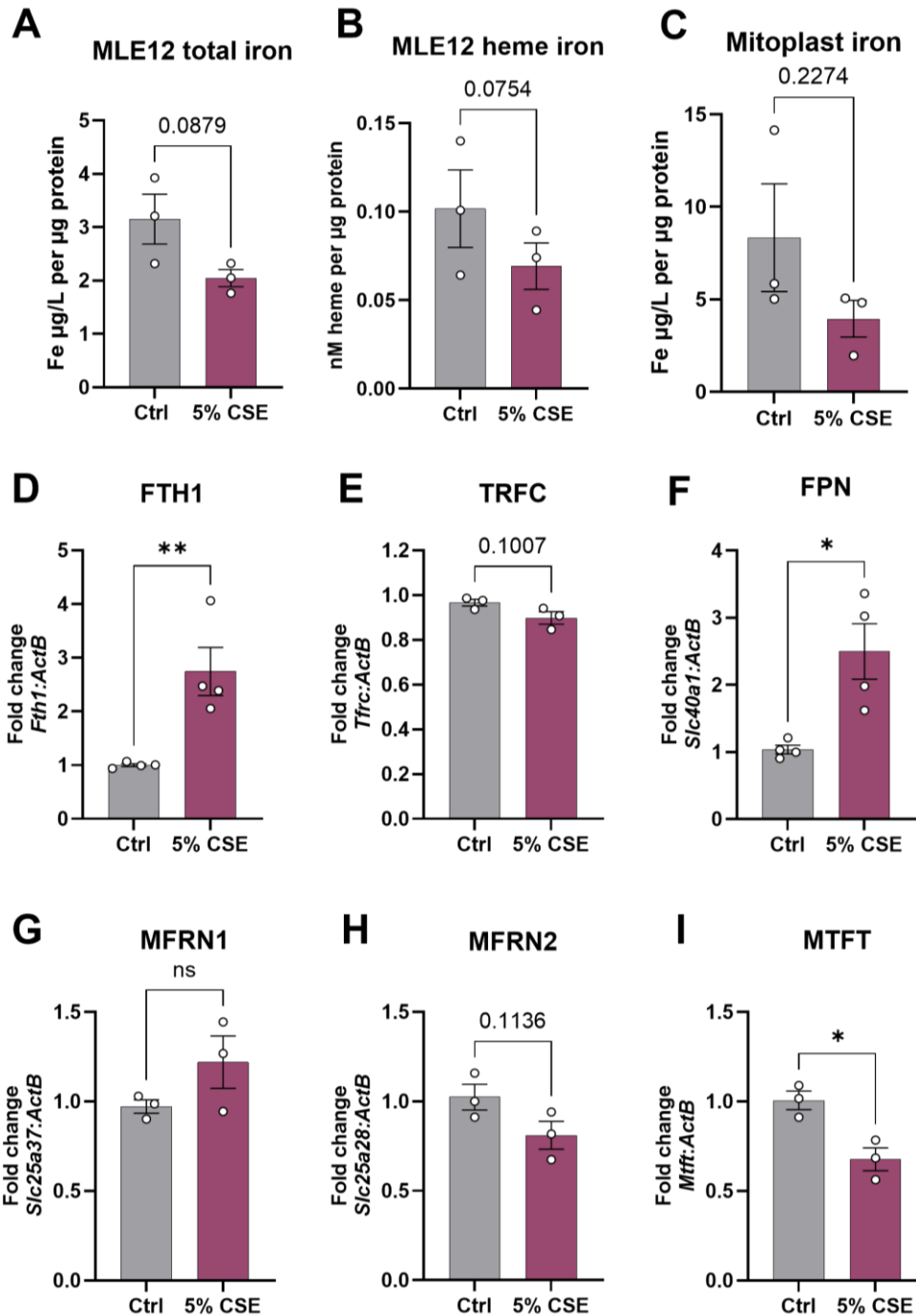


Fig 3.13: CSE may decrease cellular and mitochondrial iron in MLE12 cells: MLE12s were treated with 5% CSE or vehicle control (RPMI) for 18 hr (A) MLE12s plated in a 12 well plate were lifted through scraping, washed once, and lysed in RIPA buffer prior to digestion in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS. Each data point is representative of one biological replicate constituting the average of three technical triplicate wells of cells, normalised to protein content. (B) Intracellular heme content was measured through heme assay and normalised to protein content. (C) Mitoplasts were lysed in RIPA lysis buffer prior to digestion in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS, normalised to protein content and presented as fold change relative to untreated control. Data representative of mean $\pm$ S.E.M., $n=3$ biological replicates (D-I) Cells plated in a 6 well plate were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. All data representative of $n \geq 3$ biological replicates. All P value by t-test; * $P < 0.05$, ** $p < 0.01$, *** < 0.001 , ns = non-significant.

3.2.4 BMDMs from CS-exposed mice show transcriptomic remodelling distinct to AMs

Analysis of iron related gene expression in AMs and BMDMs originating from CS exposed mice revealed distinct changes in each cell type, with *Tfrc* the most highly expressed in AMs and *Ireb2* in BMDMs. Given the divergent expression patterns of iron-related genes in response to CS in both cell types, we next performed unbiased analyses to investigate the wider effects of CS on the AM and BMDM transcriptomes.

DeSeq2 analysis identified 481 genes which were significantly differentially expressed in AMs in response to 8 weeks CS exposure, with 313 genes significantly downregulated and 168 genes significantly upregulated (**Fig 3.14A**). *Vwf*, which encodes for von Willebrand factor, a platelet-binding glycoprotein, was the most significantly upregulated gene upon CS exposure in AMs, while *H2-eb1*, which encodes for a component of the MHC II complex, was most significantly downregulated. Gene ontology (GO) analysis using Biological Process terms revealed several immune activation related pathways to be enriched in genes downregulated in response to CS in AMs, including those pertaining to leukocyte differentiation and chemokine and cytokine response (**Fig 3.14B**). GO Biological Process analysis using genes significantly upregulated in CS failed to yield any significant pathways.

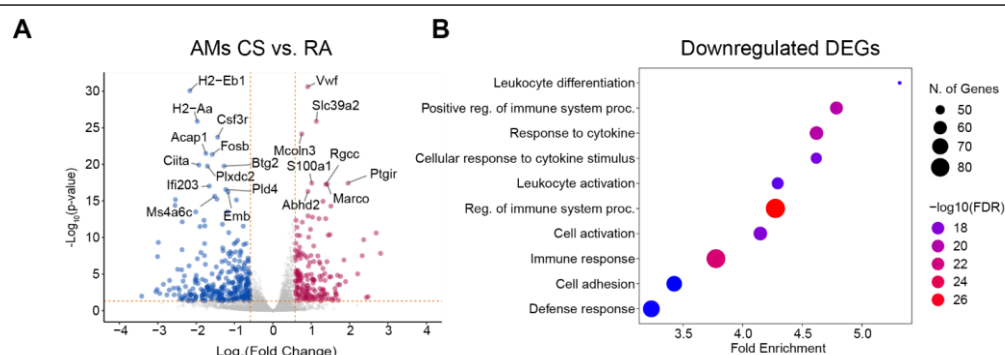


Fig 3.14: AMs from CS-exposed mice show a downregulation of genes associated with immune functions: AMs were isolated through bronchoalveolar lavage from mice exposed to 8 weeks cigarette smoke (n=4) and room air controls (n=4) and RNA isolated and sequenced. Significantly differentially expressed genes were identified through DeSeq2 analysis using an absolute Log2 fold change cutoff of 0.58 and adjusted *P* value <0.05. (A) Volcano plot of gene expression in CS versus RA: significantly upregulated genes are highlighted in red, significantly downregulated genes are highlighted in blue and non-significant genes in grey. (B) GO Biological Process analysis of significantly downregulated genes (FDR <0.05). RA = room air, CS = cigarette smoke, DEG = differentially expressed gene.

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, was used to assign a positive z-score to pathways activated by CS exposure and a negative score to those inhibited. As IPA considers the

fold change values of significantly differentially expressed genes relative to non-significant, it provides a more comprehensive picture of how the directionality of gene expression changes can combine to implicate the activation or inhibition of a particular pathway. Canonical pathway analysis yielded no significant pathways in AMs activated by CS exposure (FDR < 0.001). In line with previously published studies, significantly inhibited pathways in AMs were indicative of a blunted macrophage response to infection, including phagosome formation and pathogen-induced cytokine storm (Fig 3.15A-C). Pathways involved with antigen presentation and lymphocyte interaction and recruitment were also repressed, suggestive of a dysfunctional interaction between CS-exposed AMs and the adaptive immune system. Cumulatively, these analyses show a strong repression of the immune response upon CS exposure in AMs, particularly in the response to infection and in adaptive immune cell recruitment.

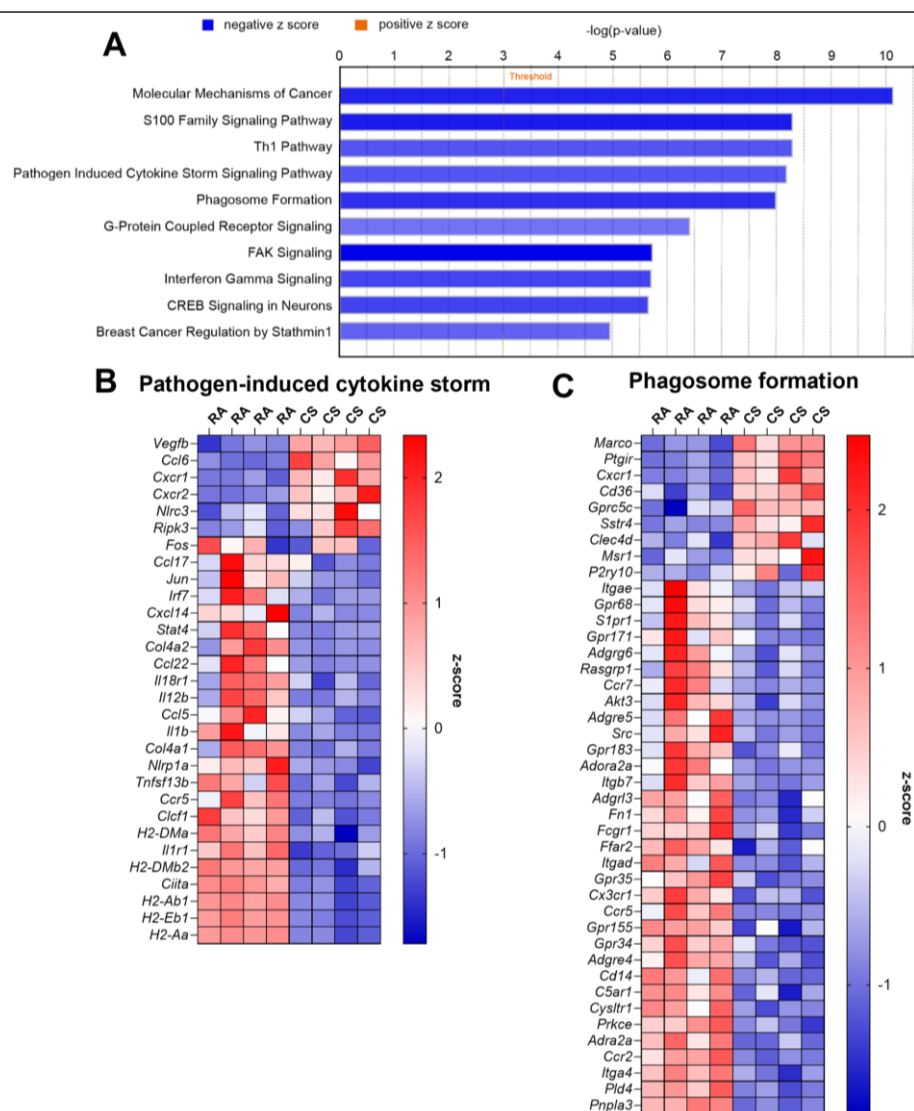


Fig 3.15: Ingenuity Pathway Analysis reveals a repression of innate immune cell function in CS AMs. (A) Canonical pathways identified by IPA in CS AMs compared to RNA and their activation z-score. Heat maps of genes significantly differentially expressed in the 'Pathogen Induced Cytokine Storm' (B) and 'Phagosome Formation' (C) pathways identified in (A).

DESeq2 analysis of BMDMs generated from the same above CS-exposed mice revealed 448 differentially expressed genes: 258 genes significantly upregulated and 190 genes significantly downregulated (**Fig 3.16A**). *Srm*, encoding for the spermidine biosynthesis gene spermidine synthase was the most significantly upregulated gene in BMDMs generated from CS-exposed mice; *Wdfy3*, the most significantly downregulated gene, encodes for an adaptor protein involved in selective macroautophagy and macrophage efferocytosis (242). Significantly upregulated genes in BMDMs from CS mice associated with nucleotide biosynthetic processes and pathways related to mitochondrial bioenergetics, including OXPHOS and ATP metabolism (**Fig 3.16B**). Significantly downregulated genes associated with GO Biological Processes relating to post-translational modification of proteins, including histone and protein lysine methylation, alongside chromatin organisation (**Fig 3.16C**).

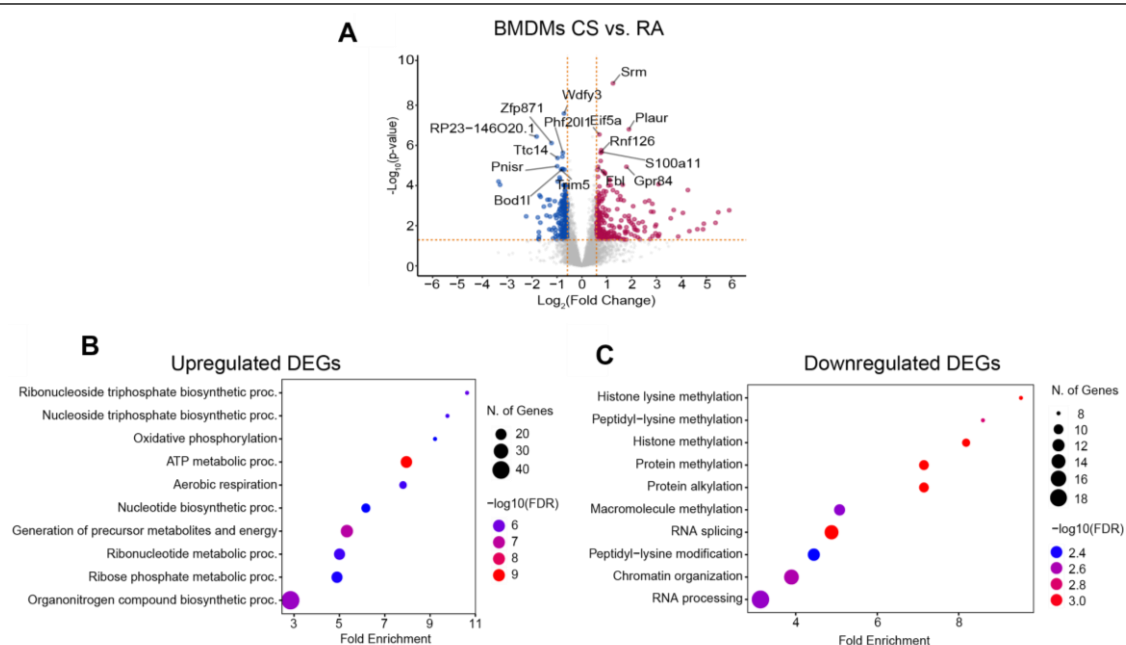


Fig 3.16: BMDMs from CS-exposed mice show altered expression of genes associated with mitochondrial bioenergetics and epigenetic modifications. BMDMs were generated from mice exposed to 8 weeks cigarette smoke (n=3) and room air controls (n=3) and RNA isolated and sequenced. Significantly differentially expressed genes were identified through DeSeq2 analysis using an absolute $\log_2\text{FC}$ cutoff of 0.58 and adjusted P value <0.05 . (A) Volcano plot of gene expression in CS BMDMs compared to RA: significantly upregulated differentially expressed genes are highlighted in red, significantly downregulated genes are highlighted in blue and non-significant genes in grey. GO Biological Process analysis of significantly upregulated (B) and downregulated (C) genes (FDR <0.05). RA = room air, CS = cigarette smoke, DEG = differentially expressed gene.

IPA identified sirtuin signalling as the most significantly enriched pathway by CS in BMDMs, showing an inhibited state (**Fig 3.17A-B**). OXPHOS was activated, with genes

encoding for several components of respiratory complexes I, II, and V enriched (**Fig 3.17C**). Cachexia signalling, a co-morbidity of COPD, was also activated (**Fig 3.17A**).

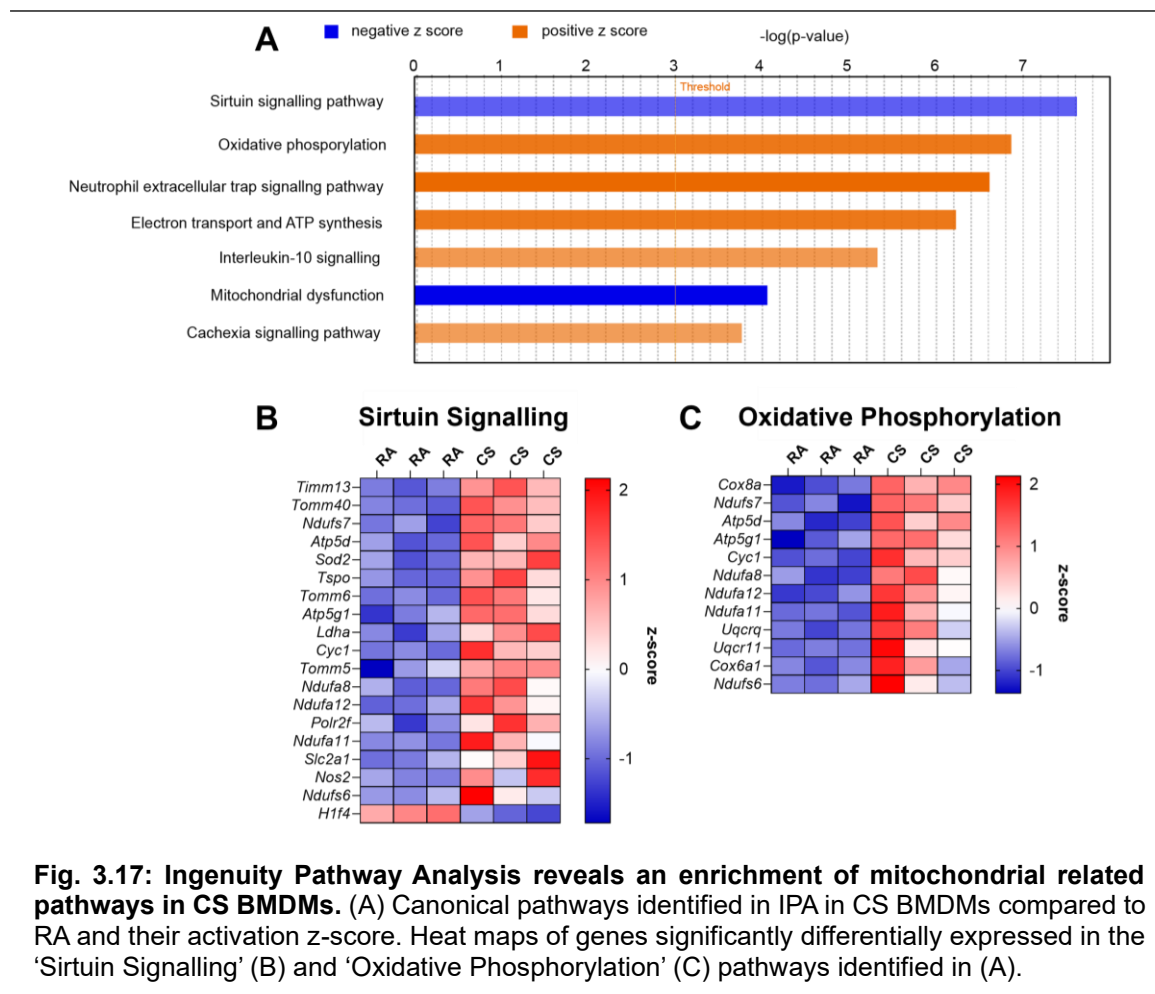


Fig. 3.17: Ingenuity Pathway Analysis reveals an enrichment of mitochondrial related pathways in CS BMDMs. (A) Canonical pathways identified in IPA in CS BMDMs compared to RA and their activation z-score. Heat maps of genes significantly differentially expressed in the ‘Sirtuin Signalling’ (B) and ‘Oxidative Phosphorylation’ (C) pathways identified in (A).

Comparing CS-induced DEGs between AMs and BMDMs, only 16 genes were commonly differentially expressed in response to CS (**Fig 3.18A**). Furthermore, only 3 of these common genes were expressed in 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 both mutually downregulated (**Fig 3.18B-C**). 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 3.18D**). Other diverging pathways relevant to COPD pathology include mitochondrial dysfunction, ROS signalling and wound healing and tissue pathways. Taken together,

these analyses show the opposing responses of lung-resident and monocyte-derived macrophages to CS.

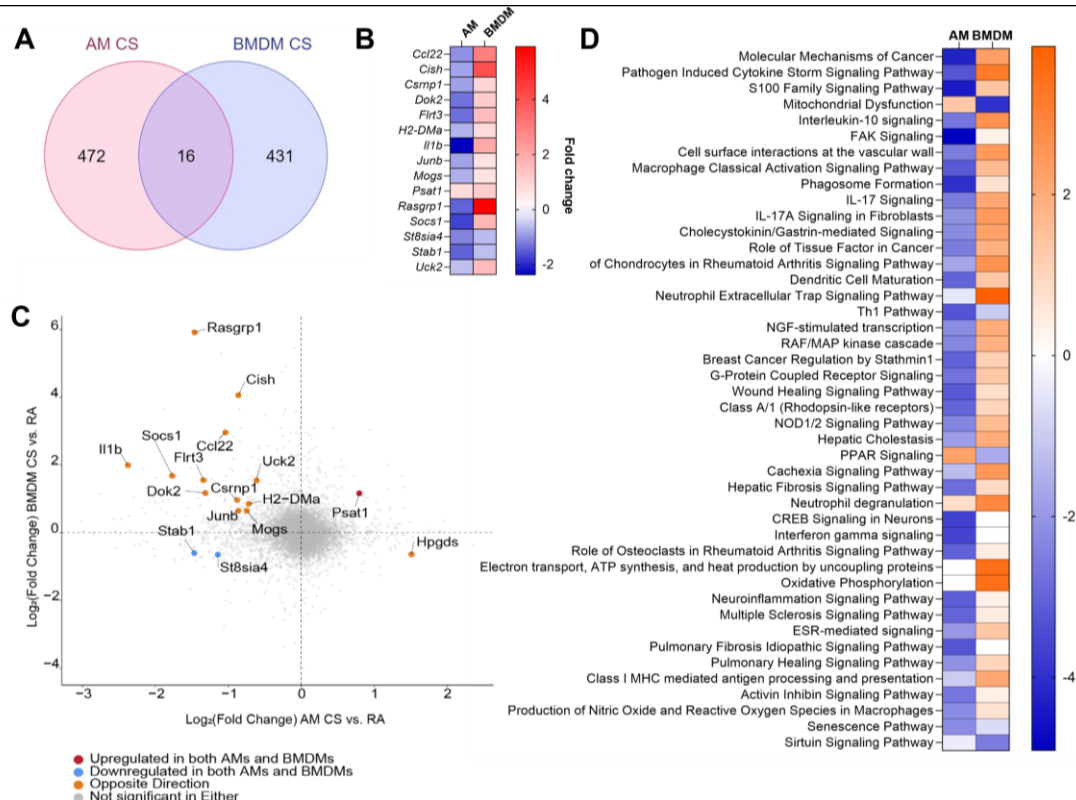


Fig. 3.18: CS exposure induces distinct transcriptomic remodelling in BMDMs compared to AMs. (A) Venn diagram of genes significantly differentially expressed in CS vs RA in AMs and BMDMs. (B) Heat map of fold change in gene expression in response to CS in genes identified as common to AMs and BMDMs in (A). (C) Log₂FC values in AMs in response to CS vs. the same in BMDMs. (D) Heat map comparing activation z-score of canonical pathways induced by CS in BMDMs and AMs significant (FDR <0.001) in either cell type.

3.2.5 Systemic monocytes from CS mice are pro-inflammatory and show epigenetic alterations

As a side observation in the above RNA-seq study independent of iron-related transcriptomic changes, we noticed the persistence of transcriptomic changes in BMDMs derived from CS-exposed mice. The presence of transcriptomic changes comparable to AMs even after 7 days in culture may indicate a long-lasting effect from CS exposure akin to innate immune memory or ‘training’ (243). Pathway analyses of genes differentially expressed in BMDMs generated from CS-exposed mice compared to RA controls implicated alterations in epigenetic and inflammatory gene expression (**Fig. 3.16-17**). As these BMDMs were terminally differentiated from bone marrow progenitors from CS-exposed mice, this suggests a capacity of CS to have a persistent effect on systemic monocytes prior to their recruitment to the directly CS-exposed lung. It is also unclear at what point in the differentiation process these changes may manifest, and whether they are unique to progenitors in the macrophage lineage. A CS-mediated effect on the

production of these macrophage progenitors (myelopoiesis) and their inflammatory state prior to their entry to the lung is also unexplored.

To query this, wild type (WT) C57BL6/J mice were exposed to CS and bone marrow isolated to profile myelopoiesis through flow cytometry*. Due to technical limitations the 8 week CS exposure model used for RNA-seq could not be repeated; instead, mice were exposed to 6 weeks CS, which induces comparable inflammatory changes (112). The absolute number of hematopoietic stem cells was significantly increased in CS-exposed mice, indicative of heightened hematopoiesis (**Fig. 3.19A**). The percentage of multipotent progenitors of the myeloid (MPP3) and the lymphoid (MPP4) lineages were unchanged (**Fig. 3.19B, C**). However, myeloid lineage-committed common myeloid progenitors (CMP) and granulocyte myeloid progenitors (GMP) trended towards increase in proportion (**Fig. 3.19D, E**) relative to RA mice, while common lymphoid progenitors (CLP) were unchanged (**Fig. 3.19F**). These results suggest increased hematopoiesis in the bone marrow in CS exposure, with hints that this may be disproportionately skewed towards myeloid cell production.

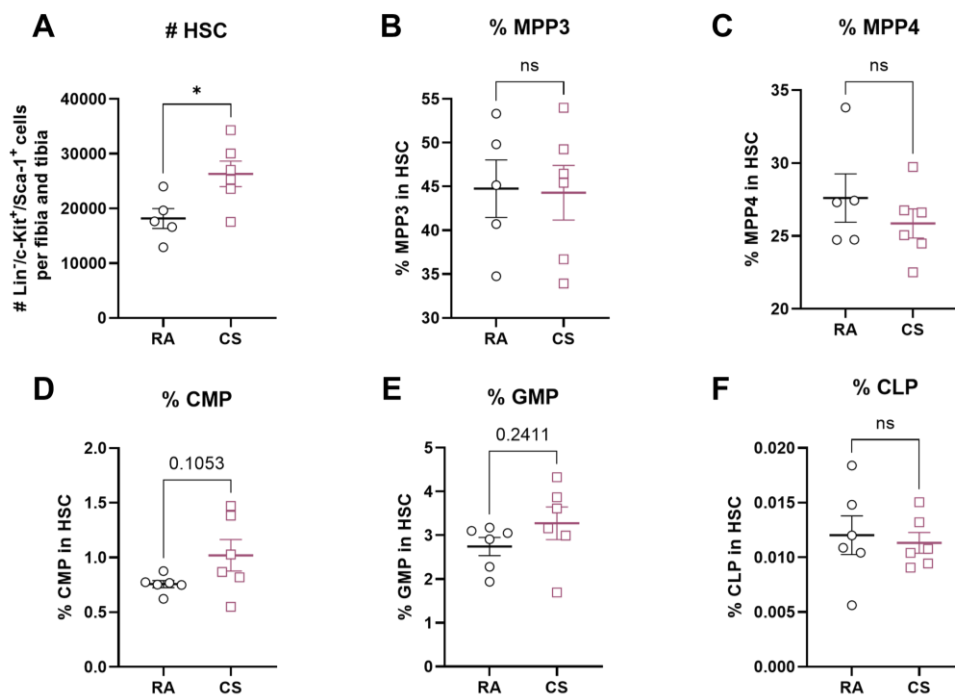


Fig. 3.19: CS exposure may increase hematopoiesis. Mice were exposed to CS for 6 weeks as described, sacrificed through CO₂ euthanasia and bone marrow isolated to profile hematopoietic progenitors. (A) Total number of HSCs in bone marrow defined as lineage negative, cKit⁺, Sca-1⁺. Proportions of MPP3 (B), MPP4 (C), CMP (D), GMP (E), CLP (F) in HSCs. Data representative of mean ± S.E.M. from one independent experiment, n=5-6 per group. P value by unpaired t-test; *= $P < 0.05$, ns = non-significant. HSC = hematopoietic stem cell, MPP = multipotent progenitors, CMP = common myeloid progenitor, GMP = granulocyte myeloid progenitor, CLP = common lymphoid progenitor.

* I preformed the CS exposure model with Sonia Gesmundo of the Cloonan lab, and euthanised mice and isolated tissue. Staining, acquisition and analysis of samples for flow cytometry was performed in collaboration with Dr. Anna Ledwith and Cian Horneck Johnston of the Sheedy Lab, Trinity College Dublin.

Blood monocytes arise from GMP cells and travel in circulation to distal tissues, where they can differentiate to macrophages (244). Blood was isolated from CS-exposed mice to profile changes in epigenetic and inflammatory profiles as observed at the transcript level in RNA-seq data, and whether these changes are common to other circulating myeloid cells like neutrophils. Pro-inflammatory monocytes (defined as Ly6C-high) from CS mice had significantly lower H3K27Ac levels (**Fig. 3.20A**), with a trend toward decrease in anti-inflammatory (Ly6C-low) monocytes (**Fig. 3.20B**). Notably, H3K27Ac levels were also significantly decreased in blood neutrophils (**Fig. 3.20C**) suggesting this loss of acetylation is common to granulocytes as well as monocytes. Next, C-C chemokine receptor type 2 (CCR2) levels were assessed, which is vital for the deployment of monocytes from the blood to subsequent tissues (245). CCR2 expression was significantly elevated in pro-inflammatory monocytes (**Fig. 3.20D**) and trended towards increase in anti-inflammatory monocytes (**Fig. 3.20E**). No change in CCR2 expression in neutrophils was observed (**Fig. 3.20F**), meaning this elevated expression of pro-inflammatory markers in CS appears restricted to monocytes in the blood. Cumulatively, these data show hematopoiesis is increased in CS and seemingly skewed towards myelopoiesis, manifesting in the deployment of pro-inflammatory, epigenetically remodelled monocytes to the blood.

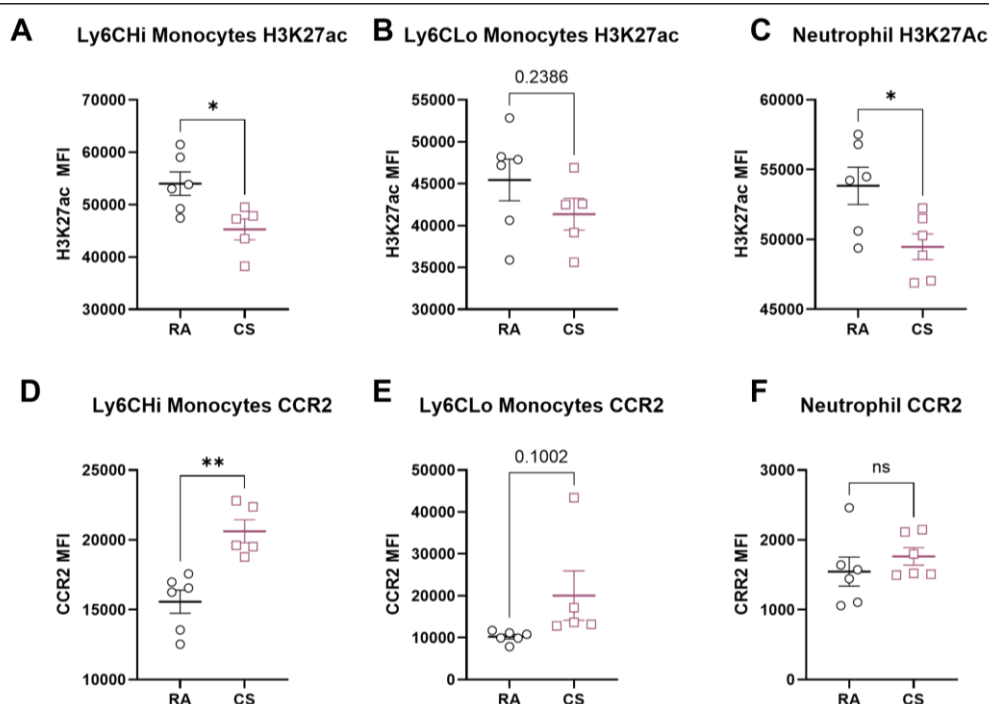


Fig. 3.20: Blood monocytes from CS mice show reduced H3K27Ac and increased CCR2 expression. Mice were exposed to 6 weeks CS as described, sacrificed through CO₂ euthanasia and blood isolated for immune cell profiling through flow cytometry. MFI H3K27Ac in (A) Ly6C-high monocytes, (B) Ly6C-low monocytes and (C) neutrophils. MFI CCR2 in (D) Ly6C-high monocytes, (E) Ly6C-low monocytes and (F) neutrophils. Data representative of mean $\pm$ S.E.M. from one independent experiment, n=5-6 per group. *P* value by unpaired t-test; *=*P*<0.05, **=*P*<0.01, ns = non-significant. MFI = mean fluorescence intensity.

3.3 Discussion

The relevance of pathogenic mitochondrial iron loading to lung immune cells in COPD is poorly understood. In this chapter, we show dysregulated expression of genes associated with mitochondrial iron metabolism in AMs isolated from smokers and individuals with COPD, including downregulation of MFRN1 and 2 gene expression. This change is mimicked in whole blood RNA-seq of current and former smokers, but contrastingly MFRN1 gene expression is significantly enriched in blood monocytes during exacerbations. Murine AMs from a CS-exposure model of COPD show elevated gene expression of FTH1 and TFRC, indicative of increased iron uptake and storage, and a trend towards decrease in MFRN1 and 2 gene expression. These changes were not observed in BMDMs generated from these CS-exposed mice, illustrating the unique effects of CS on AM iron metabolism. Treatment of a murine alveolar macrophage-like cell line with CSE *in vitro* as an experimental model of COPD allowed for absolute measurement of mitochondrial iron by GF-AAS, revealing trending increases in mitoplast iron with CSE treatment. Intriguingly, despite the lack of change in iron metabolism genes, BMDMs generated from CS mice still show transcriptomic changes relating to epigenetic remodelling, mitochondrial bioenergetics and cytokine production, with further investigation through flow cytometry suggesting these changes may occur centrally during myelopoiesis. Our results show alterations in mitochondrial iron metabolism in macrophages in human COPD for the first time, with suggestions this may extend to increased mitochondrial iron levels in AMs subjected to *in vitro* COPD.

MFRN1 and 2 are paralogous proteins which both function in iron import to the mitochondrial matrix (246). Little is known about any differences between the two, or indeed the circumstances that would drive a cell to favour expression of one gene over the other. It has been theorized that MFRN1 may be more important in erythroid tissues: murine *Slc25a37* (MFRN1) expression is elevated during erythroid maturation while *Slc25a28* (MFRN2) expression remains stable, and defects in terminal erythroid differentiation in *Slc25a37* deficient zebrafish mutants cannot be rescued by *Slc25a28* overexpression (24). While this has been interpreted in the literature as a specificity of MFRN1 for heme synthesis in erythroid tissues, MFRN1 may equally have a higher capacity for iron import than MFRN2: indeed, while the biochemistry of iron transport through MFRN1 has been characterised, MFRN2 is unclear (247). MFRN1 could therefore be favoured as a more efficient means of iron import to fuel cofactor production in metabolically demanding conditions, such as macrophage activation, than MFRN2. The striking enrichment of MFRN1 observed in peripheral human monocytes associated with frequent exacerbations supports this (**Fig. 3.6**), with most exacerbations infectious in origin

and necessitating the recruitment of monocytes reliant on iron-containing cofactors for their activation. *SLC25A37* expression is enriched in blood monocytes during septic cardiomyopathy, again implicating peripheral monocyte MFRN1 expression in the response to an infectious event (248). However, the COPDGene study does not collect microbiology-related data from enrolled individuals, so exacerbations recorded cannot be stratified by their infectious or sterile etiology (233).

Mitochondrial iron loading as a driver of disease pathogenesis has previously only been investigated in a murine CS exposure model of COPD (50). Our analyses are the first to investigate mitochondrial iron metabolism in human smoking and COPD, and in a cell type specific manner. MFRN1 and 2 expression were decreased in AMs isolated from individuals who smoke or have a COPD diagnosis (**Fig. 3.2D**), with interrogation of the COPD Cell Atlas revealing MFRN1 is enriched in monocyte-derived AMs (**Fig. 3.3A**). Monocytic MFRN1 expression deconvoluted from whole blood RNA-seq data showed a striking association with exacerbation risk (**Fig. 3.6**). Taken together, these data suggest a strong association of a peripheral mitochondrial iron-related gene signature with smoking and COPD, with *SLC25A37* expression negatively associated with smoking history and positively associated with exacerbation events. The limitations in determining absolute levels of mitochondrial iron in cell populations of low abundance poses difficulties in interpreting how gene expression of MFRN1 or MFRN2 correlates with mitochondrial iron content. Increased expression could be interpreted as an increase in mitochondrial iron, or as an attempt by a cell to increase mitochondrial iron levels when depleted, with the opposite true when MFRN1 and 2 are decreased. How this mitochondrial gene signature may relate to the consumption of mitochondrial iron for co-factor production to fuel macrophage function, and whether these cofactors are utilised distinctly in systemic monocytes versus lung-resident macrophages, would provide a more direct readout into how MFRN1 and 2 expression directly translates to mitochondrial iron usage.

Expression of MFRN1 and 2 at the mRNA level was increased in our *in vitro* model of AM smoke exposure (**Fig. 3.10, 3.11**) but decreased in AMs isolated from smokers (**Fig. 3.2A**). As we observed an increase in total mitoplast iron *in vitro* at the same dose and time, these changes are likely reflective of increased mitochondrial iron import through MFRN1 and 2 to drive this iron accumulation in the mitochondrial matrix. Accordingly, owing to chronic smoke exposure in humans versus the more acute nature of the *in vitro* model, pathological mitochondrial iron loading may have occurred potentially decades ago in these patients. This downregulated expression of MFRN1 and 2 observed in human smoking in our data is therefore likely indicative of a cell protective effort to curtail already excessive iron mitochondrial loading accrued through years of smoking; however, this is

difficult to say conclusively without raw iron measurements. Determining mitochondrial iron levels as in CSE-treated FLAMs in **Fig. 3.10A** in human AMs is difficult owing to the high amount of starting material required for subcellular fractionation-based methods: the development of more refined fluorescent probes to quantify mitochondrial iron will enable this in limited cell numbers.

Our analysis of RNA sequencing data of AMs isolated from human BALF did not detect any differences in AM iron related gene expression between smokers and smokers with COPD (**Fig. 3.2**). In the context of COPD, the molecular switch between health and disease in smokers is not understood. As we were only provided with expression data from a subset of genes involved in iron metabolism, it is difficult to determine the extent of global gene expression change in AMs between the two groups. Previous work published from our group used another subset of this data, where gene expression of genes in the human MitoCarta dataset was accessed. This identified 149 genes differentially expressed between smokers with and without COPD (155). This suggests that observed changes in iron metabolism gene expression may be specific to smoking rather than COPD, but as all COPD patients in this study are ever-smokers this is difficult to delineate in our data. COPD patients in this study were predominantly individuals presenting with GOLD stage I or II disease, also biasing our analyses towards those with an earlier stage of disease.

In our analysis of peripheral blood RNA-seq datasets in human smoking, *SLC25A37* and *SLC25A39* were mutually downregulated. *SLC25A39* encodes for an inner mitochondrial membrane protein recently characterised as an importer of glutathione into the mitochondrial matrix from the cytoplasm (249), a critical component of FeS cluster biogenesis and a key antioxidant in curtailing excessive ROS production. The ability of *SLC25A39* to import glutathione is itself regulated by iron availability: in physiological conditions *SLC25A39* is degraded by an associated protease, but in mitochondrial glutathione deficiency this degradation is inhibited through the competitive binding of an FeS cluster (53). A genetic lethality screen identified *SLC25A37* and *SLC25A39* as interacting partners and showed functional copies of both genes are essential for efficient OXPHOS (236). The downregulation of both genes in peripheral monocytes is therefore another indication of aberrant mitochondrial iron metabolism in COPD, the functional effects of which would be elucidated by further characterisation of the *SLC25A37/SLC25A39* axis in both health and disease.

Our pathway analyses of AMs isolated from an 8-week CS exposure model of experimental COPD revealed an inhibition of immune response pathways, including those associated with phagosome formation and cytokine response (**Fig. 3.15**). This is in line

with numerous functional studies from our group and others illustrating defective AM bactericidal capacities in smoking and COPD, including inefficient pro-inflammatory cytokine secretion (112) and uncontrolled ROS production (145, 250). An impairment of adaptive immune signalling was also present in CS AMs, with numerous components of the MHC II complex downregulated and Th1/Th2 signalling identified as a significantly inhibited pathway in our IPA analysis (**Fig. 3.15A**). COPD AMs have reduced antigen-presenting capacities (251) but AM-lymphocyte interactions are poorly studied in COPD, particularly given the presence of increased lymphocytes and lymphoid follicles in the COPD lung (252). Both iron and iron-containing proteins can influence

Our results from our sequencing of AMs and BMDMs in our murine CS model highlight the ability of CS to uniquely alter distal compartments compared to the directly exposed lung. CS exposure induced comparable numbers of differentially expressed genes in BMDMs compared to AMs (448 vs 481), despite the former being cultured *in vitro* for 7 days. This, in concert with the implications of histone methylation in GO analyses (**Fig. 3.16C**), suggests CS may exert systemic effects through epigenetic changes. Epigenetic modifications are known to associate with monocyte-mediated tissue destruction in COPD, with elevated expression of the arginine methyltransferase PRMT7 in monocytes leading to ferroptotic cell death in the alveolar epithelium (253). In line with this, our analysis of blood monocytes from CS mice revealed decreased levels of the H3K72Ac mark in pro-inflammatory monocytes compared to RA controls, accompanied by elevated expression of the tissue invasion marker CCR2 (**Fig. 3.20**). Cells are reliant on iron for epigenetic reprogramming, with TET and histone-lysine demethylation (KDM) enzymes requiring iron for DNA and histone demethylation, respectively (254, 255). How changes in mitochondrial iron metabolism would affect the ability of these enzymes to govern monocyte memory, and the functional effects of such a memory response in ex-smokers, remains to be seen.

Previous transcriptomic studies comparing COPD lung tissue and blood have shown enrichments of opposing gene ontology pathways in each tissue, asserting the lung and blood as distinct compartments in COPD (256). Indeed, little overlap is observed in inflammatory markers between the blood and the sputum of COPD patients, suggesting that systemic inflammation independent of inflammatory spillover from the lung may play a larger role in driving COPD pathophysiology than previously appreciated (257). Our findings show that this distinction can exist at the level of a single cell type localised to each compartment in a COPD model (**Fig. 3.18**). Importantly, BMDMs are ontogenically, morphologically and functionally distinct from AMs and as such should not be used for *in vitro* purposes to model the response of a lung resident macrophage to insult. However,

our work has demonstrated the ability of CS to exert a transcriptomic effect on BMDMs, meriting the study of BMDMs from experimental models of COPD as effector cells in their own right. Macrophages derived from recruited monocytes in COPD and smoking can uniquely orchestrate tissue damage in the lung (143, 253), and CS can affect macrophage populations at distal organs (258, 259). CS exposure increased hematopoiesis in mice (**Fig. 3.19**), with analysis of progenitor proportions suggesting this may be skewed towards myelopoiesis, concordant with previously published data from our group showing inefficient erythropoiesis in CS-exposed mice (112). Distal cues have been implicated in altering myelopoiesis, including alterations in the gut microbiota (260) and myocardial infarction (261). While monocytes were previously proposed to retain a high degree of plasticity in circulation until tissue invasion, recent studies have suggested this plasticity can be limited at the progenitor level before egress from the bone marrow (262). A central effect on immunity can affect multiple sites, as tissue-resident macrophage pools are replenished at various organs by monocytes issued to circulation from the bone marrow. CS is a risk factor for numerous cardiovascular and metabolic diseases beyond the lung, and over 75% of COPD patients present with two or more chronic diseases (263). Administration of healthy bone marrow cells to a rodent model of experimental COPD has been shown to reverse emphysema (264), but the capacity of systemic monocytes to both be effector cells in the systemic manifestations of COPD, and therapeutic targets in this, requires further study.

3.3.1 Limitations

While well powered, gene expression data from human AMs detailed in **Fig 3.2** was acquired through microarray, which cannot identify any splice variants or differential isoforms present compared to modern next generation sequencing-based methods. Only data related to iron-associated gene expression from this study was made available to our group, limiting our ability to conduct unbiased ontology and pathway analyses. All COPD patients recruited to this study were smokers, making it difficult to determine the extent of iron-related AM transcriptomic alterations in COPD patients of other etiologies. Our analyses in **Fig. 3.4** and **3.6** were generated using whole blood RNA-seq data, which we have used to infer gene expression changes in peripheral leukocytes. While the methods of these papers state that red blood cells were depleted prior to RNA isolation, a contribution of red blood cell contamination to the observed changes in iron-related gene expression cannot be ruled out, particularly in relation to genes encoding for components of the heme synthesis pathway given the reliance of erythroid tissues on this process. The optimal method of assessing changes in peripheral monocyte gene expression to combat this would lie in sequencing isolated mononuclear cells, but we are limited by the data

available publicly. As these datasets are robustly powered, our analyses remain valuable and provide insight into iron-related gene expression across COPD and smoking.

Although AMs are the predominant cell type in murine 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. BMDMs are not a true model of a monocyte: numerous single-cell studies have previously elegantly defined the ontogenies of monocyte-derived alveolar macrophages in human COPD and its experimental models. 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 differentially expressed genes in AMs and BMDMs. 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 our observed changes in BMDMs, particularly the supplementation of M-CSF to drive differentiation, are unknown. Trending increases in myelopoiesis were observed in CS mice but require further experimental power to confirm changes. Epigenetic changes were confirmed to be present in blood monocytes in CS exposure, where H3K27Ac levels were interrogated as an exemplar histone modification (**Fig. 3.20**). Future efforts should interrogate histone methylation in these monocytes to validate BMDM RNA-seq data, with a particular focus on those previously implicated in smoking and COPD like H3K27me3 and H4K20me2 (265, 266). We also did not assess DNA methylation status through 5-methylcytosine changes: hypomethylation has previously been observed in the blood of smokers (267), meaning changes are likely present in peripheral immune cells.

3.3.2 Conclusions

This chapter has identified differential expression of iron and mitochondrial iron-associated genes in macrophages across several large-scale human cohorts of smoking and COPD, in both lung and systemic compartments. Macrophages from these compartments in experimental COPD have divergent transcriptomic responses to CS exposure, which also increases hematopoiesis and alters the epigenetic profile of circulating monocytes. Our data shows changes in MFRN1 and 2 expression in human AMs for the first time alongside an ability of CS exposure to increase AM mitochondrial iron *in vitro*, deepening our understanding of AM iron loading in COPD.

Chapter 4: Iron and *S. pneumoniae*-alveolar macrophage dynamics in health and disease

4.1 Introduction

The decline in lung function in individuals with COPD is often not linear. In a significant portion of individuals, disease progression can be punctuated by episodes of acute worsening of symptoms termed exacerbations, which disproportionately accelerate loss of lung function and heighten risk of mortality (137, 268). Exacerbations are primarily driven by lower respiratory tract infection which can be both viral (rhinoviruses, respiratory syncytial virus, influenza) and bacterial (*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*) (138, 269, 270). As individuals with a history of smoking but preserved lung function can still experience exacerbations equivalent to those observed in disease (271), a significant environmental component with unknown molecular cause likely underpins exacerbation risk.

S. pneumoniae is one of the most prevalent pathogens in the COPD lung and is frequently implicated in acute exacerbations (139, 272). Detection of *S. pneumoniae* nucleic acids in blood isolated from current and former smokers also positively associates with dyspnoea (shortness of breath) in these individuals (273). While it is asymptotically carried in the upper respiratory tract in healthy individuals, susceptible individuals, including those who smoke or suffer from a chronic lung disease, are at risk of invasive pneumococcal disease through extravasation of bacteria to the lower respiratory tract (176). However, the host factors driving this increased susceptibility are poorly understood.

AM iron loading is an established hallmark of COPD. Crucially, both BALF iron levels (163) and AM hemosiderin (274) associate with exacerbation frequency in COPD. As numerous bacteria, both intracellular and extracellular, are phagocytosed by macrophages, this elevated intracellular iron will likely promote bacterial pathogenicity. *S. pneumoniae* is one of the most successful pathogens in COPD, with its lack of siderophore-producing machinery suggesting it may favour non-classical forms of iron uptake. The relationship between *S. pneumoniae* growth in COPD AMs and intracellular iron levels therefore presents an underexplored axis in the molecular mechanisms underpinning patient susceptibility to acute infection. Based on this, and findings relating to AM iron and mitochondrial iron dysregulation in **Chapter 4**, we hypothesised that ***S. pneumoniae* can use macrophage iron for its virulence, which is further promoted by the iron overload observed in COPD.**

Invasion of *S. pneumoniae* to the lower respiratory tract triggers expression of bacterial virulence factors to establish a bacterial niche. Pneumolysin, which can be secreted from pneumococci or coat the outside of the bacterial capsule, targets MRC-1⁺ immune cells like AMs in the lung to diminish NF- κ B signalling (275). This dampens pro-inflammatory cytokine production and ensuing monocyte and neutrophil recruitment to create an alveolar microenvironment permissive to *S. pneumoniae* proliferation (176). While classically characterised as an extracellular pathogen, *S. pneumoniae* possesses the necessary machinery to mount an acid stress response, suggestive of a capacity to withstand the acidic environment of the phagolysosome that is typical of intracellular bacteria (276, 277). Recent studies have outlined specific conditions and niches in which *S. pneumoniae* can replicate intracellularly, which was first characterised in a CD169⁺ population of splenic macrophages (278). To date, intracellular survival in lung-like macrophages has been exclusively in viral co-infection models, with persistence observed AMs isolated from HIV-positive individuals and in THP-1 cells previously infected with influenza virus A (279, 280). Whether this phenomenon may occur in AMs without prior insult is unknown.

COPD progression is underpinned by persistent inflammation in the airspaces, with patients showing elevated levels of the pro-inflammatory cytokines IL-6, IL-1 β and TNF- α in sputum and BALF (281). These cytokines are increased in the exhaled breath condensate of patients experiencing an acute exacerbation compared to stable COPD (282). Chemokines, including IL-8, CCL5 and CXCL10, are also increased in COPD BALF and sputum, which correlates with an increase in absolute numbers of macrophages, neutrophils, and lymphocytes (151, 283, 284). Despite this increased abundance in the host machinery necessary for bacterial killing, individuals with COPD remain highly susceptible to acute respiratory infection.

Defective phagocytosis in COPD and smoking has been reported in human AMs and monocyte-derived macrophages. This defect in phagocytosis appears to be uniquely linked to pathogens: the decreased capacity of COPD macrophages to phagocytose *S. pneumoniae*, non-typeable *H. influenzae* and *Aspergillus fumigatus* was not observed when using polystyrene beads (285, 286). However, these findings are based on the use of the nonviable heat-killed bacteria or sterile bacterial cell wall components: they therefore cannot accurately account for the response of a biologically active bacterium when internalised by a host macrophage. Bacteria have sophisticated genomic rearrangement and protein secretion systems to subvert the host immune response and facilitate intracellular survival and proliferation, which cannot be recapitulated if inactivated. Specifically in COPD, clinical isolates of non-typeable *H. influenzae* show genomic

adaptations promoting enhanced survival in the respiratory tract (287). COPD AMs also showed impaired bacterial killing when challenged with live, opsonised *S. pneumoniae* compared to healthy non-smokers (250), illustrating the importance of using live bacteria to fully unravel host-pathogen dynamics in COPD.

Acquisition of host iron is a crucial determinant of bacterial success (94). *S. pneumoniae* is relatively unique among bacteria in that it does not express its own endogenous siderophores, which are a classical means of bacterial iron uptake from the extracellular space. *S. pneumoniae* also cannot use transferrin or lactoferrin bound iron for its growth (288). Despite it being a classically extracellular bacterium, this seems to indicate *S. pneumoniae* lacks the resources to capture traditional sources of extracellular iron. This may suggest a preference for acquiring more atypical sources of iron, or through intracellular acquisition. However, little is known about how AM intracellular iron levels change with *S. pneumoniae* infection, or indeed with any bacterial infection. As with host-pathogen dynamics in COPD, studies investigating how bacteria can change host cell iron levels are limited by use of TLR ligands to infer pathogen-induced changes in host iron. Bacteria, including *S. pneumoniae*, can dynamically remodel their transcriptome and proteome to fuel success in the host-pathogen fight, meaning the use of live bacteria is crucial in identifying putative host iron sources.

4.1.1 Aims

While *S. pneumoniae* needs iron for growth, the effects of *S. pneumoniae* infection on host macrophage iron storage and utilization both at homeostasis and in the COPD disease state is poorly understood. This chapter will characterise the interaction of *S. pneumoniae* with AMs in COPD through the following specific aims (**Fig. 4.1**):

- Determining if *S. pneumoniae* can replicate intracellularly in FLAMs and human AMs.
- Measuring iron-related changes in FLAMs following *S. pneumoniae* infection.
- Investigating the effects of CS exposure on bactericidal and pro-inflammatory responses to *S. pneumoniae* in FLAMs and human AMs.

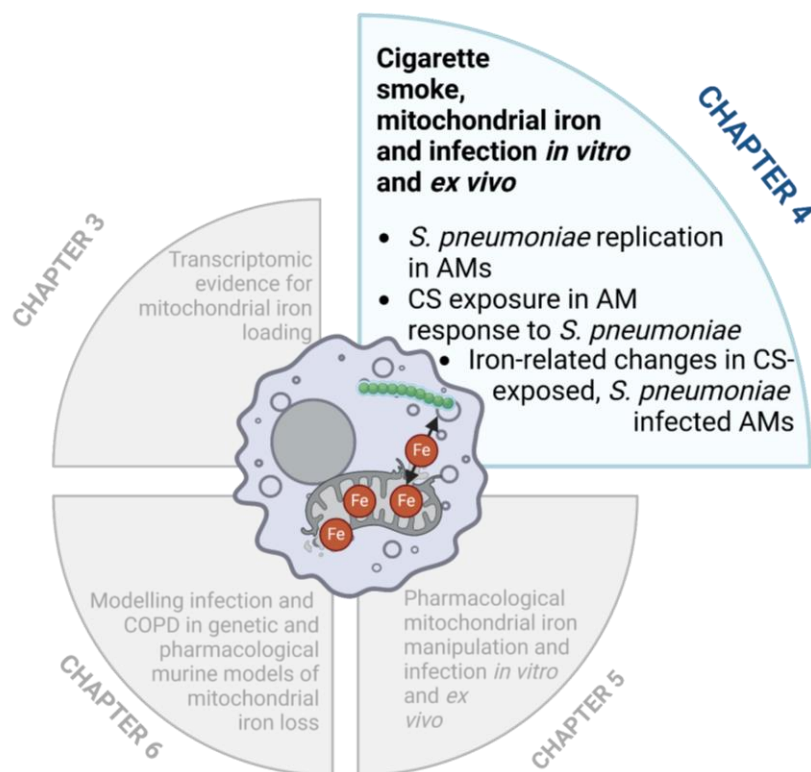


Fig. 4.1 Summary of aims of Chapter 4.

4.1 Results

4.2.1 *S. pneumoniae* can replicate intracellularly in AMs

While classically considered an extracellular bacterium, recent studies have described conditions in which *S. pneumoniae* can persist intracellularly, including in AMs isolated from HIV patients (279). To establish if intracellular survival was possible in our alveolar macrophage-like model *in vitro*, FLAMs were infected with a well-characterised laboratory serotype 2 strain of *S. pneumoniae*, D39 (289) (**Fig. 4.2A**). After an initial 30 min incubation of FLAMs with *S. pneumoniae* to allow for bacterial uptake, the antibiotic gentamicin was added to kill any extracellular non-internalised bacteria. One group of cells was lysed immediately using saponin, a naturally occurring detergent which permeabilises mammalian cells but has no effect on *S. pneumoniae* viability. Cell lysates were then diluted and streaked on agar plates to establish baseline colony forming unit (CFU) levels intracellularly. Here, and throughout, this initial timepoint is referred to as 0 hr post-infection. Fresh media was added to the remainder of cells, which were lysed at later timepoints to determine if bacteria could replicate (increased CFU compared to baseline) or were unable to persist inside FLAMs (decreased CFU compared to baseline) (**Fig. 4.2A**). At a multiplicity of infection (MOI) of both 10 and 20, *S. pneumoniae* CFU increased in a time dependent manner, with bacterial burden peaking 6 hr post-infection and decreasing after 24 hr (**Fig. 4.2B**). We hypothesized that this decrease in bacterial burden may be reflective of FLAM cell death no longer facilitating intracellular persistence. This was confirmed by measuring cell viability in FLAMs 24 hr post-infection, which was significantly decreased at an MOI of 20 compared to uninfected control (**Fig. 4.2C**). A genetically modified D39 *S. pneumoniae* fluorescent strain was used to further investigate this, gifted from Prof. Jan-Willem Veening (*University of Lausanne, Switzerland*) which expresses GFP under the control of the promoter for the *hlpA* housekeeping gene (82). Using the same gentamicin-based approach to killing non-internalised bacteria, there was a significant increase in fluorescence 1 hr post-infection at MOI 10 and 20 (**Fig. 4.2D**). To investigate whether *S. pneumoniae* can replicate intracellularly in other macrophage populations, *S. pneumoniae* infection was repeated in BMDMs and CFU analysed 1 hr post-infection. This revealed a decrease in internalised bacteria, suggestive of bacterial killing (**Fig. 4.2E**). Together, these results illustrate the ability of *S. pneumoniae* to persist and replicate intracellularly FLAMs, a phenomenon not observed in BMDMs.

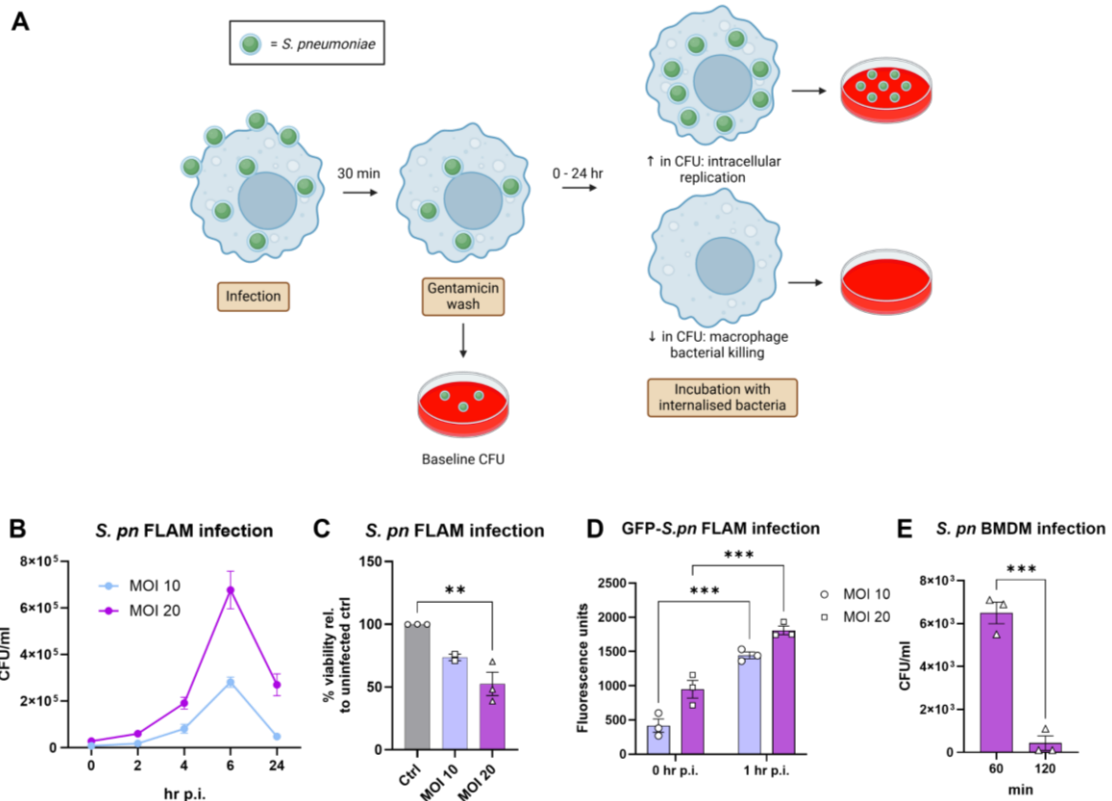


Fig 4.2: *S. pneumoniae* can persist intracellularly in FLAMs. (A) Schematic of *in vitro* infection model. FLAMs were plated at a density of 3×10^4 cells/well in a 96 well plate. Cells were washed once in PBS and media replaced containing *S. pneumoniae* diluted to the specified MOI. Following centrifugation, cells were incubated for 30 min before incubation with 200 ng/mL gentamicin to remove extracellular bacteria for 15 min. One group of cells was immediately lysed (0 hr p.i. group), serially diluted in PBS and streaked on Todd Hewitt agar plates with 5% v/v sheep's blood for CFU enumeration. Fresh media was added to other groups lysed at later timepoints. (B) Time course of FLAM *S. pneumoniae* infection, $n=3$ biological replicates conducted in technical triplicate with one representative biological replicate pictured. (C) FLAM viability assay of *S. pneumoniae* infection after 24 hr. Data presented as a percentage relative to untreated control minus blank, $n \geq 2$ biological replicates conducted in technical triplicate. (D) FLAMs were infected with GFP-*S. pneumoniae* as above and fluorescence measured at 461 nm_{ex}, 501 nm_{em} at indicated timepoints. Data presented as fluorescence units minus uninfected cell blank, $n=3$ biological replicates conducted in technical triplicate with one representative biological replicate pictured. (E) BMDMs were infected with *S. pneumoniae* (MOI=20) for 60 min, washed and fresh media replaced for a further 60 min for CFU enumeration. All data presented as mean $\pm$ S.E.M., P value by one- (C) or two- (D) way ANOVA or unpaired t-test (E). **= $P < 0.01$, ***= $P < 0.001$; MOI = multiplicity of infection, p.i. = post infection, CFU = colony forming units.

To investigate if the above findings translated to humans, AMs were isolated from bronchoalveolar lavage fluid (BALF) acquired from individuals undergoing a diagnostic bronchoscopy at Tallaght University Hospital (**Table 4.1**). During a bronchoscopy, suction tubing is lodged into a bronchus and saline flushed into the airspaces of one lung lobe, with these washings (BALF) containing AMs (~90%), alongside small numbers of other airway leukocytes and lymphocytes (290).

Parameter	Non-smoker	Ex-smoker	Smoker	Unknown
Total n	7	8	4	3
Sex, M/F, n	5/2	8/0	2/2	3/0
Age, year	46 ± 7	73 ± 4	50 ± 13	65 ± 2
Smoking, pack year	-	-	30 ± 10	-
Years since cessation	-	21 ± 14	-	-

Table 4.1: Clinical parameters of subjects enrolled in Tallaght University Hospital cohort.

Patients exhibiting signs of an ongoing respiratory infection as noted by their physician prior to or during bronchoscopy were excluded from analysis, as were those receiving ongoing steroid treatment. AMs are the predominant cell type retrieved from BALF, and their ability to adhere to plastic allows for their overnight purification from other cell populations, which remain in suspension in a cell culture plate (**Fig. 4.3A**) (291). While establishing a model of human AM infection, no phagocytosis of the D39 *S. pneumoniae* strain used in **Fig. 4.2** was observed irrespective of prior opsonisation with human serum (*data not shown*). To develop a more human-relevant model of infection, an isolate of *S. pneumoniae* subcultured from a bronchoalveolar lavage fluid sample was used (**Fig. 4.3B**). After an initial incubation of this *S. pneumoniae* isolate with human AMs for 4 hr (denoted as 0 hr post-infection), CFU numbers were comparable to the initial infection timepoint in FLAMs detailed in **Fig. 4.2**. Again, using gentamicin to kill non-internalised bacteria, one group of AMs was lysed to determine baseline CFU following this initial 4 hr incubation with bacteria. Remaining AMs were lysed after a further 24 hr incubation with fresh media to investigate bacterial burden. Considering all 22 subjects that underwent bronchoscopy, *S. pneumoniae* CFU in AMs was significantly increased 24 hr post-infection (**Fig. 4.3C**).

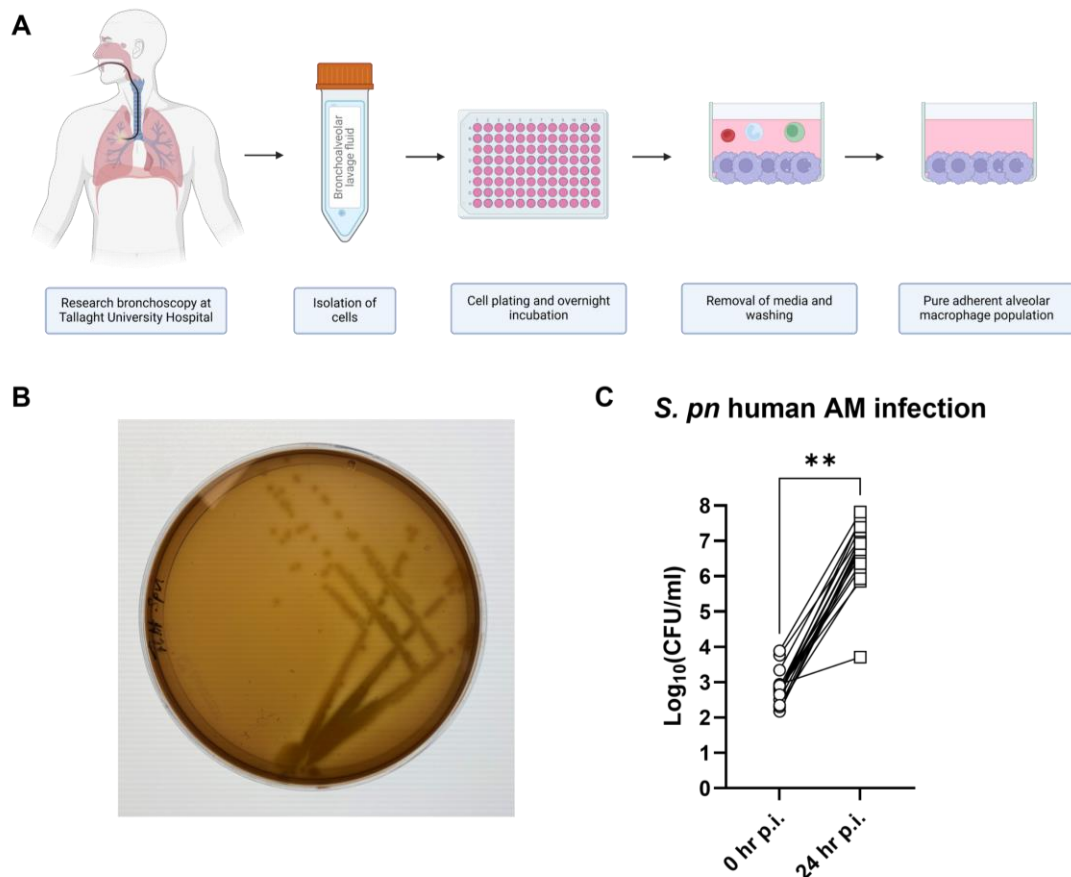


Fig. 4.3: *S. pneumoniae* can persist intracellularly in human AMs: (A) Schematic of adherence purification of AMs from bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid was acquired through bronchoscopy and cells isolated through centrifugation. Cells were washed once and RBC lysis performed as necessary prior to plating at 4×10^4 cells/well in a 96 well plate. Following overnight incubation at 37°C and 5% CO₂, media was removed, cells washed once in PBS and media replaced to remove non-adherent, non-AM cells. (B) Image of clinical *S. pneumoniae* isolate used in *ex vivo* infection model on Todd Hewitt agar with 5% v/v sheep's blood. (C) Human AMs were infected with *S. pneumoniae* as described with gentamicin used to assess intracellular persistence 24 hr after initial infection through CFU enumeration. Data representative of n=22 individual donors, datapoints at each timepoint matched. *P* value by paired t-test, **=*P*<0.01. CFU = colony forming units, p.i. = post-infection, *S. pn* = *S. pneumoniae*.

4.2.2 *S. pneumoniae* infection induces differential expression of AM cellular and mitochondrial iron-related factors

Having established that *S. pneumoniae* can replicate intracellularly in murine and human AMs in addition to its documented extracellular lifestyle, we next sought to investigate the effects of infection on AM cellular and mitochondrial iron metabolism. To do this, publicly available RNA-seq data published by Zec *et. al.* (292) was analysed. In this study, RNA-seq was performed on AMs (defined in this instance as Cd11c⁺, Siglec-F⁺ and

CD45⁺) sorted through flow cytometry from the lungs of mice intratracheally instilled with *S. pneumoniae* 72 hr prior. Using an absolute Log₂FC cutoff of 0.58 and *P* value <0.05, the expression of cellular and mitochondrial iron-related genes was analysed (**Fig. 4.4**). Relevant to cellular iron metabolism, AMs from infected mice had significantly upregulated expression of several genes including *Fth1*, *Slc11a2* (the elemental iron importer DMT1), *Slc11a1* (the phagosomal membrane iron exporter NRAMP1), and *Hamp* (encoding for the bactericidal hepcidin). Pertaining to mitochondrial iron import, *Slc25a37* (MFRN1) was significantly upregulated and *Slc25a28* (MFRN2) significantly downregulated. Also of note regarding mitochondrial iron dynamics, *Aco2* (encoding for mitochondrial aconitase) and *Ftmt* (mitochondrial ferritin) were both upregulated. *Tfrc* and *Slc40a1* (FPN) expression were unchanged relative to control (**Fig. 4.4**).

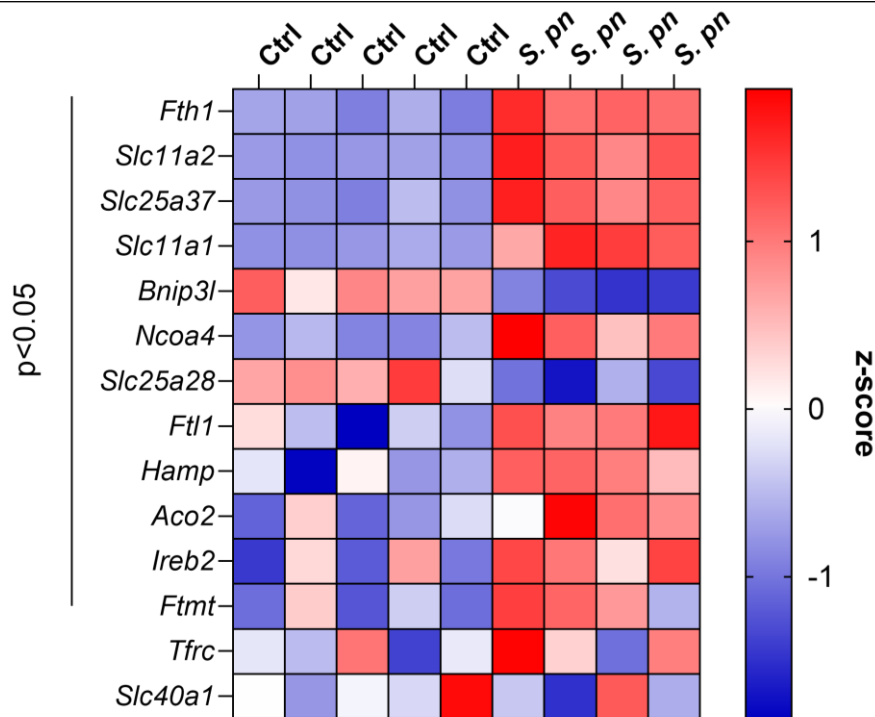


Fig. 4.4: Iron metabolism-related gene expression is altered in AMs isolated from a murine model of *S. pneumoniae* infection. Publicly available RNA-seq data of AMs isolated from mice intratracheally instilled with *S. pneumoniae* and PBS controls was downloaded from Zec *et. al.* (286). Heat map of iron-related gene expression in AMs isolated from control and *S. pneumoniae*-instilled mice, ranked in order of significance (cutoffs of absolute Log₂FC >0.58 and *P* value <0.05.) *S. pn* = *S. pneumoniae*.

Having observed the ability of *S. pneumoniae* infection to rewire cellular and mitochondrial iron metabolism in an *in vivo* infection model (**Fig. 4.4**), we next wanted to confirm these changes in our *in vitro* model of FLAM infection. While gentamicin was used previously to eliminate extracellular bacteria to interrogate intracellular replication (**Fig. 4.2**), failing to consider the capacity of extracellular bacteria to exert an effect on host iron

metabolism would not be physiologically representative. As such, gentamicin was not used to remove extracellular bacteria for ensuing experiments analysing the host response to infection. Cells were washed once in PBS following initial infection, which ensures full removal of media containing *S. pneumoniae* but leaves any bacteria associated with the cell surface intact, for a further incubation in fresh media for 4 hr. Using this approach, RNA and protein were isolated from *S. pneumoniae* infected FLAMs 4 hr post-infection to investigate changes in host iron-related gene and protein expression.

Relating to cellular iron metabolism, both FTH1 and TFRC expression increased with *S. pneumoniae* infection with increasing multiplicity of infection (**Fig. 4.5A**). This increase in *Fth1* was recapitulated at the mRNA level (**Fig. 4.5B**), while *Tfrc* expression significantly decreased (**Fig. 4.5B**). Both *Slc40a1* (FPN) and *Ncoa4* were significantly decreased (**Fig. 4.5D** and **4.5E** respectively), and *Slc11a1* and *Hmox1* (encoding for the heme-degrading heme oxygenase 1) significantly increased (**Fig. 4.5F** and **4.5G** respectively). There was no significant change in *Flvcr1a* or *Ftl* expression, with *Lcn2* (encoding for the siderophore-binding lipocalin-2) trending towards increase ($P = 0.1199$).

Regarding mitochondrial iron metabolism, *Slc25a37* (MFRN1) expression significantly increased with infection while *Slc25a28* (MFRN2) decreased (**Fig. 4.6A** and **4.6B** respectively), recapitulating *in vivo* findings in **Fig. 4.4**. Expression of *Flvcr1b* (encoding for the mitochondrial heme exporter) significantly increased relative to uninfected control (**Fig. 4.6C**), while expression of *Mtft*, *Bnip3* and *Mcu* was unchanged (**Fig. 4.6D-F**). Taken together, these results confirm the ability of *S. pneumoniae* infection *in vitro* to rewire FLAM iron-related gene expression, including through increasing MFRN1 and decreasing MFRN2 mRNA expression as observed *in vivo* (**Fig. 4.4**).

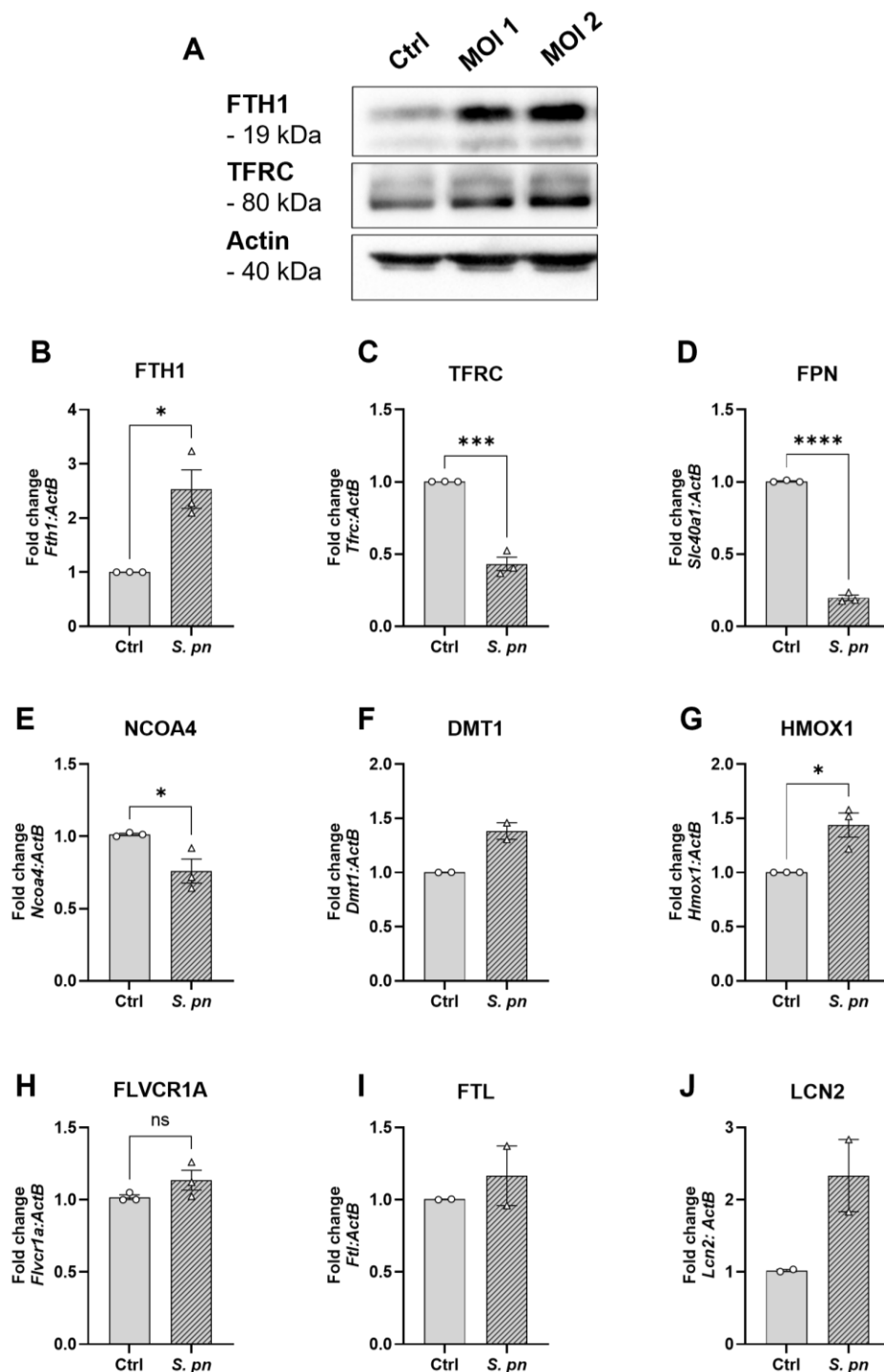


Fig. 4.5: *S. pneumoniae* infection dysregulates FLAM cellular iron metabolism-related gene and protein expression. FLAMs were infected with *S. pneumoniae* (MOI=2) for 30 min, washed once and fresh media added for a further 4 hr. (A) Cells were lifted through scraping, washed once, and lysed in RIPA buffer and SDS-PAGE and western blotting performed as described. Primary antibodies used are listed to the left of each blot, along with estimated band weight determined relative to a protein marker of known molecular weights. (B-J) Cells were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. Data representative of mean $\pm$ S.E.M., $n \geq 2$ independent experiments. *= $P < 0.05$, ***= $P < 0.001$, ****= $P < 0.0001$ by unpaired t-test. *S. pn* = *S. pneumoniae*.

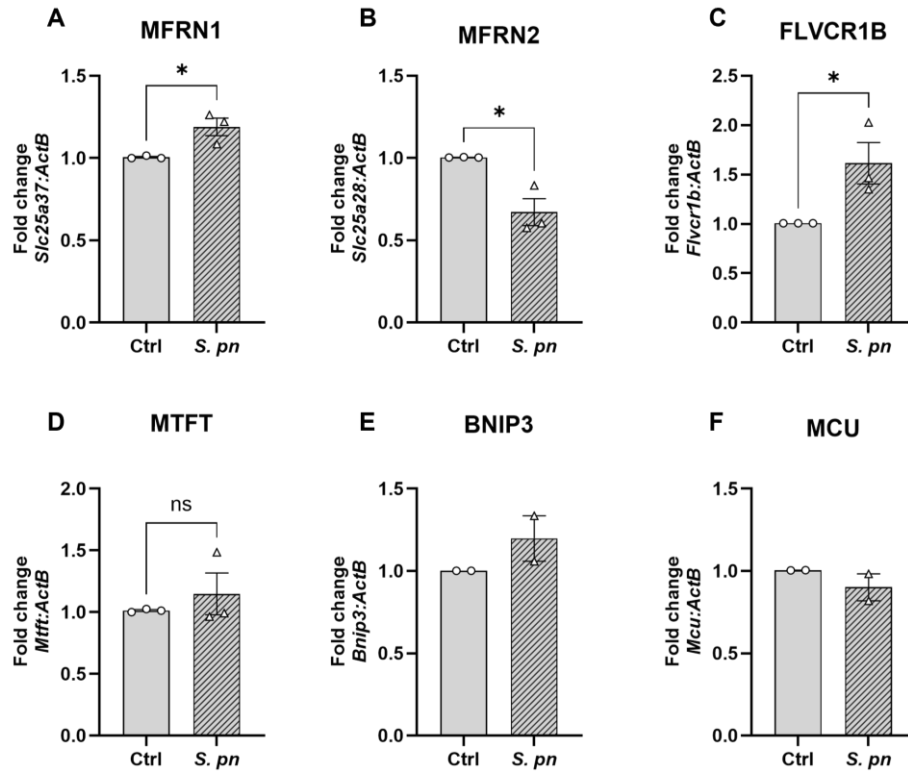


Fig 4.6: *S. pneumoniae* infection dysregulates FLAM mitochondrial iron metabolism-related gene expression. FLAMs were infected with *S. pneumoniae* (MOI=2) for 30 min, washed once and fresh media added for a further 4 hr. (A-F) Cells were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. Data representative of mean $\pm$ S.E.M., $n \geq 2$ independent experiments. $*$ = $P < 0.05$ by unpaired t-test. *S. pn* = *S. pneumoniae*.

4.2.3 *S. pneumoniae* infection decreases total FLAM iron

Next, we wanted to establish whether these changes reflected alterations in host intracellular iron levels. FLAMs were infected with *S. pneumoniae* as in **Fig. 4.5** and **Fig. 4.6** and harvested 4 hr post-infection for measurement of total intracellular iron and heme through GF-AAS and heme assay, respectively. There was no significant change in either total iron (**Fig 4.7A**) or heme (**Fig 4.7B**) at this dose and time of infection.

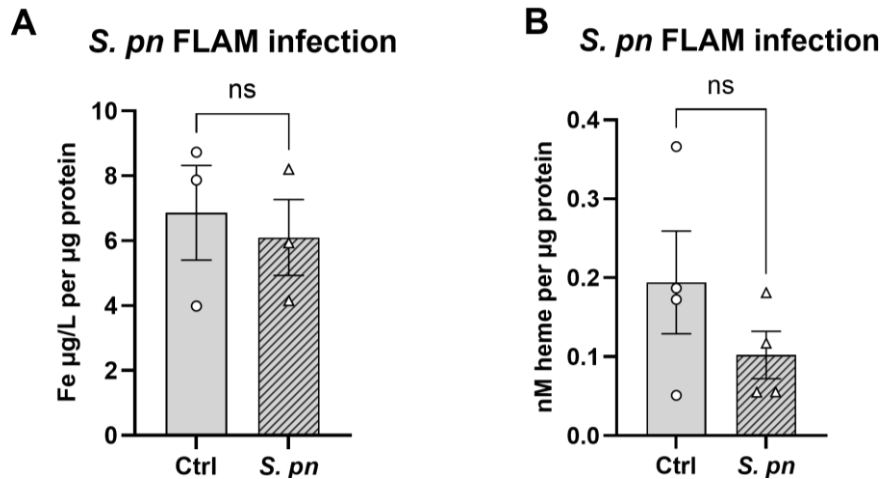


Fig. 4.7: FLAM total iron or heme does not change 4 hr post-*S. pneumoniae* infection. FLAMs were infected with *S. pneumoniae* (MOI=2) for 30 min, washed once and fresh media added for a further 4 hr. (A) Cells plated in a 12 well plate were lifted through scraping, washed once, and lysed in RIPA buffer prior to digestion in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS. Each data point is representative of one biological replicate constituting the average of three technical triplicate wells of cells presented as fold change to control, normalised to protein content measured through BCA assay. (B) Cells plated in a 6 well plate were lifted through scraping, washed once and heme assay performed as described, normalised to protein content as measured through BCA assay. Data representative of mean $\pm$ S.E.M. of $n \geq 3$ independent experiments, P value by unpaired t-test. ns = non-significant. *S. pn* = *S. pneumoniae*.

As changes were observed in several iron-related genes and proteins in **Fig. 4.5** and **4.6**, we investigated whether there may be a change in cellular iron levels at an earlier point of infection to drive, which may be masked by the increase in bacterial burden 4 hr post-infection. Total cellular iron in FLAMs was measured immediately after initial 30 min incubation with *S. pneumoniae*. This was then compared with those that had media replaced to incubate for a further 4 hr with internalised bacteria as in **Fig. 4.7**. FLAM intracellular iron decreased immediately after initial 30 min incubation with *S. pneumoniae* compared to uninfected control (**Fig. 4.8A**). In line with our results in **Fig. 4.7**, no change was observed in infected cells which were incubated for a further 4 hr compared to their uninfected control (**Fig. 4.8A**). To investigate if this observed decrease is worsened by an increased burden of infection, intracellular iron measurements immediately after this initial 30 min incubation with *S. pneumoniae* were repeated at a range of different MOIs. While not significant at lower MOIs, there was a trend towards a decrease in FLAM total iron with increasing multiplicity of infection (**Fig. 4.8B**).

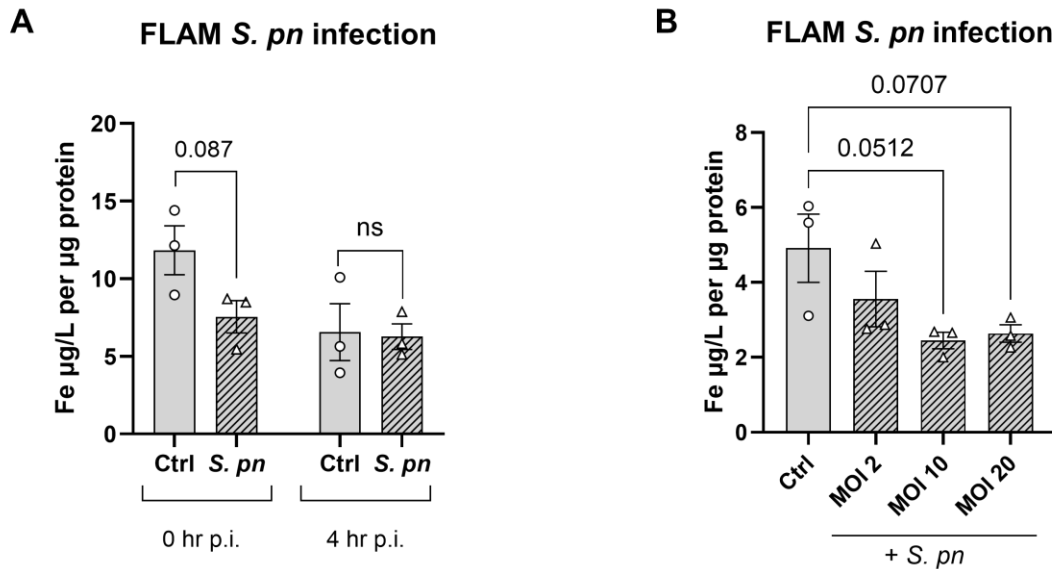


Fig. 4.8: FLAM total cellular iron levels decrease following 30 min incubation with *S. pneumoniae*. (A) FLAMs were infected with *S. pneumoniae* at MOI 2 for 30 min, with one group harvested immediately after initial infection (0 hr p.i.) and the remaining washed once and fresh media added for a further 4 hr (4 hr p.i.). (B) FLAMs plated in a 12 well plate were infected with *S. pneumoniae* at MOI 2 for 30 min, with one group harvested immediately after initial infection (0 hr p.i.). Cells were lifted through scraping, washed once, and lysed in RIPA buffer prior to digestion in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS. Each data point is representative of one biological replicate constituting the average of three technical triplicate wells of cells presented as fold change to control, normalised to protein content measured through BCA assay. Data representative of mean $\pm$ S.E.M. of 3 independent experiments, *P* value by one-way ANOVA with Tukey's multiple testing correction, ns = non-significant. p.i. = post-infection, *S. pn* = *S. pneumoniae*

To determine whether this loss of cellular iron was an autonomous cellular response to infection or a bacterial strategy to manipulate host iron storage, FLAMs were incubated with live or inactivated *S. pneumoniae*. Bacteria were inactivated through fixation, which will retain bacterial cell surface proteins, or through heating at 95°C to lyse bacteria. 30 min incubation of FLAMs with live, fixed or heat-killed bacteria at equal amounts revealed no change in intracellular total iron with either inactivated form (**Fig. 4.9A**). This suggests that intracellular iron loss may not be a conserved host immune response to receptor-mediated signalling pathways and rather may be uniquely induced upon infection with live bacteria, suggesting they maintain a capacity to influence intracellular iron that is lost with inactivation. To further interrogate intracellular iron dynamics in this model, labile Fe^{2+} iron was measured through confocal microscopy. Surprisingly, an increase in Fe^{2+} was observed upon incubation with live bacteria relative to uninfected control (**Fig. 4.9B**). This change again was not observed with inactivated *S. pneumoniae*, where Fe^{2+} levels were comparable to control. This data again illustrates the

unique impact of a live bacterium on cellular iron levels and supports a model where *S. pneumoniae* may liberate inertly stored Fe^{3+} to the labile Fe^{2+} prior to loss.

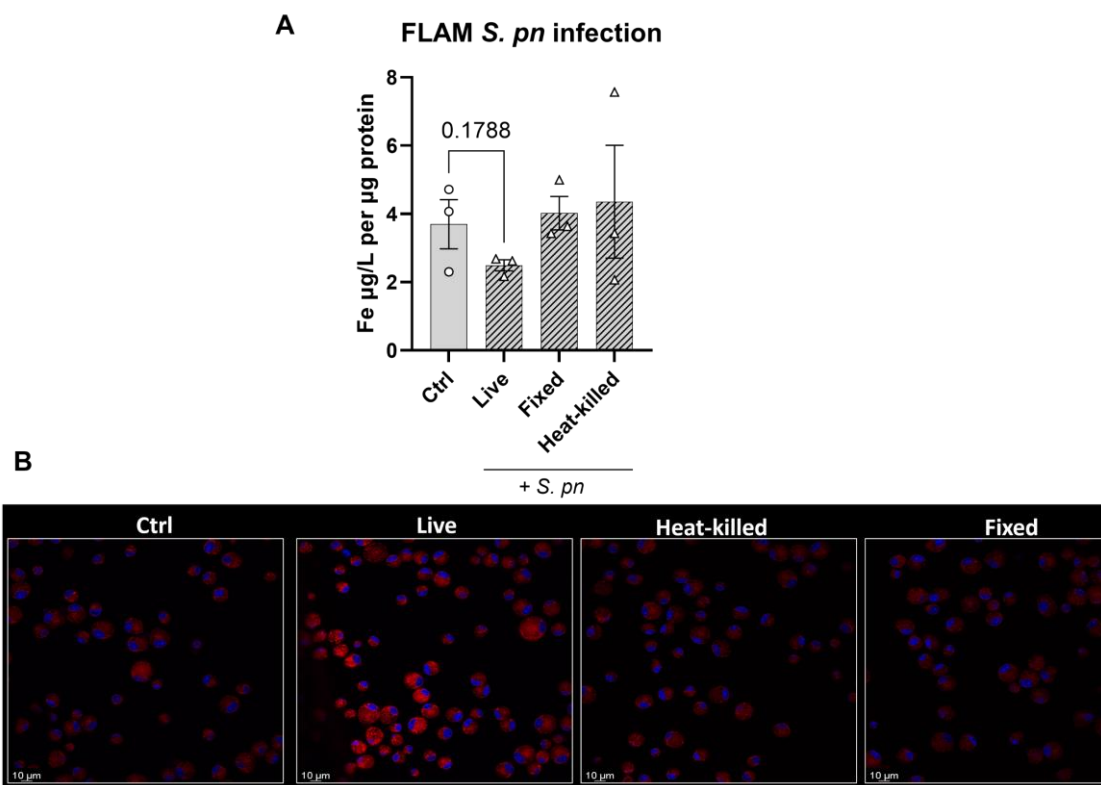


Fig. 4.9: Inactivated *S. pneumoniae* does not alter FLAM total cellular iron. (A) FLAMs were infected with live *S. pneumoniae* at MOI 2 or the equivalent number of fixed or heat-killed bacteria for 30 min, with one group harvested immediately after initial infection (0 hr p.i.). Cells were lifted through scraping, washed once, and lysed in RIPA buffer prior to digestion in 50% nitric acid for 2 hr at 70°C for iron measurement through GFAAS. Each data point represents one biological replicate constituting the average of three technical triplicate wells of cells presented as fold change to control, normalised to protein content measured through BCA assay. Data representative of mean ± S.E.M. of 3 independent experiments, *P* value by one-way ANOVA with Tukey's multiple testing correction. (B) FLAMs plated in a 35 mm glass-bottomed dish were infected as above, washed once and sequentially stained with 1 µM FerroOrange and 5 µg/mL Hoechst nuclear dye. Images were captured using a 40X magnification oil-immersion lens of a Leica SP8 scanning confocal microscope and scale bar added using the LAX Processing Suite software. Representative images for each condition pictured. *S. pn* = *S. pneumoniae*

4.2.4 *S. pneumoniae* burden of infection increases with CS exposure in murine and human AMs

The above data has described the ability of *S. pneumoniae* to persist intracellularly in both human AMs and a murine model of AMs, and to manipulate host iron and mitochondrial iron metabolism. Data in **Chapter 3** illustrated the ability of CSE to increase FLAM total and mitochondrial iron levels: considering that *S. pneumoniae* can alter host iron metabolism, we next sought to investigate how this iron-replete environment in the COPD disease state might be exploited by *S. pneumoniae* to utilise this excess in host

iron. To determine a suitable combination of CSE pretreatment and bacterial MOI to use in an infection model, FLAM viability was analysed following 18 hr pretreatment with a range of CSE concentrations followed by *S. pneumoniae* infection, as in **Fig. 4.2**, at varying MOIs. To ensure there was no confounding effect of CSE alone on bacterial viability, cells were washed with PBS after removing CSE-containing media. 5% CSE treatment for 18 hr followed by infection with an MOI of 2 was chosen as a model for further experimental use. (**Fig 4.10A**). Using this model, we next investigated how FLAM pretreatment with CSE affected *S. pneumoniae* intracellular replication, utilising the gentamicin invasion assay detailed in **Fig. 4.2**. In FLAMs pretreated with CSE, bacterial CFU increased 4 hr post-infection related to those untreated (**Fig. 4.10B**). Interestingly, there was no detectable difference in CFU following initial incubation with bacteria. This suggests that the increased bacterial burden observed 4 hr post-infection may arise from a change in the intracellular FLAM environment, rendering it more permissive for bacterial replication, rather than a direct effect on bacterial uptake.

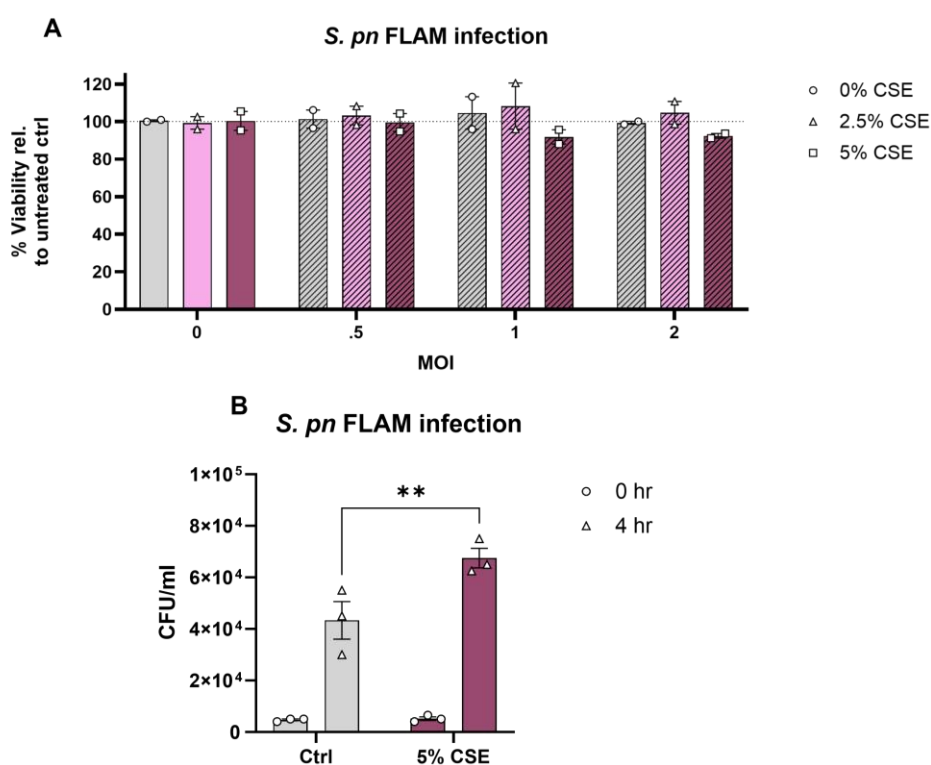


Fig. 4.10: FLAM CSE treatment enhances *S. pneumoniae* intracellular persistence. FLAMs were treated with indicated concentrations of CSE for 18 hr, washed once, and infected with *S. pneumoniae* for 4 hr as in **Fig. 4.2A**. (A) FLAM viability assay of CSE treatment and *S. pneumoniae* infection. Data presented as a percentage relative to untreated control minus blank, $n \geq 2$ independent experiments conducted in technical triplicate. (B) Bacterial burden following infection at MOI 2 measured through CFU enumeration, normalised to CFU/ml. Data representative of $n=3$ independent experiments conducted in technical triplicate, one representative experiment pictured. P value by two-way ANOVA with Tukey's multiple testing correction; **= $P < 0.001$. CFU = colony forming units, p.i. = post-infection, *S. pn* = *S. pneumoniae*.

To investigate how human smoking affects *S. pneumoniae* intracellular replication, findings in **Fig. 4.3B** were stratified by smoking status to investigate bacterial burden in non-smokers, ex-smokers and current smokers. 3 subjects for which data on smoking history was not recorded were removed from analysis (**Table 4.1**). Considering non-smokers, ex-smokers and current smokers, bacterial burden increased 0 hr (**Fig. 4.11A**) and 24 hr (**Fig. 4.11B**) post-infection in smokers compared to both non-smokers and ex-smokers. While not significant, ex-smokers exhibited a trend towards increase ($p=0.332$) in bacterial burden relative to non-smokers 24 hr post-infection (**Fig. 4.11B**).

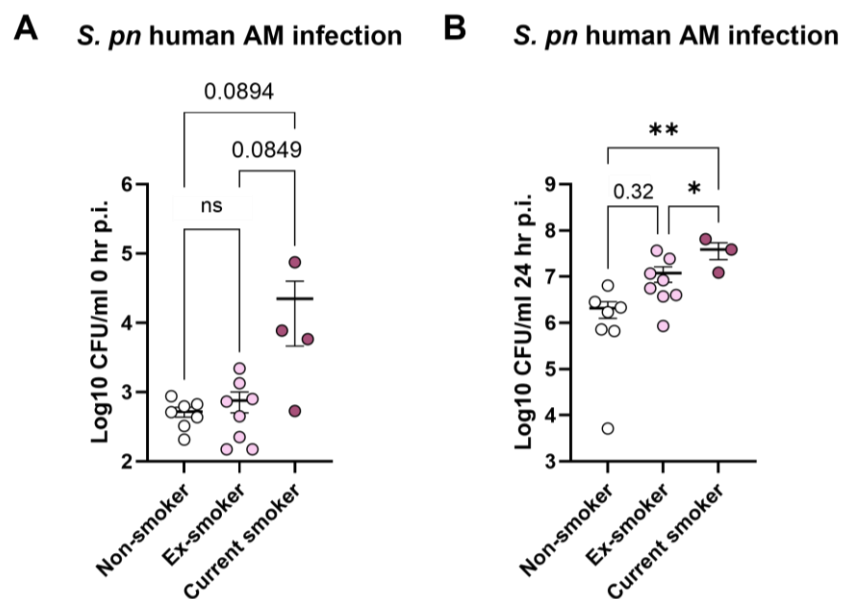


Fig. 4.11: AMs isolated from smokers show an increased bacterial burden upon infection with a clinical isolate of *S. pneumoniae*. Human AMs were isolated from BALF and infected with *S. pneumoniae* as in **Fig. 4.7A**. Bacterial burden 0 hr (A) and 24 hr (B) post-infection presented grouping patients by smoking status ($n=6$ non-smoker, $n=8$ ex-smoker, $n=4$ current smoker), P value by one-way ANOVA with Tukey's multiple testing correction. CFU = colony forming units, p.i. = post-infection, *S. pn* = *S. pneumoniae*

4.2.5 Smoking blunts AM secretion of pro-inflammatory cytokines in response to *S. pneumoniae*

We next assessed whether the increased burden of *S. pneumoniae* observed in CS AMs associated with altered secretion of pro-inflammatory. FLAM supernatants 4 hr post-infection to measure differences in pro-inflammatory cytokine production with CSE pre-treatment. Secretion of the pro-inflammatory cytokines IL-6, TNF- α and lipocalin-2 increased with *S. pneumoniae* infection in a dose-dependent manner (**Fig 4.12A-C**). Secretion of IL-6, TNF- α and lipocalin-2 was significantly decreased upon infection in cells pretreated with CSE compared to vehicle control (**Fig 4.12A-C**). Of note, lipocalin-2 was detected in the supernatants of uninfected cells, which was significantly decreased in

those pretreated with CSE compared to the untreated, uninfected control (**Fig 4.12C**). These results show CSE pretreatment blunts FLAM pro-inflammatory cytokine secretion upon *S. pneumoniae* infection, with this effect observed in the absence of infection in the case of lipocalin-2.

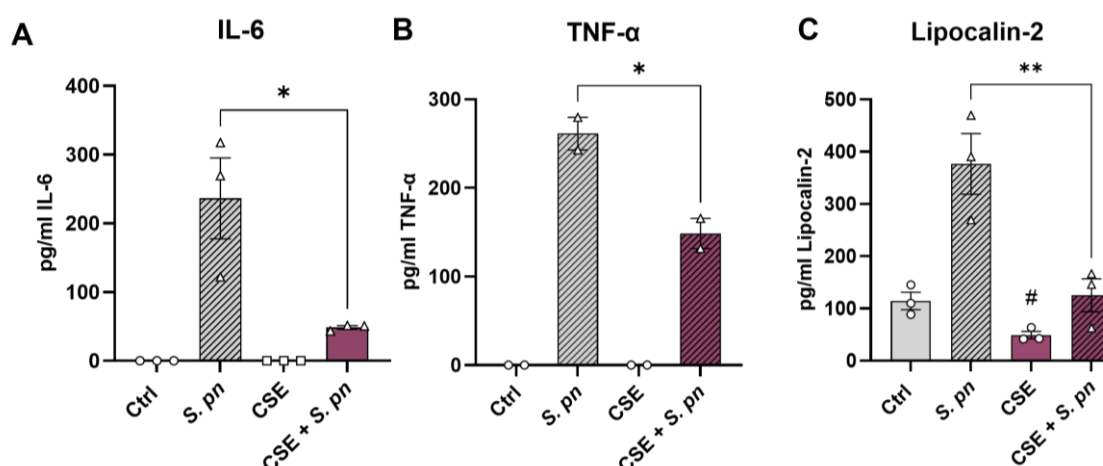


Fig. 4.12: CSE pretreatment blunts FLAM pro-inflammatory cytokine expression upon *S. pneumoniae* infection. FLAMs pretreated with 5% CSE or vehicle control (RPMI) for 18 hr were infected with *S. pneumoniae* (MOI=2) for 30 min, washed once and fresh media added. Supernatants were collected after 4 hr and IL-6, TNFα and lipocalin-2 measured by ELISA. Data presented as mean ± S.E.M., representative of n≥2 independent experiments conducted in technical triplicate. *= $P<0.05$, **= $P<0.01$ by one-way ANOVA with Tukey's multiple testing correction.

4.2.6 CSE treatment dysregulates AM iron metabolism upon *S. pneumoniae* infection.

These results have illustrated the detrimental effect of smoking on AM bactericidal responses, in both human AMs from smokers and a murine AM-like *in vitro* model of CSE treatment. Considering the profound effects of CSE treatment at this dose and time on the FLAM 'ironome' (**Fig. 1.1** and **Fig. 1.2**) detailed in **Chapter 3**, changes in cellular and mitochondrial iron model upon infection were interrogated. FLAMs were pretreated with CSE for 18 hr and infected with *S. pneumoniae* as described. RNA was isolated 4 hr post-infection to investigate expression of iron-related factors through RT-qPCR, respectively.

Considering the expression of cellular iron metabolism genes, expression of *Fth1*, *Tfrc* and *Slc40a1* (FPN) in CSE-treated, *S. pneumoniae*-infected FLAMs remained consistent compared to untreated, infected FLAMs (**Fig. 4.13A-C**). An additive effect of CSE treatment and infection on expression of *Hmox1* (**Fig. 4.13D**) and *Ftl* (**Fig. 4.13E**) was observed, while CSE blunted *S. pneumoniae*-induced gene expression of *Lcn2* (**Fig. 4.13F**), mirroring previous observations at the protein level (**Fig 4.12C**). There was no

significant change in *Flvcr1a* expression across all groups (**Fig. 4.13G**), and expression of *Dmt1* and *Ncoa4* in CSE-treated, *S. pneumoniae*-infected FLAMs was unchanged relative to control (**Fig. 4.13H** and **Fig. 4.13I** respectively).

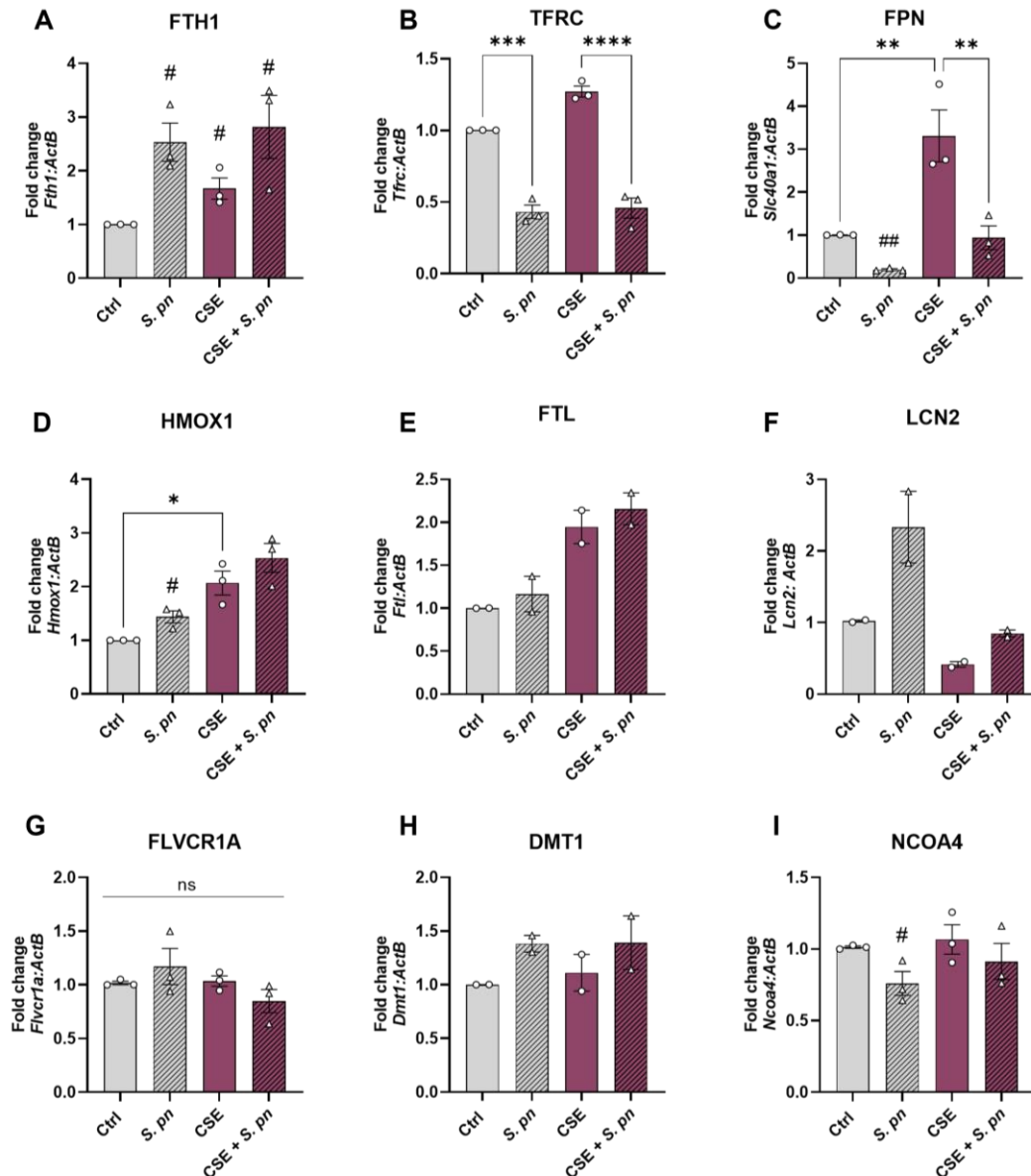


Fig. 4.13: CSE treatment dysregulates FLAM cellular iron metabolism-related gene and protein expression upon *S. pneumoniae* infection. FLAMs pretreated with 5% CSE for 18 hr were infected with *S. pneumoniae* (MOI=2) for 30 min, washed once and fresh media added for a further 4 hr. (A-I) Cells were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. Data representative of mean $\pm$ S.E.M., $n \geq 2$ independent experiments. *= $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$, ****= $P < 0.0001$ by one-way ANOVA with Tukey's multiple testing correction. #= $P < 0.05$ relative to untreated, uninfected control by unpaired t-test. Data from untreated, uninfected control (Ctrl) and untreated, *S. pneumoniae*-infected (*S. pn*) groups are from the same experiments as in **Fig. 4.4**.

Relating to mitochondrial iron metabolism, *Slc25a37* remained upregulated in *S. pneumoniae* infection with CSE pretreatment compared to infection alone (**Fig. 4.14A**), while *Slc25a28* remained downregulated (**Fig. 4.14B**). *Flvcr1b* expression was upregulated to a greater extent in *S. pneumoniae* infection with CSE pretreatment than in infection alone, indicative of a synergistic effect of CSE and infection in increasing mitochondrial heme export (**Fig. 4.14C**). No significant change in *Mtft*, *Bnip3* or *Mcu* expression was detected across all groups (**Fig. 4.14D-F**)

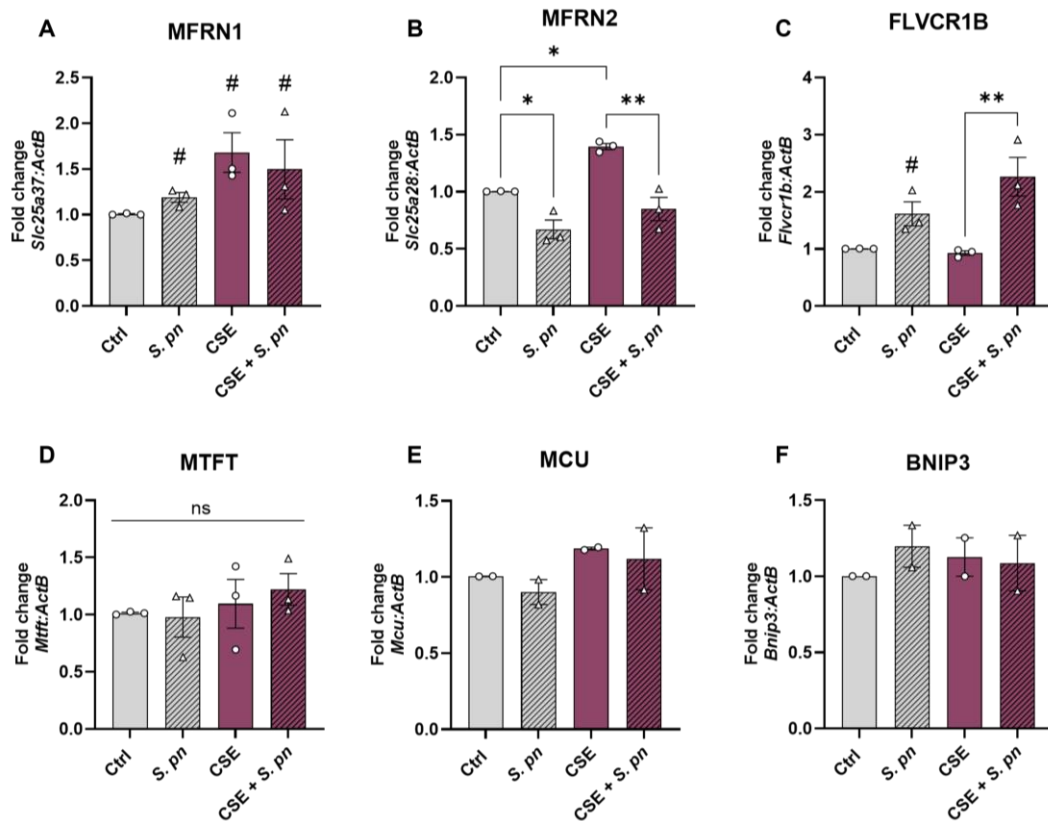


Fig. 4.14: CSE treatment dysregulates FLAM mitochondrial iron metabolism-related gene expression upon *S. pneumoniae* infection. FLAMs pretreated with 5% CSE for 18 hr were infected with *S. pneumoniae* at indicated MOIs for 30 min, washed once and fresh media added for a further 4 hr. (A-F) Cells were lysed and RNA extracted as described, with gene expression measured through RT-qPCR on reverse-transcribed cDNA and normalised to *ActB* expression as endogenous control. Data representative of mean $\pm$ S.E.M., $n \geq 2$ independent experiments. $*=P < 0.05$, $**=P < 0.01$ by one-way ANOVA with Tukey's multiple testing correction. $\#=P < 0.05$ relative to untreated, uninfected control by unpaired t-test. Data from untreated, uninfected control (Ctrl) and untreated, *S. pneumoniae*-infected (S. pn) groups are from the same experiments as in **Fig. 4.5**.

4.2.7 CSE inhibits *S. pneumoniae* growth and decreases bacterial iron levels:

Findings in **Chapter 3** described the ability of CSE, an *in vitro* model of COPD, to alter intracellular iron in FLAMs. How iron might reciprocally change in the bacteria carried in the COPD lung, like *S. pneumoniae*, is unknown. To study this, CSE was prepared in bacterial growth media and *S. pneumoniae* was cultured in varying concentrations of CSE. Bacterial growth in CSE-containing medium was slowed, but not fully inhibited (**Fig. 4.15A**). To assess the effects of CSE on bacterial iron levels, intracellular iron from *S. pneumoniae* grown in CSE was measured through GF-AAS. While not reaching significance, bacterial intracellular iron trended toward decrease ($P = 0.118$) when grown in the presence of CSE compared to normal medium control (**Fig. 4.15B**).

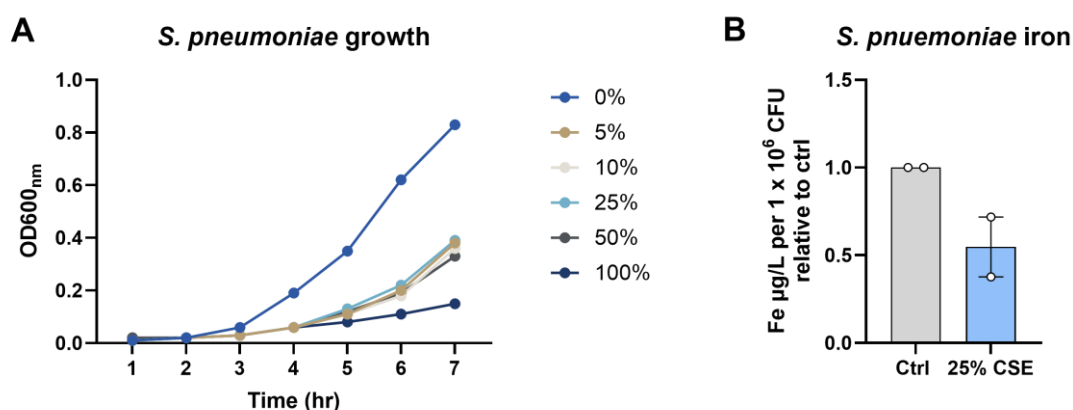


Fig. 4.15: CSE reduces *S. pneumoniae* growth and may decrease bacterial iron levels. CSE was generated in Todd Hewitt broth as described and added to fresh broth at indicated concentrations. *S. pneumoniae* was added to each culture at a starting OD600_{nm} of 0.005 and incubated at 37°C and 5% CO₂. (A) OD600_{nm} of each culture was measured at hourly intervals. Data representative of one independent experiment. (B) *S. pneumoniae* was grown from a starting OD600_{nm} of 0.005 in Todd Hewitt broth containing 0 (control) and 25% CSE for 6 hr. Bacterial pellets were resuspended in 1 mL PBS and lysed using bead homogenisation. 50 µl lysate was digested in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS, with data normalised to CFU. Data representative of mean ± S.E.M. of two independent experiments, P value by unpaired t-test.

4.3 Discussion

Acute, recurrent infections drive COPD mortality, but the contribution of AM iron loading to the burden of infection in COPD is unclear. We have shown a novel ability of *S. pneumoniae* to persist inside alveolar-like macrophages that is enhanced with CSE treatment, with these findings recapitulated in human AMs isolated from smokers. CSE pretreatment also impaired pro-inflammatory cytokine production (IL-6, TNF- α , lipocalin-2) in FLAM *S. pneumoniae* infection. *S. pneumoniae* infection altered protein and mRNA expression of key players in cellular iron metabolism with divergent responses of MFRN1 and 2 to infection. Live *S. pneumoniae* decreased FLAM total iron in a dose-dependent manner, which was not observed upon treatment with inactivated bacteria, illustrating the ability for a live bacterium to uniquely influence host iron dynamics. These findings position AMs as a potential bacterial target for iron piracy, which may be exacerbated by the iron loading present in experimental and human COPD.

Total intracellular iron levels in FLAMs significantly declined following 30 min incubation with *S. pneumoniae*, which returned to the level of uninfected control 4 hr after initial infection (**Fig. 4.7**). This iron loss appears to be a pathogenic bacterial mechanism rather than host-driven: an increase in bacterial number appeared to negatively correlate with FLAM total iron, and incubation of FLAMs with inactivated forms of *S. pneumoniae* had no effect on host iron levels (**Fig. 4.9A**). Temporally, the host has the capacity to manipulate compartmental iron levels rapidly after the onset of infection, with transferrin-capturing extracellular vesicles detected in supernatants immediately after macrophage *S. Tm* infection (293). Relevant to host intracellular iron and infection, TLR3 and 4 agonists induce a downregulation of FeS cluster assembly components (294). Our data is unique among these studies in our use of a live bacterium over sterile TLR agonists, strengthening the physiological relevance of these findings to host-pathogen interactions *in vivo*. Live bacteria have a unique metabolic influence on host macrophages compared to dead, with macrophages even retaining the ability to repurpose carbons from phagocytosed live bacteria for production of their own metabolites (295).

It is also important to note the contribution of intracellular iron from *S. pneumoniae* internalized in macrophages at the point of harvesting for iron measurement, which may be slightly inflating total intracellular iron levels from infected macrophages and thus masking a larger decrease in endogenous cellular iron, particularly after intracellular replication. *S. pneumoniae* can live both an extracellular and, as this work has shown, intracellular lifestyle (**Fig. 4.2-3**). Typically, cells employ a gene expression program promoting iron export upon infection with an intracellular bacterium, and upregulate iron

import when faced with an extracellular bacterium (296). In this instance, where cells are faced with the dual threat of intracellularly and extracellularly replicating bacteria, the macrophage nutritional immune response is less defined, as depleting iron from one compartment would only advantage bacteria in the other. Intracellular persistence by *S. pneumoniae* in this instance may represent a bacterial ‘trojan horse’ strategy for iron acquisition, where a subpopulation of bacteria that survive intracellularly lead AMs to expel iron into the extracellular space to fuel extracellular bacteria. The activity of this intracellular faction appears crucial, inducing an increase in free Fe^{2+} iron not observed with inactive bacteria (**Fig. 4.10B**). As this increase in labile iron correlates with a loss in total iron, it could be speculated that iron is being liberated by intracellular bacteria from inert storage, like cytosolic or mitochondrial ferritin, for subsequent loss from the cell. Contrary to this theory, FTH1 expression is elevated at the mRNA and protein level: as FTH1 is the subunit of ferritin responsible for the oxidation of Fe^{2+} and not iron storage, this may be an attempt to capture bioavailable iron liberated by bacteria. Immunoprecipitation of *S. pneumoniae* from FLAM lysates and investigation of its association with key players in this iron liberation, like NCOA4 or ferredoxins, would help to prove this. Notably, FerroOrange, the probe used for Fe^{2+} measurements, is not compartment specific. Further pairing of FerroOrange with fluorescently labelled *S. pneumoniae* will determine if bacteria co-localise with this liberated iron, and the precise subcellular localisation of this iron usage.

So where does this iron go? Despite the rapid loss of iron from infected cells, expression of cellular heme (FLVCR1a) and iron (FPN) exporters are decreased (**Fig. 4.5**). While iron and heme may still be exported by the two proteins, one would think such a rapid and significant loss of iron would be contingent on upregulated gene expression. FLVCR1a and ferroportin expression are both reduced in macrophages upon LPS treatment, suggesting a possible iron-retention based response to TLR4 activation (96). However, the drastic loss of iron from infected macrophages observed in this project does not align with this, hinting macrophages may lose iron through an alternative mechanism. Examples of such include the exocytosis-mediated export of ferritin (297). Curiously, *S. pneumoniae* induces export of mitochondria through extracellular vesicles in human lung tissue explants (298). Macrophages can uptake mitochondria-containing extracellular vesicles in both homeostatic (299) and pathogenic (300) contexts. Whether mitochondrial export occurs in macrophages in response to infection is unclear, but as large reserves of cellular iron are stored in mitochondria their export would likely be reflected in a significant decrease in total intracellular iron. Gene expression of MFRN1 was increased and MFRN2 decreased in infected FLAMs (**Fig. 4.6**) and in AMs sorted from *S. pneumoniae* infected mice (**Fig. 4.4**), confirming the ability of *S. pneumoniae* infection to influence mitochondrial

iron dynamics. Measuring mitochondrial mass in infected AMs would be an informative first step in determining any correlation with this loss of total intracellular iron.

We have demonstrated a novel ability of *S. pneumoniae* to survive intracellularly in human AMs *ex vivo* and a murine AM-like cell line *in vitro* (**Fig. 4.2-3**). This intracellular replication was heightened in smoking, with increased bacterial burden observed in human AMs from smokers, and in FLAMs treated with CSE (**Fig. 4.10-11**). The mechanisms by which *S. pneumoniae* persists intracellularly are unclear, and reports investigating the macrophage receptors involved in *S. pneumoniae* uptake are varied. Pneumolysin can directly target the MRC-1 to promote bacterial uptake and evade lysosomal compartments in AMs (275). The scavenger receptor MARCO is critical for AM *S. pneumoniae* uptake and killing *in vivo* (301) and its expression is reduced in COPD (302). Of particular interest to this work, *S. pneumoniae* can hijack transcytosis of the TFRC to invade brain microendothelial cells to cross the blood-brain barrier (303). TFRC expression in infected FLAMs was upregulated at the protein level but downregulated at the mRNA level (**Fig. 4.7**), which could be indicative of bacterial hijacking for internalisation into the cell with the functional consequence of occluding cellular iron import. As TFRC expression is increased in CSE-treated FLAMs (**Fig. 3.10F**), this could represent a mechanism for *S. pneumoniae* to identify and hijack cells which are importing increased amounts of iron as putative targets for iron usage.

S. pneumoniae intracellular replication was first identified in a CD169⁺ population of healthy splenic macrophages (278). All previous reports of *S. pneumoniae* persistence or replication in AMs were conducted using AMs from immunocompromised individuals only (279), making our results the first to show bacterial replication in a 'healthy' AM. Regulated AM cell death is a conserved response mechanism to *S. pneumoniae* infection (275): as our model of infection ultimately resulted in a loss of AM viability, these intracellular bacteria conserve host pathogenicity comparable to those extracellular. It is important to note that AMs do not exist in isolation in the alveolar space, and *in vivo* can issue the necessary signals to recruit additional immune cells to aid in the fight against infection while limiting tissue-destructive inflammation. Lung specific-mediators like surfactant protein A (304) can also influence AM-*S. pneumoniae* interactions, which are not considered in *in vitro* models. Still, as the ability of *S. pneumoniae* to persist inside macrophages appears unique to AMs and not other macrophage types like BMDMs (**Fig. 4.2E**) (305), our findings hold promise.

Stratification of individuals enrolled in our TUH cohort by smoking status revealed a higher burden of *S. pneumoniae* in current smokers 24 hr post-infection compared to non-

smokers, validating our *in vitro* data (**Fig. 4.11**). Intriguingly, ex-smokers also displayed a trending increase in *S. pneumoniae* burden. Ex-smokers still experience exacerbation-like events years after smoking cessation (271), but the molecular mechanisms behind this are understudied. Clarifying if alterations in cellular and mitochondrial iron metabolism can persist in AMs in these individuals to a level comparable to current smokers, as suggested in by transcriptomic data in **Fig. 3.4**, will prove informative in this regard.

COPD is an inflammatory disease accompanied by an increase in numerous pro-inflammatory cytokines and chemokines in the airspaces. However, our data shows that a model of lung-resident AMs pretreated with CSE are unable to secrete IL-6, TNF- α and lipocalin-2 in response to *S. pneumoniae* infection* (**Fig 4.12**). CSE was generated using standardised research cigarettes issued by the University of Kentucky which are certified as LPS-free, meaning a tolerising effect from previous endotoxin exposure is unlikely. These findings are supported by previous work from our lab which shows that primary murine AMs treated with CSE have decreased IL-6 gene and protein expression upon infection with a serotype 4 strain of *S. pneumoniae* (112). AMs from CS-exposed mice displayed attenuated IL-6 and TNF- α secretion in response to LPS and poly:IC stimulation (306), but the pro-inflammatory response of CS AMs to other live bacteria has not been studied. The lung-resident AM pool is typically lost during insult, with recruited immune cells continuing the fight against infection. As suggested by RNA-seq data in **Chapter 3**, the increase in cytokines observed in the COPD lung does not appear to be AM driven – examining cytokine secretion in CS-exposed BMDMs upon *S. pneumoniae* infection would help elucidate the contribution of recruited macrophages to this inflammatory burden.

We detected secretion of lipocalin-2 by FLAMs without *S. pneumoniae* infection, which was significantly reduced upon CSE pretreatment (**Fig 4.12C**). Given its siderophore binding abilities, literature studying lipocalin-2 primarily focuses upon its anti-microbial activities in the context of bacterial iron deprivation. Independent of this specific role in nutritional immunity, lipocalin-2 is important in the inflammatory response: the *Lcn2* gene promoter region contains NF- κ B -responsive elements, and lipocalin-2 is one of the most highly expressed proteins in the *S. pneumoniae*-infected lung despite the fact that *S. pneumoniae* cannot secrete siderophores (123, 292). The same study reported a lipocalin-2 dependent induction of IL-10 upon *S. pneumoniae* infection, but IL-10 could not be detected by ELISA in this instance (*data not shown*). Few studies have looked specifically into the ability of AMs to secrete lipocalin-2 at baseline, and studies measuring levels of the cytokine in BALF are confounded by the ability of other cells in the alveolar

* I have collected supernatants from *S. pneumoniae*-infected primary AMs from n=14 individuals thus far to validate these findings in humans.

microenvironment to secrete lipocalin-2 (alveolar epithelial cells, neutrophils). Expression of the *Lcn2* gene was detected in unstimulated adherence-purified AMs (307), but whether a homeostatic role for lipocalin-2 in the lung exists, and how this would be altered in the COPD disease state, remains unknown.

In line with previous observations (308), CSE had an inhibitory effect on *S. pneumoniae* growth in *in vitro* culture (**Fig. 4.15A**). Smoking is a well-appreciated risk factor for invasive pneumococcal disease (309, 310), with suggestions that CS exposure can induce *S. pneumoniae* invasion from the colonised nasopharynx to the lower respiratory tract (311, 312). Multiple bacteria show enhanced virulence in models of cigarette smoke exposure, including enhanced biofilm formation in *P. aeruginosa* (313) and increased adherence to epithelial cells in *S. aureus* (314). Studies investigating this phenotype in *S. pneumoniae* are limited, but CSE treatment *in vitro* decreased pneumolysin expression, the primary pneumococcal pore-forming virulence factor (308), suggesting *S. pneumoniae* may rely on other mechanisms to fuel its virulence in the CS-exposed lung. As *S. pneumoniae* iron levels trend towards a decrease upon CSE treatment (**Fig. 4.15B**), this could indicate that these bacteria are depleted of iron in the COPD lung and thus show an increased readiness to obtain further iron from the host. Investigating the invasiveness of CSE-treated *S. pneumoniae* in CSE-treated FLAMs would help to unravel the functional effects of this bacterial iron deficiency and shed light on the underexplored effects of CS on respiratory pathogen virulence.

4.3.1 Limitations

Human AMs were acquired from individuals undergoing research bronchoscopy at Tallaght University Hospital. During the course of this study, we were only able to capture clinical variables relating to subject age, sex, disease status and smoking status. These individuals present with a variety of idiopathic and congenital lung diseases, which may influence the ability of AMs to fight *S. pneumoniae* infection independent of individual smoking status. Owing to limited sample size in our human study, we were unable to control for the effects of age and sex on bacterial burden. All ex-smokers in this study were men, with a mean age of 73. Age is known to associate with infection risk independent of COPD status, and macrophages isolated from aged mice showed reduced clearance of *S. pneumoniae* *ex vivo* (305). Men also show increased carriage of *S. pneumoniae* compared to women, along with higher rates of invasive pneumococcal disease (315, 316). We are currently recruiting approximately 10 subjects per month in this study: increase in cohort size in the future will provide the statistical power needed to further stratify patients by disease status, age and sex to determine their influence on our findings.

Opsonisation of the D39 *S. pneumoniae* strain with human serum failed to promote its uptake. Incubation of non-capsulated strains of *S. pneumoniae* has been observed in human AMs isolated through bronchoscopy, while non-encapsulated strains showed no uptake when preincubated with as much as 80% human serum (317). As such, infection experiments in human AMs were conducted using a clinical isolate of *S. pneumoniae*, obtained from a bronchoscopy sample. While this isolate was confirmed to be *S. pneumoniae* through phenotypic and MALDI-ToF analyses (*personal correspondence, Dr. Thomas Butler*) the precise serotype remains unknown. The 19A serotype is most prevalent in Ireland (318), so determining the serotype of our TUH strain will provide further clinical relevance in our data and provide other information of relevance to the interpretation of our results, such as capsular status*.

4.3.2 Conclusions

AMs show iron loading in COPD and smoking *in vitro* and *in vivo*, but how this affects the ability of AMs to control exacerbation-associated pathogens is unclear. We have shown that *S. pneumoniae*, a disease relevant pathogen, persists inside murine alveolar-like macrophages *in vitro* and in human AMs *ex vivo*. This infection upregulates MFRN1 and downregulates MFRN2, the first time that dynamic changes in mitochondrial iron import during infection have been noted. We have shown a novel effect of *S. pneumoniae* on macrophage iron, inducing a loss of total FLAM iron and an increase in the labile iron pool which appears unique to live bacteria. Intracellular persistence is heightened in AMs from *in vitro* and *ex vivo* smoking, which associates with blunted pro-inflammatory cytokine secretion *in vitro*. Our data highlights the profound effects of smoking on AM pathogen clearance and the importance of using live bacteria to study the nutritional immune response.

* The clinical *S. pneumoniae* isolate used in this study is currently undergoing whole genome sequencing analysis to investigate this.

Chapter 5: Defining the effects of mitochondrial iron manipulation on *S. pneumoniae* infection

5.1 Introduction

Numerous pathogens hijack host mitochondria for essential nutrients (208, 319, 320), but whether changes in mitochondrial iron levels can dictate macrophage response to infection is unknown. Results from **Chapter 3** and **Chapter 4** have shown treatment of alveolar-like macrophages *in vitro* with CSE induces both cellular and mitochondrial-specific increases in total iron, which associates with an increased bacterial burden and attenuated pro-inflammatory cytokine production upon *S. pneumoniae* infection. This led us to **hypothesise that bacteria can ‘steal’ this excess iron to fuel their virulence**, which could explain the increased incidence of infection in COPD patients (321). As these macrophages show increased iron in both the cytosol and in mitochondria, how the specific subcellular localisation of iron influences the capacity of *S. pneumoniae* to persist intracellularly is unclear. To test this, we sought to discriminate between cytosolic and mitochondrial iron levels through establishing models of compartment-specific iron overload and depletion *in vitro*.

Experimental models of iron supplementation *in vitro* are commonly used in the literature to mimic iron loading *in vivo* (322, 323). Iron exists intracellularly in both its bioavailable Fe^{2+} and inert Fe^{3+} forms and can be internalised from the extracellular space by cells through TFRC or DMT1-mediated import. Ferric ammonium citrate (FAC) and ferrous lactate (FeL) are water soluble iron-containing compounds with clinical use in treatment of iron-deficiency anaemia (324). FAC been used to model intracellular iron loading *in vitro* extensively in the literature and is proven to increase iron loading in subcellular organelles, including mitochondria, in macrophages (325). FeL is less studied in comparison, but the supply of iron in its more bioavailable Fe^{2+} form offers a contrast to FAC.

Induction of mitochondrial-specific iron overload in the literature typically relies on small molecule inhibitors of the heme synthesis pathway, which preclude the use of iron in heme production resulting in its accumulation in the mitochondrial matrix (326-328). Historically, succinylacetone (SA) and N-methyl-mesoporphyrin IX (NMMIX) have been utilised to this effect *in vitro*. SA and NMMIX are both porphyrin analogues which target the heme synthesis pathway: SA through degradation of ALAS (329, 330), the enzyme catalysing the first step in heme synthesis, and NMMIX through inhibition of ferrochelatase, the enzyme which catalyses the incorporation of Fe^{2+} into protoporphyrin IX to form heme

(Fig. 1.2) (331-333). While these chemicals have been used in erythrocytes and reticuloendothelial cells previously (330), their utility in both macrophages and primary human cells is underexplored.

Decreasing intracellular iron *in vitro* is typically achieved through treatment with iron chelators, a class of iron-binding molecules. Iron chelators are both naturally occurring and synthetic molecules that bind Fe^{2+} and/or Fe^{3+} iron to restrict bioavailability for use in biological systems. Clinically, these chelators can be used to alleviate pathological iron loading through limiting oxidative damage induced by excessive free radical formation (334). In diseases where 'anaemia of inflammation' is observed like COPD, where tissue iron overload renders patients systemically iron deficient, redistributing iron pharmacologically from tissues offers an alternative approach where phlebotomy is not useful (335). There are currently three iron chelators which are approved for clinical use in iron-overload disease settings by the European Medicines Agency and the US Food and Drugs Administration: desferasirox, deferoxamine (DFO), and deferiprone (DFP). While desferasirox is not frequently used for preclinical studies due to weak chelator-iron complex stability (336), both DFO and DFP treatments are common models of iron limitation in a range of cell lines, primary cells and *in vivo* models. At physiological doses DFO is thought to be membrane impermeable, with limited reports of intracellular activity specifically in cancer cells (337), and reduces intracellular iron through binding of Fe^{3+} in the extracellular space to limit iron available for cellular uptake (338). DFP is membrane permeable in contrast and can bind both Fe^{2+} and Fe^{3+} , oxidising Fe^{2+} to inactive Fe^{3+} when bound, with a weaker binding affinity than deferoxamine potentiating cytotoxicity (339, 340).

The unique pharmacokinetic properties of DFP makes it of particular interest in COPD. These two properties lend themselves to a 'shuttling' modality intracellularly, where **DFP can remove iron from mitochondria**, lysosomes and nuclei to donate to apotransferrin (transferrin without iron bound) for iron export (340, 341). DFP can chelate iron from human monocyte-derived macrophages *ex vivo* (129), and recent work from our group illustrates the beneficial effects of DFP in restoring AM function in human smoking (173). Treatment of human AMs isolated from smokers with DFP decreases total intracellular iron levels and associated with restoration of TNF- α secretion upon LPS stimulation (173). Importantly, this study confirms the intracellular biological activity of DFP in primary human AMs, but its mechanism of action in macrophages and therapeutic use in disease-relevant infection models remain understudied.

5.1.1 Aims

Results from **Chapter 4** revealed increased *S. pneumoniae* burden in CSE-treated FLAMs, an environment which **Chapter 3** established is replete in both cellular and mitochondrial iron. This chapter will address how manipulation of cellular and mitochondrial iron levels can affect the outcomes of *S. pneumoniae* infection through the following specific aims (**Fig. 5.1**):

- Developing and characterising pharmacological models of mitochondrial iron overload and depletion in FLAMs.
- Investigating the effects of altered mitochondrial iron levels on *S. pneumoniae* intracellular burden in murine and human AMs.
- Defining if bacterial sensitivity to host mitochondrial iron levels is macrophage specific through infection of MLE12 cells with pharmacologically manipulated cellular and mitochondrial iron.

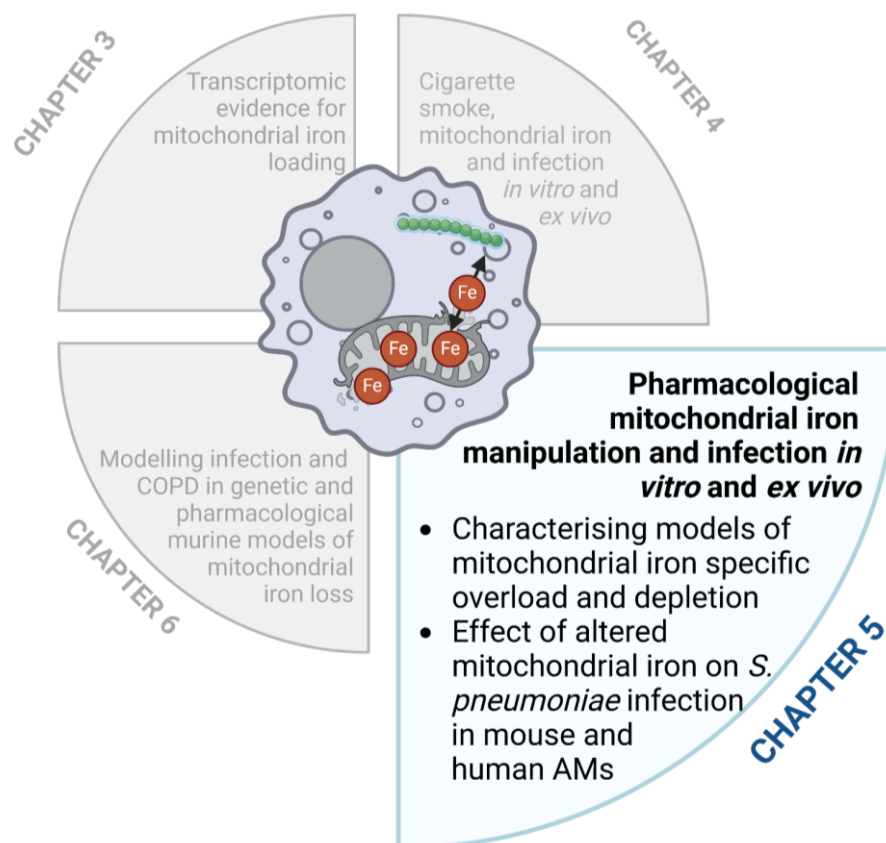


Fig. 5.1: Summary of aims of Chapter 5.

5.2 Results

5.2.1 Mitochondrial-specific alteration of FLAM iron alters whole cell iron metabolism

Our previous data has illustrated the association between CSE-induced cellular and mitochondrial iron loading and increased intracellular *S. pneumoniae* persistence in FLAMs. To further decipher how mitochondrial-specific iron loading or depletion can affect bacterial pathogenicity, we sought to establish a model of FLAMs with pharmacological induction of mitochondrial-specific iron loading and compare this to traditional methods of whole-cell iron loading and depletion.

FLAMs were treated with two compounds containing iron in its two oxidation states: ferric ammonium citrate (FAC), a source of Fe^{3+} , and ferrous lactate (FeL), a source of Fe^{2+} . Total iron in FLAMs was measured following 24 hr treatment through GF-AAS, which confirmed the ability of both compounds to induce a significant increase in cellular iron (**Fig. 5.2A**). To interrogate the mechanisms by which FAC and FeL induce iron loading, expression of iron-related proteins in these conditions was analysed through western blotting. In line with their increased iron levels, FLAMs treated with FAC and FeL showed increased expression of FTH1 and decreased expression of TFRC, indicative of reduced iron storage and uptake (**Fig. 5.2B**). An increase in NCOA4 was observed with both compounds relative to untreated control, with a slight elevation in FeL treatment compared to FAC (**Fig. 5.2B**).

Cellular iron in FLAMs was decreased through use of the iron chelators DFP and DFO. FLAMs treated with either DFP or DFO trended towards decreased whole cell iron after 24 hr, with a comparable reduction in iron from both chelators (**Fig 5.2A**). Western blotting revealed a striking loss in FTH1 expression with both chelators, supporting GF-AAS data (**Fig 5.2B**). DFP induced a strong upregulation of TFRC relative to DFO, while NCOA4 expression showed the opposite trend (**Fig 5.2B**).

We next sought to establish a model of iron loading specifically in mitochondria. To do this, two small molecule inhibitors of the heme synthesis pathway were used, succinylacetone (SA) and N-methyl-mesoporphyrin IX (NMMIX), which indirectly induce mitochondrial iron-specific loading through occluding utilisation in heme. After FLAM treatment with SA and NMMIX for 24 hr, whole cell iron levels trended towards decrease (**Fig 5.2A**). Despite this decrease in total iron, FTH1 expression increased with both compounds, along with increased levels of NCOA4 and unchanged TFRC expression (**Fig 5.2B**).

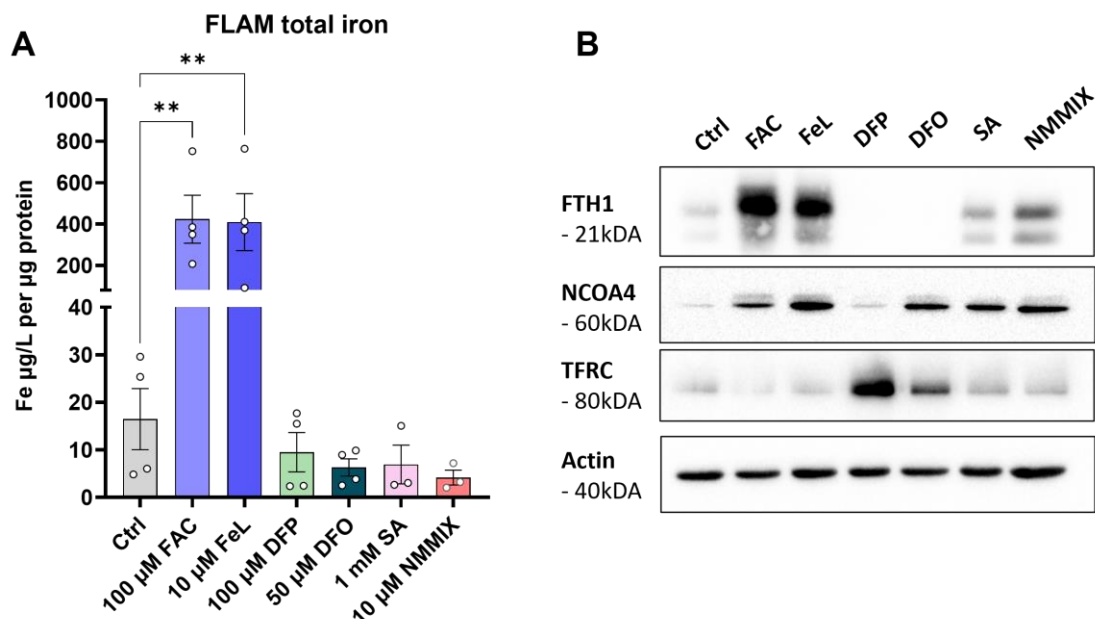


Fig 5.2: Pharmacological compartment-specific alteration of FLAM iron differentially regulates iron uptake and storage. FLAMs plated in a 12 well plate were treated with 100 µM ferric ammonium citrate (FAC), 10 µM ferrous lactate (FeL), 100 µM DFP (DFP), 50 µM deferoxamine (DFO), 1 mM succinylacetone (SA) or 10 µM N-methyl-mesoporphyrin IX (NMMIX) for 24 hr. Cells were lifted through scraping, washed once, and lysed in RIPA buffer. (A) Cell lysates were digested in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS. Data representative of mean ± S.E.M. of four independent experiments: each data point is representative of one biological replicate constituting the average of three technical triplicate wells of cells, normalised to protein content measured through BCA assay. (B) Protein as prepared in (A) was analysed through western blotting as described. Primary antibodies used are listed to the left of each blot along with estimated band weight determined relative to a protein marker of known molecular weights, with Actin presented as loading control.

Having established the ability of these compounds to manipulate FLAM whole cell iron, their effects on mitochondrial iron specifically were investigated. Mitoplasts were generated from these FLAMs and total iron measured through GF-AAS. To gain a more comprehensive understanding of subcellular iron distribution with these treatments, iron was also measured in the cytosolic fractions of these cells. Owing to technical limitations, this experiment could only be performed once. Cytosolic iron increased relative to untreated control with FAC and FeL treatment, with a higher degree of increase in FeL-treated FLAMs observed compared to FAC (**Fig. 5.3A**). Both DFP and DFO treatment reduced cytosolic total iron (**Fig. 5.3A**). Contradictory to our whole cell data, cytosolic iron increased in SA and NMMIX-treated FLAMs compared to untreated control (**Fig. 5.3A**). Total mitoplast iron trended towards increase with FAC and FeL treatment relative to untreated control (**Fig. 5.3B**). Total mitoplast iron was decreased with DFP treatment, with no change observed with DFO (**Fig. 5.3B**). SA and NMMIX both induced an increase in total mitoplast iron: NMMIX to a greater extent (**Fig. 5.3B**). To support our mitoplast total

iron data, we measured expression of ACO2 in whole cell FLAM lysates in these conditions through western blotting. This revealed an increase in ACO2 protein in conditions with increased mitoplast iron (FAC, FeL, SA, NMMIX), with no change observed in DFP or DFO treatment (**Fig. 5.3C**).

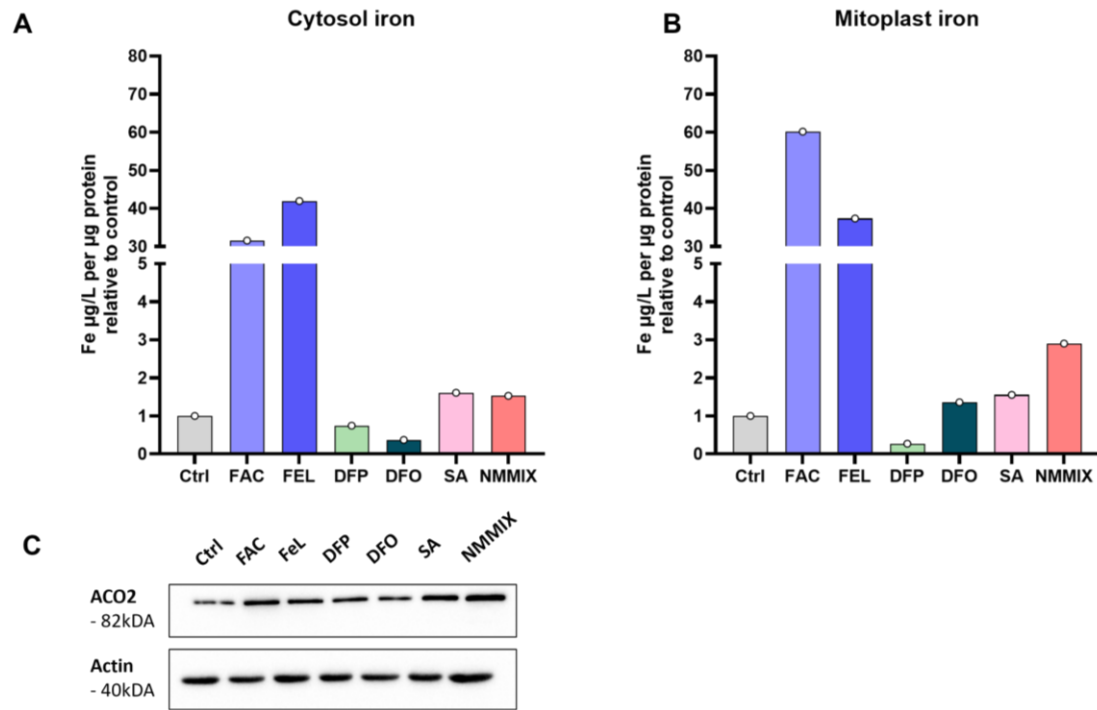


Fig 5.3 Pharmacological compartment-specific manipulation of FLAM iron may mitoplast iron: FLAMs were plated at 5×10^5 cells/mL in a 10 cm² dish and treated with 100 μM ferric ammonium citrate (FAC), 10 μM ferrous lactate (FeL), 100 μM DFP (DFP), 50 μM deferoxamine (DFO), 1 mM succinylacetone (SA) or 10 μM N-methyl-mesoporphyrin IX (NMMIX) for 24 hr, after which mitoplasts were prepared as described. (A) Cytosolic fraction and (B) mitoplast lysates were digested in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS. Each data point is representative of one biological replicate presented as fold change to control, normalised to protein content measured through BCA assay. (C) Western blotting for ACO2 was performed in whole cell lysates as prepared in **Fig. 5.2A**, with Actin presented as loading control.

We next wanted to confirm that these treatments in FLAMs do not induce cell death upon infection with *S. pneumoniae*, which was interrogated 4 hr post-infection through assaying lactate dehydrogenase (LDH) in cell supernatants as a surrogate for cell lysis relative to a killed control. There was little change in FAC, FeL, DFP, DFO, SA or NMMIX treatment either at baseline or combined with *S. pneumoniae* infection (**Fig. 5.4**).

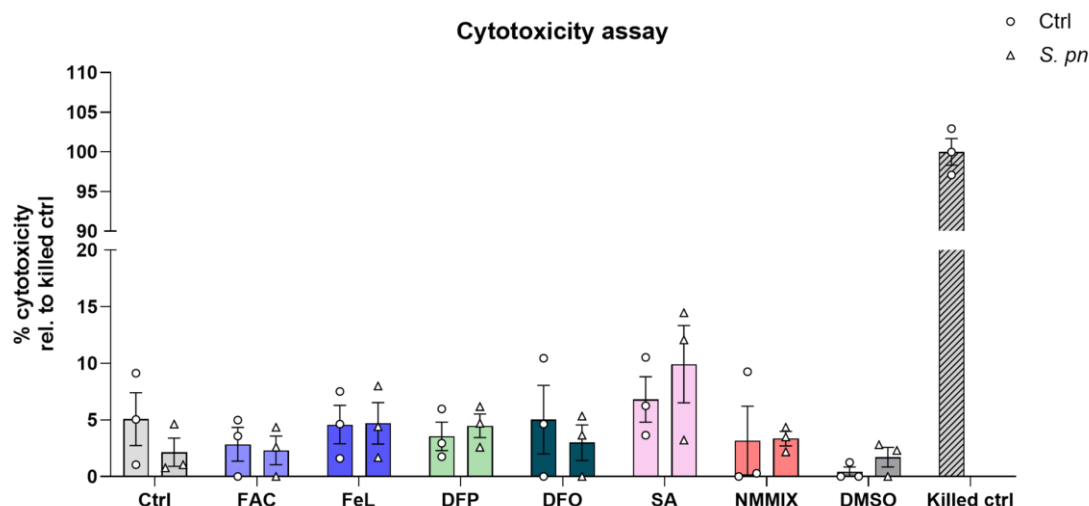


Fig 5.4: Pharmacological manipulation of FLAM iron levels does not affect cell viability upon infection. FLAMs were plated at 3×10^4 cells/well in a 96 well plate and treated with 100 μ M ferric ammonium citrate (FAC), 10 μ M ferrous lactate (FeL), 100 μ M DFP (DFP), 50 μ M deferoxamine (DFO), 1 mM succinylacetone (SA) or 10 μ M N-methyl-mesoporphyrin IX (NMMIX) for 24 hr. Cells were washed once and infected with *S. pneumoniae* at an MOI of 2 with gentamicin used as described to assess intracellular survival. LDH assay was performed as described 4 hr post-infection, with data representative of mean $\pm$ S.E.M. of one independent experiment conducted in technical triplicate relative to lysed (killed) control.

5.2.2 FLAM iron loading increases burden of *S. pneumoniae* infection

Our previous data has established the ability of FAC and FeL treatment to increase FLAM whole cell and mitochondrial total iron levels (**Fig. 5.5A**). Considering previous data showing that CSE-treated FLAMs, which also have increased whole cell and mitochondrial total iron levels (**Fig. 3.10, 3.11**), show an increased bacterial burden upon *S. pneumoniae* infection (**Fig. 4.10**), we next wanted to recapitulate this in our model of pharmacological iron loading. To do this, FLAMs treated with FAC and FeL as above were infected with *S. pneumoniae*, and intracellular burden was measured through CFU enumeration. Cells were washed once with PBS prior to infection to ensure there was no remaining FAC or FeL that could act as an extracellular iron source for bacteria. 4 hr post-infection, FLAMs treated with either FAC or FeL had increased *S. pneumoniae* CFU relative to untreated control, indicative of an increased intracellular persistence (**Fig. 5.5B**).

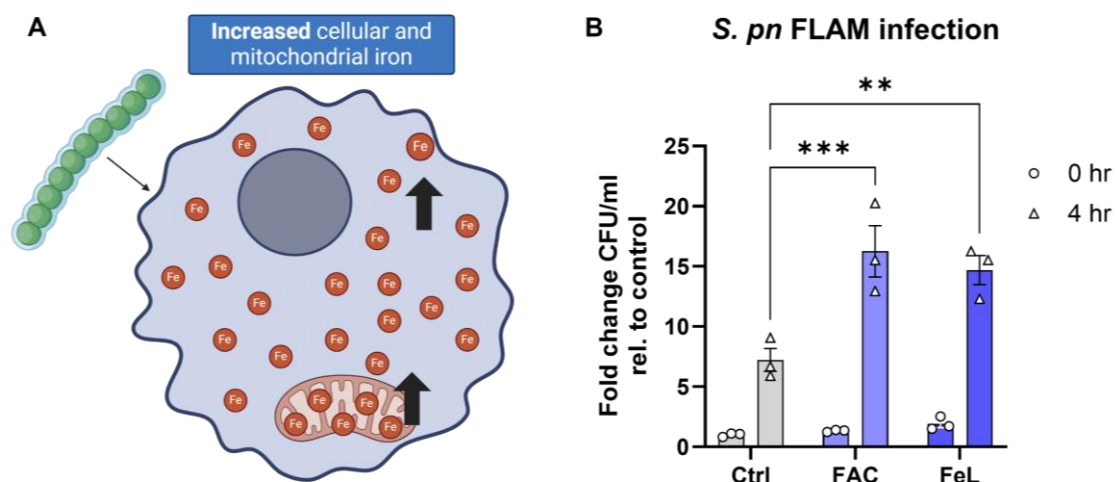


Fig 5.5: Iron loading increases burden of *S. pneumoniae* infection in FLAMs and human AMs. (A) Schematic of effects of FAC and FeL treatment on FLAM iron as in Fig. 5.2 and Fig 5.3. (B) FLAMs were treated with 100 μ M FAC, 10 μ M FeL or vehicle control (H_2O) for 24 hr, washed once, and infected with *S. pneumoniae* (MOI=2) with gentamicin used to assess intracellular persistence 4 hr after initial infection as previously described. Data presented as mean $\pm$ S.E.M. of three independent experiments performed in technical triplicate, with one representative experiment pictured. *P* value by two-way ANOVA; **= $P<0.01$, ***= $P<0.001$

5.2.3 Mitochondrial iron loading increases burden of *S. pneumoniae* infection in mouse and human AMs

As our previous results have shown that cellular iron loading increases burden of *S. pneumoniae* infection, we wanted to further determine if mitochondrial iron loading specifically could increase bacterial burden (Fig. 5.6A). To investigate this, FLAMs were treated with SA and NMMIX and infected with *S. pneumoniae* as before. In both SA and NMMIX treated FLAMs, *S. pneumoniae* CFU significantly increased 4 hr post-infection (Fig 5.6B). To confirm our findings in humans, human AMs were treated with NMMIX for 24 hr prior to infection with a clinical isolate of *S. pneumoniae*. NMMIX treatment was preferred for these experiments considering the higher increase in mitoplast iron levels observed in FLAMs with compared to SA (Fig. 5.3B). Human AMs treated with NMMIX showed significantly increased bacterial burden both 0 and 24 hr post-infection compared to untreated control, suggestive of both increased bacterial uptake (0 hr) and intracellular burden (24 hr) (Fig. 5.6C).

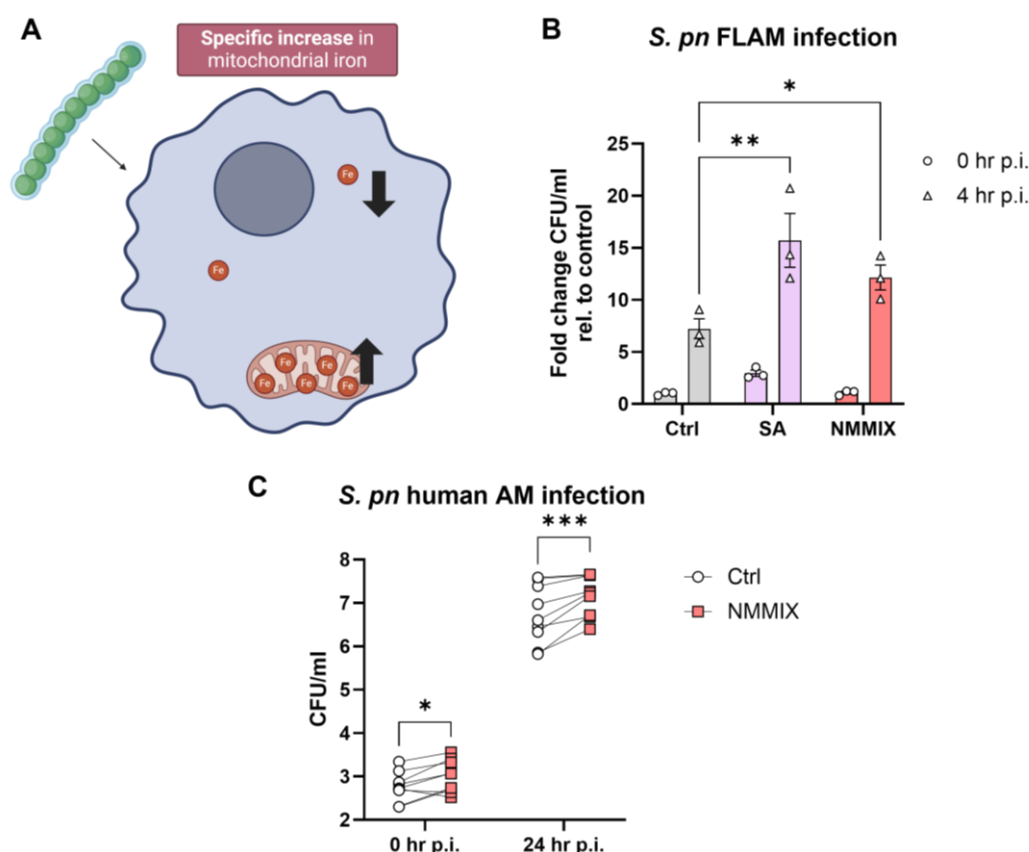


Fig 5.6: Mitochondrial-specific iron loading increases burden of *S. pneumoniae* infection in FLAMs and human AMs. (A) Schematic of effects of SA and NMMIX treatment on FLAM iron as in Fig. 5.2 and Fig 5.3. (A) FLAMs were treated with 1 mM SA, 10 μ M NMMIX or vehicle control (H_2O) for 24 hr, washed once, and infected with *S. pneumoniae* (MOI=20) with gentamicin used to assess intracellular persistence 4 hr after initial infection as previously described. Data presented as mean $\pm$ S.E.M. of three independent experiments performed in technical triplicate, with one representative experiment pictured. *P* value by two-way ANOVA; *= P <0.05, **= P <0.01. (B) Human AMs were isolated from BALF acquired from bronchoscopy, treated with 10 μ M NMMIX for 24 hr, washed once and infected with *S. pneumoniae* with gentamicin used to assess intracellular persistence 24 hr after initial infection as previously described. Data representative of 9 independent experiments from n=9 subjects, with CFU numbers from the same individual with treatment at each timepoint matched. *P* value by paired t-test; *= P <0.05, ***= P <0.001.

5.2.4 Mitochondrial iron chelation decreases bacterial burden in mouse and human AMs

Given the heightened intracellular burden observed in *S. pneumoniae* infection of FLAMs with increased mitochondrial iron, we next wanted to investigate if this could be reversed in iron-deplete conditions. FLAMs were treated with DFP and DFO as above to reduce intracellular iron (Fig. 5.7A), washed once with PBS to eliminate any effects of the compounds on extracellular bacteria independent of their intracellular iron chelation, and infected with *S. pneumoniae* as before. *S. pneumoniae* burden was significantly decreased 4 hr post-infection compared to untreated control, highlighting the ability of intracellular

iron chelation to diminish intracellular bacterial burden in FLAMs (**Fig. 5.7B**). Next, we wanted to investigate if our findings translated to human AMs. As DFP is membrane-permeable and targets mitochondria (340), DFP was favoured for human AM experiments. Human AMs were treated with 300 μ M DFP for 24 hr, a dose and time of DFP treatment previously shown by our group to reduce intracellular iron levels in human AMs isolated from smokers (173). DFP pre-treatment significantly reduced bacterial burden both 0 and 24 hr post-infection, with a profound decrease in CFU observed compared to untreated control (control median 5.63×10^6 CFU, DFP median 3.35×10^5 CFU) (**Fig. 5.7C-D**).

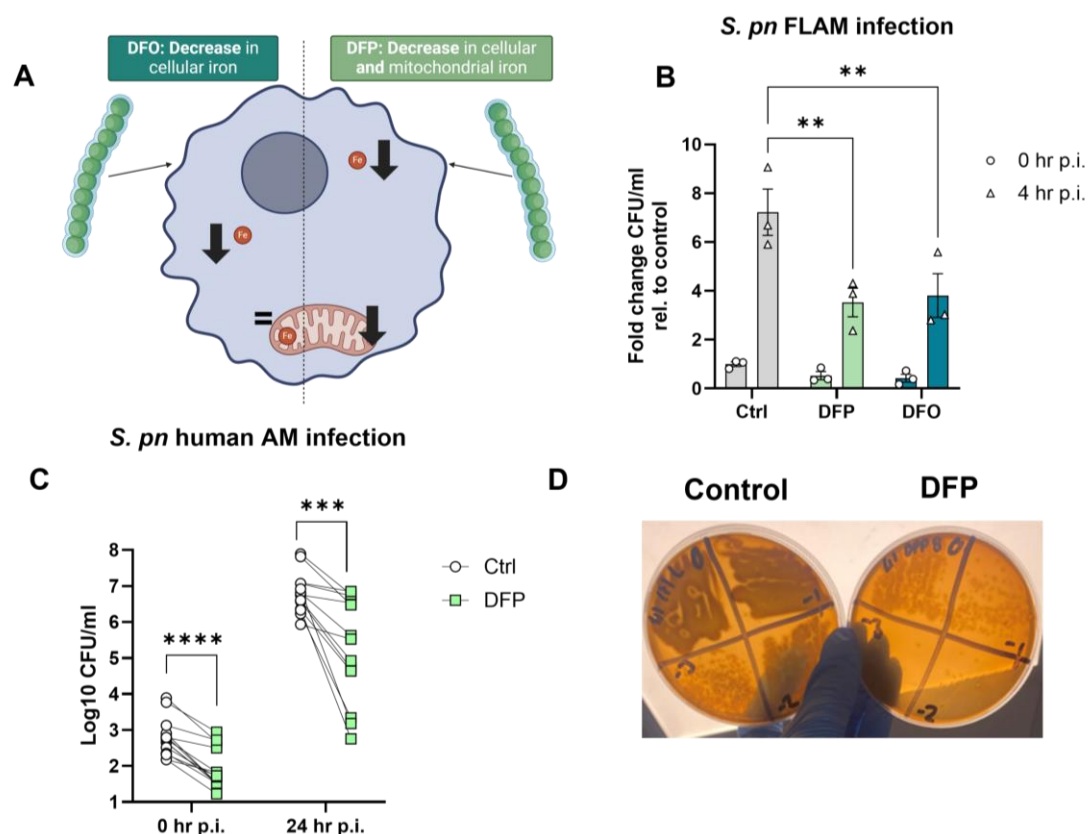


Fig 5.7: Iron chelation reduces burden of *S. pneumoniae* infection in FLAMs and human AMs. (A) Schematic of effects of DFO and DFP treatment on FLAM iron as in **Fig. 5.2** and **Fig 5.3**. (B) FLAMs were treated with 100 μ M DFP, 50 μ M DFO or vehicle control (H_2O) for 24 hr, washed once, and infected with *S. pneumoniae* (MOI=2) with gentamicin used to assess intracellular persistence 4 hr after initial infection as previously described through CFU enumeration. Data presented as mean $\pm$ S.E.M. of three independent experiments performed in technical triplicate, with one representative experiment pictured. *P* value by two-way ANOVA; **= $P < 0.01$. (B) Human AMs were isolated from BALF acquired from bronchoscopy, treated with 300 μ M DFP for 24 hr, washed once and infected with *S. pneumoniae* with gentamicin used to assess intracellular persistence 24 hr after initial infection as previously described through CFU enumeration. Data representative of 13 independent experiments from $n=13$ subjects, with CFU from the same subject with treatment at each timepoint matched. *P* value by paired t-test; ***= $P < 0.001$, ****= $P < 0.0001$. (D) Representative image of Todd Hewitt agar plates with 5% v/v sheep's blood streaked with serial dilutions of cell lysates 24 hr. p.i. from (C), beginning in top left segment with dilution factor increasing clockwise.

The above data illustrating the ability of DFP to reduce intracellular *S. pneumoniae* persistence suggest a strong relationship between mitochondrial iron chelation and decreased bacterial burden during infection. To test this hypothesis further, we collaborated with Prof. Rob Elmes (*Maynooth University, Ireland*) to characterise the ability of a novel, mitochondrially targeted derivative of DFO synthesised by his group to alleviate *S. pneumoniae* infection in FLAMs. This derivate of DFO (termed DFO-naph) contains an amide group distinct to traditional DFO which facilitates the molecule's membrane permeability and targeting to mitochondria. Additionally, DFO-naph contains a fluorophore which is quenched upon Fe^{3+} binding (*Prof. Rob Elmes, personal correspondence*). To confirm the presence of DFO-naph intracellularly, FLAMs were treated with increasing concentrations of DFO-naph for 24 hr, washed once in PBS to ensure the compound was fully removed extracellularly, and fluorescence monitored. Fluorescence increased in a dose-dependent manner with DFO-naph, confirming intracellular accumulation of the compound in FLAMs (**Fig 5.8A**). Next, the ability of DFO-naph to chelate intracellular iron in FLAMs was measured through GF-AAS. FLAMs treated with DFO-naph had trending decreases in total iron following 24 hr treatment, comparable to traditional DFO (**Fig 5.8B**). Fluorescence appeared decreased in cells pre-treated with FAC, with this quenched highlighting increased iron binding by DFO-naph in these iron-laden FLAMs (**Fig. 5.8C**). To investigate the effects of DFO-naph pretreatment on the outcomes of infection, we first confirmed that no significant cell death was observed with DFO-naph pretreatment upon *S. pneumoniae* infection compared to DMSO-treated control (**Fig 5.8D**). In line with previous results using iron chelators, *S. pneumoniae* intracellular persistence in DFO-naph treated FLAMs was significantly decreased (**Fig 5.8E**).

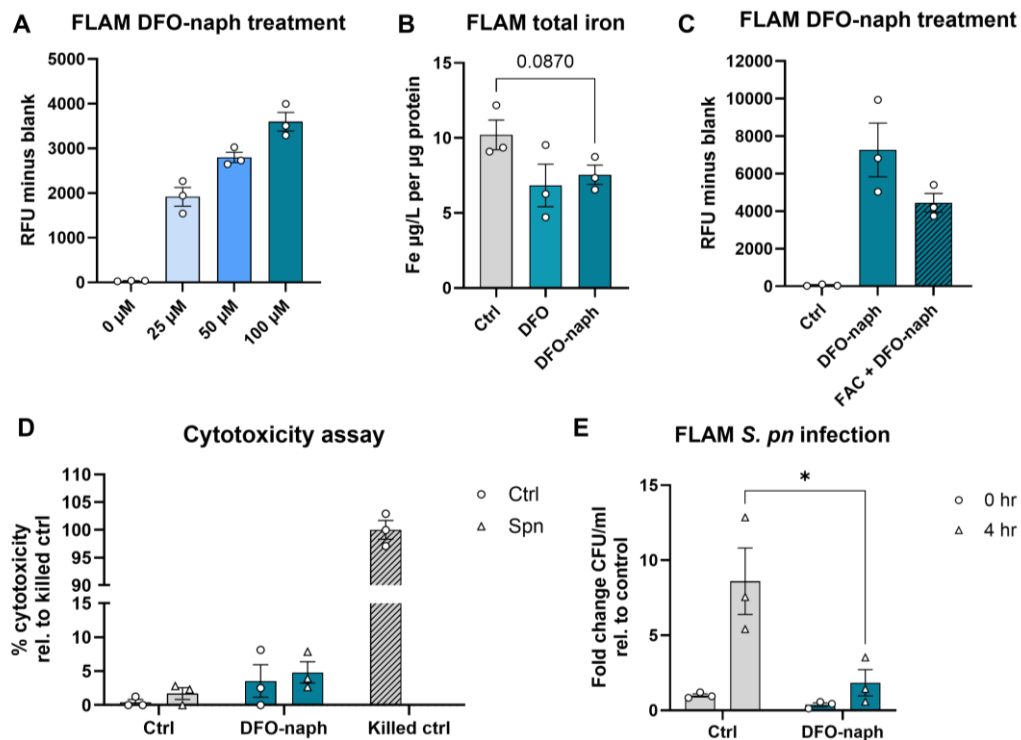


Fig 5.8: A novel iron chelator decreases burden of *S. pneumoniae* infection in FLAMs. (A) FLAMs were seeded at a density of 3×10^4 cells/well in a black-walled 96 well plate, treated with DFO-naph at indicated concentrations or vehicle control (DMSO) for 24 hr. Cells were washed once and complete FLAM media without phenol read added prior to fluorescence measurement at 435nm_{ex} , 535nm_{em} . Data presented as mean $\pm$ S.E.M representative of one independent experiment conducted in technical triplicate. (B) FLAMs were plated at 5×10^5 cells/mL in a 12 well plate and treated with $50\text{ }\mu\text{M}$ DFO, $50\text{ }\mu\text{M}$ DFO-naph or vehicle control (DMSO) for 24 hr. Cells were lifted through scraping, washed once, and lysed in RIPA buffer. Cell lysates were digested in 50% nitric acid for 2 hr at 70°C for iron measurement through GF-AAS. Each data point is representative of one biological replicate constituting the average of three technical triplicate wells of cells, normalised to protein content measured through BCA assay. Data presented as mean $\pm$ S.E.M. of three independent experiments. *P* value by unpaired t-test. (C) FLAMs were seeded at a density of 3×10^4 cells/well in a black-walled 96 well plate, treated with $100\text{ }\mu\text{M}$ FAC for 24 hr, washed once and treated with $50\text{ }\mu\text{M}$ DFO-naph. Fluorescence was measured as in (A), data presented as mean $\pm$ S.E.M representative of one independent experiment conducted in technical triplicate. (D) FLAMs were treated with $50\text{ }\mu\text{M}$ DFO-naph or vehicle control (DMSO) for 24 hr, washed once, and infected with *S. pneumoniae* (MOI=2) with LDH release (D) and intracellular persistence (E) measured 4 hr post-infection with gentamicin used to assess intracellular persistence 4 hr after initial infection through CFU enumeration. Data presented as mean $\pm$ S.E.M. of three independent experiments performed in technical triplicate, with one representative experiment pictured. *P* value by two-way ANOVA; $*=P<0.05$. CFU = colony forming units, *S. pn* = *S. pneumoniae*.

5.2.5 Pharmacological manipulation of MLE12 iron does not affect burden of *S. pneumoniae* infection

Results in this chapter have shown a positive correlation between AM mitochondrial iron levels and the burden of *S. pneumoniae* infection in both mouse and human AMs. To investigate if pharmacological manipulation of subcellular iron can similarly affect the outcomes of infection in other cell types, MLE12 cells were treated with FAC, FeL, DFP, DFO and SA* and infected with *S. pneumoniae* and intracellular persistence assessed through gentamicin invasion assay. *S. pneumoniae* replicated intracellularly in MLE12 cells, with CFU enumeration revealing a significant increase 24 hr post-infection. Contrary to FLAM data, no significant differences were observed in *S. pneumoniae* burden 24 hr post-infection in any conditions compared to the untreated control (**Fig. 5.9**).

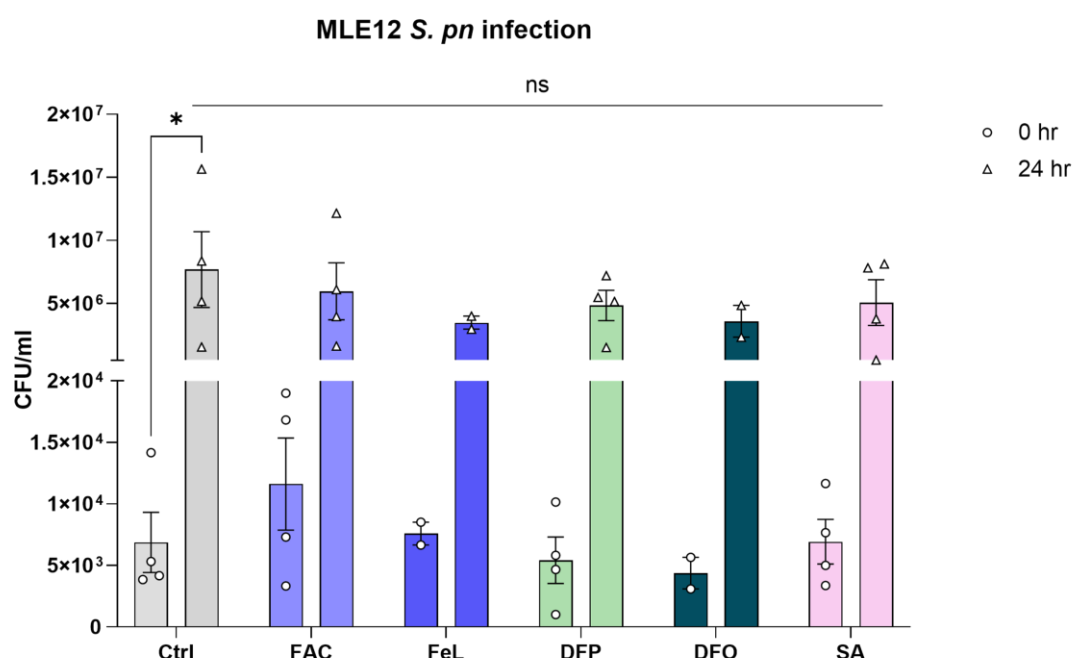


Fig. 5.9: Pharmacological manipulation of MLE12 iron levels does not affect *S. pneumoniae* intracellular replication. MLE12s were seeded at a density of 3×10^4 cells/well and treated with 100 μ M FAC, 10 μ M FeL, 10 μ M DFP, 10 μ M DFO, 1 mM SA or vehicle control for 24 hr. MLE12s were washed once and infected with *S. pneumoniae* (MOI=20) with gentamicin used as described to assess intracellular persistence. Data representative of mean $\pm$ S.E.M. of $n \geq 2$ independent experiments conducted in technical triplicate, *P* value by two-way ANOVA; *= $P < 0.05$, ns = non-significant. CFU = colony forming units, *S. pn* = *S. pneumoniae*.

* The ability of these compounds to mirror changes observed in FLAMs in **Fig. 5.2A** in MLE12s at these doses and times has been previously validated by other members of the Cloonan lab.

5.3 Discussion

How altered mitochondrial iron levels dictate macrophage function in response to infection is unknown. Using an *in vitro* model of murine AMs, we employed several chemical approaches of subcellular iron manipulation, investigated through total iron measurement in whole cell lysates and subcellular fractions. Cellular and mitochondrial-specific iron loading was pathogenic in *S. pneumoniae* infection, with our findings using the mitochondrial iron-loading agent NMMIX translated to human AMs infected *ex vivo*. FLAM iron chelation reduced the burden of *S. pneumoniae* infection, replicated using a novel mitochondrially targeted derivate of DFO. A similar reduction was observed upon *ex vivo* infection of human AMs treated with DFP. Treatment of MLE12 cells with the same iron modulators had no effect on the outcomes of *S. pneumoniae* infection, emphasising the importance of proper macrophage iron handling in the bactericidal response. These results present **mitochondrial iron as a candidate mediator of nutritional immunity in AMs** and suggest mitochondrial iron may be a druggable target in both COPD and other diseases characterised by recurrent acute infections.

To increase total cellular iron, FLAMs were treated with FAC or FeL (**Fig. 5.2A**). Iron loading was confirmed through GF-AAS, where FAC induced a marginally higher increase compared to FeL (**Fig. 5.2A**). Due to the strong effect of FeL on FLAM cell viability (*data not shown*) the concentration we used to treat cells (10 μ M) was far below that of FAC (100 μ M). Accordingly, there may be a discrepancy in the absolute amount of iron provided to FLAMs at these doses to which observed changes may be attributed. In line with this iron-loaded state, FAC and FeL treated FLAMs showed increased ferritin and decreased TFRC expression (**Fig. 5.2B**). Perhaps counterintuitively, NCOA4, the autophagy cargo receptor responsible for liberating iron from ferritin, was upregulated with both treatments compared to untreated control. NCOA4 is typically employed when intracellular iron is limited to mobilise inert Fe^{3+} iron from the ferritin nanocage to redirect it to the labile iron pool and increase bioavailable iron. In iron replete conditions, an FeS cluster is bound to the FTH1-binding domain of NCOA4, precluding binding of ferritin and limiting liberation of iron (342). Therefore, while there is an increase in NCOA4 protein in these iron loaded cells, it is likely inactive at this time. Cells could therefore maintain a certain ratio of NCOA4 relative to ferritin to ensure ferritinophagy can be efficiently employed in response to any sudden decrease in intracellular iron, which could explain elevation of the protein product in iron-loaded cells in this instance.

While this work is the first to show these treatments in macrophages can increase *S. pneumoniae* intracellular persistence, enhanced virulence of other bacteria in iron-

loaded macrophages is well described. *E. chaffeensis* proliferation and effector protein secretion was enhanced in FAC-treated THP-1 cells (103), supplementation of RAW264.7 macrophages with ferrous sulfate increased *Salmonella* Typhimurium burden (343) and FAC-treated BMDMs showed attenuated pro-inflammatory cytokine secretion and bacterial killing upon *Burkholderia pseudomallei* infection (344). Supplementation of *S. pneumoniae* culture medium with FAC failed to enhance *in vitro* growth in culture (345), suggesting that in our model *S. pneumoniae* may be utilising redistributed iron from a host source and not directly from the compound itself.

To decrease intracellular iron levels, two iron-chelating compounds were used: DFO and DFP. DFP is documented to be active at mitochondria and chelates murine whole-lung mitochondrial iron (50), with preliminary data in **Fig. 5.3B** suggesting this may also extend to AMs. The extent of DFP targets intracellularly is unknown, but DFP can directly bind and remove iron from transferrin (346). Whether DFP can extract iron bound in co-factors, like FeS clusters or heme, or in complex proteins such as ferritin, mitochondrial ferritin, or hemosiderin, remains a point of debate.

Despite their similar effects on total iron levels, DFP and DFO induced differential expression of iron metabolism related proteins relative to one another. DFP strongly increased expression of TFRC, while DFO-treated FLAMs showed elevated expression of NCOA4 (**Fig. 5.2B**). The divergent mechanisms employed to increase bioavailable iron intracellularly may reflect the modes of action of these two chelators: DFP chelates iron intracellularly, forcing cells to import transferrin-bound iron from the extracellular space, while DFO binds extracellular iron to prevent cellular import, forcing cells to liberate iron from storage. Cells are purported to sense iron in the extracellular milieu indirectly through intracellular monitoring of iron import via the IRP axis. No direct mechanism by which cells can detect extracellular iron overload or deficiency has been defined thus far that is analogous to the regulation of NCOA4-mediated ferritinophagy by FeS cluster availability (342). Given that mitochondria can sense glutathione through the presence of FeS binding to membrane solute characters like SLC25A39 (53), it would be tempting to speculate that transporters on the plasma membrane can use a similar approach.

Use of pharmacological agents documented in the literature to induce mitochondrial-specific iron loading in both FLAMs and human AMs significantly increased bacterial burden upon *S. pneumoniae* infection (**Fig. 5.6**). Importantly, this mimics observations upon FAC and FeL treatment (**Fig. 5.5**): as total intracellular iron is decreased with SA and NMMIX (**Fig. 5.2A**), the one commonality across these treatments is increased mitochondrial iron. The notion that increased bacterial proliferation in

mitochondrial iron-loaded settings could be attributed to bacterial iron theft from mitochondria is an intriguing prospect and highlights mitochondrial iron dynamics as an understudied branch of the nutritional immune response. Whether this increase facilitates bacterial growth directly through such iron piracy or has a directly host-detrimental effect on the bactericidal functions necessary to limit *S. pneumoniae* growth is an open question*, as is the generalisability of these findings to other pathogens.

Treatment of FLAMs with DFO and DFP prior to infection with *S. pneumoniae* decreased bacterial intracellular survival 4 hr post-infection (**Fig. 5.7A**). DFO has been used to investigate the effects of iron limitation on pneumococcal growth in *in vitro* culture (347), but our findings are the first to demonstrate chelation of host cell iron using either of these agents can reduce burden of *S. pneumoniae* infection in a host-pathogen model. In line with these findings, pretreatment of the RAW264.7 macrophage cell line with DFO prior to infection with *Salmonella typhimurium* reduced intracellular bacterial replication (343), and DFO treatment improved metabolic function in human macrophages infected with *M. tuberculosis* (348). The effects of DFP on host immune response have only been studied in sterile models of inflammation thus far (129, 173), and these findings are the first to suggest that targeting mitochondrial iron specifically can alleviate bacterial burden.

To confirm the translational capacity of our findings and investigate the therapeutic potential of targeting mitochondrial iron in infection, we isolated human AMs from individuals with varying smoking statuses and infected them with a clinical isolate of *S. pneumoniae* *ex vivo*. Previous studies detailing the effects of iron chelation in primary human cells in response to infection are limited. While DFO improved glycolytic control of *M. tuberculosis* infection in human MDMs (348), systemic administration to mice failed to decrease AM iron levels (349). Combined with the ability of certain pathogens to uptake iron-laden deferoxamine owing to its natural origins as a bacterial siderophore, the utility of DFO as therapeutic against infection *in vivo* may be limited (350, 351). As such, we chose to proceed with DFP treatment to chelate iron in human AMs *ex vivo*. A treatment regimen of 300 μ M DFP for 24 hr was used in line with our published work (173), which was well-tolerated by human AMs. Previous work from our group has suggested DFP may restore TNF- α production in response to LPS in human AMs from smokers (173), but these findings are the first to demonstrate DFP treatment may control bacterial infection in primary human cells (**Fig 5.7**). Increasing sample size in the future, including through enlisting COPD patients, will help to determine the specific effects of DFP and NMMIX in both smoking and COPD. Including additional readouts of the host response to infection

* I am currently addressing this through developing a streptavidin-mediated pulldown assay to isolate *S. pneumoniae* from infected cells.

in these models, such as pro-inflammatory cytokine or ROS production, will complement CFU data and further delineate the effects of mitochondrial iron manipulation on the host immune response.

The heme synthesis pathway was targeted to inhibit the rate of iron accumulation into heme, indirectly increasing levels of iron in the mitochondrial matrix. Aside from heme, mitochondrial iron is also incorporated into FeS clusters (352). As iron sulfur clusters use multiple iron atoms per molecule (ranging from 2 to 16) and heme only one, this may be a more efficient target in limiting mitochondrial iron usage and thus inducing mitochondrial iron overload. Supporting this, mice with a heterozygous loss of frataxin, a key scaffold protein in FeS cluster assembly, showed elevated levels of whole lung mitochondrial non-heme iron levels (50). No small molecule inhibitors of any mediators of FeS cluster synthesis thus far have been identified, limiting the applications of this *in vitro*.

Measuring whole cell total iron in SA and NMMIX treated FLAMs through GF-AAS revealed a loss in intracellular iron (**Fig. 5.2A**). This notion that mitochondrial iron overload can affect cytosolic iron metabolism highlights the overarching role of mitochondrial iron in normal cellular function. Beyond their traditional role as the powerhouse of the cell, extensive research over recent years has characterised mitochondria as multifaceted organelles that process and send signals to and from the cytosol to govern cellular homeostasis and response to stress. Despite the disproportionate localisation of iron-containing proteins to mitochondria relative to other subcellular compartments (353), the role of mitochondrial iron in such intracellular signalling is not known. A similar phenomenon is observed in patients with Friedrich's ataxia, who carry genetic loss-of-function mutations in frataxin. In a similar manner to our inhibition of heme synthesis, loss of FeS cluster biogenesis in these patients ensuing from inefficient frataxin activity drives accumulation of iron in the mitochondrial matrix, but depletes cytosolic iron levels compared to those from wild-type individuals (340). Mice with a genetic loss of FTH1 show reduced mitochondrial iron (354), which in line with FTH1 protein expression (**Fig. 5.2B**) suggests loss of cytosolic iron levels in SA and NMMIX treated cells could be independent of FTH1. The loss of heme as a prosthetic group for the estimated 1% of cellular proteins which utilise it as an enzymatic cofactor may also contribute to the observed decrease in whole cell iron (353).

S. pneumoniae is thought to have a particular affinity for heme iron. Supplementation of erythrocytes (355) and hemoglobin (345) enhanced pneumococcal growth in culture, and in the case of hemoglobin induced biofilm formation (356). Despite the loss of heme production that predicates mitochondrial iron loading with SA and NMMIX

treatment, *S. pneumoniae* intracellular burden was enhanced compared to untreated control (**Fig. 5.6**). Due to its lipophilic and oxidative nature, heme can prove toxic to bacteria. It has been suggested that hemoglobin may facilitate the direct membrane binding and delivery of heme to bacteria, with this sequestration in globins negating its toxicity compared to free heme in the form of hemin (345, 357). Importantly, studies detailing the beneficial effects of heme iron for *S. pneumoniae* growth were conducted through supplementation of pneumococcal growth medium: our findings are the first to study how altered intracellular macrophage iron affects pneumococcal growth, which poses different considerations for bacterial iron usage than direct addition to culture medium. Taken together, our data warrants further investigation into the viability of heme as an intracellular iron source for *S. pneumoniae*. Preincubation of *S. pneumoniae* with erythrocytes enhanced bacterial escape from neutrophil killing in an *in vitro* co-culture model, suggesting intracellular heme may be exploited by *S. pneumoniae* to some extent (355). Considering the conserved role of macrophages in erythrophagocytosis, and the suggestion that erythrocytes may contribute to AM iron loading in COPD (358), measuring *S. pneumoniae* intracellular persistence in stressed red blood cell-exposed FLAMs as a model of erythrophagocytosis will be informative.

As a complementary readout to total mitoplast iron measurement with GF-AAS, ACO2 protein expression was assessed through western blot. Binding of an FeS cluster is essential for the catalytic activity of ACO2, but unlike cellular aconitase a moonlighting role of ACO2 in the absence of an FeS cluster is not known (1). An *in silico* analysis of iron distribution in primary murine T cells predicted ACO2 as the top iron interacting protein, based on direct usage and association with other iron-containing proteins (359). This suggests ACO2 expression is likely sensitive to mitochondrial iron and may positively associate with iron levels, as suggested by our data. Measuring aconitase enzymatic activity in mitochondrial and cytosolic cellular fractions in this model would represent a further refinement of this readout and allow for simultaneous inference of mitochondrial and cytosolic iron levels.

There was no change in intracellular *S. pneumoniae* persistence in MLE12 cells in cellular or mitochondrial iron excess or deficiency. While they are not immune cells, AT2 cells have immune-like properties, such as cytokine secretion, which are engaged during infection (360). A549 cells, an AT2-like cell line, upregulate FPN gene expression and downregulate FTH1 expression in response to infection with the intracellular *Klebsiella pneumoniae*. Infection with the extracellular *E. coli* resulted in decreased FPN and increased FTH1, illustrating the capacity of these cells to differentially regulate iron metabolism in response to bacterial dynamics in a process akin to nutritional immunity

(361). While the treatment concentrations used in this study have been used in our lab previously to successfully manipulate MLE12 iron levels (*data not shown*), the extent of these changes may be lesser than observed in the FLAMs. Another important consideration is the timepoints at which bacterial burden was measured in these models. To account for the inability of AT2 cells to directly phagocytose bacteria, MLE12 cells were infected using an MOI of 20, higher than the MOI of 2 used in FLAM infection experiments. As pneumococcal intracellular replication is delayed in MLE12s compared to FLAMs, CFU were measured 24 hr post-infection, where the increased time post-treatments may diminish their effects. AT2 cells also have more mitochondria than AMs, which may further dilute the effects of treatments (362).

5.3.1 Limitations

Technical issues involving the GF-AAS used for atomic iron measurements in this study limited our ability to measure iron in mitoplasts treated with the pharmacological agents employed in this chapter, meaning data detailing cytosolic and mitoplast iron measurements from only one biological replicate is displayed in **Fig. 5.3A-B**. Further experimental work is essential to confirm the ability of these agents to manipulate mitochondrial iron levels in line with what is described in the literature in FLAMs at these described doses and times of treatment.

Previous studies have used the chelator 2, 2-bipyridine to chelate macrophage iron in *in vitro* bacterial infection models (363, 364). However, 2, 2-bipyridine is not iron-specific and binds a wide range of metal cations intracellularly, meaning observed changes cannot be attributed to a loss of iron bioavailability alone (365). To curb off-target depletion of other heavy metals, chelators that preferentially bind iron were employed. Despite this, DFO and DFP binding of other transition metal ions has been reported in limited circumstances (366, 367). Approaches that better represent the physiological levels of iron deficiency that a cell may experience in an *in vivo* system typically remove iron, which is replete in serum supplements, from cell culture media, and supply a known quantity of transferrin-bound iron (368). As the extent of pathological iron loading in COPD AMs is supraphysiological, pharmacological means were used to deplete iron in our model. Confirming that DFP treatment retains its beneficial effects in limiting infection, both in *in vitro* and *ex vivo* smoking and COPD, is an important next step.

As FLAMs and MLE12 cells were pretreated with iron-altering compounds for 24 hr prior to infection, the synergistic effects of these treatments in combination with infection was not explored. Studies have shown that direct iron limitation profoundly remodels the *S. pneumoniae* proteome (347), and interference with pneumococcal iron uptake through

substitution with the chemically-similar ruthenium decreases bacterial viability (369). Investigating simultaneous administration of iron-manipulating agents with bacteria to mimic a therapeutic regimen will be an important pre-clinical step, particularly as these findings have already highlighted the therapeutic potential of DFP when administered prophylactically*.

Treatments in which an increase in total mitoplast iron was observed associated with an increase in *S. pneumoniae* intracellular burden, leading us to hypothesise that this increased mitochondrial iron may act as a nutrient source to fuel bacterial proliferation and virulence. In experimental COPD, whole lung mitochondrial iron loading is accompanied by reduced membrane potential (50), a phenomenon observed in COPD AMs and MDMs (145, 250). To determine whether these perceived 'leakiness' of mitochondria is present in our treatment model and could enhance bacterial iron siphoning, mitochondrial number and membrane potential should be determined. Further mechanisms of mitochondrial-mediated bacterial killing, such as mtROS production, should also be assessed to determine the confounding effects of poor mitochondrial health on bacterial killing.

5.3.2 Conclusions

Results in **Chapter 4** showed increased *S. pneumoniae* intracellular persistence in CS-exposed FLAMs and human AMs, suggesting cellular and mitochondrial iron loading promotes bacterial pathogenicity. Through subcellular compartment-specific pharmacological iron manipulation, we have suggested a potential link between mitochondrial iron availability and *S. pneumoniae* infection. Pretreatment with pharmacological agents targeted the heme synthesis pathway to induce mitochondrial iron accumulation positively associated with bacterial burden in both an alveolar macrophage-like cell line and in human AMs *ex vivo*, with administration of DFP prior to infection decreasing bacterial burden in current and former smokers. Our findings suggest an as-of-yet unappreciated role of the mitochondrial iron axis in the macrophage nutritional immune response and may hold wider consequences for infection in other diseases characterised by uneven subcellular iron distribution.

* The therapeutic efficacy of DFP post-infection is currently being assessed by Ruairaidhrí Jordan, a PhD student in the Cloonan lab.

Chapter 6: Targeting macrophage mitochondrial iron *in vivo* to improve infection outcomes

6.1 Introduction

Results in **Chapter 5** showed that pretreatment with the intracellular iron chelator DFP, thought to be mitochondrially targeted, had a profound effect in limiting *S. pneumoniae* intracellular burden in both murine and human AMs. Chemical manipulation of intracellular iron in this manner allows for control over the extent of iron loading and depletion through the concentration of compound used, along with the ability to supply or target iron in a particular oxidation state. However, the effects of chemical mediators can often be dose and time dependent, and activity of these compounds at other subcellular organelles important in iron storage and metabolism such as the lysosome cannot be ruled out. Lentiviral transfection of cell lines with targeted guide RNAs can facilitate gene deletion, offering a more refined means of studying biological processes: however, macrophages are notoriously difficult to transfect *in vitro* (370), limiting the ability to genetically manipulate mitochondrial iron metabolism in FLAMs.

Furthermore, these *in vitro* models fail to recapitulate the physiological route of *S. pneumoniae* infection, which in the case of invasive pneumococcal disease is through the nasopharyngeal tract (176). Cell culture media contains elevated iron, glucose, and glutamate levels relative to the alveolar microenvironment AMs are positioned in *in vivo*, and fails to account for the presence of lipid-rich surfactant or exposure to air (81). A purified, *in vitro* AM population will therefore not be fully representative of the alveolar microenvironment encountered by *S. pneumoniae*, both in terms of cellular populations present and recruited and the contribution of the unique extracellular metabolic environment (81, 371). Similarly, *S. pneumoniae* can manipulate both immune cells and the lung microbiome to establish a bacterial niche in the lower respiratory tract, factors which are also absent *in vitro* (372). The viability of AM mitochondrial iron as a therapeutic target in *S. pneumoniae* infection therefore requires investigation *in vivo*. To assess this, we sought to create a transgenic strain of mice deficient in MFRN1 and 2, which we hypothesised would be protective in *in vivo* *S. pneumoniae* infection.

The Cre-loxP system is common approach used to develop transgenic strains of mice. Flanking of a target gene (referred to as a 'floxed' gene) with two loxP sites will result in deletion in the presence of Cre, the cell type-expression of which can be refined through placing its expression under the control of a promoter only active in a particular subset of cells (373). The amenability of the Cre-loxP system to facilitating spatial control over gene

expression therefore offers a powerful means of studying the effects of loss of one or more genes in a targeted cell population in an *in vivo* system. There are no published models of MFRN1 or 2 overexpression *in vivo*, but the Cre-loxP system has been used previously to delete MFRN1 and 2 in mice in both a global and tissue-specific manner. While heterozygotes were viable, global homozygous deletion of MFRN1 using a Cre under the control of the constitutively active *Hprt* promoter resulted in embryonic lethality, with inducible deletion in 4-week-old mice manifesting in inefficient erythropoiesis and profound anaemia (374). In contrast, mice with a global deletion of MFRN2 develop through to adulthood, but males show reduced fertility (375). Importantly, cell and tissue specific deletion of MFRN1 and/or 2 under relevant Cre drivers does not affect gross physiology or embryonic viability, meaning genetic strains carrying a targeted loss of MFRN1 and 2 can be generated and studied (374, 375).

Previously, a transgenic strain of mice carrying floxed sequences of both *Slc25a37* and *Slc25a28* on a C57BL6/J background was developed by Prof. Diane Ward's group at the University of Utah (375). In these mice, MFRN1 protein expression is ablated upon Cre expression through loxP sites flanking the second of 4 coding exons, resulting in a shorter *Slc25a37* mRNA transcript which is not translated to a protein product (374). Meanwhile, loxP sites on the 4-exon *Slc25a28* genomic sequence target exon 4, which yields a truncated MFRN2 protein product that is unable to localise to mitochondria (375). These mice have only been used once before in the literature, to generate a strain deficient in MFRN1 and 2 in the whole liver under the control of the *Alb* promoter (375). Notably, loss of MFRN1 and 2 in this context significantly decreased mitochondrial non-heme iron, confirming loss of MFRN1 and 2 can be used to directly deplete mitochondrial iron levels in a tissue-specific manner (375). Beyond this liver-specific deletion, MFRN1 and 2 have not been targeted simultaneously in a specific cell type *in vivo*.

Mitochondrial iron overload is pathogenic in a murine CS exposure model of COPD, showing a time-dependent increase in whole lung tissue (50). While this established the therapeutic benefits of iron chelation in ameliorating disease pathophysiology, a role for mitochondrial iron in disease progression at a cellular level has yet to be investigated. **Chapter 3** showed that MFRN1 and 2 gene expression is downregulated specifically in AMs isolated from smokers and individuals with COPD compared to non-smokers. Due to the varying range in pack years in the smoking group and the range of disease severities captured in the COPD group, the exact point at which MFRN1 and 2 expression appears to 'shut down' cannot be determined. Coupled with technical limitations in measuring mitochondrial iron in such a low cellular input, it is difficult to say whether this decreased expression reflects lower mitochondrial iron levels or is a

protective measure preceded by mitochondrial iron overload. As such, understanding the role of mitochondrial iron in AM response to chronic CS exposure requires an *in vivo* approach.

6.1.1 Aims

Chapter 5 suggested a correlation between *S. pneumoniae* intracellular burden and AM mitochondrial iron levels *in vitro* and *ex vivo*. This chapter will define the role of AM mitochondrial deficiency in the host response to acute respiratory infection and experimental COPD *in vivo* through the following specific aims:

- The generation and characterisation of a novel *Slc25a37^{fl/fl}*, *Slc25a28^{fl/fl}/Cd11c^{Cre+/-}* genetic strain of mice with targeted deletion of MFRN1 and 2 in AMs.
- Characterising the effects of AM-specific genetic mitochondrial iron deficiency on host and pathogen outcomes of *S. pneumoniae* infection.
- Investigating the effects of AM-specific genetic mitochondrial iron deficiency on lung pathophysiology in a CS-exposure model of experimental COPD.

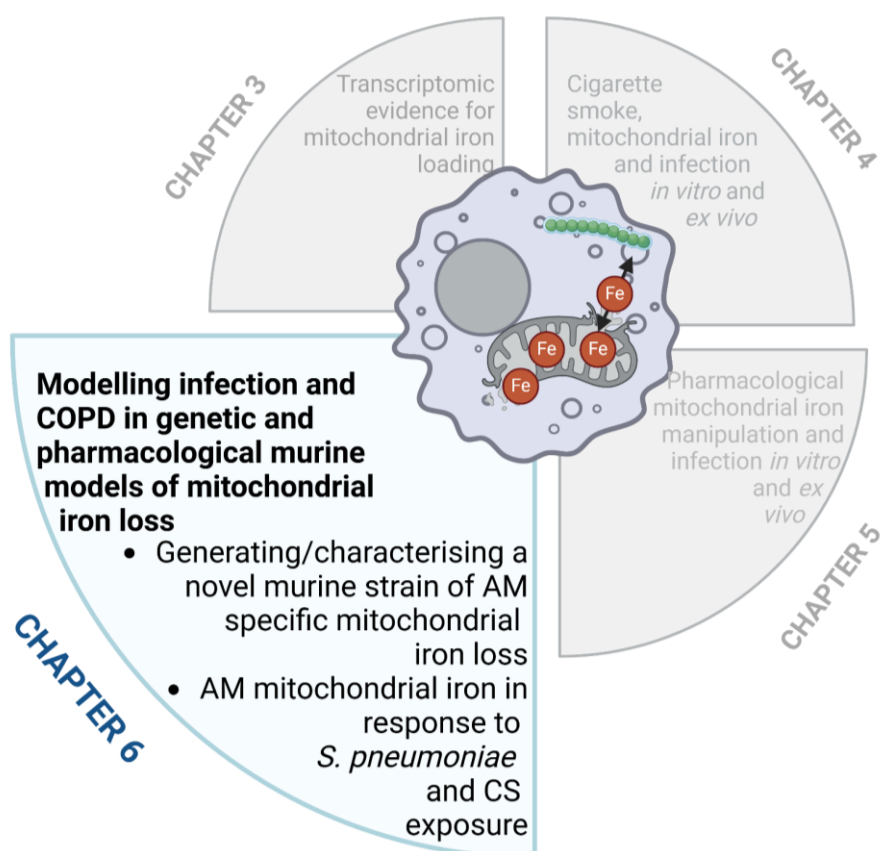


Fig. 6.1: Summary of aims of Chapter 6.

6.2 Results

6.2.1 Development of a murine model of *S. pneumoniae* infection

In **Chapter 5**, pre-treating human AMs with the mitochondrially-localised iron chelator DFP significantly reduced *S. pneumoniae* intracellular burden. To investigate if this host-beneficial effect of mitochondrial iron chelation could be observed *in vivo*, we first sought to establish an *in vivo* model of *S. pneumoniae* lower respiratory tract infection. Given that the D39 strain of *S. pneumoniae* is a decades-old, lab-adapted strain, *in vivo* experiments were performed using a strain of D39 *S. pneumoniae* subcultured from BALF isolated from an infected mouse, to ensure bacteria used were virulent *in vivo* (*data not shown*). Wild-type C57BL6/J mice were intranasally instilled 5×10^6 CFU of this mouse-adapted *S. pneumoniae* and response to infection analysed compared to PBS-instilled controls. *S. pneumoniae*-instilled mice showed a higher mean severity score over the course of infection, reflected in their increased weight loss 24 hr and 48 hr post-infection (**Fig. 6.2A-B**). To confirm that cellular infiltration to the airspaces was observed using this dose of bacteria, mice were euthanised 48 hr post infection and bronchoalveolar lavage (BAL) performed. BAL fluid (BALF) cell counts in *S. pneumoniae*-instilled mice were significantly increased compared to PBS-instilled controls at this timepoint, indicative of increased myeloid cell recruitment (**Fig. 6.2C**). There was no change in BALF protein levels between PBS and *S. pneumoniae*-instilled mice, indicating that a reasonable extent of alveolar barrier integrity was maintained during infection (**Fig. 6.2D**). Considering the observed weight loss and increased cellular infiltration to the airspaces, this dose and time of bacteria was deemed a suitable model of lower respiratory tract infection.

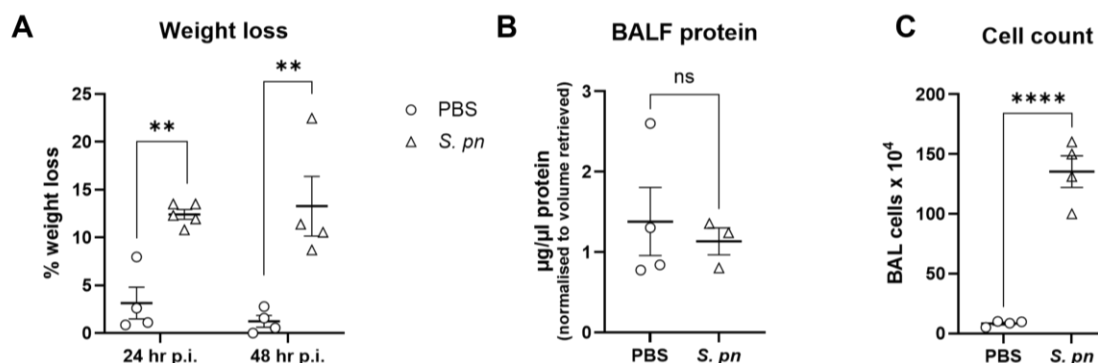


Fig. 6.2: Mice intranasally instilled with *S. pneumoniae* show worsened physiological outcomes and increased cell infiltration to the airspaces. WT C57BL6/J mice were intranasally instilled with 5×10^6 CFU of *S. pneumoniae* and euthanised by CO_2 after 48 hr. (A) Weight loss as a percentage relative to pre-installation body weight 24 hr and 48 hr post-institution in *S. pneumoniae* infected mice compared to PBS instilled controls. (B) Total BALF protein and (C) cell counts normalised to volume BALF retrieved. All data presented as mean $\pm$ S.E.M., *P* value by unpaired t-test, **= $P < 0.01$, ****= $P < 0.0001$, ns = non-significant.

6.2.2 Mice administered DFP show worse outcomes upon *S. pneumoniae* infection

Using this established model of *S. pneumoniae* infection, we sought to define the effects of prior mitochondrial iron depletion on the outcomes of infection. Wild-type (WT) C57BL/6/J mice were administered DFP systemically through their drinking water (1 mg/ml) for 3 weeks prior to intranasal instillation with *S. pneumoniae* (Fig. 6.3A)*. Mice administered DFP showed decreased survival 48 hr after *S. pneumoniae* instillation compared to mice with normal drinking water (Fig 6.3B), which associated with a minimal trend towards increased weight loss 48 hr post-infection (Fig. 6.3C).

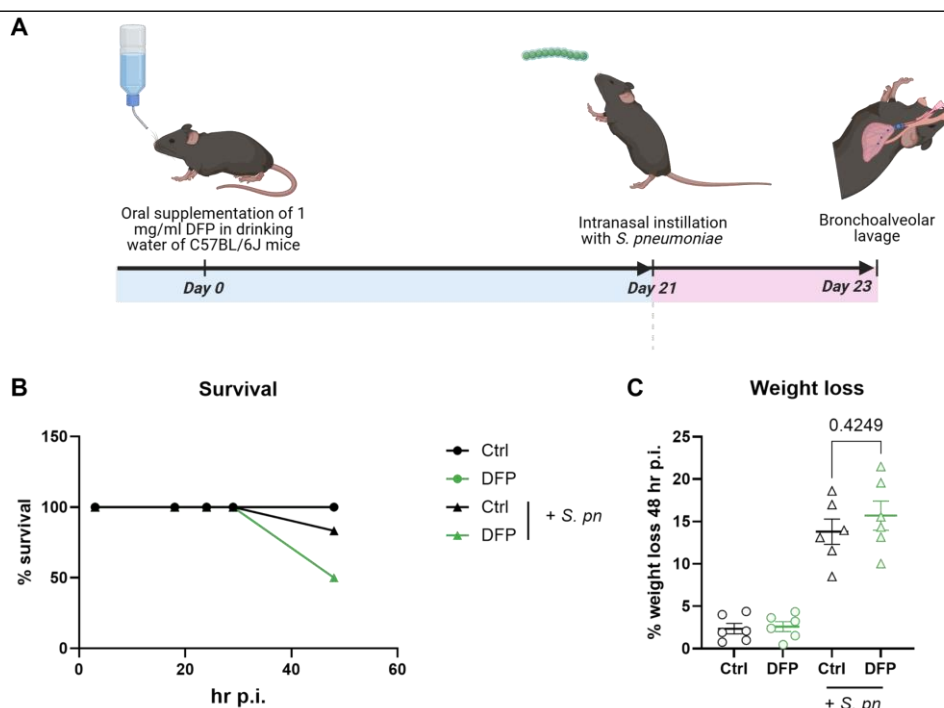


Fig 6.3: Mice administered DFP show decreased survival upon *S. pneumoniae* infection. (A) Schematic of *S. pneumoniae* infection in mice administered DFP. Mice were orally supplemented with 1 mg/mL DFP in drinking water provided *ad libitum* for 3 weeks. Mice were then returned to normal drinking water and intranasally instilled with 5×10^6 CFU *S. pneumoniae*. Mice were euthanised via CO₂ asphyxiation 48 hr post-instillation and subjected to bronchoalveolar lavage. (B) Survival of control and DFP PBS and *S. pneumoniae*-instilled mice. (C) Percentage weight loss 48 hr post-infection. Data represented as mean $\pm$ S.E.M., *P* value by one-way ANOVA with Tukey's multiple comparisons corrections. p.i. = post-infection, DFP = deferiprone, *S. pn* = *S. pneumoniae*

Following euthanasia 48 hr post-instillation, immune cell infiltration to the airspaces was investigated through bronchoalveolar lavage and differential cell staining. BALF was successfully obtained from mice which did not survive infection at volumes comparable to those that were euthanised, so differential cellular infiltration in these mice was measured and included in analyses. Mice administered DFP showed a significant increase in BALF

* Mice were administered DFP with the assistance of Ruairaidhrí Jordan of the Cloonan lab.

cell counts upon *S. pneumoniae* infection compared to control drinking water, with no difference detected between DFP and control PBS-instilled mice (**Fig. 6.4A-B**). In *S. pneumoniae* instilled mice, no significant change in the percentages of neutrophils (**Fig. 6.4C**) or macrophages/monocytes (**Fig. 6.4E**) between control and DFP-drinking water animals was detected: however, a high degree of variability was observed, including when percentages of neutrophils and macrophage/monocytes were extrapolated to determine their total numbers (**Fig. 6.4D** and **Fig. 6.4F**, respectively). This variability was reflected in the ratio of neutrophils to macrophages/monocytes, which trended towards increase in DFP-administered mice but was ultimately non-significant (**Fig. 6.4G**). These data highlight the deleterious effects of DFP administration on survival in *S. pneumoniae* infection, with further investigation needed into bacterial burden and the precise cellular mechanisms precipitating increased mortality.

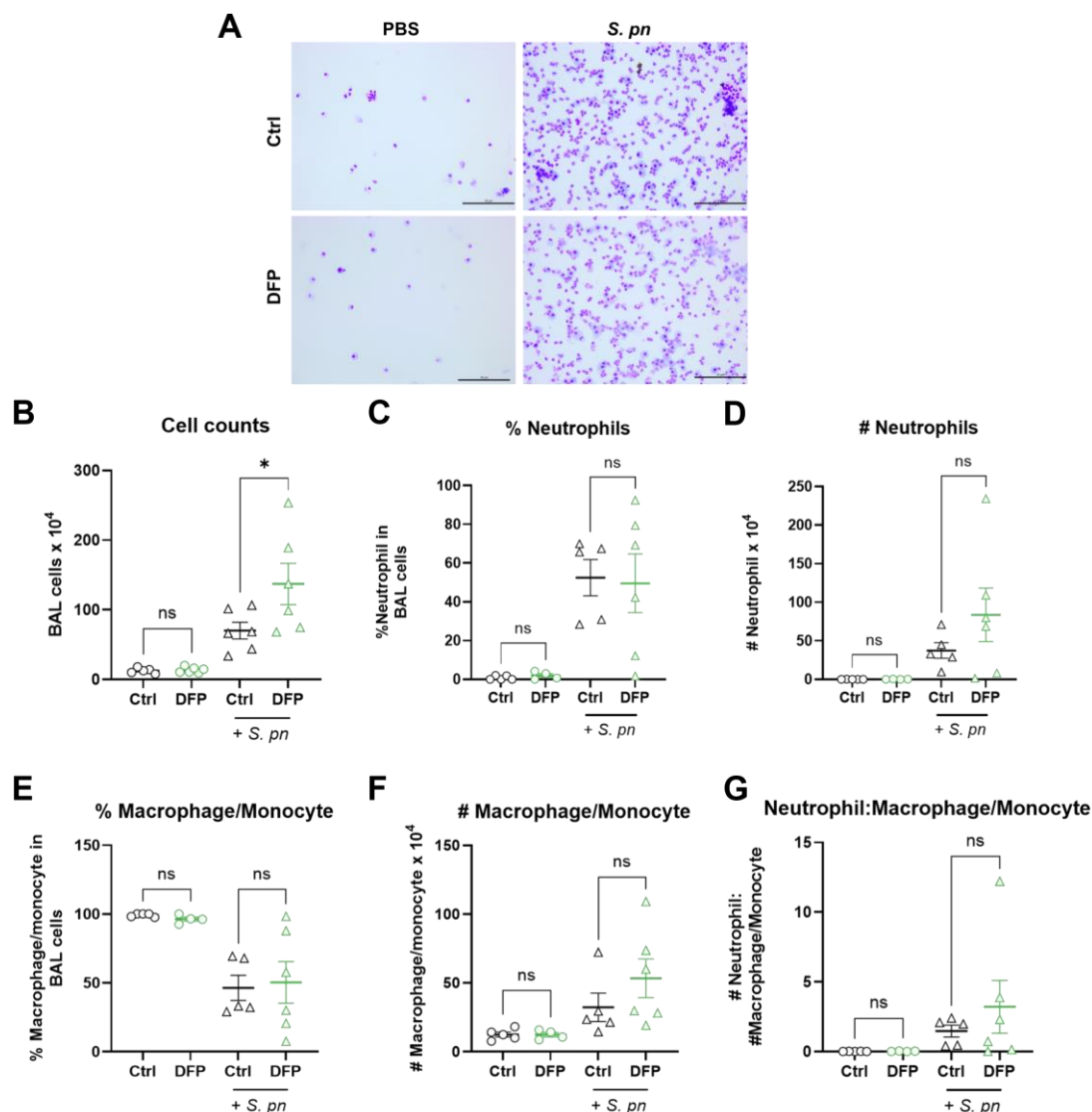


Fig. 6.4: Mice administered DFP show increased cellular infiltration upon *S. pneumoniae* infection. Mice were administered DFP and intranasally instilled with *S. pneumoniae* as (cont.).

(cont.) described. BAL cells were counted and deposited in a monolayer on a microscope slide through cytopsin, fixed and sequentially stained with eosin and methylene blue. Slides were imaged with a Leica DM 3000 LED microscope and cell differentials enumerated using ImageJ. (A) Representative cytopsin, scale bar = 10 μ m. (B) Total BAL cell counts normalised to volume of BALF retrieved. Neutrophils as a (C) percentage and (D) absolute number of BAL cells. Macrophages/monocytes as a (E) percentage and (F) absolute number of BAL cells. (G) Ratios of absolute neutrophil cell count to absolute macrophage/monocyte cell count. All data represented as mean $\pm$ S.E.M., n=6 mice per group from one independent experiment; *P* value by one-way ANOVA with Tukey's multiple testing correction; *=*P*<0.05, ns = non-significant. p.i. = post-infection, DFP = deferiprone, *S. pn* = *S. pneumoniae*

6.2.3 AMs from MFRN1/2 ^{Δ Cd11c} mice may be iron-deficient and show impaired OXPHOS

Systemic administration of DFP decreased survival upon *S. pneumoniae* infection (Fig 6.3). As in Chapter 5, DFP proved effective in controlling infection in pure AM populations *ex vivo*, we posited that localised mitochondrial iron deficiency in the lung would prove beneficial in infection whilst mitigating any off-target side effects arising from systemic mitochondrial iron depletion. To prove this, a novel transgenic strain of mice with genetic mitochondrial iron deficiency specifically targeting AMs was generated. *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl} mice were crossed with *Itgax*^{Cre+/-} mice to yield progeny with a loss of *Slc25a37* and *Slc25a28* specifically in Cd11c (*Itgax* protein product) expressing cells; hereafter referred to as MFRN1/2 ^{Δ Cd11c} mice. Cd11c is ubiquitously expressed on the AM cell surface and is a common marker used to distinguish AMs from other myeloid or structural cell populations in the lung (376). AM-specific deletion of MFRN1 and 2 expression did not manifest with any adverse physiological effects at baseline, with mice born in litter sizes and sex ratios comparable to the parental *Slc25a37*^{fl/fl}, *Slc25a28*^{fl/fl} line (Fig 6.5A-B). No differences in weight were observed in 8-week-old Cre⁺ mice compared to their Cre⁻ littermates (Fig 6.5C).

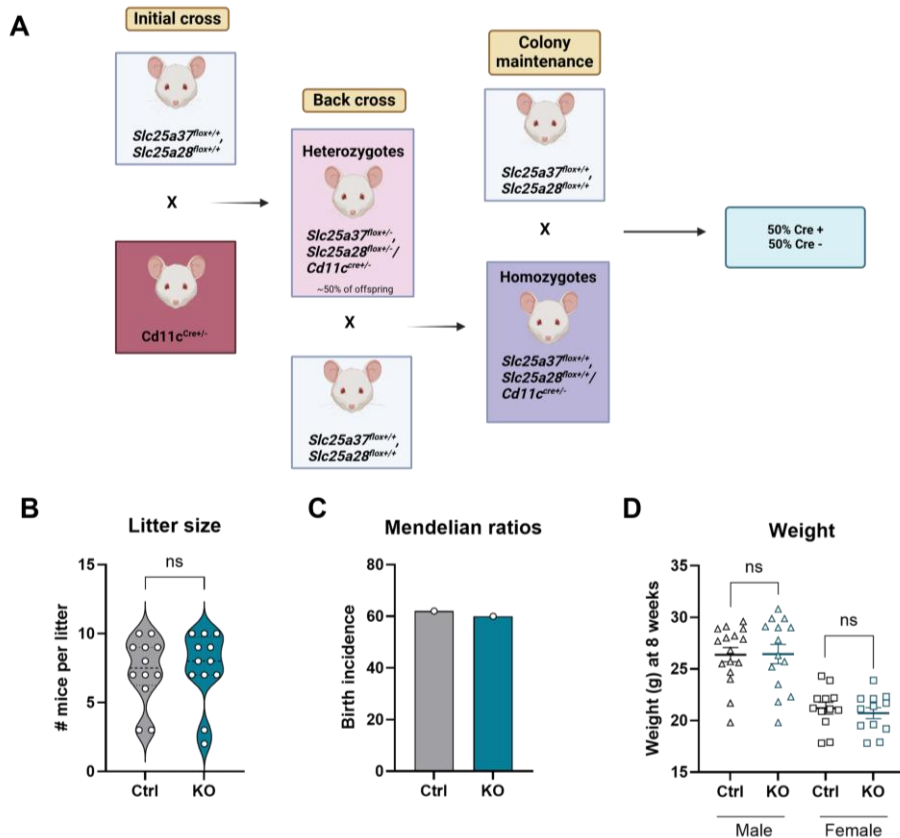


Fig 6.5: MFRN1/2^{ΔCd11c} mice display no adverse developmental effects. (A) Schematic of breeding strategy for strain generation and colony maintenance. (B) Number of mice born per litter and (C) number of male and female mice per litter compared to Cre⁻ parental line. (D) Weight of Cre⁻ (KO) and Cre⁺ (Ctrl) positive male and female mice at 8 weeks old. Data presented as mean ± S.E.M., *P* value by unpaired t-test; ns = non-significant.

To confirm loss of MFRN1 and 2 expression, AMs were isolated from these mice through bronchoalveolar lavage and gene expression analysed by RT-qPCR. *Slc25a37* expression was unchanged, with a high degree of variability between individual mice observed (**Fig. 6.6A**). *Slc25a28* expression was robustly downregulated in comparison, illustrating the efficiency of Cre expression in these cells and suggesting issues in measuring *Slc25a37* expression could be attributed to technical issues during RT-qPCR (**Fig. 6.6B**). To investigate how loss of MFRN2 may affect cellular iron status, we analysed FTH1 and TFRC gene expression: while *Fth1* was unchanged, *Tfrc* was significantly upregulated, hinting that these cells may be iron deficient (**Fig. 6.6C-D**). Cumulatively, these results confirm the successful targeting of *Slc25a28*, without any compensatory increase in *Slc25a37* expression in AMs and show this has no effect on murine development.

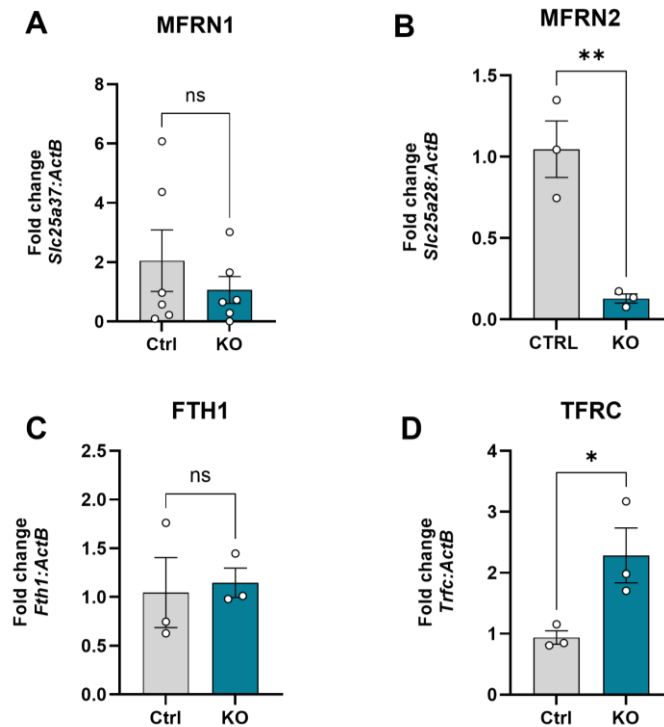


Fig 6.6: AMs from MFRN1/2 Δ Cd11c mice show upregulated expression of *Tfrc*. AMs were isolated from 8 week old MFRN1/2 Δ Cd11c (KO) and Cre⁻ (Ctrl) littermate controls through bronchoalveolar lavage. RNA was isolated and qPCR performed as described for (A) *Slc25a37*, (B) *Slc25a28*, (C) *Fth1* and (D) *Tfrc* expression, presented as fold change relative to *ActB* as endogenous control. Data presented as mean $\pm$ S.E.M., $n \geq 3$ independent replicates. *P* value by unpaired t-test; $P < 0.05 = *$, $P < 0.01 = **$, ns = non-significant.

We next sought to confirm that loss of MFRN2 expression in these AMs manifested in decreased mitochondrial iron levels. As there are approximately 100,000 AMs in the murine lung, absolute mitochondrial iron measurement through subcellular fractionation and GF-AAS as previously performed in FLAMs (**Fig. 3.11**, **Fig. 5.3**) was not possible in this instance owing to limited input. Instead, the mitochondrially targeted, Fe^{2+} selective fluorescent probe MtFluNox (377), commercially sold as Mito-FerroGreen, was explored. To validate that this probe targeted mitochondrial iron, FLAMs were treated with pharmacological modifiers of mitochondrial iron as in **Fig. 5.3** and Mito-FerroGreen fluorescence assessed through live-cell confocal microscopy. Decreased fluorescence was observed upon DFP treatment: however, despite the increases in mitoplast iron noted previously in FAC and CSE treatment via GF-AAS, Mito-FerroGreen fluorescence was quenched with either treatment compared to control (**Fig. 6.7**). As such, Mito-FerroGreen was deemed unsuitable for use in measuring mitochondrial iron.

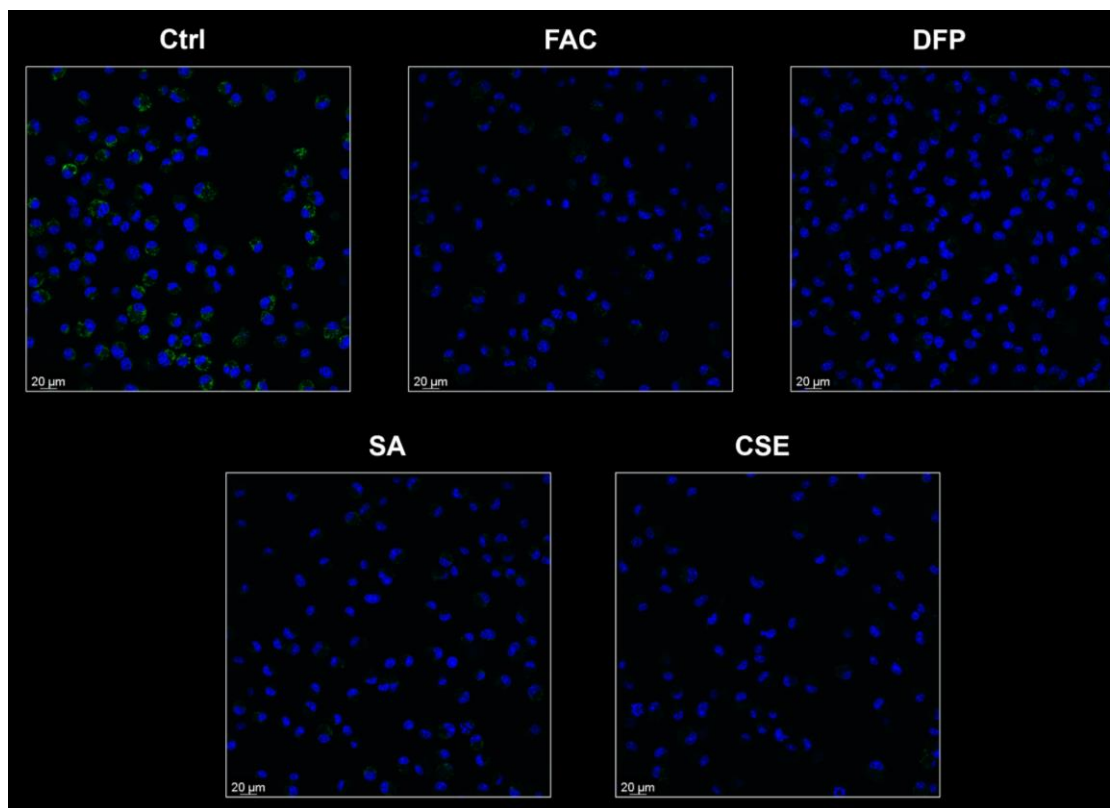


Fig. 6.7 Mito-FerroGreen fluorescence does not reflect mitochondrial iron loading or deficiency: FLAMs plated in a 35 mm glass-bottomed dish were treated with 100 μ M FAC, 100 μ M DFP or 1 mM SA for 24 hr or 5% CSE for 18 hr. FLAMs washed once and sequentially stained with 1 μ M Mito-FerroGreen and 5 μ g/mL Hoechst nuclear dye. Images were captured using a 40X magnification oil-immersion lens on a Leica SP8 scanning confocal microscope and scale bar added using the LAX Processing Suite software. Representative images for each condition pictured.

Next, the functional effects of MFRN2 loss on cellular bioenergetics were investigated. ETC complexes rely on FeS clusters for their enzymatic activity, but how loss of MFRN2 specifically affects their function in AMs is not known. To assess this, cellular metabolism was profiled through monitoring oxygen consumption rate (OCR) (**Fig. 6.8A**) and extracellular acidification rate (ECAR) (**Fig. 6.8B**) following respiratory complex inhibitor administration in AMs from MFRN1/2 Δ Cd11c mice. Compared to littermate controls, MFRN1/2-deficient AMs showed impaired basal respiration and ATP production (**Fig 6.8C-D**). MFRN1/2 Δ Cd11c AMs also showed reduced proton leak, coupling efficiency and non-mitochondrial oxygen consumption (**Fig 6.8E-G**). Spare respiratory capacity could not be reliably calculated in these cells as a robust increase in OCR was not observed upon FCCP administration at the concentration used (1 μ M) (**Fig. 6.8A**). These results highlight the profound effect of MFRN2 loss on mitochondrial bioenergetics in AMs.

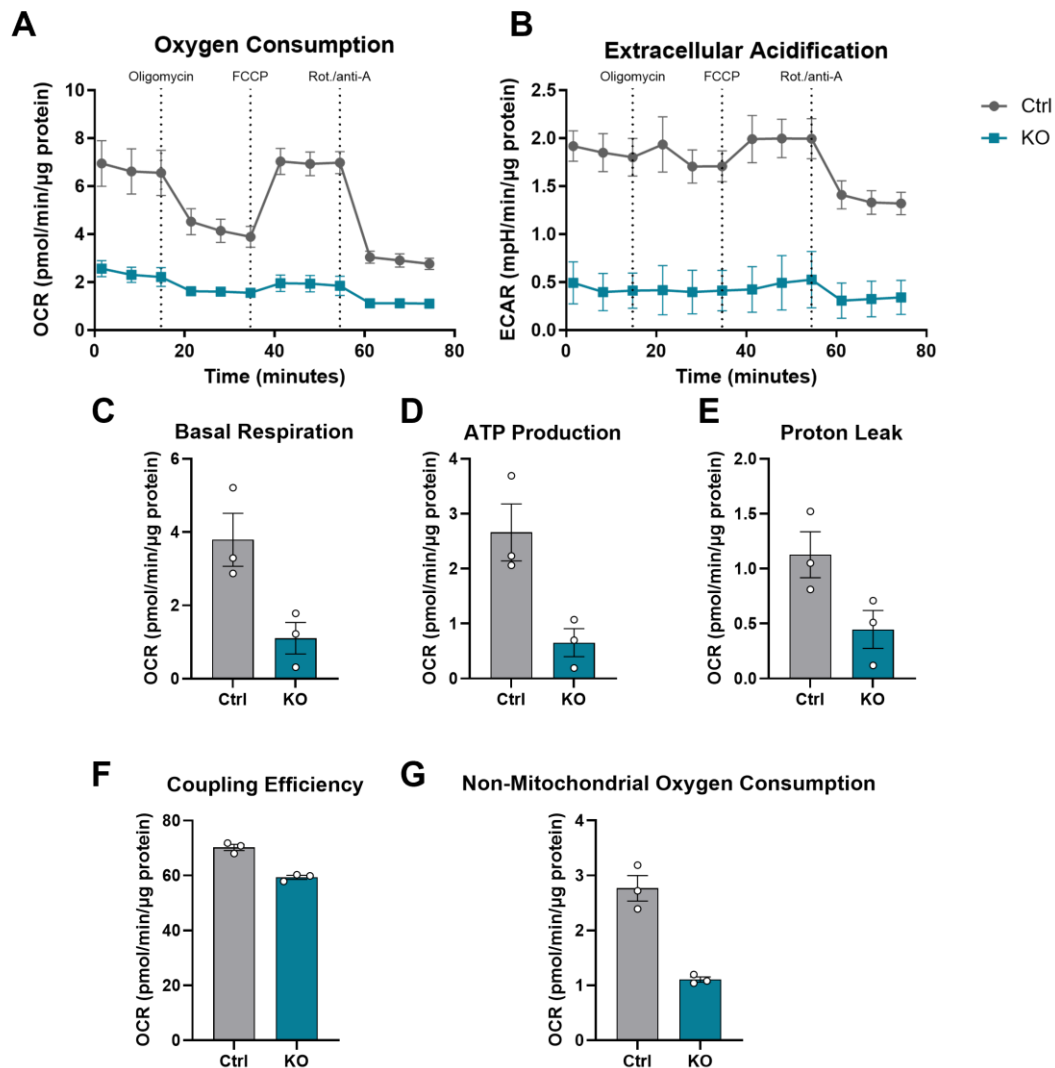


Fig 6.8: AMs from MFRN1/2^{ΔCd11c} mice show impaired OXPHOS. AMs were isolated simultaneously from 8-week-old MFRN1/2^{ΔCd11c} mice and littermate controls. OCR (A) and ECAR (B) was measured using a Seahorse Xfp analyser upon administration of 1 μM oligomycin, FCCP and rotenone/antimycin A at indicated timepoints. (C) Basal respiration, (D) ATP production, (E) proton leak, (F) coupling efficiency and (G) non-mitochondrial oxygen consumption were calculated from OCR readings as described. Data representative of mean ± S.E.M. of one independent experiment performed in technical triplicate, normalised to protein content as measured by BCA. Each data point represents a technical replicate.

6.2.4 MFRN1/2^{ΔCd11c} mice show improved outcomes following *S. pneumoniae* infection

Our earlier findings displayed the detrimental impacts of prior DFP administration upon *S. pneumoniae* infection *in vivo* (Fig. 6.3-4). Considering previous *in vitro* and *ex vivo* data suggesting the host-beneficial effects of pharmacological mitochondrial iron targeting in a pure AM population in Chapter 5, we hypothesised that AM-specific

mitochondrial iron loss would be host-protective in infection *in vivo*. MFRN1/2^{ΔCd11c} mice and littermate controls were intranasally instilled with *S. pneumoniae* as in **Fig. 6.2** and euthanised after 48 hr. MFRN1/2^{ΔCd11c} mice lost significantly less weight relative to littermate controls 48 hr post-infection (mean 5.456 and 10.27, respectively) (**Fig. 6.9A**). MFRN1/2^{ΔCd11c} mice also trended towards a decrease in spleen weight relative to littermate controls, indicative of a reduced inflammatory burden during infection (378) (**Fig. 6.9B**).

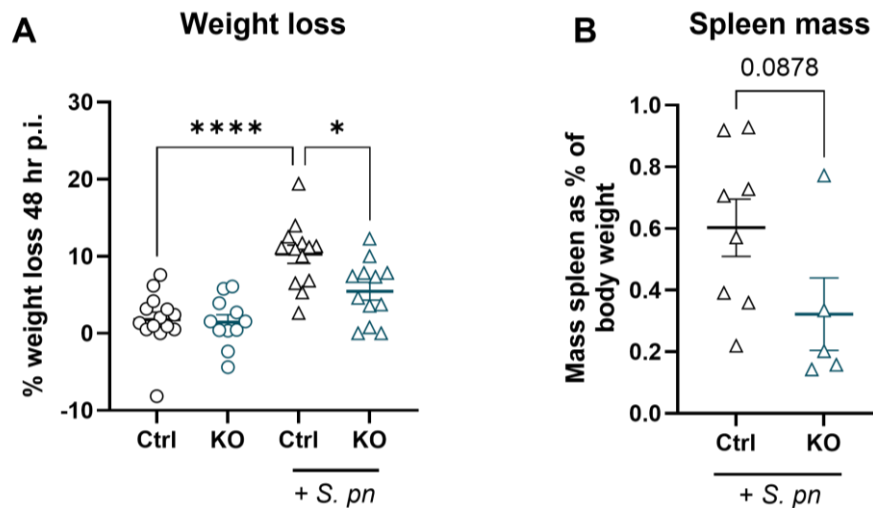


Fig 6.9: MFRN1/2^{ΔCd11c} mice show improved physiological outcomes upon *S. pneumoniae* infection. MFRN1/2^{ΔCd11c} mice and littermate controls were intranasally instilled with 5×10^6 CFU of *S. pneumoniae* and euthanised by CO₂ after 48 hr. (A) Weight loss as a percentage relative to pre-installation body weight 48 hr post-institution. (B) Spleen mass a percentage relative to pre-installation body weight 48 hr post-institution. All data represented as mean $\pm$ S.E.M. from at least one independent experiment, $n \geq 5$ per group. *P* value by one-way ANOVA with Tukey's multiple testing correction; *=*P*<0.05, ****=*P*<0.0001. p.i. = post-infection, *S. pn* = *S. pneumoniae*.

Previous data showed systemic administration of DFP enhanced cellular infiltration to the airspaces upon infection (**Fig 6.4**). To investigate if reduced cellular infiltration upon infection could be attributed to the improved outcomes observed in MFRN1/2^{ΔCd11c} mice, bronchoalveolar lavage was performed and cellular infiltration assessed. (**Fig. 6.10A**). MFRN1/2^{ΔCd11c} mice showed reduced cellular infiltration, with no significant change observed in BAL cell counts in PBS instilled MFRN1/2^{ΔCd11c} mice compared to littermate controls (**Fig. 6.10B**). Both the percentage and absolute number of infiltrating neutrophils decreased in MFRN1/2^{ΔCd11c} mice relative to infected littermates, with no neutrophilic infiltration observed in PBS-instilled MFRN1/2^{ΔCd11c} mice at baseline (**Fig 6.10C-D**). Infected MFRN1/2^{ΔCd11c} mice had a significantly higher percentage of macrophages/monocytes (**Fig 6.10E**), with no significant change observed in absolute number (*P* = 0.232) (**Fig 6.10F**). Importantly, the ratio of neutrophils to

macrophages/monocytes was significantly decreased in infected MFRN1/2^{ΔCd11c} mice compared to infected littermates (**Fig 6.10G**).

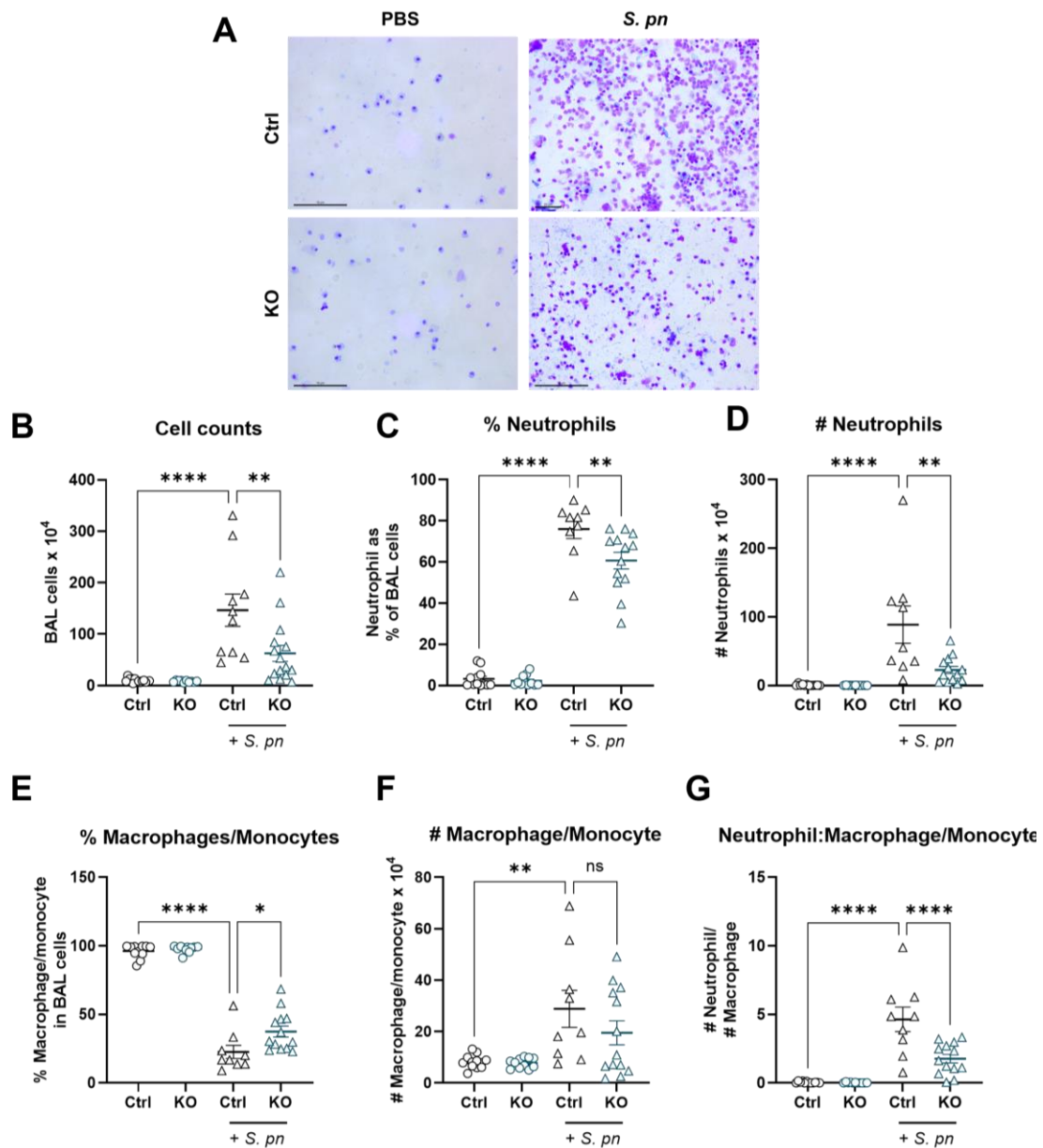


Fig 6.10: MFRN1/2^{ΔCd11c} mice show reduced monocytic and neutrophilic infiltration upon *S. pneumoniae* infection. MFRN1/2^{ΔCd11c} mice and littermate controls were intranasally instilled with 5×10^6 CFU *S. pneumoniae* and euthanised by CO₂ asphyxiation for bronchoalveolar lavage. BAL cells were counted and deposited in a monolayer on a microscope slide through cytopsin, fixed and sequentially stained with eosin and methylene blue. Slides were imaged using a Leica DM 3000 LED microscope and cell differentials enumerated using ImageJ. (A) Representative cytopsin, scale bar = 10 μ m. (B) Total BAL cell counts normalised to volume of BALF retrieved. Neutrophils as a (C) percentage and (D) absolute number of BAL cells. Macrophages/monocytes as a (E) percentage and (F) absolute number of BAL cells. (G) Ratios of absolute neutrophil cell count to absolute macrophage/monocyte cell count. All data represented as mean $\pm$ S.E.M. from two independent experiments, $n \geq 12$ per group. *P* value by one-way ANOVA with Tukey's multiple testing correction; *= $P < 0.05$, **= $P < 0.01$, ****= $P < 0.0001$, ns=non-significant. *S. pn* = *S. pneumoniae*.

We next wanted to clarify if reduced cellular infiltration in MFRN1/2^{ΔCd11c} mice correlated with a reduction in lung injury markers. As before, there was no change in BALF protein in *S. pneumoniae*-instilled mice (**Fig. 6.11A**). Next, we measured levels of BALF lipocalin-2, an important antimicrobial mediator secreted by AMs in response to *S. pneumoniae* (123). Lipocalin-2 levels appeared to trend towards decrease in infected MFRN1/2^{ΔCd11c} mice compared to infected littermates, but low sample sizes limited technical power (**Fig. 6.11B**). To assess if BALF MFRN1/2^{ΔCd11c} mice may show improved control of *S. pneumoniae* infection through enhanced extracellular iron sequestration, total iron levels were measured in BALF and whole lung tissue post-bronchoalveolar lavage. Iron levels were unchanged in all groups in both BALF and lung, hinting that the mechanisms by which MFRN1/2^{ΔCd11c} mice limit bioavailable iron in infection may be cell-intrinsic (**Fig. 6.11C-D**).

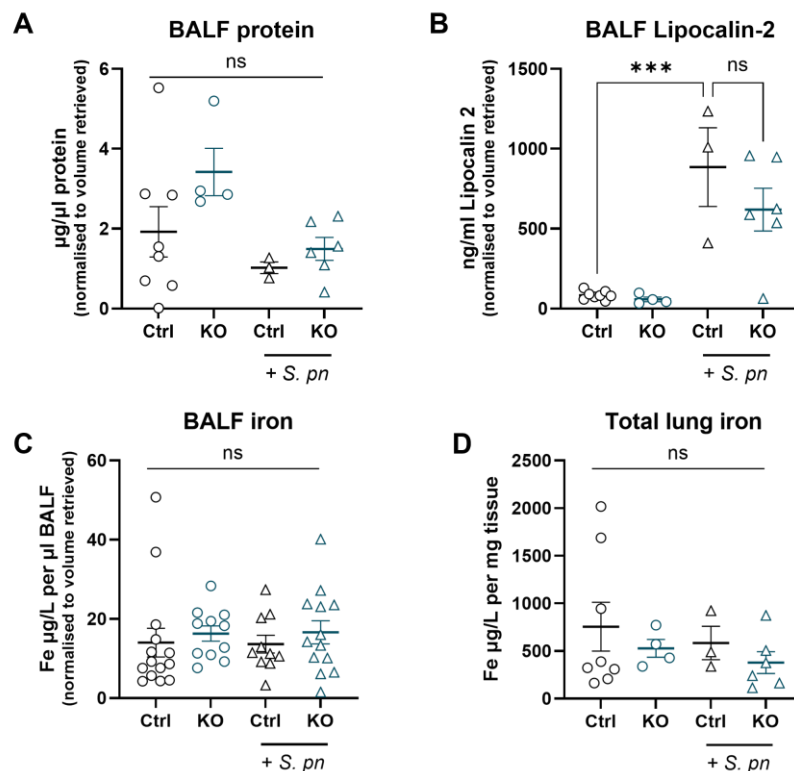


Fig 6.11: MFRN1/2^{ΔCd11c} mice show no change in lung injury markers upon *S. pneumoniae* infection. MFRN1/2^{ΔCd11c} mice and littermate controls were intranasally instilled with (cont.) 5×10^6 CFU *S. pneumoniae* and euthanised by CO₂ asphyxiation for bronchoalveolar lavage. (A) Total BALF protein was measured by BCA and normalised to volume BALF retrieved for each mouse. (B) BALF lipocalin-2 was measured through ELISA and normalised to volume BALF retrieved for each mouse. (C) BALF was digested in 50% nitric acid at 70°C for 2 hr and total BALF iron was measured by GF-AAS, normalised to volume BALF retrieved for each mouse. (D) 10 – 30 mg of lung tissue was dissected from the left lobe and digested in nitric acid for 3 hr at 70°C for total iron measurement through GF-AAS. Iron content was normalised to mass tissue digested. All data represented as mean $\pm$ S.E.M. from at least one independent experiment, $n \geq 3$ per group. P value by one-way ANOVA with Tukey's multiple testing correction; ***= $P < 0.001$, ns=non-significant. *S. pn* = *S. pneumoniae*.

Neutrophilic and monocytic infiltration to the lung is an essential host response to control infection, but sustained infiltration can induce tissue destruction and poorer host outcomes (379). To confirm that the attenuated host inflammatory response was host-beneficial and not an impairment of the bactericidal response, we measured bacterial CFU in these mice in both the lung and peripheral tissues (spleen, liver, bone marrow) to monitor bacteraemia. MFRN1/2^{ΔCd11c} mice showed a trend towards decreased whole lung CFU 48 hr post-instillation compared to littermate controls (**Fig. 6.12A**). Systemically, CFU in the spleen, liver and bone marrow trended towards decrease in MFRN1/2^{ΔCd11c} mice, suggestive of reduced bacterial dissemination (**Fig. 6.12B-D**). This suggests that the reduced inflammatory cell burden in *S. pneumoniae*-instilled MFRN1/2^{ΔCd11c} mice reflects a decreased bacterial burden and not an impaired bactericidal response. Taken together, targeted loss of MFRN2 in AMs is beneficial for the host in *S. pneumoniae* infection and appears to limit bacterial proliferation.

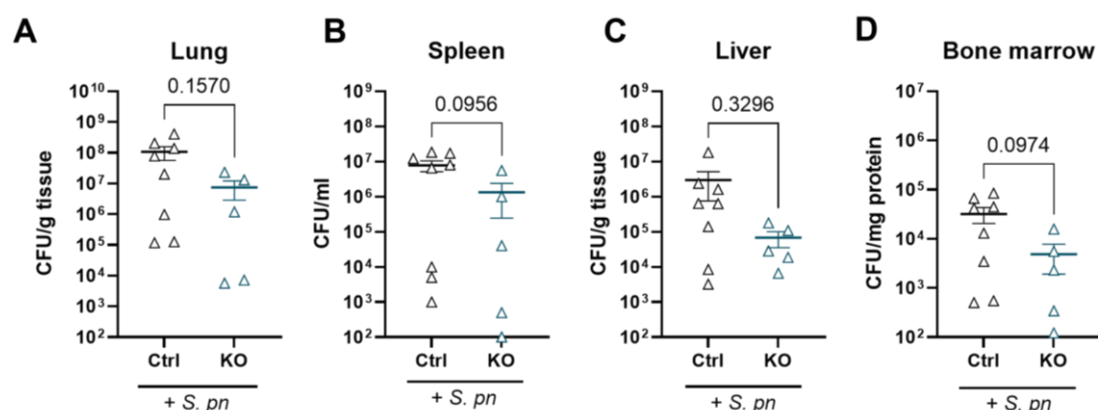


Fig 6.12: MFRN1/2^{ΔCd11c} mice may show reduced bacterial dissemination. MFRN1/2^{ΔCd11c} mice and littermate controls were intranasally instilled with 5×10^6 CFU *S. pneumoniae* and euthanised by CO₂ asphyxiation. (A) Lungs, (B) spleen and (C) liver were removed, washed with ethanol and lysed through bead homogenisation. Lysed tissue was serially diluted in PBS and plated on to Todd Hewitt agar plates with 5% v/v sheep's blood for CFU enumeration, with (A) and (C) normalised to mass of tissue homogenised. (D) Bone marrow was isolated from the femur and tibia of mice and lysed in 1% saponin. Lysate was plated on Todd Hewitt agar plates with 5% v/v sheep's blood for CFU enumeration, with CFU normalised to total protein content determined through BCA. Data represented as mean $\pm$ S.E.M. from one independent experiment, $n \geq 5$ per group. *P* value by unpaired t-test. CFU = colony forming unit, *S. pn* = *S. pneumoniae*.

6.2.5 MFRN1/2^{ΔCd11c} mice show worsened physiological outcomes following CS exposure

Whole lung mitochondrial iron loading is a recognised hallmark of experimental COPD, and depletion of mitochondrial iron through DFP administration ameliorates aberrant CS-induced immune cell recruitment to the lung (50). To investigate if AM-specific depletion of MFRN2 is similarly protective, we subjected mice to a whole-body CS

exposure model of COPD, where mice were exposed to CS for 1 hr per day, 5 days a week for a total of 6 weeks*. Over the course of CS exposure, weight gain was monitored across each group, a physiological readout which has been used previously in the literature to infer response to CS exposure (50). Relative to pre-exposure weight, there was a trend towards decreased weight gain in mice exposed to CS compared to room-air (RA) controls ($P = 0.191$ for control RA vs control CS following 6 weeks exposure) (**Fig 6.13A**). Comparing CS-exposed MFRN1/2 Δ Cd11c mice to their CS-exposed littermates, we observed a significant decrease in weight gain after 4-6 weeks of CS exposure (**Fig. 6.13B**).

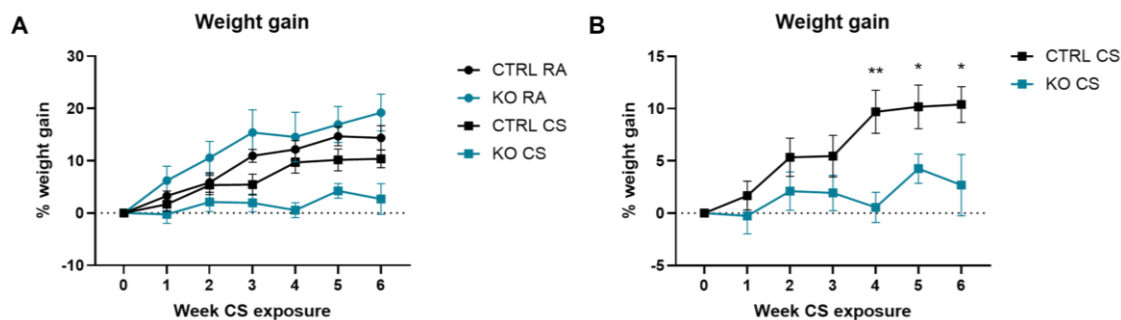


Fig. 6.13: MFRN1/2 Δ Cd11c mice show reduced weight gain over 6 weeks of CS exposure: Mice were exposed to CS for 1 hr a day, 5 days a week for a total of 6 weeks as described. Weight gain during exposure as a percentage of initial body weight for (A) MFRN1/2 Δ Cd11c and littermate control mice exposed to CS and room air and (B) MFRN1/2 Δ Cd11c and littermate control mice CS groups only. Data represented as mean $\pm$ S.E.M. of one independent experiment with $n=5$ mice per group. P value by multiple t-test; $*=P<0.05$, $**=P<0.01$.

Next, we isolated BAL cells from these mice following 6 weeks CS exposure through bronchoalveolar lavage to investigate CS-induced immune cell recruitment in MFRN1/2 Δ Cd11c mice. CS-exposed mice showed a significant increase in BAL cell counts compared to RA controls, exhibiting an approximate 2.5-fold increase (**Fig. 6.14A-B**). While not significant, MFRN1/2 Δ Cd11c mice displayed a trend toward increased BAL cell counts compared to CS-exposed littermate controls (**Fig. 6.14A-B**). Differential cell staining revealed macrophages/monocytes to be the predominant cell recruited to the airspaces, with their absolute number again trending towards increase in MFRN1/2 Δ Cd11c mice (**Fig. 6.14A, C-D**). In line with previous studies of murine CS exposure at this timepoint (112), we did not observe any neutrophilic infiltration to the airspaces in CS control mice (**Fig. 6.14E-F**). MFRN1/2 Δ Cd11c mice also did not present with any increase in neutrophils, reflected in the unchanged neutrophil:monocyte/macrophage ratio across all

* CS exposure was performed with the assistance of Sonia Gesmundo of the Cloonan lab.

groups (**Fig 6.14E-G**). Cumulatively, these results suggest that AM-specific loss of MFRN2 may be detrimental in a murine CS-exposure model of experimental COPD.

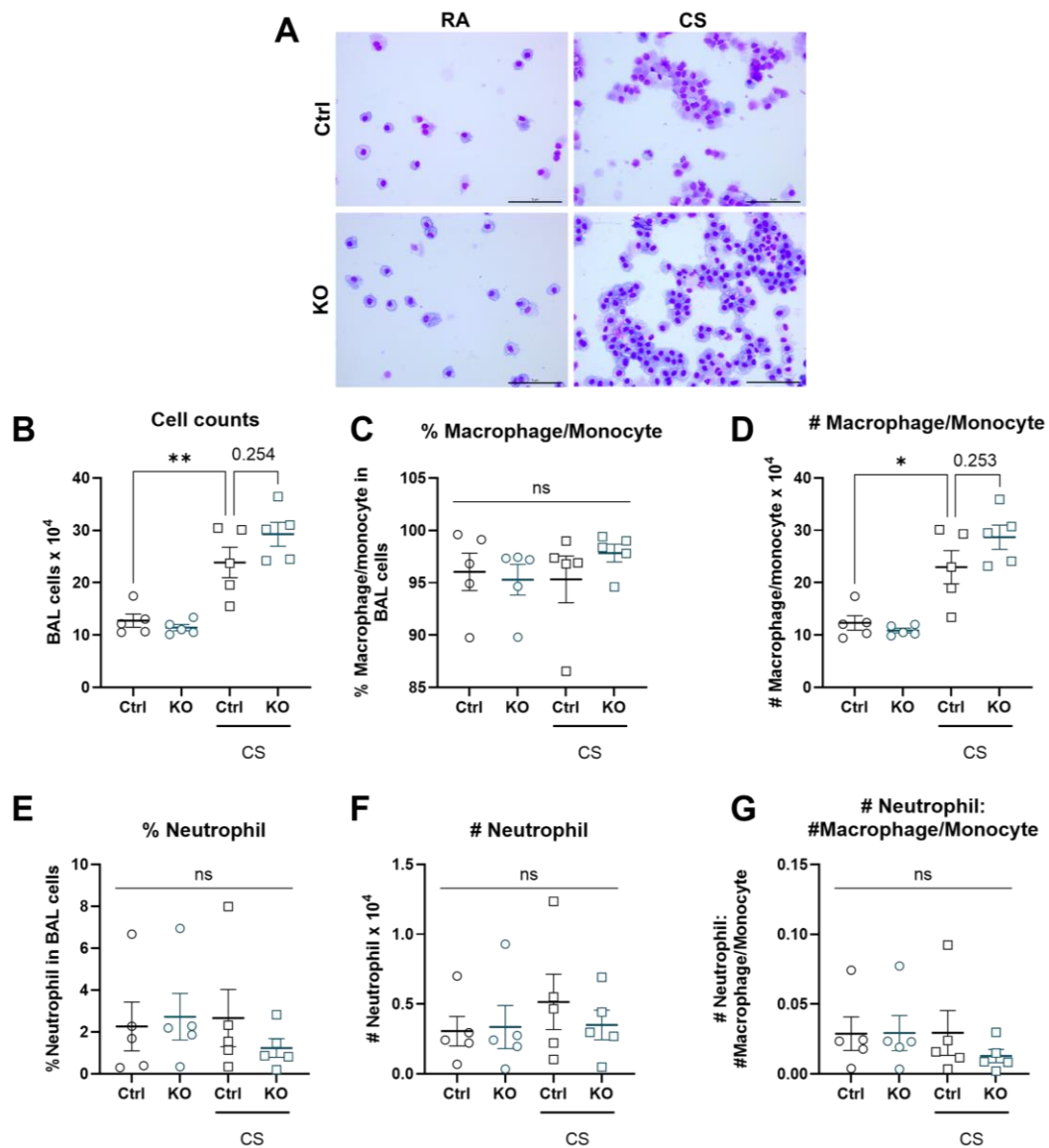


Fig. 6.14: MFRN1/2 Δ Cd11c mice may show increased cellular infiltration upon CS exposure: Mice were exposed to 150 cm³ total particulate matter of CS for 1 hr a day, 5 days a week for a total of 6 weeks as described. Mice were euthanised through CO₂ and bronchoalveolar lavage performed. BAL cells were counted and deposited in a monolayer on a microscope slide through cytopsin, fixed, and sequentially stained with eosin and methylene blue. Slides were imaged with a Leica DM 3000 LED microscope and cell differentials enumerated using ImageJ. (A) Representative cytopsins, scale bar = 5 μ m. (B) Total BAL cell counts normalised to volume of BALF retrieved. Macrophages/monocytes as a (C) percentage and (D) absolute number of BAL cells. Neutrophils as a (E) percentage and (F) absolute number of BAL cells. (G) Ratios of absolute neutrophil cell count to absolute macrophage/monocyte cell count. All data represented as mean $\pm$ S.E.M., *P* value by one-way ANOVA with Tukey's multiple testing correction. CS = cigarette smoke, BAL = bronchoalveolar lavage.

6.3 Discussion

Correct mitochondrial iron metabolism is essential for macrophage effector functions but may also function as a target for bacterial iron acquisition to fuel survival and virulence in the host. Administration of the mitochondrial iron chelator DFP prior to *S. pneumoniae* infection decreased survival, illustrating the detrimental impacts of systemic iron deficiency in the immune response to infection. As a refined model of localised mitochondrial iron deficiency, we generated a novel transgenic strain of mice targeting MFRN1 and 2 in AMs. MFRN1/2^{ΔCd11c} mice show no gross physiological changes compared to littermate controls at baseline but are deficient in OXPHOS and compensatory glycolysis as measured through Seahorse. Despite this, MFRN1/2^{ΔCd11c} mice show improved outcomes upon *S. pneumoniae* infection, with reduced neutrophilic infiltration and diminished bacterial proliferation both locally in the lung and systemically at the spleen, liver and bone marrow. Despite their protection against infection, MFRN1/2^{ΔCd11c} mice lost more weight than littermate controls and trended towards increased monocytic infiltration in a 6-week CS-exposure model of experimental COPD. Our findings represent the first model of MFRN2 deficiency in an immune cell *in vivo* and illustrate the beneficial effects of localised mitochondrial iron deficiency in respiratory infection, suggesting a divergent role of AM mitochondrial iron import in the response to acute and chronic insult.

To the best of our knowledge, no genetic strain of mice with a targeted deletion of MFRN1 or MFRN2 in any immune cell has been generated previously. The sole study which has targeted mitochondrial iron metabolism genetically in immune cells *in vivo* utilised models of targeted *Cisd1* and *Ftmt* overexpression in adipose tissue-resident macrophages (380). *Cisd1* encodes for the mitoNEET protein, which contains an FeS cluster that can be donated to IRP1, providing the catalytic core necessary for its switch to ACO1 and function in the TCA cycle in iron replete conditions (381). However, a capacity of mitoNEET to directly participate in iron trafficking to and from the mitochondria has not been proven, and the mechanisms by which its deletion depletes mitochondrial iron are unclear. Expression of MFRN1 and 2 was not studied in this model, meaning that an upregulation of iron import to fuel cofactor production cannot be ruled out. *Ftmt*, encoding for mitochondrial ferritin, is understudied in terms of its iron binding and storage capacity, and a protein capable of liberating iron from mitochondrial ferritin akin to NCOA4 has not been identified. Increased levels of the *Ftmt* protein product in the mitochondrial matrix may arbitrarily bind more iron, but again an upregulation of MFRN 1 and 2 expression to increase labile iron import and bypass storage is possible. As MFRN1 is the only mitochondrial iron metabolism protein for which the biochemistry of iron transport has been

described (247), we chose this as our target for deletion. Previous models of genetic deletion have illustrated an additive effect of loss of either MFRN1 or 2 on mitochondrial iron deficiency: as such, to prevent a compensatory increase in iron import through MFRN2, we also targeted it for deletion (375). Through directly targeting the ability of mitochondria to import iron, we believe our model represents the most robust means of ensuring efficient, permanent mitochondrial iron depletion that will directly inhibit production of iron-associated cofactors.

We chose to target AMs for gene deletion through placing Cre under the control of the Cd11c promoter. Cd11c is ubiquitously expressed in AMs, both of fetal and recruited origin. This is reflected in the efficiency of our gene deletion in BAL cells isolated from MFRN1/2 Δ Cd11c mice, with a greater than 90% reduction in MFRN2 gene expression observed in MFRN1/2 Δ Cd11c mice compared to littermate controls (**Fig. 6.6B**). While commonly used in the literature to target AMs (382-384), Cd11c-Cre mice also show Cre activity in dendritic cells (385, 386). A recent study characterising the cellular localisation of Cd11c-driven Cre also observed activity in tissue-resident CD4⁺ and CD8⁺ T cells (387). Few populations of cells will physiologically express a unique protein, and as such targeting Cre expression to a single cell population is very difficult. Other Cre drivers common in the literature that target AMs include LysM-Cre mice, which target all cells of the myeloid lineage, and Csfr1-Cre mice, which targets all mononuclear phagocytes (388, 389). Deletion of MFRN1 and 2 in AMs using either of these drivers would target systemic macrophages and myeloid cell populations, with Cre activity even observed in 25% of alveolar epithelial type II cells of LysM-Cre mice (389). Considering this, in our opinion Cd11c-Cre represents the most AM-specific Cre driver available commercially, limiting confounding deletion in recruited myeloid cells and other lung-resident immune and structural cells*.

In line with studies using other tissue specific Cre drivers to delete MFRN1 and/or 2, we observed no effect on murine development at baseline, with mice born in normal Mendelian ratios (**Fig. 6.5**). However, AMs from MFRN1/2 Δ Cd11c show severely attenuated OXPHOS as measured using a Seahorse extracellular flux analyser, with similarly diminished ECAR suggesting an inability to upregulate compensatory glycolysis (**Fig. 6.8**). Electron transfer is a heavily iron-reliant process. Respiratory complexes I, II and III contain multiple catalytic FeS clusters, while complexes III and IV also bind heme (390). Whether ETC activity is directly impaired in our model is unclear, but specific deletion of

* I am currently generating a fluorescent reporter mouse to confirm the extent of Cd11c-Cre driven MFRN1 and 2 deletion across immune cell types in our model. MFRN1/2 Δ Cd11c mice are being crossed with Rosa26-GFP mice, which carry an eGFP sequence with a loxP-flanked stop codon that is excised in Cd11c-expressing cells to allow for translation of the fluorescent protein.

Slc25a37 and *Slc25a28* in the liver resulted in diminished hepatocyte expression of all 5 respiratory complexes and a reduction in complex I and II enzymatic activity (375). The decrease in OXPHOS in our model is therefore likely driven by reduced FeS cluster production caused by mitochondrial iron depletion, impairing the enzymatic activity of the respiratory complexes. Both genetic mitochondrial iron deficiency and overload attenuated OXPHOS in adipose tissue resident macrophages (380). ECAR, a surrogate marker of glycolysis, was also decreased in these AMs (**Fig. 6.8B**). This suggests that these AMs lack the capacity to switch to anaerobic metabolism for ATP generation but requires further study through administration of 2-deoxyglucose and subsequent ECAR measurement to properly interrogate glycolytic capacity. AMs are uniquely reliant on OXPHOS at steady state and have limited glycolytic capacity compared to infiltrating monocytes (85), and specific triggers that can induce their switch to an alternative source of energy production are poorly researched (371). AMs can also employ fatty acid oxidation for ATP generation at steady state (391), important in their turnover of lipid-rich surfactant, which would be important to investigate in the future.

AMs from *MFRN1/2^{ΔCd11c}* mice show a significant upregulation of *Tfrc* mRNA expression, but no change in *Fth1* (**Fig. 6.6C-D**). While it would be tempting to speculate that increased *Tfrc* expression reflects cellular iron deficiency, measurement of total cellular iron is necessary to confirm this. As illustrated in FLAM CSE treatment (**Fig. 3.1**) and DFP treatment (**Fig 5.2B**) in earlier chapters, elevated *Tfrc* expression can be indicative of increased or decreased total cellular iron levels. Regardless, the notion that iron deficiency localised to a subcellular compartment can affect iron uptake from the extracellular space is intriguing and raises questions about the mechanism of cytosolic sensing of depleted mitochondrial iron levels. Limited FeS cluster biogenesis inducing the switch of cellular aconitase to IRP1, which in turn stabilises *Tfrc* mRNA, would appear a likely mechanism (392). Still, other ways a cell may attempt to increase bioavailable iron intracellularly, such as ferritinophagy, must be investigated.

We developed and characterised a model of acute lower respiratory tract *S. pneumoniae* infection (**Fig. 6.2**). Intranasal instillation was preferred over the more invasive intratracheal instillation to mimic the natural progression of *S. pneumoniae* from the upper to lower respiratory tract (176). For intranasal instillation, mice were anaesthetised through subcutaneous injection of ketamine/xylazine. Anaesthesia was administered subcutaneously as a refinement to the commonly used intraperitoneal injection to minimise attrition rate from anaesthesia. While inhaled isoflurane is less invasive and has a shorter recovery time, intranasal instillation is more efficient in mice administered ketamine/xylazine compared to isoflurane, inducing a higher degree of

cellular infiltration indicating enhanced delivery of the nasally instilled vehicle to the lower respiratory tract (393). *S. pneumoniae*-instilled mice showed increased weight loss and cellular infiltration to the airspaces (**Fig. 6.2**), both of which are standard in murine pneumonia and validate our model of infection (394, 395).

MFRN1/2^{ΔCd11c} mice showed decreased cellular infiltration to the airspaces when challenged with *S. pneumoniae* (**Fig. 6.11A-B**). This decrease can largely be attributed to diminished neutrophil recruitment, illustrated in the reduction in their total number and in the ratio of neutrophils to monocytes/macrophages (**Fig. 6.11G**). As mice were only infected for 48 hr, changes in the adaptive immune response could not be assessed. AMs recruit neutrophils to help in bacterial killing through secretion of specific chemokines such as CXCL1 and 2 (396, 397). This recruitment is key in the resolution of infection, with an impaired neutrophil response associated with worsened mortality in infection (397-399). Conversely, persistent neutrophilic inflammation is tissue destructive, and the *S. pneumoniae* virulence factor pneumolysin can trigger sustained neutrophil recruitment to disrupt the alveolar epithelial barrier and allow for its dissemination systemically (379). Considering the decrease in lung and systemic bacterial load at this timepoint (**Fig. 6.12**), the decrease in neutrophils in MFRN1/2^{ΔCd11c} mice likely reflects a reduced capacity of bacteria to proliferate in the lung upon initial infection, negating both the need for high numbers of neutrophil recruitment and the ability of *S. pneumoniae* to hijack this response. Analysis of BALF chemokine levels in this model would help to solidify this.

Restriction of bioavailable iron is a fundamental macrophage response upon infection (94). We hypothesised that AM mitochondrial iron is targeted by *S. pneumoniae* as a nutrient source to fuel its virulence, and that loss of AM mitochondrial iron would be favourable to the host and deleterious to the pathogen. In line with *in vitro* findings with DFP treatment in isolated human and mouse AMs prior to infection in **Chapter 5**, targeting mitochondrial iron in AMs reduced bacterial burden *in vivo* (**Fig. 6.12**). This AM specific mitochondrial iron depletion was beneficial for host outcomes of infection and associated with reduced weight loss and cellular infiltration (**Fig 6.9, 6.10**). This positions mitochondrial iron as a potentially novel arm of the nutritional immune response in the lung, where in an infection context depriving bacteria of host mitochondrial iron as a nutrient source trumps any potential detriments of this mitochondrial iron loss for the host, such as diminished mitochondrial bioenergetics (**Fig. 6.8**). Extracellular BALF iron in MFRN1/2^{ΔCd11c} mice was unchanged, suggesting the mechanism by which mitochondrial iron-deficient AMs limit iron availability to bacteria is something cell-intrinsic and not driven by changes in iron in the alveolar niche (**Fig. 6.11A**). Investigating the expression of *S. pneumoniae* iron uptake genes in this model through either a fluorescent reporter-based

system or dual host-pathogen RNA-sequencing would confirm the bactericidal effects of intracellular iron restriction in our model.

Equally, the possibility that mitochondrial iron deficiency in these AMs may drive secretion of other soluble mediators with antimicrobial effects cannot be ruled out. Mitochondrial iron depletion has been suggested to drive macrophages towards a more anti-inflammatory phenotype associated with increased secretion of anti-inflammatory TGF- β (129). Only the pro-inflammatory lipocalin-2 was analysed, where no significant decrease was detected in infected MFRN1/2 Δ Cd11c mice compared to their infected littermates (**Fig. 6.11B**). This lack of significance can be attributed to an underpowered littermate *S. pneumoniae*-instilled group, where due to technical limitations only 3 mice could be analysed. Lipocalin-2 can be secreted by barrier cells, including goblet cells and cells of the alveolar epithelium, so analysis of cytokines unique to immune cells in future will be more informative. Importantly, elevated levels of lipocalin-2 in BALF from individuals with *S. pneumoniae*-mediated community-acquired pneumonia associated with increased mortality (123, 400). Further experimental power in our lipocalin-2 measurements will determine if this trend holds in MFRN1/2 Δ Cd11c mice.

Normal mitochondrial function and iron homeostasis are both essential arms of the macrophage response to infection. Upon activation, numerous metabolites within the TCA cycle and beyond can exert direct anti-inflammatory and bactericidal activities (76). The generation of these metabolites is largely governed by iron-driven enzymatic reactions, through FeS clusters housed in enzymes like succinate dehydrogenase and aconitase (129). Equally, several of these metabolic intermediates can be consumed by invading pathogens to facilitate their own virulence (320). Our preliminary results have suggested a loss of OXPHOS in AMs from MFRN1/2 Δ Cd11c mice, suggesting that in this model mitochondrial iron deprivation holds profound consequences for cellular metabolism. Further experimental work should involve the supplementation of MFRN1/2 Δ Cd11c mice with metabolites to assess if the observed protective effects of mitochondrial iron targeting in *S. pneumoniae* infection are as a direct result of limiting mitochondrial iron availability and not the availability of a downstream metabolite. Similarly, metabolomic analysis of AMs from MFRN1/2 Δ Cd11c mice at baseline will help to clarify whether mitochondrial iron targeting can boost macrophage immunometabolism to enhance bacterial clearance, as previously suggested by a study investigating the effects of DFP in human monocyte-derived macrophages (129).

Intriguingly, we detected *S. pneumoniae* in the bone marrow of infected mice (**Fig. 6.12D**). In line with our local ethical approval, mice were euthanised 48 hr post-infection

before they exhibit 'severe' disease based on severity scoring. The extent of dissemination observed suggests that mice likely do not recover from instillation with this dose of *S. pneumoniae*. While this may mean that the presence of *S. pneumoniae* in the bone marrow in this instance is symptomatic of widespread bacteraemia, *S. aureus* and *M. tuberculosis*, bacteria associated with more chronic infections, have been detected in the bone marrow previously (401, 402). Repeating infection with a lower, non-lethal dose of bacteria will elucidate if *S. pneumoniae* can persist in the bone marrow in a similar manner.

In contrast to our genetic model, targeting mitochondrial iron through administration of DFP worsened host outcomes of infection, with decreased survival and an increase in cellular infiltration to the lung observed (**Fig. 6.3, Fig. 6.4**). As DFP was administered systemically, iron deficiency is likely present in many cell types and tissues far beyond our AM-targeted genetic model. Iron deficiency is the most prevalent micronutrient deficiency globally, and iron deficient individuals show attenuated antibody production following infection and vaccination, hinting at immune cell impairment. Mice deficient in FTH1 specifically in the myeloid compartment show heightened lung pathology upon *M. tuberculosis* infection (354). Specifically in COPD, anaemia is a predictor of mortality in patients who have been hospitalised with an exacerbation (403). Despite increased cellular infiltration in DFP mice there was no significant difference in absolute neutrophil number, or neutrophil:monocyte/macrophage ratio (**Fig. 6.4**). This may suggest an impairment of central myelopoiesis by iron limitation to yield recruitment of functionally impaired immune cells to the lung, which perpetuate tissue damage and are unable to efficiently fight infection. Iron deficiency disproportionately effects neutrophil development relative to monocytes, with mice administered a hepcidin analogue showing impaired granulopoiesis but not monopoiesis (404). Neutrophil maturation, phagocytosis and cytokine production is impaired in iron-limited conditions (404, 405). Conversely, in a rat model of glomerulonephritis, a sterile model of kidney inflammation, mice given a therapeutic course of DFP treatment commencing 4 days after disease induction showed reduced inflammatory cell infiltration and improved kidney function (129). DFP treatment reduced pancreatic lesions in a tumour model, with a reduction in infiltrating monocytes specifically compared to other myeloid cell populations (406). Therapeutic administration of DFP during CS exposure was also protective against inflammatory cytokine production and emphysema development (50). As these pathologies are largely mediated by recruited monocytes and not neutrophils, this may explain why DFP is protective in these instances and not during acute infection. The high variability of data because of the deaths observed in the DFP group necessitates further experimental investigation before this can be concluded.

The timing and route of DFP administration used is also an important consideration. Mice were administered DFP in their drinking water for 21 days to develop a prophylactic model of mitochondrial iron loss (**Fig. 6.3**), mimicking what was beneficial in our *ex vivo* *S. pneumoniae* infection of human AMs in **Chapter 5**. Models which have successfully used DFP as a therapeutic against inflammatory disease progression *in vivo* all provided DFP after initial onset of disease (50, 129). In contrast to these sterile models of disease, a direct bactericidal effect of DFP on *S. pneumoniae* cannot be excluded. To ensure no confounding direct bactericidal effect, mice were returned to normal drinking water for the duration of *S. pneumoniae* infection. As our previous data in human and mouse AM models of infection have shown, DFP has a profoundly beneficial effect when administered to pure AM populations prior to infection. The reliance on neutrophil recruitment for bacterial clearance *in vivo*, which are functionally impaired in iron-limited conditions and could therefore be detrimental to infection outcomes upon recruitment (404), makes it difficult to fully decipher how DFP is affecting AMs in this model. Development of a more targeted delivery model of DFP to the lungs will limit these off-target effects and will help to elucidate its effects on infection outcomes more locally.

In a 6-week CS exposure model, MFRN1/2^{ΔCd11c} mice showed impaired weight gain and a trending increase in cellular infiltration to the airspaces (**Fig. 6.13, 14**). As expected, these infiltrating cells were monocytes/macrophages, and we observed no change in absolute neutrophil number or ratio of neutrophils to monocytes/macrophages with CS (**Fig. 6.13**). Previous animal studies investigating the role of lung mitochondrial iron in experimental COPD used systemic genetic or pharmacological depletion approaches, making it difficult to estimate the contribution of AMs specifically to the observed phenotypes. Mice globally deficient in frataxin show increased mitochondrial iron levels in whole lung tissue, which associated with impaired mucociliary clearance and elevated whole-lung IL-6 upon 4-week CS exposure (50). Whole lung mitochondrial iron in MFRN1/2^{ΔCd11c} mice at baseline was not investigated, but total iron levels in lung tissue are unchanged relative to control, making any pre-existing increase in mitochondrial iron levels unlikely (**Fig. 6.11D**). Confirmation of the trends observed in monocyte recruitment in CS-exposed MFRN1/2^{ΔCd11c} through increasing statistical power and further investigation of the tissue distribution of iron in our model will help to determine if AM mitochondrial iron depletion does indeed have a direct pathological effect in CS.

Monocytes recruited to the lung add to the resident AM pool in smoking and COPD (144). It is possible that these monocytes could accumulate iron in CS exposure and would not be targeted by Cd11c-Cre initially until they fully assume an AM-like identity. In both human and experimental COPD, heterogeneity in AM iron loading is observed, and only a

subpopulation appears susceptible to pathological iron loading (164, 165). The reasons behind this are not known, but an inherent variability in the mechanisms controlling iron flux between subpopulations of AMs has been hinted at previously: mice with a loss of function mutation in ferroportin exhibited iron loading in only a subset of AMs (407). It could be possible that differential AM ontogeny manifests in reliance on distinct programs of iron metabolism, which endows subpopulations with unique susceptibilities to smoking-induced iron, and mitochondrial iron, loading. The Cd11c-Cre expressing cells targeted for mitochondrial iron depletion in our model may not be the cellular subpopulation where mitochondrial iron loading is pathological in COPD: further defining the contribution of monocyte mitochondrial iron to detrimental outcomes of CS exposure using more myeloid-targeted Cre drivers may be important.

The seemingly detrimental role of AM mitochondrial iron deficiency in CS exposure contrasts the beneficial effects elicited in *S. pneumoniae* infection (**Fig 6.9-12** and **Fig 6.13-14**, respectively). Work from our group has previously illustrated divergent roles of genetically manipulated AM iron metabolism, through hepcidin deficiency, in response to CS exposure and *S. pneumoniae* infection (112). The opposing phenotypes could be explained by the cell types recruited in each model. Both monocyte and neutrophil recruitment to the airspaces are hallmarks of the host response to acute infection, but in human and murine COPD and smoking monocytes are the primary myeloid cells in the airspaces, which will ultimately also lose MFRN1 and 2 expression over time in our model (144, 399). The acute nature of *S. pneumoniae* infection may only mean mitochondrial iron depletion is of short-term benefit to AMs uniquely in bacterial infection, whereas constitutive loss under the chronic stress of CS exposure is detrimental. Examining the effects of *S. pneumoniae* infection in CS-exposed MFRN1/2^{ΔCd11c} mice will characterise if the protective effect of AM mitochondrial iron depletion in infection trumps its seemingly detrimental effect in chronic CS exposure. Use of a more long-term model of respiratory infection through pathogens such as *P. aeruginosa* or non-tuberculosis mycobacteria would also elucidate if the benefit of mitochondrial iron depletion in the nutritional immune response is maintained in chronic infection (408, 409).

6.3.1 Limitations

We were unable to show a loss of *Slc25a37* expression in BAL cells isolated from MFRN1/2^{ΔCd11c} mice through RT-qPCR (**Fig. 6.6A**). A significant loss of *Slc25a28* was observed and there was no compensatory increase in *Slc25a37*, meaning Cre recombinase appears to be acting appropriately at LoxP sites in this system. As the RNA used for these measurements was extracted from BAL cells and not a purified Cd11c-

positive population, it is possible a contaminating cell type not expressing Cd11c may be contributing levels of the *Slc25a37* transcript masking its loss in Cd11c-positive cells. Similarly, the design and use of new primer pairs against *Slc25a37*, or its measurement through bulk RNA sequencing of purified cell populations, will clarify whether this result is a technical artefact of the RT-qPCR reaction.

As discussed Cd11c expression is not limited to AMs, and an impact of off-target MFRN1 and 2 genetic deletion on other immune cell populations cannot be ruled out. Dendritic cells, which Cd11c-Cre is used to target in the literature, can also contribute to neutrophil recruitment in inflammation (410). Equally, as only targeted tissue-resident AMs were targeted with the Cd11c-Cre driver, recruited monocytes may dilute this phenotype, particularly in our infection model where the short time course means these recruited macrophages likely do not assume a fully AM-like phenotype and express Cd11c. Deletion of MFRN1 and 2 using a monocyte-specific Cre driver, such as *Cxcr3*, would assess the impact of mitochondrial iron deficiency in these recruited monocyte-derived macrophages. We targeted the two universally acknowledged mitochondrial iron importers, MFRN1 and 2, to deplete iron levels in the mitochondrial matrix through inhibiting mitochondrial iron import. Deletion of the MFRN1 and 2 paralogues in *S. cerevisiae* only presented with a phenotype under iron limited conditions, suggesting another means to import iron into the mitochondria in homeostatic conditions may exist (411). Other factors theorised to import mitochondrial iron include the mitochondrial calcium uniporter, but this has only been observed using a non-physiological 'overdose' treatment with paracetamol (412). Still, expression should be assessed in our model to rule out any potential compensatory import.

As before, the expression of iron-related factors in these cells was only assessed at the mRNA level through RT-qPCR. Measuring protein expression using even the limited number of iron-associated primary antibodies available would be technically difficult due to the low abundance of AMs *in vivo* and would require extensive pooling of mice. Measurement of TFRC expression through flow cytometry has been made possible in recent years through the development of fluorescently conjugated primary antibodies, which would aid validation of gene expression data. Assessing MFRN1 protein expression via confocal microscopy using an anti-MFRN1 primary antibody and a fluorescently conjugated secondary antibody will help to elucidate whether its expression is lost in AMs in lieu of robust RT-qPCR data.

We were unable to prove that loss of MFRN1 and 2 expression results in decreased mitochondrial iron in AMs from MFRN1/2^{ΔCd11c} mice (**Fig. 6.7**). Mito-FerroGreen, a

fluorescent probe purported to bind mitochondrially localised Fe^{2+} , did not recapitulate GF-AAS findings in FLAMs in pharmacological models of mitochondrial iron manipulation (**Fig. 5.3**). GF-AAS measures all valencies of iron, and Mito-FerroGreen is described as Fe^{2+} specific (377). As the extent of mitoplast iron increase observed in FAC treatment upon GF-AAS measurement was so high (~ 60-fold increase), this discrepancy between the two techniques is not a plausible reason for the lack of increase in Mito-FerroGreen expression. Deletion of MFRN1 and 2 in the liver, using the same parental *Slc25a37^{fl/fl}*, *Slc25a28^{fl/fl}* line as in this study, manifested in significantly reduced mitochondrial non-heme iron in whole liver tissue, confirming that MFRN1 and 2 can be targeted *in vivo* to deplete mitochondrial iron. Further development of more refined methods of mitochondrial iron measurement will facilitate measurement of mitochondrial iron levels in cell populations of lower abundance, like AMs.

Importantly, the effects of mitochondrial iron depletion on cellular and mitochondrial health (measurement of mitochondrial copy number, membrane potential, mtROS etc.) in AMs from MFRN1/2 Δ^{Cd11c} mice also remain to be determined. Genetic models of mitochondrial iron deficiency have associated with reduced membrane potential at baseline, and treatment of human macrophages with DFP induces mitophagy (413). To confirm that our observed changes in OXPHOS are a direct result of mitochondrial iron depletion and not due to damage or loss of mitochondria, this should be assessed in the future. Similarly, systemic iron homeostasis in MFRN1/2 Δ^{Cd11c} mice should be assessed through measurements of serum hemoglobin and transferrin saturation to confirm whether systemic iron deficiency may contribute to the observed phenotypes. Assessing liver hepcidin levels will also elucidate whether baseline levels of the bactericidal peptide hepcidin may influence the response MFRN1/2 Δ^{Cd11c} mice to infection.

When investigating cellular infiltration to the airspaces in both *S. pneumoniae* infection and CS exposure, myeloid cell populations present were determined through manual counting of differentially stained BAL cell cytopsin preparations. While MFRN1/2 Δ^{Cd11c} mice had a higher percentage of monocytes/macrophages post-infection compared to littermate controls, the proportion of these that are recruited relative to lung-resident AMs could not be assessed. AM cell death is a regulated, front-line response to respiratory infection. This deprives intracellular bacteria of an intracellular niche and activates neutrophils to promote enhanced bacterial killing (414). A more in-depth analysis of the cellular populations in the airspaces through flow cytometry would allow us to assess whether earlier AM death in MFRN1/2 Δ^{Cd11c} mice may precipitate more efficient cell recruitment to the airspaces to control infection. This would also allow for more robust profiling of cellular infiltration in DFP-treated mice upon infection.

To assess the phenotypic effects of MFRN2 loss upon infection, we investigated cellular infiltration to the airspaces and lung and systemic CFU in MFRN1/2^{ΔCd11c} mice compared to littermate control 48 hr post-infection. Using additional timepoints in future will help provide more mechanistic insight into how MFRN1/2^{ΔCd11c} mice are protected in infection, including how the kinetics of immune cell recruitment might aid bacterial clearance and/or dissemination.

6.3.2 Conclusions

We have presented for the first time an *in vivo* model of MFRN 1 and 2 deficiency targeted to an immune cell and illustrated the profound effect of mitochondrial iron loss on AM metabolism. Unlike mice depleted of mitochondrial iron systemically through DFP, MFRN1/2^{ΔCd11c} mice showed improved outcomes of *S. pneumoniae* infection, namely decreased weight loss, immune cell recruitment and trending decreases in bacterial dissemination. The protective effect of localised AM iron depletion against *S. pneumoniae* infection did not extend to a CS exposure model of experimental COPD, where mice showed impaired weight gain and trending increases in cellular infiltration to the lung. Our findings highlight the protective effect of localised mitochondrial iron depletion on the outcomes of infection in the lung and warrant further investigation into the interplay between CS exposure and infection in mitochondrial iron dynamics.

Chapter 7: Discussion

7.1 General discussion

Mitochondrial iron, a key fuel in cellular metabolism, is pathogenically elevated in a murine CS-exposure model of experimental COPD. We hypothesised that this mitochondrial iron loading extends to alveolar macrophages, and that these iron-laden macrophages are sites of iron piracy by bacteria to fuel their proliferation and virulence which renders patients increasingly susceptible to infection. This thesis aimed to define mitochondrial iron levels in alveolar macrophages in COPD and smoking, and to determine if the burden of the common respiratory pathogen *S. pneumoniae* in AMs was sensitive to mitochondrial iron levels. Using a series of *in vitro*, *ex vivo* and *in vivo* approaches, this question was comprehensively addressed across humans, mice and cell lines. Interrogation of transcriptomic data, both publicly available and generated over the course of this work, highlighted the unique transcriptomic remodelling induced by CS in AMs, which in CSE-treated FLAMs associated with cellular and total mitochondrial iron increase. *In vitro* and *in vivo* smoke exposure impaired AM response to *S. pneumoniae* infection, and infection induced iron loss in AMs. Pharmacological models of mitochondrial iron loading and deficiency revealed burden of *S. pneumoniae* infection to correlate with mitochondrial iron levels in FLAMs and human AMs and illustrating the therapeutic potential of DFP in improving response to infection in smoking. Novel transgenic mice with a targeted deletion of MFRN1 and 2 in AMs showed reduced inflammatory cell and bacterial burden in *S. pneumoniae* infection, with the protective effect of localised mitochondrial iron depletion contrasted to the detrimental effects of systemic iron depletion with DFP. The findings of this thesis have advanced our knowledge which we propose is a novel regulator of the nutritional immune response to infection in AMs. Proactively targeting mitochondrial iron in these patients therefore represents an unexplored therapeutic avenue to combat bacterial infection, both in COPD and potentially in other diseases characterised by predisposition to acute infection.

7.1.1 Mitochondrial iron, respiratory infection and disease

We have identified a novel ability of the mitochondrial iron axis to dictate the response of AMs to infection. This positions mitochondrial iron as a putative nutrient source for bacteria, which, most importantly, can be successfully targeted to diminish intracellular bacterial proliferation. These findings are particularly relevant in the setting of COPD, as our results in **Chapter 3** have shown pathological mitochondrial iron loading previously identified in the whole lung in experimental COPD extends to AMs treated with CSE. Gene

expression analysis in human AMs from smokers and COPD hinted at a historical accumulation of mitochondrial iron, representing the first time that alterations in the mitochondrial iron axis have been noted in any lung cell in human smoking and COPD. We proved in **Chapter 4** that these AMs from smokers, and CSE-treated models, have a diminished capacity to control intracellular *S. pneumoniae* infection, which results in **Chapter 5** suggested can be improved with depletion of mitochondrial iron prior to infection and worsened by its increase. We confirmed our findings are applicable *in vivo* in **Chapter 6** through instilling *S. pneumoniae* to a novel murine strain with targeted deficiency of MFRN2 in AMs, which manifested in reduced inflammatory cell burden and bacterial survival intracellularly. As these mice were not protected from the detrimental effects of CS exposure, the value of mitochondrial iron deficiency to the host response appears not to be in enhancing immune cell function, but rather in depriving *S. pneumoniae* of a nutrient needed for growth. Many arms of the nutritional immune response are a double-edged sword, where the evolved host mechanisms to sequester away iron are the targets of equally sophisticated bacterial iron theft (94). We propose that another such mechanism is host iron storage in the mitochondrial matrix, where the fuel macrophages rely on for their effector functions in infection is the very target of bacterial iron piracy.

There is a widespread acknowledgement of the endosymbiotic origin of mitochondria, where an ingested prokaryote co-evolved with its host archaeon to become the mitochondria-containing eukaryotic cell (415). Signs of this prokaryotic origin of mitochondria remain evident in the evolved eukaryotic cell, like their possession of an extranuclear genome, and their ability to grow and divide independently of cell division through mitochondrial fission. Another putative relic of this prokaryotic origin is the arsenal of mechanisms mitochondria possess to sense bacterial infection and mount a response autonomous of the cell as a whole, including an mtDNA-encoded bactericidal peptide (416, 417). Reciprocally, virtually all successful intracellular pathogens can target mitochondrial function specifically, perhaps as mitochondria are the parts of the cell whose prokaryotic ancestry means bacteria can recognise the most. Common to mitochondria and prokaryotes still is a reliance on the acquisition and import of metals, like iron, to use in cofactor production, the maintenance of oxidative balance and to ensure metabolic health (418). This positions bacteria and mitochondria as the main consumers of host metals, and therefore rival competitors in their acquisition. In this work, we have demonstrated in mice and humans an ability of *S. pneumoniae* to respond to elevated or depleted host macrophage mitochondrial iron levels, likely in response to obtaining iron to boost intracellular persistence. Importantly, we have demonstrated this in AMs, a cell type highly relevant to host-pathogen dynamics *in vivo*. Dissecting the bacterial-mitochondrial fight for

iron further from the pathogen perspective will be vital to investigate the receptors used by *S. pneumoniae* to uptake iron, the tracking of this iron across generations of bacteria to confirm its use in driving bacterial intracellular survival, and if these mechanisms to respond to mitochondrial iron are conserved across other bacteria.

CS exposure increases mitochondrial iron levels in whole lung tissue in a time-dependent manner (50). Administration of DFP to chelate this mitochondrial iron for the final 4 weeks of an 8-week smoke exposure regimen increased murine weight gain and was protective against inflammatory cell infiltration (50). **Chapter 4** showed that acute CSE treatment recapitulates this mitochondrial iron loading specifically in an alveolar macrophage-like cell line, and AMs isolated from individuals who currently smoke or have COPD were shown to have significantly downregulated expression of MFRN1 and 2 in **Chapter 3**. Mimicking this loss of MFRN1 and 2 in AMs specifically was detrimental in murine CS exposure as observed in **Chapter 6**, contrasting the beneficial effects of pharmacologically depleting mitochondrial iron in CS exposure previously observed (50). These divergent findings highlight the importance of timing of mitochondrial iron depletion in the host to elicit protective effects. Systemically, both anaemia and non-anaemic iron deficiency are associated with worsened outcomes of COPD, and higher serum iron appears protective against smoking-induced lung function decline (403, 419). Locally in the lung, mice administered deferoxamine to induce a two-fold depletion in lung tissue iron prior to 8-week CS exposure showed elevated airway cellular infiltration, and more severe emphysema and air trapping compared to iron-replete controls (420). This data is in line with the detrimental role of pre-existing mitochondrial iron loss in AMs in our murine model. The ability to import and mobilise mitochondrial iron may therefore be an essential step in the initial response to smoking or equivalent environmental pressures, to ensure the production of cofactors necessary for cells to mount a metabolic and oxidative response. At a certain point, this iron may become trapped in the mitochondrial matrix, potentially through conversion of mitochondrial iron to an inert, unusable form by particulate matter, or indeed hydroxyl radicals produced by excess mitochondrial iron itself (421). Over time, a cytoprotective downregulation of MFRN1 and 2 expression in AMs may be an attempt to limit further import, supported by the decreased expression observed in AMs from human smoking and COPD (**Fig. 3.2, Fig. 7.1**). Equally, the cellular mechanisms of sensing mitochondrial iron may become defective, as evidenced by the accumulation of the regulatory IRP2 in the smoking and COPD lung (50, 158).

Our findings do not exclude AMs for being the cell population in the lung where mitochondrial iron loading can be pathological in smoking. Rather, they emphasise the unique capabilities of changes in the AM mitochondrial iron axis to exert an effect on the

alveolar niche, which positions them as an attractive therapeutic target. As MFRN1/2^{ΔCd11c} mice constitutively express Cre recombinase, AMs are deficient in mitochondrial iron when CS exposure begins. Generating a modified transgenic strain where Cre expression could be induced to delete MFRN1 and 2 during CS exposure would address if timing of AM mitochondrial iron depletion is indeed crucial in dictating this phenotype.

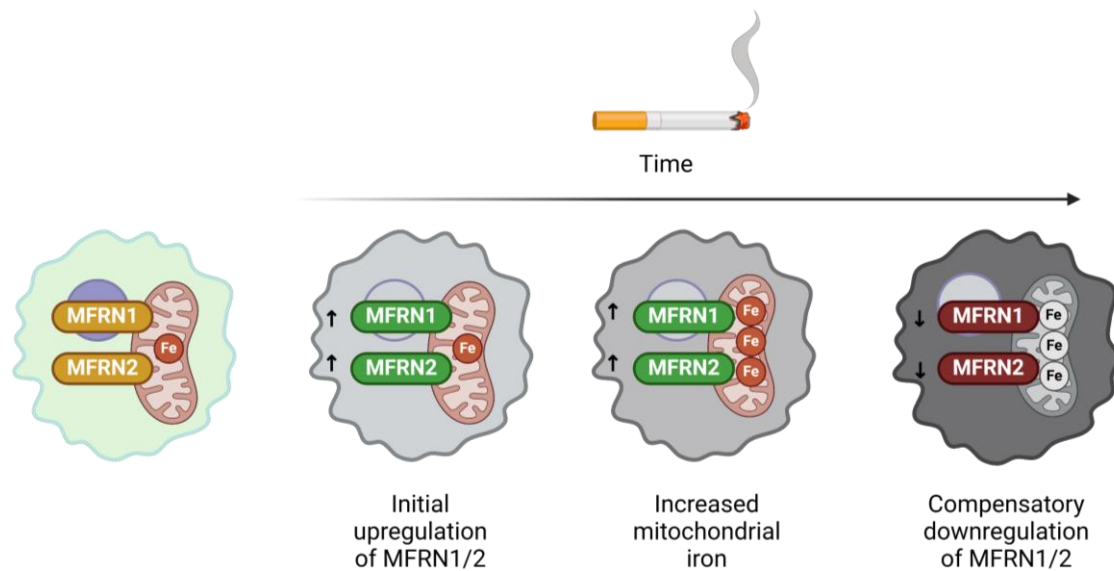


Fig. 7.1: Proposed model for observed changes in AM mitochondrial iron in smoking/COPD.

In **Chapter 4**, we showed that FLAMs lose approximately half of their total iron upon *S. pneumoniae* infection, a phenomenon that was only observed when using live bacteria. This means that this iron loss is potentially a pathogenic bacterial mechanism, independent of TLR-mediated immune signalling which inactivated bacteria remain capable of activating. What form this iron takes, and where in the cell it comes from, remain open questions. As we have described a novel capacity of *S. pneumoniae* to survive inside AMs, its intracellular niche is as-of-yet unknown: determining this will provide key insight into the source of cellular iron used. As **Chapter 5** and **Chapter 6** illustrated the capacity of mitochondrial iron to dictate infection outcomes, one could speculate *S. pneumoniae* may be mitochondrially localised or targeting mitochondria for iron through effector protein secretion. Bacterially secreted molecules can accumulate in mitochondria in the absence of direct bacterial localisation (422) and the *S. pneumoniae* virulence factor pneumolysin can directly damage mitochondria (305). Furthermore, mitochondria appear motile during infection and can be weaponised by the host: mitochondria are recruited to the macrophage phagosome upon TLR activation to aid in bacterial killing via mtROS production (80) and to the neutrophil phagosome upon *S. aureus* infection to induce NETosis via bacterial lactate sensing and degradation (423). Whether this proximity to

mitochondria can be exploited by bacteria for iron acquisition, either through inducing mitochondrial damage or exploiting the flux of iron from cytosol to mitochondria, is unknown. The precise subcellular localisation of a bacterium has consequences for host iron sequestration, highlighting the importance of interrogating this in our model. In the case of *Listeria monocytogenes*, macrophages can differentially express FPN depending on bacterial localisation to the cytosol or the phagosome (424). As we only observed this phenotype using the gram-positive *S. pneumoniae*, determining the universal relevance of this iron loss across host-pathogen interactions between different bacteria (gram negative, mycobacteria) is an important next step. Intriguingly, gram-negative bacteria are more closely phylogenetically related to mitochondria than gram-positive (425, 426) – whether this manifests in more successful exploitation of mitochondria by a gram-positive bacterium is unknown.

Several questions remain surrounding the mobilisation and utilisation of iron at mitochondria, which hold important consequences for this work. The precise chaperoning mechanism that delivers iron to the OMM has not been conclusively described, and the crossing of the OMM by iron through VDAC porins is assumed, but not proven. It is unknown if mitochondrial ferritin is comprised of multiple subunits like cytosolic ferritin, and if the ratio of these can similarly change to meet tissue-specific iron demands. It has been suggested that recycling of iron from mitochondria is regulated by mitophagy (406), but whether cells can mobilise iron from mitochondria in demanding conditions independently to heme and FeS cluster synthesis is unknown. Increasing the depth of our knowledge of mitochondrial iron dynamics will expand the repertoire of potential bacterial targets in mitochondrial iron piracy.

7.1.2 Assessing the cellular ironome

In both *in vitro* and *in vivo* models, we used a combination of techniques to measure iron in varying bound forms and oxidation states, alongside measuring expression of key players in cellular and mitochondrial iron metabolism through western blotting and RT-qPCR. Our findings illustrate the benefits of using a combination of techniques to characterise the cellular ‘ironome’, and caution against using gene/protein expression alone to infer total iron levels. For instance, TFRC expression is upregulated with both DFP treatment, which reduces total iron, and CSE treatment, which increases total iron. Literature which uses TFRC expression alone to infer cellular iron deficiency should therefore be interpreted with caution, as our data suggests expression likely reflects the flux of iron through a cell at a moment in time rather than absolute levels of iron stored. Pairing TFRC expression with measurement of transferrin-bound iron uptake through a

fluorescently conjugated transferrin derivative offers a more comprehensive measurement of TFRC activity (427). Measurement of atomic iron levels through spectroscopic means is most definitive method of measuring iron across all bound states, cofactors and valencies. We used GF-AAS, which measures iron levels in biological samples in a label-free manner, where atomic iron has been liberated through nitric acid. Inductively coupled plasma mass spectrometry (ICP-MS) goes beyond to measure absolute amounts of atomic iron at both its Fe^{2+} and Fe^{3+} valencies, providing further information on iron bioavailability. While these techniques are hugely informative other, more crude methods of measuring iron in limited circumstances, can still be beneficial in validating gene or protein expression data. Perls' Prussian blue stain can detect Fe^{3+} in histological preparations, fluorescence-based probes like calcein-AM and FerroOrange can assess labile Fe^{2+} , and both heme and non-heme iron assays can provide information on the ratio of heme-bound to non-heme bound iron. Such methods, while recognising their limitations, can prove informative in contextualising iron-related gene and protein expression in lieu of the more technically limiting GF-AAS and ICP-MS.

The limited throughput of these methods can be prohibitive to studying cells which are low in abundance, populations in which iron uptake is non-isogenic, and makes any screening approach which reliably uses changes in intracellular iron levels as a readout technically difficult. GF-AAS can measure total atomic amounts of iron in a reasonably low input of cells and provides absolute iron measurements that cannot be inferred by expression data, but is prone to contamination during sample preparation and measurement from environmental iron, illustrated by the variability in data throughout this thesis. Where feasible, we used the average of three technical triplicate wells of cells given a particular treatment, which improves experimental reproducibility but hinders high-throughput applications. GF-AAS will also liberate all forms of cellular iron, some of which may be inertly stored in proteins and functionally inaccessible to the cell for usage. Fluorescent probes for iron measurement through confocal microscopy or flow cytometry have value in allowing for the discrimination between Fe^{2+} and Fe^{3+} iron: however, fixation of cells and tissues had been associated with leeching of metal ions (428). This means these probes must be used in live cells which is a difficult task in isolated *in vivo* cell populations, or in experimental conditions where time may rapidly shift observed phenotypes, like *S. pneumoniae* infection. While a recent study suggested FerroOrange can be used in a plate reader format (429), this would still only detect the labile iron pool, which our data in **Chapter 4** shows does not necessarily correlate with total iron levels. Of note, recent expansions in single cell technologies have reached metal-based analytical techniques. ICP-MS has been used successfully to measure arsenic (430), calcium, zinc

and magnesium (428) at femtogram resolution in single-cells, a useful technological advance in studying iron distribution in limited populations of cells.

7.1.3 The lung-bone marrow axis

As an aside to our primary experimental aims, across several experiments and models we observed the capacity of a stimulus administered locally to the lung to induce distal changes at the bone marrow *in vivo*. In *S. pneumoniae* murine infection, viable bacteria were cultured from the bone marrow (**Fig. 6.12D**). *S. pneumoniae* can invade erythrocytes *in vitro*, which may explain its transport to the bone marrow (355). But as erythrocytes were depleted from bone marrow preparations, our results suggest *S. pneumoniae* may persist in, or at least associate with, hematopoietic progenitor cells. Bone marrow stromal cells in both humans and mice can produce the antimicrobial peptide hepcidin (431), suggesting that the bone marrow has evolutionarily conserved antimicrobial capacities. This could represent a bacterial niche (402), or a pathogenic bacterial mechanism to impair myelopoiesis and diminish myeloid cell production and effector mechanisms at distal tissues, further perpetuating infection: given the short timeframe of infection, the latter appears more plausible. *M. tuberculosis* can persist in the bone marrow and detrimentally remodel myelopoiesis through inducing necroptosis specifically in CMP and GMPs, coincidentally though a reduction in mitochondrial iron (432).

In **Chapter 3**, we showed *in vivo* CS exposure enhanced haematopoiesis in the bone marrow, which manifested in an increased proportion of pro-inflammatory Ly6C-high circulating monocytes. These monocytes showed decreased H3K27Ac deposition relative to RA controls, which was also observed in circulating neutrophils (**Fig. 3.19-20**). Importantly, this suggests that epigenetic changes may occur in a common precursor of these two granulocyte precursors. Work from our group has previously described inefficient erythropoiesis in CS-exposed mice (112), but how this is signalled to the bone marrow following CS inhalation to the lung is unclear. Soluble mediators such as IL-1 β (433), IFN- γ (434) and CCL2 (435) can all mediate communication to or from the bone marrow. Cell-to-cell communication is also an intriguing possibility: particulate matter has been detected in the bone marrow (436) and macrophages can transfer cell and membrane components to HSCs by trogocytosis (437). AMs can migrate into the interstitium to offload internalised *M. tuberculosis* to neutrophils (438), and tissue-resident cardiac macrophages can travel to the brain following heart injury to influence sleep pattern, illustrating the ability of tissue-associated macrophages to influence function at

distal tissues (439). Whether a more pathological interaction could exist in AMs where they mediate the transfer of sterile contents to the bone marrow remains to be seen.

Transcriptomic analysis of BMDMs, differentiated from bone marrow progenitor cells from these CS-exposed mice, revealed that these changes manifested in a distinct gene expression programme associated with upregulated metabolic pathways and downregulated epigenetic pathways (**Fig. 3.6**). BMDMs are a common model of an *in vitro* effector macrophage derived from systemic monocytes issued from the bone marrow. While our model of differentiation *ex vivo* was not physiological, the value of our work is in that these changes compared to RA mice persist. This is despite the elimination of CS exposure and the provision of supraphysiological levels of nutrients in cell culture media. The persistence of this CS ‘memory’ could hold important consequences for ex-smokers. Smoking is known to be associated with systemic inflammation: smokers show higher numbers of circulating leukocytes, elevated blood cytokine levels and enhanced rates of transplanted organ rejection, all of which are sustained even after smoking cessation (271, 440). Ex-smokers can still experience exacerbation-like events, suggesting this elevated inflammation corresponds to a functional impairment of macrophage function in line with our observations in *ex vivo* infection of AMs (**Fig. 4.11**).

Intriguingly, functional hematopoiesis requires large amounts of iron and is impaired in iron-deficient conditions (441). Work from our group has previously shown depleted iron in the bone marrow of CS-exposed mice associates with inefficient erythropoiesis (112). Our data is the first to suggest changes in myelopoiesis may occur in CS exposure, leading to the emigration of epigenetically altered monocytes into circulation. Enzymes which deposit and remove epigenetic marks are dependent on iron for their function, and iron deficiency impairs B cell proliferation through inhibition of KDM2B, KDM3B and KDM4B-mediated histone lysine demethylation (442), also inhibiting KDM3B activity in hepatocytes (443). Further cell intrinsic measures of iron metabolism should be used to determine if changes in iron may be linked to this epigenetic remodelling.

7.2 Limitations and future perspectives

The findings of this thesis have several limitations, which present novel opportunities for future studies further defining the convergence between mitochondrial iron, respiratory disease and infection.

This work successfully optimised the use of FLAMs to model COPD *in vitro*, recapitulating unique AM phenotypes of blunted pro-inflammatory cytokine secretion and iron loading upon CSE treatment. The ability of FLAMs to replicate *ex vivo* permits the

study of an alveolar-like macrophage using methods and techniques which are otherwise inhibited by the low yield of primary AMs from mice, such as mitoplast isolation or western blotting. However, these cells are differentiated from fetal liver progenitors *ex vivo*, in the absence of several lung-intrinsic factors that could affect development like surfactant and the air-liquid interface. As such, they do not represent a bona-fide AM developed *in vivo* and isolated directly from the lung. Key findings should be replicated in primary murine AMs in the future where possible, like the sensitivity of *S. pneumoniae* intracellular survival to host mitochondrial iron levels and the ability of *S. pneumoniae* to induce iron loss.

Both *in vitro* and *in vivo*, we used CS to model COPD. COPD etiology is heterogeneous and is suggested to contain a significant genetic component, alongside risk from urban pollution and particulate matter inhalation (137). Still, smoking remains the primary risk factor for COPD development and, as a prototypical exposure, is the most common laboratory model of COPD, standardised globally through research cigarette production at the University of Kentucky. As we generated CSE in aqueous cell culture media, this will not capture the substantial lipophilic components of cigarettes which have been posited to be responsible for oxidative cellular damage. *In vitro* models of non-tobacco-based environmental exposures include concentrated ambient particles to mimic particulate air pollution (444). In agreement with our CS data, diminished host defence against *S. pneumoniae* has been observed in wood chip smoke exposure (445) and particulate matter exposure (444) models of COPD. Of note, efforts are currently being made by our group and others to develop *in vitro* and *in vivo* models of vape exposure in response to the surge in popularity in e-cigarette use globally and nationally. As e-cigarette use is in its infancy compared to use of tobacco-based products, this research is imperative to pre-emptively understand long-term consequences.

To model COPD *in vivo*, we used a well-characterised model of murine CS exposure. Notable anatomical differences exist between the mouse and human lung: mice have four lobes on their right lung and one on their left, compared to three right and two left in humans. Mice were exposed to whole-body CS, meaning the possibility that CS deposits are also ingested through grooming cannot be ruled out. Nose-only exposure models of CS are also used in the literature (446), but as this requires animals to be restrained it is not concordant with local ethical guidelines. Other murine models of COPD include the intratracheal or oropharyngeal instillation of proteases, like elastase, to induce emphysematous-like lesions in the lung (447). While this leads mice to develop emphysema in a shorter amount of time, this is not a physiological model of exposure like CS and as such should be used as a complementary approach.

In infection models, iron was predominantly characterised and manipulated in host cells and not in the pathogen. Measuring gene expression of pneumococcal iron uptake factors like *piuA*, *pitB* and *spbhp1* when internalised by host cells will elucidate how mitochondrial iron manipulation associates with pathogen iron uptake and in what form (labile iron, heme iron, etc.). Recent advances in next-generation RNA sequencing methods have allowed for capturing of the host and pathogen transcriptome simultaneously, even *in vivo*, which would allow for dual monitoring of host and pathogen iron handling dynamics in infection (448, 449). Measuring total iron content in *S. pneumoniae* isolated from mitochondrial iron replete and deplete host cells would provide the most robust assessment of bacterial iron piracy, however isolation of internalised *S. pneumoniae* from infected cells through fractionation approaches is hindered by the similarities in size between *S. pneumoniae* and mitochondria (~25 µm). Bacterial pulldown approaches from mammalian cell lysates using a biotin-conjugated antibody against the pneumococcal capsule would bypass this, but magnetic cell-sorting beads are laden with iron, which may influence bacterial iron levels prior to measurement. Most informative for this research would be a technology capable of tracking the movement of labelled iron from host to pathogen without a requirement for downstream processing, to prove increased levels of mitochondrial iron correlate with increased levels of host iron theft from the pathogen. Radiolabelled iron was detected in *M. tuberculosis* when provided bound to transferrin in *in vitro* culture (450), but the transfer of labelled iron from mammalian cells to pathogen has not been described.

To determine the extent of lung injury in our models, we predominantly relied upon measurement of inflammatory cell recruitment. Cell populations present in the airspaces during CS exposure and *S. pneumoniae* infections were identified and enumerated through the preparation of differentially stained BAL cytospin preparations. Further characterising BAL cellularity in these conditions through flow cytometry would provide increasing depth to our analyses and allow for the discrimination of resident AM and recruited monocyte populations. Complementing this with histological staining of inflated lung sections and measurements of physiological function, like lung elastance, would further solidify findings and provide a more robust measurement of injury.

During this work, we developed a pipeline of BALF acquisition from patients undergoing clinical bronchoscopy at Tallaght University Hospital. This facilitated the isolation of AMs from current and ex-smokers and comparison of their response to *S. pneumoniae* infection to non-smoker controls. This data is limited by our inability to control for patient disease status. Subjects in this clinic present with a wide range of pulmonary issues and diseases, including sarcoidosis, pulmonary fibrosis and interstitial lung disease:

unfortunately, we did not capture any COPD subjects during this study. Bronchoscopy is a powerful diagnostic tool for investigating changes in cytology or microbial dynamics but is an invasive procedure and in Ireland is not frequently used to monitor COPD, particularly in its later stages. Efforts are currently being made to collaborate with a wider range of physicians to enrol more individuals with COPD.

An important player in bacterial iron dynamics during infection which was not addressed during this thesis is the lung microbiome. Although *S. pneumoniae* cannot synthesise its own siderophores, it expresses cell surface receptors capable of binding siderophores secreted by other bacterial species, such as hydroxamate and tetradentate siderophores, and even host-derived catecholamines like norepinephrine (451-453). The ability of *S. pneumoniae* to directly utilise these siderophores as fuel sources is yet to be observed but has been described in other bacteria, with a siderophore-deficient *P. aeruginosa* mutant capable of using siderophores derived from other bacteria for biofilm formation *in vitro* (196). BALF iron is increased in smoking and COPD (163), which accompanies a shift in microbial diversity where genera such as *Streptococcus*, *Moraxella*, *Hemophilus*, *Pseudomonas* and *Prevotella* increase in abundance (454-456). Three species of these genera, *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*, are among the most common bacterial drivers of COPD exacerbations. Fascinatingly, none of these bacteria can produce their own siderophores and appear limited in the host iron sources they are able to use (for instance *S. pneumoniae* cannot utilise iron from transferrin or lactoferrin *in vitro*). This suggests that these bacteria may favour more atypical iron sources, illustrated by work in this thesis defining *S. pneumoniae* sensitivity to macrophage mitochondrial iron content. Further research into the alternative iron sources favoured by these pathogens, and how the lung microbiome and its dysbiosis in disease affects the availability of such, will provide interesting insight into the molecular mechanisms underpinning the success of these bacteria in COPD. Viral co-infection is another common risk factor of invasive pneumococcal disease and can promote aspiration of *S. pneumoniae* from the nasopharynx to the lungs (457). As prior viral infection has also been observed to liberate iron in the alveolar niche for bacterial consumption (458), investigating the inter-kingdom transfer of iron in bacterial infection and its consequences for COPD would prove interesting.

A greater appreciation in recent times has emerged regarding the convergence of iron and immunometabolism, the study of shifts in immune cell metabolism following differential activation to fuel effector function (75). Key to this is the existence of several proteins capable of moonlighting in iron sensing and immunometabolism processes under certain conditions, with the conversion of cellular aconitase (ACO1) to IRP1 upon FeS

loss, and the heme-chaperoning capabilities of GAPDH, the most studied (1, 459). Pertaining to the TCA cycle, citrate and ATP can chelate Fe^{2+} (129, 460), highlighting the ability of cellular metabolism to affect iron independently to enzymatic use. Despite these observations, little experimental evidence exists detailing how macrophages can mobilise and utilise mitochondrial iron to fuel metabolism in response to activation. Apart from the MFRN1/2^{ΔCd11c} mice generated during this thesis, no transgenic strains targeting mitochondrial iron import in an immune cell exist. The functional effects of iron loss or excess on macrophage metabolism have been primarily studied using Seahorse extracellular flux studies - although informative, this offers little mechanistic insight into how iron can mechanistically promote metabolic states. Metabolomics has only been performed once in an iron altered-macrophage, in DFP-treated human MDMs (129). Despite the central role of mitochondria in macrophage activation, little at all is known about how mitochondrial iron levels change to power a metabolic shift, and how that can change the incorporation of iron as a co-factor into metabolic players. Any technique capable of combining mass spectrometry with measurement of protein-bound, radiolabelled iron, like approaches using ¹³C incorporation in cellular metabolomics, would fuel huge mechanistic insight to this.

7.3 Translational capacity of findings

There is currently no known cure for COPD. Current strategies to combat disease development merely rely on public health messaging to deter cigarette smoking, with no reliable biomarkers capable of predicting disease development in susceptible individuals. The prevalence of cigarette smoking is declining globally, but disease risk attributed to smoking is estimated to be surpassed by that of indoor and outdoor pollution within the next two decades (132). Treatment approaches for COPD typically comprise of bronchodilators and corticosteroids mainly targeting symptoms of cough and sputum production, none of which can halt, or reverse, airway destruction. The Lancet Commission on COPD has called for novel therapeutic insights centred on the basic molecular mechanisms that underpin disease progression and development (461). Despite the widespread acknowledgement of immune dysfunction in COPD, few existing treatments focus on repairing the immune response: existing biologics merely target eosinophils and type II inflammation, which is only present in approximately 20% of patients. Monocytic infiltration is tissue destructive in COPD (143), and infiltrating monocytes associate with emphysematous lesions. Lung function decline is accelerated by acute exacerbation events often defined by patient inability to mount a sufficient immune response against invading pathogens (137). Current treatments may even actively hinder this - COPD patients using inhaled glucocorticoids show higher incidences of *S. pneumoniae*-driven

pneumonia, linked to an increase in AM efferocytosis resulting in diminished bacterial killing (462, 463). Despite pre-clinical studies in rats showing transplantation of healthy bone marrow to CS-exposed recipients can directly reverse emphysema (264), restoration of macrophage function in COPD remains an underexplored therapeutic avenue.

For the first time, we have shown that iron chelation can improve the ability of a human macrophage to control proliferation of a disease-relevant pathogen. DFP is one of three iron chelators approved for use by the EMA, including deferoxamine and deferasirox. Our mouse models highlight the protective role of mitochondrial iron depletion locally in the lung to infection, but the detrimental consequences of systemic chelation, illustrate the need to consider the administration route of a therapeutic chelator. In its use as a therapeutic for beta-thalassemia, DFP is approved for intravenous administration. However, we have described the detrimental effects of systemic DFP administration on *S. pneumoniae* infection outcomes in **Chapter 6**, and both high and low plasma iron levels associate with infection risk (464). As such, there is a need to further explore the possibility of DFP as an inhaled therapeutic to ensure its activity is directed to the iron-laden COPD lung, where we have proved its efficacy in humans in improving lung-resident AM bactericidal function. DFP has a short half-life and is rapidly excreted from the body, making it an ideal candidate for time and location-controlled administration. Frequent exacerbators constitute a unique endotype of COPD, who we have shown in **Chapter 3** can be identified based on a mitochondrial iron-related transcriptomic signature. Prophylactic use of an inhaled form of DFP would be of particular benefit in these patients, given the striking effect human AM *ex vivo* pretreatment with DFP had in limiting bacterial proliferation*. Still, DFP improved bronchitis and emphysema in experimental COPD in a sterile context, meaning inhaled DFP may also be universally beneficial across COPD etiologies whilst mitigating off-target side effects through systemic administration.

Our findings may also be applicable to other infection and disease settings beyond COPD. Iron is a fundamental requirement for growth of all pathogens, with the exception of *Borrelia burgdorferi* (465) and *Lactobacillus plantarum* (466). Intracellular iron chelation can also elicit off-target effects on other bacterial nutrient sources. For instance, treatment of primary human macrophages with DFP drives lipid synthesis and droplet formation (129, 467), along with increased itaconate production (129). Both lipid droplets (208, 468) and itaconate themselves (469) can be utilised by pathogens as fuel sources, meaning further preclinical research must explore the beneficial effects of DFP on host macrophages in response to a broader range of pathogens. Of further relevance, *S. pneumoniae* is the

* Investigation of nebulised DFP as an inhaled therapeutic in both respiratory infection and COPD is an active area of research in the Cloonan lab.

fourth-highest ranked pathogen in terms of mortality attributed to antimicrobial resistance globally, rendering common antibiotics ineffective in combating invasive pneumonia (470). Consequently, there is an unmet need for urgent therapeutics targeting new aspects of bacterial pathogenesis, with this thesis illustrating that bacterial iron uptake is one such target. Individuals with primary mitochondrial disease are at heightened risk of bacterial respiratory tract infection (471, 472). While the exact mechanisms behind this risk are unknown, the benefit of DFP treatment in COPD models with mitochondrial dysfunction warrants further investigation in these patients (50). Nutritional immunity has been recently characterised in a sterile context in several cancers, where cancer cells deploy lipocalin-2 to capture iron and promote tumour growth (473, 474). Iron chelation is host-beneficial in this situation: engineering of a mutant *E. coli* strain overexpressing the siderophore enterobactin sterically hindered cancer cell iron uptake through lipocalin-2, resulting in tumour shrinkage (475). Elevated MFRN1 mRNA levels associate with poorer prognoses in human pancreatic cancer (406) and glioblastoma (476), suggesting mitochondrial iron chelation could also be tumour-suppressive in these instances.

7.4 Concluding remarks

In this thesis, we employed *in vitro*, *ex vivo* and *in vivo* approaches to interrogate the contribution of AM mitochondrial iron in acute infection and respiratory disease, where we hypothesised that AM mitochondrial iron overload is host-detrimental and can be exploited by an invading pathogen for iron siphoning. These findings implicate mitochondrial iron as a candidate mediator of macrophage response to infection for the first time, highlighting an as-of-yet underappreciated role for mitochondrial iron dynamics in the host immune response and carrying implications for host-pathogen interactions beyond COPD.

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Chapter 9: Papers resulting from research

The below papers were published as part of this PhD work, and are attached in subsequent pages:

Original research:

Faherty L, Zhang WZ, Salih MM, Robinson EK, Perez E, Kim K, Carpenter S, Cloonan SM. Transcriptomic analysis reveals distinct effects of cigarette smoke on murine airspace and bone-marrow derived macrophages. *Respir Res.* 2024 Aug 24;25(1):322. doi: 10.1186/s12931-024-02939-3.

Review articles:

Healy C, Munoz-Wolf N, Strydom J, **Faherty L**, Williams NC, Kenny S, Donnelly SC, Cloonan SM. Nutritional immunity: the impact of metals on lung immune cells and the airway microbiome during chronic respiratory disease. *Respir Res.* 2021 Apr 29;22(1):133. doi: 10.1186/s12931-021-01722-y.

Faherty L, Kenny S, Cloonan SM. Iron and mitochondria in the susceptibility, pathogenesis and progression of COPD. *Clin Sci (Lond).* 2023 Feb 14;137(3):219-237. doi: 10.1042/CS20210504.

CORRESPONDENCE

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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/m³) 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 CO₂ 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 Bio 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://ggvolcano.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

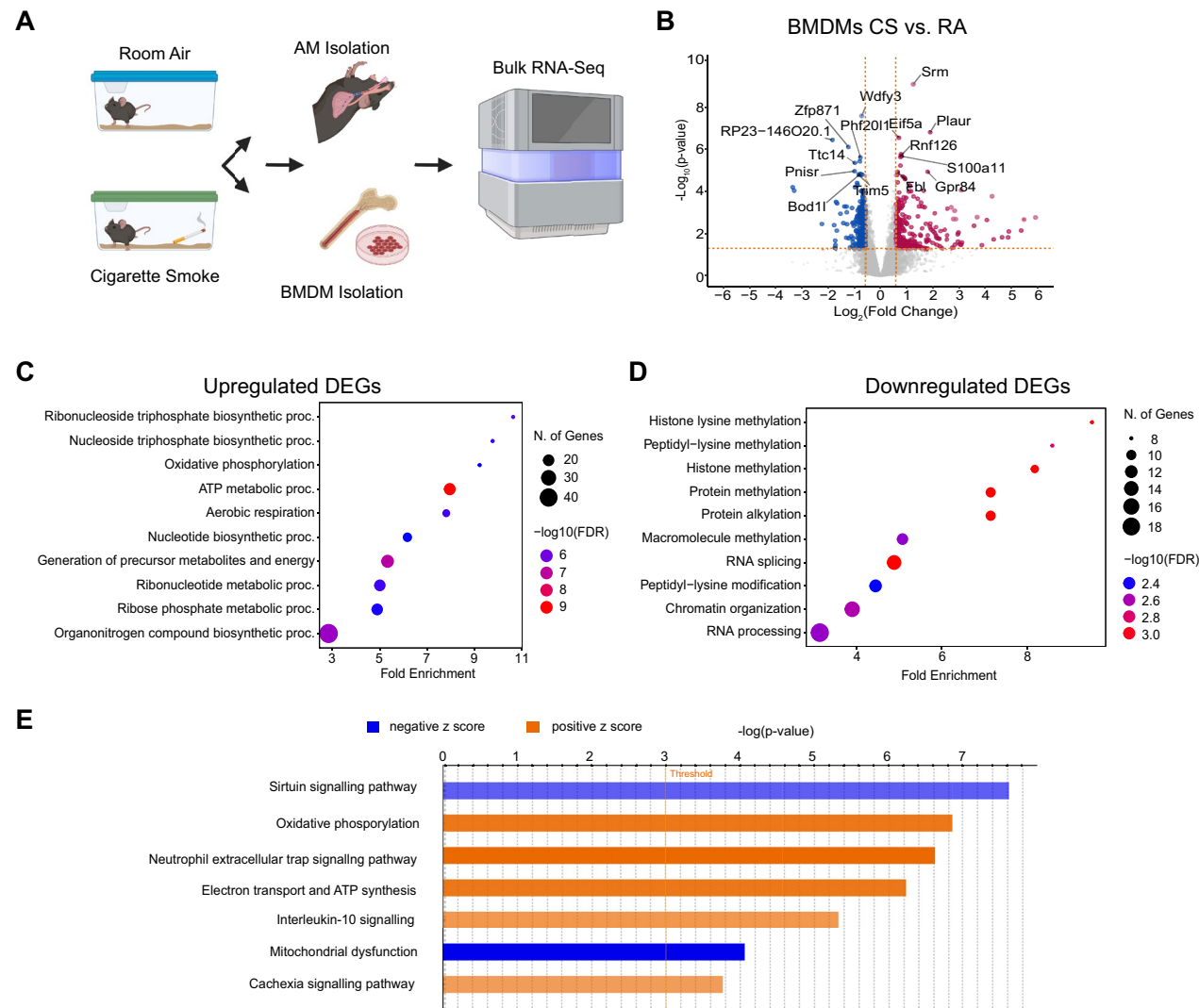


Fig. 1 CS exposure induces transcriptomic remodelling in BMDMs. **A** Schematic of experiment (created using Biorender). **B** Volcano plot of BMDM differentially expressed genes (DEGs) in CS-exposed mice (n = 3) compared to room air (RA) controls (n = 3). Biological processes associated with BMDM CS-induced 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-ebl1*, 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

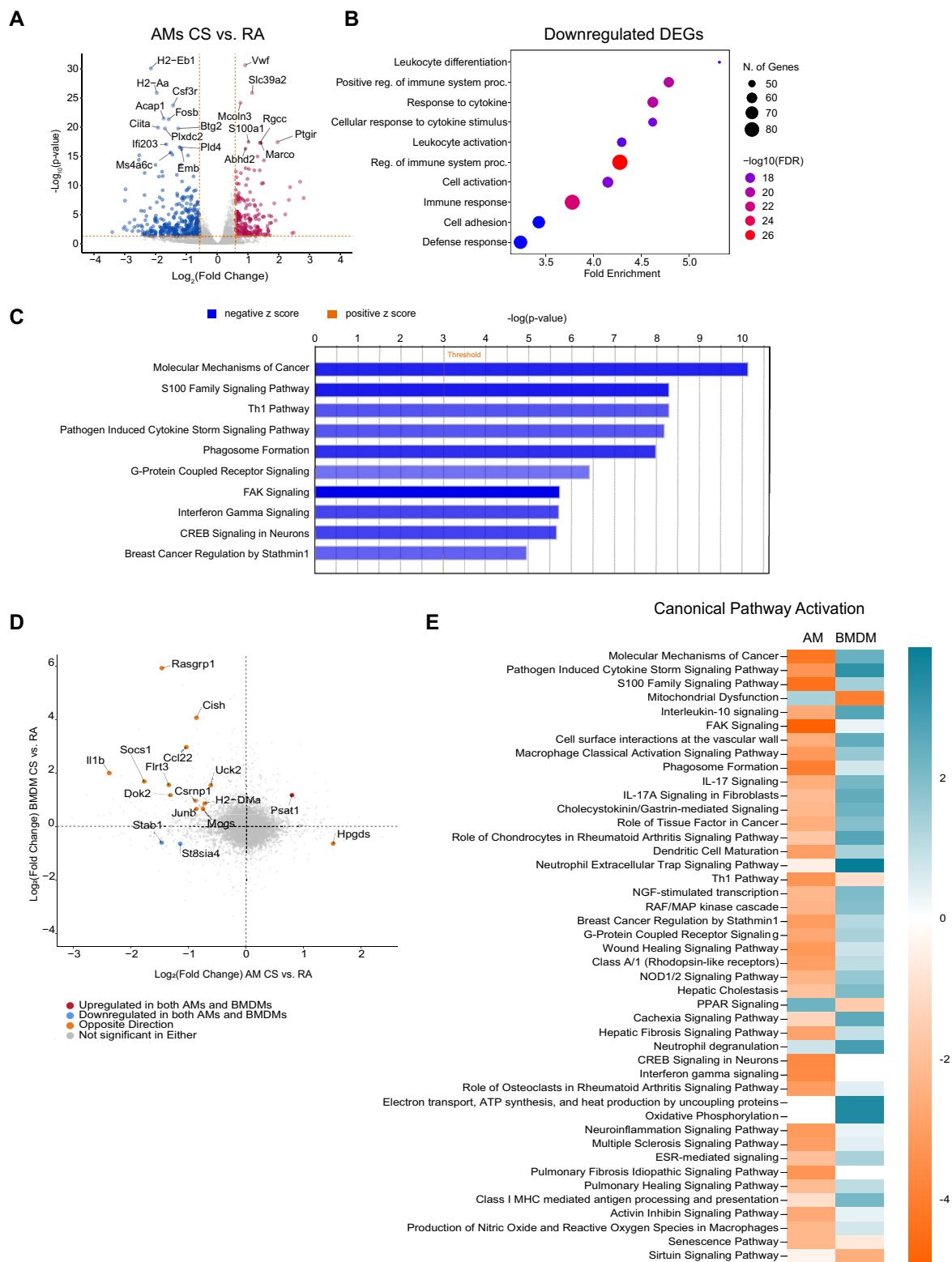


Fig. 2 CS exposure induces distinct transcriptomic remodelling in BMDMs compared to AMs. **A** Volcano plot of AM DEGs in CS-exposed mice (n=4) compared to RA (n=4). **B** Biological processes associated with AM CS-induced downregulated DEGs. **C** Canonical pathways produced from Ingenuity Pathway Analysis 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 adaptive immune 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 heightened 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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REVIEW

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Nutritional immunity: the impact of metals on lung immune cells and the airway microbiome during chronic respiratory disease

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Abstract

Nutritional immunity is the sequestration of bioavailable trace metals such as iron, zinc and copper by the host to limit pathogenicity by invading microorganisms. As one of the most conserved activities of the innate immune system, limiting the availability of free trace metals by cells of the immune system serves not only to conceal these vital nutrients from invading bacteria but also operates to tightly regulate host immune cell responses and function. In the setting of chronic lung disease, the regulation of trace metals by the host is often disrupted, leading to the altered availability of these nutrients to commensal and invading opportunistic pathogenic microbes. Similarly, alterations in the uptake, secretion, turnover and redox activity of these vitally important metals has significant repercussions for immune cell function including the response to and resolution of infection. This review will discuss the intricate role of nutritional immunity in host immune cells of the lung and how changes in this fundamental process as a result of chronic lung disease may alter the airway microbiome, disease progression and the response to infection.

Keywords: Nutritional immunity, Microbiome, Immunity, Metals, Iron, COPD, Asthma, IPF, Mycobacteria

Background

Respiratory disease remains a global leading cause of death despite the advancements made by respiratory research [1]. Furthermore, those with poor respiratory health have a greatly diminished quality of life and recurrent hospitalizations. To alleviate the immense burden of respiratory disease we need a greater understanding of the resident and recruited immune cells in the lungs, their role in respiratory disease, and their complex interplay with the lung microbiome and with invading pathogenic microorganisms. Metabolite and nutrient availability in the lung, whether produced by the host or the microbiota, are likely to have a crucial role in the progression of

lung disease as many immune cells require these specific nutrients to fuel their immune function when activated.

Nutritional immunity is classically defined as the sequestration of trace metals, most notably iron, by the host organism in an effort to block bacterial metal acquisition and thus limit disease progression during infection. Trace metals are essential to all forms of life. Most organisms require manganese, cobalt, iron, copper, nickel, selenium and zinc. Metal cofactors serve both catalytic and structural roles in a range of biological processes. With the exception of zinc, these metals are redox-active and this property contributes both to their catalytic activities and toxicity. Iron is the most abundant element on Earth and is also the most widely used metal in biological processes. This dependence on iron for most organisms makes it a vital currency in the context of host–pathogen interactions. The host takes advantage of this by

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sequestering iron during infection as a means of blocking iron acquisition by the invading microorganism, whereas bacteria have evolved mechanisms to steal iron from the host despite host iron sequestration [2]. Many respiratory pathogens such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Haemophilus influenzae* require iron and use several iron acquisition strategies [3–6]. Similarly, the commensals that comprise the microbiome require iron and other metals to support their metabolism. Thus, iron homeostasis and iron sequestration in the lung is crucial to controlling lung infections and to support a normal microbiome [7, 8]. Though nutritional immunity was first described and best characterized for iron, the sequestration, and intoxication of other metals including, zinc, manganese and copper has also been described [2, 9].

While the concept of nutritional immunity has traditionally encompassed the sequestration of metals from microbes, the innate and adaptive immune systems can also actively utilize metals to facilitate bactericidal function. For example, the host utilizes copper to exert a bactericidal effect through both redox-dependent and independent mechanisms limiting bacterial growth and facilitating bacterial death [10–12]. Similarly, the S100 family of calcium-binding host proteins, primarily calprotectin, are commonly found at the site of infection, where they can chelate free zinc, iron, nickel, copper and manganese and exert bactericidal activity in addition to nutrient deprivation having a pleiotropic role in the nutritional immune response [13]. Less ubiquitous metals also have a role to play: selenium, as part of selenoproteins, is important in defence against viral replication, and host manganese can reduce superoxide dismutase (SOD) activity in pathogens [14]. The proper storage, metabolism, and utilisation of these metals by immune cells is therefore critical both in facilitating immune cell function and depriving microbes of the necessary nutrients for survival and proliferation in the host. Intriguingly, pathogen reliance on the acquisition of host-derived metal supports further investigations into therapeutic avenues for metal chelators in chronic and acute lung infection.

Finally, metabolites created through metal-dependent metabolic pathways are also essential for the function of immune cells such as macrophages, neutrophils and T-cells [15]. As our understanding of the rapidly emerging field of immunometabolism expands, we are now beginning to appreciate that trace metal biology may also be essential for the correct functioning of key metabolic pathways (e.g., glycolysis, fatty acid oxidation, the tricarboxylic acid cycle, etc.) engaged by immune cells. We could therefore expand the meaning of nutritional immunity to encompass not just the battle over trace metals

but also the role of all metabolites and nutrients that are dependent on metal biology important for host pathogen interactions.

This review will discuss the key roles for metals in lung immune cell function, how metals are altered and dysregulated in chronic respiratory disease and the evidence if any, for the role of metals in dictating the repertoire of commensal bacteria present in the lung as well as the role of nutritional immunity in the response of the lung to infection.

Metals, innate immune cells of the lung and response to infection

Metal cofactors serve both catalytic and structural roles in a range of biological processes and play an important role in the development, maturation and function of immune cells. The role of metals in the biology of immune cells that are present in the lung is discussed below.

Monocytes and macrophages

Macrophages are a heterogeneous family of professional phagocytes and are the most abundant immune cell present in the lung under homeostatic conditions [16]. Their key role in the respiratory immune response is highlighted by macrophage dysfunction contributing to chronic lung diseases including chronic obstructive pulmonary disease (COPD) [17], asthma [18] and cystic fibrosis [19]. Their phagocytic abilities make them excellent surveillance cells both in the context of lung homeostasis and inflammation or infection. Tissue resident macrophages maintain immune homeostasis by carrying out essential housekeeping roles such as tissue repair while also acting as a first line of defence against microbial infections [20]. There are two major classes of lung resident macrophage populations. Alveolar macrophages (AMs) are the most abundant and are easily characterised for their low levels of the phagocytic receptor CD11b, their autofluorescent nature and high levels of the integrin CD11c and the lectin SiglecF [21]. AMs originate from foetal liver cells in the embryonic yolk sac where under the influence of granulocyte macrophage-colony stimulating factor (GM-CSF) they remain and sustain themselves within the alveoli [21, 22]. AMs reside within the lumen of the alveolus in close proximity to the alveolar epithelium and are directly exposed to air and the environment. AMs phagocytose antigens but also maintain homeostasis in the lung by catabolizing surfactant, removing particles as well as limiting inflammation [23, 24]. Originally thought to be sessile, AMs have recently been shown to be motile and to continuously crawl and cleanse all alveoli of particulate matter [25]. They also have the ability to communicate immunosuppressive

signals to alveolar epithelial cells (AEC) [26]. AMs act as first line defence for respiratory pathogens, including bacteria and viruses such as *Streptococcus pneumoniae*, *Mycobacterium tuberculosis* and *influenza* [27–29]. During infection AMs limit the inflammatory response by producing anti-inflammatory cytokines and by promoting tissue repair upon pathogen clearance [30, 31].

The second major resident lung macrophage population is the interstitial macrophage (IM) which is comprised of three phenotypically distinct subpopulations identified by their differential expression of CD11c, major histocompatibility complex (MHC)II and the mannose receptor CD206 [32, 33]. While their precise location in the lung remains controversial, IMs have been found in the lung parenchyma, both in the interstitium of the alveoli and the bronchovascular bundles with some specific subpopulations associated with nerves and blood vessels [32]. IMs originate and are maintained by circulating progenitor cells. They are smaller than AMs, immunoregulatory, and capable of antigen presentation [34].

In the setting of injury, inflammation, disease or with aging, resident AMs and IMs are often depleted and require the assistance of monocyte-derived macrophages that infiltrate the lung [35–38]. Once in the lung these infiltrating macrophages are defined by niche-derived tissue-instructive signals that trigger expression of specific differentiation programs, thus tailoring a particular lung specific functional identity [39].

Macrophages, iron and heme

Macrophages have been termed the “ferrostat” of tissue iron homeostasis [40]. Macrophages are vital for systemic iron homeostasis; supplying, sequestering or recycling iron as needed for erythropoiesis, bacteriostasis and erythrophagocytosis and constitute the main iron reservoir among immune cells and the third most important in the body after haemoglobin and liver ferritin stores [41]. Tissue-resident macrophages sequester and secrete iron on demand regulating local iron availability and modulating the tissue microenvironment, contributing to cellular and tissue function [40]. The exposure of AMs to a multitude of exogenous and endogenous sources in the lung, position AMs as key regulators of iron in the lung. As such, the storage, metabolism and detoxification of iron by AMs is paramount in their protection of alveoli against oxidative damage and maintenance of their innate immune functions (Table 1).

Following gut absorption of dietary and heme-conjugated iron, ferric iron (Fe^{3+}) is bound to the glycoprotein transferrin for systemic circulation. Uptake of iron by AMs is mediated through the iron transporters transferrin receptor 1 (TFR1) and divalent metal transporter 1 (DMT1); AMs also express the additional iron uptake

proteins including low-density lipoprotein receptor-related protein 1 (LRP1) and the zinc uptake receptor ZIP-14 (Fig. 1) [42–45]. Once inside the cell, iron dissociates from transferrin and is reduced to ferrous iron (Fe^{2+}) for storage in ferritin, a ‘nanocage’ like structure for safe storage of iron within the cytoplasm [46]. The transmembrane protein ferroportin (FPN) exports ferrous iron from AMs into the extracellular space, where it is oxidised and bound to transferrin in serum [42]. FPN is the only known exporter of elemental iron, allowing the release of iron into the circulation and to other cell types. In the lungs FPN is highly expressed in epithelial cells and AMs [47, 48]. Decreased levels of FPN are observed in response to infection, in a bid to reduce cellular iron efflux and extracellular iron levels [49, 50]. The systemic iron regulator hepcidin, expressed in response to pro-inflammatory cytokines and bone morphogenic protein 6 signalling, inhibits FPN to reduce circulating iron (Fig. 1) [51, 52]. While hepcidin does not seem to significantly contribute to AM lung iron trafficking [53], AMs produce endogenous hepcidin in response to challenge with the endotoxin lipopolysaccharide (LPS), potentially to sequester iron intracellularly through FPN degradation [53]. Indeed, this AM-produced hepcidin has proved essential for AM bactericidal function [54].

Heme (iron-protoporphyrin IX) is an essential metal-locofactor and signalling molecule across all cell types. While in highest demand in erythroid cells, heme synthesis is an evolutionarily conserved process occurring in virtually all eukaryotic cell types. De novo heme synthesis is a multienzyme process which starts and culminates in the mitochondria, with the first step of glycine and succinyl CoA condensation by 5-aminoevulinate synthase (ALAS) constituting the rate-limiting step in non-erythroid cells like AMs [55]. The hydrophobicity and cytotoxicity of heme necessitates carefully regulated handling in the cell, with such mechanisms of paramount importance in macrophages owing to their roles in erythrophagocytosis and heme degradation [56]. Unlike reticuloendothelial macrophages, which recycle iron from senescent erythrocytes via erythrophagocytosis [57] AMs in the alveoli do not seem to necessitate erythrophagocytosis at homeostasis. Instead, AMs uptake haemoglobin-haptoglobin and heme-hemopexin complexes via CD163 and CD63 respectively. However, AMs of patients exhibiting lung haemorrhage show iron overload, suggesting during increased heme burden AMs possess some erythrophagocytic capability [58]. AMs play important roles regulating the iron pool by sensing free heme (the iron-containing porphyrin, key to O_2 transport and storage among other biological processes) at concentrations reflective of pulmonary haemorrhage. AMs utilize heme to produce reactive oxygen species (ROS) and nitric oxide (NO)

Table 1 Metal homeostasis within immune cells

	Iron	Zinc	Copper
Macrophages	DMT1 [42] Transferrin Receptor 1 (TFR1) [45] Slc39a14 (ZIP14) [77] Lactoferrin receptor (Lfr) [43] CD163 [45] CD64 [220] Ferroportin (FPN) [50] Lipocalin-2 (LCN-2) [63] Nramp-1 [45] Hepcidin [53]	SLC39A (ZIP) Slc39a2 [77] Slc39a4 [77] Slc39a14 [77] SLC30A (ZnTs) Slc30a1 [77] Slc30a3 [77] Slc30a5 [77]	CTR1 [84] ATP7A [84]
Neutrophils	Nramp-1 [63] Lipocalin-2 [63] Myeloperoxidase [113] Ceruloplasmin [119] Lactoferrin [110]	Calprotectin (S100A8/S100A9 heterodimer) [13] Calgranulin C (S100A12) [124]	Calgranulin C (S100A12) [124] ATP7A [118] ATP7B [118] Ceruloplasmin [119]
NK cells	TfR1 (CD71) [183] Lactoferrin receptor (Lfr) [185]	KIR receptor [188] Surface receptors with tyrosine phosphorylation sites [190]	Unknown
Dendritic cells	Unknown	ZIPs and ZNTs [102]	Unknown
Basophils	Unknown	Methallothioneins (MTs) [158]	Unknown
Eosinophils	Eosinophil peroxidase (EPX) [152]	Unknown	Unknown
Mast cells	Unknown	Unknown	Ctr2 [161]
$\gamma\delta$ T cells	Lactoferrin receptor (Lfr) [195] Hereditary hemochromatosis susceptibility gene (HFE) [197, 198] B2-microglobulin (β m-2) [198]	Unknown	Unknown
iNKT cells	Ferroportin (FPN) [201] Hepcidin [201]	Unknown	Unknown
MAIT cells	Unknown	Unknown	Unknown
T cells	TfR1 (CD71) [209] Lactoferrin receptor (LFR) [195]	Unknown	Unknown
B cells	TfR1 (CD71) [209]	Unknown	Unknown

enhancing their bactericidal and phagocytic capabilities [59]. Importantly, erythrophagocytosis also upregulates the expression of heme oxygenase 1 (HO-1) in macrophages leading to heme degradation. Heme catabolism by HO-1 exerts anti-inflammatory effects through the products of heme degradation: bilirubin (arising from the reduction of biliverdin), carbon dioxide and ferritin all of which possess anti-inflammatory activity [60–62]. This illustrates the importance of iron metabolism, particularly of the HO-1/heme axis in maintaining AM function and lung homeostasis.

Iron-sequestration is of particular importance to limit pathogen outgrowth in the lungs. AMs act as the first line of defence against respiratory pathogens and contribute to limiting iron availability to pathogens. An example is the ability of AMs to secrete the siderophore lipocalin-2 (LCN-2) during infection that binds to enterobactin-type and mycobacterial siderophores to sequester free iron (Fig. 2) [63]. Macrophage polarisation also differentially regulates iron-response in AMs. Iron accumulation in M1-polarized AMs promotes a bacteriostatic response

to the anaemia of chronic infection and simulates expression of pro-inflammatory cytokines, potentially through hepcidin-mediated FPN downregulation [64]. In contrast, M2 cells favour iron release linked to upregulation of FPN a phenotype that may be driven by upregulation of HO-1 [65]. Reciprocally, iron can also modulate monocyte polarization. In vivo murine models of iron overload drive macrophage polarization to favour the M2 phenotype mitigating pro-inflammatory responses [66]. However, in vitro studies of macrophages isolated from chronic venous leg ulcers showed excessive erythrophagocytosis and an ensuing high intracellular iron concentration to promote M1 polarization [67]. Likely, iron concentration is crucial in modulating polarisation – the tissue iron deposition exhibited in venous leg ulcers is extremely high, whilst the dietary iron supplementation approach used in the murine studies provides a moderate dose [66]. While the effects of iron overload in the lung on macrophage polarization remain unclear, it will be interesting to see if such a concentration-dependent mechanism is also observed.

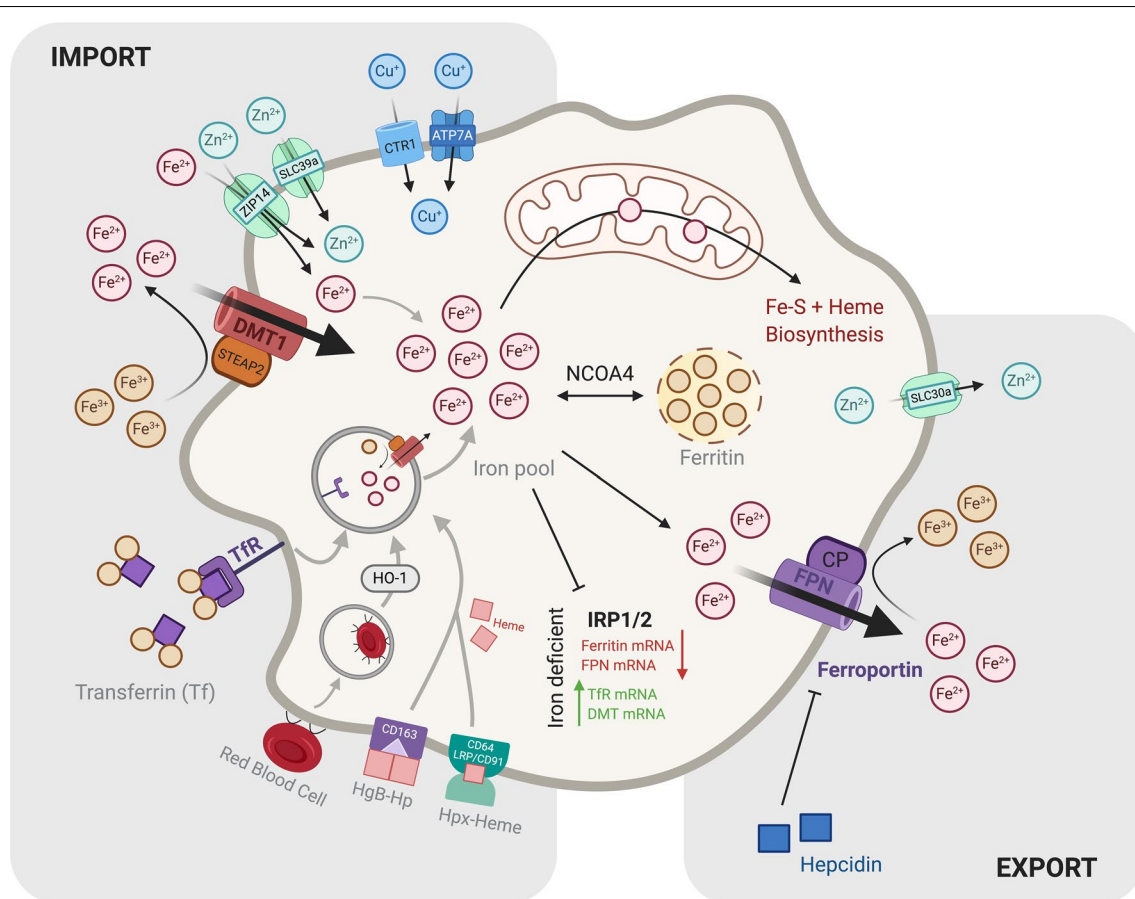
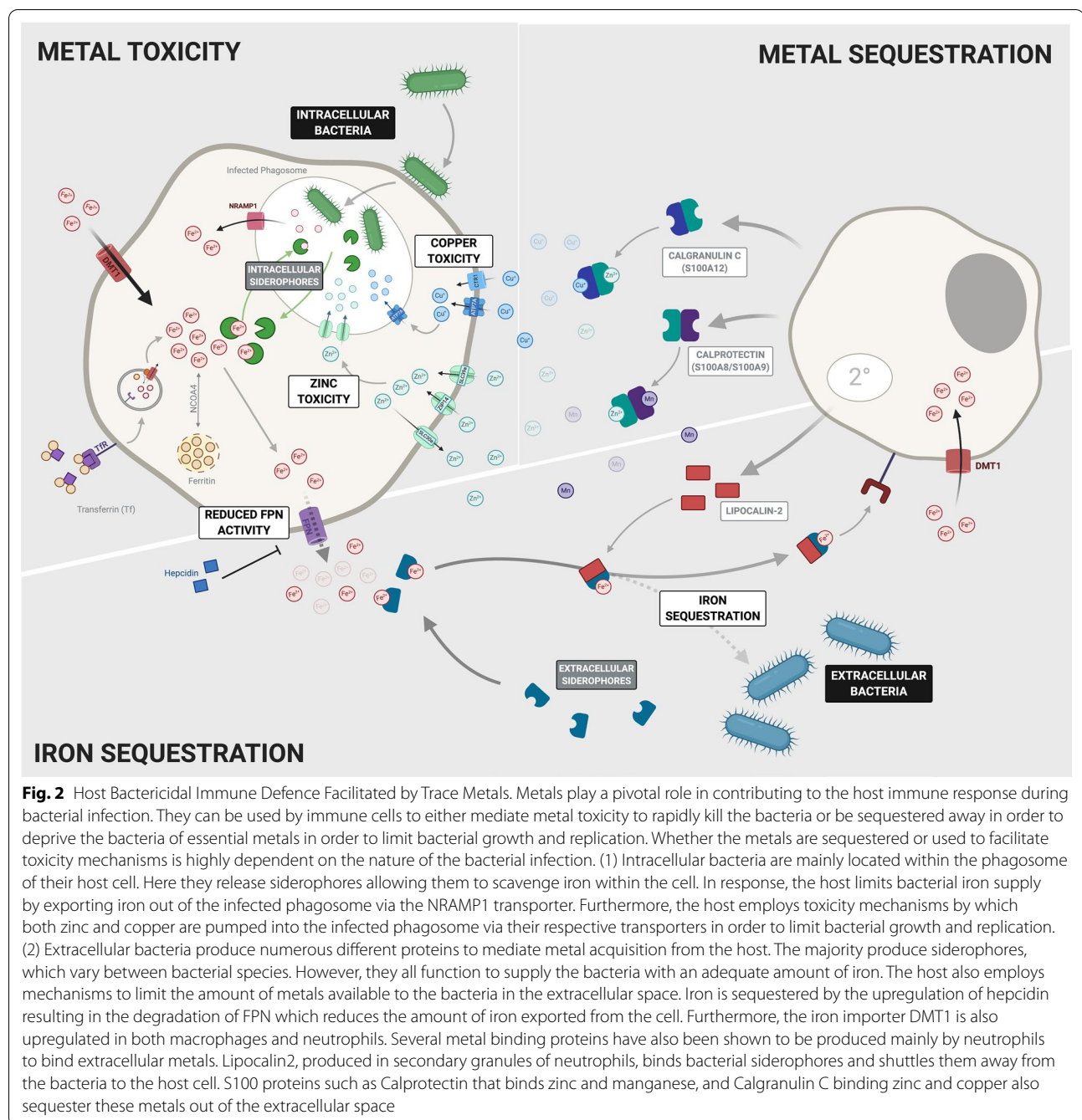


Fig. 1 Macrophage Metal Metabolism. This brief summary of the metal metabolism of a generic macrophage highlights several mechanisms by which a macrophage can obtain metals. There are numerous metal transport proteins and receptors involved to ensure an adequate supply of these essential metals to the macrophage. (1) Macrophages obtain iron from various sources. Free circulating iron is first reduced from ferric to ferrous iron and then imported into the macrophage via protein transporters such as DMT1 and ZIP14. Transferrin-bound iron is bound to the transferrin receptor on the cell surface and imported via receptor-mediated endocytosis. Some macrophages also have the ability to obtain iron via erythrophagocytosis while others express receptors such as CD163 and CD64 allowing for the uptake of haemoglobin-haptoglobin and heme-hemopexin complexes, respectively. Once iron enters the macrophage it has numerous fates depending on the activation state of the cell. It can be stored in its ferrous state within the ferritin complex or utilized in the mitochondria for Fe-S cluster and heme biosynthesis. Iron is exported out of the macrophage through the only known iron export protein, ferroportin (FPN) whose expression is regulated by the hormone hepcidin. Further post-transcriptional regulation of intracellular iron levels is carried out by cytosolic regulatory proteins IRP1 and IRP2. (2) Intracellular zinc supply is mediated by two families of transport proteins; SLC39A (ZIPs) import zinc into the cell while SLC30A mediates zinc export out of the cell. (3) The copper importers Ctr1 and ATPase metal pump, ATP7A are two known copper transporters within the macrophage and gets upregulated to facilitate copper mediated host defence mechanisms

Macrophages carry out a vast array of effector functions and must be able to adopt several activation states by changing their metabolism to fuel these functions [68]. The influence of macrophage immunometabolism in determining an inflammatory or anti-inflammatory phenotype have been extensively reviewed [69]. However little attention has been given to the role that metals play in the regulation of immunometabolic networks. Iron-sulfur (Fe-S) clusters play an essential role in macrophage metabolism by acting as cofactors to essential metabolic proteins and mediating the electron transport

chain. For example, the mitochondrial enzyme lipoic acid synthase (LIAS) requires Fe-S clusters to aid in the synthesis of lipoamide-requiring enzymes such as pyruvate dehydrogenase (PDH) and α -ketoglutarate dehydrogenase (α -KGDH). Furthermore, the proteins involved in electron transport, such as ferredoxins also require Fe-S clusters as a part of their redox-active centres to ultimately produce ATP [70]. In response to inflammatory stimuli Fe-S clusters are also regulated. LPS/interferon- γ (IFN- γ) stimulation of murine bone marrow derived macrophages (BMDMs) and RAW264.7 macrophages



downregulates two key Fe-S cluster biosynthesis proteins (cysteine desulfurase Nfs1 and its partner IscU) possibly to avoid damage to Fe-S clusters by NO [71]. In vivo stimulation with *Mycobacterium bovis* Bacille Calmette Guerin (BCG) or in vitro stimulation with IFN- γ inhibits aconitase activity in macrophages by removing an iron atom for the Fe-S prosthetic group of aconitase which is essential for its catalytic activity in the TCA cycle. This inhibition of aconitase coincides with the downregulation

of mitochondrial respiration upon its activation and also a compensatory increase of glycolysis [72]. Similarly, acute iron deprivation of primary human macrophages enhances glycolysis by upregulating hypoxia-inducible factor 1- α (HIF-1 α) regulated genes while downregulating oxidative phosphorylation (OX PHOS) via the iron-responsive transcription factor ATF-4. Furthermore, when these iron-deprived cells were stimulated with LPS their proinflammatory functions were impaired [73].

Macrophages, zinc and copper

Like iron, zinc is an essential cofactor for life. Vertebrates have evolved complex transport and buffering systems to maintain zinc homeostasis. The balance of zinc homeostasis is crucial during infection to block access to zinc from invading microorganisms, but also to ensure the function of immune cells. Zinc deficiency in humans is associated with impaired response to several infectious diseases [74, 75]. Zinc deficiency leads to reduced phagocytic ability of macrophages, while zinc supplementation can improve phagocytosis but the molecular mechanisms behind how zinc affects this process is not yet known [74–76]. Cellular zinc homeostasis is regulated by two families of zinc transporters, the SLC39A importers (ZIPs) and the SLC30A exporters (ZnTs). Zinc plays an important role in macrophage efferocytosis involving the coordinated action of the ZIP1 and ZIP2 importers [77]. The roles of other zinc transporters in different infection contexts is not yet known but the exporter SLC39A10 is vital for mediating zinc homeostasis in macrophages in response to LPS stimulation resulting in increased macrophage survival (Fig. 2) [78]. *Slc30a1* (*ZnT1*) is induced in human macrophages during *M. tuberculosis* infection and zinc toxicity is a strategy used by *M. tuberculosis* infected macrophages [79]. Zinc accumulation was also observed in phagosomes containing *E. coli* contributing to its killing, suggesting that this may be a general antimicrobial strategy employed by macrophages [80].

Copper is used as an essential cofactor in an array of biological processes in many forms of life, including microorganisms. Copper deficiency increases susceptibility to many types of infections indicating an important role for this metal in host defence [10, 81, 82]. Furthermore, the prevalence of copper resistance mechanisms in pathogenic microbes highlights the importance of copper toxicity as a defence strategy in mammals. Macrophages exploit both the essentiality and toxicity of copper to defend against microbes. As a host defence strategy, copper levels rise within phagosomes of peritoneal macrophages upon infection with mycobacteria [83]. The copper importer Ctr1 and the ATPase copper pump ATP7A are induced by IFN- γ in macrophages. The ATPase pump is trafficked to phagosome compartments and is required for the bactericidal activity of macrophages (Fig. 2) [84]. Copper toxicity is a host defence strategy against other respiratory pathogens such as *S. pneumoniae* as pulmonary macrophages were shown to be more efficient at clearing pneumococcal bacteria lacking the CopA copper efflux pump [85]. Conversely, IFN- γ activated macrophages reduce copper levels in phagosomes to control the fungal pathogen *Histoplasma capsulatum* [86]. Fungi have high tolerance to copper and so copper toxicity as a defence strategy is not suitable

for these micro-organisms, thus macrophages restrict this essential nutrient from fungi within the phagosome.

Macrophages and other trace metals

The role of other trace metals in macrophage biology is poorly studied and has mainly focused deciphering additional metal binding capabilities of proteins implicated in iron biology. In addition to iron, the membrane transporter NRAMP1 also has affinity for manganese, transporting it across the phagosomal membrane [87]. Transferrin has been shown to bind manganese in vitro and is the main manganese-positive protein in circulation in vitro, although whether macrophages can take up manganese-laden transferrin through TFR1 remains to be defined [88].

While numerous selenoproteins key in cellular processes have been characterised, their precise roles in processes governing the macrophage immune response are poorly defined. Selenoprotein methionine sulfoxide reductase B1 (MsrB1) expression is induced in macrophages upon LPS stimulation, where it regulates actin assembly and production of anti-inflammatory cytokines [89]. This anti-inflammatory role is concordant with observations that dietary selenium supplementation drives macrophages towards the alternatively activated M2 phenotype in an IL-4 dependent manner [90]. Macrophages deficient in the selenocysteine tRNA showed diminished migration in vitro, but no change in inflammatory response [91]. Targeting of specific selenoproteins in future studies will allow a greater understanding of the intricacies of selenium regulation in macrophages.

Dendritic cells

Dendritic cells (DCs) play a crucial role in the initiation and regulation of the immune response by functioning as powerful antigen presenting cells. DCs resident in the lungs can be divided into three subsets that are of independent origin and have distinct functions depending on their location [92]. Regardless of their subtype, DCs develop in the bone marrow from hematopoietic stem cells into early progenitor cells where they then branch off into their specific subsets [93]. Conventional DCs (cDCs) in the lung express either CD103 or CD11b. CD103⁺ DCs (cDC1) are located in the respiratory epithelium where they sample contents of the conducting airways by projecting their long dendrites between epithelial cells into the airway lumen. In contrast, CD11b⁺ DCs (cDC2) are lodged under the basement membrane of the lamina propria [94]. Once the cDCs sample an antigen, they migrate the draining lymph nodes where they present the processed antigen to naïve T cells which drives specific T cell polarization and proliferation [95].

Monocyte-derived DCs (moDC) are recruited to the lung during infection or inflammation to help drive the immune response. The existence of these DCs remains controversial as they express markers such as CD11c, CD64 and the MAR-1 antibody which can also represent a macrophage cell population [94, 96]. Plasmacytoid DCs (pDC) secrete large amounts of type I interferon (IFN) during viral infections and have been located in the lymphoid follicles of the small airways [97, 98]. Furthermore, pDCs also play an immunoregulatory role by sensitising T helper (Th) type-2 cells to harmless antigens in the lung while suppressing the generation of effector T cells. However, this tolerance is suggested to be suppressed when the pDCs interact with a virus and produce IFNs [99].

Dendritic cells and metals

Not much is known about the role that metals may play in the function of dendritic cells. Iron levels affect the development of DCs from bone marrow progenitors whereby high iron conditions used to culture bone marrow cells leads to a defective development of moDCs that are unable to respond efficiently to LPS [100]. On the other hand, iron deficiency in vitro also leads to the improperly differentiated moDCs that are unable to stimulate T cells [101]. Interestingly, reduced intracellular free iron levels promote the activation of DCs. Stimulation of DCs with LPS leads to an NF κ B dependent increase of ferritin.

Zinc has also been implicated in DCs response to stimulation. A study identified a link between toll like receptor (TLR) signalling and zinc homeostasis in these immune cells. Stimulation of DCs with the TLR4 ligand LPS lead to altered expression of zinc transporters (both importers and exporters), and a decrease in intracellular free zinc [102]. Treating DCs with a zinc chelator resulted in the same effects as LPS stimulation, in terms of upregulation of MHC class II and costimulatory molecules. Further investigations are needed to understand the link between DC zinc homeostasis and their response to TLR stimulation.

Neutrophils

Neutrophils are short-lived, highly mobile phagocytes that constitute hallmarks of acute infection. As granulocytes neutrophils have enzyme-filled granules that they use to fight off infections in addition to their phagocytic capabilities and synthesis of chromatin-derived neutrophil extracellular traps (NETs) [103]. Despite their prominent phagocytic role in the response to acute infection, neutrophil function is remarkably plastic, with secreted effectors driving pro- and anti-inflammatory neutrophil function [104–106]. Neutrophils also possess the capacity to modulate the functionality of other immune cells through secreted effector proteins, with the cleavage

of TLRs and cytokines by neutrophil elastase associated with altered macrophage function [107]. Given its frequent exposure to environmental pathogens, it is no surprise that the lung is a major neutrophil reservoir. Studies in a rabbit model have shown the upregulation of P-selectin by AECs in response to internal and external stimuli binds P-selectin glycoprotein ligand-1 on circulating neutrophils, facilitating further binding and subsequently tissue extravasation [108]. The majority of marginated neutrophils in the lung reside in the capillary bed, expressing the chemokine receptor (CXCR) 4 to promote their retention [109]. Such margination in the microvasculature positions a large pool to kill inhaled bacterial, fungal and viral pathogens through phagocytosis, NETosis and release of antimicrobial cytokines [107].

Neutrophils and iron

Iron is central to numerous neutrophil inflammatory responses. Neutrophils secrete LCN2 from their secondary granules to bind bacterial siderophores and limit their iron acquisition, and express NRAMP1 to sequester free iron from serum during infection [63]. Studies in the iron transport protein lactotransferrin (lactoferrin)-deficient mice have illustrated a role of neutrophil-secreted lactoferrin in modulating the oxidative burst response [110]. Iron-unsaturated lactoferrin was shown to inhibit human neutrophil apoptosis in vitro, suggesting a role in chronic neutrophilic inflammation [111]. Binding of recombinant human lactoferrin to human neutrophils in vitro induces neutrophil activation although the precise mechanisms of lactoferrin binding to the neutrophil surface remain uncharacterised [112]. Neutrophils also express the heme-containing myeloperoxidase, which catalyses the generation of hypochlorite anions to drive degranulation and NETosis [113].

Iron availability may also influence metabolic reprogramming in neutrophils. HIF-1 α is regulated by a family of prolyl hydroxylases (PHDs) and under normoxic conditions the PHDs rapidly hydroxylate HIF-1 α and so target it for degradation [114]. *Phd2*^{-/-} neutrophils have higher ATP levels, increased abundance of pentose phosphate pathway intermediates, more glycogen stores and a greater glycolytic capacity [115]. This suggests that neutrophils, like macrophages, require HIF-1 α to engage in glycolysis. Low oxygen tension is not the only way to block HIF-1 α hydroxylation, as PHDs also require α -ketoglutarate, Fe²⁺ and ascorbate. Increased expression of HIF-2 α , regulated in a similar manner to HIF-1 α , has been linked to decreased death of neutrophils by apoptosis, and iron chelators have been shown to inhibit apoptosis in neutrophils [116]. Heme metabolism has also been implicated in neutrophil function during respiratory infections as HO-1 expression in the lung is

also associated with reduced neutrophil recruitment in murine models of LPS-induced pulmonary inflammation [117].

Neutrophils and other metals

Copper is required for proper NETosis, with mice carrying mutations in the copper transporters ATP7A and ATP7B showing a reduced capacity for NET release during systemic inflammation [118]. Ceruloplasmin, a Cu^{2+} dependent ferroxidase elevated in serum during acute infection, is secreted by human neutrophils to upregulate ROS in a potentially bactericidal mechanism [119]. Calprotectin is released from the cell during degranulation as part of NETs, where it is essential for antifungal activity [13].

Neutrophil expression of protein effectors binding other trace metals is crucial in their bactericidal function. Zinc deficiency induces expression of the zinc-binding heterodimer calprotectin and detrimentally impacts neutrophil phagocytic capacity through impairing ROS function, along with oxidative burst, degranulation and cytokine production [120]. Indeed, zinc signalling has been shown to be vital in NET formation by neutrophils and granulocytes: both zinc excess and deficiency inhibit superoxide production in neutrophils, a prerequisite for NET formation [121]. Neutrophils express calprotectin which can bind zinc and manganese, attenuating the effects of zinc-associated enzymes such as matrix metalloproteases and manganese-dependent bacterial superoxide defences [122, 123]. Neutrophils also express calgranulin C (S100A12), which can bind copper and zinc to exert an antimicrobial effect and trigger monocyte recruitment [124]. In addition to iron and zinc, calprotectin can also chelate manganese in a manner that both sequesters the nutrient from pathogens and facilitates antimicrobial activity, with recombinant calprotectin defective in manganese binding unable to exert antifungal activity against *Aspergillus fumigatus* [125]. Indeed, calprotectin-mediated manganese and zinc sequestration is a host immune strategy against the *S. aureus* superoxide defence, enhancing the susceptibility of the bacterium to neutrophil killing [122]. Little is known about the role of selenium in neutrophil function, although increased dietary intake may protect neutrophils from oxidative damage [126].

Other granulocytes

Mast cells

Mast cells are granulocytic leukocytes that mediate inflammation through degranulation in response to infection, allergens and tissue injury [127]. Mast cells are unique among leukocytes in that they are released from the bone marrow as CD34^+ mast cell progenitors

and do not terminally differentiate until their recruitment to tissue [128]. These immature progenitors are minimally granulated and reach maturation upon tissue homing, where stem cell factor and other tissue-specific factors drive MCs towards a pro-inflammatory, anti-inflammatory or an immunosuppressive phenotype [129]. Mast cell activation is regulated by several cell surface receptors, including TLRs, cytokine and chemokine receptors, hormone receptors and $\text{Fc}\epsilon\text{RI}$ and $\text{Fc}\gamma$ receptors. The ensuing combination of stimuli facilitates a pleiotropic role for mast cells in the immune response and leads to the secretion of a wide range of effector proteins: preformed mediators rapidly released upon activation ($\text{TNF-}\alpha$, histamine and proteoglycans), and mediators synthesised following activation (lipid mediators, growth factors, cytokines and chemokines) [130–132]. Mast cell frequency in the lung at homeostasis is low and interspecies variation in mast cell populations have made the homing process difficult to study, although the identification of a multitude of mast cell chemoattractants means it likely occurs through a regulated recruitment process [133, 134].

Basophils

Basophils similar to mast cells contain granules within their cytoplasm [135], express the high affinity receptor for IgE ($\text{Fc}\epsilon\text{RI}$) and release proinflammatory mediators such as histamine when this receptor becomes cross-linked. Both are derived from CD34^+ haematopoietic stem cells located in the bone marrow [136]. Despite their similarities, basophils and mast cells are morphologically and biochemically distinct cell types [137]. The type 2 immune response is initiated by the immune system when in contact with parasites which increases IgE production and the number of eosinophils, basophils and CD4^+ T cells [138]. Basophils drive this type 2 response by producing the cytokine IL-4 [139] but also plays a role in allergic reaction and airway inflammation. Basophils have quite a short lifespan and so they must be constantly replenished by the bone marrow. For many years it was accepted that basophils are present in circulation and only migrate to tissue in response to certain inflammatory signals [140]. However, murine studies have proposed the idea of tissue-resident basophils after it was discovered that basophils seem to be present at all stages of lung development [141]. Furthermore, these lung-resident basophils differ phenotypically from peripheral blood basophils and are driven by IL-33 and GM-CSF produced in the lung microenvironment. Localised in the alveoli, intriguingly basophils also seem to be responsible for guiding the development and function of AMs [141].

Eosinophils

Eosinophils are bone marrow-derived, granule containing, pro-inflammatory leukocytes. Terminally differentiated in circulation, eosinophils act as effectors in host defence from microbial and metazoan infection and allergic reaction, primarily through the activity of cationic proteins resident in the eosinophilic granule. Eosinophils comprise a mere 1–3% of circulating leukocytes and readily migrate to the lung during inflammation. Following their IL-5-induced proliferation and mobilization in the bone marrow, eosinophil recruitment into the lung is driven by local expression of the chemotactic factor eotaxin [142]. Lung-resident eosinophils reside in the parenchyma and display a marker expression pattern (Siglec-F^{mid}CD62L⁺CD101^{low}) distinct from those recruited to the airways (Siglec-F^{hi}CD62L⁺CD101^{hi}) although functional differences between the two subsets remain uncharacterised [143]. Murine models of chronic allergic asthma have shown secretion of CCL17 and CCL22 by dendritic cells recruits circulating eosinophils to the lung in response to allergic inflammation, where eosinophils recruit further DCs and Th2 cells [144, 145]. During viral respiratory infection, expression of TLR7 allows eosinophils to detect pathogen associated molecular patterns (PAMPs) and exert direct antiviral effects through degranulation and cytokine and superoxide production [146].

Metals and granulocyte function

There are many reports correlating altered iron metabolism with allergy and atopic airway hyperreactivity. There is a higher prevalence of allergic diseases in those with diseases causing high systemic iron levels [147]. As basophils, eosinophils and mast cells play a role in allergy, the altered iron homeostasis likely impacts the function of these cells, contributing to pathology. Reducing iron levels, by administration of an iron chelator reduces IgE serum levels in a rat model of Th2 mediated autoimmunity [148]. Iron supplementation results in a significant decrease of airway eosinophils in an ovalbumin-driven allergic asthma mouse model [149]. Neutrophils utilize the metalloprotein myeloperoxidase (MPO), which is a hemeprotein that plays a role host defence via oxidation [150]. Similarly, eosinophils have eosinophil peroxidase (EPX), which is a two-chain hemeprotein and has been shown to have homology to MPO in neutrophils [151]. Efficient iron stores and uptake is said to be required for the generation of EPX suggesting iron levels are important for eosinophil function and activity [152].

HIF-1 α , whose activation is iron dependent was shown to accumulate after basophils were stimulated with anti-IgE [153]. Accumulation of HIF-1 α is suggested to be linked to the reaction of basophils to the hypoxic

environment and stress-induced conditions. Whether intracellular iron levels in basophils affects HIF1 α and cell activation as it does in macrophages and dendritic cells remains to be determined. This would be an interesting avenue to explore as altered iron levels are linked with allergic inflammation.

Reduced zinc levels are associated with several allergic diseases including bronchial asthma and chronic rhinosinusitis [154–156]. Zinc is required in mast cells for degranulation and the production of cytokines and mast cell granules are reported to contain high levels of zinc [157]. Fc ϵ RI is expressed on the surface of mast cells and basophils, and its activation leads to the release of cytokines (including IL-4) and chemical mediators. The requirement of zinc in basophil function has not yet been fully elucidated however the regulation of intracellular zinc levels by metallothioneins (MTs) has an important role in Fc ϵ RI-driven calcineurin/nuclear factor of T cell signalling and IL-4 signalling in basophils [158]. Zinc suppresses eosinophilic inflammation, increased numbers of eosinophils in bronchoalveolar lavage fluid (BALF) are observed in zinc deficiency while zinc supplementation reduces the numbers of eosinophils in BALF, similar to what is observed with iron [159, 160]. However, the molecular mechanism driving this zinc-associated eosinophil recruitment to the lungs is yet to be elucidated. While copper has been implicated in mast cell maturation, little remains known about the role of copper and other metals in granulocyte function [161].

Innate lymphocytes

In addition to the innate and adaptive defences, the lung harbours innate-like lymphocytes types that constitute the second and third tiers of defence. Lung tissue-resident innate-lymphocytes include innate lymphocyte cells (ILCs) which include NK cells, invariant natural killer cells (iNKT), MR1-restricted T (MAIT) cells and $\gamma\delta$ T cells.

Natural killer (NK) cells are short-lived innate effectors that belong to type 1 innate lymphoid cells, and account for 5–25% of total CD45⁺Lin[−] cells in the lungs [162, 163]. NK cell activation is mediated through expression of inhibitory and activating receptors. Inhibitory receptors (killer cell-immunoglobulin like receptor (KIR), CD94:NKG2A, ILT-2) recognise host major histocompatibility complex class I molecules to limit cytotoxicity towards the host and facilitate self-tolerance in the ‘missing self’ response [164]. Activating receptors (TLRs, NKG2D) recognise increased ligand expression on target cells to mediate the ‘induced self’ response [165]. NK cells migrate from the bone marrow to the lung. In humans, NK cells are found in the lung parenchyma whereas in mice they have been found in the alveoli during influenza

infection [166, 167]. In the human lung NK cells constitute several heterogeneous populations. CD16⁺ CD56^{dim} cells constitute 80% of lung NK cells and are a hyporesponsive, mature, cytotoxic population that express KIR [168]. Whether the lung contains a tissue resident population of NK cells has been debated. Marquardt et al. showed lung NK cells lack expression of the CD69 tissue resident marker; however, Yamamoto et al. showed that blocking NK cell recruitment has no effect on NK ability to control lung tumour growth, suggesting lung-resident NK cells are essential in controlling metastasis [167, 169]. Parabiotic mouse studies have shown this lung-resident population to be low relative to other tissues with a specific tissue and functional signal, potentially contributing to barrier functions in the lung [170]. Murine lung NK cells also have diminished proliferative capacity and cytotoxic activity than splenic NK cells [171], suggesting that the lung microenvironment may inhibit NK cytotoxic potential to prevent airway damage. NK cells play important roles in response to infection and cancer. Murine models have illustrated their importance against respiratory viruses, bacteria and fungi [172]. Their precise contribution during human lung infections is unclear, but they are important in the early response to influenza [173]. Loss-of-function mutations leading to NK cell deficiencies uncovered an increased susceptibility to recurrent bacterial respiratory infections [174]. NK cells have also been implicated in asthma [172] and COPD [172].

$\gamma\delta$ T cells are a subset of unconventional or innate-like T cells that are characterized by the expression of a γ and δ chain T cell receptor (TCR) which require MHC for antigen recognition. $\gamma\delta$ T cells are abundant in mucosal surfaces including the lungs [175] where they swiftly react to conserved non-peptide antigens and produce large amounts of cytokines. In the lungs, $\gamma\delta$ T can constitute up to 20% of resident lymphocytes and contribute to maintain tissue homeostasis and fight infections and cancer. These cells play important roles against bacterial pathogens including *S. pneumoniae*, *B. pertussis*, *M. tuberculosis*, viruses i.e., influenza, and some fungi. Likewise, they promote protection in models of cancer. However, these cells can also contribute to pathology in asthma and lung fibrosis [176].

In addition to NK cells, ILCs include a heterogeneous population of other innate lymphocytes described both in mice and humans. These include lymphoid tissue inducer (LTi) cells and three subsets of 'helper' ILCs (ILC1, ILC2 and ILC3) which unlike NK cells, lack cytotoxic properties and secrete higher amounts of effector cytokines. Lung helper ILCs have been extensively reviewed [177]; these cells are present across all different parts of the respiratory tract and lungs, including upper respiratory tract, lung parenchyma and bronchoalveolar

spaces, where they contribute to homeostasis, pathogen clearance and also pathology [177].

Invariant natural killer cells (iNKT) and MR1-restricted T (MAIT) cells are "innate-like" unconventional T cells that reside in the lung and other mucosal surfaces and play important roles during infection by recognizing non-peptidic antigens [178]. iNKT cells are $\alpha\beta$ T cells which recognize glycosphingolipids and other microbial-derived glycolipids presented in CD1d molecules. iNKT cells can swiftly produce a range of inflammatory cytokines when activated and type I iNKT also present cytolytic activity against cancerous and virally infected cells [178, 179]. Lung type I NKT cells are mainly resident within the parenchyma (NKT17, which produce IL-17) or in the vasculature (NKT1 and NKT2) [180, 181]. MAIT cells recognize microbial-derived metabolites [178] and produce cytokines including IFN- γ , TNF- α , IL-17A, and IL-22 aiding in bacterial killing [182]. Their role and precise localization in the lung in homeostasis remains to be determined.

Metals and innate lymphocytes

The influence of metals on innate lymphocytes has been less studied and seem to vary among different innate lymphocytes subsets.

NK cells and Iron

In contrast to monocytes and polymorphonuclear cells, little is known about a potential role for an iron-related nutritional immune response in NK cell function, although recent studies suggest a role for iron signalling pathways in NK cell activation. Specifically, expression of the transferrin receptor CD71 is upregulated in the NK cell surface during their maturation in the murine bone marrow and during activation with poly(I:C) [183]. Basal expression of CD71 in human NK cells at homeostasis is low and increases in response to cytokine treatment in vitro, although how this potential to increase iron uptake drives the activated NK cell phenotype is unclear [184]. Lactoferrin increases NK cell cytotoxicity in vitro, which could imply an antimicrobial role for NK cells in infection in response to neutrophil and macrophage secretion of lactoferrin [185]. NK cells of patients exhibiting systemic iron overload in myelodysplastic syndromes displayed upregulated c-Jun N-terminal kinase (JNK) and downregulated p38 expression, suggesting a role for iron in NK signal transduction pathways [186]. It is interesting to note that perturbed iron homeostasis in NK target cells affects NK cell recognition—iron depletion and ferritin heavy chain (FTH) in primary cancer cells was observed to increase NK cell targeting [187].

NK cells and other metals

Zinc signalling has also been suggested as a mediator of the NK cell phenotype. Recognition of surface MHC-1 on endogenous cells by the NK cell KIR is essential for NK cell-mediated killing. Zinc is essential for KIR multimerization to form this 'NK cell synapse' [188]. Zinc supplementation *in vitro* stimulates the differentiation of human-derived CD34⁺ progenitors to NK cells [189]. NK cells also possess several surface receptors with tyrosine phosphorylation sites, suggesting a role for zinc in signal transduction; the cytotoxic capabilities of NK cells are reduced in zinc deficiency and increased in zinc excess, with the precise signalling pathways driving such a change remaining unclear [190]. Roles for other trace metals such as copper or manganese in NK cell biology are difficult to decipher; the few studies attempting to ascertain this were largely performed *in vitro* and thus may not hold significant physiological relevance [191]. Manganese supplementation promotes NK cell anti-tumoral activity *in vivo* [192]. Manganese also activated the adhesion protein lymphocyte function-associated antigen (LFA)-1, essential for NK cell cytotoxicity [191]. *In vitro* supplementation with selenium increases splenic NK cell cytotoxicity [193]. Further *in vivo* studies are required to ascertain the exact role of manganese and selenium on NK cell function and the biological pathways implicated.

iNKT, MAIT, $\gamma\delta$ T cells and metals

Limited data on $\gamma\delta$ T cells suggest that variations in iron availability may not affect these cells as much as they affect their adaptive lymphocyte counterparts. $\gamma\delta$ T cells express the TFR1 (or CD71) which is required by $\alpha\beta$ CD4⁺ and CD8⁺ T cells during proliferation. While blocking TFR1 with an anti-CD71 antibody blocks proliferation of T cells, $\gamma\delta$ T cell proliferation is not affected, suggesting these cells are either less dependent on iron or rely on other mechanisms for obtaining iron [194]. Indeed, activated $\gamma\delta$ T cells express high levels of the lactoferrin receptor (Lfr). Like transferrin, lactoferrin can bind 2 ferric ions and provide Lfr-expressing cells of an iron source; in addition lactoferrin possesses antimicrobial activity [195]. Unlike transferrin, which is present at high concentrations in plasma, lactoferrin is abundant in secretions like breast milk, and fluids covering the linings of mucosae like saliva, mucus and BALF [195, 196]. Addition of lactoferrin to *in vitro* cultures increase the proliferation of $\gamma\delta$ T cells upon mitogen stimulation. Given the important role of $\gamma\delta$ T cells in defence against mucosal pathogens, high expression of Lfr may be an adaptation for acquisition of lactoferrin-bound iron in mucosal sites including the lungs.

A functional link between the hereditary hemochromatosis (HH) susceptibility gene HFE (also known as homeostatic iron regulator), iron status and adaptive T cell function has been suggested [197]. However, if or how HFE regulates $\gamma\delta$ T cells remains unknown. HFE encodes a non-classical MHC molecule; as such the protein may have the ability to form complexes with β 2-microglobulin (β 2-m) [198]. Interestingly, mice deficient in β 2-m display iron overload patterns similar to HH [199] and have enhanced homeostatic proliferation of $\gamma\delta$ T cells. Since HFE deficiency is linked to iron overload, it is possible to hypothesize that excess iron could be linked to the altered $\gamma\delta$ T cell proliferation in these mice and maybe also in HH patients. In addition to the altered lymphocyte ratios, some HH patients may have altered iNKT numbers. Iron overload is associated with reduced numbers of iNKT cells, which was more notable in untreated patients [200]. Intriguingly, the regulation of iron levels and iNKT activation/proliferation seems to be two-way: iron levels affect iNKT numbers, and iNKT cells can affect iron homeostasis. *In vivo*, activation of mouse iNKT cells by injection of their prototypical antigen α -galactosylceramide (α -GalCer), not only induces iNKT proliferation but also promotes early hepcidin expression while suppressing FPN. Activation of iNKT cells also lead to an early peak of serum iron followed by accumulation of iron in the spleen and liver. These effects were abolished in *J α 18^{-/-}* mice which particularly lack iNKT cells. This indicates that iron and iNKT cells reciprocally regulate each other [201]. More research is needed to understand the effects of iron and other metals on MAIT cells and other groups of innate lymphocytes.

Metals and adaptive immune cells of the lung

Adaptive lymphocytes (B and T cells)

T and B cells are at the centre of cellular adaptive immune responses. These cells are virtually capable to recognize infinite antigens in a highly specific manner thanks to their TCR and B cell receptors (BCR) and offer long-term protection against infection thanks to the generation of effector and tissue resident memory T lymphocytes and long-lived antibody producing plasma cells. Adaptive lymphocytes can be found in different lung compartments. Conventional CD4⁺ and CD8⁺ T lymphocytes populate the mucociliary epithelium of the conducting airways in the trachea and bronchi. In contrast, B cells (mostly IgA⁺) and most CD4⁺ T cells are found in the bronchial lamina propria. Early studies also reported the presence of lymphocytes in the lung interstitium in similar numbers to those found in the circulation [202]. In addition, an intravascular pool has been identified. T CD4⁺, T CD8⁺ and B lymphocytes have been isolated

from the alveolar spaces. B cells constitute only 5–10% of the total lymphocytes isolated in BALF [202].

T and B lymphocytes can also accumulate in tertiary lymphoid structures (TLS) in the lungs. Bronchial Associated Lymphoid Tissue (iBALT) are the main TLS in the lung and serve as priming structures for B and T cells. iBALT organization is similar to secondary lymphoid organs, presenting a T cell zone and a B cell follicle. These structures are readily detectable in children but only inducible and transient in adults. Once iBALT is formed, it can be maintained in the lungs for months even in the absence of the original stimulus that triggered it and serves as a transient lymphoid structure to recruit naïve lymphocytes from the blood and to promote their interaction with local antigens in the airways to favour activation and differentiation into effector cells. The iBALT is formed near the basal side of the bronchial epithelium and in close association with the pulmonary blood vessels in response to infection or other inflammatory stimuli [203]. iBALT formation is protective against acute viral and bacterial respiratory infections and it has been also shown to contribute during chronic *M. tuberculosis* infection. However, activation of lymphocytes in TLS can also contribute to airway damage. For instance, these structures tend to form near small airways in COPD patients and are associated with more severe forms of the disease [204].

Lung resident memory T cells (T_{RM}) protect against infection by expanding rapidly upon challenge. $CD4^+ T_{RM}$ reside in the lung parenchyma, whereas $CD8^+ T_{RM}$ are found in parenchyma and the airway epithelium. $CD4^+ T_{RM}$ are maintained over longer periods of time while $CD8^+ T_{RM}$ decay and must be replenished from circulating T effector memory cells [205]. A subset of memory $CD69^+ CD103^+$ T regulatory (Tregs) cells expressing FoxP3 are also found in the lungs of mice and humans [206, 207]. Antigen-specific memory Tregs differentiate in response to certain respiratory infections such as influenza and can persist in the lungs after the pathogen has been cleared. These cells can contribute to limit the damage upon reencounter with the pathogen [207] and can limit the pro-fibrotic potential of $CD44^{hi} CD69^+ CD103^{lo} CD4^+$ T cells [208].

Metals and adaptive immune cells

Trace metals including iron and zinc, as well selenium can influence adaptive lymphocyte biology and function. As for most immune cells, iron is key to T and B lymphocytes. Both express TFR1 or CD71 which allows them to acquire transferrin- Fe^{3+} via endocytosis. The acquired iron is used as a cofactor in several enzymes and a small proportion is stored in ferritin or remains as part of the labile iron pool in the cell; although the iron reserve

pool in lymphocytes is very limited compared to other immune cells [209]. In addition to TFR1, lymphocytes also express LfRs upon activation which are particularly abundant on $\gamma\delta$ T cells [195].

Both iron overload and iron deficiency impact adaptive immunity and lymphocyte function but the different T helper and cytotoxic lymphocyte subsets display different responses to iron perturbations and their dependence on TFR1 iron uptake. Mice fed an iron-rich diet show lower IFN- γ production and impaired delayed-type hypersensitivity responses, whereas iron-deficient diets are linked to impaired T-cell proliferation [209]. In vitro, blocking TFR1 with antibodies causes the arrest of helper T cell (Th)1 cell proliferation, whereas Th2 cells do not seem to be affected. In contrast, supplementation of culture media with iron and transferrin boosts lymphocyte proliferation induced by polyclonal activators such as concanavalin A, phytohemagglutinin or LPS [210]. Supporting the importance of transferrin and iron for lymphocytes in the lungs, transferrin levels in the BALF of patients with COPD and sarcoidosis correlate with the number of lymphocytes in the BALF [211].

Iron levels are key to adaptive responses to pathogens and vaccines; anemia, iron or increased hepcidin and low serum iron caused by a mutation in TMPRSS6 predict reduced responses to rubella, diphtheria, pertussis, *H. influenzae* type B and pneumococcal vaccines [212, 213]. Importantly, in humans another homozygous mutation in *TFRC* (the gene encoding TFR1) results in defective TFR1 iron internalization. Homozygous carriers of this mutation display a combined immunodeficiency syndrome that results from impaired T and B cell proliferation, defective class-switching and lower antibody production, demonstrating the importance of iron uptake and TFR1 for adaptive response and lymphocyte's function [214].

T cells are also affected by iron overload as seen in certain cohorts of patients with HH or transfusion-dependent thalassemia. A subgroup of thalassemia patients present with unusually low counts of $CD8^+$ T cells, which can improve after subcutaneous chelation therapy with the iron chelator deferoxamine (DFO) [215]. A similar phenotype has been observed in HH patients. HFE competes with transferrin for binding to TFR1 and downregulating iron cellular uptake. Iron-sensing via the HFE-TFR1 axis ultimately induces hepcidin transcription blocking iron efflux. HH is associated to the partial or total loss of hepcidin, which results in excessive iron uptake and accumulation in the tissues. HH patients present anomalies in $CD8^+$ T cells and altered $CD4^+/CD8^+$ T cell ratios; in particular HH is associated with lower numbers of $CD8^+$ T cells in the circulation and liver and a defect

in the generation of CD8⁺ memory cells [197]. Altered cytokine profiles are also seen in HH patients, where CD8⁺ T cells increase the production of IL-10 and IL-4 contributing to Th2 polarization of the adaptive response. Although HFE is a non-classical MHC Ib molecule it does not appear to have antigen presenting capabilities; however, a role for HFE in the antigen presentation process cannot be ruled out. TFR1 interacts with the TCR ζ chain, which is involved in signal transduction upon antigen recognition [216]. Therefore, by competing with transferrin, and depending on iron availability HFE could regulate T cell activation. In addition to the altered lymphocyte ratios, some HH patients may have altered iNKT numbers. Iron overload was associated with reduced numbers of iNKT cells, which was more notable in untreated patients [200].

The balance between iron and selenium also seems important to the development of T cell responses. Iron metabolism is key to survival of CD8⁺ T cells as Fe²⁺ mediates a type of cell death known as ferroptosis in CD8⁺ T cells. During ferroptosis, Fe²⁺ reacts with H₂O₂ produced by mitochondrial respiration driving the formation of free hydroxyl radical (OH $\cdot$) which promotes lipid peroxidation and death. The glutathione peroxidase 4 (Gpx4) is a selenoenzyme that acts as a major scavenger of phospholipid hydroperoxides and is essential to prevent ferroptosis. Gpx4 is essential for homeostasis of both CD4⁺ and CD8⁺ T cells, as mice lacking Gpx4 have impaired proliferate T cell responses during a viral challenge or *Leishmania* infection [217]. Selenium has been shown to be important for both cellular and humoral immune responses, although cell-mediated immunity seems to depend more on selenium. Selenium can enhance responsiveness of lymphocytes to IL-2 by inducing the upregulation of IL-2 receptors, therefore promoting proliferation, cytotoxic activity and antibody production [218]. High selenium diets promote Th1 differentiation in mice. Whereas selenium deficiency has been linked to more severe influenza in mice [219]. Selenium deficiency leads to reduction in the expression of selenoprotein K (SelK), which is expressed in the endoplasmic reticulum membrane of many immune cells including T cells. SelK deficiency impairs proliferation of T cells as it impairs crucial Ca²⁺ fluxes upon TCR activation. SelK deficiency also affects neutrophil and macrophage function increasing susceptibility to a viral challenge. Zinc (Zn²⁺) is required for thymic development of T lymphocytes. Zn²⁺ deficiency leads to impaired Th1 responses while promoting Th17 differentiation and IL-1 β production [219].

Host nutrient availability and the microbiome of the lung

The human body harbours over 100 trillion microorganisms that live in a commensal relationship with their host, mostly at barrier surfaces and mucosae including the gut, the airways, skin and genitourinary tract. Imbalances in the composition of the human microbiota have been increasingly linked to pathologies such as allergy, asthma, chronic inflammation and autoimmunity. While the gut has been the most studied and characterized niche in terms of the microbiome, in recent years the importance of other niches including the lung microbiome, became apparent. We are only now learning that the lung is colonized by a complex and dynamic microbiota. While progress in understanding how changes in the lung microbiome contribute to disease has been limited due to sampling, technical and analytical problems, 16S sequencing studies have demonstrated distinctive differences in the lung microbiome between health and disease [220]. Despite the low density of the lung microbiota, the extraordinary diversity of interacting microbiota is evident, and it is the change or decline in this diversity that is often associated with the progression of disease. To date, no particular bacterial genera have been implicated in lung disease and the variability in the diversity of the species detected in the upper versus lower respiratory tract as well as the regional variations in the host environment (e.g., mucus or surfactant secretion, pH, nutrient or oxygen availability) has also limited our understanding of the contribution of the respiratory microbiome in lung disease [221].

It appears that the airway microbiota in healthy lungs is dominated by *Bacteroidetes* and with prominent genera including *Prevotella*, *Veillonella* and *Streptococcus* [222–224], all of which are dependent on metals for growth and survival [9, 225, 226]. Approximately 30% of all proteins in bacteria depend on metals for their function [226] and changes in host metal availability alters bacterial diversity and abundance in vivo [227–236], however there have been few studies examining the effect of metal availability directly on lung microbe populations [237]. In addition, whether or not changes in host metal availability in the lung directly modifies the lung microbiome which in turn contributes to the development and progression of chronic or acute respiratory disease is an evolving question.

Similarly, it has become evident that it is not only the microbial communities but also the metabolites they produce that can influence host susceptibility to certain diseases [238]. Despite the advances in microbiome research, our knowledge of the airway microbiome is still way behind compared to that of the gut microbiome. And as such, lung microbiota composition, microbiota

derived metabolites and how they could contribute to lung inflammation, immunity and disease is only in its infancy [239, 240]. Alterations in bacterial burden, gut microbial species and the metabolites they produce are associated with altered inflammation and immunity in the lungs as well as the development of lung diseases. This interaction, known as the gut-lung axis allows the access of gut-derived bacterial components, hormones, microbial metabolites, endotoxins and cytokines to the lung niche via the bloodstream, also influencing immune cell trafficking. Future research of the lung microbiota in the context of both healthy and diseased airways will likely discover the causes and consequences of altered lung microbiota in lung disease and identify microbial derived metabolites that play important roles in these processes. Whether or not microbe derived metal regulators interfere with the host immune responses in the lung therefore requires significant attention. In the below sections we will discuss the existing evidence for alterations in host metal availability in chronic respiratory disease and the evidence, if any, for the role trace metals have to play in the nutritional immune response or to changes in the lung microbiome.

Nutritional immunity and the host lung microbiome in chronic respiratory disease

Asthma

Asthma is a chronic and heterogeneous disease of the airways characterized by airway hyperreactivity, difficulty breathing, cough, wheezing and chest tightness. It is estimated that 300 million people are affected with asthma and that ~250,000 die as a result each year. The pathogenesis of asthma is complex with innate and adaptive cells acting together with epithelial cells to induce airway hyperreactivity. A broad clinical spectrum of phenotypes associated to different underlying immune mechanisms (endotypes) have been described [241, 242]. Asthma can be divided in two subsets: the eosinophilic type mainly controlled by Th2 or ILC2s, and the neutrophilic type which is characterized by a strong Th17 component and can present as steroid-resistant asthma. *Eosinophilic asthma* can be categorized as Th2^{hi} and Th2^{lo} component depending on the presence of IL-4, IL-5 and IL-13 being produced and the number of eosinophils in blood and lungs [243]. In addition to Th2 cells, ILC2 can also contribute to the pathology of asthma and produce high amounts of type 2 cytokines. These cells have been identified in samples of blood but also BALF and sputum of asthmatic patients [177]. ILC2 also drive mucus hypersecretion, mucus cell metaplasia, fibrosis and inflammation which are driven by IL-13, eosinophils and monocytes [244]. Late onset and steroid-resistant asthma phenotypes are associated with IL-17 driven neutrophilic

asthma and present with a more irreversible airway obstruction. Besides Th17 cells, cell including $\gamma\delta$ T cells, NKT cells and ILC3 can contribute to IL-17A, IL-17F and IL-22 production amplifying the pathology [242, 245].

T helper responses in asthma are supported by DCs that are directly activated by allergens and other environmental insults or via the damaged epithelium. Allergens including house dust mites, spores, cat dander and others can have protease activity that damages epithelial cells and triggers protease-activated receptors and triggers the release of damage associated molecular patterns (DAMPs) that lead to the secretion of innate responses via IL-33, TSLP, TLR4 and C-type lectin receptors among others [242]. Among lung DCs conventional DC expressing CD11b and SIRP1 α and that rely on interferon regulatory factor (IRF)4 are the most important for allergic sensitization [246, 247]. DCs and epithelial cells not only contribute to sensitization to allergens but also play a role in ongoing asthma.

The lung microbiome and asthma

The time from the perinatal period up to the first years of life is key to the establishment of a “healthy” microbiome. The composition of the microbiome at all anatomical sites is dynamically shaped by the interactions between symbionts, pathogens, the immune system, nutrition and the environment. Disturbances during the critical period of establishment of this ecosystem may determine future pathological manifestations [248]. Several retrospective and longitudinal studies suggest this is the case for asthma and airway hyperreactivity, where aberrant immune development results from environmental exposures that may influence the airway microbiota. The analysis of the airway microbiome in infants revealed an association between the airway microbial composition and the risk of developing asthma within the first 6 years of life. Enrichment of the upper airway microbiome with taxa including *Veillonella*, *Prevotella*, and *Gemella*, was associated with increased risk of asthma and a characteristic immune profile in the airway which was also independently associated with increased asthma risk within 6 years. While the study cannot prove causation, it suggests a potential link between an early shift in the airway microbiome composition and immune perturbation which could lead to asthma later in life [249].

Analysis of different respiratory samples in healthy vs asthmatic adults suggests that the lungs of asthmatic patients receiving corticosteroid therapy are dominated by the phylum Proteobacteria which include the genera of potential pathogens including *Haemophilus*, *Moraxella*, and *Neisseria* [250]. Response to steroids is accompanied by an increase in Actinobacteria, whereas *Kelbsiella* is linked to severe asthma [250]. In addition, patients

with severe neutrophilic asthma receiving high doses of inhaled corticosteroids display lower airway microbiome diversity, enrichment with *Moraxella* and *Haemophilus* and a reduction in *Streptococcus*, *Gemella* and *Porphyromonas* [248]. While airway microbiome profiles seem to correlate with severity of the disease and may even predict response to corticosteroids, it is not possible to discriminate whether these changes are promoted by the interactions between commensals and the immune system or induced by the steroid treatment. In a group of steroid-naïve asthmatic patients, members of the Sphingomonadaceae family and species of *Haemophilus*, *Neisseria*, *Fusobacterium*, and *Porphyromonas* were enriched in the bronchi compared to healthy controls, whereas members of the *Mogibacteriaceae* family and *Lactobacillales* order were lower. Importantly, steroid treatment caused a shift in the balance of the bronchial microbiome and differences were linked to responsiveness to treatment [251].

In addition to the local airway and lung microbiome, the distant gut microbiome and the gut-lung axis of immune regulation have been implicated in the pathogenesis of asthma. Significant reduction in species of the genera *Lachnospira*, *Veillonella*, *Faecalibacterium* and *Rothia* in the gut of 3-month-old infants correlated with elevated risk of developing asthma [252]. Mechanistically, high risk of asthma and gut dysbiosis has been linked to loss of short-chain fatty acid (SCFA)-producing bacteria which ferment soluble fibre into these highly volatile metabolites (e.g., acetate, propionate, butyrate) known for their immunoregulatory properties [253]. Higher levels of butyrate and propionate in the stool of 1-year old toddlers associate with less atopic sensitization and low risk of asthma between the age of 5 and 6 [254] and supplementation with soluble fibre inulin, associates with clinical improvement of asthma in adults and an increase in *Bifidobacteria* which degrade inulin into the SCFA acetate and lactate [255].

Intestinal bacteria can also produce metabolites that may be detrimental for lung function. For example, histamine-producing bacteria are more abundant in the gut of asthmatic patients compared to healthy controls and this histamine has been linked to airway hyperreactivity [248]. Shifts in the microbial populations in the airways and in the gut seem to be associated with asthma endotypes and severity, the causal link between development of the disease and dysbiosis remains to be proven. The current evidence supports mechanisms of immune modulation mediated by microbial-derived metabolites. While an inverse correlation between SCFA in the gut and asthma severity exists, whether significant concentrations of SCFA can reach the bloodstream or the lungs remains questioned. It would be interesting to determine

if changes in the lung microbiome influence local production of these metabolites to directly modulate the lung response to allergens and other environmental insults that could promote asthma.

Trace metals, nutritional immunity and asthma

Epidemiological studies have shown a correlation between iron deficiency and atopic disease, involving iron metabolism in the regulation of the immune response to allergens and atopic airway hyperreactivity [256, 257]. A large cross-sectional study in children and adolescents in the US, found a strong association between anaemia and atopic disease, including eczema or asthma [257]. The prevalence of asthma decreases with age and the iron reserve is higher in adults compared to children supporting the idea that the higher incidence of asthma and atopic disease in early life is linked to iron deficiency. In adulthood, men have higher levels of iron compared to women and asthma primarily affects women, again supporting a role of iron in prevention of asthma [41]. Though, the relationship between iron and asthma is not straightforward: increased iron stores in the form of ferritin have been associated with decreased odds of asthma, whereas higher tissue iron (lower serum soluble transferrin receptor) and lower body iron have been linked to lower lung function [256].

The iron storage capacity of Th2 cells is higher than Th1 as the latter have a lower iron labile pool [258]. Iron chelation affects Th1 cytokine production including IFN- γ , IL-12 and IL-18, but not Th2 cytokines [259]. Therefore, a limited iron supply during stimulation of lymphocytes by allergens may favour development of Th2 responses over Th1 and predispose to allergic sensitization [41]. Severe and moderate asthma patients present low cell-free (non-heme) iron levels in BALF which correlate with lower lung function measured as (forced expiratory volume in 1 s (FEV1), whereas iron-loaded cells numbers are increased and also show increased expression of *DMT1* and *TFR1*. These features were recapitulated by a murine model of house dust mite asthma where macrophages display the highest expression of *Tfr1* and also upregulate *Il13*, suggesting that by accumulating iron they orchestrate the type 2 response in the lungs [260]. The relationship between iron metabolism and pathophysiology of asthma represents a dichotomy: while low systemic and local iron levels in BALF correlate with severity of asthma, cellular iron overload is also linked to the pathology of the disease.

Importantly, most allergens of mammalian origin including those in dander, urine, fur, and saliva of animals, belong to the lipocalin family of proteins and have the capacity to sequester bacterial siderophore-iron complexes. Some of these allergens promote Th2

responses when not bound to iron, suggesting that iron deficiency may potentiate the stimulatory potential of lipocalin-like allergens [261, 262]. In addition, to promoting Th2 responses directly, allergens may change the microbial ecosystem of the gut and/or the airways by sequestering bacterial siderophores and altering the availability of iron to certain bacteria, therefore inducing dysbiosis. Supporting this notion, supplementation of dietary iron in rats favoured the production of the SCFA butyrate compared to animals fed an iron deficient diet [263]. This suggests that iron availability can influence the composition of the microbiome and the production of immunomodulatory metabolites such as SCFA that are important modulators of type 2 allergic responses and asthma [41].

The role of other metals in the pathophysiology of asthma is less clear but serum concentrations of trace elements including copper, zinc and selenium have been found to be altered in individuals suffering from asthma compared to healthy controls. Imbalances in selenium have been implicated in allergic inflammation. Curiously, while mice fed on high and low selenium diets show low incidence of allergic inflammation in an ovalbumin sensitization model, intermediate doses of selenium were associated with heightened inflammation. This suggests that the modulation of the allergic response by selenium in the lungs is not linear and may involve several intermediate enzymes and other unknown mechanisms [264].

Two independent studies reported elevated serum levels of copper in adults suffering from asthma compared to healthy controls, whereas zinc and selenium were lower and magnesium remained unchanged [155, 265]. While the mechanisms by which alterations in trace metal concentration contribute to the pathology are unclear, given the role of copper and zinc in regulation of redox metabolism, altered levels of these metals may contribute to imbalances in oxidative stress in asthma. Indeed, asthmatic patients present higher levels of NO and nitrated products than healthy individuals, and these correlate with the severity of the disease. Non-enzymatic decomposition of S-nitrosothiols (RSNO) is mainly catalyzed by Cu^{2+} ions leading to generation of NO and the corresponding disulphide [266]. Therefore, it is possible to postulate that elevated copper levels in asthma may accelerate RSNO consumption with the concomitant increase in NO. Supporting this hypothesis, RSNO deficiency in the airways has been linked to asthmatic respiratory failure in children which suggests that RSNO metabolism and in particular their copper-mediated catalytic decomposition could be therapeutic targets in asthma [267].

Chronic obstructive pulmonary disease

COPD is a chronic inflammatory lung disease associated with cigarette smoke or other environmental exposures. As a leading cause of death worldwide, COPD encompasses chronic bronchitis and emphysema [268, 269] and involves an aberrant immune and inflammatory responses to the inhalation of noxious particles [269, 270], with chronic inflammation persisting for many years after [204]. The definitive molecular mechanisms underlying the chronic inflammatory changes observed in COPD remain to be determined, however there is overwhelming evidence implicating AMs in this process [204, 271, 272]. AMs exposed to cigarette smoke have been shown to ineffectively clear respiratory pathogens, damaged epithelial cells as well as having defective responses to activating stimuli [273–276]. Strikingly, there is a 25-fold increase in the number of AMs in the lung parenchyma and alveolar space in COPD, which correlates with disease severity [277, 278] and areas of lung destruction [272], highlighting their central role in disease pathogenesis. Neutrophilic inflammation is also a hallmark of COPD and is associated with bacterial infection [279] whereas eosinophilic inflammation is present in a subgroup of COPD patients [280], and is associated with less bacterial infection [281]. Aberrant regulation of the immune response is a clear factor in the development and progression of COPD however little is known regarding the microbiome or nutritional immunity in this process.

The lung microbiome and COPD

Microbiome changes have emerged as a contributing factor in COPD progression, clinical phenotypes, severity and long-term mortality [282–284]. Studies comparing sputum and BALF microbiota between stable COPD patients and healthy controls have identified a change in microbial diversity with an increased abundance of *Moraxella*, *Streptococcus*, *Proteobacteria*, *Veillonella*, *Eubacterium* and *Prevotella* in disease [283–289]. Other studies of BALF report no difference in *Streptococcus* [290]. Conversely, increased *Proteobacteria* and reduced *Firmicutes*, *Bacteroidetes*, *Streptococcus*, *Haemophilus influenza* and *Prevotella spp.* have been documented in COPD lung tissue explants [291]. During an exacerbation event (periods of symptom worsening and reduced lung function) shifts in bacterial composition, characterized by a relative increase in *Proteobacteria* that falls in response to antibiotics have also been observed [292, 293]. Some of the above studies suggest that these changes may be independent of smoking status and that real changes in microbial diversity may be more apparent upon examination of associations in specific COPD endotypes [289] such as the neutrophilic inflammatory

endotype [283, 294]. Intriguingly the faecal microbiome and COPD patients is also distinct from those of healthy individuals, with *Streptococcus* sp000187445, *Streptococcus vestibularis* and multiple members of the family *Lachnospiraceae* correlating with reduced lung function [295]. During an acute exacerbation event the faecal microbiome has also been shown to display a lower relative abundance of *Firmicutes* and *Actinobacteria* with an increase in *Bacteroidetes* and *Proteobacteria* [296]. The importance of the gut microbiota in COPD development is further highlighted by the reversal of murine smoke-induced inflammatory emphysema via faecal microbiota transplantation or a high-fibre diet, possibly via the beneficial effects of SCFAs [297].

Trace metals, nutritional immunity and COPD

Genome wide association studies of COPD patients implicate a role for abnormal iron metabolism in COPD [298–302]. Current and former smokers have abnormally high levels of iron in sputum, BALF in exhaled breath condensate, and in AMs, compared to non-smoking controls [211, 303–312]. Anaemia and non-anaemic iron deficiency often accompany COPD [313] with anaemia being an independent predictor of mortality [314–316], and with iron-deficient COPD patients having more exacerbations than control subjects [317]. Smoke exposure also reduces hepcidin expression in murine models. COPD patients have an inappropriate suppression of hepcidin in response to iron deficiency, with less hepcidin expression in severe end-stage disease [54, 316, 318]. This may or may not be related to findings that iron accumulates inside cells and tissues of in vitro and in vivo COPD models [304, 305, 309]. Whether increased cellular iron is pathogenic [319] or a protective [307, 320] stratagem should consider the lung and systemic iron regulation as separate entities. Both the use of iron chelators directly in the lung as well as administering intravenous ferric carboxymaltose to iron deficient COPD patients may have beneficial effects [319, 321].

As previously mentioned, iron plays an important role in the functional response of macrophages and other immune cells of the lung and similarly iron regulatory pathways play an important role in the response of these cells to smoke [54, 304, 319]. Specifically, cigarette smoke increases the expression of FPN, ferritin and the TFR1 on AMs and inhibits hepcidin induction by LPS as well as inducing ferroptosis [54, 322]. However, whether or not the above changes in macrophage iron regulation in the lung as a result of smoke or COPD progression alters the interplay between immune cells and the lung microbiome remains to be determined.

Zinc levels have also been shown to play an important role in COPD. COPD patients have lower levels of zinc

[77, 323] and smokers with low dietary zinc intake have an increased incidence of COPD [324]. The zinc transporter ZIP8 is also increased at mRNA and protein levels in the lungs of chronic smokers [325] and zinc deficiency potentiates the effects of smoke on the epithelial cell barrier function [326, 327]. Depletion of zinc in adult mice resulted in a significant increase in lung cadmium burden and permanent lung tissue loss following prolonged smoke exposure [328]. Zinc also regulates the immune response to smoke whereby airway inflammation is exaggerated in zinc deficient mice [329] and zinc supplementation reduces AM numbers in smoke-exposed mice [330]. Zinc protects against cadmium toxicity and loss of zinc in AMs leads to the toxic accumulation of cadmium in the AMs of smokers [331]. Functionally, loss of zinc inhibits efficient efferocytosis of apoptotic epithelial cells by AMs [77]. Whether or not a loss in zinc levels alters the microbiome of the lung or the response of immune cells such as AMs to infection remains to be determined.

Copper also plays a role in the development of COPD. Copper deficiency induces emphysema in animal models [332–334] and the EBC of stable COPD patients contains lower copper levels than of controls an observation that is positively related to FEV1 in individuals with COPD [335]. Intriguingly, individuals with Menkes disease, an X-linked recessive disorder of mutations in the intracellular copper-transporter ATP7A display an increased incidence in emphysema [336, 337]. A loss of copper directly impacts AEC integrity and copper directly regulates elastin synthesis via the copper dependent enzyme lysyl oxidase [334, 338]. However, whether or not copper levels in lung immune cells dictate function or regulate responses to cigarette smoke remains to be determined. Finally, selenium responsive genes are altered in individuals with COPD [339] and COPD patients with higher selenium have a higher FEV1 [340]. Selenium and manganese levels may be beneficial before the clinical onset of COPD in smokers [341] and manganese levels are higher in COPD patients with severe disease when compared to control smokers [342] however the role of selenium or manganese in disease pathogenesis is unknown.

Cystic fibrosis

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator gene (CFTR). The *CFTR* gene encodes a protein that mainly acts as a chloride channel that transports ions across the apical membrane of epithelial cells. This disease is characterized by progressive lung disease, malnutrition, growth defects and several other presentations [343]. In the lungs, CF is characterised by mucus accumulation and obstructive lung disease. Reduced mucus clearance leads to higher bacterial loads in the

lower airways and recurrent respiratory infections are the main cause of morbidity and mortality in CF patients, with *Pseudomonas aeruginosa* being one of most common pathogens in CF.

The lung microbiome and CF

The altered landscape of the CF airways results in a dysbiosis of the airway microbiome, however which specific factors of the CF lung microenvironment drive this change in the microbiota still remains unknown [344]. The onset of the CF microbiome involves an increase in bacterial burden and a reduction in microbial species diversity. Infants and children with CF have a higher microbial diversity which is lost with age, disease progression, and the domination of CF pathogens, most notably *P. aeruginosa* [345–349]. In advanced disease the lower airways consist of mostly homogenous populations of CF pathogens [350]. The main taxa found within CF microbiomes are *Streptococcus*, *Prevotella*, *Veillonella*, *Rothia*, *Actinomyces*, *Gemella*, *Granulicatella*, *Fusobacterium*, *Neisseria*, *Atopobium* and *Porphyromonas* [351, 352]. Diversity appears to serve as a marker for lung function as reduced species richness correlates with reduced lung function [351, 353]. Several studies have examined the airway microbiome in CF patients during exacerbation [351, 354, 355] and have observed that overall microbial community structure remains the same during stable periods and exacerbations and that the extent of changes observed during exacerbation are dependent on the community composition and diversity at baseline. In one study the relative abundance of *Gemella* was increased in most of the patients during exacerbation and was the most altered genus between baseline and exacerbation [354]. In this same study a subset of patients had substantial changes in microbial community structure during exacerbation in that those that had *Pseudomonas* dominated microbiota communities at baseline became more diverse at exacerbation. Furthermore, the presence of anaerobes in the microbiota is associated with reduced inflammation and higher lung function at early exacerbation compared to *Pseudomonas* [356]. While many studies have identified the constituents of the microbial community in the CF lung and during exacerbation and stable periods, there are few mechanistic insights into how these microbial residents of the airways can contribute to this disease. A recent study quantified bacterial active translation in the sputum from CF patients and found that active bacteria (i.e., those actively translating proteins) represent only a subset of those captured by conventional sequencing [357]. By adopting a dormant phenotype in which reduced cellular activity endows a temporary multi-drug resistant phenotype, the inactive bacteria subpopulations could persist during antibiotic

challenge. While the common residents of the CF airway microbiota are now known, their physiology and how they interact with other members of the microbiota and with their airway microenvironment remain to be more fully explored in order to understand how CF drives altered microbial community in the airways and how the CF microbiota subsequently contributes to CF disease progression.

Trace metals, nutritional immunity and CF

Systemic iron deficiency is prevalent among adults and children with CF [358–364]. However iron levels are elevated in the airways in CF with higher iron levels detected in the sputum and within AM and IMs [365–368]. With macrophages being key regulators of extracellular iron levels, they represent a likely source contributing to increased airway iron. Indeed, CF macrophages have recently been shown to have altered iron metabolism that can be corrected with CFTR modulators ivacaftor and lumacaftor [369]. Furthermore, airway epithelial cells expressing $\Delta F508$ -CFTR, the most common CFTR mutation observed in CF, have been shown to have altered iron homeostasis and release more iron than those expressing WT-CFTR [370, 371]. This increased iron in the airways provides easier access of this vital nutrient to bacteria present in the airways, including *P. aeruginosa*. Iron not only is essential for growth, but it also regulates biofilm formation in this respiratory pathogen [369, 370, 372]. This has a major impact on the ability of these bacteria to persist in the lungs as bacteria within biofilms are much more difficult to clear by host immunity and by antibiotic treatment. With persistent *P. aeruginosa* lung infections driving increased mortality in CF patients, reducing high iron levels in the lungs or depriving *P. aeruginosa* of iron appears to be a sensible strategy to control this CF pathogen. Gallium has been used to treat *P. aeruginosa* in CF, this metal was shown to and inhibit growth of *P. aeruginosa* in sputum and improved lung function in people with CF and chronic infection by interfering with bacterial iron metabolism [373]. Gallium compounds have also been shown to inhibit *in vitro* and *in vivo* growth of *Mycobacterium abscessus* by disrupting iron uptake in this emerging important pathogen in CF [374].

Zinc deficiency has been observed in CF patients, especially those with malabsorption and impaired growth. The serum or plasma levels of subsets of CF patients, both child and adult, have been observed to be lower than healthy controls in several studies [375, 376]. Zinc supplementation has been used to treat CF patients, but studies have shown conflicting results, some have shown that zinc supplementation leads to improved lung function and reduced need for antibiotics and

hospitalisations, while others showed no benefits for zinc supplementation [377–381]. Interestingly, while zinc levels in serum can be low, zinc levels in the airways of CFs are reported to be higher [382, 383]. The prevalence and the impact of altered zinc homeostasis in CF is not yet well defined, however zinc levels affect microbial community structure in CF. Explants of CF lungs had *S.aureus* and *P. aeruginosa* co-existing in calprotectin enriched regions. As calprotectin chelates zinc, regions in the lungs enriched with this protein will have lower zinc levels which causes *P. aeruginosa* to reduce its production of antistaphylococcal factor thus allowing for the co-existence of this bacterial species [384].

Studies have found reduced activities of copper enzymes from cells (mononuclear cells, neutrophils and erythrocytes) in CF patients, possibly indicating reduced copper availability within these cells [385, 386]. Interestingly copper supplementation does not counteract this copper deficiency in CF patients indicating an altered copper metabolism that cannot be corrected simply by supplementation [387]. One study reported that copper levels are elevated in the sputum in CF [382]. The cause and consequences of altered copper homeostasis in CF are yet to be determined. Selenium deficiency is observed in CF patients [388–391] but the levels of selenium in the CF lung have not been reported.

Non cystic fibrosis (CF) Bronchiectasis

Bronchiectasis refers to the permanent widening of the bronchi and usually presents clinically with coughing, sputum production and recurring respiratory infections, along with other symptoms. The widening of the bronchi leads to impaired mucociliary clearance and failure to effectively clear microbes and mucus leading to persistent infection and inflammation. Bronchiectasis is complex and heterogenous and can be the final common feature of many infectious, inflammatory, and allergic disorders [392].

The microbiome and bronchiectasis

Several studies of the lung microbiome in bronchiectasis have observed alterations. A longitudinal study of non-CF bronchiectasis patients found that the airway microbiomes remained stable over time and that patients with lower diversity of the microbial community were more likely to experience a subsequent decline in lung function [393]. This study also reported that the microbiomes of patients that were dominated by *Pseudomonas* differed greatly from patients whose microbiomes are *Haemophilus* dominated and that antibiotic treatment did not affect their microbiome composition. However, a larger study observed that disease severity is reduced with lower microbiome diversity [394]. Another study

reported no significant changes in microbial diversity and during or after exacerbations, but also observed stability in the microbiomes of patients over time (6 months) even with antibiotic therapy [395]. This study also highlighted the differences in culture versus sequencing to identify the composition of the airway microbiota, notably *H. influenzae* is not detected well by culture. A study which compared bronchiectasis patients treated with low dose of a macrolide antibiotic (erythromycin) with a placebo control found that patients with *Haemophilus* dominated microbiomes were associated with fewer exacerbations but both *Pseudomonas* and *Haemophilus* dominated microbiomes were associated with lower lung function [396, 397]. Infections caused by non-tuberculous mycobacteria (NTM) are often seen in patients with bronchiectasis. These bacteria are found in the environment and usually do not cause disease but can establish chronic infections in people with underlying conditions. The role of NTM in the lung microbiome and how it may interact with other species in the microbiota of bronchiectasis patients, or even in healthy individuals, is not yet known. Limitations of sequencing approaches commonly used contribute to this lack of knowledge and studies have shown that 16S rRNA gene sequencing is not sensitive enough for *Mycobacteria* [398, 399]. Using a more sensitive technique for mycobacteria, a study was able to identify a non-tuberculous ‘mycobacteriome’ in the mouth and upper respiratory tract of healthy individuals [399]. A study involving non-CF bronchiectasis patients with high prevalence of NTM used this mycobacteriome sequencing approach for the lower airways of 20 patients [398]. They found that in the lower airways of NTM positive patients, taxa identified as oral commensals were associated with increased inflammatory biomarkers. Studies with larger cohorts and using sensitive sequencing methods for mycobacteria are required to further investigate the role of NTM in the microbiome, particularly in the context of bronchiectasis.

Trace metals, nutritional immunity and bronchiectasis

The roles that metals such as iron, zinc and copper may play in non-cystic fibrosis bronchiectasis is not known and there are only a few reports measuring the levels of these metals in patients with this chronic lung disease. Some reports have observed that altered trace metals levels in non-CF bronchiectasis patients. Specifically, serum zinc levels have been reported to be lower in bronchiectasis patients when compared to healthy controls, but an earlier study did not find significant differences in serum zinc levels and found that even though zinc supplementation resulted in increased zinc serum levels no clinical improvement was observed [400, 401]. Two studies have detected higher levels of iron and zinc in the sputum of

non-CF bronchiectasis patients compared to controls [382, 383]. Bacteria that cause recurrent infections in non-CF bronchiectasis could benefit from easier access to essential trace metals in the airways, supporting their growth. Thus further study of the source of these trace metals in the airways and their role in both the pathology of non-CF bronchiectasis, and in respiratory infections in those with chronic lung disease needs to be further investigated.

Lung cancer

Lung cancer is one of the most aggressive and lethal cancers, with low treatment success and many complications. The most common form of lung cancer is non-small cell lung cancer which is subdivided into three main types; adenocarcinoma (AD) which arises in the peripheral bronchi, squamous cell (SC) which arises in the main bronchi and large-cell undifferentiated carcinoma [402]. The less common form of lung cancer is small cell lung cancer (SCLC) which accounts for ~15% of lung cancers is characterised by its neuroendocrine features [403]. Previously, lung cancer was thought to be non-immunogenic due to failed immunotherapies but has recently been revisited as being correlated with various immune cells and responses, particularly with inflammatory responses being integral to cancer progression [404]. Commonly during early stages of lung tumour generation an influx of immune cells such as leukocytes into the tumour and around the tumour microenvironment (TME) are observed [405]. The main source of this immune inflammation comes from M2 macrophages, which have been shown to promote angiogenesis, tissue remodelling and repair for the malignant cells [406].

The microbiome and lung cancer

Studies testing patients with lung cancer found strong associations between the presence of malignancy and high densities of *Haemophilus influenzae*, *Acidovorax*, *Klebsiella*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis* and *Granulicatella adiacens* [224, 407]. Furthermore, comparison between the taxa present in SCLC and AD revealed specific taxa such as *Acidovorax*, *Klebsiella*, *Rhodoferrax*, *Tepidimonas* and *Anaerococcus* were more abundant in SCLC [408]. Possibly through an environmental influence, the density of commensal bacteria was reduced within the lungs, allowing for more insidious opportunistic species to proliferate and drive lung inflammation. Microbial profiles have been suggested as a potential biomarker for lung cancer. Using 16S RNA sequencing and real time PCR Yan et al. (2015) successfully linked microbiota present in the saliva to lung cancer. Both *Capnocytophaga* and *Veillonella* were significantly higher in patients with lung

cancer [409]. However, the sample size of this study was relatively small so further investigations into the affiliation between microbial composition in saliva and lung cancer is needed. In more recent studies, comparisons of the microbial profile between patients with emphysema and or lung cancer noted that the microbial composition of lung cancer tissue samples were distinctly different from both controls and patients with emphysema [410]. Furthermore, preliminary analysis of the microbiome in the sputum identified six further bacterial species that were significantly more abundant in lung cancer samples compared to controls [411]. It is clear that there is link between microbial composition and disease status, however, it is noted that the above studies attempted to analyse the lung microbiome indirectly through analysing the microbial composition in either saliva or sputum. Further direct analysis of the lung microbiome are needed to establish a stronger link between the microbial profile within the lung and lung cancer.

The gut microbiome has also been identified to play potential roles in the pathobiology of lung cancer. For example, *H.pylori* has been suggested to play a role in lung cancer pathology [412, 413] with patients with lung cancer possibly having a significantly higher rate of seropositivity for antibodies against *H.pylori* [414], however these findings have not been well replicated [415, 416]. The mechanisms behind bacterial burden and lung cancer include; the production of bacterio-toxins and pro-inflammatory factors, which could ultimately lead to the DNA damage and mutation causing malignancy, as well as a hyperinflammatory immune response [417, 418]. Also of note, evidence from patients with lung cancer has suggested antibiotic use during treatment to have a largely negative effect on tumour regression, with antibiotics only strengthening the pathogenic bacteria and staving off healthy commensal species [419]. Taken together it is clear that both commensal and infiltrating bacteria play a strong role in the carcinogenesis of lung cancer. Further investigations should attempt to directly assess the lung microbiome, for example, through BALF samples collected via endotracheal tube extraction to avoid contaminants from both mouth and upper airways [349].

Trace metals, nutritional immunity and lung cancer

Disturbed metal homeostasis has been previously proposed as a biomarker of lung cancer [420]. Specifically, increased level of copper and decreased level of zinc in the serum has been a successful indicator for lung cancer when compared to healthy controls [421]. Increased serum copper may aid in tumour progression through angiogenesis, while decreased zinc may lead to unprecedented changes in cell cycles and apoptosis [422]. In

contrast, increased plasma zinc levels correlated with a lower risk of lung cancer [423]. Higher levels of zinc were also shown to regulate eight different cancer genes and suggested to prevent cancer progression through decreasing telomere attrition [423].

Iron accumulation has been seen in several cancers and has even been suggested as a potential target for cancer therapy [424]. In the context of lung cancer, ferritin levels in patients are significantly elevated when compared to controls and patients with COPD [425]. Increased expression of other iron regulators like LCN2 and TFR1 have also been observed in lung cancer patients [426, 427]. Furthermore, down-regulation of LCN2 or TFR1 in murine models for adenocarcinoma was shown to significantly suppress the growth of the tumours [426, 427]. Higher IL-6 levels in lung cancer patients [428] may be associated with the upregulation of hepcidin, resulting in decreased iron influx and induced cancer-related anaemia [429]. miR-20a, upregulated in non-small cell lung cancer (NSCLC), and can negatively regulate FPN, which may promote proliferation of cancer cells indirectly through the retention of iron [430]. Iron has also been reported to induce cancer stem cells and aggressive phenotypes through ROS generation in human lung cancer cells [431].

The TME has been characterized as a key player in tumour growth and progression [432]. Tumour associated macrophages (TAMs) are the most commonly found immune cell in the TME and have been shown to integrate an M2 'like' phenotype to promote tumour growth and proliferation [405]. In contrast, a subset of TAMs, iron-loaded TAMs (iTAMs), were characterized as tumoricidal due to their ability to switch the polarization of M2 macrophages to pro-inflammatory M1 macrophages when exposed to iron or heme [433, 434]. Furthermore, AD patients with higher levels of iTAMs were shown to have an overall better survival in comparison to controls [435]. Little is known about where the surplus of iron is coming from, although some studies have suggested cigarette smoking may be a contributing factor [304]. Metagenomic sequencing of microbial profiles in the sputum of lung cancer patients have also identified increases in iron siderophore sensors and receptors [411]. M2 'like' TAMs produce higher levels of FPN and lower levels of ferritin when compared to M1 macrophages. Furthermore, it was suggested that M2 'like' TAMs contribute to tumour progression through their "iron-recycling" phenotype and fulfil the high iron demand from tumour cells by exporting iron into the TME [433]. Interestingly, the treatment of cells with exogenous iron can prevent liver metastases by inducing a pro-inflammatory M1 polarization in macrophages [436].

Links between other trace-elements, such as selenium and manganese with cancer have also been explored in the literature. While there are no reports on altered levels of manganese in lung cancer, some studies have reported an increase in manganese SOD (MnSOD) activity to be correlated with the disease [437–439]. Genetic analysis demonstrated that polymorphisms in the MnSOD gene were shown to contribute to a higher risk of lung cancer [440]. It is thought that these polymorphisms accelerate tumour progression due to the reduced ability to defend against oxidative stress. In more recent studies, over expression of an isoform of MnSOD in cancer cells was shown to increase tumour invasion in vitro [441]. The altered expression and mutations of MnSOD seen in lung cancer implicate an important role for mitigating oxidative stress in order to suppress tumour progression.

Finally, the role of selenium in the pathogenesis and progression of lung cancer is controversial with some studies demonstrating a lower risk of lung cancer with higher exposure to selenium [442] and others reporting no correlation between dietary selenium and lung cancer risk a [443, 444]. Further metallomic and observational analysis are needed to clarify whether these metals are associated with the pathogenesis of lung cancer.

Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a form of interstitial lung disease, characterised by a gradual, irreversible decline in lung function [445]. Symptoms include gradual dyspnea and a non-productive cough. The disease is diagnosed via CT or lung biopsy to show the presence of usual interstitial pneumonia. As 'idiopathic' would suggest, the exact cause of the disease is unknown. IPF tends to affect those aged 60 and above, often with a history of smoking, and is more common in men than women. Environmental, occupational and genetic risk factors may also play a role in the onset of IPF. It is thought that persistent damage and abnormal repair to the alveolar epithelium drives the disease. Treatments for IPF are limited, and none can reverse the fibrosis. The current strategy focuses on slowing the progression of the disease and two antifibrotics have been approved, nintedanib and pirfenidone [446].

The microbiome and IPF

Active infection in patients with IPF is a high risk for mortality, and as antibiotics can improve quality of life for IPF patients, this would suggest that the microbiome can negatively affect disease progression [447, 448]. One study showed bacterial burden in BALF from IPF patients to be twice that of healthy controls, and this increase has been shown to correlate with worsening disease progression [449, 450]. The microbiota of IPF patients has

been shown to be less diverse than healthy controls, with increased number of *Streptococcus*, *Haemophilus*, *Neisseria*, and *Veillonella* [449]. The *Streptococcus* and *Staphylococcus* taxa in particular may be linked to increased mortality, though subsequent studies have not supported this [449, 451, 452]. Instead, it may be the increased bacterial load, rather than particular species that is detrimental [449]. Interestingly, germ free mice had reduced mortality in a bleomycin-induced pulmonary fibrosis model despite similar levels of fibrosis, with the authors of the study speculating that the microbiome may alter the balance between Th1 and Th2 responses [450]. In a study comparing the difference in the microbiomes of patients with either stable IPF or those undergoing an acute exacerbation, those with stable IPF had a reduced bacterial load in their BALF fluid [452]. With increased bacterial load comes epithelial cell damage and increased expression of pro-inflammatory cytokines leading to inflammation and fibrosis [453]. Genetics may play a role determining the increased bacterial burden. Mucin 5B is one of a family of proteins that when combined with water forms mucus. Individuals with a polymorphism in the promoter region of the *MUC5B* gene, a gene associated with susceptibility to IPF have a higher bacterial burden than those lacking this polymorphism [449].

As bacteria must acquire metals essential for their viability from their host the altered metal profile in the lung during IPF, as discussed below, may affect bacterial growth. *Streptococcus pneumoniae*, for example, requires manganese and iron from its host, while high levels of copper are toxic [454]. Growth of both *Haemophilus influenzae* and *H. parainfluenzae* can be inhibited by the addition of the iron chelator, deferoxamine, to the growth media [455]. Whether the changes seen in metal availability in the lung drive infection during IPF, or whether they occur due to infection or the disease itself is unknown. Further study would be required, but as metal availability can affect the growth of the bacterial species that may exacerbate IPF, monitoring metal levels in BALF may be an indication of how susceptible patients with IPF are to bacterial infection.

Trace metals, nutritional immunity and IPF

Exposure to different metals, whether in an environmental or occupation settings, has been linked to the development of lung fibrosis and is considered a risk factor in the development of IPF [456]. The availability and metabolism of several metals have also been implicated in IPF. BALF from IPF patients showed lower levels of chromium, nickel, manganese and zinc when compared to healthy controls, and the authors of this study speculated that this may be due to increased oxidative stress as these metals are involved in the antioxidant response

[457]. Of the metals implicated in IPF, iron has been the most widely studied. Iron homeostasis is known to be altered in IPF patients as increased iron deposition is seen, which may also lead to increased oxidative stress in the lung [458, 459]. The same study also showed iron and copper to be elevated in BALF of IPF patients [457].

AMs may play a role in driving fibrosis [460]. Iron accumulation in macrophages leads to persistent activation in other diseases, and as iron is known to accumulate in AMs this may also contribute to damage in IPF [461]. BALF cells from IPF patients have increased iron-dependent ROS generation [461]. A higher proportion of AMs in IPF patients lack expression of TFR1, when compared to healthy controls [462]. Consequently, a higher level of transferrin is found in BALF and TFR1^{neg} AMs were shown to be unable to take up transferrin. The inability to sequester iron may lead to an increase in bacterial growth in the lung, for example *Staphylococcus aureus* can use transferrin bound iron as a growth factor [463]. The impairment in phagocytosis seen in these TFR1^{neg} AMs may also allow for increased bacterial growth. Coupled with this possible increase in bacterial burden due to a lack of TFR1 expression, the TFR1^{neg} AMs also had an altered gene expression profile that could be described as profibrotic. This increased proportion of TFR1^{neg} AMs was also linked to reduced survival in IPF patients [462].

Other metalloproteins may also contribute to fibrosis. Lysyl oxidase-like 2 (LOXL2), a copper-dependent monoamine oxidase, has been linked to pulmonary fibrosis [464]. LOXL2 plays an essential role in matrix remodeling and fibrogenesis, but in both bleomycin treated mice and patients with IPF, LOXL2 expression is increased [465, 466]. The transforming growth factor (TGF)- β /Smad signalling pathway has been implicated in the onset of IPF, and signalling through this pathway in fibroblasts from bleomycin challenged mice was reduced when LOXL2 was silenced with siRNA [464]. Targeting LOXL2 with an inhibitory monoclonal antibody has also been shown to be protective in bleomycin exposed mice [467]. SODs convert superoxide radicals into the less damaging hydrogen peroxide and oxygen [468]. Mammalian cells possess three isoforms: the cytosolic copper-zinc SOD1, the mitochondrial manganese-dependent SOD2, and the extracellular copper-zinc SOD3. Macrophages from patients who had been exposed to asbestos have high levels of the copper, zinc-SOD/SOD1, and produce high levels of H₂O₂, while SOD1^{-/-} mice were protected from developing pulmonary fibrosis following intratracheal exposure to asbestos [469]. Follow up studies showed that SOD1, via its generation of H₂O₂, drives a M2 macrophage phenotype among AMs, which is associated with the development of fibrosis [469, 470].

Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is characterized by increased pulmonary arterial pressure associated with remodelling of the pulmonary arteries that leads to right ventricular hypertrophy and failure increasing the incidence of right heart failure and death [471]. Variants of pulmonary hypertension (PH) affect over one hundred million people globally [472]. Immune cells play an important role in the development of PAH supported by the observation that dysregulated lymphocytes, AMs, DCs, and mast cells frequently accumulate in the perivascular regions and in the pulmonary arterial vascular lesions found in human PAH tissue samples [473].

The microbiome and PAH

There have been few studies assessing the airway microbiome in individuals with PAH. However, a recent study described the difference in abundance of microbiota in pharyngeal swabs showing a significantly higher proportion of *Streptococcus*, *Lautropia*, and *Ralstonia* in patients with PH than reference subjects [474]. Gut microbiota differences have also been noted in PAH and experimental models for PAH have documented alterations in faecal microbiota composition with rodents displaying a three-fold increase in *Firmicutes*-to-*Bacteroidetes* ratio [475, 476].

Trace metals, nutritional immunity and PAH

Iron deficiency and/or anaemia is prevalent in PH patients, significantly affecting morbidity and mortality of PH patients [477, 478]. Similarly PH is a major cause of morbidity and mortality in patients with hemoglobinopathies and chronic haemolytic anaemias including sickle cell disease (SCD) and thalassemia [479]. Mutations (G208C) in NFU1, a mitochondrial protein that is involved in the biosynthesis of iron-sulfur clusters develop PAH in ~ 70% of cases [480]. Iron accumulates in the lungs and AMs of individuals with PH [481]. Iron handling, in particular the hepcidin/FPN axis has been shown to be important in the vascular remodelling and endothelial cell dysfunction associated with PAH and loss of the iron regulatory protein Irf1 leads to the development of PH in mice [482–484]. While the role of iron in vascular endothelial and smooth muscle cells has been well studied; little is known regarding the role of iron in the perivascular immune cell infiltrates observed in PH. Circulating macrophages recruited to the lung that contribute to pulmonary vascular remodelling in PH have a distinct iron and HO-1 rich phenotype driven by haemoglobin [485], however whether other immune cells

also display alterations in iron handling remains to be determined.

Increases in both zinc and copper transport have also been implicated in pulmonary vascular homeostasis [486, 487] and selenium levels may be lower in individuals with PH [488]. Pulmonary hypertension can be triggered by chronic hypoxia. Hypoxia has been shown to cause an NO-dependent increase in labile zinc in mouse lung endothelial cells from isolated perfused lungs (IPL) [489]. Hypoxic restriction is attenuated in IPL from mice treated with a zinc chelator suggesting a role for chelatable zinc in modulating hypoxic pulmonary vasoconstriction. Furthermore, zinc homeostasis has been implicated in PH as disruption of the zinc importer ZIP12 (Slc39a12) gene attenuates the development of PH in rats housed in a hypoxic environment [486]. Increased expression of copper-related genes, copper uptake transporter (CTR1), copper efflux pump ATP7A, and lysyl oxidase (a copper-dependent enzyme), was observed in mice with hypoxia induced PAH. Increased expression of CTR1 has also been observed in response to hypoxia within macrophages [490]. However, administration of a low copper diet in experimental models of PH had no effect on right ventricular (RV) failure [491]. Dietary administration of selenium to broiler chickens prevented RV hypertrophy associated with PH syndrome [492] suggesting some possible benefit for PH. Little is known regarding the role of these trace metals in immune cell function in the lung or interaction with the airway microbiome in PAH.

Mycobacterial lung infections: tuberculosis and non-tuberculous mycobacteria (NTM)

Lung infections caused by mycobacteria cause a significant global health burden. *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), causes 9 million new infections each year and over 1 million deaths [493]. While *Mtb* is a human adapted pathogen, non-tuberculous mycobacteria (NTM) consist of over 200 species of environmental mycobacteria, some of which can opportunistically infect humans. NTM lung infections most often occur in those with underlying conditions including COPD, cystic fibrosis and bronchiectasis. NTM infections are less prevalent than TB but the prevalence of these infections is increasing globally [494]. This is concerning as the treatment for NTM requires multi-drug regimen lasting over a year with only a 50–80% success rate [495]. Furthermore, recurring infections after treatment completion are common.

Mycobacteria and trace metal acquisition

Pathogenic mycobacteria must be able to acquire nutrients to support growth, survival and persistence within the host [496]. These intracellular pathogens acquire

iron by stealing it from their host through the production of siderophores as well as utilisation of host iron-binding proteins heme, haemoglobin and transferrin [497]. Siderophores are compounds produced by some microorganisms with a high-affinity for iron in response to iron deficient conditions [498]. There are three types of mycobacterial siderophores: mycobactin, carboxymycobactin and exochelin. Mycobactin is associated with the bacterial cell envelope and transports iron through the cell envelope into the cytoplasm. The structure of mycobactin varies between bacterial species. However, its general lipid-soluble structure allows the binding of one iron atom per molecule at a very high affinity [498, 499]. Carboxymycobactin has a very similar structure to mycobactin but it is modified by the addition of a carboxylic acid group, making it more hydrophilic, thus it is an extracellular siderophore that is secreted by the bacteria. Exochelins are extracellular siderophores and are produced only by fast growing mycobacteria. Their structure consists of ornithine-derived hydroxamates as iron coordination sites allowing them to acquire iron from insoluble sources such as ferric oxide and ferritin [499].

Mycobacteria also have alternative mechanisms to siderophores to obtain iron from their host [500]. Heme is one of the largest human iron stores and as a result it is often exploited by bacteria as a source of iron. *M. tuberculosis* is capable of importing heme and either extracting the iron if needed or storing the heme. *M. tuberculosis* also requires heme itself and encodes its own heme biosynthesis pathway, which is essential if an external heme source is not provided [501–503]. Mycobacteria can also use haemoglobin as an iron source to support their growth. The growth supported by haemoglobin was accompanied by an decrease in siderophore production, suggesting an alternative iron-acquisition system [504].

Pathogenic bacteria must be able to safely store intracellular iron once it has been acquired from the host and like vertebrates, they use ferritins and bacterioferritins (Bfrs) to do so. Typically, ferritins assemble into spherical particles or ‘nanocages’ consisting of 24 subunits with a ferroxidase centre within each subunit. *M. tuberculosis* has both ferritin and Bfrs (BfrA and BfrB). The expression of *bfrA* and *bfrB* genes is regulated by the iron sensitive regulator IdeR such that ferritins levels adapt to the level of iron [505]. Deletion of BfrA and BfrB from *M. tuberculosis* results in a decreased ability to withstand oxidative stress, antibiotic treatment and survival within human macrophages. Loss of both genes leads to reduced virulence in a guinea pig model of TB infection and loss of *bfrB* lead to an inability for *M. tuberculosis* to persist in mice [506, 507]. These studies show the iron storage system of *M. tuberculosis* to be an attractive drug target, as it not only is required for full virulence but also

to withstand antibiotic treatment (including the front-line TB drug isoniazid). *M. avium* encodes only one ferritin homologue (BfrA) which is less important to iron homeostasis and virulence in *M. tuberculosis*. As *M. avium* most often causes infections in those with chronic lung diseases such as COPD, CF and non-CF bronchiectasis where iron levels in the lungs are increased, one could speculate that iron storage within *M. avium* is particularly crucial to persist in the host and thus we could speculate that the BfrA homologue in *M. avium* could be important for this opportunistic pathogen in dealing with abundant iron. Interestingly, the crystal structure of Bfr from *M. smegmatis* (which is 87% identical to its homologue in *M. tuberculosis*) revealed that it contained zinc in its di-nuclear metal binding site [508]. The biological implications of this and whether this is also true for the Bfrs in other mycobacteria have not yet been explored.

TB and the microbiome

Studies have shown that *M. tuberculosis* infection affects both the lung and the gut microbiome of its host. However, the role of other mycobacterial species on the microbiome still needs to be elucidated. Interactions between the microbial communities of the gut and lung of an infected host can influence the outcome of *M. tuberculosis* disease progression and response to treatment [509]. The microbiota of sputum samples from TB patients were more diverse compared to respiratory secretions of healthy participants. Bacteria such as *Pseudomonas* and *Cupriavidus* were exclusive to TB patients and could influence the onset or development of infection [510]. BALF samples of TB patients showed a similar diversity in the lower respiratory tract with *Cupriavidus* bacteria possibly acting as a cofactor to secondary TB infections [511]. Furthermore, oral microbes present in the lungs of HIV patients on anti-retroviral therapy produce SCFAs, such as butyrate and propionate that increased patient susceptibility to *M. tuberculosis* infections by inhibiting the production of IFN- γ and IL-17A cytokines [512].

Mice infected with *M. tuberculosis* by aerosol lost their gut microbial diversity and recovered with a different microbial composition [513]. Furthermore, the administration of first-line TB antibiotics almost immediately (within 1 week) altered the intestinal microbiota of *M. tuberculosis* infected mice with distinct and long lasting effects [514]. Faecal samples from clinical pulmonary TB patients had dysbiosis of their gut microbiota with a significant decrease in the SCFA-producing bacteria. A decrease in SCFA-producing bacteria have also been found in systemic inflammatory disease and therefore, loss of these bacteria in TB patients may indicate systemic inflammation and an impaired immune response

[515]. There have been attempts to create a classification model using the abundance of gut microbes to discriminate between healthy and diseased patients [515].

Trace metals, nutritional immunity and mycobacteria

Elemental analysis of the phagosomes of peritoneal macrophages of C57BL/6 mice infected with *M. avium*, *M. tuberculosis* or *M. smegmatis* showed that the iron concentration of the phagosomes containing the pathogenic mycobacteria was significantly higher [83]. Radioactive iron-loaded transferrin was used to reveal that the infected macrophages acquire extracellular iron and delivers it to vacuoles containing *M. avium* through the transferrin receptor. However, when the macrophages were activated with IFN- γ before infection, iron accumulation by the pathogenic mycobacterial phagosomes was prevented. IFN- γ is known to downregulate the transferrin receptor in order to reduce the iron pool and enhance macrophage activity. Furthermore, when the macrophages were treated after infection, there was no change in iron concentration indicating macrophage anergy to IFN- γ activation [83]. Iron is extracted from transferrin by mycobactins but holo-transferrin can also be taken up by *M. tuberculosis* within macrophages [516].

The *Nramp1/Slc11a1* gene is associated with the controlling intracellular pathogens such as *Mycobacteria*, *Salmonella* and *Leishmania*. Several studies have reported polymorphisms in *Nramp1* which are associated with increased susceptibility to tuberculosis and leprosy (caused by *M. leprae*) in humans [517–519]. NRAMP1 is a transmembrane protein localised to lysosomal membranes of phagocytic cells and is recruited to the phagosome upon infection [520–522]. It belongs to a family of divalent cation transporters and functional studies have shown that it transports iron out of the phagosome [523–526]. A recent report found that NRAMP1 restricts *Salmonella* growth in mice through magnesium deprivation, but whether this is via magnesium being directly transported by NRAMP1 has not yet been determined [527]. NRAMP1 is also known to have roles in nitric oxide production, phagosome maturation and induction of lipocalin 2 [528–530]. Despite studies in macrophages showing that NRAMP1 can restrict mycobacterial growth, deletion of *Nramp1* in mice infected with *M. tuberculosis* did not result in increased bacterial burden [531]. This is in contrast to *M. avium*, where expression of the *Nramp1*^{D169} allele (which results in deficient function and/or expression) in mice results in these animals being highly susceptible to *M. avium* [532]. *M. avium* growth increased in a dose-dependent manner with iron administration in mice expressing the functional *Nramp1*^{G169} allele indicated that excess iron can impair or overcome the function of the NRAMP1 protein

[532]. Further studies have argued that NRAMP1 in fact transports iron into the phagosome upon its recruitment to aid mycobacterial killing by catalysing the Fenton/Haber–Weiss reaction to produce hydroxyl radicals [533]. Further investigation is required to fully elucidate the role of NRAMP1 during mycobacterial infections and whether the importance of its role differs depending on the mycobacterial species causing infection.

Murine BMDM infected with *M. avium* upregulated the transcription of heavy-chain (H)-ferritin in response to the stimulation of toll-like receptor-2. This could play a role in starving the mycobacterium of iron by storing iron atoms away from the pathogen while also driving macrophage polarisation towards a glycolytic M1 antibacterial phenotype via HIF1 α [534, 535]. Iron deficiency induced stabilisation of the transcription factor HIF-1 α promoting sustained IFN- γ -mediated polarization towards the M1 phenotype in vivo and the addition of the iron chelator deferoxamine to *M. tuberculosis*-infected human primary macrophages promoted the M1 phenotype in vitro [535, 536]. LCN2 is involved in the innate immune response and can bind to the bacterial siderophore-iron complex, carboxymycobactin and restricts bacterial growth. In vitro studies showed that Lcn2 restricts the growth of *M. avium* in vitro while the mycobacteria also induced Lcn2 production by murine BMDMs [537]. Further in vivo studies with LCN2, myeloid differentiation primary response (MyD88) and TIR-domain-containing adapter-inducing interferon- β (TRIF) knock out mice confirmed this observation [537]. LCN2 was elevated and reduced *M. avium* growth in the blood of infected mice. Since *M. avium* is an intracellular pathogen, subcellular imaging showed that the mycobacteria avoid LCN2-mediated immunity by residing in the Rab11 + endocytic recycling pathway which still allows them access to transferrin [537]. The Rab5 and Rab7 GTPases were also investigated as they control early and late endosomal fusion, respectively. A well-known strategy that pathogenic mycobacteria employ to persist within macrophages is by inhibiting phagosomal maturation. In vitro studies using murine BMDM revealed that those expressing the dominant negative Rab5, Rab5(S34N), limited the concentration of iron within the *M. avium* phagosome which resulted in the phagosome maturing and ultimately killing the mycobacteria when compared to wild-type Rab5 BMDM. Therefore, early endosomal fusion is required for the mycobacteria to persist in these early phagosomal compartments but *M. avium* requires an adequate iron supply to prevent further phagosome maturation [538].

Copper is essential to mycobacteria with its role as a required co-factor in the *aa3*-type cytochrome c oxidase, which is essential for *M. tuberculosis* growth. However,

most studies on copper in mycobacteria have focused on resistance to copper stress as copper toxicity in the macrophage phagosome is a known antimycobacterial strategy employed by the host. Several studies have characterised the transcriptional response to copper but the exact mechanisms of copper uptake and not yet fully defined [539]. However, copper uptake is known to be mediated by outer membrane porins in mycobacteria [540]. Other membrane proteins have been identified, such as CtpV which acts as a copper efflux pump and MctB which regulates intracellular copper levels, as being important for *M. tuberculosis* copper resistance and virulence [541, 542]. *M. tuberculosis* senses copper via two copper responsive transcriptional regulators CsoR and RicR [543, 544]. Both of these regulators control genes that contribute to copper resistance and virulence of *M. tuberculosis*. DNA binding of CsoR is affected by binding to copper and derepresses the expression of an operon which includes *ctpV*, in response to high copper levels. RicR regulates a copper induced regulon including the methallothionein *mymT* a copper binding protein that helps to protect against copper toxicity [545]. Mutation of RicR resulting in an inability to respond to copper led to increased copper sensitivity and attenuated virulence in mice suggesting that this regulon is important for protecting *M. tuberculosis* against copper stress during infection [546]. The toxicity of copper in bacteria is thought to be due to copper removing iron from iron sulfur clusters, indeed, transcriptomic data from *M. tuberculosis* under copper stress also implicates damage of iron sulfur cluster enzymes [12, 539].

Zinc is an essential nutrient for mycobacteria and so access to this metal must be crucial for establishing and persisting in the host [547]. Mycobacteria can sense zinc levels and adapt to allow for persistence during zinc limited conditions [548–550]. The zinc import systems in mycobacteria are not clearly defined but are likely to consist of unspecific transport divalent cations transporters from the CorA, MgtE, ZIP, NiCo and Pit families of uptake systems. P-type ATPases may also be able to import zinc ions into the cell [551]. Export of zinc to maintain zinc homeostasis during exposure to high zinc levels is an important resistance mechanism as *M. tuberculosis* infected macrophages import high amounts of zinc into the phagosome. *M. tuberculosis* increases the expression of a P-type ATPase CtpC in response to infection and exposure to zinc. Loss of this ATPase leads to increased zinc levels within the bacteria and a reduced capacity to grow within macrophages [79]. Another gene Rv3929, which is encoded in the same operon as CtpC, is upregulated in response to zinc stress. This gene encodes a putative metallochaperone that could potentially supply zinc to the CtpC transporter. Zinc sequestration may

also occur to restrict the growth of extracellular bacteria present in the necrotic granulomas in active TB as S100 proteins are produced at high levels by neutrophils in the granulomas of TB patients [552]. Zinc starvation has been shown to lead to ribosome hibernation in *M. smegmatis* [553]. This ribosome hibernation results in antibiotic tolerance of *M. tuberculosis* in mouse lungs and so while zinc sequestration may restrict the growth of the bacteria it may also potentially cause the bacteria to be more difficult to treat and eradicate during infection [554].

Manganese, nickel and cobalt are also essential nutrients for *M. tuberculosis*. CtpC was also found to transport manganese allowing for metalation of secreted proteins, and deletion of CtpC resulted in altered manganese homeostasis and increased sensitivity to oxidative stress [555]. Nickel is used as a co-factor for the *M. tuberculosis* urease UreA [556, 557]. Mycobacteria are some of the few bacteria that can synthesize vitamin B₁₂, a complex cobalt containing molecule, and have three vitamin B₁₂-dependent enzymes [558–560]. However, the mechanisms by which these metals are taken up by mycobacteria not yet known. Host sequestration of manganese (and zinc) by S100 proteins, including calprotectin, a chelator of extracellular iron and zinc, is induced in response to bacterial infections [561]. Sequestration of extracellular manganese and zinc is likely to occur via S100 proteins as they are highly produced by neutrophils in the lung granulomas in TB patients [552]. This would likely impact the growth of extracellular bacteria that are present within necrotic granulomas in active TB, but further studies are required to clearly determine the role of these metals in *M. tuberculosis* virulence.

Therapy

An alteration in metal homeostasis, either systemically or within the lung compartment is associated with the pathogenesis and poor outcomes in several respiratory diseases. Therefore, correcting metal dysregulation could provide a novel approach to treat these diseases, potential counteracting one of the driving forces in the pathogenesis of these diseases. The potential of using iron chelators to reduce pulmonary iron overload in chronic lung diseases such as COPD, CF and IPF are an exciting prospect [319, 562–564]. Reducing iron overload specifically in the lung in chronic lung diseases could help to counteract the metal dysregulation driving chronic inflammation and also potentially limit the respiratory infections that commonly occur in people living with these conditions by depriving airway microbes from this essential nutrient. However, as iron deficiency and anaemia is prevalent in the majority of these diseases, any direct targeting of iron in the lung must avoid reducing systemic iron levels further and re-supplementing systemic iron in iron deficient

Table 2 Studies investigating the role of metals in respiratory diseases and potential treatments targeting metal homeostasis/dysregulation

Disease	Metal	Human studies	Therapy	Animal studies
Asthma	Iron	Anaemia is associated with asthma [258] Ferritin stores associated with decreased incidence of asthma [257] Lower non-haem iron in BAL, and increased number of iron-loaded cells seen in asthma patients [261] <i>DMT1</i> and <i>TFR1</i> expression increased in airway tissue of patients with severe asthma [261]	IV iron administration to correct iron deficiency in asthma patients [567]	Mice: In house dust-mite model, macrophages with highest <i>Tfr1</i> also upregulate <i>IL13</i> [261] Mice: Iron supplementation decreased airway eosinophilia and type 2 cytokines in ovalbumin-induced allergic asthma model [149] Rats: Dietary iron supplementation causes increased butyrate [264, 568]
	Zinc		Zinc supplementation in children with asthma [569] Iron chelators to reduce lung iron overload [320, 561–563]	
COPD	Iron	Iron metabolism altered in COPD [299–303] Current/former smokers have high iron in sputum, exhaled breath condensate, AMs [211, 304–313] Anaemia and non-anaemic iron deficiency often accompany COPD [314] Anaemia can predict mortality [315–317] Iron-deficient patients have more exacerbations [318] COPD AMs do not suppress hepcidin in response to iron deficiency [317] AMs from smokers have higher iron than non-smokers [310]		Rats: Iron accumulates in respiratory epithelial cells after smoke exposure smoke [305] Mice: Smoke exposure reduces hepcidin expression in COPD models [54] Mice: Cigarette smoke increases expression of <i>FPN</i> , ferritin and the <i>TFR1</i> on AMs and inhibits hepcidin induction by LPS [54]
	Zinc	Zinc lower in COPD patients [77, 324] Low dietary zinc in smokers linked to higher incidence of COPD [325] Chronic smokers have increased ZIP8 expression [326] Zinc deficiency allows cadmium to accumulate to toxic levels in smoker's AMs [332]		Mice: Zinc depletion caused increased lung cadmium burden and permanent lung damage [329] Mice: Zinc deficiency causes increased airway inflammation when exposed to smoke [331] Mice: Zinc supplementation decreases AM numbers after smoke exposure [331]
Copper		COPD patients with stable disease have lower copper in EBC than healthy non-smokers [336] Patients with Menkes disease have higher incidence of emphysema [337, 338]	Administration of copper/heparin [337]	Copper deficiency induces emphysema in several animal models [333–335] Rats: Copper deficiency reduces AEC integrity [335]
	Selenium	Selenium responsive genes altered in COPD patients [340] Patients with higher selenium have a higher FEV1 [341]		
Manganese		Patients with severe COPD have higher manganese levels [343]		

Table 2 (continued)

Disease	Metal	Human studies	Therapy	Animal studies
Cystic Fibrosis	Iron	Systemic iron deficiency common [359–365] Increased iron in airways and sputum, and within AMs and IMs [366–369, 383] CF macrophages have altered iron metabolism [370] ΔF508-CFTR expressing AECs have altered iron homeostasis, and release more iron [371, 372] Serum and plasma zinc are decreased [376, 377]	Gallium administration to CF patients with chronic infection to improve lung function [374]	
	Zinc	Zinc is elevated in CF airway [383, 384] Regions in the lung enriched with calprotectin allow <i>S.aureus</i> to coexist with <i>P.aeruginosa</i> [385]		
	Copper	Increased copper in sputum [383] Reduced activity of copper enzymes [386, 387]		
Non-cystic fibrosis bronchiectasis	Selenium	Selenium deficiency is observed [389–392]		
	Iron	Elevated iron in sputum [383, 384]		
	Zinc	Elevated zinc in sputum [383, 384] Zinc may be reduced or unchanged in serum [401, 402]		
Lung Cancer	Iron	Patients have higher ferritin levels [426] Increased LCN2 and TFR seen [427, 428] Increased miR-20a expression in NSCLC inhibits ferroportin expression [431]	Iron deprivation, iron supplementation to suppress tumours [425] Anti-transferrin therapy [425]	Mice: Downregulation of LCN2 or TFR in adenocarcinoma model suppressed tumour growth [427, 428] Mice: Iron given i.v. inhibited tumour growth [437]
	Zinc	Decreased serum zinc is a biomarker of lung cancer [422] Increased plasma zinc correspond with lower risk of lung cancer [424]		
	Copper	Increased copper in serum [422]		
	Manganese	Increased MnSOD activity [438–440] Polymorphisms in MnSOD linked to higher risk of lung cancer [441]		

Table 2 (continued)

Disease	Metal	Human studies	Therapy	Animal studies
IPF	Iron	Patients have increased iron deposition [459, 460] Iron is elevated in BAL fluid [458] BAL cells have increased iron-dependent ROS generation [462]	Iron chelators to reduce lung iron overload [320, 561–563]	
	Zinc	Reduced zinc in BAL fluid [458]		Mice: CuZn-SOD/SOD1 deficient mice are protected from asbestos induced lung injury [470]
	Copper	Copper is elevated in BAL fluid [458] LOXL2 expression is increased [467]		Mice: LOXL2 expression increased by bleomycin [466] Mice: LOXL2 inhibitory antibody protects against bleomycin induced lung injury [468]
	Other	Chromium, nickel, manganese lower in BAL fluid [458]		
PAH	Iron	Iron deficiency and/or anaemia common in PH patients [478, 479] PH is a major cause of mortality in those with chronic haemolytic anaemias [480] Iron accumulation in lungs and AMs of PH patients [482] 70% of those with G208C mutation in NFU1 develop PAH [481]	Iron supplementation in iron deficient patients [322]	Mice: Loss of IRP1 leads to PH [485] Mice: Expression of hepcidin-resistant FPN in smooth muscle causes PAH [483]
	Other	Zinc and copper transport implicated in pulmonary vascular homeostasis [487, 488] Selenium may be elevated in PH [489]		Mice: Low copper diet in PH model has no effect on RV failure [490] Chicken: Dietary supplementation of selenium in PH model prevented RV hypertrophy [491]

Table 2 (continued)

Disease	Metal	Human studies	Therapy	Animal studies
Mycobacterial infections (TB & NTM)	Iron		Gallium compounds to inhibit bacterial iron dependent proteins and siderophores [375, 564, 565] Modulation of iron to support host TB defences [7, 534] Pyrazolopyrimidinone (PZP)—anti mycobacterial compound chelates intracellular iron [570] Synthetic fluorescent iron chelators restrict <i>M. avium</i> within macrophages [571]	Mice: Infected macrophages take up iron and deliver it to phagosomes containing mycobacteria via TFR1 [83] Mice: <i>Nramp1</i> ^{pl69} (functionally deficient) expression increases susceptibility to <i>M. avium</i> infection in mice [531] Mice: Expression of functional NRAMP1 had <i>M. avium</i> growth that increased with iron administration [531] Mice: BMDMs infected with <i>M. avium</i> upregulate H-ferritin[533] Mice: <i>M. avium</i> infection induced LCN2, which in turn can limit <i>M. avium</i> growth [536]
	Zinc	Zinc is essential for mycobacteria [546] Neutrophils in necrotic granulomas produce S100 proteins, possibly to sequester zinc and inhibit bacterial growth [551] Zinc deficiency in <i>M. smegmatis</i> causes ribosomal hibernation [552]		Mice: Zinc starvation causing ribosome hibernation in <i>M. tuberculosis</i> may lead to antibiotic tolerance [553]
	Copper	Copper responsive transcription factors control genes important for <i>M. tuberculosis</i> virulence [542, 543]		Mice: RicR mutation reduced <i>M. tuberculosis</i> virulence [545]
	Other	Manganese, nickel and cobalt are essential nutrients for <i>M. tuberculosis</i> [554]		

and anaemic patients may benefit overall survival and exercise capacity. Another approach to counteracting high iron in the lung is the use of gallium, a non-essential metal that acts as an iron mimetic. Gallium containing compounds have been shown to improve the lung function of CF patients with chronic infections. Gallium compounds have been shown to inhibit bacterial iron dependent processes in major respiratory pathogens, *P. aeruginosa* and *M. tuberculosis*, *M. avium*, and *M. abscessus* [373, 374, 565, 566]. As gallium can replace iron in iron dependent bacterial proteins but is not redox active it interferes with function of iron-dependent essential processes such as the respiratory chain and kills the bacteria. Metal metabolism dysfunction could also potentially be corrected very easily by dietary supplementation, such as selenium supplementation for influenza and zinc supplementation for asthma. However, these treatments are likely to only have a beneficial effect in patients with deficiencies in these metals and so personalised treatment approaches are vital.

Immunonutrition is a novel concept where patients are given a personalized diet rich in vitamins and minerals such as zinc, selenium and iron to modulate immune responses in order to improve disease outcome and recovery. The modulation of the gut microbiota by immunonutrition has been reviewed as a potential therapeutic strategy for obese COVID-19 patients [567]. This novel idea of utilizing the gut-lung axis might also have potential influences on the outcome of bacterial infections such as *M. tuberculosis* that rely on metals such as iron for their replication and growth. Perhaps a personalized diet to deprive the bacteria of certain metals may one day be a potential therapeutic strategy for pulmonary bacterial infections. An overview of potential therapies for respiratory disease by targeting metal homeostasis and dysregulation are presented in Table 2.

Conclusion

The lungs are a unique compartment in the body in that they are constantly exposed to the environment. We are constantly breathing in air from our surroundings which consists of microorganisms, particles, chemicals, and pollutants. The resident immune cells within this niche must be able to deal with the influx of microbes and particles without causing inflammation. Unfortunately, environmental, lifestyle and genetic factors can lead to chronic lung inflammation resulting in an array of lung diseases that result in obstruction and damage to the airways and a decline in lung function. Furthermore, recurrent respiratory infections are common among those with chronic lung disease and contribute to progressive decline in lung function. The role of metabolites and nutrients present in lung

microenvironment are just beginning to be investigated and appreciated. Metals essential to most organisms, such as iron, copper and zinc are found to be altered in several lung diseases. These metals play important roles both in the function of immune cells in the lung and in the microbes present in the airways. There remains much to be explored on the cause and source of this metal dysregulation during lung disease, as well as the exact mechanistic consequences of this on the immune cells and the constituents of the airway microbiota. We believe that targeting disturbed metal metabolism seen in many lung diseases is a novel approach that could potentially address both immune-driven pathology and the increased occurrence of respiratory infections. We hope that future work will further our understanding of the complex interactions between lung immunity, the lung microbiome and the lung microenvironment and will lead to much improved treatments for the many diseases of the lung that yet remain to be curable.

Abbreviations

2DG: 2-Deoxy-D-glucose; AD: Adenocarcinoma; AEC: Alveolar epithelial cells; ALAS: 5-Aminoolevulinic synthase; AM: Alveolar macrophage; ATP: Adenosine triphosphate; BALF: Bronchoalveolar lavage fluid; BCR: B cell receptor; Bfr: Bacterioferritin; BMDM: Bone marrow derived macrophage; cDCs: Conventional dendritic cells; CF: Cystic fibrosis; CFTR: CF transmembrane conductance regulator; COPD: Chronic obstructive pulmonary disease; CXCR: Chemokine receptor; DAMP: Damage associated molecular patterns; DCs: Dendritic cells; DMT1: Divalent metal transporter 1; EBC: Exhaled breath condensate; EPX: Eosinophil peroxidase; FEV1: Forced expiratory volume in 1s; FPN: Ferroportin; FTH: Ferritin heavy chain; GM-CSF: Granulocyte macrophage-colony stimulating factor; Gpx4: Glutathione peroxidase 4; HFE: Hereditary hemochromatosis (HH) susceptibility gene; HIF-1 α : Hypoxia inducible factor-1 α ; HO-1: Heme oxygenase 1; iBALT: Bronchial associated lymphoid tissue; IFN- γ : Interferon- γ ; ILCs: Innate leukocyte cells; IM: Interstitial macrophage; iNKT: Invariant natural killer cells; IPF: Idiopathic pulmonary fibrosis; IPL: Isolated perfused lungs; IRF: Interferon regulatory factor; iTAMs: Iron-loaded TAMs; JNK: C-Jun N-terminal kinase; KIR: Killer cell-immunoglobulin like receptor; LCN-2: Lipocalin-2; LFA: Lymphocyte function-associated antigen; LfR: Lactoferrin receptor; Lipoic acid synthase; LOXL2: Lysyl oxidase-like 2; LPS: Lipopolysaccharide; LRP1: Lipoprotein receptor related protein 1; LT: Lymphoid tissue inducer; MAIT: MR1-restricted T cells; MHC: Major histocompatibility complex; MnSOD: Manganese superoxide dismutase; moDCs: Monocyte-derived dendritic cells; MPO: Myeloperoxidase; mRNA: Messenger ribonucleic acid; MsrB1: Methionine sulfoxide reductase B1; MTs: Metallothioneins; MyD88: Myeloid differentiation primary response; NETs: Neutrophil extracellular traps; NK: Natural killer; NO: Nitric oxide; NRAM1: Natural resistance associated macrophage protein 1; NTM: Non-tuberculous mycobacteria; OH $\cdot$: Hydroxyl radical; OX PHOS: Oxidative phosphorylation; PAH: Pulmonary arterial hypertension; pDCs: Plasmacytoid DCs; PDH: Pyruvate dehydrogenase; PH: Pulmonary hypertension; PHDs: Prolyl hydroxylases; RNSO: S-nitrosothiols; ROS: Reactive oxygen species; SC: Squamous cell; SCD: Sickle cell disease; SCFA: Short chain fatty acid; SCLC: Small cell lung cancer; SelK: Selenoprotein K; SOD: Superoxide dismutase; STAT: Signal transducer and activator of transcription; TAMs: Tumour associated macrophages; TB: Tuberculosis; TCR: T cell receptor; TGF: Transforming growth factor; Th: Helper T cell; TLR: Toll like receptor; TLS: Tertiary lymphoid structures; TME: Tumour microenvironment; Tregs: T regulatory cells; TFR1: Transferrin receptor 1; TRIF: TIR-domain-containing adapter-inducing interferon- β ; T_{RM}: Lung resident memory T cells; tRNA: Transfer ribonucleic acid; α KGDH: α -Ketoglutarate dehydrogenase; β 2-m: β 2-Microglobulin.

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Authors' contributions

Conception: SMC; Preparation of manuscript: CH, NMW, SK, NCW, JS, LF, SMC; Manuscript revision and editing: SMC, CH, NMW, SCD; Figure preparation: JS; Table preparation: LF, NCW, CH. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interests

The authors have no competing interests to declare.

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Review Article

Iron and mitochondria in the susceptibility, pathogenesis and progression of COPD

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Chronic obstructive pulmonary disease (COPD) is a debilitating lung disease characterised by airflow limitation, chronic bronchitis, emphysema and airway remodelling. Cigarette smoke is considered the primary risk factor for the development of COPD; however, genetic factors, host responses and infection also play an important role. Accumulating evidence highlights a role for iron dyshomeostasis and cellular iron accumulation in the lung as a key contributing factor in the development and pathogenesis of COPD. Recent studies have also shown that mitochondria, the central players in cellular iron utilisation, are dysfunctional in respiratory cells in individuals with COPD, with alterations in mitochondrial bioenergetics and dynamics driving disease progression. Understanding the molecular mechanisms underlying the dysfunction of mitochondria and cellular iron metabolism in the lung may unveil potential novel investigational avenues and therapeutic targets to aid in the treatment of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory respiratory disease, and the third leading cause of death worldwide. The disease is characterised by emphysema, or destruction of alveoli, chronic bronchitis, and destruction and remodelling of the small airways. Disease pathogenesis is punctuated by exacerbation events where patients experience a rapid and significant decline in lung function, often driven by acute respiratory infections [1]. While cigarette smoking is widely accepted to be the primary risk factor for COPD development [2], up to 30% of COPD patients have never smoked, illustrating the heterogeneity of risk factors associated with disease susceptibility [3]. COPD disproportionately affects those in low- and middle-income countries, which account for an estimated 80% of COPD deaths worldwide [4]. Available pharmacological interventions for COPD are merely symptomatic and aimed at the alleviation of exacerbation events. No current treatment exists that can directly stop or reverse disease progression [5]. Considering the above, a more comprehensive understanding of the exact molecular mechanisms underpinning disease susceptibility and pathogenesis is urgently needed to identify both preventative strategies and novel therapeutic targets to cure the disease.

Mitochondria are double membrane bound organelles, present in virtually all eukaryotic cells. In addition to their traditional role as the cell's powerhouse, mitochondria are also integral to processes key for normal homeostasis, such as fatty acid and protein synthesis, calcium signalling and regulated cell death. Disturbances to mitochondrial morphology and function have been reported in several respiratory cells in COPD, including cells of the innate immune system. Given the high energy demands of certain lung cell types, such as type II alveolar epithelial (AT2) cells in surfactant production, mitochondrial dysfunction may have profound consequences for lung physiology [6]. In conjunction with a reduced energy capacity, perturbations in mitochondrial function can cause significant oxidative damage to cells through uncontrolled production of mitochondrial reactive oxygen species (ROS) which can lead to cellular stress, senescence and ultimately cell death.

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Iron is an essential element for all forms of life and is required for numerous key mammalian cell functions, including respiration, DNA synthesis and cell division. The ability of iron to readily switch between oxidation states facilitates its role as a catalyst, although the ensuing generation of free radicals through Fenton chemistry requires careful handling and storage of iron to limit cytotoxicity. Mitochondria are the main storage and utilisation sites for iron in the cell, producing the key metallocofactors iron-sulfur clusters (FeS clusters) and heme [7]. The lung constitutes a unique environment for iron handling, through exposure to both exogenous iron inhaled in particulate matter, and to endogenous iron accessed through the pulmonary vasculature. Additionally, the prevalence of respiratory infections requires careful handling of iron by the immune cells of the lung to limit its availability to pathogens for their own proliferation and virulence [8]. As a result, altered iron homeostasis can have deleterious consequences in the lung.

This review will detail the role and function of mitochondria and iron metabolism in normal lung physiology and how their dysfunction is associated with COPD pathogenesis. Any available evidence for the interplay between mitochondrial function and cellular iron metabolism will also be discussed. Finally, this review will provide an up to date summary of the possible therapeutic ways to target either mitochondrial dysfunction or iron metabolism in the lung and the possible implications for COPD therapy.

Mitochondria in the lung at homeostasis

Residing in most eukaryotic cells, mitochondria are essential organelles that command numerous processes underpinning normal cellular homeostasis, ranging from the regulation of cellular bioenergetics to heme metabolism and innate immune signalling. Considered to be of bacterial origin, mitochondria possess a unique double-membrane structure [9]: the outer mitochondrial membrane (OMM) allows for passive transport of metabolites [10], while the inner mitochondrial membrane (IMM) is a highly vaginated diffusion barrier, with the transport of metabolites and ions facilitated by mitochondrial carrier proteins [11]. Mitochondria also possess their own unique, maternally inherited circular genome (mtDNA), which encodes several essential components of the electron transport chain (ETC), which generates adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) [12]. Interestingly, an additional isoform of ETC complex IV is present in the lung, which increases the oxygen binding activity of cytochrome *c* oxidase compared with other tissues and is a key metabolic determinant of maximal ATP production in the healthy lung [13]. The ETC also regulates the production of mitochondrial reactive oxygen species (mtROS), which act as important second messengers under normal physiological conditions and can be harnessed by innate immune cells of the lung to facilitate oxidative burst and aid phagocytosis.

Adaptations in mitochondrial bioenergetics and dynamics are characteristic of lung cells with high metabolic activity, such as those lining the alveolar and bronchial epithelia. The number, position and size of mitochondria in respiratory epithelial cells is dependent on the cell type and their respective function. In ciliated cells, such as those of the bronchial epithelium, the mitochondria localise to the apical side of the cell to support the production of mucus and beating of cilia [14]. Interestingly, secretory cells of the mouse tracheal epithelium are reported to have enlarged mitochondria when compared with ciliated cells; however, the precise purpose of this phenomenon remains unclear [15]. AT2 cells have also been reported to reduce the number and size of their mitochondria upon transdifferentiation, and to yield AT1 cells with decreased bioenergetic demands [16,17]. However, the mitochondrial calcium uniporter has recently been shown to play a key role in A2-to-A1 differentiation, with its deletion inhibiting alveolar regeneration following pneumococcal pneumonia, suggesting that the dogma that mitochondria are lost during transdifferentiation may be outdated [18]. In AT2 cells, mitochondria reside closer to the nucleus, facilitating the high ATP needs of these cells allowing them to efficiently produce, secrete and recycle pulmonary surfactant, a key component in immune cell signalling and the maintenance of alveolar integrity [6]. Surfactant is composed of 90% lipids, the production of which is reliant on production of acetyl CoA by mitochondria to drive *de novo* lipid synthesis: under conditions of cellular stress, mitochondria of AT2 cells have been suggested to transform into lamellar bodies to further surfactant production [19,20]. These high ATP needs are also dependent on mitochondrial fusion, where the GTPases mitofusin 1 (MFN1) and mitofusin 2 (MFN2) promote the joining of two mitochondria, allowing for the exchange of genetic and nutritional material [21]. An increase in fusion leads to the production of elongated mitochondrial network, favoured by cells with a high reliance on OXPHOS like AT2: as such, MFN1 and are 2 vital to their normal homeostatic function [22,23]. Recently, it was shown the AT2 cells are also capable of lactate consumption as a substrate for rapid oxidative ATP production [24]; however, the effects of lactate consumption on mitochondrial fusion remain unclear. In contrast, cells which favour glycolysis, including rapidly dividing cells such as lung fibroblasts and those of the pulmonary endothelial vasculature, show an increase in mitochondrial fission and display a more punctate-appearing network of mitochondria [25]. Until recently, mitochondria in endothelial cells (ECs) were

thought to rely on glycolysis to generate 85% of their ATP requirements [26]. However, emerging evidence shows that EC mitochondria are metabolically active [27], have an important signalling role [28], and their dynamic behaviors orchestrate EC homeostasis [29].

In the lung, tissue-resident cells of the innate immune system can be found in the lung parenchyma and alveolar space, with further leukocytes recruited to the lung in response to injury or infection [30]. The metabolic response of innate immune cells is highly heterogeneous and is primarily dependent on both the cell type and the local microenvironment. Alveolar macrophages (AMs) are faced with a local microenvironment containing low levels of glucose and high levels of lipid-rich pulmonary surfactant; as a result, AMs present with lower levels of glycolysis and favour OXPHOS pathways as a mechanism to fuel high levels of lipid catabolism [31,32]. In contrast, recruited leukocytes may be less reliant on OXPHOS, with pro-inflammatory stimuli shown to induce a switch towards glycolysis as a fuel source. The ensuing breaks in the TCA cycle can lead to the accumulation of intermediate metabolites which aid pro-inflammatory functions: for example, succinate activates hypoxia inducible factor 1 α (HIF-1 α) to drive expression of inflammatory genes [33]. However, the precise effects of the lung microenvironment and its unique nutrient composition on the mitochondrial metabolism of these recruited cells remains poorly understood. In addition, little remains known about the preferred metabolic state of interstitial macrophages and the influence of their proximity to the pulmonary vasculature on such, or macrophages of the central airways, which encounter increased levels of mucin glycoproteins which coat the airway epithelium. For a more in-depth review on the effects of mitochondrial metabolism on the immune response, readers are referred elsewhere [34,35].

Iron metabolism in the lung at homeostasis

Iron is an essential element for all forms of eukaryotic and prokaryotic life, key in mediating processes such as DNA synthesis and repair, OXPHOS, erythropoiesis, and the host immune response to infection. Systemically, the majority of iron is bound to hemoglobin and utilised at the bone marrow to fuel erythropoiesis, with iron recycled through erythrophagocytosis as mediated through heme oxygenase 1 [36]. Its wide range of oxidation states and ensuing redox activity facilitates its role as a catalytic component of metalloproteins, primarily through iron-sulfur (FeS) clusters and heme. Cellular iron exists primarily in the ferric (Fe³⁺) and the more bioavailable ferrous (Fe²⁺) state, with oxidation/reduction reactions governing the interchangeability between these two states [37]. This redox activity can have detrimental consequences for the cell: free Fe²⁺ can form toxic reactive oxygen species (ROS) via the Fenton/Haber–Weiss reaction. Thus, in its free ion form, iron must be carefully sequestered into proteins to prevent its toxicity; as such, dysregulation of iron homeostasis can have dire consequences for the lung.

The upper respiratory tract is exposed to up to 25 μ g of exogenous iron per day, which can increase in areas of high urbanisation [38,39]. Up to 27% of lung iron is detected in bound or unbound forms in bronchoalveolar lavage fluid (BALF) [40]: soluble iron-carrying mediators include the systemic iron transport protein transferrin, the glycoprotein lactoferrin and the iron siderophore lipocalin-2 [41]. Chelation of free iron by these extracellular mediators is key not only in limiting cytotoxicity to the alveolar epithelium to ensure proper gas exchange but also in limiting bioavailable iron to respiratory pathogens. Indeed, secretion of lipocalin-2 by epithelial cells and lung-resident innate immune cells can bind siderophores (microbial iron-scavenging proteins) to sequester iron from respiratory pathogens, with lactoferrin also reported to have bactericidal functions [42–45].

Transferrin and non-transferrin bound iron is primarily imported by lung epithelial and immune cells through transferrin receptor 1 (CD71) and divalent metal transporter 1 (DMT1), respectively, with additional iron import also facilitated through the zinc uptake receptors ZRT/IRT-like protein 8 and 14 (ZIP8 and ZIP14) and low-density lipoprotein-related protein 1 (LRP1) [46–49] (Figure 1). Lung resident macrophages and neutrophils can also import iron through natural resistance-associated macrophage protein 1 (NRAMP1). Little is known about the way lung cells utilise heme iron, although higher levels of iron are observed in AMs of patients experiencing lung haemorrhage, suggesting AMs have a capacity for erythrophagocytosis [50,51]. Following its cellular uptake, iron enters the labile iron pool where it can be further transported to organelles such as the mitochondria for metabolic utilisation. Cytosolic iron can be inertly stored in the ferritin nanocage composed of heavy and light-chains. The metallochaperone poly C binding proteins 1 and 2 (PCBP1 and 2) can aid in iron delivery to ferritin, while nuclear receptor coactivator 4 (NCOA4) aids in the degradation of ferritin to release free iron in a process termed ferritinophagy [52,53].

The cytosolic iron regulatory proteins 1 and 2 (IRP1 and IRP2) regulate cellular iron metabolism. Through their binding of conserved hairpin loop structures termed iron response elements (IRE) in the untranslated regions of mRNA transcripts with a role in iron metabolism, cellular labile iron storage can be increased or decreased to meet need. IRP1/2 can bind the 5' UTR of iron-associated mRNA transcripts to downregulate expression (ferritin, ferroportin) and the 3' UTR of iron retention-associated genes (transferrin, DMT1) to increase intracellular iron content.

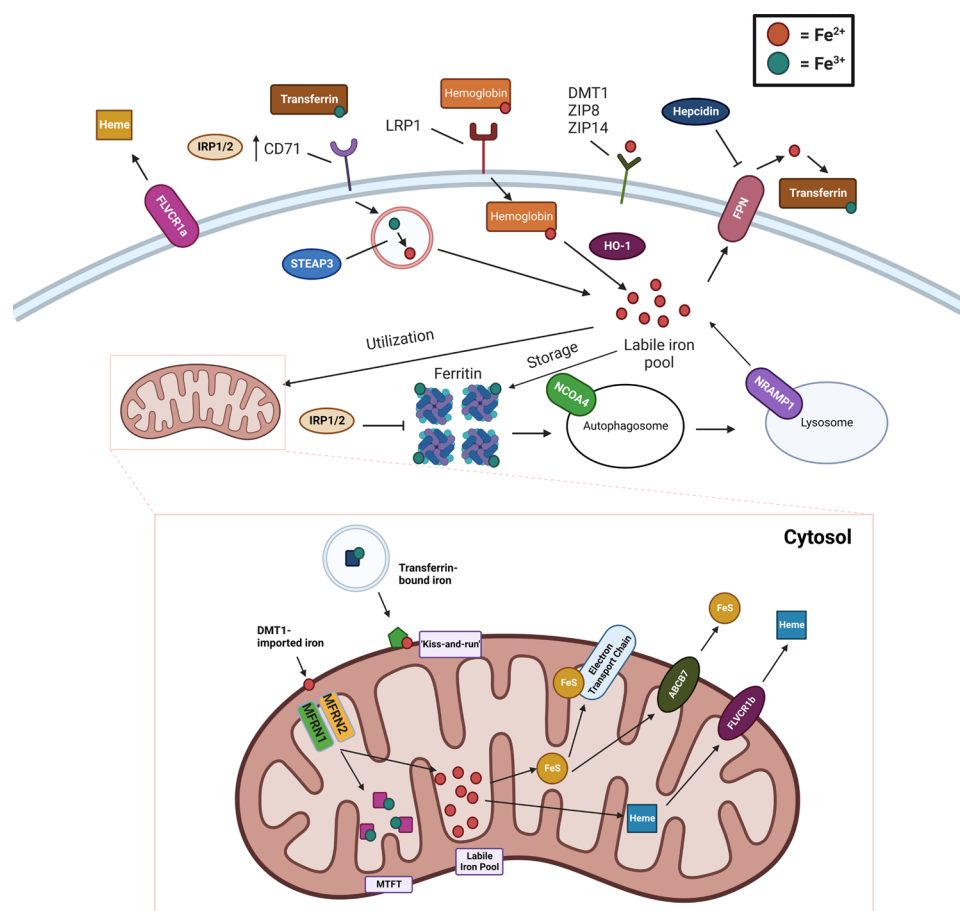


Figure 1. Cellular and mitochondrial iron metabolism

Free circulating ferrous (Fe^{2+}) iron can be imported to the cell through protein transporters including DMT1, ZIP8 and ZIP14, while transferrin-bound ferric (Fe^{3+}) iron is imported by the transferrin receptor (CD71) and reduced to ferrous iron by STEAP3. Hemoglobin is imported by LRP1 and ferrous iron liberated through HO-1. Free iron enters the labile iron pool, where it has numerous fates depending on cell activation state. Iron can be inertly stored in ferritin, which can be degraded via lysosomal NCOA4-mediated ferritinophagy and used to replenish the labile iron pool via NRAMP1. Iron is delivered to the OMM through two mechanisms: delivery of DMT1-imported iron, or endosomal delivery of transferrin bound iron via an unknown chaperone termed 'kiss-and-run'. Iron import through the IMM is mediated by MFRN1 and MFRN2. One inside the mitochondria, iron can be stored in MTFT or in the mitochondrial labile iron pool where it can be utilised for FeS or heme synthesis, which are exported to the cytosol through ABCB7 and FLVCR1b, respectively. Iron and heme iron are exported from the cell through ferroportin (FPN) and FLVCR1a respectively, with the former regulated by hepcidin. The iron regulatory proteins IRP1 and IRP2 post-transcriptionally regulate iron metabolism genes to increase or decrease intracellular iron stores depending on cellular or systemic cues.

IRP1 senses intracellular iron levels through its 4 Fe-S clusters: under iron replete conditions, IRP1 acts as an aconitase, but loss of this cluster in iron deplete conditions facilitates its IRE binding and post-transcriptional regulation of iron metabolism [7,54].

Ferrous iron is exported from lung cells into the extracellular space by the transmembrane protein ferroportin, the only documented iron exporter, where it is bound to circulating transferrin in serum following its oxidation [55]. Hepcidin, a small peptide hormone released from the liver, can induce the endocytosis and lysosomal degradation of ferroportin to decrease iron export from cells in response to systemic iron levels [56–58]. AMs have also been observed to express endogenous hepcidin in response to LPS, potentially as a mechanism to sequester iron intracellularly [59]. This endogenous hepcidin has proved essential for AM bactericidal function, with murine AMs from a hepcidin deficient model showing reduced secretion of $\text{TNF-}\alpha$ and IL-6, and proving unable to control the growth of *Streptococcus pneumoniae* *in vitro* [60]. In murine models exhibiting a global loss of hepcidin expression, only

certain populations of AMs exhibited subsequent iron loading, suggesting that ferroportin may be differentially expressed between AM subsets, or indeed that a different lung-specific mechanism may exist to regulate ferroportin expression [49].

Mitochondrial iron metabolism

Mitochondria constitute the primary iron storage and utilisation sites in the cell (Figure 1). The precise mechanism for delivery of ferrous iron to the mitochondria is unknown, with two methods proposed. The first is the reduction of ferric iron in the endosome, followed by its DMT1-mediated transport across the cell membrane and transport to the OMM by an unidentified chaperone protein [61]. The second is the direct endosomal delivery of transferrin-bound iron, referred to as ‘kiss and run’ [62]. The presence of voltage-dependent ion channels in the OMM makes it extremely permeable to small ions, facilitating the diffuse transport of iron. In contrast, the tight diffusion barrier of the IMM necessitates the presence of hydrophobic carrier proteins for ion transport. Mitoferrin 1 and 2 (MFRN1 and MFRN2) are the only known mitochondrial iron transporters, originally characterised through their yeast orthologues [63]. The exact molecular details surrounding iron transport by the mitoferrins remain unclear, although the two appear to possess complementary functionalities. Expression patterns suggest that MFRN1 is the predominant mitochondrial iron transporter in erythroid cells, with MFRN2 thought to be preferred in non-erythroid cells such as those of the lung [64,65]. While MFRN1 and 2 are known to be expressed in the lung epithelium and AMs, little is known about both at baseline or in response to varying insults.

Upon its import, mitochondrial iron can be utilised for heme synthesis, a key signalling molecule and metallocofactor in cellular reactions including mitochondrial respiration and biogenesis and DNA synthesis [7]. While bioenergetic demand for heme synthesis is highest in erythroid cells, it is an evolutionarily conserved process and occurs in all mammalian cells, including those of the lung. The process both begins and ends at the mitochondrion, starting with the condensation of acetyl CoA and glycine by δ -aminolevulinic acid, which is catalysed by 5-aminolevulinase synthase 1 isoform in the lung, and culminating in the mitochondrial matrix with the insertion of Fe^{2+} into protoporphyrin IX as catalysed by ferrochelatase [66]. Mitochondria are also the site of FeS cluster synthesis, metallocofactors which are key components of the respiratory chain complexes and also contribute to cellular iron sensing, ribosome generation and DNA synthesis and repair [67]. The precise processes governing heme biosynthesis and signalling and FeS cluster assembly and biogenesis are extensively reviewed elsewhere [7,68–71]. Mitochondria are thought to export heme and FeS clusters through feline leukaemia virus subgroup c receptor 1 isoform b (FLVCR1b) and ATP binding cassette subfamily B member 7 (ABCB7), although the precise role of each in the lung is uncharacterised [72,73]. Mitochondria contain a small labile iron pool for utilisation in these processes and can also store iron in the mitochondrial matrix in the mitochondrial form of ferritin (MTFT). While its precise function remains broadly unknown, MTFT is expressed in the bronchial epithelium [74].

Mitochondria and iron in the pathogenesis of COPD

COPD remains a global health problem. Its cost, both in terms of economic burden and life years lost, highlights the need for a deeper understanding of disease pathogenesis at a molecular level to better inform therapeutic design. An impaired inflammatory response is central to the progressive and irreversible decline in lung function observed in COPD, with numerous cytokines (such as interleukin-8 [IL-8] and tumour necrosis factor α [TNF- α]) and chemokines (such as CCL2 and CCL3) elevated in the BALF of individuals with COPD [75]. Cells of the airway epithelium can show enhanced oxidative stress and impaired ciliary beating and repair processes, accompanied by increased mucus production and alterations in surfactant composition [76–80]. Defective lung fibroblast function is believed to associate with impaired tissue repair: small airway fibroblasts show elevated profibrotic markers and cellular senescence and possess a pro-inflammatory phenotype [81–83]. Up-regulation of surface adhesion markers by the pulmonary vasculature associates with significant monocytic and neutrophilic infiltration, with secreted neutrophil elastase and matrix metalloproteases causative of tissue destruction and obstruction of the small airways [84–87]. Up to a 20-fold increase of macrophages is observed in the COPD lung associated with an increase in recruited monocytes; however, both these recruited monocytes and resident AMs display inefficient phagocytic capabilities, facilitating bacterial colonisation in the lower respiratory tract which may drive acute exacerbation events [88,89]. AMs isolated from individuals with COPD also display defective clearance of apoptotic cells, including those of the alveolar epithelium: this is more prevalent in current smokers, potentially due to the consistent alteration of AM cell surface proteins from cigarette smoke exposure [90,91]. This inability to remove apoptotic cells, such as epithelial cells, can promote further lung damage through secondary necrosis and the release of inflammatory mediators [92].

Table 1 Altered mitochondrial metabolism in COPD

Mitochondrial process	Process name	Location	Changes in lung cells in COPD	Reference
Bioenergetics	Aerobic/anaerobic glycolysis	BECs	Increased anaerobic glycolysis in response to CSE <i>in vitro</i>	[101]
	Fatty acid β -oxidation (FAO)	AT2	Increased in CS-exposed mice	[100]
		BEC	Increased in response to CSE <i>in vitro</i>	[99]
		AMs	Differentially expressed genes involved in FAO in COPD	[97]
	TCA	Basal Cells	Decreased levels of succinate and acetyl CoA in smokers	[98]
	OXPHOS	BECs	Increased expression of OXPHOS genes <i>in vivo</i> in COPD BECs, <i>in vitro</i>	[102]
mtDAMPS	mtDNA	Plasma	Increased circulating mtDNA	[105]
		Peripheral leukocytes	Decreased copy number	[107]
		AT2	Decreased copy number in emphysematic patients	[108]
	mtROS	BECs	Increased in response to CSE <i>in vitro</i>	[22,99,110]
		AMs	Increased in COPD AMs	[109]
		AT2	Increased in response to CSE <i>in vitro</i> , emphysemic patients	[76,108]
Dynamics	Mitophagy	BECs	Induced in response to CSE <i>in vitro</i> , increased in emphysematic patients	[22,110,111]
		AT2	Increased in emphysematic patients	[108]
		Fibroblasts	Impaired in response to CSE <i>ex vivo</i>	[112]
		BECs	Impaired in response to CSE <i>in vitro</i>	[22,102]
	Fission/Fusion	AT2	Impaired fission and fusion in emphysemic patients; hyper-fusion in response to CSE <i>in vitro</i>	[108,116]
		AMs	Differentially expressed genes involved in fission and fusion processes in COPD compared with healthy smokers	[97]
		AMs	Differentially expressed genes involved in biogenesis in patients	[97]
	Biogenesis	BEC	Increased in patients with severe COPD	[102]
		AT2	Decreased in emphysemic patients	[108]
		Whole Lung	Decreased in patients with severe COPD	[113]

Abbreviations: AM, alveolar macrophage; AT2, alveolar type 2 epithelial cell; BEC, bronchial epithelial cell.

The dearth of available therapeutics aimed at directly inhibiting disease progression highlights the need for a deeper understanding of the pathways driving this excessive inflammation, tissue destruction and susceptibility to infection.

Mitochondrial dysfunction and COPD

As mentioned, mitochondria lie at the centre of cellular function in respiratory cells; through mitochondrial metabolism, signalling and biogenesis, mitochondria harness the power to control the activation and function of both the innate and adaptive immune response [35,93]. Changes and dysfunction in these mitochondrial mechanisms have been linked to the pathogenesis of several respiratory diseases, including COPD (Table 1) [6,94,95].

Mitochondrial bioenergetics

In COPD, altered metabolic responses of respiratory cells are associated with cellular senescence, injury and death. For example, AMs present with an altered metabolic and polarisation state when compared with healthy controls [96] with mitochondrial-related genes, including genes involved in mitochondrial biogenesis and FAO, significantly altered in smokers with COPD when compared with healthy smokers [97]. COPD AMs also display increased proton leak, reduced mitochondrial respiration and defective compensatory glycolysis, with AMs from healthy smoker controls exhibiting increased glycolysis at baseline [97]. This would suggest that healthy smokers attempt to adapt their metabolism as a protective mechanism whereas COPD AMs lack this response and are unable to initiate metabolic responses, perhaps due to mitochondrial dysfunction, thus rendering the cell energy deficient and unable to carry out its normal function.

The metabolic shift of lung airway epithelial cells (LAECs) in COPD is heterogenous and poorly understood. Primary basal cells (BC) have smoke-induced altered energy metabolic profiles, with decreased expression of key components of the TCA cycle [98]. Exposure of a human bronchial epithelial cell (BEC) line to cigarette smoke extract (CSE) *in vitro* increased FAO metabolism as a mechanism of promoting ROS and cell death [99]. Similarly, AT2 cells isolated from smoke exposed mice have increased fatty acid oxidation (FAO) as a mechanism to overcome glycolytic impairment [100]. In contrast, a more recent study showed differentiated primary BECs shifted to anaerobic glycolysis in response to smoke exposure [101]. Furthermore, CSE exposure was shown to robustly induce OXPHOS proteins in an immortal BEC line [102]. The discrepancies between the above studies ultimately lies in the differences between the cell types and the experimental design, with both studies using different concentrations of CSE. Furthermore, both studies rely on cigarette smoke which could be the cause of the observed phenotype, and therefore, whether increased FAO or glycolysis correlates with disease pathogenesis remains unknown. It could be suggested that in the acute stage of CS-exposure, LAECs attempt to overcome CS-induced stress by up-regulating metabolic pathways to fuel repair mechanisms and when these responses are lost cellular injury and death persists beyond repair. Interestingly, reduced FAO metabolism in murine AT2 cells in combination with a pro-inflammatory environment associate with apoptosis of AT2 cells [103]. As COPD is a chronic inflammatory disease, it would be interesting to see whether a similar phenotype is observed for AT2 cells isolated from COPD patients. Taken together these studies highlight the need for further investigations into the metabolic profile of LAECs in COPD. In the future, studies should include both healthy non-smoker and healthy smoker controls, while also accounting for different disease stages as responses may differ at end-stages of COPD. Furthermore, more cell specific studies are needed to differentiate between the metabolic response of different LAECs.

MtDAMPS

As discussed, mitochondria can influence immune cells through the release of various signals including the release of mitochondrial damage associated molecular patterns (DAMPs) [35]. One such signal, intracellular mtDNA can activate and sustain an immune response by acting as a second messenger in the activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome [104]. In contrast, during disease circulating extracellular mtDNA has deleterious effects in the body, with increased mtDNA levels linked to disease severity in several inflammatory diseases including COPD [105,106]. Intracellular mtDNA copy number has also been suggested to associate with mitochondrial dysfunction in COPD [107,108]. Isolated peripheral leukocytes from patients with COPD have a significantly lower mtDNA copy number when compared with healthy non-smokers [107]. However, when compared with healthy smokers this decrease in copy number was not significant, although the trend remained. Of note, COPD donors were at least 10 years older than healthy donors which could be confounding the results as mtDNA copy number has previously been shown to decrease with age. Similarly, AT2 cells isolated from patients with emphysema were shown to have decreased mtDNA number when compared with healthy smokers and non-smokers [108]. Reduced mtDNA levels correlated with increased superoxide generation and mtDNA damage in AT2 cells [108]. Taken together these results suggest that mtDNA copy number negatively correlates with COPD, with mtDNA copy number in AT2 cells linked with cellular dysfunction.

In addition to mitochondrial DAMPs, mtROS generated from the electron transport chain can also influence the cells of the innate immune system [6,35]. In biology, mtROS acts as a double-edged sword: it is an essential intermediate messenger in several cell signalling pathways but when produced in excessive amounts it becomes toxic to cells. As a result, sophisticated mechanisms have developed whereby mtROS can be scavenged to maintain homeostasis. In disease these mechanisms can become dysfunctional resulting in aberrant ROS production, mtDNA damage and cellular injury. In COPD, increased oxidative burden from cigarette smoke and altered mitochondrial membrane potential are both linked to increased mtROS production in AMs, AT2s and LAECs [22,76,95,99,108–110]. Interestingly, increased baseline mtROS levels in COPD AMs were linked to reduced bacterial clearance [109]. In contrast, mtROS levels in monocyte derived macrophages (MDM) from patients with COPD were shown to have unchanged mtROS levels at baseline, but after bacterial challenge levels increased significantly when compared with control, suggesting that mtROS scavenging machinery in COPD macrophages are dysfunctional [95]. The differences observed are most likely due to the difference between tissue macrophages and MDM, with AMs in the alveolar niche being exposed to other agents that can influence their basal mtROS levels. Irrespective of the differences, both studies highlight how mtROS levels in macrophages disrupt their response to bacteria, with both increased basal levels and impaired clearance of mtROS after infection reducing their phagocytic capacity.

Mitochondrial dynamics

Several studies have provided evidence for abnormalities in mitochondrial quality control mechanisms, including mitochondrial fission, fusion and mitophagy, which can have deleterious effects and contribute to disease pathogenesis. One such study reported higher mtROS in BECs treated with CSE, inducing PINK1 expression and phosphorylation of Drp1, to potentially induce mitophagy [22]. In agreement with this, isolated AT2 cells from individuals with emphysema also have mtROS-associated mitophagy [108], suggesting that increased mitophagy may contribute to the pathogenic responses observed in COPD. In contrast, increased mtROS was suggested to drive insufficient mitophagy which ultimately leads to the accumulation of damaged mitochondria, resulting in cellular senescence of AT2 cells [110–112]. Whether mtROS is a cause or a consequence of impaired mitophagy still remains up for discussion; however, mitochondrial regulation and maintenance are evidently a pathogenic sequence observed in COPD. Further investigations into early stages of the disease are needed to help understand how mtROS and impaired mitophagy contribute directly to disease progression.

Altered expression of genes and proteins involved in mitochondrial biogenesis has also been observed in COPD [97,102,108,113]. The expression of PGC-1 α , a stimulator of mitochondrial biogenesis, is significantly higher in primary BEC isolated from patients with severe COPD [102] and in peripheral lung tissue from mild COPD patients [113]. Interestingly, PGC-1 α levels were reported to decrease with disease severity, suggesting decreased biogenesis associates with severe stages of the disease [113]. In agreement with this, isolated AT2 cells from patients with emphysema had significantly decreased expression levels of mitochondrial transcription factor A (TFAM), a regulator of mitochondrial biogenesis, when compared with healthy smokers [108]. Taken together these studies would suggest that during the acute stages of the disease, biogenesis acts as a potential protective mechanism; however, as the disease progresses mitochondria can no longer compensate for the damage through biogenesis. Recently, it was shown that CSE impairs the sirtuin 1 (SIRT1)/ factor forkhead box O3 (FOXO3) axis, inducing a pro-inflammatory response in primary BECs [114]. Interestingly, overexpression of SIRT1 was shown to be protective against CS-induced senescence by activating mitochondrial biogenesis via the deacetylation of FOXO3 [115]. This study highlights the potential protective mechanism of SIRT1 induced biogenesis; however, SIRT1 also has other cellular targets and therefore further investigations are needed before concluding that increased biogenesis plays a protective role in CS-induced senescence.

In alignment with impaired biogenesis, fission and fusion events also appear altered in COPD. As mentioned previously, smoke treated BECs present with significantly lower levels of phosphorylated-DRP1 (p-DRP1), a key regulator of fission, when compared with controls [22]. In agreement, p-DRP1 and FIS1 levels, a receptor for DRP1, were also significantly lower in AT2 cells isolated from patients with emphysema when compared with healthy non-smokers [108]. In conjunction with impaired fission machinery, hyperfusion of the mitochondria has previously been documented in COPD [102,116], with murine AT2 cells exposed to CSE showing increased expression of mitofusin 2 (MFN2) [116]. In agreement, primary BECs treated with CS had upregulated mitofusin 1 (MFN1) levels [101]. In contrast, AT2 cells isolated from patients with emphysema had decreased expression of MFN1 [108]. These observed differences are probably a factor of disease state in the cells and models. Early up-regulation of mitofusin regulators in response to smoke may be a mechanism to compensate for the mitochondrial injury at the beginning of the disease, with their loss as disease progresses resulting in cellular damage and death.

Iron and COPD

Genetic and epidemiological associations of iron and COPD

Numerous genetic and epidemiological studies have highlighted the role of iron and aberrant iron metabolism in the susceptibility to COPD. Genome wide association studies of COPD patients compared with smoking controls have implicated *IREB2*, encoding the iron regulatory protein IRP2, as a COPD susceptibility gene, with *IREB2* mRNA and IRP2 protein levels found to be elevated in lung tissue of COPD patients [117–122]. Variants in *IREB2* have also been found to associate with differential DNA methylation of the gene in COPD patients [123]. Interestingly, *IREB2* is located in a cluster of genes on chromosome 15q, some of which encode for several components of the nicotinic acetylcholine receptor. Despite this, the association between *IREB2* and COPD status has also been reported to be independent of smoking [124–126]. Polymorphisms in *IREB2* also associate with COPD in individuals carrying mutations in *SERPINA1*, causative of α anti-trypsin 1 deficiency [124]. Exposure to exogenous iron has been associated with increased susceptibility to COPD, with subjects working in iron ore mines and iron foundries revealed to be at increased risk of development of COPD, alongside persistent respiratory symptoms including chronic cough and airway inflammation [127–129]. While the precise relationship between *IREB2* polymorphisms and smoking remains

Table 2 Altered iron metabolism in COPD

Iron-related factor	Location	Change	Reference
Divalent metal transport 1 (DMT1)	BECs	Increased mRNA in response to CSE <i>in vitro</i>	[136]
Ferritin	AT2s	Increased mRNA in response to CSE <i>in vitro</i>	[55,136,145,183]
	BALF	Increased in COPD BALF	
Ferroportin	Whole lung	Increased mRNA expression in COPD lung tissue	[55,60]
	AMs	Increased mRNA/protein expression, increased release <i>ex vivo</i>	
	Whole lung	Increased mRNA expression in COPD lung tissue, CS-exposed mice	
Hepcidin	AMs	Increased mRNA expression in CS-exposed mice, unchanged in patient AMs	[60,184]
	Plasma	Decreased mRNA expression in COPD patients, CS-exposed mice	
	Whole lung	Decreased mRNA expression in CS-exposed mice	
Iron regulatory protein 2 (IRP2)	AMs	Increased mRNA expression in patient AMs	[135]
	Whole lung	Increased mRNA/protein expression in COPD lung tissue, CS-exposed mice	
Lipocalin-2	Plasma	Increased in COPD plasma	[185,186]
	BALF	Increased in COPD BALF	
Mitoferrin 2 (MFRN2)	Whole lung	Increased mRNA expression in CS-exposed mice	[135]
Transferrin	Whole lung	Increased in COPD lung tissue	[55,152]
	AMs	Decreased in COPD AMs	
Transferrin receptor (TFRC)	Whole lung	Increased in CS-exposed mice	[55,152]
	AMs	Increased in COPD AMs	

Abbreviations: AM, alveolar macrophage; AT2, alveolar type 2 epithelial cell; BEC, bronchial epithelial cell.

to be determined, genetically perturbed iron metabolism and environmental exposure evidently underpin disease susceptibility perhaps in a sub-set of individuals.

Iron driven cellular dysfunction in COPD

Lung iron accumulation is a widely acknowledged pathogenic phenomenon in COPD. The origin of this iron remains unclear: while smoking one cigarette exposes the lung to approximately 0.3 µg of iron, removal of iron from CSE was observed to have no effect on AM expression of iron related genes *in vitro*, suggesting the contribution of CS iron may be minimal [39,60]. Redox active iron is decreased in the exhaled breath condensate of COPD patients compared with healthy smoker controls, indicative of increased sequestration in lung tissue, and non-heme iron, ferritin, lactoferrin and lipocalin-2 are increased in patient sputum and BALF [130,131] (Table 2). Alveolar macrophages are the predominant iron-positive cell type in the COPD lung, with the percentage of iron-loaded macrophages negatively correlating with disease severity [55,132,133]. Iron loading is also observed in the respiratory epithelium – cigarette smoke extract was observed to increase the cellular labile iron pool in a bronchial epithelial cell line, while ferritin staining in human COPD tissue showed increased iron storage in the alveolar epithelium [134–136]. Increased cellular iron loading is known to drive ROS production and oxidative stress, and both iron and heme-loaded monocytes show deficiencies in phagocytosis and cytokine production *in vitro* [137–140], and non-heme iron overloading impairs monocyte to macrophage differentiation [141]. Increased levels of hemosiderin (a partially digested ferritin complex) in patient AMs was found to positively correlate with infection-driven exacerbation events and BALF IL-6 levels [142]. COPD macrophages show deficiencies in the phagocytosis of major respiratory pathogens including *Streptococcus pneumoniae* and *Haemophilus influenzae* but the contribution of iron loading to this phenotype remains incompletely understood [89,95,109].

Nutritional immunity and the role of dysregulated iron in infection

The exposure of the lung to inhaled pathogens such as those listed above necessitates the careful handling and sequestration of trace metals by the host. Immune cells in the lung, primarily AMs and neutrophils, employ a number of methods to sequester free iron and limit bacterial outgrowth in a phenomenon termed nutritional immunity [8]. Dysregulation of the nutritional immune response is deleterious to the host, both in terms of fuelling pathogen virulence and the detrimental effect on immune cell function. Numerous iron-binding proteins show altered expression patterns in the COPD lung, including lipocalin-2 and transferrin (Table 2). CS inhibits AM hepcidin production *in vivo*, which is associated with blunted cytokine production following LPS challenge *ex vivo*. Hepcidin-deficient AMs

showed reduced clearance of *S. pneumoniae in vivo*, but the exact bactericidal function of hepcidin in the lung is unclear [60]. While they are not immune cells, AT2 cells have known immunogenic properties including the ability to secrete numerous cytokines and to regulate immune cell function through production of pulmonary surfactant and granulocyte macrophage-stimulating colony factor (GM-CSF) [6,143]. A recent report detailed the capacity of the human A549 alveolar epithelial cell line to differentially regulate iron metabolism in response to specific bacteria *in vitro*; however, the effect of pre-existing iron loading in the alveolar epithelium on this host-pathogen interaction remains undetermined [144]. Extracellularly, BALF iron and ferritin increase with exacerbations, suggesting increased bioavailability of iron may fuel acute infection-driven exacerbation events [145].

Iron deficiency and COPD

Systemically, iron deficiency is a common manifestation of COPD. Anaemia is commonly comorbid with COPD and associates with worse morbidity and mortality, with increased serum iron levels linked to a resistance to COPD development in a Japanese cohort of smokers [146–148]. In line with this, a recent study observed mice fed a low-iron diet to induce anaemia to have poorer outcomes in response to an acute course of CS exposure, displaying increased immune cell infiltration and heightened emphysema compared with controls fed a normal diet [149]. The discrepancies between the pathological outcome of lung and systemic iron loading in COPD was further highlighted in a large-scale patient cohort study examining the association between iron parameters in the lung and in plasma and symptom severity and early disease progression. Despite increased BALF ferritin and non-heme iron, COPD patients show no change in plasma ferritin levels compared with healthy smoker controls, and a strong association with exacerbation risk and lung function decline was observed for increased lung ferritin levels, but not for that of plasma [145]. However, using this same data set, anaemia was found to associate with worse exercise capacity, greater dyspnoea, and greater disease severity in participants with COPD, particularly among those with comorbid chronic cardiac and metabolic diseases [148]. Taken together, these studies indicate that systemic and local lung iron-loading status ensure the two serve as two distinct compartments in COPD, with opposing roles for iron in disease pathogenesis in both, and that any therapeutic interventions aimed at manipulating iron levels must take this into account.

COPD and ferroptosis

Ferroptosis, a recently characterised form of necrotic regulated cell death, relies on the iron-driven peroxidation of phospholipids through Fenton chemistry-produced ROS [150]. A recent study demonstrated expression of Acyl-CoA Synthetase Long Chain Family Member 4 (ACSL4), which activates polyunsaturated fatty acids that later undergo peroxidation, is increased in patient lung tissue and murine models of emphysema [151]. Conditioned media from LPS-stimulated monocytes was observed to induce increased ACSL4 expression in an AT2 cell line *in vitro*, suggesting infiltration of inflammatory monocytes to the COPD lung drives ferroptotic cell death in the alveolar epithelium [152]. An increase in cellular iron via ferritinophagy has also been linked to ferroptosis in COPD, with siRNA mediated knockdown of NCOA4 in a murine model of acute smoke exposure significantly decreasing lipid peroxidation in bronchial epithelial cells [153]. Interestingly, mitochondrial morphology is also altered in ferroptosis, with mitochondrial swelling, a decrease in cristae and ruptured outer mitochondrial membranes observed [154,155]. However, little is known about the involvement of mitochondrial iron, if any, in ferroptosis in COPD or indeed at baseline. Much remains to be deciphered surrounding the role of ferroptosis in COPD, particularly in the landscape of lung iron overload and the specific cell types involved.

Mitochondrial iron and COPD

As discussed, mitochondria play a central role in governing cellular metabolism. The central role of iron in catalysing key metabolic reaction leaves their function intricately linked to correct iron homeostasis. Reciprocally, mitochondria, as the primary site of cellular iron storage and utilisation, are vital in the detoxification and sequestration of iron to limit free radical-induced cytotoxicity. Normal cellular iron homeostasis has proven essential for mitochondrial function, with inhibition of NCOA4-mediated ferritinophagy reducing mitochondrial membrane potential and respiration, illustrating the importance of normal iron turnover in mitochondrial function [156]. The oxidation state of MitoNEET, an iron-containing OMM protein, regulates gating of the OMM transporter voltage-dependent anion channel 1 (VDAC1), highlighting the role for iron-containing proteins in normal cytosolic-mitochondrial cross-talk [157]. Mitochondrial iron can also regulate mitochondrial dynamics: suppression of the Fe-S clusters harboured in aconitase induces mitochondrial swelling and oxidative stress in *C. elegans*, while loss of frataxin induces mitophagy in *Drosophila* [158,159]. Iron chelation through chemical means can also be detrimental to mitochondria. Deferasirox treatment was observed to induce mitochondrial swelling and partial uncoupling of the mitochondria

membrane in cells of the kidney proximal tubule *in vivo*, and deferiprone and deferoxamine treatment can induce BNIP3-dependent mitophagy [159,160]. Inhibition of heme synthesis in mouse tissue was also observed to reduce expression of subunits of the respiratory complexes and alter cyclooxygenase enzymatic activity [161]. Conversely, a high iron diet associates with a decrease in mitochondrial respiration *in vivo* [162]. Mitochondrial iron uptake is also linked to mitochondrial function, with a recent study employing a CRISPR screen approach revealing MFRN1-mediated mitochondrial iron uptake to functionally co-ordinate with mitochondrial glutathione import via SLC25A39, which is critical in ensuring proper OXPHOS [163]. Evidently, proper iron metabolism and mitochondrial function are heavily reliant on one another, although the crosstalk between the two in disease remains poorly studied.

In recent years, the combined role of mitochondrial and iron dysfunction has become better appreciated in COPD. Cellular iron loading, as discussed above, is a well-appreciated phenomenon in COPD. While ferritin levels are known to be increased in the lung, little else is known about the precise subcellular location of iron and its subsequent pathogenic effects in disease. Further light has been shed upon this recently with the implication of mitochondrial iron loading in COPD. Following its identification as a risk factor in COPD, IRP2 was identified as a regulator of mitochondrial iron, with loss of IRP2 protective against CS-driven mitochondrial dysfunction. CS-exposed mice displayed increased levels of non-heme and heme mitochondrial iron in whole lung homogenates, accompanied by an increase in MFRN2 expression and a loss of frataxin expression. Mice deficient in frataxin, a key enzyme in iron-sulfur cluster biogenesis, as a model of mitochondrial iron overload were found to display higher pulmonary inflammation at baseline. Loss of IRP2 was found to be protective against CS-induced mitochondrial iron loading and dysregulated expression of MFRN2, and chelation of mitochondrial iron in models of emphysema with the chelator deferiprone was observed to alleviate pulmonary inflammation and improve mucociliary clearance [135]. Mitochondrial iron dynamics have also proven important in the pathogenesis of numerous comorbidities associated with COPD. Loss of Rieske iron-sulfur protein, a component of the respiratory chain complex III, has shown to protect against hypoxia induced ROS driven pulmonary hypertension, a condition comorbid in approximately 90% of COPD patients [164,165]. COPD patients with iron deficiency and systemic inflammation show dysregulated expression of the mitophagy receptors BCL2/Adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L) in skeletal muscle tissue, both of which are proposed to play a role in iron loss-driven mitophagy [166–168]. While the link between mitochondrial iron loading and COPD pathogenesis is clear, questions remain surrounding the specific cellular factors contributing to such and the precise cell types involved.

Therapeutics and conclusions

Mitochondrial dysfunction is widely accepted as a hallmark of not only COPD pathogenesis, but also across a plethora of pulmonary diseases and diseases of ageing. As our knowledge of the molecular mechanisms of this dysfunction has increased, so has the availability of targeted therapeutics. Senotherapy with drugs targeting cellular senescence have shown promise in *in vitro* models of COPD, with metformin and rapamycin capable of reducing the senescence-associated secretory phenotype *in vitro* in lung epithelial cells, and in improving symptoms of emphysema in murine models [169–171]. Inhibition of mitophagy through Mdivi-1 and quercetin protected LAECs against CSE-induced necrotic and apoptotic cell death, respectively [22,172]. The mitochondrially targeted antioxidants MitoTEMPO and MitoQ, which can scavenge mtROS, have been observed to inhibit the effects of CSE-induced mitochondrial dysfunction including mitochondrial fragmentation, senescence, and excess mitophagy [22,112,173]. However, mtROS are essential at baseline for cell signalling, and are also key in the function of innate immune cells, including macrophages and neutrophils, so the potential for a deleterious effect globally must be considered.

Given the extent of iron loading observed in the COPD lung, depletion of iron using chelators represents an intriguing therapeutic option. Iron chelators including deferiprone and hydrogen sulfide have shown promise in alleviating emphysema and pulmonary inflammation in murine models [135,174]. Heparin has also been explored as a therapeutic target in diseases associated with aberrant iron metabolism, with heparin mimetics used to successfully treat mice with diseases of iron overload such as hemochromatosis [175]. However, the global effects of decreasing systemic iron levels through these means must be considered, particularly given the prevalence of iron deficiency and anaemia in COPD patients and its association with mortality [146–148]. Iron is emerging as a vital component for immune memory T cell and neutrophil function, further highlighting the need to exert caution when considering systemic iron chelation approaches for any inflammatory disease [176–178]. Additionally, the iron chelator deferoxamine has been observed to act as a fungal siderophore *in vitro*, and the *Escherichia coli* siderophore enterobactin was shown to induce mitochondrial iron loading in model organisms and a human fibroblast cell line. Therefore, the effects of manipulating lung iron levels must also take into account potential effects on the lung microbiome and the effects that further dysbiosis could hold for lung iron loading [179]. Gallium derivatives have shown promise in

directly targeting bacterial iron metabolism in respiratory pathogens: redox-inactive gallium displaces iron ions in iron dependent bacterial proteins, stopping processes dependent on iron redox reactivity, and can also competitively bind bacterial siderophores [8,180,181]. Gallium nitrate, when used in a Phase 2 Study for patients with cystic fibrosis (IGNITE Study), did not have a significant impact on lung function (defined as a >5% increase in FEV1) when compared with placebo at Day 28. However, for participants who were culture positive for *Pseudomonas aeruginosa* at baseline, participants treated with IV gallium nitrate had a significant reduction in the amount of *Pseudomonas aeruginosa* from sputum when compared with those participants who received placebo (NCT02354859). Recently, gallium protoporphyrin has shown promise as an antimicrobial for non-typeable *Haemophilus influenzae* (NTHI) in COPD patients [182] showing potential that this approach may help to improve NTHI driven exacerbations in COPD. However, as with use of chelators, limiting the amount of iron available to lung-based pathogens may lead to further dysbiosis of the lung microbiome, which may in turn promote further lung injury upon acute infection events.

The heavy economic burden associated with admitting and treating patients for repeat exacerbation events will only be worsened in coming years accounting for increased urbanisation and an ageing population. Evidently, mitochondrial dysfunction and altered iron metabolism contribute significantly to the susceptibility to, and the pathogenesis of, COPD. Further investigation into the precise molecular mechanisms governing the two will delineate novel therapeutic avenues in the treatment of COPD, and indeed across diseases characterised by defective mitochondrial and iron metabolism.

Data Availability

NA

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRedit Author Contribution

Lynne Faherty: Writing—original draft, Writing—review & editing. **Sarah Kenny:** Writing—original draft, Writing—review & editing. **Suzanne M. Cloonan:** Supervision, Writing—original draft, Writing—review & editing.

Abbreviations

AM, alveolar macrophage; AT2, alveolar type 2 epithelial cell; ATP, adenosine triphosphate; BALF, bronchoalveolar lavage fluid; BC, basal cells; BEC, bronchial epithelial cell; COPD, chronic obstructive pulmonary disease; CSE, cigarette smoke extract; ETC, electron transport chain; FAO, fatty acid oxidation; GM-CSF, granulocyte macrophage-stimulating colony factor; IMM, inner mitochondrial membrane; LAEC, lung airway epithelial cell; OMM, outer mitochondrial membrane; OXPHOS, oxidative phosphorylation.

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