

Light microscopy applied to study defective macrophage phagocytosis in chronic obstructive pulmonary disease

A thesis submitted to Imperial College London for the
degree of Doctor of Philosophy (PhD)

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Statement of Originality

I confirm that I have performed all the experiments and analysis reported in this thesis, unless otherwise stated.

For the analysis of the cytoskeleton (chapter 5) and mitochondrial features (chapter 6), custom MATLAB code was created by made Dr Yuriy Alexandrov (Photonics group, Imperial College London). Work performed in chapter 6 was performed at the Facility for Imaging by Light Microscopy (FILM) at Imperial College London.

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Abstract

Chronic obstructive pulmonary disease (COPD) is a progressive lung condition characterised by airflow limitation, primarily caused by cigarette smoking and other air pollutants, which results in irreversible damage to the lungs. This leads to alveolar destruction, reduced lung elasticity, narrowing of the small airways and fibrosis, and is associated with an increased number of phagocytic immune cells, including macrophages and neutrophils. Despite increased number of phagocytes, approximately half of COPD patients exhibit bacterial colonisation that are associated with increased numbers of exacerbations that often lead to hospitalisation. Bacterial colonisation may be explained, in part, by defective phagocytosis of pathogens by macrophages, although the mechanism involved is unclear.

Light microscopy is widely used to study cell biology, although the ability to image cellular processes within cells and microorganisms is conventionally limited by the diffraction of light, as well as additional considerations of signal to noise that impacts the imaging speed and contrast. However, advances in light microscopy, including computational and physical techniques, to improve spatial resolution and image contrast prompted the investigation of their potential use in exploring phagocytosis. This project initially explored the potential of super-resolved microscopy and computational techniques, to allow image acquisition at a resolution of less than 20nm. These techniques can be used to identify and image sub-cellular proteins and cellular processes previously unobtainable, including phagocytosis. This thesis aimed to utilise live, cell imaging and super-resolved microscopy techniques to identify differences in morphology, cytoskeletal proteins, and mitochondria in macrophages. The most practically useful technique was found to be wide-field epifluorescence imaging with

computational deconvolution, which was applied to study macrophages from COPD subjects, smokers and non-smoking control subjects with single cell analysis.

This work confirmed that COPD macrophages were poorer at phagocytosing bacteria, and this was associated with structural changes in COPD macrophages. Using high-resolution microscopy, COPD macrophages were shown to be larger compared to non-smoker cells, with significantly longer microtubules observed in both lung tissue-derived and blood monocyte-derived macrophages (MDM), together with a reduction in actin-containing podosomes.

Mitochondria function can be a key factor that affects cytoskeletal restructuring in cells, with the mitochondria membrane potential being important in the function of ATP generation, with mitochondria under normal conditions displaying a polarised phenotype, whereby they are highly negatively charged, due to the influx of electrons required for the electron transport chain. However, a significantly depolarised mitochondria is the result of a more positively charged membrane potential, which can result in impaired ATP production and cause cell death, which is associated with several pathologies. Work performed in this thesis using microscopy explored mitochondrial differences in COPD MDM, with a significantly depolarised mitochondrial membrane potential ($\Delta\Psi_m$) compared to non-smoker controls, but no difference in mitochondria reactive oxygen species (mROS). Mitochondrial antioxidants, SkQ1 and PQQ, were able to improve bacterial phagocytosis in COPD MDM by up to 60% but had no effect on $\Delta\Psi_m$ or mROS.

Work in this thesis has shown the use of microscopy and single cell image analysis can detect differences in key structural proteins and the mitochondria, whilst capable of being used as a screening tool for phagocytic correctors such as mitochondrial antioxidants, which were shown to increase phagocytosis with no alterations in mitochondrial function.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
α	Alpha
AATD	Alpha-1 antitrypsin deficiency
<i>actB</i>	Beta-actin gene
AF	AlexaFluor
AM	Alveolar macrophage
ANOVA	Analysis of variance
Arp2/3	Actin related protein 2/3
α TAT1	α -tubulin acetyltransferase 1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
β	Beta
BAL	Bronchoalveolar lavage
BEAS-2B	Human bronchial epithelial cell line
BHI	Brain heart infusion
C3b	Complement component 3b
CD	Cluster of differentiation
CFU	Colony-forming units
Cl ⁻	Calcium ion
CMLE	Classic maximum likelihood estimation
CO ₂	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CR	Complement receptor
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
$\Delta\Psi_m$	Mitochondrial membrane potential
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl sulfoxide
Drp1	Dynamin-related protein 1
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
F	Filamentous
FCS	Foetal calf serum
FcR	Fc receptor
FcγR	Fc gamma receptor
FEV ₁	Forced expiratory volume in 1 second
FSC	Forward scatter
FVC	Forced vital capacity
γ	Gamma
G	Globular
G1	Gradient 1
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GGM	Gaussian gradient modulus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOLD	Global initiative for chronic obstructive lung disease
GTP	Guanosine triphosphate
GWAS	Genome-wide association study
H ⁺	Hydrogen ion
H ₂ O ₂	Hydrogen peroxide
HBSS	Hanks' Balanced Salt Solution
HDAC	Histone deacetylase
<i>H. influenzae</i>	<i>Haemophilus influenzae</i>
HO-1	Heme oxygenase-1
HPC	High-performance computing
HSC	Hematopoietic stem cell

HyD	Hybrid detector
ICS	Inhaled corticosteroids
IFN- γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin
<i>IREB2</i>	Iron-responsive element-binding protein 2
ITAM	Immunoreceptor tyrosine-based activation motif
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
λ	Lambda, wavelength of illumination
LB	Luria-Bertani
LD	Linear density of zero-crossings
LPS	Lipopolysaccharide
LSCM	Laser scanning confocal microscopy
LTA	Lipoteichoic acid
LTB4	Leukotriene B4
MAPK1	Mitogen-activated protein kinase 1
MARCO	Macrophage receptor with collagenous structure
MBL	Mannose-binding lectin
MDM	Monocyte-derived macrophage
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinases
MOI	Multiplicity of infection
mRNA	Messenger RNA
mROS	Mitochondrial reactive oxygen species
mtDNA	Mitochondria DNA
MTT	3-(4, 5-Dimethylthiazol2-yl)-2,5 diphenyltetrazolium bromide
NA	Numerical aperture
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO ₃	Sodium bicarbonate
NF- κ B	Nuclear factor-kappa B
NHS	Succinimidyl

Nrf2	Nuclear factor erythroid 2–related factor 2
NS	Non-smoker
NT	Non-treated
O ₂ ⁻	Superoxide
OD	Optical density
<i>pac</i>	Puromycin N-acetyltransferase
PALM	Photo-activated localisation microscopy
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered solution
PDE4	Phosphodiesterase-4
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PMA	Phorbol-12-myristate-13-acetate
PMT	Photomultiplier tube
PQQ	Pyrroloquinoline Quinone
PRR	Pattern recognition receptors
PSF	Point spread function
RFU	Relative fluorescence intensity
Rho	Ras homolog
ROI	Region of interest
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
sCMOS	Scientific complementary metal–oxide–semiconductor
scRNAseq	Single cell RNA sequencing
SEM	Standard error of the mean
SIM	Structured illumination microscopy
SiR	Silicon rhodamine
SIRT	Sirtuin
SkQ1	10-(6-plastoquinonyl) decyltriphenylphosphonium

SMLM	Single molecular localisation microscopy
SNP	Single-nucleotide polymorphisms
S.O.C	Super optimal broth with catabolite repression
SOD	Superoxide dismutase
<i>S. pneumoniae</i>	<i>Streptococcus pneumoniae</i>
SR	Scavenger receptor
SRM	Super-resolved microscopy
SSC	Side scatter
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
SVI	Scientific Volume Imaging
SyK	Spleen tyrosine kinase
TGF β 1	Transforming growth factor beta 1
THP-1	Human monocytic cell line
TIRF	Total internal reflection fluorescence
TLR	Toll-like receptor
TM	Tissue macrophages
TNF- α	Tumour necrosis factor-alpha
TPP+	Decyl-triphenylphosphonium cation
Treg	Regulatory T-cell
V-ATPase	Vacuolar-type ATPase
v/v	Volume/volume
w/v	Weight/volume
WASP	Wiskott-Aldrich syndrome protein

Publications

Journals

Lightley, J., Görlitz, F., Kumar, S., Kalita, R., Kolbeinsson, A., Garcia, E., Alexandrov, Y., Bousgouni, V., Wysoczanski, R., Barnes, P.J., Donnelly, L.E., Bakal, C., Dunsby, C., Neil, M.A.A., Flaxman, S., French, P.M.W. (2021) Robust deep learning optical autofocus system applied to automated multiwell plate single molecule localization microscopy. *J Microsc.* DOI: 10.1111/jmi.13020.

Conference Abstracts

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Chapter 1: Introduction

1.1 Overview of Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a largely irreversible and progressive lung disease, characterised by airflow limitation due to abnormal changes to the airway and alveolar, which is primarily caused by exposure to noxious particles and gases (GOLD, 2022). COPD is an umbrella term for three distinct pathologies of lung disease: chronic bronchitis, small airways disease and emphysema (Gordon, 2016). COPD has a worldwide prevalence of approximately 250 million people and is responsible for over 3 million deaths a year, which currently accounts for 5% of worldwide mortality (Jarhyan et al., 2022). As such, COPD places a large burden on global healthcare systems, not only due to the high mortality rate, but also due to significant morbidity associated with COPD. COPD is estimated to cost up to 40 billion euros per annum in the European Union (GOLD, 2022), equating to 6% of the total healthcare budget, and accounts for over 50% of all respiratory disease cost. As COPD is an irreversible disease there are currently no curative treatment options, and management of the disease requires lifelong support and regular medical intervention.

1.2 Causes of COPD

COPD is caused by a number of factors, with the major instigators being inhalation of particulates, primarily cigarette smoke (Burkhardt & Pankow, 2014), however COPD occurs in people who have never smoked (Lamprecht et al., 2011). Other environmental and occupational exposures can cause lung irritation and drive lung damage causing COPD, and include air pollution, chemical fumes and dusts (GOLD, 2022). In developing countries, the use of biomass burners as a primary cooking source is the largest cause of COPD (Salvi &

Barnes, 2009), highlighting that the inhalation of noxious gases and particles is the primary cause of COPD, and is not isolated to just cigarette smoke.

1.3 Risk factors for COPD

There are risk factors and genetic susceptibilities that contribute to the likelihood of developing COPD. One of the major risk factors for COPD is a history of cigarette smoking (Bai et al., 2017), but also COPD is a disease associated with premature aging (Barnes et al., 2015). Lung function is known to naturally decline with age (Figure 1.1), reaching peak lung performance at approximately 25 years old followed by progressive decline (Sharma & Goodwin, 2006); however, there is a more rapid decline in this lung function in patients with COPD indicative of premature ageing (Fletcher & Peto, 1977).

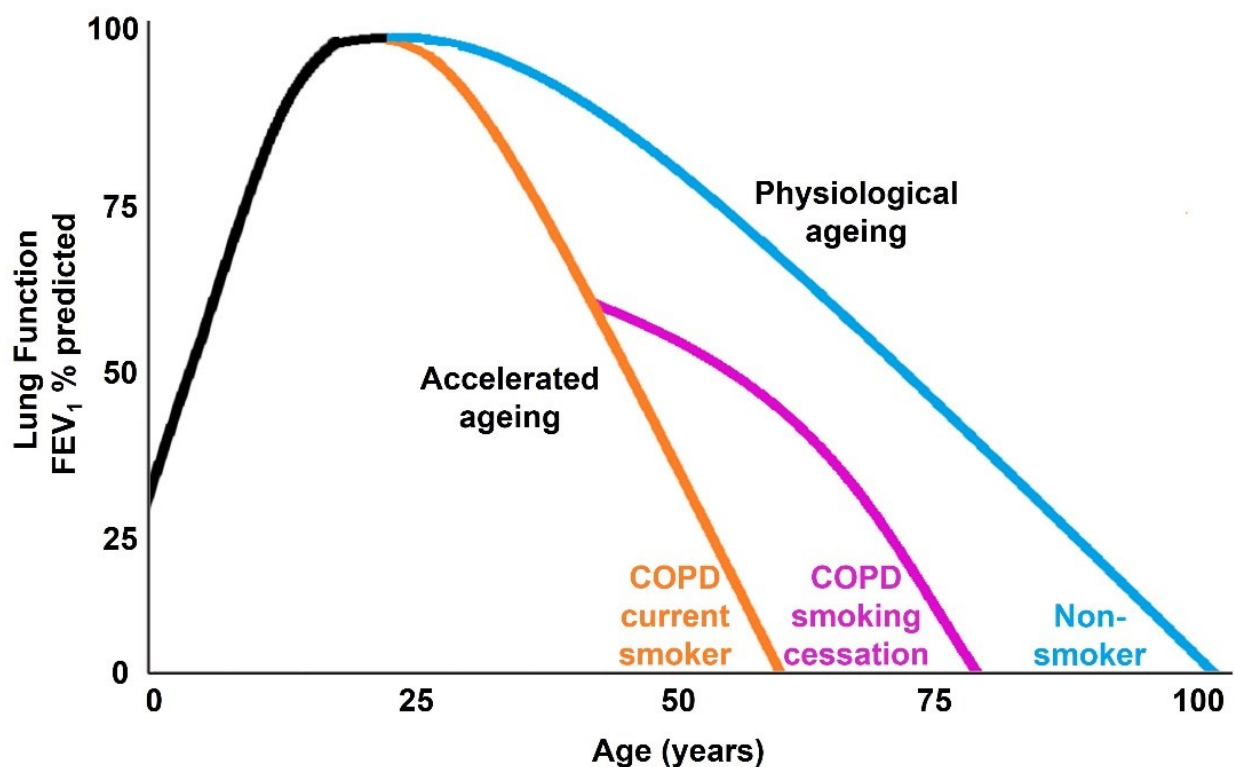


Figure 1.1: Diagram representing lung function decline with age. Lungs reach peak performance at age 25 followed by natural lung decline, however COPD accelerates this significantly. FEV₁: Forced expiratory volume in 1 second. Adapted from (Agustí & Hogg, 2019).

There is increasing evidence that factors arising during pregnancy and birth impede normal lung development leading to an increased risk of COPD. These include being exposed to tobacco *in utero* by maternal smoking during pregnancy (Beyer, Mitfessel & Gillissen, 2009; McEvoy & Spindel, 2017), preterm birth (Kwinta & Pietrzyk, 2010; Simpson et al., 2018) and low birthweight (Balte et al., 2016; Barker et al., 1991). COPD is more prevalent in patients who have a history of severe lung infections during childhood (Hayden et al., 2015; Savran & Ulrik, 2018), as history of lung inflammation that predisposes further inflammatory conditions such as COPD. Patients with asthma are also significantly more likely to develop COPD, with studies showing up to a 30-fold increased likelihood of suffering airway obstruction (Lange et al., 1998; McGeachie, 2017; Tagiyeva et al., 2016).

A known rare cause of COPD is the genetic disorder alpha-1 antitrypsin deficiency (AATD). This is commonly associated with early onset COPD, often before the age of 50, or in patients with no history of smoking (Craig & Henao, 2018). Alpha-1 antitrypsin is a protease inhibitor, where a genetic mutation results in reduced circulating levels of this protein, leading to lung lower lobe emphysema but can also present with liver disease (Brode, Ling & Chapman, 2012; Santos & Turner, 2020).

A genetic component for COPD has been shown by genome-wide association studies (GWAS), which have identified a number of single nucleotide polymorphisms (SNP) showing an association with susceptibility for COPD (Cho et al., 2014; Lee et al., 2020). These include major histocompatibility complex 2 (*HLA-DQB2*), which could be an indicator of the increased inflammation in COPD (Kubysheva et al., 2018) and iron-responsive element-binding protein 2 (*IREB2*), where increased iron-products have been identified in the lung of COPD patients and correlated with exacerbations (Zhang et al., 2020a). One large study exploring samples

from the UK Biobank identified 95 variants, confirming 52 previously reported and revealing 43 novel variants (Wain et al., 2017), showing the extent of the genetic risk in COPD. As well as identifying variants that pose a possible risk of COPD, these GWAS studies may also indicate potential pathways for therapeutic targets.

1.4 Diagnosis of COPD

COPD diagnosis is primarily clinical, with common symptoms including chronic cough, excessive sputum production and shortness of breath (dyspnoea) on exertion (Burkhardt & Pankow, 2014). This will usually be accompanied by a patient history of cigarette smoking or exposure to other environmental or occupational irritants (Gentry & Gentry, 2017). A COPD diagnosis can be confirmed with lung function testing, including spirometry performed post-bronchodilator. The lung function parameters measured are the forced expiratory volume in 1 second (FEV₁), which measures the volume of air exhaled in the first second after maximal inhalation, and forced vital capacity (FVC), which is the total volume of air exhaled forcibly after maximal inhalation. Using these measurements and information from the patient history, including height, age and gender, the FEV₁ predicted percentage can be calculated, as well as the FEV₁/FVC ratio. Spirometry values for the FEV₁/FVC ratio of <0.7 is an indicator of airflow limitation and therefore COPD (Barnes et al., 2015), with FEV₁ predicted percentage used to classify COPD subjects based on severity of disease, as specified by the Global Initiative for Chronic Obstructive Lung Disease (GOLD), ranging from GOLD 1 - 4; FEV₁ predicted % for GOLD 1 (mild COPD, >80%), GOLD 2 (moderate COPD, 79-50%), GOLD 3 (severe COPD, 49-30%) and GOLD 4 (very severe COPD, <30%) (GOLD, 2022). The use of spirometry in determining severity of COPD can be used clinically to establish the need for

additional inhaled treatments, although the choice of therapy is based on symptoms and history of exacerbations (GOLD 2022).

1.5 Pathology and pathogenesis of COPD

COPD encompasses three pathologies: chronic bronchitis, small airways disease and emphysema. However these pathophysiologies are not separate, with COPD often being described as a spectrum, whereby patients often have overlapping characteristics from each (Kim & Criner, 2013). Chronic inflammation in the lung of COPD patients is associated with increased numbers of phagocytic immune cells, including macrophages and neutrophils (Barnes, 2016; Sethi & Murphy, 2008), yet COPD patients are often colonised with airway pathogens, predominantly *Haemophilus influenzae* and *Streptococcus pneumoniae* (Patel et al., 2002; Wedzicha, Mackay & Singh, 2013). This paradox of having an increased number of phagocytic immune cells within the lung, yet a reduced ability to clear bacteria may be due to a defective phagocytic function in COPD macrophages (Berenson et al., 2006; Taylor et al., 2010). Not only do these macrophages fail to clear bacterial pathogens, there are also defects in clearance of apoptotic cells (Bewley et al., 2016; Hodge et al., 2007) and fungal spores (Wrench et al., 2018). However, the mechanism for this defective phagocytosis has yet to be discovered.

1.5.1 Chronic Bronchitis

Chronic bronchitis is defined as a chronic cough and excess sputum production for 3 months per year over a consecutive two-year period and is manifest as mucus hypersecretion and a productive cough (GOLD, 2022). Not all COPD patients have chronic bronchitis and patients with chronic bronchitis may not always develop airflow limitation. Chronic bronchitis patients have an accelerated decline in lung function and an increased risk of exacerbations, results in

an increased mortality rate (Kim et al., 2011). Overall, symptom severity correlates with faster lung decline and poor long-term prognosis.

Mucus hypersecretion is due to increased numbers (hyperplasia) and size (hypertrophy) of goblet cells in the small airway epithelium (Ramos, Krahne & Kim, 2014), leading to mucus hypersecretion, an overproduction of mucus in response to inflammatory stimuli (Kim et al., 2011) are seen in chronic bronchitis. These changes in airway epithelial architecture is primarily caused by exposure to cigarette smoke or other harmful irritants, as well as bacterial or viral infections (Shaykhiev, 2019). These produce a pro-inflammatory response from epithelial cells of the small airways to release cytokines such as chemokine (C-X-C motif) ligand 8 (CXCL-8) and tumour necrosis factor-alpha (TNF- α) (Mercer et al., 2006). There is also reduced clearance of mucus from the airways, due to reduced ciliary function of ciliated cells (Ancel et al., 2021) and reduced peak expiratory flow (Kim & Criner, 2013) further restricting airflow.

1.5.2 Small Airways Disease

The small airways are terminal respiratory bronchioles measuring <2mm in internal diameter, in the periphery of the lung branching tree resulting with a vastly increased surface area and leading to alveolar sacs where gas exchange occurs (Singh, 2017). Inhaled particulates and gases target the mucosal surface and the small airways. Prolonged exposure to these harmful particles leads to immune infiltration and subsequent chronic inflammation, which promotes airway remodelling, narrowing and often complete destruction of the small airways (Hogg et al., 2004; Koo et al., 2018). Damage to the peripheral small airways results in a significant increase in small airways resistance associated with small airways disease of COPD, of up to 40-fold when compared to the small airways of healthy controls (Hogg, McDonough & Suzuki,

2013; McDonough et al., 2011). This is caused by both the reduction in number of small airways and the narrowing of remaining bronchioles (Hogg, McDonough & Suzuki, 2013). Small airways disease can be attributed to early stages of COPD, prior to emphysematous destruction of the alveoli (Hogg, McDonough & Suzuki, 2013; McDonough et al., 2011).

1.5.3 Emphysema

Emphysema is destruction of the alveolae (Sharafkhaneh, Hanania & Kim, 2008) due to excessive proteolytic activity from the vastly increased number of immune cells, including macrophages and neutrophils in COPD (Goldklang & Stockley, 2016). There is a protease-antiprotease imbalance, whereby there are higher concentrations of extracellular matrix (ECM) degrading proteases in the lung compared to protease inhibitors resulting in excessive degradation of lung tissue (Shapiro, 2003). Macrophages and neutrophils are major sources of proteases within the lung. This leads to elevated secretion of several proteases responsible for degradation of the ECM, including matrix metalloproteinases (MMP)s such as MMP-2, 8 and -9, neutrophil serine proteinases such as neutrophil elastase, cathepsin G and proteinase 3, and cysteine proteinases such as cathepsins S and L (Shapiro, 2003).

Cigarette smoke exposure also produces oxidative stress, which can drive emphysema (Kirkham & Barnes, 2013). The generation of reactive oxygen species (ROS) activates pro-inflammatory pathways, via the transcription factor nuclear factor-kappa B (NF- κ B), driving expression and release of multiple inflammatory cytokines and proteases (Barnes et al., 2015), whilst also inhibiting anti-proteases, such as α -1-antitrypsin, producing additional ECM breakdown (Pandey, De & Mishra, 2017).

1.6 Current treatment and therapy of COPD

Current treatment options for COPD treat symptoms rather than being disease modifying, as COPD results in irreversible damage to the lung parenchyma and airways. The main purpose of COPD treatment is to reduce or relieve symptoms, to increase exercise tolerance, slow down disease progression, prevent exacerbations, and reduce mortality (Pauwels et al., 2001; GOLD, 2022).

1.6.1 Pharmacological intervention

There are several pharmacological interventions used in the treatment of COPD, including the use of bronchodilators with inhaled beta-2 (β_2)-agonists and antimuscarinics (anticholinergics) commonly used to induce smooth muscle relaxation (Barnes, 2002).

Other drugs used in COPD including inhaled corticosteroids (ICS) to reduce inflammation (Gan, Man & Sin, 2005), although their efficacy is poor as the inflammation in COPD is largely steroid-resistant (Barnes, 2000). ICS are currently recommended into reduce exacerbations in patients with increased blood eosinophils, but have no effects on symptoms, disease progression or mortality (GOLD 2022). Oral corticosteroids are used to treat acute exacerbations of COPD that are not triggered by bacterial infection, when antibiotics are preferred (Cazzola & Hanania, 2006).

Phosphodiesterase-4 (PDE4) inhibitors may improve lung function and reduce exacerbations (Chong, Leung & Poole, 2017). The PDE4 inhibitor roflumilast has a small effect in reducing exacerbations in COPD patients but is poorly tolerated as side effects, such as headaches, nausea and diarrhoea are common, so it is little used.

1.6.2 Non-pharmacological intervention

Current non-pharmacological intervention will typically involve cessation of smoking, which is usually adopted upon a diagnosis of COPD. Cessation of smoking has been shown to reduce COPD disease progression by minimising the accelerated lung function decline (van der Meer et al., 2003) and is shown to be the most effective option at reducing mortality (Bai et al., 2017). Once COPD disease is present the inflammation in the lung is relatively self-perpetuating (Sharafkhaneh, Hanania & Kim, 2008; Yao & Rahman, 2009), and lung function will not go back to 'normal' non-smoking levels of lung decline. Nevertheless, the minimisation of continued insult to the lung is still the only intervention that clearly reduces disease progression in COPD (Bollmeier & Hartmann, 2020; Candela et al., 2019).

An increase in regular exercise is also suggested to help increase pulmonary function, but in more severe disease a structured program of pulmonary rehabilitation, including regular physiotherapy, may be required to improve exercise capacity and quality of life in patients (Lacasse et al., 2007). In some patients with very severe COPD with hypoxaemia yet long-term oxygen therapy may also be indicated.

1.6.3 Surgical intervention

There are surgical interventions that have been shown to produce positive outcomes in patient survival and quality of life. Techniques include lung volume reduction surgery, and endoscopic lung volume reduction by implantation of an endobronchial valve resulting provide clinical benefits in highly selected emphysema patients (Gompelmann, Eberhardt & Herth, 2015).

1.7 Pathophysiology of COPD

COPD is complex with many cell types involved in pathology of the disease, with crosstalk between these cells providing migratory signals for immune cell infiltration and activation of pro-inflammatory pathways (Fig. 1.2).

There is a significant immune cell component to the disease, which presents with an abundance of macrophages, neutrophils and T-lymphocytes, which are collectively responsible for chronic inflammation, remodelling and destruction of the lung tissue and airways observed in COPD (Barnes, 2014; O'Donnell et al., 2006). Structural cells including epithelial, endothelial and fibroblasts have all also been implicated in the involvement and disease progression of COPD.

Epithelial cells and immune cells such as macrophages, when exposed to cigarette smoke release pro-inflammatory cytokines including TNF- α , chemotactic factors such as CXCL8, leukotriene B₄ (LTB₄) and CCL2 (Mercer et al., 2006). The infiltration of macrophages and neutrophils to the site of inflammation are in turn activated and produce proteases which can break down ECM in the lung parenchyma, promote fibrosis and loss of small airways and stimulate mucus hypersecretion (Pandey, De & Mishra, 2017).

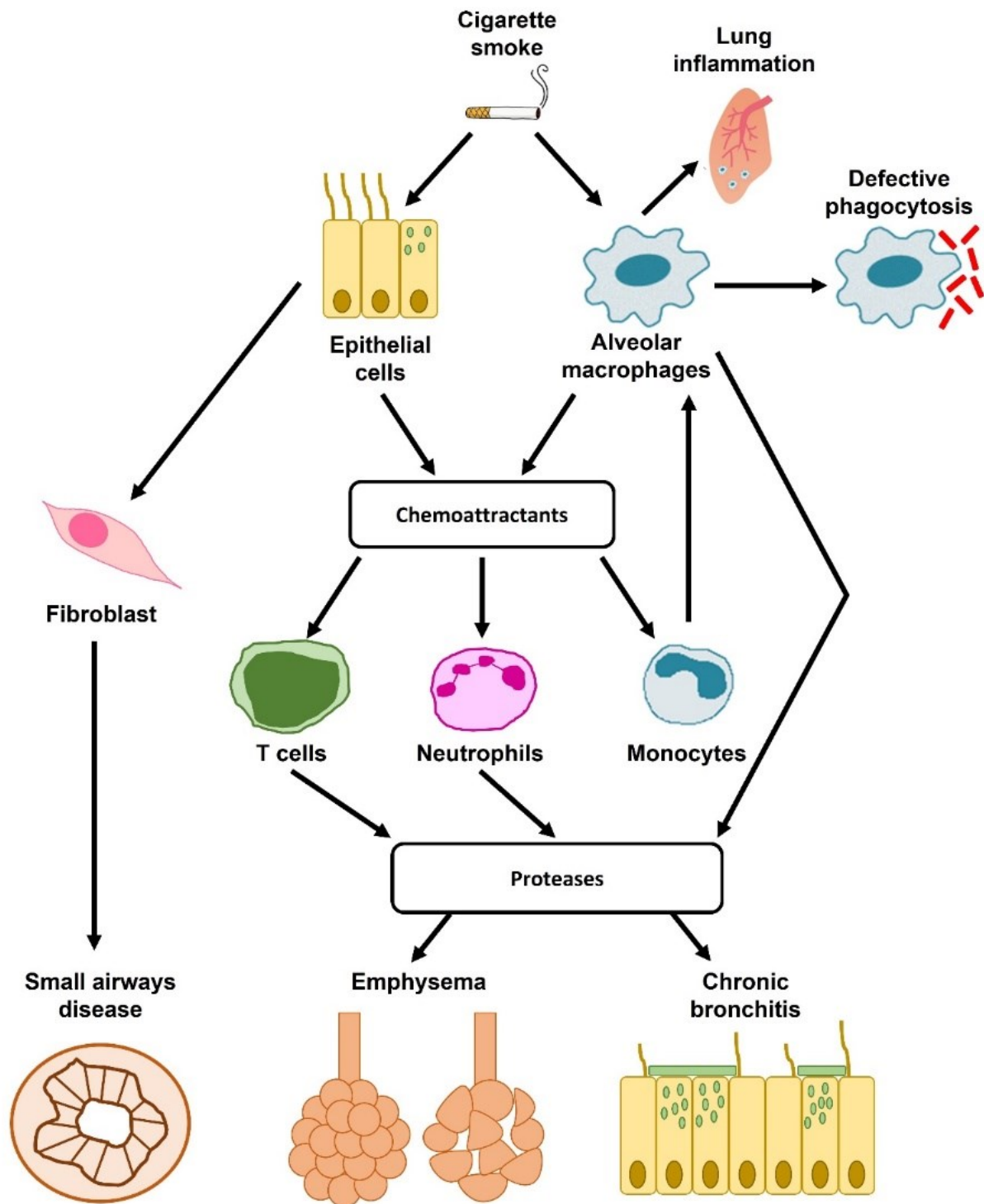


Figure 1.2 Overview of cellular involvement in pathology of COPD. There is a wide variety of cells involved in the pathologies of COPD, with significant interplay between different cell populations. Inflammation causes release of inflammatory cytokines and chemoattractants which recruits numerous immune cells to the lung. Release of proteases results in destruction of the lung parenchyma and promotes excessive mucus secretion.

1.7.1 Epithelial cells

Epithelial cells line all major organs in the body and provide a physical barrier to the external environment (Glaser et al., 2019). In the airway, the epithelial layer consists of ciliated epithelial cells, goblet cells and basal cells, all forming a continuous layer connecting the basement membrane to the external environment (Crystal et al., 2008). The epithelium is directly involved in the recruitment of immune cells to infiltrate the tissue in response to stimulus such as infection and inflammation (Stadnyk, 1994). In COPD, epithelial cells are chronically exposed to cigarette smoke and secrete numerous pro-inflammatory cytokines and chemokines including interleukin (IL)-1 β , IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and CXCL8 (Mercer et al., 2006), which are involved in directing the immune response to the inflammatory insult. Cigarette smoke also contains a number of particulates and oxidants that produce both oxidative stress and chronic inflammation responsible for morphological and functional changes to the epithelium in COPD (Randell, 2006). This results in ROS-mediated disruption of epithelial cell tight junctions, shortening of cilia leading to a reduction in mucociliary clearance and goblet cell metaplasia with subsequent mucus hypersecretion (Fig. 1.3) (Gohy et al., 2019), all contributing to the pathology of COPD. Alveolar epithelial cell apoptosis is prevalent in COPD (Yokohori et al., 2004) possibly due to cytotoxic lymphocyte derived granzymes and perforins (Demedts et al., 2006). Loss of these cells leads to break down of alveolar walls and enlargement of the alveolar air sacs, presenting as emphysema (Segura-Valdez et al., 2000)

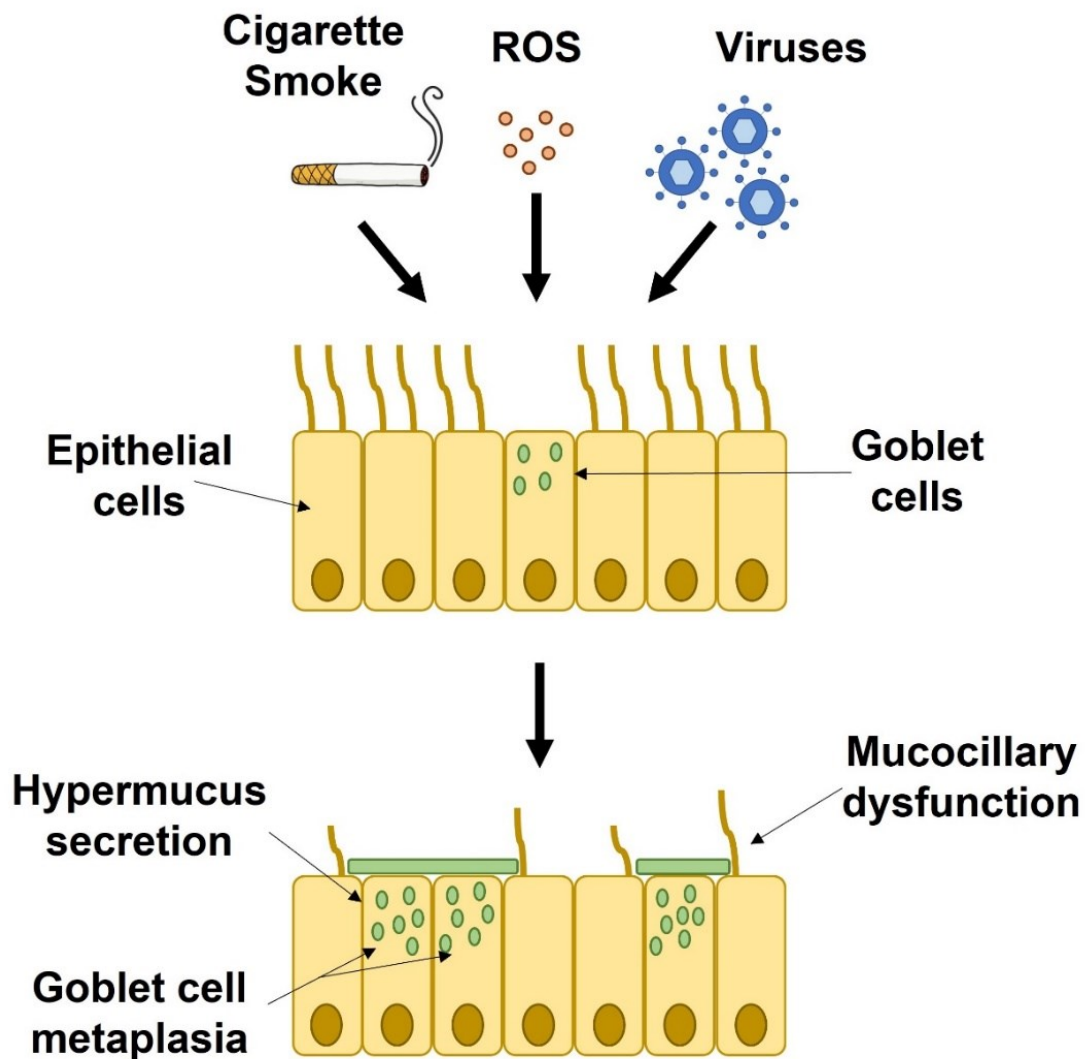


Figure 1.3: Overview of changes to the airway epithelium in COPD. Epithelial cells of the airway are exposed to cigarette smoke, reactive oxygen species and viruses leading to chronic inflammation leading to morphological changes. Goblet metaplasia leads to mucus hypersecretion, whilst shortening of the cilia leads to disruption to the mucociliary clearance and an accumulation of mucus in the airway lumen.

1.7.2 Endothelial cells

Endothelial cells form a single layer that line blood vessels (Krüger-Genge et al., 2019). These cells form tight junctions and produce connective tissue surrounding these vessels as well as many important roles, including haemostasis, thrombosis, fibrinolysis (Krüger-Genge et al.,

2019). In COPD there is endothelial dysfunction, including damage to the endothelial lining and subsequently the extracellular matrix (Szucs et al., 2019). This can result in vasoconstriction and hypoxia, leading to pro-thrombotic conditions and co-morbidities of cardiovascular diseases including pulmonary hypertension and atherosclerosis. Endothelial repair is affected in COPD, particularly emphysema (Doyle et al., 2017), with the loss of endothelial cells in vascular remodelling being a potential therapeutic target.

1.7.3 Fibroblasts

Fibroblasts are an abundant cell type within the stroma of tissues that are responsible for the homeostatic maintenance of the ECM (Dick, Miao & Limaïem, 2022). As such fibroblasts are important in wound healing and angiogenesis, but also play a critical role in inflammation and recruitment of immune cells into the tissue (Plikus et al., 2021). Fibroblasts are stimulated by cytokines including TNF- α , transforming growth factor beta 1 (TGF β 1) as well as IL-1 β , IL-6 and IL-13, which can induce proliferation (Chung, 2001; Feghali & Wright, 1997; Scotton & Chambers, 2007). Once activated, fibroblasts release cytokines and chemokines, including TGF β 1, IL-1 β and ROS. COPD fibroblasts also exhibit a pro-inflammatory phenotype with upregulation of secretory cytokines and mediators including IL-6, CXCL8, MMP-1 and versican (Zhang et al., 2012), responsible for recruitment and activation of leukocytes to the tissue during inflammation (Wight et al., 2020).

1.7.4 Lymphocytes

Lymphocytes are immune cells that originate from haematopoietic stem cells (HSC) in the foetal liver and adult bone marrow (Cano & Lopera, 2013) and are responsible for the adaptive immune response, consisting of B- and T-lymphocytes (Alberts et al., 2002a). B-cells are mostly conserved in the bone marrow and are responsible for the production of

antibodies, where upon presentation of an antigen will differentiate into plasma cells capable of mass production and secretion of antibodies specific for that antigen (Cyster & Allen, 2019). Lymphocytes are able to migrate to the thymus where they become T-cells which can differentiate into different subsets including; CD4+ helper T-cells which release cytokines that can activate the innate immune response or stimulate B cell differentiation (Luckheeram et al., 2012), CD8+ cytotoxic T-cells which are responsible for the killing of infected, apoptotic and cancer cells (Andersen et al., 2006) and regulatory T-cells (Treg) which help to regulate immune responses (Vignali, Collison & Workman, 2008). These subtypes circulate in the blood and lymphatic system where they can infiltrate into tissues.

The immune response in COPD is considered to be largely driven by innate immunity, however, there is involvement of the adaptive immune response with increased lymphocyte numbers in sputum of COPD subjects (Chrysofakis et al., 2004). There are increased numbers of CD4+ cells, and CD8+ (cytotoxic) T-cells (O'Shaughnessy et al., 1997), with this increase in CD8+ T-cells significantly correlating with reduced FEV₁ and airflow limitation (Saetta et al., 1999). This increased number of cytotoxic T-cells exhibited high expression levels perforin and cytotoxic proteins (Chrysofakis et al., 2004; Williams, Todd & Fairclough, 2021), this can result in apoptosis of type II alveolar epithelial cells contributing to emphysema (Plataki et al., 2006; Hodge et al., 2005). T-helper 17 (Th17) cells are a subset of CD4+ T helper cells which produce the pro-inflammatory cytokine IL-17 (Tesmer et al., 2008), and have been implicated in COPD with increased numbers of Th17 cells in the airways of COPD patients (Di Stefano et al., 2009).

1.7.5 Neutrophils

Neutrophils are an abundant phagocytic cell found in the blood, comprising approximately 70% of all circulating leucocytes, which migrate into major tissues as a rapid response to

infection or inflammation (Jasper et al., 2019). There is an increased number of neutrophils in the lungs of COPD patients, regardless of clinical presentation of disease (Keatings et al., 1996), that correlates with disease severity (Pilette et al., 2007), reduced FEV₁ (Donaldson et al., 2005) and progression of chronic bronchitis (Thompson et al., 1989). Neutrophil recruitment to the lung is mediated by CXCL1 and CXCL8, which act on the highly expressed chemokine (C-X-C motif) receptor (CXCR)2 receptor (Singh, 2015). Neutrophils migrating into the lung also release a number of proteases such as neutrophil elastase, cathepsin G, proteinase-3, MMP-8 and -9 (Pandey, De & Mishra, 2017) that degrade key components of ECM. Neutrophils are also implicated in mucus hypersecretion, as neutrophil derived serine proteinases are potent stimulators of mucus secretion (McKelvey et al., 2021). There is currently no consensus concerning defective phagocytosis by neutrophils in COPD (Müns, Rubinstein & Singer, 1995; Prieto et al., 2001). Neutrophil migration is impaired in COPD, with neutrophil migration being faster but less accurate than in healthy people, whilst also forming less pseudopodia (Sapey et al., 2011). This could explain increased ECM destruction during migration, as misdirected migration whilst secreting proteases will lead to excessive degradation of the tissue contributing to emphysema.

1.7.6 Macrophages

Macrophages are a highly specialised phagocytic cell involved in the innate immune response and play an important role in the removal of pathogens and apoptotic cells (Wynn, Chawla & Pollard, 2013). There is a population of macrophages found in all tissues that are referred to as tissue resident macrophages, which originate during embryonic development and due to their ability to self-replicate will persist into adulthood (Ginhoux et al., 2010). In addition, circulating monocytes can be recruited to the tissue by specific stimuli, including cytokines,

growth factors and bacterial lipopolysaccharide (LPS), where they differentiate into macrophages to replenish the population within the tissue (Epelman et al., 2014).

1.7.6.1 Macrophage characterisation and phenotype

Macrophages phenotype is an interesting debated topic, originally thought to display distinct phenotypes, either M1 or M2 (Mantovani et al., 2004; Mantovani, Sica & Locati, 2005) it is more commonly accepted that macrophages are part of a spectrum depending on their activation state and the micro-environment (Martinez & Gordon, 2014). Macrophages can still be broadly be characterised as pro-inflammatory, known as classically activated or anti-inflammatory known as alternatively activated (Atri, Guerfali & Laouini, 2018; Gordon & Plüddemann, 2017). However, macrophages exhibit plasticity with the ability to alter phenotype depending on the micro-environment, (Das et al., 2015). Macrophages displaying a pro-inflammatory phenotype release of pro-inflammatory cytokines such as TNF- α , interferon- gamma (IFN- γ) and IL-6 and are involved in the recruitment of other immune cells to the site of infection by the release of chemokines including CXCL8 (Chen et al., 2017; Hamidzadeh et al., 2017). Anti-inflammatory macrophages secrete cytokines including IL-10 and TGF- β and suppress activated macrophages and other immune cells (Mosser & Edwards, 2008). Anti-inflammatory macrophages have a more resolving phenotype where the downregulation of pro-inflammatory cytokine production and promotion of tissue repair (Koh & DiPietro, 2011).

1.7.6.2 Macrophages in the lung

There are distinct populations of macrophages found within the lung, which play a key role in homeostasis through regulation of immune responses to infection and inflammation (Hou et al., 2021). Macrophages within the lung are broadly characterised as alveolar macrophages

(AM), located in the alveolar cavity, or interstitial macrophages, located in the interstitium (Hou et al., 2021). These populations can then be further subdivided into a tissue resident subset, which originate from embryonic development and can regenerate their population through self-replicative processes, or a monocyte-derived subset, recruited from the blood and differentiated in macrophages (Yamasaki & van Eeden, 2018). Under conditions of homeostasis the macrophage lung population is rarely influenced by peripheral blood monocytes, however under states of infection and inflammation, if alveolar macrophage number are diminished it can be repopulated by monocytes recruited to the lungs that differentiate into monocyte-derived macrophages (MDM) (Yona et al., 2013; Hashimoto et al., 2013).

1.7.6.3 Macrophages in COPD

Macrophages play a key role in the pathology of COPD, causing much of the chronic inflammation and ECM breakdown withing the disease (O'Donnell et al., 2006). There is an abundance of macrophages found within the COPD lung, however they are known to have defective phagocytosis, which is associated with increased bacterial colonisation and exacerbations (Taylor et al., 2010; Singh et al., 2021).

Some studies of alveolar macrophage surface markers show increased expression of CD14 in an expanded population of 'small' macrophages compared to non-smokers (Frankenberger et al., 2004), and increased expression of the proliferation marker Ki-67 in smokers, both with and without COPD (Hodge et al., 2007). As CD14 is more highly expressed in peripheral monocytes compared to alveolar macrophages (Jubrail, Kurian & Niedergang, 2017) this gives rise to the hypothesis that COPD caused by exposure to cigarette smoke results in an increased immature population of undifferentiated CD14+ macrophages from the peripheral

blood that replenish the tissue resident macrophage population. This immature macrophage population could explain the reduced phagocytic ability in COPD (Bazzan et al., 2017). Other studies have also shown reduced expression of surface markers including CD14, CD163 and CD203, whilst also releasing more MMP-9 and an insensitivity to glucocorticosteroids (Chana et al., 2014). This again reveals the potential of different subsets of macrophages being present in the COPD lung, which show increased production of pro-inflammatory mediators and reduced scavenger receptor markers, which could explain defects in phagocytosis.

Analysis of macrophages in COPD at a single cell level have found a number of key differences, with single cell RNA sequencing (scRNAseq) revealing differences in alveolar macrophage markers associated with chemotaxis and inflammation, as well as increased expression of metallothioneins (Sauler et al., 2022). This highlights differences in macrophage populations in the lungs of COPD patients compared to healthy controls (Kapellos et al., 2018), however the involvement of metallothioneins in COPD is unclear, as they produce an antioxidant effect by scavenging ROS and seem to convey a protective effect against lung destruction in emphysema (Tanaka et al., 2022).

1.7.6.3.1 Macrophage-derived inflammation

In COPD, it is likely that the increased numbers of macrophages are derived from circulating monocytes recruited to the lung by chemoattractants such as CCL2, and CXCL1 which are highly expressed in COPD sputum (Traves et al, 2002). These increased numbers of macrophages are activated by cigarette smoke and release pro-inflammatory mediators and chemoattractants, including TNF- α , CXCL1, CXCL8, CCL2, LTB₄, and ROS (Fig. 1.4) (Akata & van Eeden, 2020) further perpetuating chronic inflammation. Macrophages also release a number of MMPs, which degrade ECM (Russell et al., 2002). In healthy conditions, MMP are important

in key cell processes including migration, proliferation, and apoptosis (Cabral-Pacheco et al., 2020). However, in COPD the recruitment of excess macrophages to the lung and subsequent activation results in release of MMPs such as MMP-2 and -9, which are responsible for the degradation of the lung matrix (Finlay et al., 1997).

1.7.6.3.2 Defective phagocytosis

There are numerous studies showing that COPD macrophages display reduced uptake of phagocytic prey when compared macrophages from non-smokers and smokers, including gram-positive bacteria such as *Streptococcus pneumoniae* (*S. pneumoniae*) (Taylor et al., 2010; Belchamber et al., 2019), gram-negative bacteria including *Escherichia coli* (*E. coli*) (Prieto et al., 2001) or *Haemophilus influenzae* (*H. influenzae*) (Taylor et al., 2010; Berenson et al., 2006), fungal spores (Wrench et al., 2018) and apoptotic cells such as airway epithelial cells (Hodge et al., 2003). Overall, there is a weight of information that macrophages from COPD subjects are defective in phagocytosis, likely affected by harmful compounds in cigarette smoke or oxidative stress, however the mechanism for this defect is currently still unclear. As such, targeting defective macrophage phagocytosis may lead to novel treatments for COPD.

Recently the phagocytic receptor, siglec-1, also known as CD169, a scavenger receptor associated with tissue resident macrophage populations, has been shown to be decreased in COPD alveolar macrophages compared to cells from non-smokers or healthy smokers (Tanno et al., 2020). As CD169 is known to be involved in the process of pathogen recognition and capture during phagocytosis as well as antigen presentation (Liu, Xia & Qiu, 2021; Grabowska et al., 2018), therefore decreased cell surface expression of CD169 could at least partially explain reduced phagocytosis from lung resident macrophage populations. As reduced

expression of a key phagocytosis receptor could lead to reduced recognition and subsequent internalisation of pathogens in the lung. However, this does not fully explain the systemically reduced phagocytosis from MDM experiments (Belchamber et al., 2019; Taylor et al., 2010).

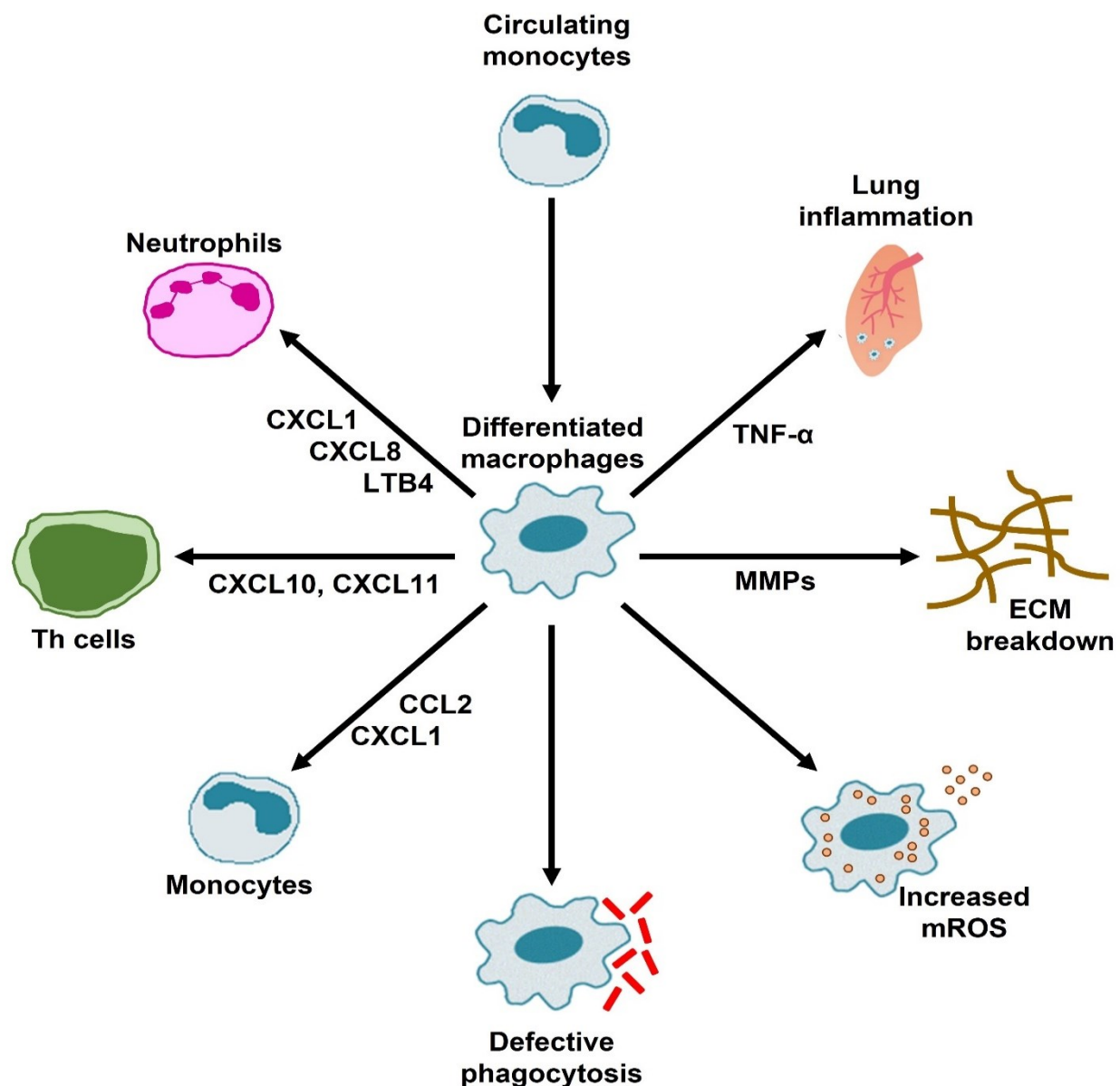


Figure 1.4: Overview of macrophage involvement in COPD. Circulating monocytes are recruited to the site of infection or inflammation, differentiating into macrophages. Macrophages are responsible for the release of pro-inflammatory cytokines including TNF- α , responsible for lung inflammation, and MMPs responsible for extracellular matrix degradation. Macrophages also release chemoattractants responsible for recruitment of neutrophils, monocytes, and T helper (Th) cells to the lung.

1.8 Phagocytosis

Phagocytosis is a complex multi-stage process, whereby specialised phagocytic immune cells, including monocytes, macrophages and neutrophils can detect and destroy foreign particles, pathogens, and apoptotic cells (Rosales and Uribe-Querol, 2017). Recognition of the pathogen (Fig. 1.5 A) activates signalling pathways that result in internalisation of the pathogen through cytoskeletal rearrangement (Fig. 1.5 B), most notably involving actin, whereby cellular protrusions engulf the pathogen into the phagosome (Fig. 1.5 C) (Mylvaganam, Freeman & Grinstein, 2021). The phagosome then undergoes maturation leading to merger with the lysosome, that contains hydrolytic enzymes (Levin, Grinstein & Canton, 2016). This fusion forms a phagolysosome (Fig. 1.5 D), and the pathogen is degraded (Fig. 1.5 E) (Levin, Grinstein & Canton, 2016; Rosales & Uribe-Querol, 2017).

It is important that phagocytic cells recognise a wide variety of foreign particles, microorganisms, and apoptotic cells, and this requires many discrete receptors that will distinguish these from self-cells (Rosales & Uribe-Querol, 2017). Phagocytosis receptors can be separated into two classes: non-opsonised, which directly recognise epitopes on the target organism (Taylor et al., 2005), or opsonised, where the phagocyte recognises opsonins, which are host-derived molecules, such as antibodies or complement, that bind to the foreign particle to target them for internalisation (Ofek et al., 1995).

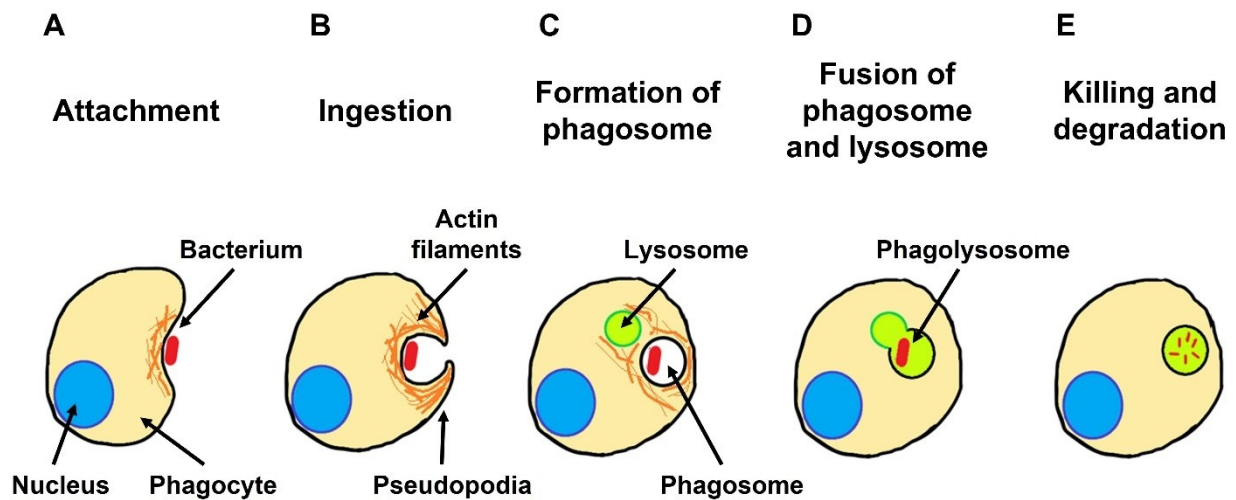


Figure 1.5: Summary of the major stages during the process of phagocytosis. A microorganism is detected by a phagocyte, whereby receptor binding occurs. This produces downstream signalling that activated cytoskeletal restructure of actin filaments to form pseudopodia which engulf the microorganism into a vesicle called the phagosome. The phagosome will mature and fuse with the lysosome where the microorganism is degraded by hydrolytic enzymes and antimicrobial proteins.

1.8.1 Non-opsonic phagocytosis

Non-opsonic receptors bind directly to molecular targets on the surface of foreign objects to stimulate phagocytosis (Rosales & Uribe-Querol, 2017). There is a huge variety of these receptors, but some important examples include mannose receptor (CD206) which binds to mannan, macrophage receptor with collagenous structure (MARCO) which binds to bacteria and is highly expressed on alveolar macrophages (Arredouani et al., 2004), CD33 which binds to lectins and scavenger receptor A (SR-A) which can bind to LPS and lipoteichoic acid (LTA) (Rosales & Uribe-Querol, 2017; Taylor et al., 2005). Scavenger receptors are a family of 10 cell-surface receptors, classed as A-J, which are capable of detecting a wide variety of

pathogen-associated molecular patterns (PAMP) from bacterial and fungal species, where successful binding resulting in induction of phagocytosis (Alquraini & Khoury, 2020).

Toll-like receptors (TLR) are a group of 10 pattern recognition receptors (PRR)s expressed on the surface of immune cells such as macrophages and dendritic cells, but also in fibroblasts and epithelial cells (Kawasaki & Kawai, 2014). These TLRs mainly detect foreign components found in the membranes of microorganisms, such as LTA, mannan and peptidoglycans, which can be detected by TLR2, LPS which can be detected by TLR4, and bacterial flagellin to which TLR5 binds (Kawasaki & Kawai, 2014). However, these receptors do not act as phagocytic receptors themselves, but are responsible for the initiation of pro-inflammatory cytokines and chemokine secretion, including TNF- α and IL-1 β (Kawai & Akira, 2010) and can work in conjunction with phagocytic receptors to enhance phagocytosis by increasing degranulation and respiratory burst (Iwasaki & Medzhitov, 2015). TLR activation also results in induction of a phagocytic gene program, which upregulates genes for scavenger receptors, including MARCO and SR-A, as well as receptors involved in opsonic phagocytosis (Doyle et al., 2004).

1.8.2 Opsonic phagocytosis

Opsonic receptors recognise elements of the hosts defence system rather than the pathogens themselves. These include the Fc receptors (FcR) which bind to the constant Fc region of antibodies such as immunoglobulin G (IgG) (Thau, Asuka & Mahajan, 2022), and complement receptors (CR) that bind to complement (Janeway et al., 2001). Complement-mediated phagocytosis performed by macrophages and neutrophils is an important initial response to bacterial infection and occurs through three pathways: the classical pathway involving antibody activation of complement proteins, the lectin pathway where mannose-binding lectin (MBL) released from the liver activates complement, and the alternative pathway

where components of bacterial and fungal cell walls activate complement proteins (Dustin, 2016; Thau, Asuka & Mahajan, 2022). All three pathways generate complement component 3b (C3b), which is produced by proteolytic cleavage of inactive C3 protein by C3 convertase into the active forms C3a and C3b (Dustin, 2016). C3b then binds to the surface of the microorganism, which is then detectable by phagocytic cells including macrophages and neutrophils (Yuste et al., 2008).

Following engagement of phagocytosis receptors, downstream signalling activates cytoskeletal remodelling (Rosales & Uribe-Querol, 2017). In the case of FcR binding to an antibody opsonised microorganism, the cytosolic region of these receptors, known as the immunoreceptor tyrosine-based activation motif (ITAM), is phosphorylated by the Src-family kinases, which in turn allows co-localised spleen tyrosine kinase (SyK) to bind to and activate ITAM (Mylvaganam, Freeman & Grinstein, 2021). SyK then phosphorylates phosphatidylinositol 3-kinase (PI3K) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn activates GTPase Rac (May & Machesky, 2001). Rac when activated is responsible for actin remodelling, and this process can produce pseudopodia and phagosomes (May & Machesky, 2001).

Complement-mediated phagocytosis relies on a different internalisation mechanism compared to FcR-mediated phagocytosis. Instead of the cellular extension to internalise pathogens, complement-mediated phagocytosis is characterised by the sinking of the microorganism into the plasma membrane (Aderem & Underhill, 1999). This process involves remodelling of the cytoskeleton through the GTPase Rho, to remodel actin at the phagocytic cup (Uribe-Querol & Rosales, 2020; Schulz et al., 2019).

1.8.3 Phagocytic killing

Once the microorganism has been internalised into a phagosome, the process of phagosome maturation occurs (Canton, 2014; Kinchen & Ravichandran, 2008). During maturation, the phagosome becomes acidified by the influx of hydrogen ions (H^+) by vacuolar-type ATPase (V-ATPase) (Marshansky & Futai, 2008) while it is being internally trafficked by cytoskeletal rearrangement towards lysosomes (Levin, Grinstein & Canton, 2016). The lysosome contains hydrolytic enzymes, including proteases, lysozymes and lipases, that degrade key components of microorganisms or apoptotic cells (Kinchen & Ravichandran, 2008). ROS such as superoxide (O_2^-) are produced by NADPH oxidase complexes located on the phagolysosomal membrane (Minakami & Sumimotoa, 2006) that then dismutate into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) (Wang et al., 2018). H_2O_2 can react with calcium ions (Cl^-) to form hypochlorous acid, a potent microbicidal agent capable of destroying pathogens (Uribe-Querol & Rosales, 2020). The phagolysosome is also highly acidic, at pH 4.5, which helps facilitate the degradation of the microorganism (Kinchen & Ravichandran, 2008).

1.9 The cytoskeleton

The cytoskeleton consists of a complex network of proteins, including actin and tubulin (Fletcher & Mullins, 2010) and maintains cellular structure and resists deformation, whilst allowing the cell to move and change shape (Alberts et al., 2002b). Thus, it is intrinsic to the process of phagocytosis.

1.9.1 Actin

Actin is the most abundantly expressed cytoskeletal protein in eukaryotic cells and is integral to cellular function and movement (Cooper, 2000b). Actin participates in more protein-

protein interactions than any other protein (Dominguez & Holmes, 2011) and is highly regulated. Actin occurs in two forms: monomeric or globular actin (G-actin) and filamentous actin (F-actin). There are different isoforms of actin monomers: alpha (α) found in various muscle cells, beta (β) in non-muscle cells and gamma (γ) also found in muscle cells, with these differing by a few amino acids (Dominguez & Holmes, 2011). The formation of F-actin requires polymerisation of G-actin into dimers and trimers, before continual addition of monomers to both ends forms filamentous strands of F-actin (Fig. 1.6).

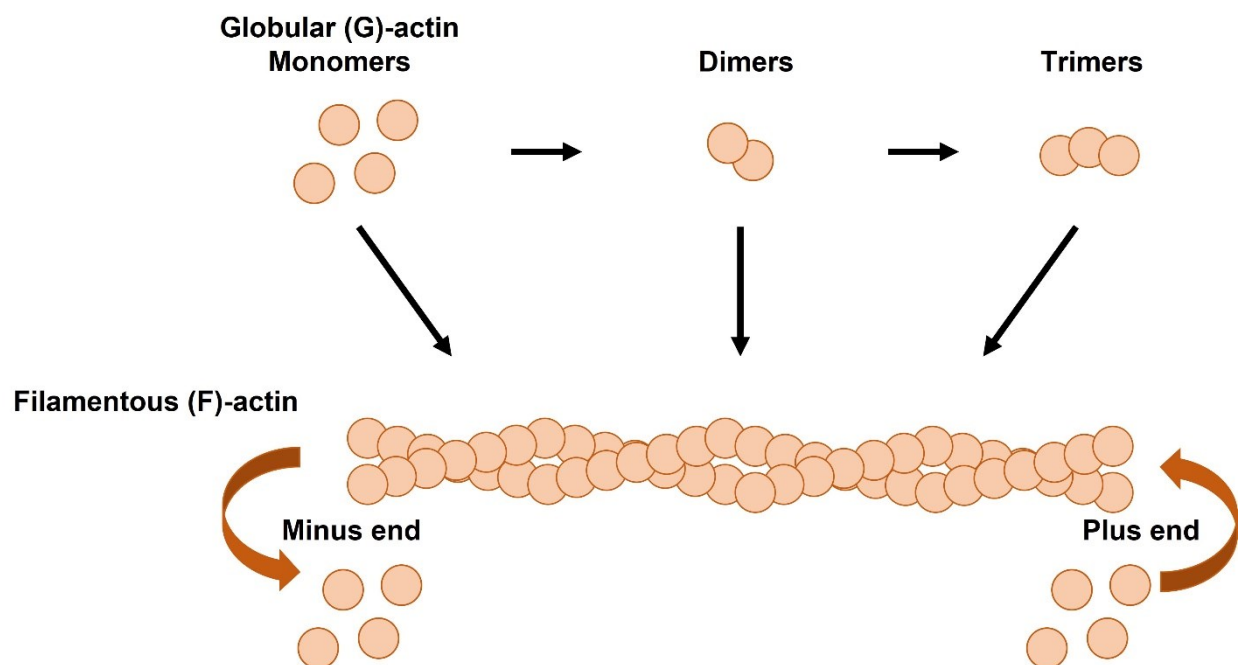


Figure 1.6: Diagram representing filamentous actin production, structure, and maintenance. Globular actin monomers polymerise to form dimers or trimers, with continued addition of monomers to either end forms filamentous (F)-actin. F-actin is under constant addition of monomers at both ends, yet actin polymerisation occurs faster at the plus end compared to the minus end. To maintain F-actin in a steady-state, there is a net addition of G-actin at the plus end and a net dissociation at the minus end.

F-actin is dynamic and undergoes constant association and dissociation, with actin monomers polymerising to the plus end of F-actin at a greater speed compared to the minus end (Wegner

& Isenberg, 1983). To maintain a constant steady-state, there needs to be a net addition of G-actin onto the plus end, with a comparative net loss of G-actin from the minus end, known as “treadmilling”. Cell movement involves the rapid assembly/disassembly of actin filaments, controlled by the actin related protein 2/3 (Arp2/3) complex that is activated by the Wiskott-Aldrich syndrome protein (WASP) (Blanchoin et al., 2000). This results in actin polymerisation to produce rapidly branching actin filaments from the cytoplasm towards the plasma membrane, forming extending pseudopodia that allow the cell to ‘crawl’ (Fig. 1.7) (Pollard & Borisy, 2003). Actin is also involved in the trafficking of organelles and intracellular vesicles, serving as a track, facilitating movement, and internalisation of vesicles (Pollard & Cooper, 2009) (Semenova et al., 2008). For experimental inhibition of actin polymerisation, a potent fungal toxin, cytochalasin D is extensively used, which binds to the end of F-actin, preventing the association or disassociation of monomeric G-actin (Casella, Flanagan & Lin, 1981).

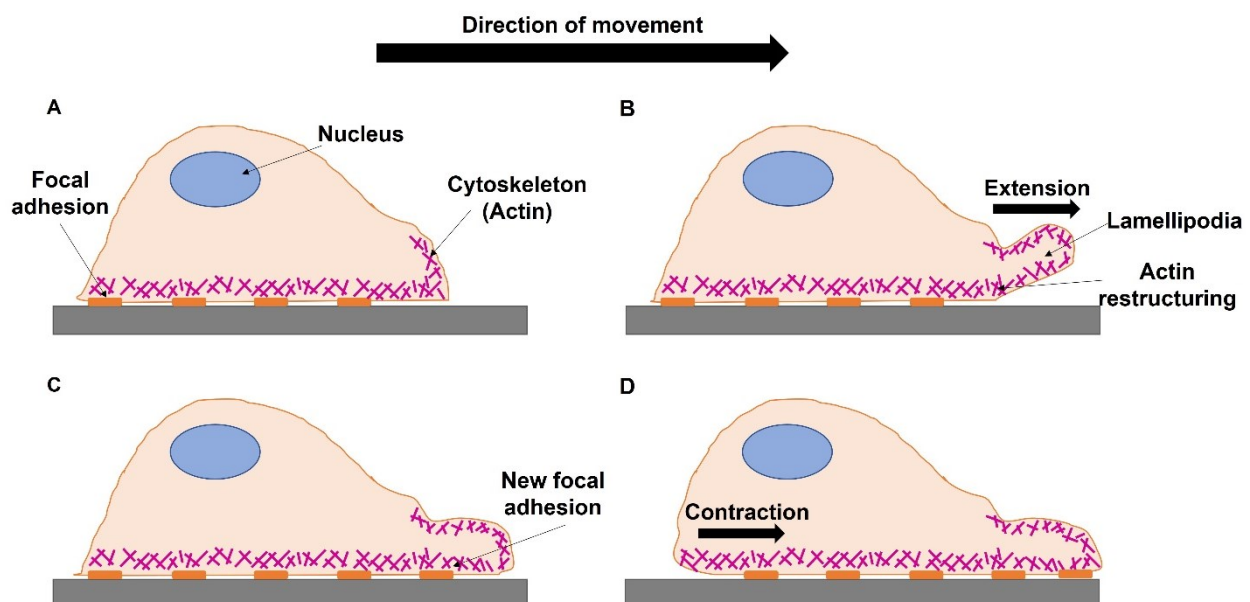


Figure 1.7: Diagram representing actin restructuring for cell movement. Cytoskeletal rearrangement produces the formation of cellular protrusions, lamellipodia (B). This produces a ‘crawling’ effect at one end of a cell (C), with contraction at the other end resulting in cell movement (D).

1.9.2 Tubulin

Tubulin is a highly conserved protein and a key component of the cytoskeletal network. Tubulin is a dimer of α - and β -tubulin, forming dynamic microtubules that are part of the structural framework of the cell and are directly involved in mobility, restructuring of the cell for division, intracellular transport and trafficking (Cooper, 2000a). Microtubules are linear filaments comprised of 13 tubulin dimers that assemble to form cylindrical hollow rods approximately 25 nm in diameter, with a 14 nm hollow centre (Fig. 1.8).

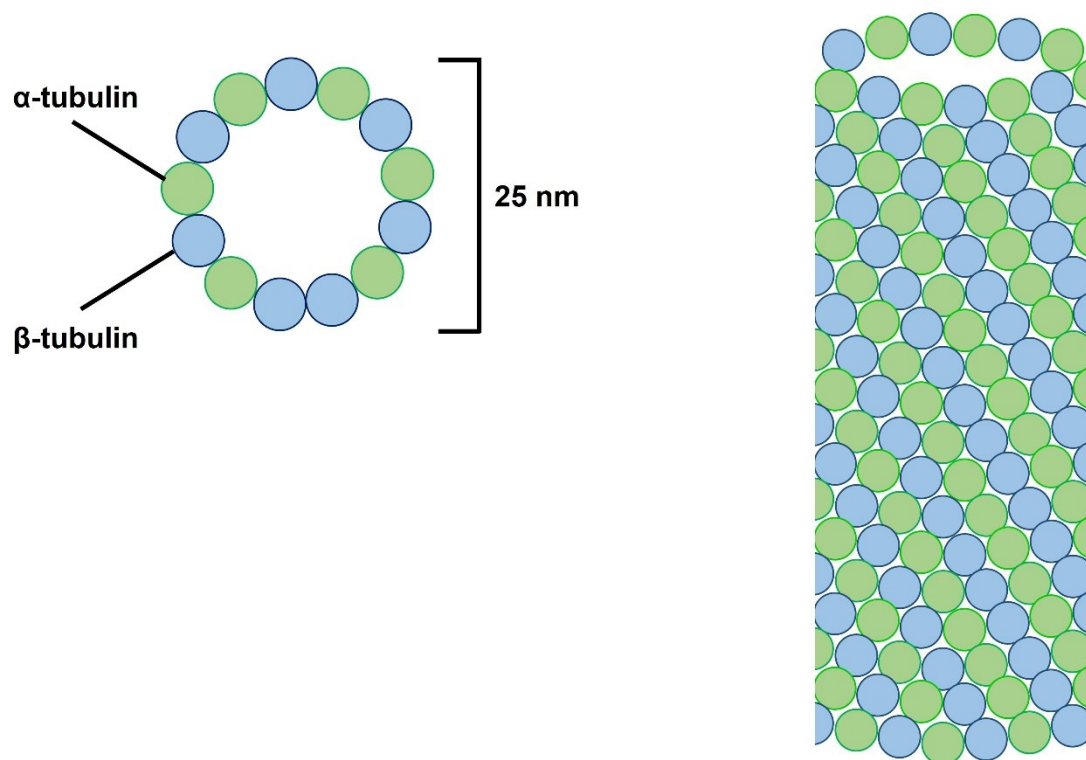


Figure 1.8: Diagram representing microtubule formation. Microtubules are formed by 13 tubulin dimers composed of α - and β -tubulin polymerising to form a 25 nm diameter rod around a 14 nm hollow core.

Microtubules also undergo treadmilling, in a similar manner to that described for actin, whereby guanosine-5'-triphosphate (GTP)-bound tubulin is continually added to the plus end

of a microtubule, with concurrent loss of guanosine diphosphate (GDP) bound tubulin from the minus end (Alberts et al., 2002d). This treadmilling effect, which is driven by GTP hydrolysis, controls the rate at which microtubules grow or shrink and is known as dynamic instability. The constant turnover and redistribution of tubulin to form new microtubules results in microtubules having a short half-life of minutes within the cell, hence can be difficult to track over longer time frames (Brouhard & Rice, 2018).

Microtubules act as tracks for intracellular trafficking of vesicles and cargo by motor proteins, including dynein and kinesin (Goodson & Jonasson, 2018). Dynein moves cargo towards the minus-end of microtubules, whereas kinesin moves towards the plus-end, therefore these motor proteins function in tandem to ensure cargo is trafficked to the correct end of microtubule (Ferro et al., 2019).

For experimental inhibition of tubulin polymerisation, an antineoplastic and anti-mitotic agent, nocodazole can be used. Nocodazole binds to β -tubulin to prevent assembly and disassembly of the microtubule (Vasquez et al., 1997). This disruption in microtubule dynamics causes temporary cell cycle arrest by impairing mitotic spindle formation during metaphase and these prevents cell division (Beswick, Ambrose & Wagner, 2006).

1.10 Role of the cytoskeleton in phagocytosis

The cytoskeleton plays a critical role in macrophages, where cell movement and the production of pseudopodia are required to detect pathogens and undergo phagocytosis. Phagocytosis is known to be an actin driven process (Groves et al., 2008), whereby actin is polymerised locally during binding of opsonised particles to pathogens through Fc gamma receptors (Fc γ R) (Aderem & Underhill, 1999) and complement receptor three (CR3) prior to internalisation (May et al., 2000). Activation of Fc γ R activates cytoskeletal remodelling (Cox

et al., 1999; Richards & Endres, 2014), resulting in depression of the membrane to form the phagocytic cup, and formation of pseudopodia to engulf the pathogen (Beningo & Wang, 2002). FcR-mediated phagocytosis is known solely involved the actin cytoskeletal network, however the microtubule network is involved in CR-mediated phagocytosis, which is known to require both the actin and tubulin cytoskeleton (Uribe-Querol & Rosales, 2020). The use of microtubule inhibitors; nocodazole and taxol, which disrupt microtubule polymerisation, have been shown to reduce CR-mediated but not FcR-mediated phagocytosis in macrophages (Newman, Mikus & Tucci, 1991; Gilberti & Knecht, 2015).

As the cytoskeleton is key to the process of phagocytosis in macrophages, dysfunction of these processes may account for the defective response observed in COPD cells. However, it is challenging to image the cytoskeleton in primary macrophages in real time and therefore to study such dysfunction.

1.11 Mitochondria

Rearrangement of the cytoskeleton requires ATP. This can be produced by mitochondria through the electron transport chain (Jahraus et al., 2001), or through glycolysis (Bonora et al., 2012). Glycolysis produces relatively low amounts of ATP, whereas oxidative phosphorylation produces more ATP molecules (Bonora et al., 2012). Under oxygenated conditions most energy is supplied through oxidative phosphorylation, however in conditions of hypoxia there is a compensatory switch towards glycolysis (Zheng, 2012), and both processes work in tandem to ensure adequate ATP supply within the cells. Both forms of ATP generation are important in a number of cellular processes, including differential macrophage activation in the lung by inflammatory stimuli (Ogger & Byrne, 2021).

The production of ATP through these processes is important for phagocytosis in macrophages and neutrophils (Borregaard & Herlin, 1982; Leeper-Woodford & Mills, 1992). Mitochondria are the 'powerhouse' of eukaryotic cells. Mitochondria are organelles that uniquely contain their own distinct DNA (mitochondria DNA, mtDNA), and are formed of a double membrane, with the inner membrane containing numerous folds called cristae, that extend into the cellular matrix and greatly increase surface area for the primary mitochondrial function of ATP generation through the electron transport chain (ETC) (Alberts et al., 2002c).

In phagocytes, motility and formation of lamellipodia to sense the environment for the detection of foreign objects are key components requiring ATP (Svitkina, 2018), and the process of phagocytosis itself requires ATP to fuel the restructuring of the cytoskeleton for formation of pseudopodia, internalisation and intracellular vesicle trafficking (Groves et al., 2008).

1.11.1 Defective mitochondria in COPD

There is evidence for mitochondrial dysfunction in COPD, consistent with the evidence that accelerated ageing is a key driving mechanism (Cloonan et al., 2020). Mitochondrial number has been estimated using mtDNA copy number and is decreased in COPD peripheral leukocytes compared to non-smokers (Liu et al., 2015). Airway epithelial cells isolated from GOLD4 COPD patients have marked changes in mitochondrial morphology compared to non-smokers, including elongation and swelling of the mitochondria, as well as depletion of cristae with increased branching and fragmentation (Hoffmann et al., 2013) but this has not been explored in macrophages from these patients.

The differences in mitochondrial number and morphology displayed in COPD cells can be explained by defective autophagy, specifically the detection and elimination of defective

mitochondria, known as mitophagy (Barnes, Baker & Donnelly, 2022). COPD cells are unable to eliminate dysfunctional or damaged mitochondria. Impaired autophagy in COPD may also explain defective phagocytosis, as alveolar macrophages from smokers were shown to have altered autophagy, resulting in an accumulation of mitochondria with altered function which was linked to a reduction in phagocytic capabilities when compared to non-smoker subjects (Monick et al., 2010).

Importantly, studies have shown that numerous cell types in COPD patients present with decreased mitochondrial membrane potential ($\Delta\Psi_m$), including cells in bronchial biopsies (Haji et al., 2020), airway smooth muscle (Wiegman et al., 2015) and alveolar macrophages and MDM (Belchamber et al., 2019). This decrease in $\Delta\Psi_m$ results in a depolarised phenotype, which is an indicator of mitochondrial dysfunction or an inactive mitochondrion (Walsh et al., 2017), and is linked to apoptosis of the cell (Gao et al., 2012; Wang & Youle, 2009). During depolarisation, the mitochondria becomes more positively charged and is no longer able to accept H^+ from the intermembrane space as part of the ETC gradient (Wyatt & Buckler, 2004). This results in reduced oxidative phosphorylation and might switch the cell to a less efficient form of ATP generation, such as glycolysis (Zhao, Dennery & Yao, 2018). The decreased $\Delta\Psi_m$ in COPD cells is also associated with increased mitochondrial reactive oxygen species (mROS) production (Bewley et al., 2017; Wiegman et al., 2015).

The mitochondrial production of ATP is critical in phagocytosis, as phosphate depletion resulting in a reduced ATP, leads to a significant reduction in the phagocytosis of bacteria in leukocytes (Kiersztejn et al., 1992). As such, mitochondrial dysfunction is a key target for investigation into defective phagocytosis observed in COPD macrophages.

Release of mROS is a marker of endogenous oxidative stress in COPD, as such the use of mitochondria antioxidants, for scavenging and reduction of mROS, are interesting therapeutic options in COPD. The use of antioxidants have rebalance mitochondria membrane potential *in vitro*, for example in airway smooth muscle cells exposed to ozone to produce oxidative stress (Wiegman et al., 2015) but their effect on macrophages is less clear. One such antioxidant, sulforaphane, has been shown to produce an anti-inflammatory effect by activating the nuclear factor erythroid 2–related factor 2 (Nrf2) pathway to increase heme oxygenase-1 (HO-1) and inhibit NF- κ B, resulting in reduced secretion of IL-1 β and TNF- α levels in macrophages (Santín-Márquez et al., 2019)

1.12 Models of macrophages

When investigating the immune function of macrophages, the most biologically relevant models are primary macrophages isolated from the relevant tissues. These can be collected from tissue resident populations such as lung tissue macrophages (TM) or alveolar macrophages (AM) isolated from resected lung tissue, from bronchoalveolar lavage (BAL) fluid or from sputum samples (Nieto & Alcaraz, 1995; Shaykhiev et al., 2009; Tomar et al., 2016).

Tissue resident macrophages in the lung originate during embryonic development (Wynn, Chawla & Pollard, 2013; Yona et al., 2013), and are a self-regulating population that is adapted to the lung microenvironmental niche (Martin et al., 2021). During infections and inflammatory conditions, the tissue resident macrophage population may become depleted and can be restored by infiltrating monocytes to the lung, differentiating into MDM (Martin et al., 2021). The use of monocytes isolated from peripheral blood and differentiated *in vitro*

into macrophages is a well-established technique in studying macrophage populations (Belchamber et al., 2019; Taylor et al., 2010).

However, there are limitations to using these primary cells. For production of MDM, monocytes are isolated and cultured from whole blood, requiring an incubation time of over two weeks to differentiate them to MDM (Alteri et al., 2022). Obtaining alveolar or tissue macrophages is more difficult due to the invasive nature of the isolation procedures, such as BAL or isolation from lung tissue obtained by surgical resection, usually for lung cancer, making it additionally harder to obtain healthy non-smoker samples. The number of macrophages isolated from these procedures also varies between subjects, with COPD subjects having significantly more phagocytic cells in the lung when compared to non-smokers (Hogg et al., 2004), resulting in a potential discrepancy in isolated cell number. Overall, these invasive procedures generate more variable, and often lower, numbers of isolated macrophages when compared to generating MDM. For experimental convenience, therefore, cell lines may also be used, including immortalised cell populations of interest that can yield inferences relating to the specific cell type.

Due to the heterogeneity of macrophages, bulk or ensemble analysis, such as plate fluorometry, are useful at looking at the total expression of the macrophage population but are unable to distinguish different subsets in macrophage population. An alternative is to perform microscopy, which can allow each individual cell to be visualised and therefore enable single cell analyses that may detect differences in behaviour of subsets of macrophages.

1.13 Fluorescence microscopy

Fluorescence microscopy is a term for microscopy techniques whereby imaging of a fluorescent material or chemical compound known as a fluorophore occurs. The fluorescence is the result of the sample being illuminated with radiation at a wavelength of light that is absorbed by the fluorophore, resulting in excitation from the ground state to a higher excited state. This is followed by emission of photons by the fluorophore, where light is emitted at a longer wavelength than that originally absorbed, known as Stokes shift (Abbyad et al., 2007). These emitted photons are described as fluorescence. This process of absorbing and emitting photons results from transitions of electrons between energy levels and is often visualised in a Jablonski diagram, as illustrated in Fig. 1.9, showing single photon excitation (A), multiphoton excitation (B) and fluorescent and non-fluorescent states that can be utilised in certain super-resolved microscopy techniques.

Fluorescence microscopy entails recording an image of fluorescence emission across the field of view. This can utilise a wide variety of fluorophores such as endogenous intrinsically fluorescent molecules, genetically expressed fluorescent proteins and exogenous fluorescent labels such as small molecule dyes. The latter may bind preferentially to specific components in tissue or may be conjugated to antibodies that bind specific proteins (i.e., immunofluorescence).

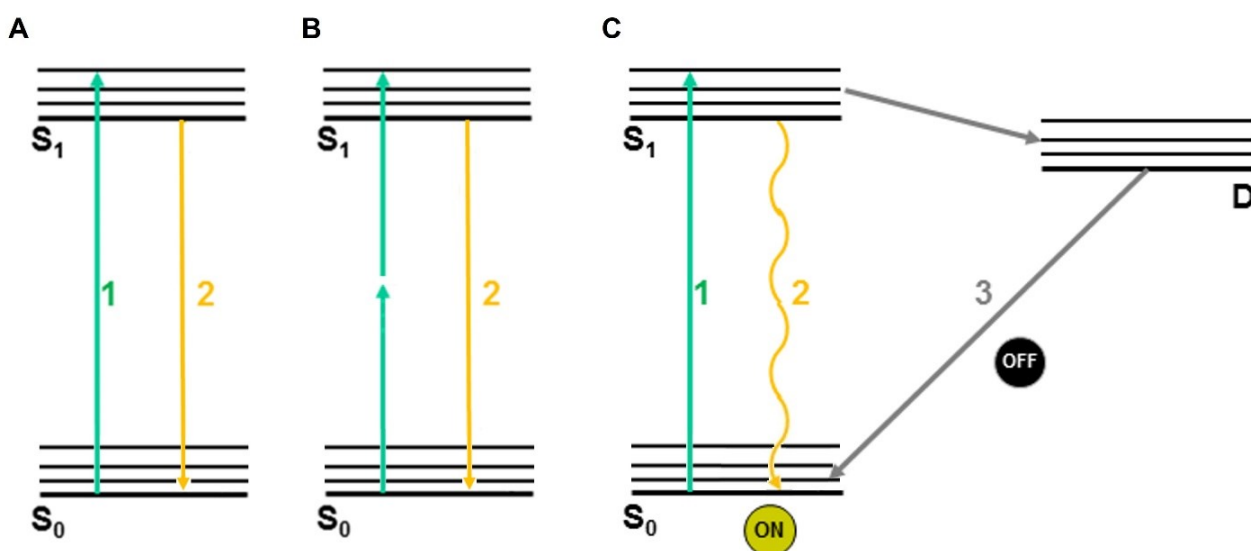


Figure 1.9: Simplified Jablonski diagrams illustrating fluorescent and non-fluorescent states.

There is a manifold of rotational and vibrational energy levels (parallel horizontal lines) associated with each electronic energy level of a fluorophore. In equilibrium the electrons preferentially occupy the lowest energy levels. A fluorophore can be excited from the S_0 ground state, the lowest energy levels, by absorption of one or more photons. Excited fluorophores rapidly thermalise such that electrons occupy the lowest rotational/vibrational energy levels, from where they can decay (by internal conversion or emission of a photon) to any of the rotational/vibrational levels associated with S_0 (shown as line 2 in A-C). From here the fluorophores return to thermal equilibrium, occupying the lowest S_0 levels. The range of possible transition (photon) energies corresponds to the excitation and emission spectra for different fluorophores. Excitation from S_0 to each S_1 state requires absorbance of a specific amount of energy, which can be achieved by single photon (A) or multiphoton excitation (B). Energy can also be transferred through intersystem crossing from the excited singlet state S_1 to a non-fluorescent (“dark”) triplet state, from where the fluorophore can relax to the S_0 ground state by emitting a photon (this low probability emission is called “phosphorescence”) or via a non-radiative pathway (line 3) (photon).

1.13.1 Fluorescence labelling

Cell and tissue components can exhibit innate fluorescence, known as autofluorescence, which can be used to detect cells or other structures in an unstained sample, but more commonly autofluorescence is considered a problem, as it can present an unwanted (and often unknown) background to fluorescence images. There are examples of autofluorescence relevant to this thesis, e.g., alveolar macrophages from smokers were found to be significantly more autofluorescent compared to non-smoker cells (Sköld et al., 1989) and this has been used to identify subpopulations of macrophages (Liégeois et al., 2022).

A common way of incorporating fluorescent markers into cells for the purpose of imaging is the use of fluorescent proteins, such as green fluorescent protein (GFP) (Kremers et al., 2011), which can be fused to specific proteins of interest. This involves genetic manipulation of the cell using techniques such as DNA transfection or viral transduction, to incorporate genes encoding a specific protein tagged with a fluorescent marker, (Chudakov et al., 2010). Fluorescent proteins can be used in live cell imaging experiments; however, these fluorophores can be sensitive to their local environment and are prone to photobleaching, limiting their use (Hirano et al., 2022; Cranfill et al., 2016).

One of the most common and widely used form of exogenous fluorescent labelling for imaging is antibody staining. Antibodies are not cell permeable, so this method is best suited for labelled cell surface proteins but it can still be used to label internal targets following permeabilisation and, as such, antibody labelling is usually performed on fixed cells (Im et al., 2019). The process of antibody labelling can either be performed using a primary antibody directly conjugated to a fluorophore (direct labelling) or using primary antibody staining followed by a secondary antibody, specific for the primary antibody, with a conjugated

fluorescent dye (indirect labelling) (Im et al., 2019). A major benefit of antibody labelling for cell imaging is target specificity. There are a large range of fluorescent dyes available to perform this type of imaging, many of which have been chemically designed to be very bright with good signal to noise ratio, and have improved photostability so are more resistant to photobleaching, such as the Alexa fluor (AF) dyes (Berlier et al., 2003).

Finally, another commonly used technique is the direct use of fluorescent dyes that intrinsically bind specific tissue components, such as silicon rhodamine (SiR) dyes. These dyes are cell permeable, and after diffusing through the cell membrane can chemically bind to specific targets, allowing their use for live cell imaging (Bucevičius et al., 2020). SiR dyes offer bright fluorescence, good signal to noise ratio and good photostability, giving rise to their use in super-resolved microscopy (Kanagasundaram et al., 2019).

1.14 Cell microscopy techniques

The ability to image and track live cells by optical microscopy is a well-established cell biology technique. Fluorescence microscopy has reached single molecule sensitivity with widely available detectors and there are many fluorescent probes that can label specific organelles within cells. However, conventional optical microscopy is limited in spatial resolution by the diffraction of light (Abbe, 1873), which prevents all the image information (spatial frequencies) conveyed by light scattered or emitted by a sample being transferred to the detector. Formally, the limit to the spatial resolution is related to the point spread function (PSF) of the microscope, which is the image of an infinitesimally small object. The effect of limited spatial resolution is to “blur” the image when compared to the object itself. Mathematically, this “blurred” image results from a convolution of the PSF with every emitter or scatterer in the object.

1.14.1 Spatial resolution in optical microscopes

The resolution of a microscope can be defined as the smallest separation distance between two distinct point objects at which they can be individually distinguished (Patterson, 2009) (Fig. 1.10). This is limited by the wavelength of the light being used to image the object and the numerical aperture of the objective lens. It was first defined in terms of the maximum spatial frequency that could be captured by a microscope, leading to the following formulae

for lateral resolution (Abbe, 1882):
$$\Delta_{xy} = \frac{\lambda}{2NA}$$

Where λ is the wavelength of illumination and NA is the numerical aperture (NA) of the microscope objective lens. NA is equal to $n \sin \theta$, where n is the refractive index of the imaging medium and θ is the aperture angle. Optical microscopes have developed to the point where the numerical apertures of objective lenses for live cell imaging are close to their maximum possible value (NA~1.5) and the shortest wavelengths that can be used are already limited by considerations of phototoxicity and the ability to excite fluorescent probes of interest (Valli et al., 2021; Magidson & Khodjakov, 2013).

Using light at 500 nm and an objective lens of NA=1.5, Abbe's formula gives a diffraction-limited lateral resolution of ~170 nm. A more precise analysis of spatial resolution in optical instruments with an effective circular aperture (e.g. microscopes) that limits the maximum spatial frequency results in a PSF corresponding to the Fourier transform of that circular aperture, which is an Airy function (Hecht, 2012). Lord Rayleigh proposed the lateral spatial resolution criterion to be defined as the separation of two just-resolved PSF when the maximum of one PSF coincided with the first minimum of the other, as illustrated in Figure 1.10.

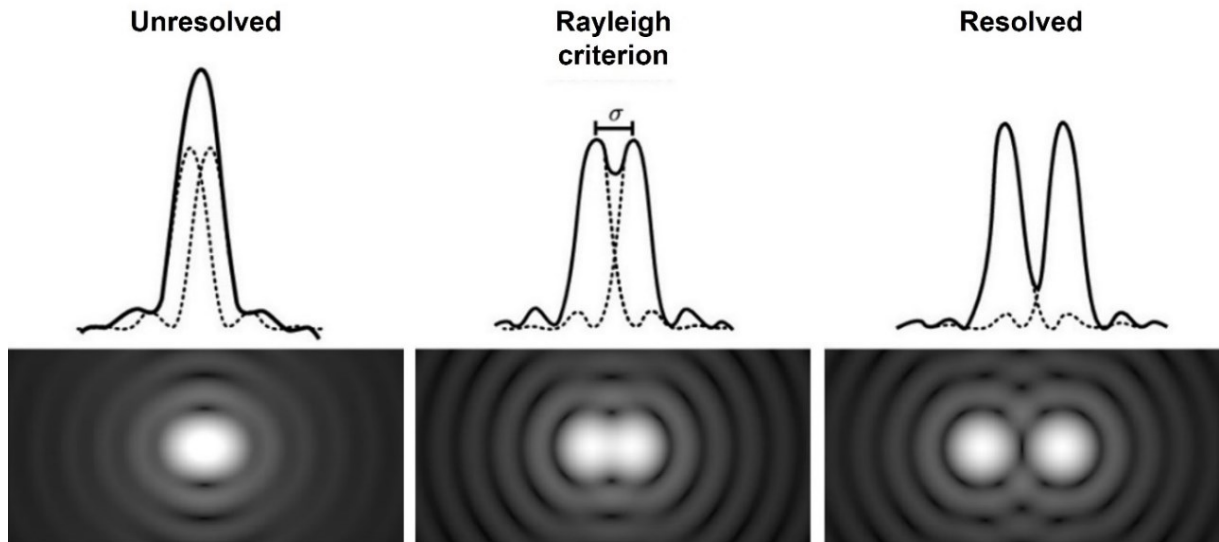


Figure 1.10: Diagram representing the resolution of point objects. When the PSF overlap such that they cannot be distinguished from each other the points become unresolved. As points are further separated the PSF overlap can be distinguished. The centre figure illustrates the Rayleigh criterion where the maximum of one PSF coincides with the first minimum of the other.

This leads to lateral resolution (Rayleigh, 1879): $\Delta_{xy} = \frac{0.61\lambda}{NA}$

Axial resolution (Rayleigh, 1879): $\Delta_z = \frac{2\lambda n}{(NA)^2}$

From these formulas the typical practical resolution achievable with an objective lens of $NA = 1.5$ is approximately ~ 200 nm (lateral) and of ~ 500 nm (axial) (Sanderson et al., 2014).

Note that in a conventional wide-field microscope there is no optical sectioning and the formula for Δz more properly describes the depth of field, i.e., the axial distance over which a plane in the object stays in focus. When imaging an extended object, a wide-field microscope image will also include contributions from out-of-focus light that will blur the recorded image. This out-of-focus light can be physically blocked during image acquisition using an optical sectioning technique, such as confocal microscopy (Elliott, 2020) or multiphoton microscopy (Young et al., 2015).

1.14.2 Laser scanning confocal and multiphoton microscopy

Laser scanning confocal microscopy (LSCM) uses point illumination with a confocal pinhole to produce a single point of detection, whereby light detected from the sample will be primarily collected from a diffraction-limited spot in the focal plane (Elliott, 2020). This suppression of fluorescence from outside of that focal plane improves the signal to noise ratio and can improve the spatial resolution (under the assumption of an infinitely small pinhole). The image is acquired by scanning point illumination over the field of view to produce a thin optically sectioned image. Sample scanning can be achieved using a motorised stage or beam scanning can be implemented, e.g. using fast electronically scanned mirrors (Elliott, 2020).

Multiphoton techniques involve the simultaneous excitation of a fluorophore by multiple photons within an attosecond (10^{-18} s), with the sum of the photon energies matching or exceeding that required for single-photon excitation from the S_0 ground state to the S_1 excited state (Benninger & Piston, 2013). The energy of a photon is inversely proportional to the wavelength, therefore, the wavelength of light used for two-photon excitation can be double that required for single-photon excitation (Dunn & Young, 2006). Multiphoton microscopy can provide increased imaging depth (due to reduced absorption and scattering of longer wavelength photons) and reduced phototoxicity, as the sample is only excited in a single optical section.

1.14.3 Deconvolution microscopy

Instead of acquiring optically sectioned images, the out-of-focus light can be removed from wide-field fluorescence images by computational post-processing to provide in-focus sectioned images using deconvolution (Sibarita, 2005), which can be similar to those that would be obtained using confocal microscopy with the same microscope objective lens.

Deconvolution software tools are available that can be applied post-acquisition to single wide-field images or to z-stacks. As well as removing out-of-focus blur (e.g., resulting from fluorescence above or below the focal plane) of wide-field fluorescence images, they may also improve the image quality – approaching that of confocal microscopy (Guo et al., 2020). In general, deconvolved wide-field fluorescence images can present improved spatial resolution compared to wide-field images, although this will depend on the signal-to-noise ratio of the images and the nature of the sample.

Deconvolution entails computationally deconvolving the PSF from the acquired image data and so deconvolution algorithms require a mathematical description of the PSF (Ikoma et al., 2018). The PSF is a function of the optical properties of the microscope and the sample mounting and so information concerning these parameters may be used to calculate a theoretical PSF, or an experimentally measured PSF may be used, e.g. measured by imaging a sample of beads that are much smaller than the PSF (Guo et al., 2020).

For the work reported in this thesis, I applied commercial deconvolution software (Huygens Essential software version 19.04, Scientific Volume Imaging, SVI) to single wide-field fluorescence images using a theoretical PSF derived from within the software by a PSF generator algorithm.

1.15 Super-resolved microscopy

The diffraction-limited resolution of a microscope was thought to be a fundamental limit to optical microscopy, that could not be practically surpassed. However, advances in detectors, computational and optical techniques have enabled this resolution barrier to be overcome, giving rise to the field of super-resolution microscopy (SRM), which can enable optical microscopes to resolve structures down to below 10 nm (Patterson, 2009).

There are many different methods of SRM including structured illumination microscopy (SIM) (Gustafsson, 2000, 2005), stimulated emission depletion microscopy (STED) (Hell & Wichmann, 1994; Klar et al., 2000) and single molecule localisation microscopy (SMLM) techniques such as photo-activated localisation microscopy (PALM) (Betzig et al., 2006; Hess, Girirajan & Mason, 2006) and stochastic optical reconstruction microscopy (STORM) (Rust, Bates & Zhuang, 2006). These techniques and their relative advantages and performance are reviewed in (Galbraith & Galbraith, 2011; Prakash et al., 2022). The techniques explored for the work reported in this thesis are briefly outlined below.

1.15.1 Stochastic Optical Reconstruction Microscopy

SMLM techniques such as PALM or STORM acquire wide-field fluorescence images of fluorophores whose emission varies stochastically over time and space. This stochastic “blinking” of fluorophores labelling a sample is typically achieved using special photo-switchable fluorophore, such as photo-activated fluorescent proteins (Betzig et al., 2006) or molecular constructs incorporating fluorescent dyes (Rust, Bates & Zhuang, 2006). Alternatively, in an approach described as “*dSTORM*”, stochastic blinking can be induced in standard fluorophore dyes through the use of a chemical buffer containing an oxygen scavenger (Heilemann et al., 2008; van de Linde et al., 2011). If the emitting fluorophores are sufficiently sparse, they can be treated as individual single molecules within the field of view and their lateral positions can be accurately determined by finding the centre of their

respective PSF in the image to a precision given by:

$$\Delta_{xy} = \frac{\lambda}{2NA} \frac{1}{\sqrt{N}}$$

where N is the number of detected photons in the PSF.

Thus, the ability to sequentially activate different, sparse subsets of the photo-switchable fluorophores that are separated by a distance greater than a diffraction limited PSF (Rust, Bates & Zhuang, 2006) essentially allows each fluorophore to be imaged individually. The collection of temporally separated sets of localisation co-ordinates for each fluorophore can be combined to produce a map of every stochastically blinking fluorescent molecule, which can be reconstructed as a super-resolved image. The resolution of a SMLM image is determined by the ability to localise individual emitters, which depends on the number of photons collected and the PSF of the microscope (Xu, Ma & Liu, 2017). To collect sufficient photons for significant enhancement of the resolution, SMLM entails the acquisition of many thousands of images of the blinking fluorophores, which can typically take several minutes per field of view. The scope for motion artefacts on this timescale, and the relatively high excitation powers required, particularly for STORM, often make extended live cell SMLM impractical. However, SMLM of fixed cells, often labelled with dyes using antibodies, is relatively straightforward and STORM – particularly dSTORM, which can be implemented with standard dyes excited at a single wavelength – has become a widely used technique.

For the SMLM work reported in this thesis, I used the technique of *easySTORM* (Kwakwa et al., 2016) developed in the Physics Department at Imperial College London. *EasySTORM* is a cost-effective implementation of *dSTORM* utilising multimode diode lasers for excitation to provide large (>125 μm diameter) fields of view. During my PhD, *easySTORM* was being developed in a parallel project in the Physics Department and I worked with this project to explore the potential to apply this to study phagocytosis.

1.16 Hypothesis

The hypothesis addressed by the work reported in this thesis was that optical microscopy of live macrophages undergoing phagocytosis may be able to determine differences between COPD patients and controls in cell structure and function, including the cytoskeletal network and dysfunctional mitochondria, correlated with the defective phagocytosis observed in COPD, and that this approach could be used to discover drugs that can improve phagocytosis.

1.17 Aims

- To develop a protocol enabling live cell optical microscopy of macrophages undergoing phagocytosis.
- To compare different techniques used in the assessment of phagocytosis, to determine the advantages of using optical microscopy.
- To apply widefield microscopy techniques, image analysis and bacterial quantification to confirm defective phagocytosis in COPD in both MDM and tissue macrophages.
- To compare differential phagocytic responses between the two macrophage models.
- To apply image analysis to determine any morphological differences in macrophages between subject groups.
- To investigate whether differences in cytoskeletal proteins (specifically tubulin and actin) to determine any that may account for defective phagocytosis in COPD macrophages.
- To apply microscopy, image analysis and bacterial quantification to compare mitochondrial function between subject groups, and to investigate if mitochondrial function can be modulated with the addition of mitochondrial antioxidants.

Chapter 2: Materials and Methods

This chapter outlines the materials and methods generally used in the experiments reported in the subsequent chapters. Where there are specific differences in the materials and methods used, these are indicated in the respective chapters.

2.1 Materials

3-(4, 5-Dimethylthiazol2-yl)-2,5 diphenyltetrazolium bromide (MTT)	Sigma-Aldrich, Dorset, UK
5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1)	Fisher Scientific, Loughborough, UK
10-(6-plastoquinonyl) decyltriphenylphosphonium (SkQ1)	Cayman Chemical, Michigan, USA
Agar plates	Sigma-Aldrich, Dorset, UK
AlexaFluor 405	Fisher Scientific, Loughborough, UK
AlexaFluor 488	Fisher Scientific, Loughborough, UK
AlexaFluor 647	Fisher Scientific, Loughborough, UK
Amphotericin	Sigma-Aldrich, Dorset, UK
BEAS-2B cells, Bronchial epithelial cells	ATCC, LGC, Middlesex UK
BD polystyrene tubes	Beckton Dickinson, Oxford, UK
Brain Heart Infusion broth	Sigma-Aldrich, Dorset, UK
Cell dissociation buffer	Sigma-Aldrich, Dorset, UK
Cell scraper	Sigma-Aldrich, Dorset, UK
Columbia Agar with Chocolate Horse Blood and Bacitracin	Fisher Scientific, Loughborough, UK
Costar 24-well tissue culture plates	Fisher Scientific, Loughborough, UK

Costar 96-well black tissue culture plates	Fisher Scientific, Loughborough, UK
Cytochalasin D	Sigma-Aldrich, Dorset, UK
Dextran	Sigma-Aldrich, Dorset, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Dorset, UK
DL-Lactate	Sigma-Aldrich, Dorset, UK
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich, Dorset, UK
Dulbecco's Phosphate-buffered saline (PBS)	Sigma-Aldrich, Dorset, UK
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Dorset, UK
Falcon tubes	Sarstedt, Numbrecht, GER
Fluorescent Activated Cell Sorting clean	Beckton Dickinson, Oxford, UK
Fluorescent Activated Cell Sorting flow	Beckton Dickinson, Oxford, UK
Fluorescent Activated Cell Sorting rinse	Beckton Dickinson, Oxford, UK
Foetal calf serum (FCS)	Invitrogen, Paisley, UK
Gloves	Starlab, Milton Keynes, UK
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	R&D Systems, Abingdon, UK
<i>Haemophilus influenzae</i> strain 1479	ATCC, Teddington, UK
Hanks' Balanced Salt Solution (HBSS)	Sigma-Aldrich, Dorset, UK
HiSpeed Plasmid Plus Maxi Kit	QIAGEN, Hilden, GER
Ibidi μ -Slide 8 well chamber slides	Thistle Scientific, Glasgow, UK
LB broth	Sigma-Aldrich, Dorset, UK
L-glutamine	Sigma-Aldrich, Dorset, UK
Lipofectamine LTX	Fisher Scientific, Loughborough, UK
Luria-Bertani (LB) Agar	Fisher Scientific, Loughborough, UK
Mercaptamine	Sigma-Aldrich, Dorset, UK
Methanol	Sigma-Aldrich, Dorset, UK

MitoSox Red	Fisher Scientific, Loughborough, UK
MitoTracker Red CMXRos	Fisher Scientific, Loughborough, UK
Multimode laser diode source	Cairn Research Ltd, Kent, UK
NanoDrop spectrophotometer	Fisher Scientific, Loughborough, UK
Nocodazole	Sigma-Aldrich, Dorset, UK
One Shot® TOP10 chemically competent <i>Escherichia coli</i> (<i>E. coli</i>)	Fisher Scientific, Loughborough, UK
OptoTIRF module	Cairn Research Ltd, Kent, UK
Oxrase Enzyme	Sigma-Aldrich, Dorset, UK
Parafilm	Sigma-Aldrich, Dorset, UK
Paraformaldehyde	Sigma-Aldrich, Dorset, UK
Pasteur pipette	Sarstedt, Numbrecht, GER
Penicillin/Streptomycin	Sigma-Aldrich, Dorset, UK
Percoll	GE Healthcare, Buckinghamshire, UK
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich, Dorset, UK
Polybrene	Sigma-Aldrich, Dorset, UK
Poly-L-lysine	Sigma-Aldrich, Dorset, UK
Pyrroloquinoline Quinone (PQQ)	Sigma-Aldrich, Dorset, UK
Roswell Park Memorial Institute 1640 (RPMI)	Sigma-Aldrich, Dorset, UK
Saline	Baxter Healthcare Ltd, Norfolk, UK
Silicon Rhodamine (SiR)-actin and -tubulin	Spirochrome, Stein am Rhein, SWI
Super Optimal broth with Catabolite repression (SOC)	Fisher Scientific, Loughborough, UK
SNAP-tag	NEB, Massachusetts, USA
THP-1 cells	ATCC, LGC, Middlesex UK
Tissue culture flasks	Fisher Scientific, Loughborough, UK
Triton-X	Sigma-Aldrich, Dorset, UK

2.2 Methods

2.2.1 Cell culture of BEAS-2B human airway epithelial cell line

An immortalized, non-tumorigenic cell line of human bronchial epithelial cells, BEAS-2B, were purchased from American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM L-glutamine. Cells were adhered to T125 tissue cultures flasks in an incubator at 37°C, 5% (v/v) carbon dioxide (CO₂).

For transfection, staining and imaging; between 2-4 x10⁴ cells, depending on experimental design, were plated into ibidi µ-Slide 8 well chamber slides, pre-coated with poly-L-lysine, and adhered for 24 h.

2.2.2 Cell culture of THP-1 monocytic cell line

A human monocytic cell line derived from an acute monocytic leukaemia patient, THP-1, were purchased from ATCC. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI) media supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 10 mg/mL penicillin/streptomycin. Cells were cultured in suspension at an initial density between 2-4 x 10⁵ cells/mL in T125 tissue culture flasks at 37°C, 5% (v/v) CO₂ with subculturing every 2-3 days before cell concentration reached 8 x10⁵ cells/mL, as THP-1 doubling time is approximately 35 - 50 h (Chanput, Mes & Wichers, 2014).

For transfection, staining and imaging, cells were plated between 1-2.5 x10⁴ cells per well in ibidi µ-Slide 8 well chamber slides in 200 µL of media and treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h to induce macrophage phenotype (Aldo et al., 2013; Daigneault et al., 2010).

2.2.3 Subject Recruitment

2.2.3.1 Whole blood samples

Non-smoking, smoking and COPD subjects, were recruited at the Royal Brompton Hospital Respiratory Biomedical Research Unit, London or St Mary's Hospital, London by research nurses and/or clinical research fellow. All subjects provided informed written consent in accordance with NHS National Research Service, London-Brent (13/LO/0354) ethical approval, as well as providing detailed smoking histories and spirometry testing to measure baseline lung function including FEV₁ and FVC.

All subject groups were age matched, with recruitment age range between 45 and 80 years with inclusion criteria (Table 2.1). Exclusion criteria for the study include any active or pre-existing lung disease excluding COPD, recent chest infections in the preceding six weeks to sample collection, exacerbation of COPD, positive allergy tests and pregnancy/breastfeeding.

COPD subjects were diagnosed according to GOLD criteria, with a FEV₁/FVC ratio <0.7 and FEV₁ % predicted <80%. With non-smoker and smoker subjects group showing normal lung function results with FEV₁:FVC ratio > 0.7 and FEV₁ predicted percentage > 80% (Table 2.1). Subjects were aged 45-80 years and were age-matched between groups.

2.2.3.2 Lung tissue samples

Lung tissue samples were provided from the Royal Brompton Hospital, London from patients undergoing lung resection surgery for lung carcinoma treatment. All subjects recruited provided informed written consent in accordance with the NHS National Research Service, 20/SC/0142, as well as providing detailed smoking histories and spirometry testing to measure lung function (Table 2.1).

Subject group	Age (years)	Lung function	Smoking History
Non-smoker (NS)	45 - 80	Normal lung function FEV ₁ /FVC > 0.7 FEV ₁ predicted % > 80%	No history of smoking 0 pack years
Smoker	45 - 80	Normal lung function FEV ₁ /FVC > 0.7 FEV ₁ predicted % > 85%	Current or ex-smoker > 10 pack-years
COPD	45 - 80	Diagnosis of COPD (GOLD 1 – 4) FEV ₁ /FVC < 0.7 FEV ₁ % predicted < 80%	Current or ex-smoker > 10 pack-years

Table 2.1. Inclusion criteria for blood and tissue samples. Patients recruited to the study for either blood samples or resected lung tissue were age matched between groups and provided consent for patient history and lung function data to be acquired.

2.2.4 Isolation of primary monocytes and differentiation to MDM

Blood was donated from human volunteers; 60 ml of blood was collected by venepuncture into three 20 mL syringes containing 500 µL 0.5 M ethylenediaminetetraacetic acid (EDTA) and diluted 1:1 with Hanks' Balanced Salt Solution (HBSS); erythrocytes were removed by sedimentation using 6% (w/v) dextran. Peripheral blood mononuclear cells (PBMC) were isolated by gradient density centrifugation using Percoll at gradients of 55, 67 and 81% (v/v) and centrifuged at 1000g for 30 min at 20°C with no brake. The leukocyte rich PBMC layer, interface between 55% and 67% (v/v) Percoll gradients, was collected with a Pasteur pipette into a fresh Falcon tube and washed twice in HBSS before being resuspended in RPMI 1640

media supplemented with 10% (v/v) FCS, 2 mM L-glutamine and 100 µg/mL penicillin/streptomycin.

Monocytes were counted, using Kimura stain to identify mononuclear cells, and were plated at 100 µL/well for 8-well chamber slides, 96-well plates or 500 µL per well for 24-well plates, for 2 h to allow monocytes to adhere. Media was aspirated to remove non-adherent cells and fresh RPMI 1640 containing 2 ng/mL GM-CSF was added to wells. Cells were incubated at 37°C, 5% (v/v) CO₂ for 12 days to allow differentiation into MDM with media being removed and replaced with fresh media containing GM-CSF on days 4 and 7, following previous work (Belchamber et al., 2019; Taylor et al., 2010).

2.2.5 Isolation and culture of tissue macrophages from resected lung tissue

Macrophages were isolated from resected lung tissue from human volunteers. Macroscopically normal lung tissue as defined by a pathologist was obtained from patients undergoing surgical resection for carcinoma, as previously described (Smith et al., 2004, 2006). Tissue was flushed with RPMI 1640 supplemented with 5 mM EDTA, 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine and 2.5 µg/mL amphotericin, to lavage cells and collected into a Sterilin tube. Cells were centrifuged at 300g for 5 min at room temperature to pellet the cells, before removing the supernatant and resuspending the cells in RPMI 1640 supplemented with 5 mM EDTA, 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine and 2.5 µg/mL amphotericin. Macrophages were isolated by gradient density centrifugation using Percoll gradients of 25% and 65% (v/v) and centrifuged at 500g for 30 min at 20°C with no brake.

The macrophage fraction was collected at the 25 - 65% interface and washed in phosphate-buffered saline (PBS) before macrophages were counted and resuspended in RPMI 1640

supplemented with 5 mM EDTA, 100 µg/mL penicillin/streptomycin, 2 mM L-glutamine and 2.5 µg/mL amphotericin with the volume adjusted to give a macrophage density of 1×10^6 macrophages/mL. Cells were then plated at 100 µL/well for 8-well chamber slides and 96-well plates or 500 µL per well for 24-well plates and left overnight to allow cells to adhere. Media was aspirated the next day to remove non-adherent cells and replaced with fresh RPMI 1640. Previous work has shown macrophage preparation produced >95% purity determined by Kimura staining and immunocytochemistry labelling of anti-CD68 antibody (Smith et al., 2004; Tudhope et al., 2008).

2.2.6 Preparation of Fluorescently Labelled Bacteria

2.2.6.1 Bacteria Culture and Heat Killing

H. influenzae, strain 1479, isolated from a COPD patient, was streaked onto Columbia agar with chocolate horse blood and bacitracin and incubated at 37°C for 24 h. A single colony was harvested and inoculated into 25 mL of brain heart infusion (BHI) broth for an 8 h incubation at 37°C in a shaker incubator at a constant rotation of 150 rpm. Bacteria were transferred into 500 mL of broth for a further 12 h incubation under the same conditions. Optical density (OD) measured using a spectrophotometer at $\lambda = 600$ nm was monitored to determine growth rate and calculate colony-forming units (CFU) (Fig. 2.1), with bacteria being harvested once an OD of 1.2 had been reached, to ensure bacterial growth was not inhibited by lack of nutrients and overpopulation, cells were collected in the exponential phase to ensure cells had not entering the death-phase.

During all stages of bacterial growth, agar plates were streaked and incubated at 37°C overnight to ensure a pure live population of bacteria was present. Broth was centrifuged for 20 min at 2500g to pellet bacteria. This was resuspended in 20 mL HBSS before heat killing

the bacteria by incubation for 2 h at 70°C. An agar plate was streaked and incubated at 37°C overnight to confirm no live bacteria were present after heat killing. Heat killed bacteria stock were frozen at -80°C before labelling with a fluorescent dye.

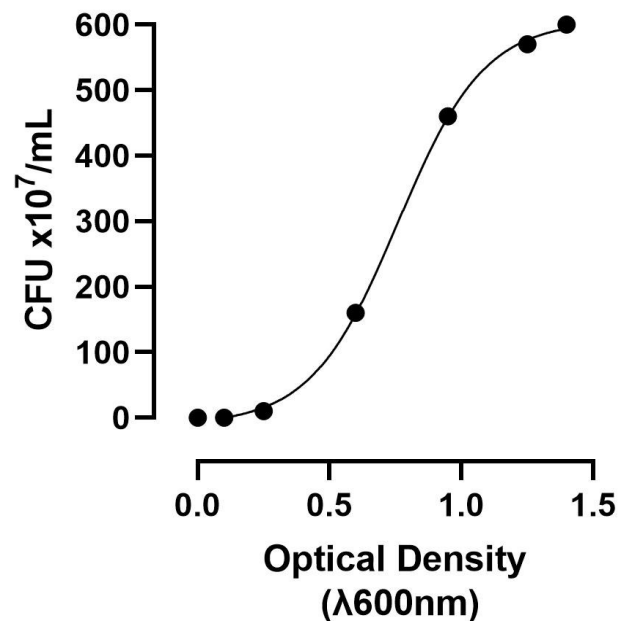


Figure 2.1. Bacterial growth curve over time for *H. influenzae*. A single colony of *H. influenzae* was inoculated in BHI broth and incubated at 37°C, 5% (v/v) CO₂ under constant rotation. Optical density was measured every hour at $\lambda 600\text{nm}$ in a spectrophotometer to determine CFU.

2.2.6.2 Fluorescent Labelling of Bacteria

Heat-killed bacteria were thawed prior to centrifuging at 2500g for 10 min and resuspended in 15 mL of 0.1 M sodium bicarbonate buffer (NaHCO₃), pH 8.3, as a slightly basic solution is required to maximise the amide bonding between the amine-reactive reagent to proteins. Bacteria were sonicated for 5 min before incubation with 150 μL of AlexaFluor (AF) 405 or 488 NHS (Succinimidyl) Ester, 10 mg/mL in dimethyl sulfoxide (DMSO) on a windmill rotator overnight protected from light. Bacteria were then washed three times by centrifugation and resuspension of the pellet in sterile PBS to remove any free dye from the supernatant. The

labelled bacteria were resuspended in 15 mL of sterile PBS, before aliquoting into 1 mL Eppendorf tubes at a final concentration of 5×10^9 CFU/mL and frozen at -20°C .

2.2.6.3 Preparation of bacteria for phagocytosis assays

Frozen stock of the heat-killed fluorescently labelled *H. influenzae* as described in 2.2.6.2 were thawed at room temperature and sonicated for 10 min. Bacteria were diluted 1:10 in fresh RPMI warmed to 37°C , to produce a working concentration of 5×10^8 CFU/mL. For use in subsequent cell exposure experiments, media was removed from cells, THP-1, MDM or tissue macrophages, and replaced with media containing diluted *H. influenzae* for up to 1 h.

2.2.7 Production of SNAP-tag DNA plasmids for transfection experiments

One Shot® TOP10 chemically competent *Escherichia coli* (*E. coli*) were transformed with SNAP-tag plasmid DNA. *E. coli* were thawed on ice and incubated with plasmid DNA for 30 min on ice before heat shocking at 42°C for 45 s. 1 mL of Super Optimal broth with Catabolite repression (SOC) was added to the vial and incubated for 1 h at 37°C in shaking incubator. Agar plates were prepared in advance by dissolving 32 g Luria-Bertani (LB) agar powder in 1 L deionised water before autoclaving and cooled before adding 100 μL ampicillin. Transformed *E. coli* were streaked on LB agar plates containing ampicillin and incubated at 37°C overnight. A single colony was removed and cultured in LB broth overnight at 37°C in shaking incubator. LB broth was centrifuged at $6000g$, 4°C for 30 min to pellet bacteria and plasmid DNA was isolated using the HiSpeed Plasmid Plus Maxi Kit according to manufacturer's instructions.

The bacterial pellet was resuspended in 10 mL buffer P1, before adding 10 mL of buffer P2 and mixed by inverting before incubating at room temperature for 5 min, resulting in a blue solution. 10 mL of prechilled Buffer P3 was added and mixed by inverting until the solution turned colourless. The lysate was added into a QIAfilter Cartridge and incubated at room

temperature for 10 min, before using a plunger to filter the cell lysate into a HiSpeed Tip. The HiSpeed Tip was washed with 60 mL Buffer QC, before DNA was eluted with 15 mL Buffer QF. DNA was precipitated by adding 10.5 mL isopropanol and incubated for 5 min at room temperature. The eluate-isopropanol mixture was then filtered through a QIAprecipitator Module attached to a 30 mL syringe, before washing the QIAprecipitator Module by passing 2 mL 70% (v/v) ethanol through the syringe. The QIAprecipitator was dried and attached to a 5 mL syringe, before 1 mL of Buffer TE was added to elute DNA into a collection tube using a plunger under constant pressure. Plasmid DNA quality and quantity was assessed using a NanoDrop spectrophotometer.

2.2.8 Transfection of plasmid DNA into BEAS-2B and THP-1 cell lines

BEAS-2B or THP-1 cells treated with PMA were cultured in 24-well plates at a density of $5-10 \times 10^4$ cells per well for 24 h prior to transfection experiments. Varying amounts of the transfection reagent, Lipofectamine LTX, 1 - 5 μ L was added to 50 μ L of serum free media in an Eppendorf tube. Plasmid DNA was diluted in serum free media, with between 1 - 5 μ g added to 250 μ L media in an Eppendorf tube, before 5 μ L of PLUS reagent was added. The diluted DNA was mixed with the diluted Lipofectamine LTX Reagent at a 1:1 ratio and incubated at room temperature for 5 min to produce DNA-lipid complexes. The DNA-lipid complex was added to the adherent cells and incubated for between 24 and 72 h at 37°C, 5% (v/v) CO₂.

2.2.9 Transduction of GFP using lentiviral vector into BEAS-2B and THP-1 cell lines

Lentiviral particles encompassing DNA for green fluorescence protein (GFP)- β actin (actB) gene was kindly donated by Dr J. Baker (particles were provided at 35000 copies/ μ L). The lentiviral construct contains the puromycin N-acetyltransferase (pac) gene from *Streptomyces*

alboniger, which allows positive selection of transfected cells with the use of puromycin dihydrochloride. Puromycin is an aminonucleoside antibiotic which is a potent inhibitor of protein synthesis by producing premature termination of translation in cells. Treatment of cells with low concentrations of puromycin produces rapid cell death, yet cells which have been successfully transduced with the pac gene will confer resistance to puromycin, allowing the generation of a puromycin-resistant stable cell line.

BEAS-2B and THP-1 cells were plated into either 6-well tissue culture plates at a density of 2×10^5 cells per well or 24-well tissue culture plates at a density of $5 - 10 \times 10^4$ cells per well and adhered for 24 h. Lentivirus particles were added in varying amounts from 1 μ L to 20 μ L. To increase transduction efficiency, a small positively charge molecule, polybrene was added at 10 μ g/mL, which binds to cell surfaces to neutralise surface charge, increasing binding of pseudoviral capsids to the cellular membrane (Davis, Morgan & Yarmush, 2002).

Cells were incubated for 72 h at 37°C, 5% (v/v) CO₂ before being washed and re-plated in T75 flasks. Cells were assessed by microscopy and flow cytometry through multiple consecutive passages to determine if a stable GFP-actin cell line had been produced.

2.2.10 Preparation and use of fluorescent dyes and stains

2.2.10.1 Silicone Rhodamine (SiR) dyes

SiR-tubulin and SiR-actin were prepared by dissolving the content of the vial, 50 nmol, into 50 μ L of DMSO to make a 1 mM stock solution, which was stored at -20°C. The stock solution was diluted 1:1000 in fresh media to make a working solution of 1 μ M. BEAS-2B, THP-1, MDM and tissue macrophages were incubated with SiR-tubulin or SiR-actin for 1 h at 37°C, 5% (v/v) CO₂. Cells were washed three times with fresh media to remove excess SiR dye from sample

prior to incubation with *H. influenzae* at 37°C, 5% (v/v) CO₂ for up to 1 h prior to cells being imaged live.

2.2.10.2 MitoTracker Red CMXRos

MitoTracker was used to identify mitochondria in the cytoplasm. MitoTracker was prepared by dissolving 50 µg into 94.07 µL of DMSO to produce a 1 mM stock solution. The stock solution was diluted 1:2000 in fresh media to make a working solution of 500 nM. THP-1, MDM and tissue macrophages were incubated for 30 min at 37°C, 5% (v/v) CO₂ before three washes with fresh media to remove excess MitoTracker, prior to bacterial incubation with *H. influenzae* for up to 1 h. Macrophages were washed three times to remove unbound bacteria and were immediately imaged live by confocal microscopy.

2.2.10.3 MitoSox Red

MitoSox was used to measure mROS production. MitoSox was prepared by dissolving 50 µg into 13 µL of DMSO to produce a 5 mM stock solution. The stock solution was diluted 1:1000 in fresh media to make a working solution of 5 µM. THP-1, MDM and tissue macrophages were incubated with 5 µM MitoSox solution for 15 min at 37°C, 5% (v/v) CO₂, prior to three washes with fresh media to removed excess MitoSox. After staining, macrophages were exposed to *H. influenzae* for up to 1 h before being washed three times to remove unbound bacteria and imaged live by confocal microscopy.

2.2.10.4 JC-1

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was used to measure mitochondrial potential and was prepared by dissolving 30 µg of JC-1 into 230 µL of DMSO to produce a 129.8 µg/mL (200 µM) stock solution.

The stock solution was diluted 1:26 in fresh media to make a working solution of 5 µg/mL (7.7 µM). THP-1, MDM and tissue macrophages were incubated for 30 min at 37°C, 5% (v/v) CO₂ prior to three washes with fresh media to remove excess JC-1 dye before cells were incubated with bacteria and imaged.

2.2.11 Preparation and use of cytoskeletal inhibitors

2.2.11.1 Cytochalasin D

Cytochalasin D was used to inhibit actin. Cytochalasin D was prepared by dissolving 1 mg into 197 µL of DMSO to produce a stock solution of 10 mM, which was aliquoted and stored at -20°C. Stock cytochalasin D was diluted 1:1000 in fresh media for a working concentration of 10 µM. THP-1 and MDM were preincubated with 10 µM cytochalasin D for 1 h at 37°C, 5% (v/v) CO₂ before incubation with bacteria for imaging experiments.

2.2.11.2 Nocodazole

Nocodazole was used as a tubulin inhibitor and was prepared by dissolving 10 mg into 3.32 mL of DMSO to produce a stock solution of 10 mM, which was aliquoted and stored at -20°C. Stock nocodazole was diluted 1:100 in fresh media for a working concentration of 100 µM. THP-1 and MDM were preincubated with 100 µM nocodazole for 1 h at 37°C, 5% (v/v) CO₂ before incubation with bacteria for imaging experiments.

2.2.12 Preparation and use of mitochondrial antioxidants

2.2.12.1 Pyrroloquinoline Quinone (PQQ)

A stock solution of PQQ was made by dissolving 10 mg into 1 mL of DMSO to produce a concentration of 26.73 mM. 3.74 µL of stock solution was dissolved into 1 mL of fresh media to make a concentration of 100 µM from which a serial dilution in media was performed to

produce working concentrations of 500 nM, 100 nM and 50 nM. MDM were preincubated with 50 nM - 500 nM PQQ, for either 1 h or 24 h at 37°C, 5% (v/v) CO₂. MDM were then exposed with *H. influenzae* for up to 1 h, before three washes to remove unbound bacteria. MDM were then stained with MitoTracker, MitoSox or JC-1 as previously described and imaged live by confocal microscopy.

2.2.12.2 10-(6-plastoquinonyl) decyltriphenylphosphonium (SkQ1)

A stock solution of SkQ1 was made by dissolving 5 mg into 1.62 mL of DMSO to produce a concentration of 5 mM. 2 µL of stock solution was dissolved into 1 mL of fresh media to make a concentration of 10 µM from which, a serial dilution was performed in media to produce working concentrations of 500nM, 100 nM and 50 nM. MDM were preincubated with 50 nM - 500 nM SkQ1, for either 1 h or 24 h at 37°C, 5% (v/v) CO₂. MDM were then incubated with *H. influenzae* for up to 1 h, before three washes to remove unbound bacteria. MDM were then stained with MitoTracker, MitoSox or JC-1 as previously described and imaged live by confocal microscopy.

2.2.13 Measurement of macrophage phagocytosis using fluorimetry

THP-1 or MDM were plated in Costar 96-well black plates at a density of 1 x10⁶ cells/well. Cells were incubated with *H. influenzae* fluorescently labelled with AF488 for up to 1 h at 37°C, 5% (v/v) CO₂. Following bacterial incubation, cells were washed three times in D-PBS to remove unbound bacteria, before 100 µL trypan blue was added per well for 2 min to quench extracellular fluorescence. Trypan blue was removed from each well and fluorescence was measured for AF488, excitation λ480 nm/emission λ520 nm using a BMG Fluostar plate reader, with relative fluorescence intensity (RFU) used to quantify phagocytosis (Liang et al., 2014)

2.2.14 Measurement of cell viability

Cells plated into 96-well plates were incubated with 50 μ L of 0.1% (w/v) 3-(4, 5-Dimethylthiazol2-yl)-2,5 diphenyltetrazolium bromide (MTT) and incubated for 30 min at 37°C, 5% (v/v) CO₂. MTT was removed from plate prior to adding 50 μ L of DMSO to lyse the cells, with absorbance being read on a Spectramax photometer at λ 570nm. Non-treated cells were considered 100% viable.

2.2.15 Measurement of phagocytosis by flow cytometry

Adherent BEAS-2B, THP-1 cells or MDM were removed from the plates using cell dissociation buffer incubated for 10 min at 37°C, 5% (v/v) CO₂ before gentle scraping. Cells were collected into Eppendorf tubes and centrifuged at 300g for 5 min. Cell pellets were re-suspended in fresh PBS and collected into FACS tubes.

Flow cytometry was performed on a BD FACSCanto with cell populations determined and gated by forward scatter area (FSC-A) and side scatter area (SSC-A), before doublets were removed by gating for FSC-A and forward scattered height (FSC-H), seen in Fig 2.2 A-B. Fluorophore gating were adjusted using unstained control samples of cells.

Fluorophores and cell stains were detected using appropriate filters, depending on excitation and emission wavelengths, with *H. influenzae* labelled with AF488. All data analysis was performed using FlowJo v10, and measurements were taken as percentage of GFP positive cells.

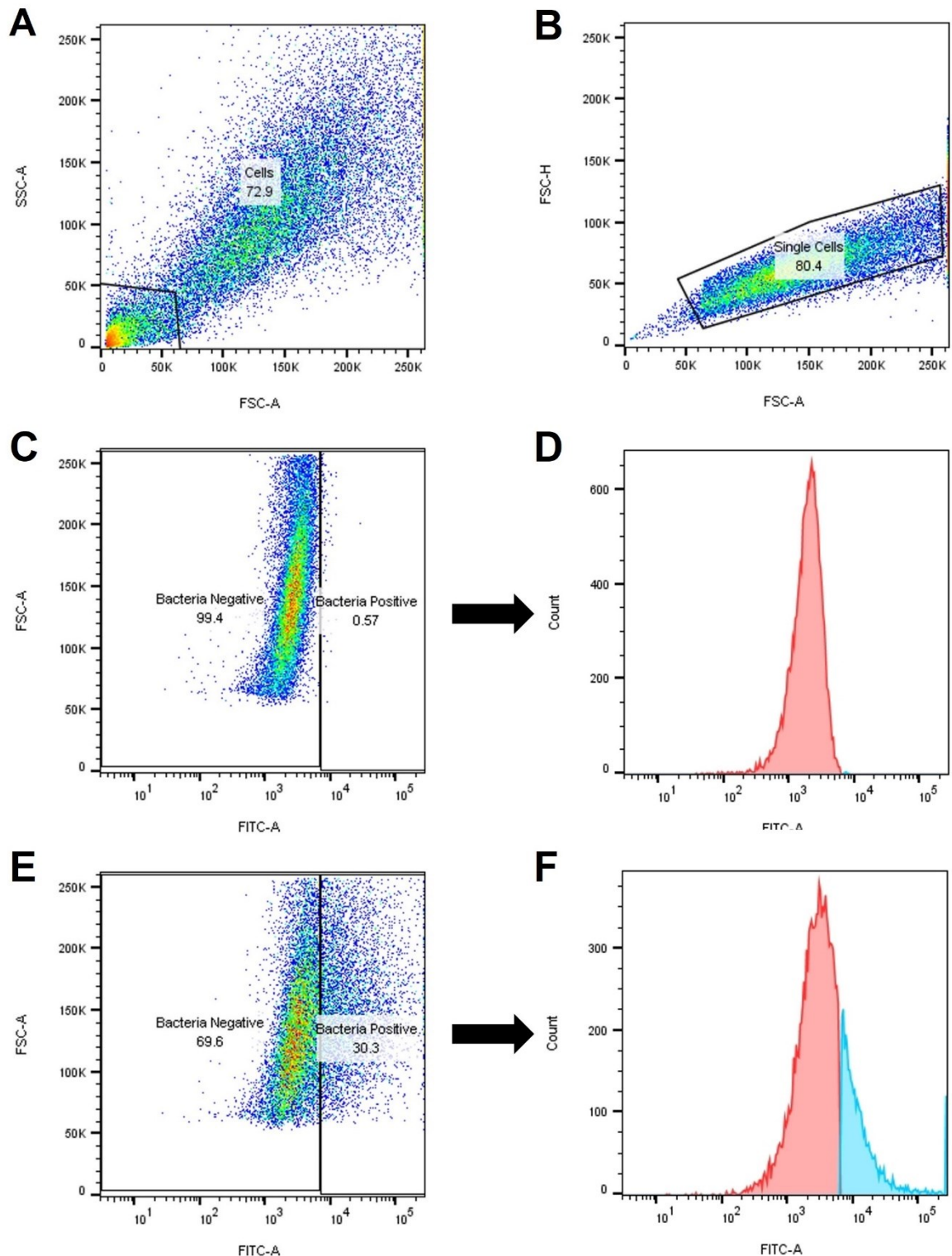


Figure 2.2. Flow cytometry analysis and gating strategy of MDM phagocytosis. (A) Cell populations were gated on forward scatter area (FSC-A) and side scatter area (SSC-A) with doublets removed by gating forward scatter height (FSC-H) against FSC-A (B). A control population with no bacteria present (C) shown as the red histogram (D), was used to identify, and set the gate for cells positive for bacteria (E), shown as blue region of histogram (F).

2.2.16 Visualisation and imaging of cells by epi-fluorescent widefield microscopy

BEAS-2B, THP-1, MDM or tissue macrophages were imaged in 8-well chamber slides using a 100x, 1.46 NA oil lens, using the same microscope set up as STORM imaging in 2.2.16.4. Cells were imaged at the interface of the chamber slide to visualise key cytoskeletal protein structure, including tubulin in microtubules and actin podosomes structures. Cells labelled with AF488 were excited using a 462nm laser, and AF647 were excited at 635nm, all using a Cairn multimode laser diode source, with a typical excitation intensity of 0.5 kW/cm² with a frame integration rate between 10 ms and 50 ms. The microscope, camera and excitation lasers were all controlled during image acquisition using the MicroManager software.

2.2.17 Visualisation and imaging of cells by confocal microscopy

8-well chamber slides were imaged using a 63x, 1.40 NA oil lens in Leica SP8 inverted confocal microscope housed within a temperature-controlled incubation chamber. For imaging experiments, both photomultiplier tube (PMT) and hybrid detectors (HyD) were used for fluorescence detection, and PMT detector was used for brightfield. Depending on fluorescence labelling, the sample was excited with the following lasers and corresponding wavelengths: Diode - 405 nm, Argon - 458, 488 and 514 nm and HeNe - 633 nm. Laser intensity, gain and offset were optimised for each fluorescent dye and kept constant across imaging experiments. The microscope, excitation lasers and detectors were all controlled during image acquisition using the Leica software, LASX.

2.2.18 Deconvolution of widefield images

Single epi-fluorescent widefield images were converted to tiff. file formats and deconvolved using Huygens Essential software version 19.04, Scientific Volume Imaging (SVI). Each imaging channel had settings optimised based upon excitation wavelength, 488nm and 647nm, with

deconvolution using the classic maximum likelihood estimation (CMLE) algorithm and signal-to-noise ratio between 20 and 30, maximum of 40 iterations, and stopping criterion of 0.01. The PSF used for deconvolution was a theoretical PSF generated by the Huygens software.

2.2.19 Post-acquisition processing of widefield and confocal images

Post-acquisition image processing was performed on all imaging data sets using ImageJ. Images were imported into ImageJ and processed to have consistent brightness and contrast across imaging experiments and to reduce background noise, with all files saved in the TIF file format.

Images of MDM or tissue macrophages were also analysed using custom MATLAB code by Dr Yuriy Alexandrov, to perform an unbiased analysis of cell morphology and markers for the cytoskeleton and mitochondria. Cell segmentation was performed for samples stained for actin or tubulin, by using a 3-scale log detection complemented with a median-normalisation σ_d texture (Fig. 2.3) (Guglielmi et al., 2021).

Measurements including cell area and perimeter were calculated using the known pixel range of the camera converted into μm . Cell shape was determined by eccentricity, which can be used to define the 'roundness' of a cell. Values are given between 0 and 1, with 0 being a perfect circle and a value of 1 being a parabola, values in-between 0 - 1 denotes the range of elongation, with higher values being more of an ellipse. For segmentation of subcellular cytoskeletal structures, punctate segmentation was performed using a peak-sensitive local detection and fibres using a fibre-selective enhancement mask (Fig. 2.3).

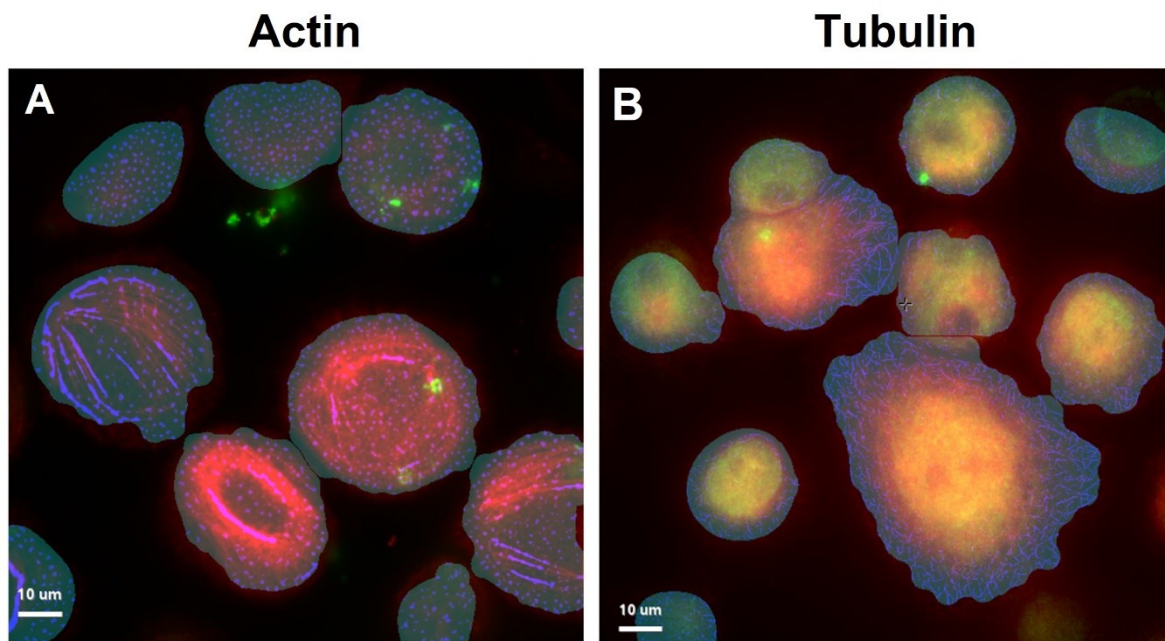


Figure 2.3. Representative images of segmentation and texture analysis. Cell segmentation on cells stained for actin (A) and tubulin (B) was performed, with subsequent analysis of the cytoskeleton proteins. Red: **actin or tubulin**, Green: *H. influenzae*, Blue: **fibre or punctate segmentation**. Scale bar = 10µm.

To analyse mitochondrial morphology, analysis of image texture was performed by assessing the Gaussian gradient modulus (GGM) from the acquired confocal images. The GGM quantifies the rate of directional change of intensity in the image within a pre-defined characteristic scale, as demonstrated in Fig. 2.4, with a fine-scale using a 1-pixel scale equivalent to 0.18 µm (G1) and a coarse-scale over a 6-pixel scale equal to 1.08 µm (G2). This can be used to assess the degree of roughness, i.e., how rough or smooth the detected edge is, with a higher value of G1 or G2 representing a rougher edge. In this case, G1 and G2 can be used to assess the smoothness of the mitochondrial edge morphology (Song et al., 2019).

Another fine-scale reference image was prepared by the spatial domain high-pass filtering with 3 µm Gaussian mask, to estimate linear density of zero-crossings (LD). This is a texture analysis which quantifies the signal of the stain, with low LD score indicating a coarser or

denser signal (Corcoran et al., 2019). This can be used to assess mitochondrial morphology in relation to compaction or decompaction of the mitochondrial staining, which can be related to aggregation or fragmentation (Hoffmann et al., 2013).

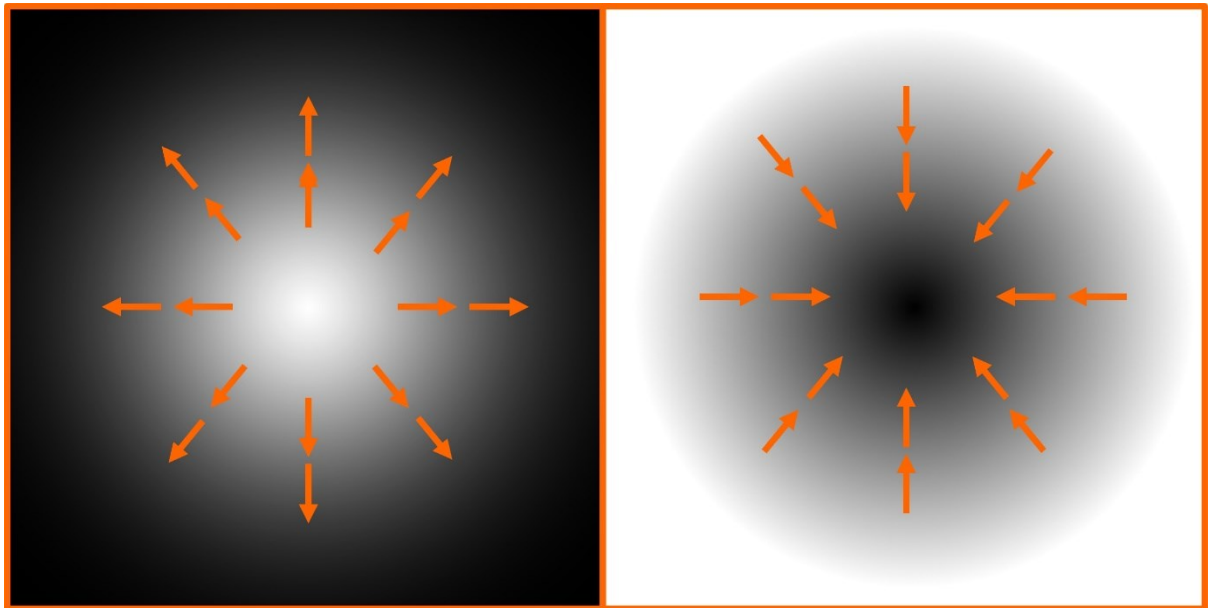


Figure 2.4. Representative diagram of Gaussian gradient.

2.2.20 Measurement of phagocytosis by quantification of bacteria in imaging experiments

Bacterial quantification was performed using *MicrobeJ*, a free ImageJ plugin (Ducret, Quardokus & Brun, 2016). Image files were opened in ImageJ, where masks were drawn around each individual cell to define a region of interest (ROI) using the brightfield channel or the cytoskeleton channel to determine the cell membrane (Fig. 2.5 B). Each individual cell was processed through *MicrobeJ*, identifying individual bacterial cells within the predetermined cell masks (Fig. 2.5 C). Segmentation was set to identify individual bacterial cells where clusters of bacteria are imaged within the cell.

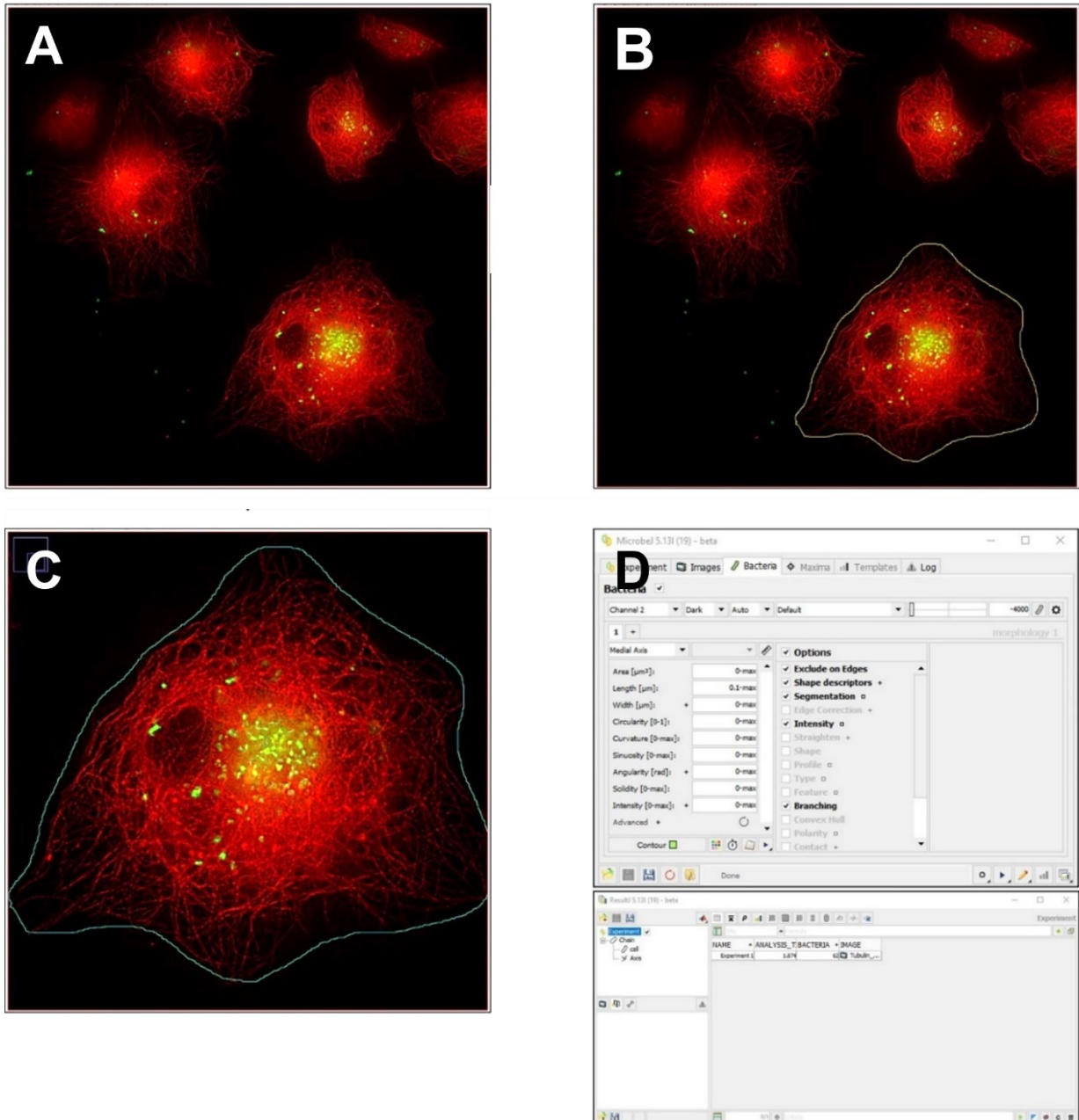


Figure 2.5. Overview of automated bacterial quantification by *MicrobeJ*. (A) Representative image of live MDM stained for tubulin with SiR-dye and incubated with *H. influenzae* for 60 min. (B) Cytoskeletal proteins were visualised in 635 nm channel and used to identify the cell border, where ROI masks were drawn around each individual cell. (C) Single cells were analysed for cell morphology properties including shape, size, and fluorescence intensity. (D) Within ROI, parameters were defined in *MicrobeJ* GUI and optimised for bacterial detection and quantification using cell segmentation, fluorescence intensity and branching detection.

2.2.21 STORM Imaging of MDM and tissue macrophages

2.2.21.1 Sample fixation

THP-1, MDM and tissue macrophages were fixed with 400 mL 100% (v/v) ice-cold methanol for 10 min, followed by 4% (v/v) paraformaldehyde (PFA) in PBS for 15 min at room temperature. Cells were washed three times with PBS supplemented with 0.05% (v/v) Triton-X. Cells were then blocked for 2 h PBS supplemented with 0.05% (v/v) Triton-X and 10% (v/v) FCS.

2.2.21.2 Staining of cytoskeleton proteins in macrophages by antibodies

THP-1, MDM and tissue macrophages were stained with primary monoclonal antibody for α -tubulin at a 1:1000 dilution in blocking solution for 1 h at room temperature. Cells were washed three times with PBS supplemented with 0.05% Triton-X, before incubation with an anti-mouse secondary antibody conjugated with either AF488 or 647 at a 1:2000 dilution in PBS with 0.05% (v/v) Triton-X and 10% (v/v) FCS for 20 min at room temperature. Cells were washed three times in PBS with 0.05% (v/v) Triton-X, before three washes with PBS.

2.2.21.3 Preparation of imaging buffer for dSTORM imaging

Imaging buffer was made fresh prior to each imaging session, 400 μ L of buffer per well, consisting of 50 mM mercaptamine, 10 mM DL-Lactate in PBS pH 8.7. Subsequently, 10 μ L of Oxyrase enzyme was added to each well before the chamber slide was sealed with Parafilm.

2.2.21.4 STORM Image acquisition

BEAS-2B, THP-1, MDM and tissue macrophages plated into 8-well chamber slides were imaged using a 100x, 1.46 NA oil lens in a Carl Zeiss Axiovert 200 epifluorescence microscope, configured for easySTORM (Kwakwa et al., 2016), using a total internal reflection fluorescence

(TIRF) illumination module, OptoTIRF, with a Photometrics Prime 95B scientific complementary metal–oxide–semiconductor (sCMOS) camera. After identifying a field of view, the sample was allowed to settle for 20 min to reduce thermal focal drift during acquisition. Cells labelled with AF488 were excited using a 462 nm laser, and AF647 were excited at 635 nm, all using a multimode laser diode source, with a typical excitation intensity of between 0.5 - 5 kW/cm² until stochastic blinking was observed. For each STORM image 10,000 frames were acquired using frame integration between 10 ms and 50 ms to achieve sufficient localisation density to reconstruct a super-resolved image. The microscope, camera and excitation lasers were all controlled during image acquisition using the MicroManager software (Edelstein et al., 2014), with the raw data saved in the OME.TIFF file format.

2.2.21.5 96-well automated STORM imaging

THP-1 or MDM plated into 96-well plates, were imaged in 400 µL of STORM imaging buffer per well, to maximise blinking rates over long imaging acquisition times, using a custom-built autofocus microscope for automated dSTORM (Lightley et al., 2021). Images were acquired using a 100x, 1.46NA oil lens in a Olympus ix81 microscope frame with an andor zyla 5.5 sCMOS camera. As described in 2.2.16.4, the microscope, camera, and excitation lasers were all controlled during image acquisition using the MicroManager software, with the raw data saved in the OME.TIFF file format.

2.2.21.6 Reconstruction and processing of STORM images

SMLM image data analysis entails localisations of thousands of emitters over thousands of image-frames and requires significant computation resources. The work reported here was mainly undertaken using the open source software package, ThunderSTORM (Ovesný et al., 2014), implemented in a parallelised configuration on a high-performance computing (HPC)

cluster (Munro et al., 2019). The software for this parallelised implementation of ThunderSTORM is available from https://github.com/ImperialCollegeLondon/HPC_STORM.. Fluorophore localisation and subsequent visualisation and analysis utilised the ImageJ open-source image processing platform with the Bio-formats and ThunderSTORM plug-ins.

Four parallel nodes on the HPC were used to analyse the *easySTORM* data, which was divided into four interleaved datasets, implemented using the “awk” command in UNIX, and shared equally between the available cluster nodes (Munro et al., 2019). The four datasets were compiled as separate localisation tables, which were merged using the "sort" command in UNIX, before filtering, drift correction and visualisation using the ThunderSTORM plug-in on ImageJ.

2.2.22 Statistical Analysis

All statistical analysis was performed using Graphpad Prism version 9.3.1 with all data shown represented as mean $\pm$ standard error of the mean (SEM) or individual values.

The number of repeated experiments or subjects per group is represented by the ‘n’ values, with all statistical tests performed annotated in figure legends and statistical significance determined to be reached with $p < 0.05$.

For analysis between groups, analysis of variance (ANOVA) with multiple comparisons was performed. For correlation statistics, Spearman’s rank correlation coefficient was performed.

Chapter 3: Exploration of techniques for the measurement and analysis of phagocytosis in macrophages

This chapter outlines the initial exploration of the potential for fluorescence techniques to be applied to study phagocytosis, including an investigation of different techniques to fluorescently label cytoskeletal proteins for live cell imaging and a comparison of plate fluorometry, flow cytometry and fluorescence microscopy applied to the quantitative analysis of phagocytosis. I also describe some preliminary work exploring the potential for the study of phagocytosis of new super-resolved microscopy tools based on dSTORM being developed by colleagues in the Physics Department, Imperial College London.

3.1 Introduction

Despite COPD patients having increased numbers of phagocytic cells within the lung (Barnes, 2016; Sethi & Murphy, 2008), defective phagocytic capabilities of macrophages means they are often colonised with common respiratory pathogens such as *H. influenzae* and *S. pneumoniae* (Patel et al., 2002; Wedzicha, Mackay & Singh, 2013). Phagocytosis is routinely measured using plate fluorometry and flow cytometry, usually represented as the relative amount of fluorescence compared to a negative control sample. These techniques have their advantages of being relatively high throughput and can easily distinguish between those cells which have and have not phagocytosed. However, the precise number of bacteria which have been phagocytosed is not quantifiable, and these techniques often do not have the sensitivity to detect morphological changes in cells. In comparison, the use of microscopy and fluorescently labelled bacteria can quantify the number of internalised bacteria within a sample at the single cell level, which allows elucidation of any potential differences in the macrophage population with respect to phagocytosis, alongside changes in the cytoskeleton.

Changes in the cytoskeleton are required for cell movement and phagocytosis (Aderem & Underhill, 1999; Groves et al., 2008), and so any alterations in reorganisation of the cytoskeleton in these cells could be a factor explaining defective phagocytosis in COPD. However, currently defective phagocytosis has not been directly linked with differences in fundamental cytoskeletal proteins such as tubulin and actin. The analysis of live macrophages undergoing phagocytosis gives the potential for visualising changes in the cytoskeletal, which could be involved in the defective nature of phagocytosis in COPD.

Initial experiments were performed to optimise microscopy techniques using cell lines. The bronchial epithelial cell line, BEAS-2B, was used as a positive control for transfection experiments due to their ease to transfect and manipulate using molecular biology techniques. The monocyte cell line, THP-1, differentiate towards a macrophage-like phenotype upon treatment with PMA (Schwende et al., 1996) and can phagocytose both Gram-positive and Gram-negative bacteria (Kapetanovic et al., 2007; Schiff et al., 1997). THP-1 cells have been extensively used as a model for macrophages (Ackerman et al., 2011; Chanput, Mes & Wichers, 2014) with PMA differentiation shown to produce a phenotype similar to MDM, as assessed by cell surface markers, mitochondrial and lysosomal numbers and the inflammatory cytokine and phagocytic response (Daigneault et al., 2010).

This chapter discusses several imaging methodologies that were explored to image fluorescently labelled cytoskeletal proteins in macrophages undergoing phagocytosis. Fluorescence labelling of fixed cells could be realised using antibodies (i.e., immunofluorescence), while labelling proteins for live cell imaging required the use of dyes designed to target specific organelles in the cell or the expression of fluorescent proteins conjugated to the proteins of interest. The latter approach requires transfection of foreign

nucleic acid, in the form of DNA or RNA, into a cell, as such transfections are a useful tool allowing the over-expression or the suppression of a targeted gene, by the introduction of plasmid DNA or a small interfering RNA respectively (Keller et al., 2018) into live cells.

However, macrophages are a hard-to-transfect cell type, due to their primary function of detecting and destroying foreign objects and pathogens (Dokka et al., 2000). Macrophages contain a number of DNase molecules (Wilber, Lu & Schneider, 2002), making transfection of a DNA plasmid by standard liposomal transfection reagents difficult. For these reasons, transfection of primary macrophages remains highly challenging and such primary cells are generally fixed before fluorescence imaging. For live cell imaging studies, model cell lines such as THP-1 cells are typically used.

3.1.1 Hypothesis

The use of microscopy and image analysis will allow analysis of phagocytosis using small population of cells, allowing differences in phagocytosis to be determined.

3.1.2 Aims

- To produce a protocol that will allow live cell imaging of macrophages undergoing phagocytosis.
- Compare different techniques used in the assessment of phagocytosis, to determine the advantages of using microscopy.
- Use widefield microscopy, image analysis and bacterial quantification to quantify phagocytosis in macrophage-like cells.
- Determine if microscopy can be used to determine differences in the cytoskeletal between subject groups.

3.2 Methods

3.2.1 Cell culture of BEAS-2B cell line

BEAS-2B cells were cultured as described in section 2.2.1 and seeded at a density of $2-4 \times 10^4$ cells into 8-well chamber slides or 6-well plates, pre-coated with poly-L-lysine.

3.2.2 Cell culture of THP-1 cell line

THP-1 cells were cultured as described in section 2.2.2 and seeded at a density of between $1-2.5 \times 10^4$ cell into 8-well chamber slides before differentiated with 10 ng/mL of PMA for 48 h to produce adherent cells with a macrophage-like phenotype.

3.2.4 Lentiviral transduction of GFP-actin

BEAS-2B cells seeded into 6-well plates were transduced with lentiviral particles containing a DNA for GFP-actin for 72h at 37°C, 5% (v/v) CO₂.

3.2.5 Transfection of cells with DNA-plasmid

BEAS-2B or THP-1 cells seeded into 8-well chamber slides were incubated with lipofectamine reagent encompassing plasmid DNA at varying concentrations (100 - 1000 ng) for 48h.

3.2.6 Transfection of cells with mRNA

BEAS-2B or THP-1 cells seeded into 8-well chamber slides were incubated with actin-mRNA transfection reagent, Viromer Cytostain, at varying concentrations (50 - 200 ng) for 48 h.

3.2.7 Antibody staining

THP-1 cells seeded into 8-well chamber slides were fixed and permeabilised with 100% (v/v) methanol. Cells were incubated with a primary antibody for alpha-tubulin, followed by secondary antibody conjugated to fluorescent probe.

3.2.8 Silicone Rhodamine cell staining

THP-1 cells seeded into 8-well chamber slides were incubated with 1 μ M of SiR-actin or SiR-tubulin for 60 min at 37°C, 5% CO₂ (v/v) prior to washing with PBS to remove excess free stain.

3.2.9 Epi-fluorescent widefield microscopy

Epi-fluorescent widefield microscopy was performed on BEAS-2B or THP-1 cells (described in 2.2.16)

3.2.10 Deconvolution of widefield microscopy

Widefield images, saved as .TIFF files were analysed using Huygens deconvolution software (described in 2.2.18).

3.2.11 Stochastic Optical Reconstruction Microscopy (STORM)

STORM was performed on fixed or live BEAS-2B or THP-1 cells (described in 2.2.21). Cells were imaged in STORM imaging buffer containing oxyrase as an oxygen scavenger.

3.2.12 Measurement of phagocytosis by plate fluorimetry

THP-1 cells seeded into 96-well plates were incubated with heat killed *H. influenzae* labelled with AF488 and phagocytosis measured by plate fluorimetry (described in section 2.2.13).

3.2.13 Measurement of phagocytosis by flow cytometry

THP-1 cells seeded into 24-well plates were incubated with heat killed *H. influenzae* labelled with AF488 and phagocytosis was measured by flow cytometry (described in section 2.2.15).

3.3 Results

Labelling cytoskeletal proteins in cell lines for live cell fluorescence imaging

3.3.1 Labelling actin in with GFP using lentiviral transduction

To first optimise the live imaging of bacterial phagocytosis, various techniques to detect cytoskeletal changes in cells using high content microscopy were used. Initially, experiments were performed using BEAS2B cells, as they are an easy to transfect cell line.,

BEAS-2B cells were cultured and transduced using lentiviral particles encompassing DNA for GFP-actin. A multiplicity of infection (MOI) of 1.5 was used (personal communication Dr J Baker) and as such, 10 μ l of lentiviral particles were added to BEAS-2B cells and incubated for 72 h. To increase the GFP positive population percentage and produce a stable GFP-actin cell line, resistance selection was performed using varying concentrations of puromycin dihydrochloride, between 1 μ g/ml to 10 μ g/ml.

GFP expression was quantified by flow cytometry (Fig. 3.1. A) and showed 88.2 and 90.8% of BEAS-2B cells positive for GFP, with 2 μ g puromycin producing the highest percentage of positive cells. After this confirmation of positive GFP expression within the cell population, the cell line was expanded, and stocks maintained. To confirm GFP-actin was stably expressed widefield epi-fluorescent imaging was performed to ensure visualisation of the actin protein could be performed on daughter cells. Representative widefield images show BEAS-2B positive for the expression of GFP-actin, with actin filaments observed (Fig. 3.1. B and C).

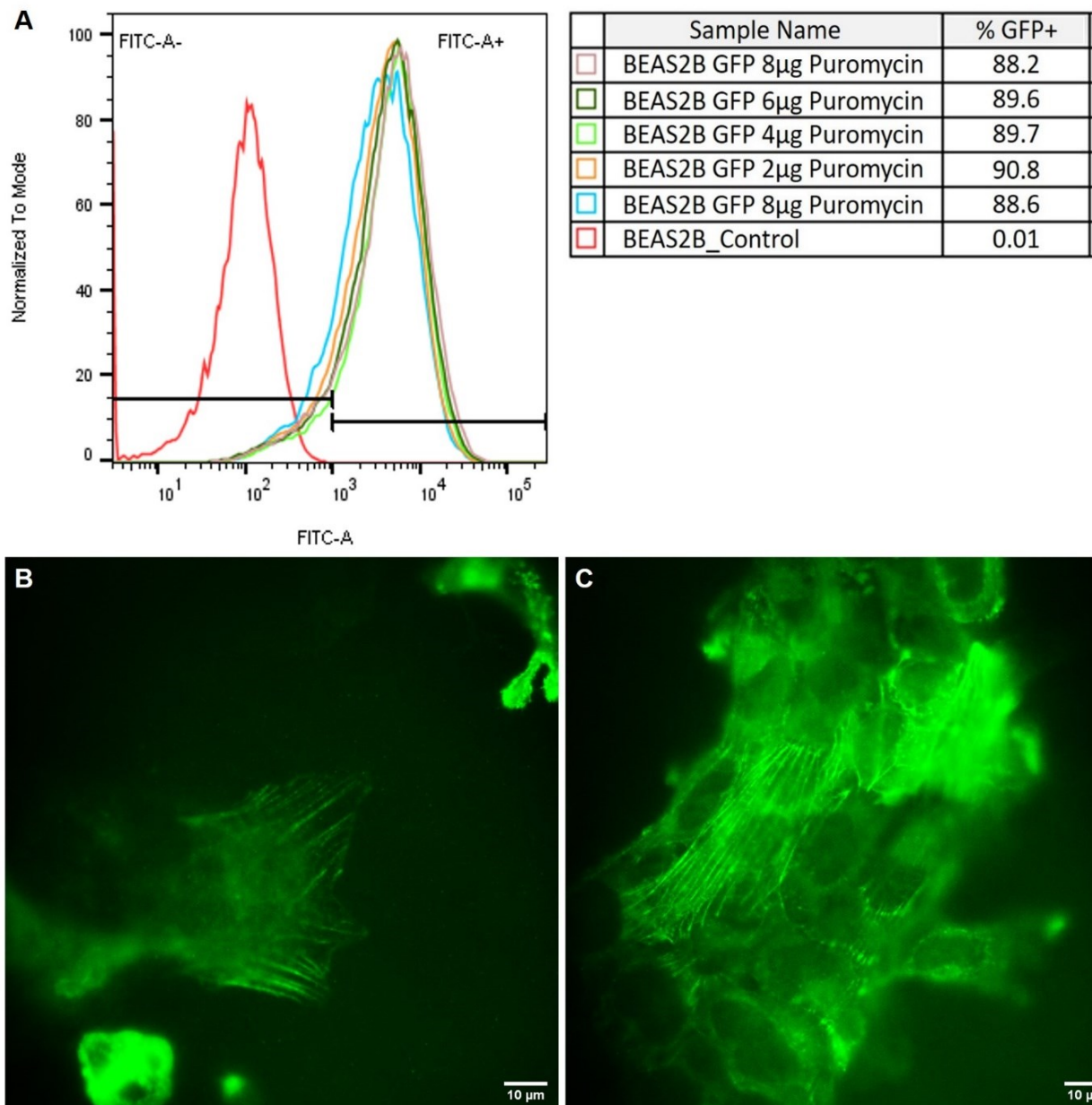


Figure 3.1. Confirmation of stable GFP-Actin BEAS-2B cell line. BEAS-2B cells were transduced with a lentivirus expressing GFP-Actin, with the GFP+ population quantified using flow cytometry (A) and imaged using widefield epi-fluorescence microscopy (B and C). Scale bar shows 10μm for widefield. Representative of three experiments.

3.3.3 SNAP-tag labelling of tubulin in BEAS-2B and THP-1 cells

SNAP-tag labelling of actin was explored as an alternative to GFP labelling for both BEAS-2B cells and THP-1 cells. Cells were transfected using lipofectamine with varying concentrations of a DNA plasmid for SNAP-tag labelled tubulin (100, 300 and 1000 ng) for 48h, followed by incubation with the 1 μ M SNAP-substrate, SNAP-Cell® 647-SiR for 1h. Widefield fluorescence imaging was then performed on live cells and cells fixed with methanol.

Figure 3.3 shows representative widefield fluorescent images of BEAS-2B cells transfected with the plasmid DNA, either live (Fig. 3.2 A,B) or fixed with 100% (v/v) methanol (Fig. 3.2 C,D). Widefield fluorescence images are shown (Fig. 3.2 A,C) with their corresponding deconvolved image (Fig. 3.2 B,D) calculated using the Huygens deconvolution software. Increasing plasmid concentrations led to more extensive fluorescence images of SiR/SNAP-labelled tubulin in BEAS-2B cells (Fig. 3.2 A1 - A4). The fixation of the cells using methanol resulted in less clear fluorescence images of the tubulin structures as seen (Fig. 3.2 C,D), although the tubulin structures were visible at the highest concentration of plasmid DNA (Fig. 3.2 C4 and D4).

This protocol was repeated in differentiated THP-1 cells however, no concentration of plasmid DNA in either fixed or unfixed cells produced fluorescence images of tubulin structure (Fig. 3.3), i.e., no fluorescence was observed above the background cellular autofluorescence levels (Fig. 3.3 A-B).

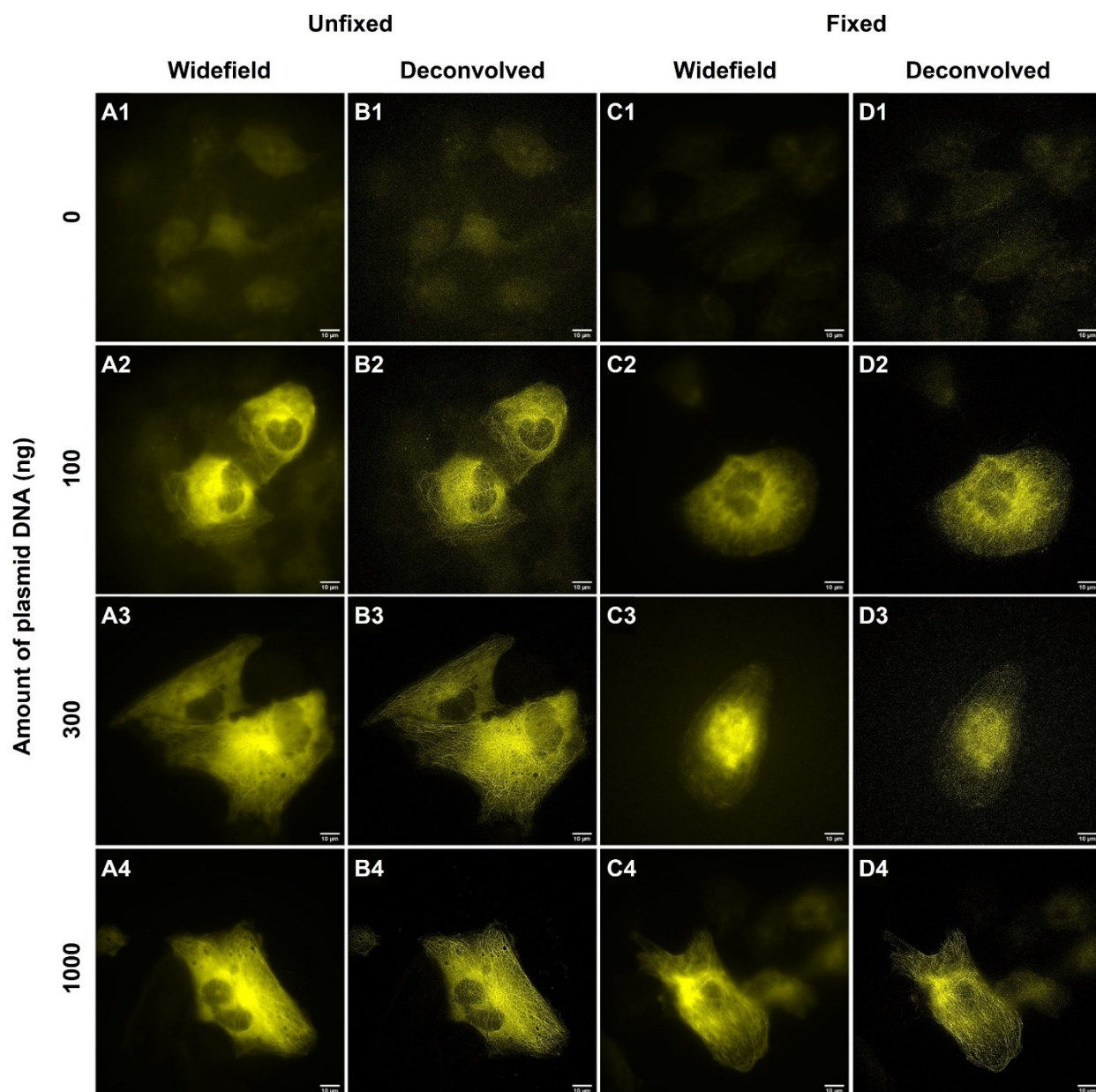


Figure 3.2. Comparison of SNAP-tag transfection on live and fixed BEAS-2B cells for widefield microscopy. BEAS-2B were transfected with SNAP-tag plasmid DNA specific for tubulin at indicated amounts for 48h, then incubation with 1 μ M SNAP-substrate for 1h. Cells were then either left unfixed or fixed with 100% (v/v) methanol before being imaged using widefield microscopy and post-acquisition deconvolution was performed. Scale bar shows 10 μ m in widefield images.

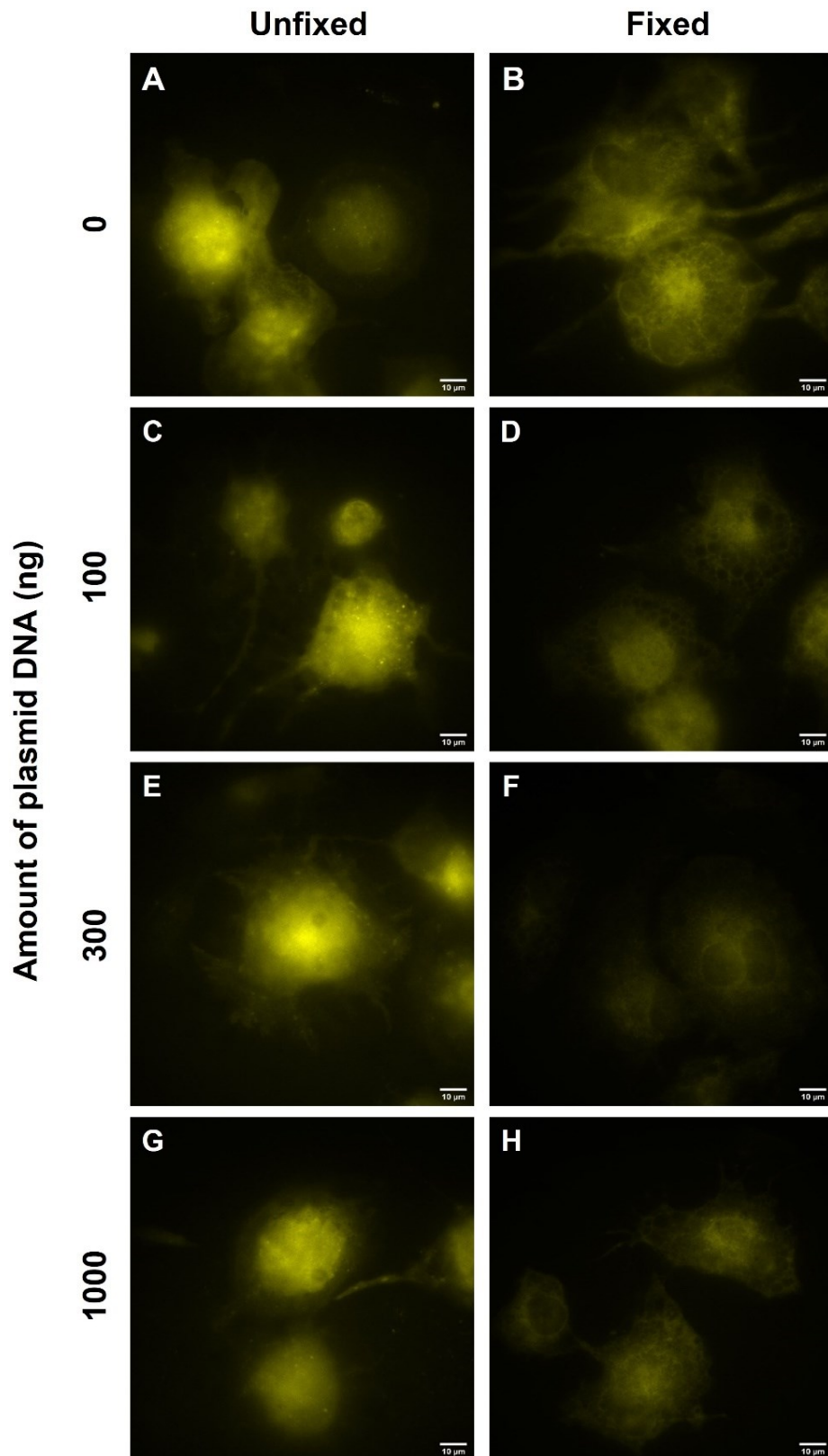


Figure 3.3. Comparison of SNAP-tag transfection on live and fixed THP-1 cells for widefield microscopy. THP-1 differentiated with PMA for 48 h towards a macrophage-like phenotype were transfected with SNAP-tag plasmid DNA specific for tubulin at indicated amounts for 48h, then incubation with 1 μ M SNAP-substrate for 1h. Cells were then either left unfixed or fixed with 100% (v/v) methanol before being imaged using widefield microscopy. Scale bar shows 10 μ m.

3.3.4 Labelling actin in BEAS-2B and THP-1 cells using Cytostain mRNA transfection

As the SNAP-tag transfection was unsuccessful in THP-1 cells, the use of a mRNA transfection agent (Viromer Cytostain), was investigated with BEAS-2B and THP-1 cells, to see if RNA encoding actin labelled with mCherry could be incorporated into the cell.

Live BEAS-2B cells were cultured and transfected using Cytostain-actin at varying concentrations (50, 100 and 200 ng), before being imaged using widefield epi-fluorescent microscopy. A distinctive, actin filament structure was observed in these cells (Fig. 3.4) and the labelling efficiency was insensitive to the different concentrations of mRNA transfection reagent used.

However, when applying the same procedure to THP-1 cells differentiated towards a macrophage-like phenotype, no distinct actin structure was observed for any of the transfection conditions tried (Fig. 3.5).

When comparing the mRNA transfection of BEAS-2B (Fig. 3.4) and THP-1 cells (Fig. 3.5), both cell types display a non-distinct fluorescence intensity not localised to a specific cellular structure, but instead diffusely located within the cytoplasm of the cell. This is likely due to a significant amount of background fluorescence from the mRNA plasmid, meaning this type of staining technique is not suitable for the investigation of phagocytosis in macrophages.

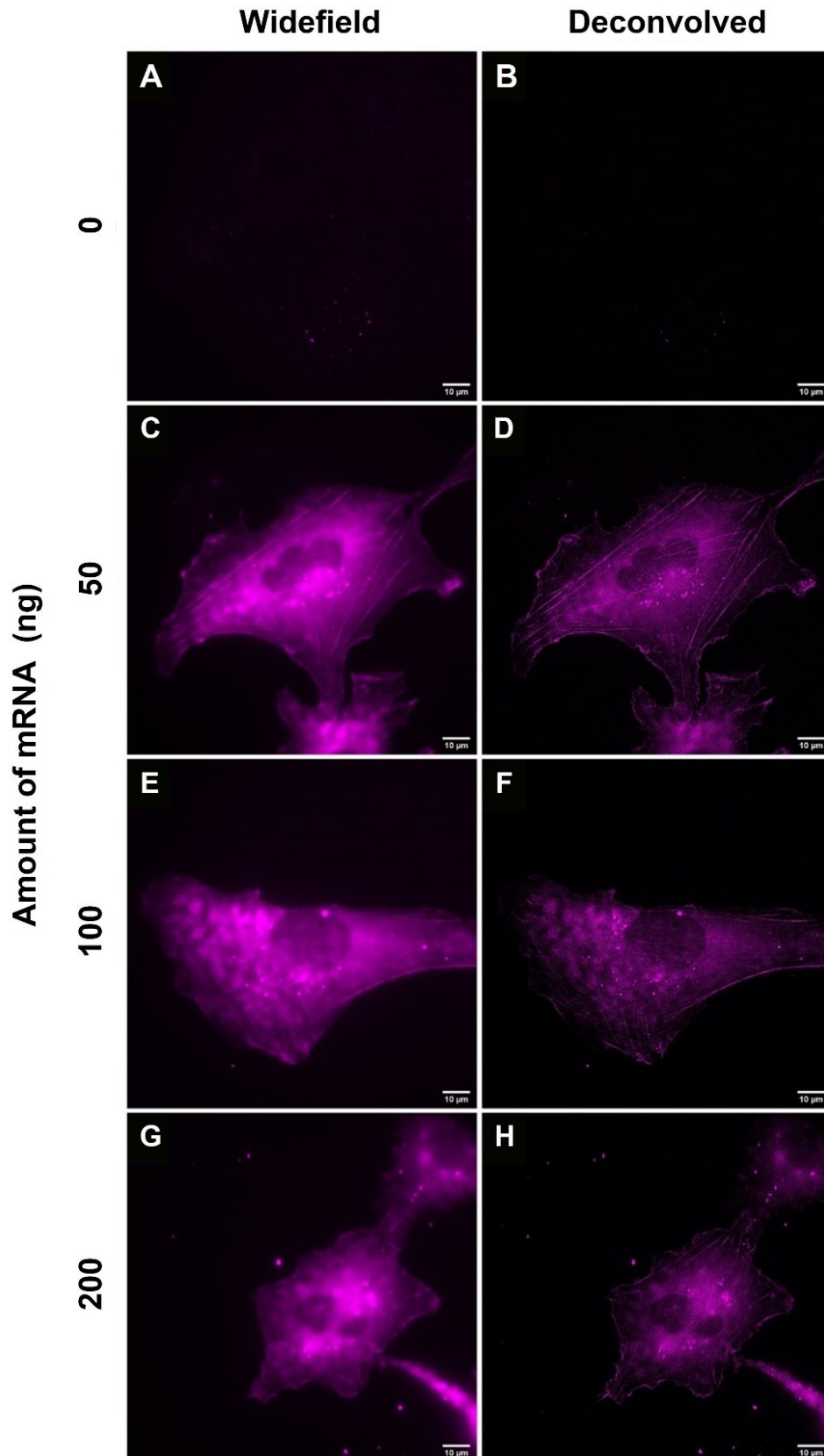


Figure 3.4. Comparison of mRNA transfection on live BEAS-2B cells for widefield microscopy. BEAS-2B were transfected with mRNA specific for actin at indicated amounts for 48h. Cells were imaged live by widefield microscopy and post-acquisition deconvolution was performed. Scale bar shows 10μm in widefield images.

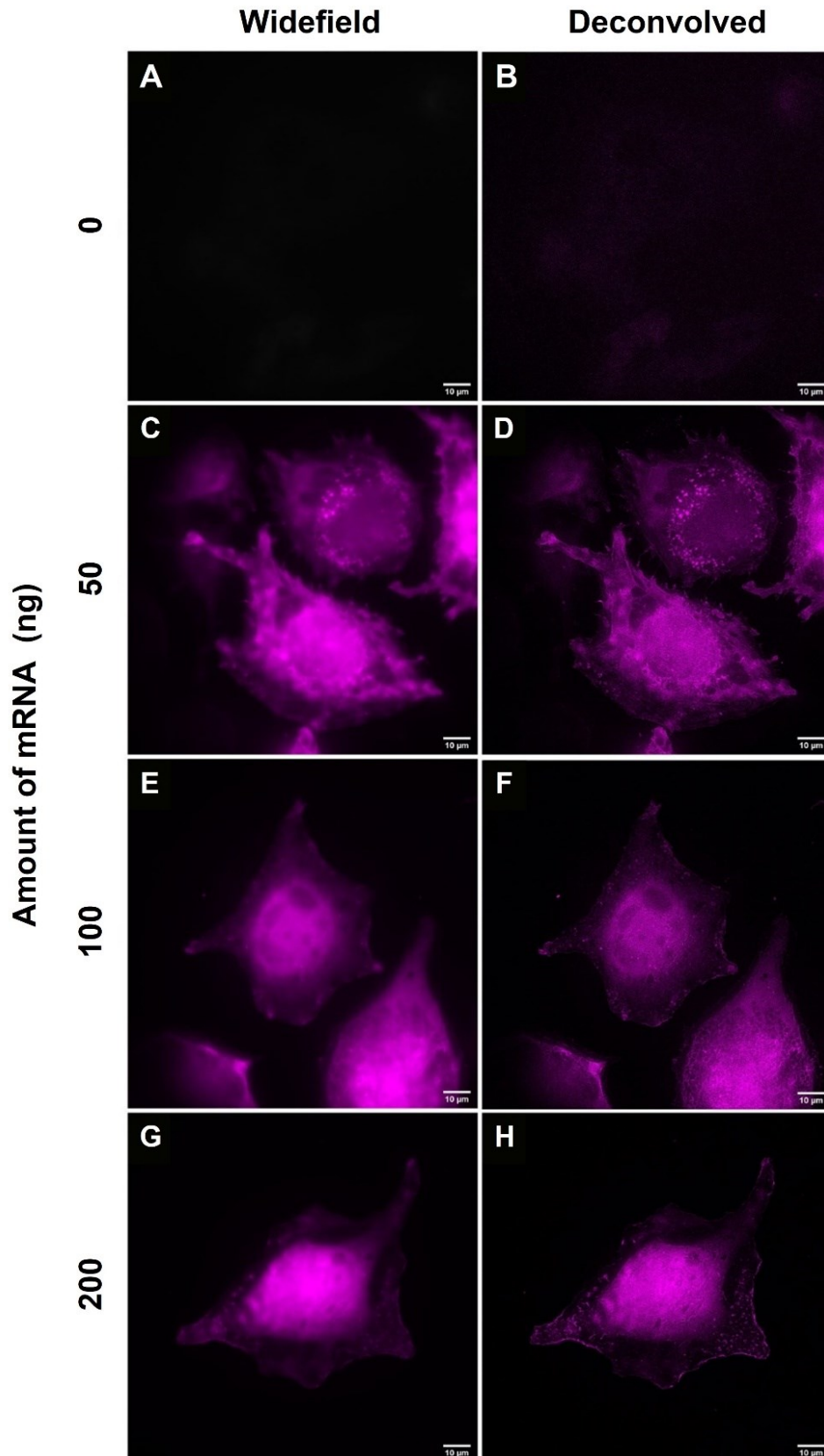


Figure 3.5. Comparison of mRNA transfection on live THP-1 cells for widefield microscopy. THP-1 differentiated with PMA towards a macrophage-like phenotype were transfected with mRNA for actin, at indicated amounts for 48 h. Cells were imaged live by widefield microscopy and post-acquisition deconvolution was performed. Scale bar shows 10μm in widefield images.

3.3.5 Labelling cytoskeleton in BEAS-2B and THP-1 cells with Silicon Rhodamine dyes

Next, the use of silicone-rhodamine (SiR)-dyes to directly stain cytoskeletal structures in BEAS-2B and THP-1 cells was examined as an alternative to transfection. Specific variants of SiR-dyes have been developed to bind to tubulin or actin, that undergo a conformational change which produces the fluorescence signal (Lukinavičius et al., 2014). As unbound dye does not fluoresce, they provide low background fluorescence and a high signal-to-noise ratio.

To explore the potential of SiR dyes, BEAS-2B and THP-1 cells were seeded into 8-well chamber slides and incubated with 1 μ M of either SiR-actin or SiR-tubulin for 60 min at 37°C, 5% CO₂ (v/v), prior to washing with PBS to remove excess free stain (described in Section 2.2.10.1). These SiR-labelled cells were then imaged live in cell culture media using a wide-field epifluorescence microscope with an excitation laser at 635 nm. Figure 3.6 A and B shows deconvolved wide-field fluorescence images of BEAS-2B cells presenting microtubule structures labelled with the SiR-tubulin dye (Fig. 3.6 A) and actin structures labelled with SiR-actin (Fig. 3.6 B). The same SiR labelling protocol was applied on THP-1 cells differentiated towards a macrophage-like phenotype. Figure 3.6(C,D) shows the deconvolved widefield fluorescence images of the THP-1 cells presenting the expected tubulin (Fig. 3.6 C) and actin (Fig. 3.6 D) morphology, consistent with previous literature (McKayed & Simpson, 2013; Schvartz et al., 2017).

Figure 3.7 compares the widefield (Fig. 3.7 A) and deconvolved (Fig 3.7 C) SiR-tubulin fluorescence images of the THP-1 cells with magnified regions of interest (Fig. 3.7 B,D), that illustrated the improvement in spatial resolution of the deconvolved images compared to the wide-field image, presenting apparent tubulin fibre diameters of ~220 nm and ~450 nm respectively.

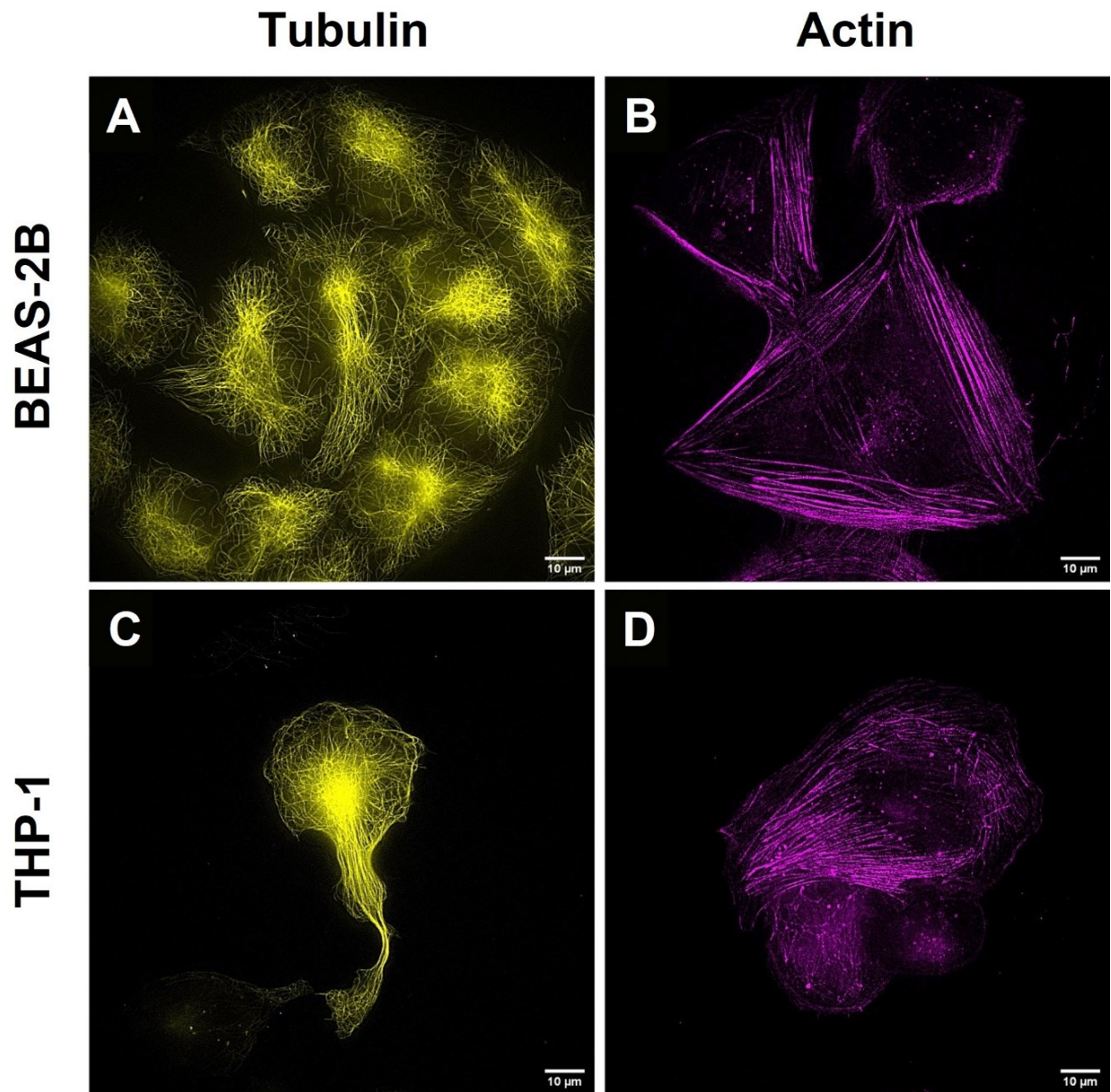


Figure 3.6. Image comparison of BEAS-2B and THP-1 cells stained with silicone rhodamine dyes. BEAS-2B (A-B) or THP-1 (C-D) cells were adhered to 8-well chamber slides, before cells were stained for 60 min with 1 μ M SiR-dye specific for actin or tubulin. Cells were washed and imaged live by epi-fluorescent widefield microscopy and deconvolved using Huygens software. Representative images show tubulin (A and C) and actin (B and D), scale bar shows 10 μ m.

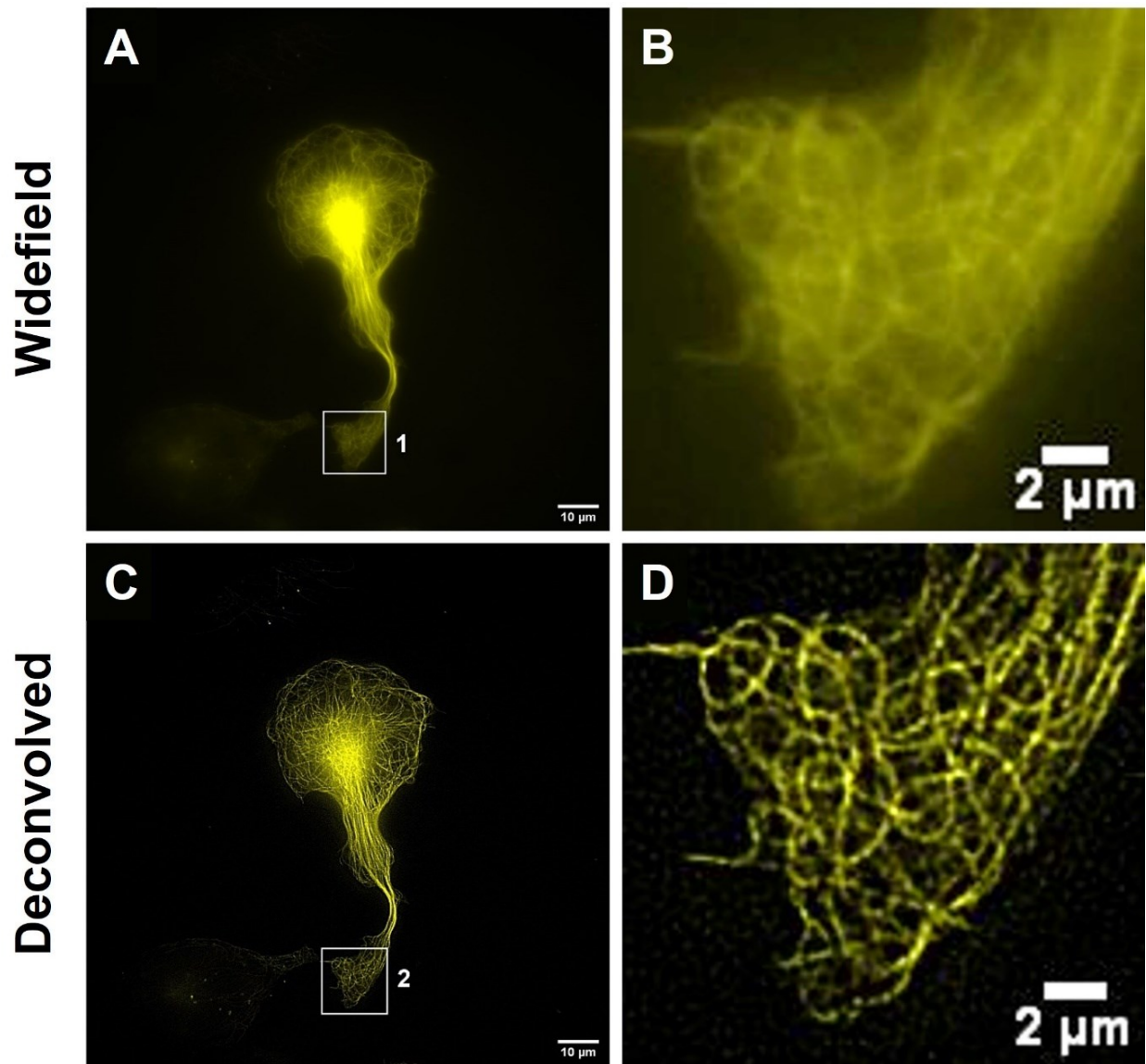


Figure 3.7. Comparison of widefield and deconvolved images for morphological analysis. THP-1 cells differentiated towards a macrophage-like phenotype with PMA for 48h were adhered to 8-well chamber slides, before cells were stained for 60 min with 1 μ M SiR-dye specific for actin or tubulin. Cells were washed and imaged live by epi-fluorescent widefield microscopy and deconvolved using Huygens software. Representative images show widefield (A) and deconvolved (C), scale bar shows 10 μ m, with respective magnified region shown (B and D), scale bar shows 2 μ m.

3.3.6 Visualisation of action of cytoskeletal inhibitors in SiR-tubulin labelled THP-1 cells

Since SiR labelling proved the most effective technique for live cell imaging of THP-1 cells, whether fluorescence microscopy could detect changes in structure of cytoskeletal proteins induced by cytoskeletal inhibitors, to mimic potential defects that may be observed in COPD macrophages was investigated. THP-1 cells were incubated with nocodazole, a tubulin inhibitor, and cytochalasin D, an actin inhibitor, prior to incubation with *H. influenzae*. These cells were then imaged live (Fig. 3.8), and bacterial quantification was performed using *MicrobeJ* to assess phagocytosis and whether inhibition of cytoskeletal rearrangement altered the response.

Incubation of THP-1 cells with 1 μ M nocodazole for 60 min led to an almost complete disruption of visible microtubules within the cell (Fig 3.8 B). There was still fluorescent staining above background levels, yet this was not localised and instead displayed a diffuse staining of the cytoplasm.

Incubation of THP-1 cells with 1 μ M cytochalasin D for 60 min led to a change in the number and distribution of the distinct punctate morphology, with large aggregates of punctate structures when compared to more evenly distributed actin structures seen when no actin inhibition is present (Fig. 3.8 C).

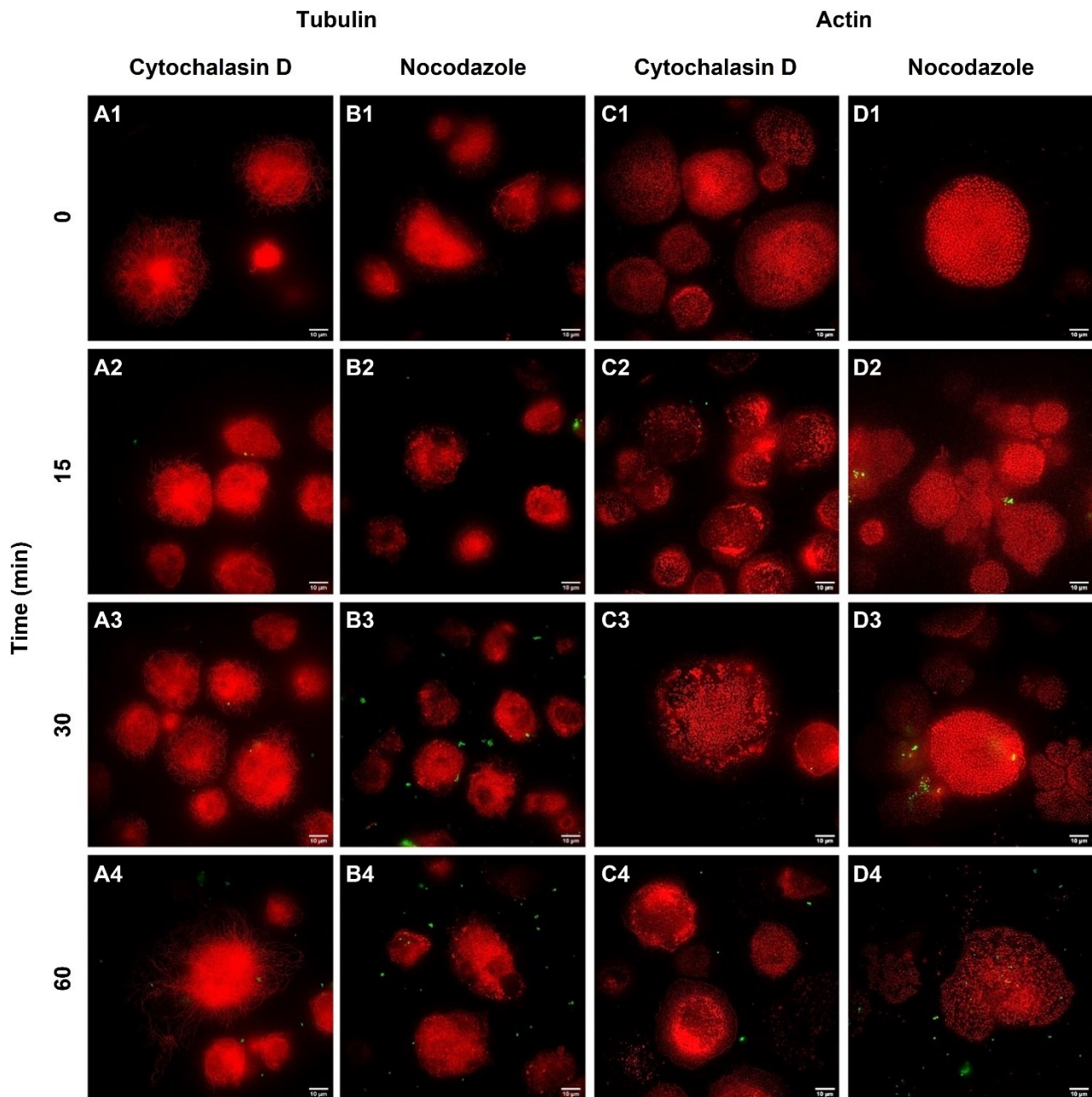


Figure 3.8. Visualisation of cytoskeletal inhibitors in THP-1 cells. THP-1 cells were differentiated towards a macrophage-like phenotype in the presence of PMA for 48 h. Cells were pre-incubated with nocodazole or cytochalasin D for 60 min, incubated with *H. influenzae* for up to 60 min and then stained for tubulin or actin with silicon rhodamine dye. Cells were imaged by epi-fluorescent widefield microscopy with a 1.46NA, 100x objective. Images were deconvolved using Huygens software. Representative images selected and 10μM scale shown. Red: tubulin or actin, Green: *H. influenzae*.

3.4 Comparison of fluorescence microscopy with plate fluorimetry and flow cytometry to quantify phagocytosis for measurement of phagocytosis

To determine whether fluorescence microscopy could be used to detect differences in the phagocytic capacity of macrophages with improved sensitivity compared to other methods, THP-1 cells differentiated towards a macrophage-like phenotype were incubated with heat killed *H. influenzae* labelled with AF488, and phagocytosis was measured by plate fluorimetry, flow cytometry and fluorescence microscopy with deconvolution.

As shown in Fig. 3.9 A-D, all three techniques could detect increases in phagocytosis after 60 min incubation of bacteria compared to baseline ($p < 0.01$), however, when comparing between the techniques there are some noticeable differences. When analysing mean percentage of cells positive for phagocytosis, flow cytometry produced mean measurements of $39 \pm 1\%$ (Fig. 3.9 B), when compared to analysed by widefield microscopy which produced $59 \pm 6\%$ (Fig. 3.9 C). This 20% difference in phagocytosis percentage is likely due to the sensitivity of detection between the two techniques, with flow cytometry giving lower sensitivity but greater throughput capabilities, compared to widefield microscopy which can detect more individual bacteria within the cell volume but can only be performed on a smaller number of cells.

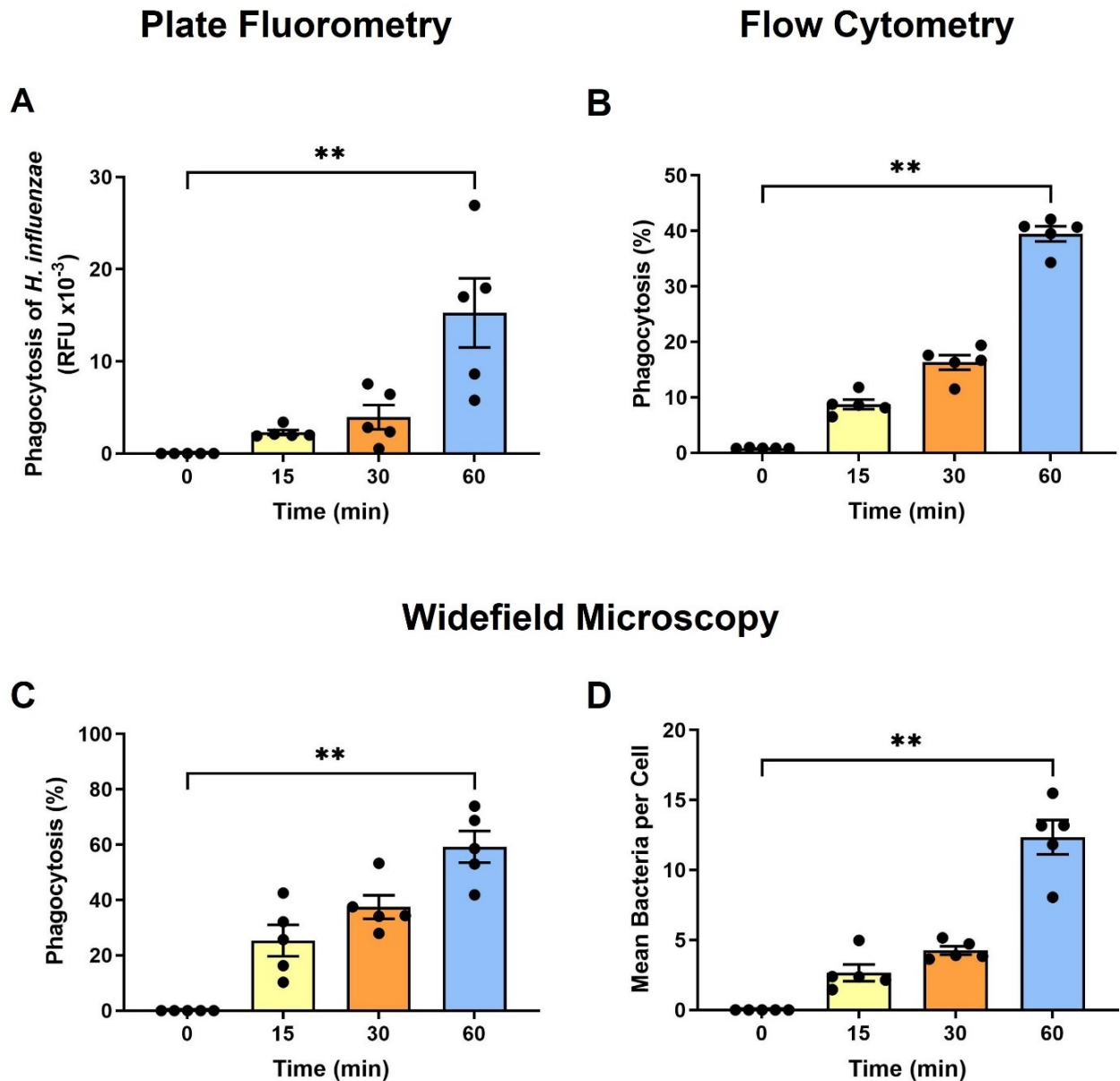


Figure 3.9. Comparison of techniques for the measurement of phagocytosis. THP-1 cells (n = 5) were differentiated towards a macrophage-like phenotype in the presence of PMA for 48 h. Cells were incubated with *H. influenzae* for up to 60 min. Phagocytosis was then measured by plate fluorometry as assessed by relative fluorescence intensity (A), by flow cytometry assessed by phagocytosis percentage (B), or widefield microscopy assessed by phagocytosis percentage (C) and mean number of bacteria per cell (D). Data analysed a one-way ANOVA with Friedman multiple comparisons test. ** p<0.01.

3.5 Quantifying the effect of cytoskeletal inhibitors on phagocytosis

To explore the potential of fluorescence microscopy to investigate changes in cytoskeletal processes that may be involved in phagocytosis, the cytoskeletal proteins tubulin and actin were artificially disrupted with the use of cytoskeletal inhibitors, nocodazole and cytochalasin D, and their impact on phagocytosis measured by plate fluorometry, flow cytometry and fluorescence microscopy with deconvolution.

There was a reduction in uptake of bacteria when cells were incubated with cytochalasin D or nocodazole when compared to the control cells when assessed by plate fluorometry (Fig. 3.10 A), flow cytometry (Fig. 3.10 B) and fluorescence microscopy (Fig. 3.10 C and D). When both cytoskeletal inhibitors were used in combination, there was total inhibition of phagocytosis as assessed by all techniques (Fig. 3.10 A-D).

To see if these differences observed could be explained by increased cell death, cell viability was assessed using the MTT assay (van Meerloo, Kaspers & Cloos, 2011). Neither cytochalasin D nor nocodazole alone caused a reduction in viability when compared to the control, however when both inhibitors were used in combination there was a reduction in viability of approximately 10% when compared to the untreated control or DMSO control groups (Fig. 3.10 E).

Similar to results shown in fig. 3.9, when comparing mean percentage of cells positive for phagocytosis there were observable differences between techniques. Flow cytometry produced a lower phagocytosis percentage at $23 \pm 1\%$, compared to widefield microscopy at $62 \pm 6\%$. This again shows potential differences in the sensitivity between these two techniques, with flow cytometry potentially being unable to discriminate signal from individual bacteria from background fluorescence.

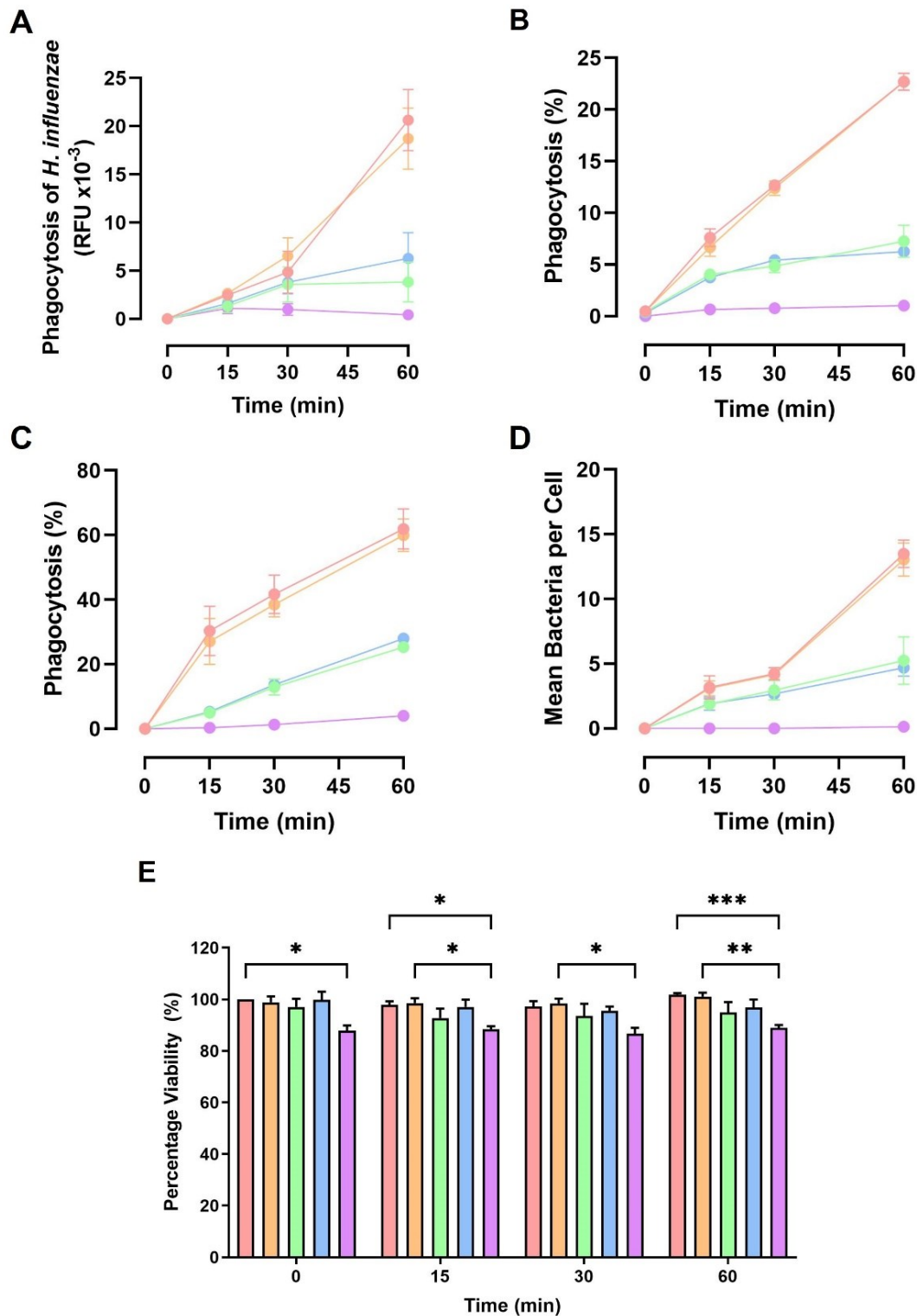


Figure 3.10. Measurement of phagocytosis by different techniques following cytoskeletal inhibition. THP-1 cells ($n = 3$) differentiated towards a macrophage-like phenotype by PMA for 48h were left in media (■) or pre-incubated with DMSO (■), 100 μ M nocodazole (■), 10 μ M cytochalasin D (■) or both inhibitors (■) for 60 min, prior to incubation with *H. influenzae* for up to 60 min. Cells were stained for tubulin or actin with SiR dyes and phagocytosis was measured by plate fluorometry (A), flow cytometry (B) or widefield microscopy (C and D). MTT viability test was also performed (E). Data analysed a two-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.6 Exploring STORM to image cytoskeletal proteins

In parallel to this project, colleagues in the Physics Department were developing super-resolved microscopes based on their *easySTORM* approach. Therefore, the potential of STORM to image phagocytosis was investigated. Currently STORM requires image data acquisitions of several minutes with high excitation intensities and so is typically not practical for live cell studies, as such most of the *easySTORM* work at Imperial, samples are fixed and immunolabelled, usually with Alexa dyes.

To explore the potential of *dSTORM* for bacterial quantification, the performance of *easySTORM* was compared with wide-field fluorescence microscopy and with deconvolution of the widefield images using *MicrobeJ*, the open-source *ImageJ* plugin for bacterial quantification, (Ducret, Quardokus & Brun, 2016).

Representative images with a 10µm scale bar are presented in Figure 3.11, which shows that the widefield fluorescence imaging provides the lowest spatial resolution (Fig. 3.11 A and B) for which the *MicrobeJ* analysis could not detect individual bacteria but reported larger clusters up to 7 µm in length (Fig. 3.11 C). The deconvolved wide-field fluorescence images yielded improved spatial resolution (Fig. 3.11 D and E) and *MicrobeJ* reported bacteria sizes ranging from 0.1 to 1.4µm (Fig. 3.11 F). *EasySTORM* provided the highest spatial resolution images (Fig. 3.11 G and H), resolving individual each bacterium and *MicrobeJ* reported bacteria sizes ranging from 0.1 to 0.8 µm.

Widefield fluorescence imaging is the quickest imaging modality providing snapshot acquisitions, including of live cells, and requiring minimal post-acquisition image processing but offers the lowest resolution and can miss significant information when quantifying bacteria. While *easySTORM* provides the highest image resolution and enables the most

precise bacterial quantification, it is currently only practical with fixed cells. Additionally, the image data analysis is computationally intensive and can require hours to process a few fields of view. Deconvolved widefield fluorescence microscopy can be considered a middle ground between these two modalities, requiring the same image data acquisition time as a widefield fluorescence image although the post-acquisition processing time using the deconvolution software can take several minutes per field of view. The improved resolution compared to widefield fluorescence imaging and the improved capacity for bacterial quantification, together with the capability for live cell imaging made this the technique of choice for the cell biology studies reported in subsequent chapters of this thesis. Nevertheless, the potential of super-resolved microscopy to study phagocytosis and bacterial infection is significant and justifies further work to develop it.

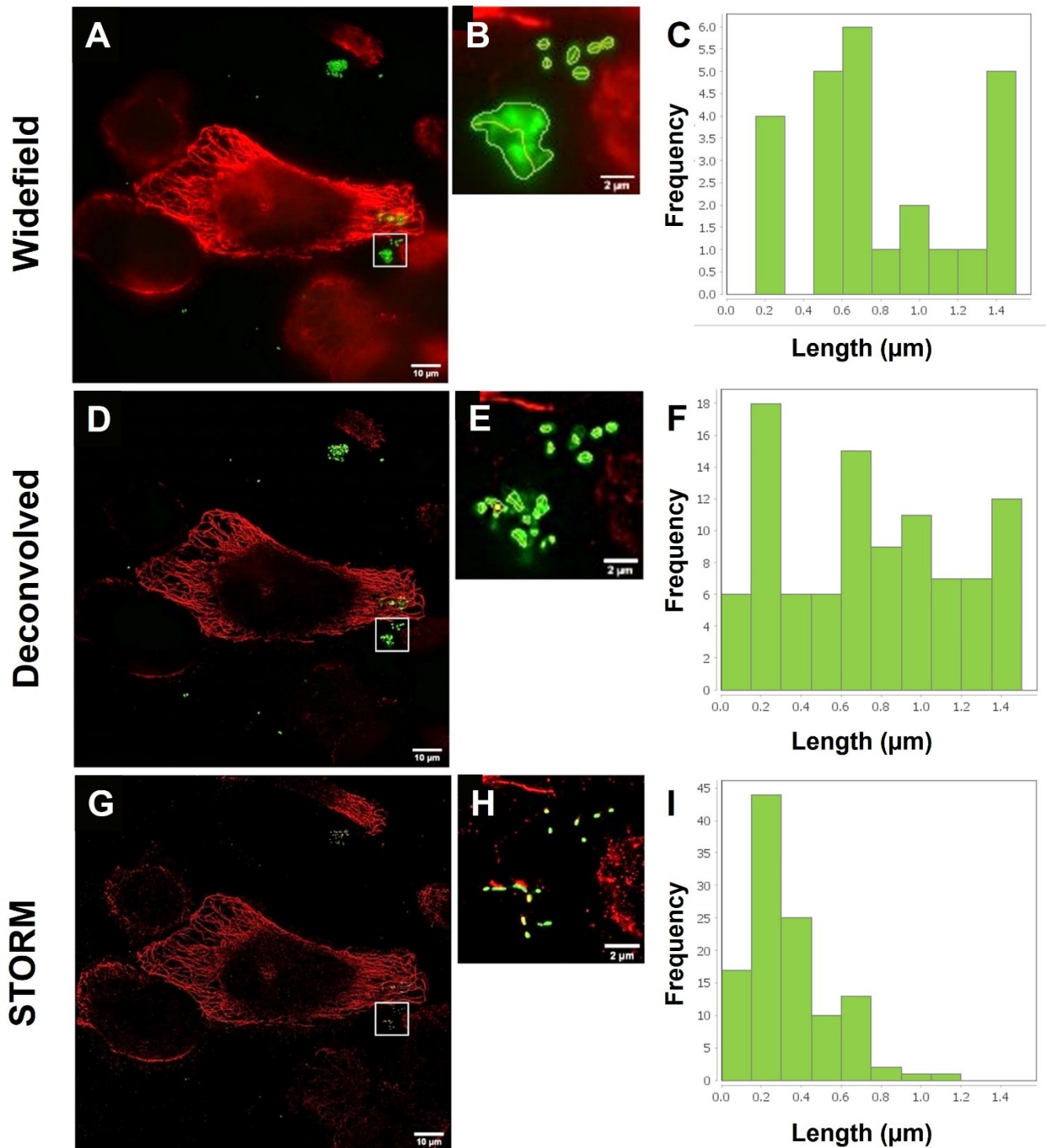


Figure 3.11. Comparison of bacterial quantification by microscopy technique. THP-1 cells were differentiated towards a macrophage-like phenotype in the presence of PMA for 48 h. Cells were incubated with *H. influenzae* labelled with Alexa 488 for 60 min, then fixed and antibody stained for α -tubulin before secondary labelling with Alexa 647. Cells were imaged by widefield fluorescence microscopy with a 1.46NA, 100x objective, followed by *easySTORM*. Red: tubulin, Green: *H. influenzae*. Bacterial quantification and length analysis was performed using *MicrobeJ* on images from widefield (A-C), deconvolved (D-F) or STORM (G-I).

3.6.1 Automated multiwell plate *easySTORM*

For systematic quantitative studies of phagocytosis or any other super-resolved, cell-based assay, it is desirable to automate. Therefore, after optimising imaging for higher throughput to achieve readouts with more statistical power and to enable the direct comparison of different experimental parameters. To this end, the Photonics Group in the Physics Department have been developing automated multiwell plate *easySTORM* microscopy and this was explored briefly to assess its potential to study phagocytosis. The full experimental details are published in Lightley et al, 2021, from which data shown here in Figure 3.12 is reproduced.

For this experiment THP-1 cells were seeded and differentiated towards a macrophage-like phenotype into a 96-well plate, where they were incubated with *H. influenzae* labelled with AF488 for up to 60 min in 15-min intervals, before washing to remove non-bound bacteria. The cells were then fixed with 100% (v/v) methanol and stained with a primary monoclonal antibody for α -tubulin labelled with AF647. They were then automatically imaged in 400 μ L of STORM imaging buffer per well, using a custom-built autofocus-enabled fluorescence microscope on which automated *easySTORM* was implemented (Lightley et al., 2021) using a 100x, 1.46 NA oil objective lens in a Olympus IX81 microscope system controlled by the open-source *MicroManager* software, with the raw image data saved in the OME.TIFF file format.

This initial automated *easySTORM* experiment was promising, but the prototype microscope was not sufficiently reliable or available to be used routinely. Furthermore, *easySTORM* works best with immunostained fixed cells, which is a limitation for some studies - although the ability to fix cells at different time points in different wells does enable time-resolved studies, and the immunostaining could be applied to primary macrophages.

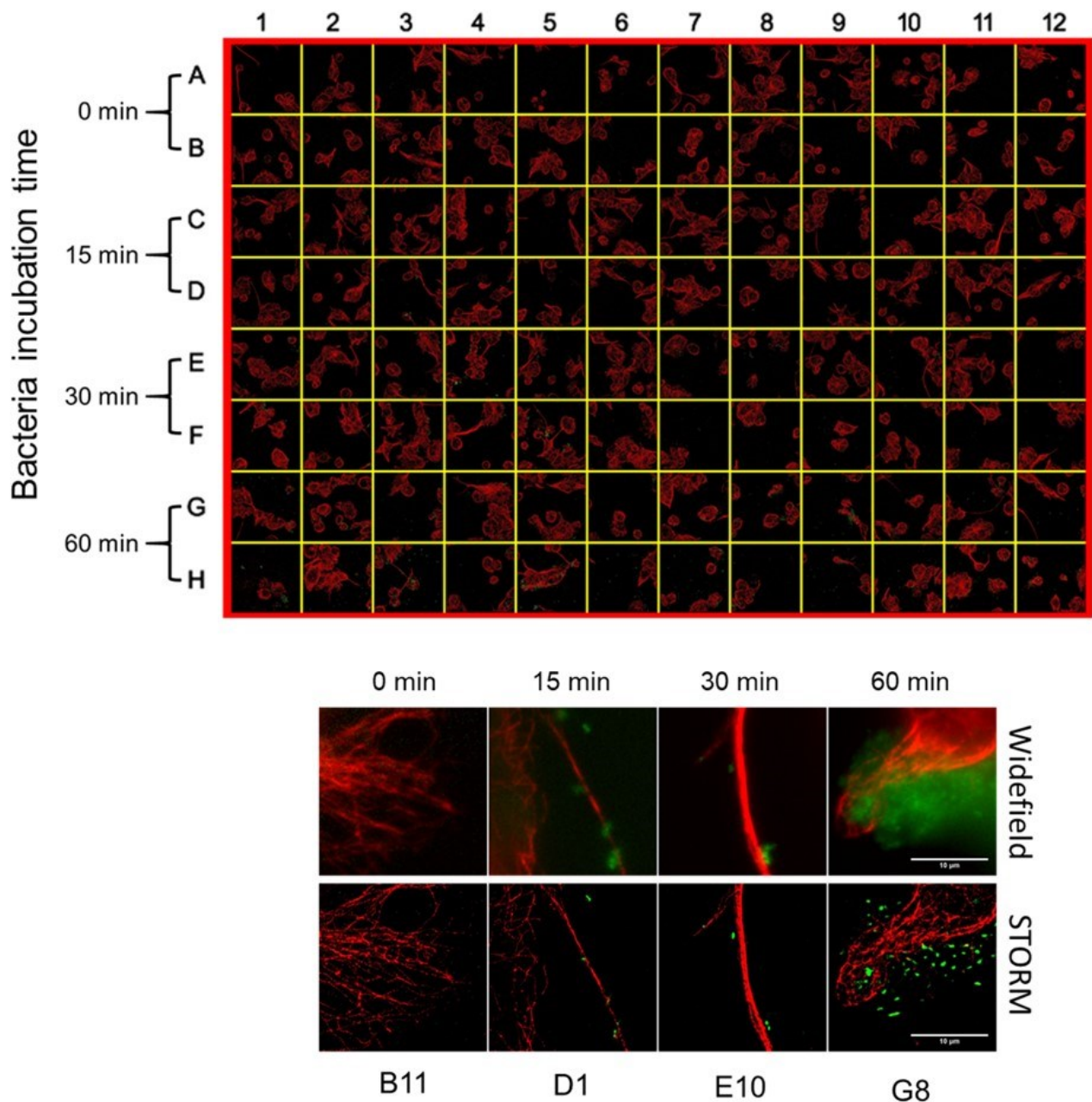


Figure 3.12. Use of automated *easySTORM* for visualisation and measurement of macrophages. Array of automatically acquired *easySTORM* images of THP-1 cells incubated with *Streptococcus pneumoniae* bacteria labelled with AF488 for a range of time intervals and fixed for imaging. The alpha-tubulin in the THP-1 cells is labelled with AF647 (red) and the bacteria are labelled with AF488 (green). Representative *easySTORM* images are shown in their respective wells (top) with exemplar expanded wide-field and *easySTORM* images (bottom) illustrating the change in bacteria distribution with increasing incubation time.

3.4.2 Analysis of silicon rhodamine dyes for live STORM imaging of BEAS-2B cells

To see if *easySTORM* could be implemented with live cells, the potential of using SiR-dyes previously shown to label both actin and tubulin staining in live BEAS-2B and THP-1 cells was investigated. There are numerous studies utilising SiR-dyes in live SRM, typically focusing on STED (Butkevich et al., 2016; Thompson et al., 2017), however their use for photo-activated super-resolved techniques is less well documented, due to rhodamine derived photoactivatable dyes often being non-cell permeable and have problems with solubility, leading to their use in fixed cells (Frei et al., 2019). There have been advances in the production of photoswitchable SiR-dyes, however these have required significant chemical engineering and are not commercially available (Frei et al., 2019; Tyson et al., 2021).

To test this use of SiR-dyes, BEAS-2B cells were incubated with SiR-dye for 60 min, prior to incubation in STORM imaging buffer and imaged by epi-fluorescence widefield microscopy and then *easySTORM* on the same region of interest. For *easySTORM* imaging, 5000 frames were acquired and processed by ThunderSTORM. The widefield fluorescence image was deconvolved post-acquisition using Huygens software. This allowed the same region of interest to be compared by three imaging techniques, widefield, deconvolved and STORM.

The use of SiR-dyes produced comparable images by widefield (Fig. 3.13 A and D) and higher resolution images by deconvolved widefield (Fig. 3.13 B and E). The use of SiR-dyes achieved stochastic blinking when imaged in STORM buffer, from which acquisition of 5000 frames was sufficient to reconstruct a super-resolved image of tubulin (Fig. 3.13 C) and actin (Fig. 3.13 F). However, the *easySTORM* image quality was not as good as that obtained using the usual (Alexa Fluor) STORM dyes. This may have been due to cellular movement or thermal drift during the image acquisition time (~few minutes), which is a recognised challenge live cell

STORM, as are phototoxic cell effects due to the high excitation intensities over this time (Sigal et al., 2018; Tosheva et al., 2020). Live STORM has previously been reported (Bálint et al., 2013) and future improvements of SMLM should allow quicker image data acquisition of live cells (Jones et al., 2011). Nevertheless, any cell movement during the image data acquisition can result in motion artefacts that may be significant for super-resolved imaging.

Given the state-of-the-art at the time of undertaking this PhD project, the comparison of the wide-field fluorescence, deconvolved and *easySTORM* images shown in Figure 3.13 again supported the decision to proceed with deconvolved widefield fluorescence microscopy for the work reported in the subsequent chapters of this thesis.

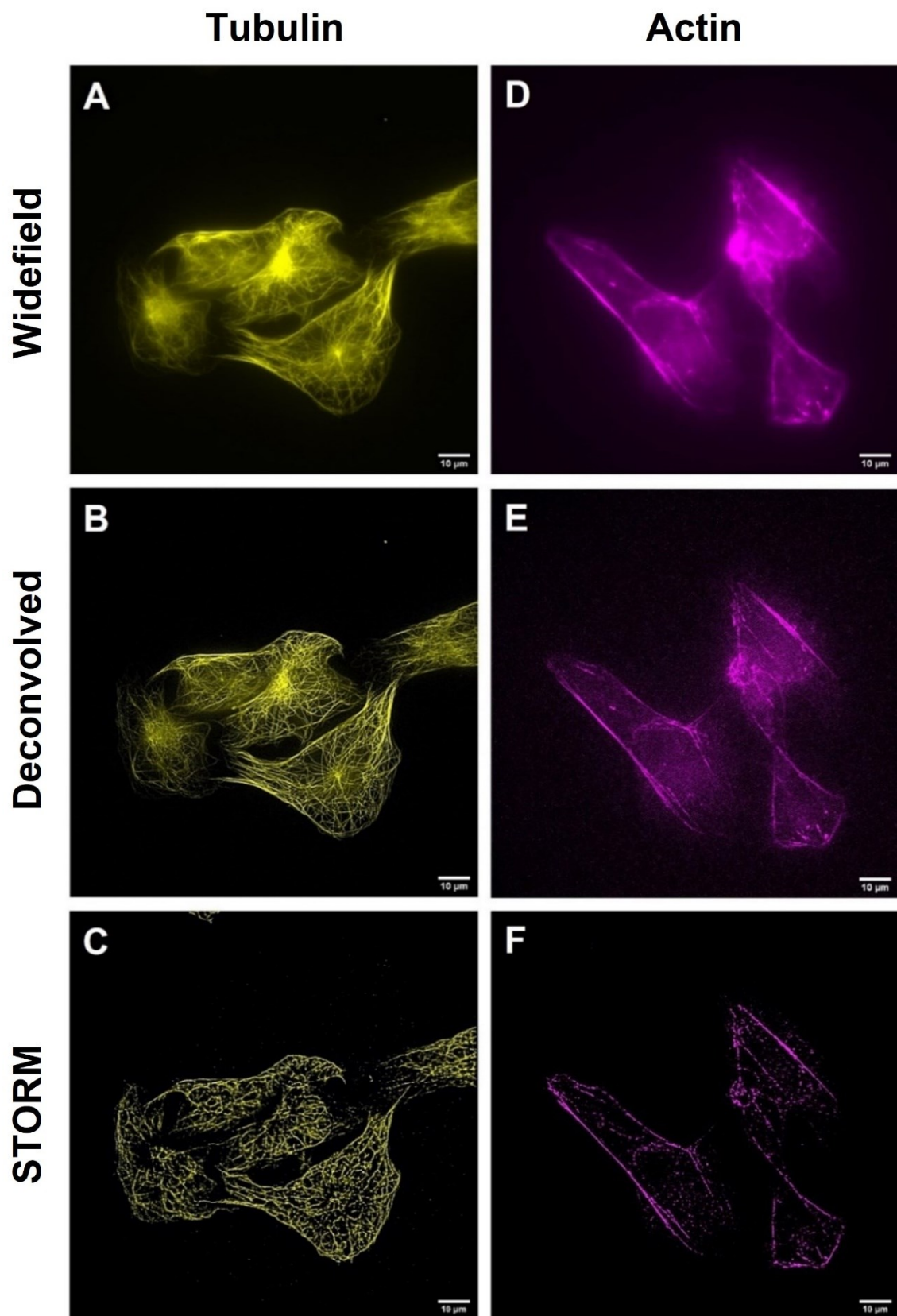


Figure 3.13. Comparison of live cell imaging by widefield, deconvolved and STORM. BEAS-2B cells adhered to 8-well chamber slides were stained for 60 min with 1 μ M SiR-dye specific for tubulin (A-C) or actin (D-F). Cells were washed and imaged live by epi-fluorescent widefield and sequential STORM, followed by post-acquisition deconvolution with Huygens software. Representative images show widefield (A and D), deconvolved widefield (B and E) and STORM (C and F). Scale bars show 10 μ m.

3.4 Discussion

This chapter aimed to design an improved protocol to quantify phagocytosis by macrophages. Initially, the aim was to utilise the ability to image live macrophages undergoing phagocytosis, to allow areas of specific interest, such as the cytoskeleton, to be visualised and quantified. The use of fluorescent proteins or transfection of DNA tags are useful tools to label endogenous proteins within a cell, which has advantages whilst performing microscopy to track dynamics over time.

Super-resolved microscopy techniques such as STORM offer greatly improved resolution compared to standard widefield microscopy, however, with STORM being a form of SMLM, it requires the use of either a photoswitchable fluorophore or a specific buffer which can produce stochastic blinking. Photoswitchable fluorophores usually come in the form of antibodies conjugated with the specific fluorescent probe, and therefore would require fixation and permeabilisation of the cell to perform antibody staining. This was not the intent of this project, so work was performed to attempt to introduce a fluorescent protein or DNA tag into relevant cell types, to attempt to use live cell STORM imaging. A significant problem to overcome is that macrophages have consistently been shown to be difficult to transfect, either in the form of RNA or DNA (Keller et al., 2018), however the benefits would allow the continued development towards super-resolving imaging of macrophages. Therefore, BEAS-2B cells were used first to establish the imaging techniques.

The introduction of a fluorescent protein or a SNAP-tag labelled protein was initially attempted, to allow live cell imaging without the need for fixation or antibodies. However after several attempts at introducing different genetic vector into cells an alternative option was selected, as there are numerous papers utilising SiR-dyes in live SRM, typically focusing

on STED microscopy, where problems with solubility lead to their use in fixed cells (Frei et al., 2019).

Experiments measuring phagocytosis involving plate fluorometry and flow cytometry have their merits, but advances in microscopy allows observation using a much smaller number of cells, allowing the study of rare cell populations or difficult to obtain samples to be explored. Comparison experiments between plate fluorometry, flow cytometry and microscopy were designed to use 1×10^5 cells. For microscopy, confluent cells in an 8-well chamber slide required acquisition of a low number of ROI to identify a minimum of 100 cells per subject to be analysed, compared to 1000s of cells required for flow cytometry or plate fluorometry. Additionally, flow cytometry required adherent cells to be removed from the plate post-phagocytosis before cell events were acquired on the flow cytometer, which can have some effect on cell viability and the cell membrane (Batista et al., 2010).

It is important to note how each technique assesses phagocytosis, plate fluorometry calculates phagocytosis by relative fluorescence intensity, where the amount of fluorescence gives a relative value for phagocytosis when compared to a baseline control. Flow cytometry can assess phagocytosis in two ways; the percentage of cells positive for bacteria or as mean fluorescence intensity (MFI), which again uses a measure of relative fluorescence compared to baseline to calculate a relative value for phagocytosis. The microscopy used however can give phagocytosis as a percentage of cells positive for bacteria, similar to flow cytometry, but with the additional benefit of being able to visualise internalised bacteria within the cell, which allows the number of internalised bacteria to be quantified instead of assessing the relative fluorescence, giving a clear advantage of this technique over the others investigated.

Additionally, when measuring phagocytosis by the percentage of cells positive for internalising at least one bacterium, image analysis of microscopy images produced a mean phagocytosis percentage in THP-1 cells at around 60%, when compared to similar analysis by flow cytometry, which resulted in 40% cells positive for phagocytosis. This difference of around 20% was consistent through several experimental replicates, and likely highlights a difference in the sensitivity of the detection of individual or small numbers of bacteria within the cell volume, again revealing an advantage of microscopy over other detection methods.

This technique also allows further analysis of specific proteins of interest, in this study that will include cytoskeletal proteins, actin and tubulin, as well as an investigation into mitochondria activity within macrophages. The measurements of phagocytosis, both as a percentage of cells positive for bacteria and the number of internalised bacteria, can then be correlated to morphological markers.

The quantification of bacteria within the different imaging modalities, correlated with imaging resolution. With STORM giving the best spatial resolution allowing individual bacteria to be distinguished from each other, however STORM also produces the lowest temporal resolution, often taking tens of minutes to acquire enough frames to reconstruct a super-resolved image in multiple fluorescence channels. This added with other technical limitations of super-resolved microscopy including fixation and buffer conditions. In comparison, deconvolved widefield microscopy can be performed on live cells with vastly increased temporal resolution compared to STORM, and offers improved spatial resolution compared to standard epi-fluorescent widefield, which still allowed quantification of individual bacterium. Therefore, as work in this thesis wanting to perform live cell imaging, deconvolved widefield was the preferred technique.

Chapter 4: Comparison of phagocytosis in MDM and tissue macrophages between non-smoker, ex-smoker and COPD subjects

This chapter outlines the use of microscopy and image analysis for the investigation of defective phagocytosis in COPD MDM and lung tissue-derived macrophages.

4.1 Introduction

Previous work has shown that alveolar macrophage and MDM from COPD subjects show a significant reduction in phagocytosis of bacteria compared to cells from non-smokers (Berenson et al., 2006) (Taylor et al., 2010). However, the mechanism underlying this defect in phagocytosis is currently unknown. Work explored in this chapter will investigate both macrophages isolated from lung tissue and MDM differentiated from blood, and phagocytosis will be assessed by microscopy and image analysis. Deconvolved widefield images will be used to give sufficient resolution to identify and quantify the internalised bacteria within the macrophage volume. This will allow phagocytosis to be measured as both a percentage of cells positive for internalised bacteria, as well as the mean number of internalised bacteria, allowing a more comprehensive view of phagocytosis in non-smoker and COPD groups for both MDM and tissue macrophages.

4.1.1 Hypothesis

Microscopy and image analysis will confirm and quantify defective phagocytosis to *H. influenzae* in COPD macrophages.

4.1.2 Aims

- Use widefield microscopy, image analysis and bacterial quantification to confirm defective phagocytosis in COPD in both MDM and tissue macrophages.
- Compare differential phagocytic responses between the two macrophage models.

4.2 Methods

4.2.1 Subject selection

All subjects were recruited under inclusion/exclusion criteria with written, informed consent (described in 2.2.3)

4.2.2 Isolation and culture of MDM

MDM were isolated and cultured from non-smoker and COPD subjects (described in 2.2.3.1). MDM were plated at 1×10^6 cells/well into 8-well chamber slides with media supplemented with 2 ng/ml GM-CSF.

4.2.3 Isolation and culture of tissue macrophages

Tissue macrophages were isolated and cultured from non-smoker, ex-smoker and COPD subjects (described in 2.2.3.2). TM were plated at 1×10^6 cells/well into 8-well chamber slides pre-treated with poly-L-lysine.

4.2.4 Measurement of phagocytosis by microscopy

MDM or TM were incubated with 5×10^8 CFU/ml heat killed *H. influenzae* labelled with AF488 for up to 60 min (described in 2.2.6.3), before being imaged live by epi-fluorescent widefield microscopy (2.2.16) with images deconvolved using Huygens software (2.2.18).

4.2.5 Bacterial quantification by image analysis

Images of MDM or TM saved as .tiff files were inputted into *MicrobeJ* for cell segmentation and bacterial quantification was performed (described in 2.2.20).

4.2.6 Statistical analysis

All data analyses were performed using GraphPad Prism software version 9.3.1. All data are expressed as mean $\pm$ SEM. For correlation analysis, Spearman's rank correlation coefficient was performed with all r and p values stated. For grouped analysis, 2-way ANOVA with Sidak's multiple comparisons test were performed, with p value stated.

4.3 Results

4.3.1 Subject demographics for MDM and tissue macrophage models

Two models for studying macrophages were used, blood was collected from non-smoker and COPD subjects to generate MDM, and tissue macrophages isolated from resected lung tissue from non-smoker, ex-smoker, and COPD subjects.

There was no significant difference in age between subject groups for experiments performed on MDM or tissue macrophages (Table 4.1). There was no significant difference in smoking history, between COPD and ex-smoker subjects, however there was a significant difference in smoking history between non-smoker and COPD subjects in the MDM.

The COPD subjects MDM had significantly lower lung function compared to non-smokers. For the tissue macrophages subject demographics, despite showing similar trends as the MDM subjects, with COPD subjects displaying reduced FEV₁, FEV₁ % predicted and FEV₁: FVC ratio, these differences did not reach significance likely due to smaller 'n' in the COPD group.

	MDM		Tissue Macrophages		
	Non-smoker N = 7	COPD N = 8	Non-smoker N = 4	Ex-smoker N = 4	COPD N = 3
Age	65.67 ± 2.50	69.86 ± 2.13	69.75 ± 4.42	71.00 ± 6.06	76.67 ± 4.84
Gender (M:F)	4:3	2:5	1:3	3:1	3:0
Smoking History (Pack years)	0.00 ± 0.00	35.14 ± 5.23	0.00 ± 0.00	48.75 ± 24.86	46.33 ± 8.76
FEV₁ (L)	3.01 ± 0.20	1.58 ± 0.19 **	2.54 ± 0.29	3.03 ± 0.20	1.21 ± 0.28
FEV₁ % predicted	101.2 ± 4.26	68.34 ± 10.33 *	107.10 ± 5.79	102.80 ± 10.45	48.27 ± 15.14
FVC (L)	3.97 ± 0.31	2.56 ± 0.27 **	3.40 ± 0.39	4.07 ± 0.31	3.54 ± 0.06
FEV₁:FVC ratio	0.77 ± 0.04	0.62 ± 0.04 *	0.75 ± 0.02	0.75 ± 0.4	0.34 ± 0.08

Table 4.1. Subject demographics for macrophages isolated from blood samples (MDM) and tissue macrophages between subject groups. * p < 0.05, ** p < 0.01

4.3.2 Analysis of phagocytosis

4.3.2.1 Visualisation and comparison of *H. influenzae* phagocytosis by MDM between subject groups using microscopy and image analysis

Epi-fluorescent widefield microscopy was performed on MDM exposed to *H. influenzae* for up to 60 min and components of the cytoskeleton observed by staining with SiR dyes. Images were taken to observe a minimum of 100 cells per timepoint, per sample. Representative images for MDM stained with actin (Fig. 4.1 A and B) and tubulin (Fig. 4.1 C and D) show bacteria within the cell and appear to show reduced uptake in COPD samples compared to non-smokers.

Using this technique, firstly quantification of uptake of fluorescently labelled *H. influenzae* was compared between MDM from NS subjects and those with COPD (Fig. 4.2) to confirm the dysfunction observed using non-imaging methodology (Taylor et al, 2010). Image analysis of epi-fluorescent microscopy showed there was a reduction in the number of MDM phagocytosing bacteria in COPD MDM compared to non-smokers (Fig. 4.2 E) at 60 min. Further image analysis measured the number of bacteria taken up within a cell, and again, COPD MDM showed a significant reduction in the mean number of *H. influenzae* phagocytosed compared to non-smokers (Fig. 4.2 F) at 60 min.

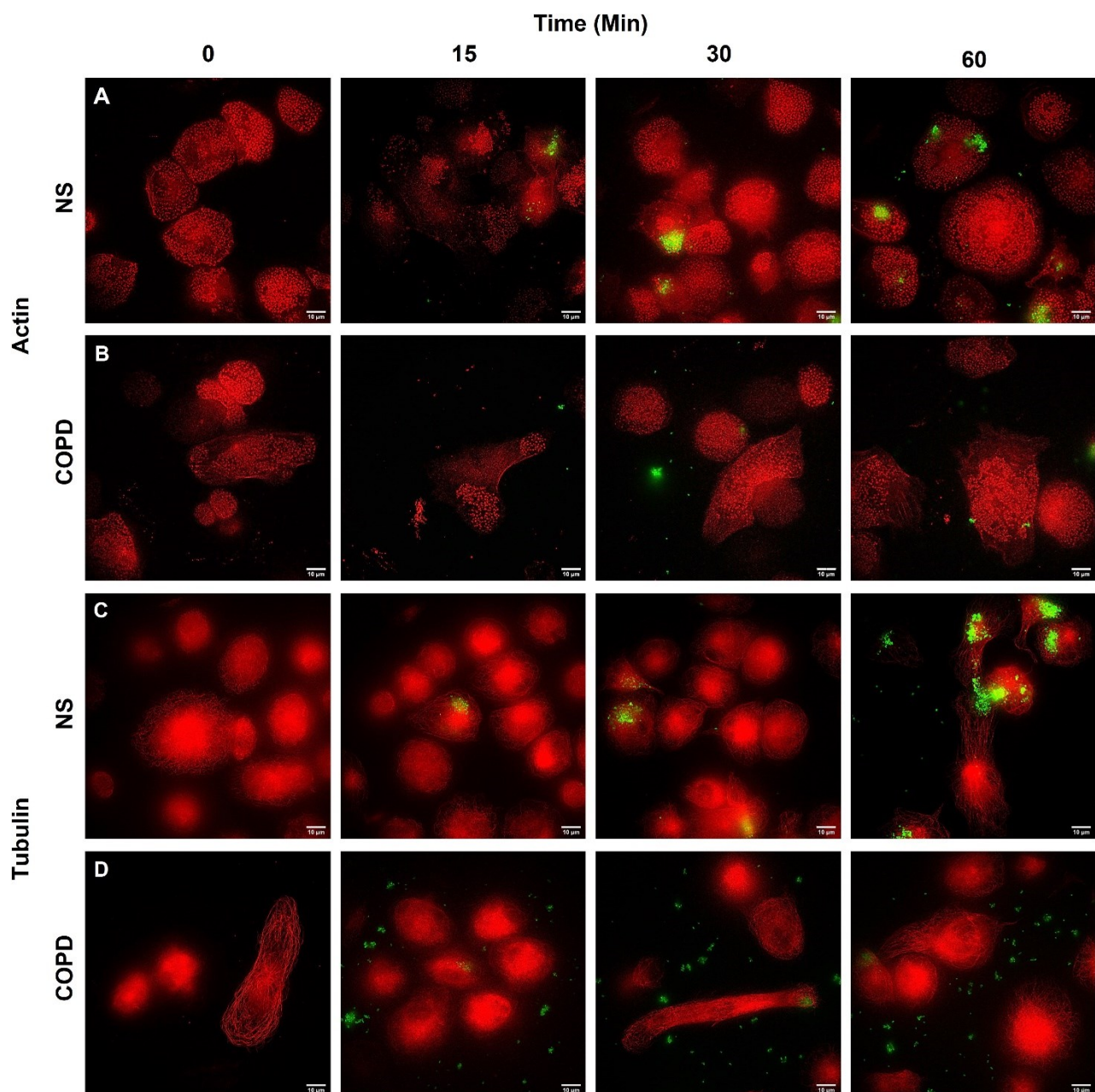


Figure 4.1. Visualisation of phagocytosis of *H. influenzae* by subject MDM. MDM from non-smokers (NS) and COPD subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* fluorescently labelled with Alexa fluor® 488 for up to 60 min. Cells were stained for actin (A-B) or tubulin (C-D) with silicon rhodamine dye Alexa Fluor® 647 specific for F-actin or α -tubulin respectively. Cells undergoing phagocytosis were imaged by epi-fluorescent widefield microscopy with a 1.46NA, 100x objective. Images were deconvolved using Huygens software. Representative images selected and 10µm scale shown. Red: **actin or tubulin**, Green: *H. influenzae*.

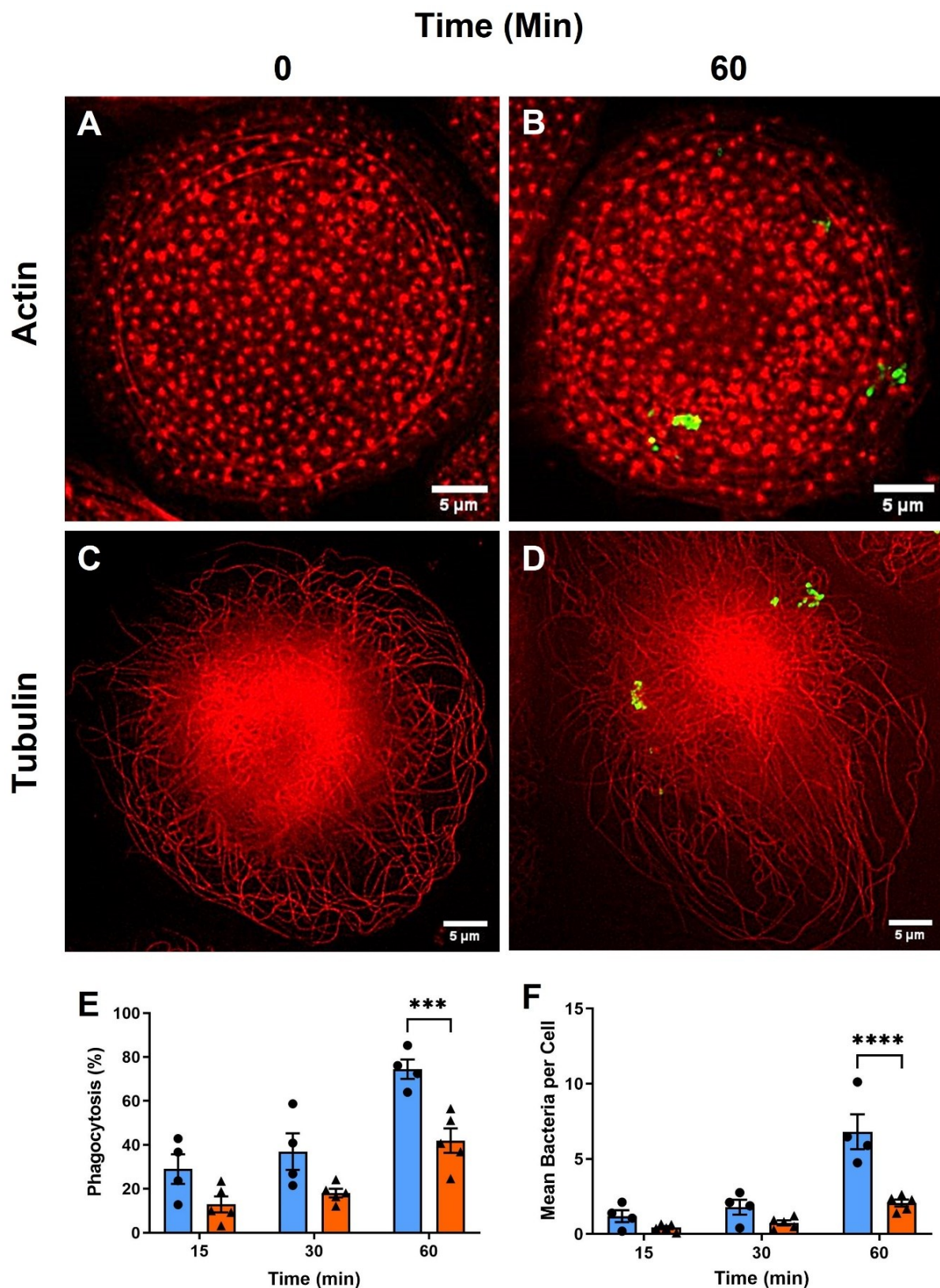


Figure 4.2. Measurement of phagocytosis of *H. influenzae* by subject MDM. Representative images of single MDM $\pm$ *H. influenzae*, 5 μ m scale shown. Red: **actin or tubulin**, Green: *H. influenzae*. Phagocytosis was measured with 100 cells per time point analysed for each subject, NS (■, n = 4) and COPD (■, n = 5). Data presented as mean $\pm$ SEM for phagocytosis as a percentage of cells positive for internalisation (E) and mean number of bacteria internalised (F). Data were analysed using a 2-way ANOVA with Sidaks multiple comparisons test. *** $p < 0.001$, **** $p < 0.0001$

4.3.2.2 Visualisation and comparison of *H. influenzae* phagocytosis by tissue macrophages between subject groups using microscopy and image analysis

Having successfully established methodology to image MDM at high resolution, similar techniques were then used to image lung tissue macrophages isolated from non-smoker, ex-smoker and COPD subjects following exposure to *H. influenzae* for up to 60 min. Representative images for tissue macrophages stained with actin (Fig. 4.3 A-C) and tubulin (Fig. 4.3 D-F) show bacteria within the cell volume and appear to show reduced phagocytosis in COPD samples compared to non-smokers.

Tissue macrophages isolated directly from COPD resected lung tissue and exposed to *H. influenzae* displayed no significant difference in phagocytosis of *H. influenzae* compared to non-smokers when analysing percentage of macrophages positive for internalised *H. influenzae* at any time point (Fig. 4.4 E). However, defective phagocytosis was observed in COPD and ex-smoker tissue macrophages when comparing the mean number of bacteria internalised when compared to non-smokers macrophages (Fig. 4.4 F), similar to that observed in MDM.

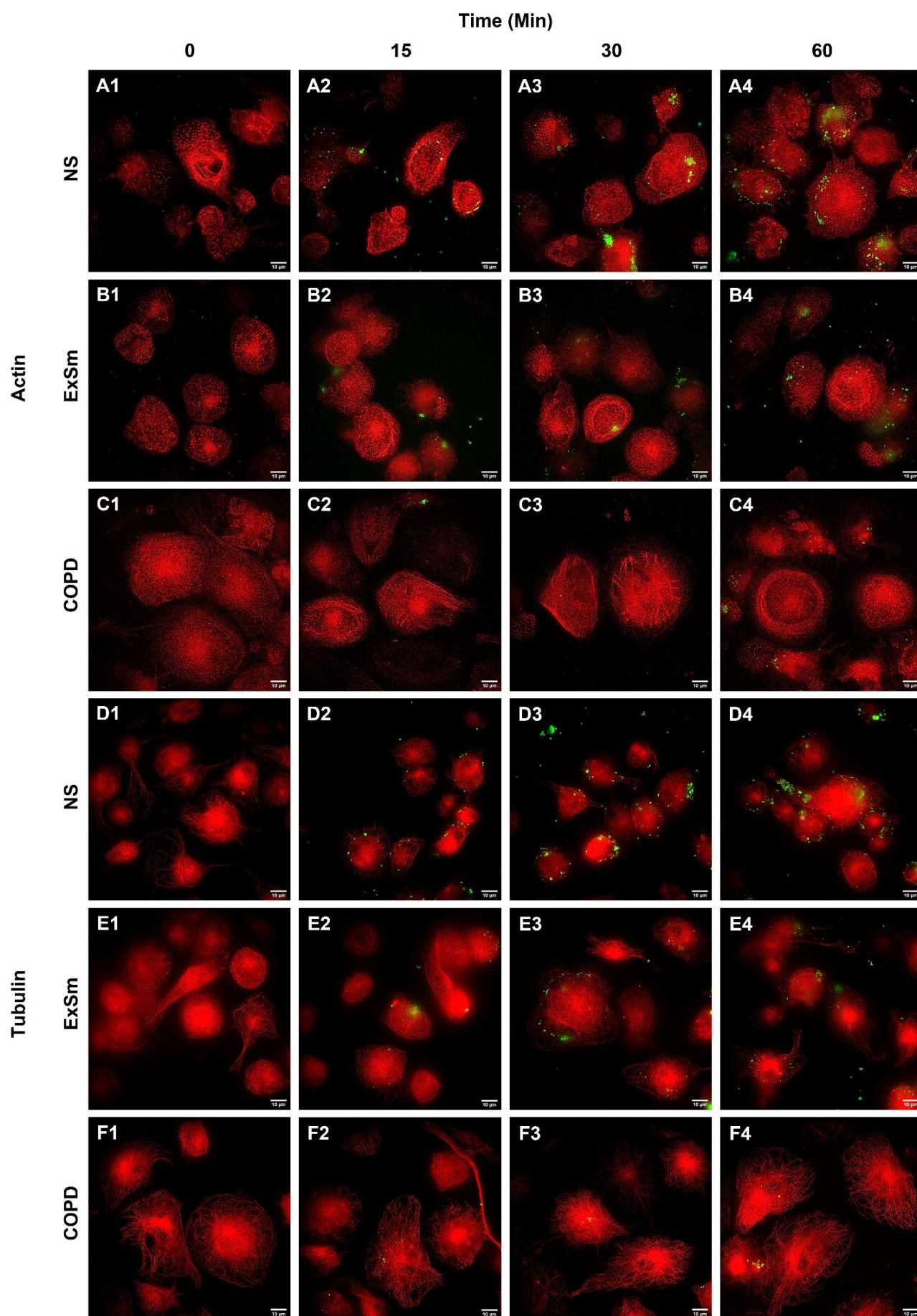


Figure 4.3. Visualisation of the cytoskeleton and *H. influenzae* phagocytosis by tissue macrophages. Tissue macrophages from non-smokers (NS), ex-smoker (ExSm) and COPD subjects were isolated from resected lung tissue. Cells were incubated with *H. influenzae* fluorescently labelled with Alexa fluor® 488 for up to 60 min. Cells were stained for actin (A-C) or tubulin (D-F) with silicon rhodamine dye Alexa Fluor® 647 specific for F-actin and α -tubulin respectively. Phagocytosis was measured by epi-fluorescent widefield microscopy with a 1.46NA, 100x objective. Images were deconvolved using Huygens software. Representative images selected and 10 μ m scale shown. Red: **actin or tubulin**, Green: *H. influenzae*.

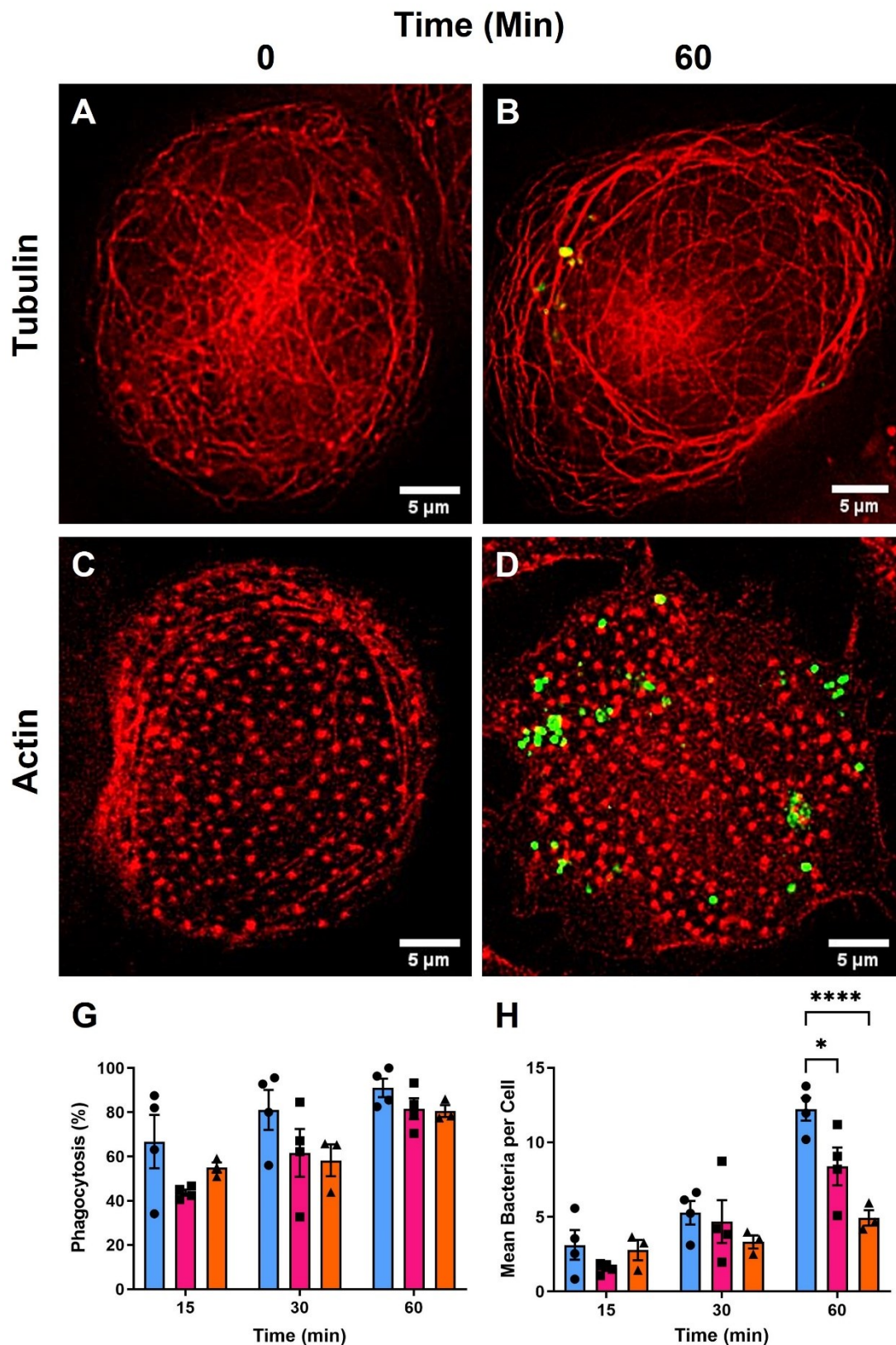


Figure 4.4. Measurement of *H. influenzae* phagocytosis by tissue macrophages (TM). Representative images of TM $\pm$ *H. influenzae*, 5μm scale shown. Red: actin or tubulin, Green: *H. influenzae*. Phagocytosis was measured with 100 cells per time point analysed for each subject, NS (■, n = 4), ExSm (■, n = 4) and COPD (■, n = 3). Data presented as mean $\pm$ SEM for phagocytosis as a percentage of cells positive for internalisation (G) and mean number of bacteria internalised (H). Data analysed by a 2-way ANOVA with Sidak's multiple comparisons test. * p<0.05, **** p<0.0001

4.3.2.3 Comparison of phagocytosis between MDM and tissue macrophages

Both the MDM and tissue macrophage models showed significant decreases in the phagocytic capabilities of COPD cells when compared to cells from non-smokers. To directly compare between the two models, phagocytosis as measured by percentage of macrophages positive for *H. influenzae* (phagocytosis %) and the mean number of *H. influenzae* internalised by a single macrophage was compared between MDM and tissue macrophages.

There were significant differences between the MDM and tissue macrophage models when comparing the percentage of cells able to phagocytose. When macrophages were incubated with *H. influenzae*, there was a greater proportion of tissue macrophages able to phagocytose at least a single bacterium when compared to MDM, for non-smokers (Fig. 4.5 A). This occurred for all samples incubated with *H. influenzae* except for non-smokers with 60 min incubation, where the difference between MDM and tissue macrophage phagocytosis did not reach significance.

When phagocytosis was measured as the mean number of bacteria internalised per cell, there was again significant differences between the MDM and tissue macrophage models, with tissue macrophages internalising more bacteria than MDM. Non-smoker tissue macrophages internalised more *H. influenzae* compared to MDM with a 30 min ($p = 0.0014$) and 60 min ($p < 0.0001$) incubation, whereas there was only a significant increase observed in COPD was with a 60 min incubation ($p = 0.0176$) (Fig. 4.5 B).

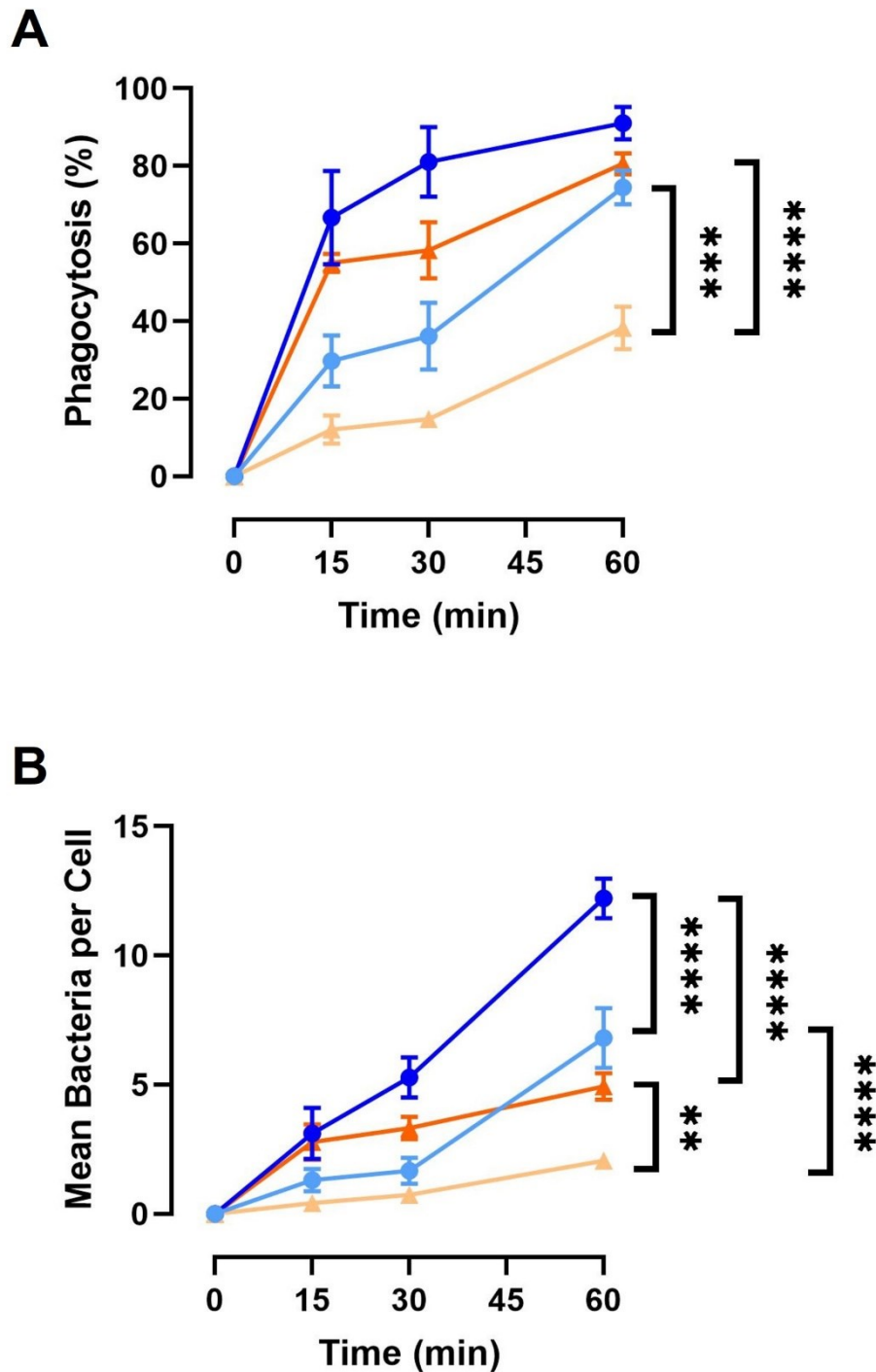


Figure 4.5. Comparison of macrophage models of phagocytosis using imaging analysis. MDM from non-smokers (■, n = 4) and COPD (■, n = 5) were compared to tissue macrophages from non-smoker (■, n = 4) and COPD (■, n = 3) subjects. Cells were incubated with *H. influenzae* for up to 60 min. Phagocytosis was measured with 100 cells per time point analysed for each subject in sample groups. Data presented as mean ± SEM for phagocytosis as a percentage of cells positive for internalisation (A) and mean number of bacteria internalised (B). Data were analysed using a 2-way ANOVA with Tukey's multiple comparisons test. ** p<0.01, *** p<0.001, **** p<0.0001.

4.3.3 Relationship of subject demographics and phagocytosis

To determine if bacterial phagocytosis of *H. influenzae* by MDM or tissue macrophages was directly related to any subject demographics, correlation analyses were performed between phagocytosis and subject characteristics.

4.3.3.1 Age

There was no significant correlation between age and MDM phagocytosis for non-smokers or COPD subjects independently or together (Fig. 4.6 A). Similarly, there were no correlations of age and tissue macrophage phagocytosis either when analysing subject groups independently or together (Fig. 4.6 B).

4.3.3.2 Sex

There was no effect of sex on MDM phagocytosis irrespective of whether cells were derived from non-smokers and COPD subjects independently (Fig. 4.6 C). Similarly, there was no effect of sex when phagocytosis of tissue macrophages was assessed when analysing subject groups independently or together (Fig. 4.6 D).

4.3.3.3 Smoking history

There were no significant correlations between smoking history and phagocytosis for either MDM or tissue macrophages (Figs. 4.6 E and F).

4.3.3.4 FEV₁

Patients with COPD are diagnosed based on lung function tests and therefore, the relationship between a function of innate immunity such as macrophage phagocytosis and lung function parameters were investigated. There were no correlations between MDM phagocytosis and lung function when cells from non-smoker and COPD subjects were analysed independently. However, when analysed together there was a significant positive correlation of FEV₁ and phagocytosis either measured by the percentage of cells that phagocytosed ($r = 0.82$, $p = 0.01$) or by how many of *H. influenzae* were internalised ($r = 0.78$, $p = 0.02$) (Fig. 4.6 G). In contrast, there were no correlations between FEV₁ and phagocytosis for tissue macrophages when analysing groups independently or together (Fig. 4.6 H).

4.3.3.5 FVC

Lung volume as measured by FVC is another measure of lung function and the relationship between FVC and phagocytosis was also investigated. As seen for FEV₁, there was no correlation between phagocytosis and FVC for when analysing non-smoker and COPD groups independently. However, when groups were analysed together there was a significant positive correlation between the percentage of cells phagocytosing and FVC ($r = 0.72$, $p = 0.04$) but this did not occur when the number of *H. influenzae* internalised/cell was analysed either as subject groups independently or together (Fig. 4.6 I). As seen for FEV₁, there were no significant correlations between FVC and phagocytosis for tissue macrophages when analysing groups independently or together (Fig. 4.6 J).

4.3.3.6 FEV₁:FVC ratio

FEV₁/FVC reflects airway obstruction and a value of <0.7 is used in the diagnosis of COPD therefore the relationship between airway obstruction and phagocytosis was explored. For MDM, there were no correlations between FEV₁/FVC and phagocytosis when analysing non-smoker and COPD groups independently, however when analysing the groups together there was a significant positive correlation with the percentage of cells capable of phagocytosis ($r = 0.83$, $p = 0.01$). When analysing correlation between FEV₁:FVC ratio and mean number of *H. influenzae* internalised by MDM, there was a no significant correlations when analysing non-smoker and COPD groups independently or together (Fig. 4.6 K). There were no significant correlations between FEV₁:FVC ratio and phagocytosis for tissue macrophages when analysing groups independently or together, for phagocytosis as a percentage of macrophages positive for *H. influenzae* (phagocytosis %). However, there was a weak positive correlation between FEV₁:FVC ratio and mean number of *H. influenzae* internalised, when the groups were analysed together ($r = 0.66$, $p = 0.03$) (Fig. 4.6 L).

MDM

Tissue Macrophages

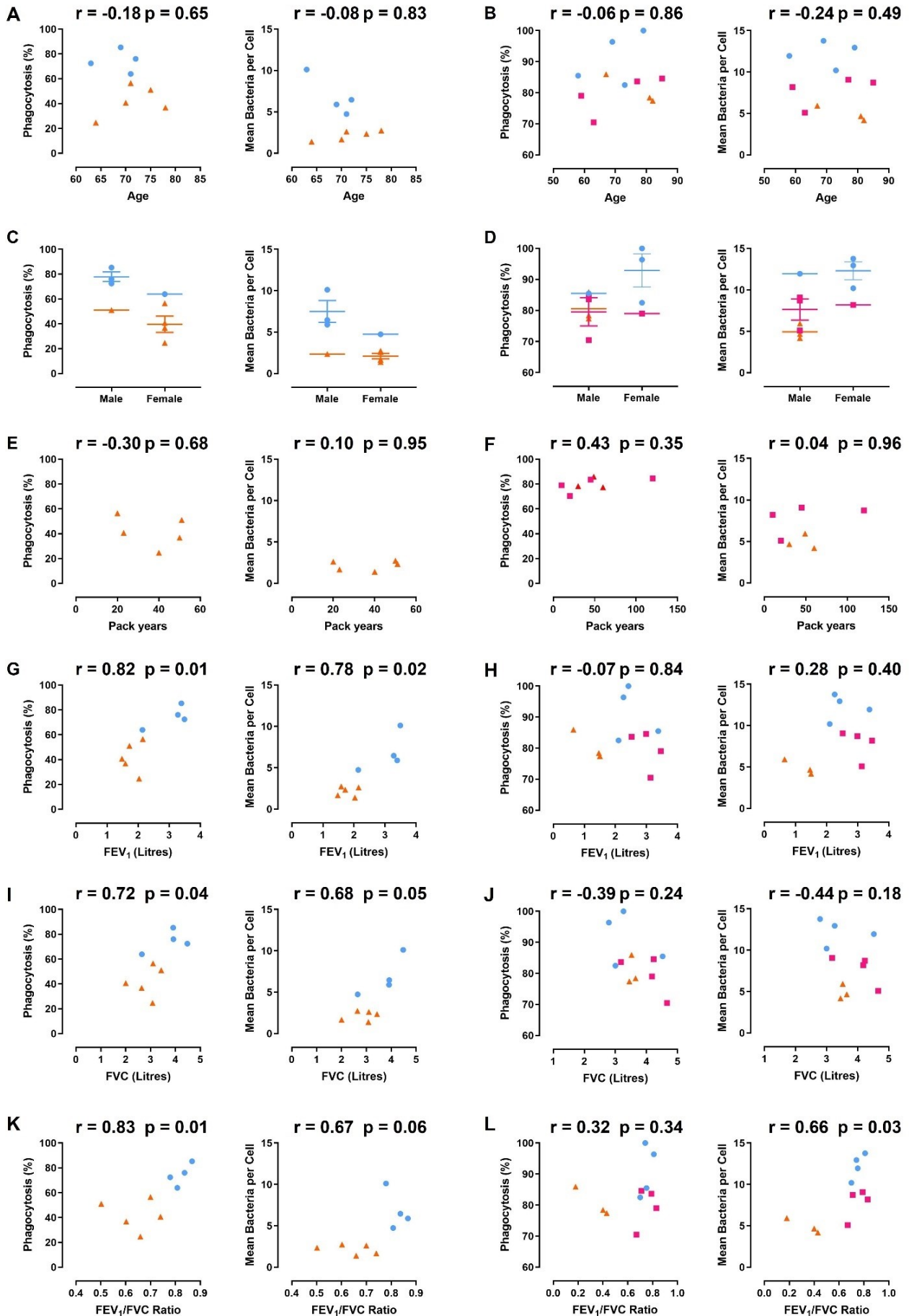


Figure 4.6. Correlation between subject demographics and measurements of phagocytosis of *H. influenzae* by MDM and tissue macrophages. MDM from non-smokers (●, n = 4) and COPD (▲, n = 5) subjects were differentiated in the presence of GM-CSF for 12 days (A, C, E, G, I and K) or tissue macrophages from non-smokers (●, n = 4), ex-smokers (■, n = 4) and COPD (▲, n = 3) subjects were isolated from resected lung tissue (B, D, F, H, J and L). Cells were incubated with *H. influenzae* for up to 60 min, with phagocytosis quantified as percentage of cells positive for bacteria and mean bacteria per cell. Data presented as Spearman rank correlation coefficients (r) and associated p value of phagocytosis against age (A-B), gender (C-D), pack years (E-F), FEV₁ (G-H), FVC (I-J) and FEV₁/FVC ratio (K-L).

4.4 Discussion

Macrophages isolated from the lungs of COPD patients show reduced phagocytosis of bacteria compared to cells from non-smokers and healthy smokers (Berenson et al., 2006; Bewley et al., 2018). This effect has also been reported in MDM (Taylor et al., 2010; Bewley et al., 2018) and confirmed in this chapter. It was important to confirm this defect in the samples used herein before novel analysis of macrophage morphology and the cytoskeleton. When measuring phagocytosis as the percentage of macrophages internalising a least a single bacterium, MDM from COPD subjects showed a significant reduction in phagocytosis of *H. influenzae* after 60 min, confirming the data of Taylor et al 2010, who used a different method to detect phagocytosis. However, analysis of phagocytosis using measurements for the percentage of cells positive for internalisation did not produce an observed difference in tissue macrophages between non-smokers and COPD subjects, where a trend was seen but did not reach significance, likely due to fewer samples. A power calculation was performed using these data and with a power of 80%, α of 0.05 and a 1:1 enrolment rate, resulted in a predicated 'n' value of 20 (10 non-smoker and 10 COPD subjects required).

With the advancement in microscopy and image analysis tools, including the automated quantification of bacteria, single-cell image analysis allowed the number of bacteria internalised by individual macrophages to be quantified. This offers an improvement when compared to other techniques such as flow cytometry and plate based fluorometry, which analyse internalised bacterial as a product of the overall fluorescence intensity but gives little insight into number of bacteria or their location within the macrophage. Importantly, when assessing the mean number of bacteria internalised, both COPD MDM and tissue macrophages showed significantly reduced phagocytosis of *H. influenzae* compared to non-

smokers. This clarifies that the defect in phagocytosis observed in COPD is due to how many bacteria the macrophages are able to internalise, not the percentage of cells capable of internalisation.

Tissue macrophages from both non-smokers and COPD subjects were shown to phagocytose significantly more bacteria when compared to MDM from the same groups. Additionally, an important observation is that COPD tissue macrophages had a higher percentage of cells positive for phagocytosis and a comparable mean number of internalised bacteria when compared to non-smoker MDM, disproving a theory that tissue macrophages within the lung fail to phagocytose due to already being full. These differences observed between tissue macrophages and MDM, also validates the use of the MDM to further investigate defects in phagocytosis.

These differences in the phagocytic capabilities between MDM and tissue macrophages are in keeping with the literature and could be due to the macrophages having different phenotypes, as they have been isolated from different microenvironment (Vlahos & Bozinovski, 2014). Macrophages isolated from the lung have been differentiated in the lung microenvironment, where the tissue resident population differs in lineage to blood-derived macrophages. Tissue macrophages have also likely been exposed to pathogens and other chemicals, leading to activation of these cell and are more highly specialised phagocytic cells (Yamasaki & van Eeden, 2018). Macrophages display different phenotypes within a population, resulting in a heterogeneous response to bacterial prey within the lung, and is largely based on activation state and the local microenvironment. This can be alluded to in this data set, as seen by the varied phagocytic capabilities of macrophages within the total population, as some cells are able to phagocytose many bacteria compared to other

macrophages which are unable to phagocytose at all. These data refute the suggestion that alveolar macrophages from COPD subjects fail to phagocytose because they are replete and supports the use of MDM to investigate the phagocytic defect in these cells.

Another explanation for the differences between the two models shown in this thesis could be linked to the length of time the macrophages were incubated with bacteria. As the work performed in this chapter examined differences in phagocytosis between subjects with COPD and controls at early timepoints of the phagocytosis process, up to 60 min. When these experiments are performed for longer periods of time, such as incubation with either *H. influenzae* or *S. pneumoniae* for 4 hours, the MDM model shows no difference in the percentage of macrophages positive for phagocytosis between non-smoker, smoker, and COPD subject groups, as determined by flow cytometry (Belchamber et al., 2019). However, the same study showed that the number of internalised bacteria is significantly decreased in COPD subjects compared to non-smokers after 4-hour incubation, as assessed by MFI. This shows that the percentage of COPD macrophages able to phagocytose bacteria is comparable with non-smoker subjects, however the COPD macrophages internalise less bacteria per cell (Belchamber et al., 2019). This is in keeping with findings from this thesis, where both MDM and tissue macrophages are capable of phagocytosis, but COPD cells are unable to internalise as many bacteria compared to cells from non-smokers. Data in this thesis shows that phagocytosis in COPD MDM is slower during the first 60 min of bacterial incubation compared to non-smoker cells, which could indicate a dysfunction in the initiation of phagocytosis or in the continued progression in the process after initial internalisation of at least a single bacterium. This could be described as the COPD macrophages having a lag-phase compared to non-smokers, where they are unable to phagocytose bacteria initially before recovering over time, and this could be due to a defect in the initial recognition of bacterial or

cytoskeletal changes necessary for phagocytosis to occur. However, they do not reach the levels of intake of bacteria observed in cells from non-smokers.

When analysing correlations between subject demographics and measurements of phagocytosis, there were no significant correlations in any subject group for both macrophage models in relation to age, gender, or smoking history. As this study used age-matched groups there was unlikely to be a difference in correlations between subject groups. The prevalence of COPD is known to increase with age, (Raherison & Girodet, 2009), as such phagocytosis is known to have a negative correlation with age (Li, 2013; Nagel et al., 1986). No correlations were seen in smoking history. Smoking is one of the major causes of COPD and is known to have a direct effect on phagocytosis, where differences can be observed in COPD subjects based on their smoking status, with current smokers having reduced phagocytosis compared to ex-smokers (Hodge et al., 2007). There were correlations between lung function variables and phagocytosis, with FEV₁, FVC and FEV₁:FVC ratio all showing significant positive correlations with phagocytosis, when all subjects were analysed together but not within the COPD subjects separately.

The work performed in this chapter confirms the use of deconvolved epi-fluorescence widefield microscopy, followed by image analysis and bacterial quantification, to perform comparative analysis of phagocytosis in MDM and tissue macrophages from subject groups. This methodology can detect significant differences in phagocytosis between non-smoker and COPD groups using as few as 100 cells per sample.

Chapter 5: Comparison of cellular morphology and the cytoskeletal network in macrophages between non-smoker and COPD subjects

This chapter outlines the use of fluorescence microscopy techniques to study cytoskeletal changes of actin and tubulin in macrophages undergoing phagocytosis.

5.1 Introduction

Macrophages are highly motile cells, as they need to migrate to areas of inflammation and infection to detect and destroy pathogens (Liu et al., 2020). As such the intracellular cytoskeletal network needs to constantly remodel to allow movement and phagocytosis. Currently, defective mechanisms involving the cytoskeleton have yet to be identified in COPD macrophages.

The process of phagocytosis is a multi-stage process (see Fig. 1.5), starting with detection of the pathogen by cell surface phagocytic receptors, followed by ingestion and formation of the phagosome before the phagosome and lysosome are trafficked to merge to form the phagolysosome where bacterial degradation occurs (Uribe-Querol & Rosales, 2020). Phagocytosis requires significant involvement of the cytoskeleton, and dynamic rearrangement. For example, dysregulation of actin in macrophages affects bacterial uptake in receptor-mediated phagocytosis (Li et al., 2017). The microtubule network is also constantly remodelled and is important during phagocytosis. Phagocytosis is mainly considered an actin driven process (May & Machesky, 2001), but the involvement of microtubules has been shown to contribute to CR-mediated but not FcR-mediated phagocytosis (Allen & Aderem, 1996; Rougerie, Miskolci & Cox, 2013), as disruption of the tubulin cytoskeleton by the inhibitor nocodazole only disrupted CR3-mediated phagocytosis,

but has no effect on FcR-mediated phagocytosis of IgG beads (Allen & Aderem, 1996). Additionally, the microtubule network forms the framework to transport the phagosome and lysosome after bacterial internalisation towards the formation of the phagolysosome (Harrison & Grinstein, 2002; Jubrail, Kurian & Niedergang, 2017).

With the cytoskeletal network being integral for cell motility and phagocytosis, it is important to understand if any differences can be observed in morphology and structural arrangement of the key proteins: tubulin and actin. Any morphological differences observed by microscopy could be used to elucidate a potential mechanism for the defective phagocytosis observed in COPD macrophages.

5.1.1 Hypothesis

Microscopy and image analysis will reveal differences in cellular morphology and the arrangement of cytoskeletal proteins; actin and tubulin, in COPD macrophages, which are associated with defective phagocytosis of *H. influenzae*.

5.1.2 Aims

- Using image analysis to determine any morphological differences in macrophages between subject groups.
- Investigate differences in cytoskeletal proteins; tubulin and actin, to determine any differences which could explain defective phagocytosis observed in COPD macrophages.

5.2 Methods

5.2.1 Subject selection

All subjects were recruited under inclusion/exclusion criteria with written, informed consent (described in 2.2.3).

5.2.2 Isolation and culture of MDM

MDM were isolated and cultured from non-smoker and COPD subjects (described in 2.2.3.1). Monocytes were plated at 1×10^6 cells/well into 8-well chamber slides, pre-treated with poly-L-lysine, cultured for 12 days in RPMI 1640 media supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 10 mg/ml penicillin/streptomycin and 2 ng/ml GM-CSF, with media being changed on days 4 and 7.

5.2.3 Isolation and culture of tissue macrophages

TM were isolated from non-smoker, ex-smoker and COPD subjects (described in 2.2.3.2). TM were plated at 1×10^6 cells/well into 8-well chamber slides, pre-treated with poly-L-lysine and cultured in RPMI 1640 media supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 10 mg/ml penicillin/streptomycin with media replaced prior to experiment.

5.2.4 Measurement of cell parameters by microscopy

MDM or TM were incubated with 5×10^8 CFU/ml heat killed *H. influenzae* labelled with AF488 for up to 60 min (described in 2.2.6.3) The cytoskeleton proteins, tubulin or actin, were labelled using SiR-dyes (described in 2.2.10.1), followed by live epi-fluorescent widefield microscopy (2.2.16) and image deconvolution using Huygens software (2.2.18).

5.2.5 Image analysis of cell parameters of macrophages undergoing phagocytosis

Images of MDM or tissue macrophages were analysed using ImageJ or MATLAB (described in 2.2.19). Measurements were calculated using the known pixel range of the camera converted into μm .

5.2.6 Statistical analysis

All data analyses were performed using GraphPad Prism software version 9.3.1. All data are expressed as mean $\pm$ SEM. For correlation analysis, Spearman's rank correlation coefficient was performed with all r and p values stated. For grouped analysis, 2-way ANOVA with Sidak's multiple comparisons test were performed, with p value stated.

5.3 Results

5.3.1 Analysis of macrophage morphology

Having showed differential phagocytic responses between non-smoker and COPD MDM and tissue macrophages, cell morphology was investigated to determine if there were differences in cell area, perimeter, and shape between cells from those with and without disease.

5.3.1.1 Analysis of cell morphological attributes

Epi-fluorescent widefield imaging showed no significant difference in cell area between COPD and non-smoker MDM either at baseline or following exposure to bacteria (Fig. 5.1 A). In contrast, tissue macrophages from COPD subjects had a significantly larger cell area than cells from control subjects at baseline (Fig. 5.1 B). This was also seen when looking at cell perimeter, with no differences observed in MDM (Fig. 5.1 C) but significantly larger perimeter in COPD tissue macrophages compared to non-smoker cells at baseline (Fig. 5.1 D).

These size differences observed in tissue macrophages were lost within 15 min exposure to *H. influenzae* but were restored during the 60 min incubation with bacteria. No difference in cell eccentricity was observed in MDM (Fig. 5.1 E) or tissue macrophages (Fig. 5.1 F).

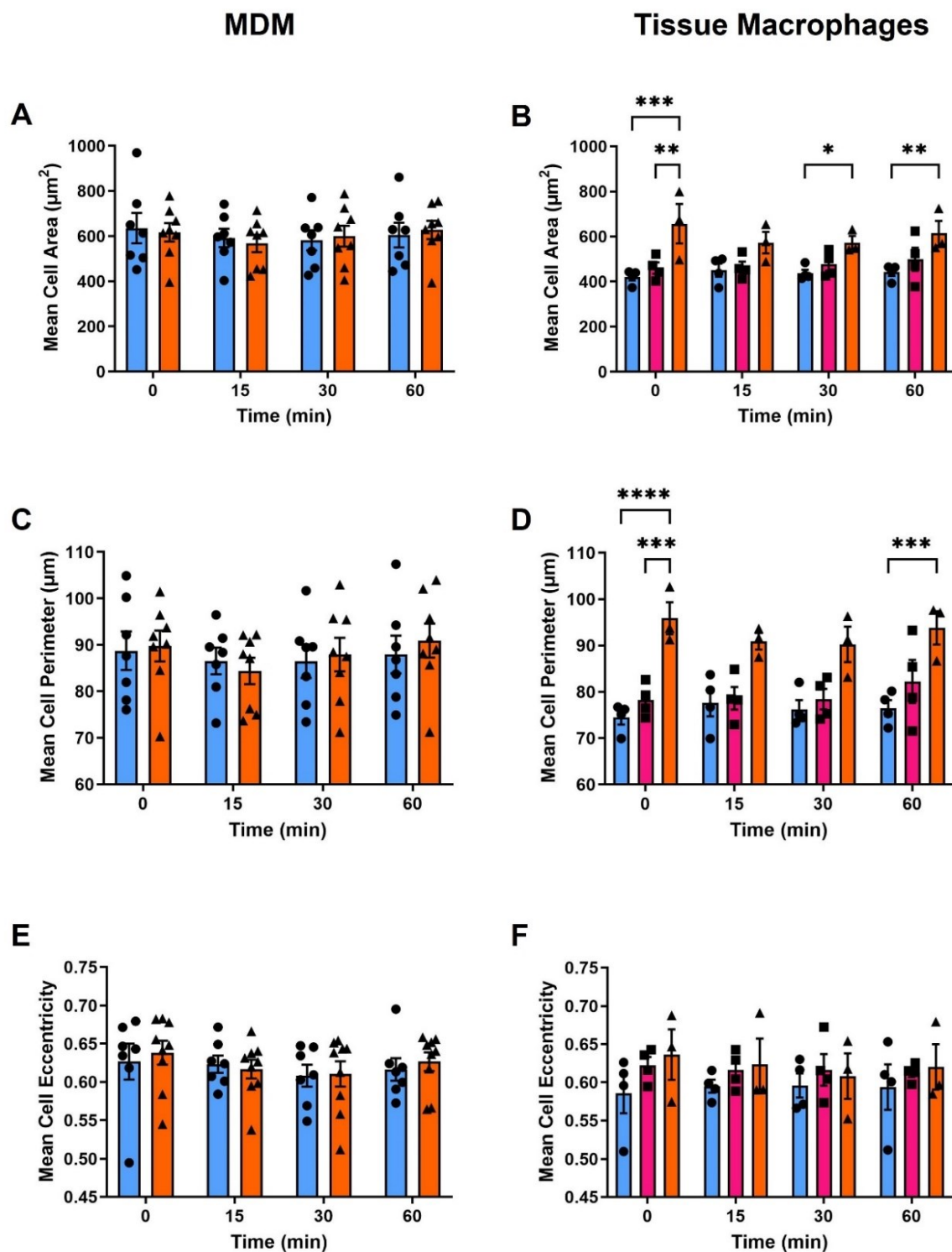


Figure 5.1. Comparison of cell morphology across subject groups. MDM from non-smokers (■, n = 7) and COPD (■, n = 9) subjects were differentiated in the presence of GM-CSF for 12 days (A, C and E) or tissue macrophages from non-smokers (■, n = 4), ex-smokers (■, n = 4) and COPD (■, n = 3) subjects were isolated from resected lung tissue (B, D and F). Cells were incubated with *H. influenzae* for up to 60 min. Data displayed showing cell area (A-B), cell perimeter (C-D) and cell eccentricity (E-F). Data presented as mean $\pm$ SEM. Data were analysed using a 2-way ANOVA with Sidak's multiple comparisons test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

5.3.1.2 Comparison of macrophage morphology between COPD and controls using pooled subject groups

From the data above, tissue macrophages from COPD patients are larger than cells from non-smokers. However, image analysis examines single cells and by pooling data from several subjects, the relationship between function and cell size can be investigated in a larger population and can also allow for comparison between disease and control samples.

Using this analysis there was no difference in MDM cell area between subject groups at baseline or following exposure to *H. influenzae* (Figs. 5.2 A), yet the differences observed in tissue macrophages, where COPD cells were larger compared to non-smokers, were present at baseline and was maintained during exposure to *H. influenzae* for up to 60 min (Fig. 5.2 B) This was also observed when analysing perimeter, with no differences seen in MDM (Fig. 5.2 C) but COPD tissue macrophages were larger than non-smokers with or without incubation with bacteria (Fig. 5.2 D).

With this analysis, non-smoker MDM also showed a lower eccentricity, thus displaying a more circular morphology, than cells from COPD subjects (Fig 5.2 E), but this was lost following exposure to bacteria. Tissue macrophages had no significant differences in eccentricity across subject groups (Fig. 5.2 F).

No difference in cell area, perimeter or eccentricity was observed when data was separated by cells that did or did not phagocytose bacteria.

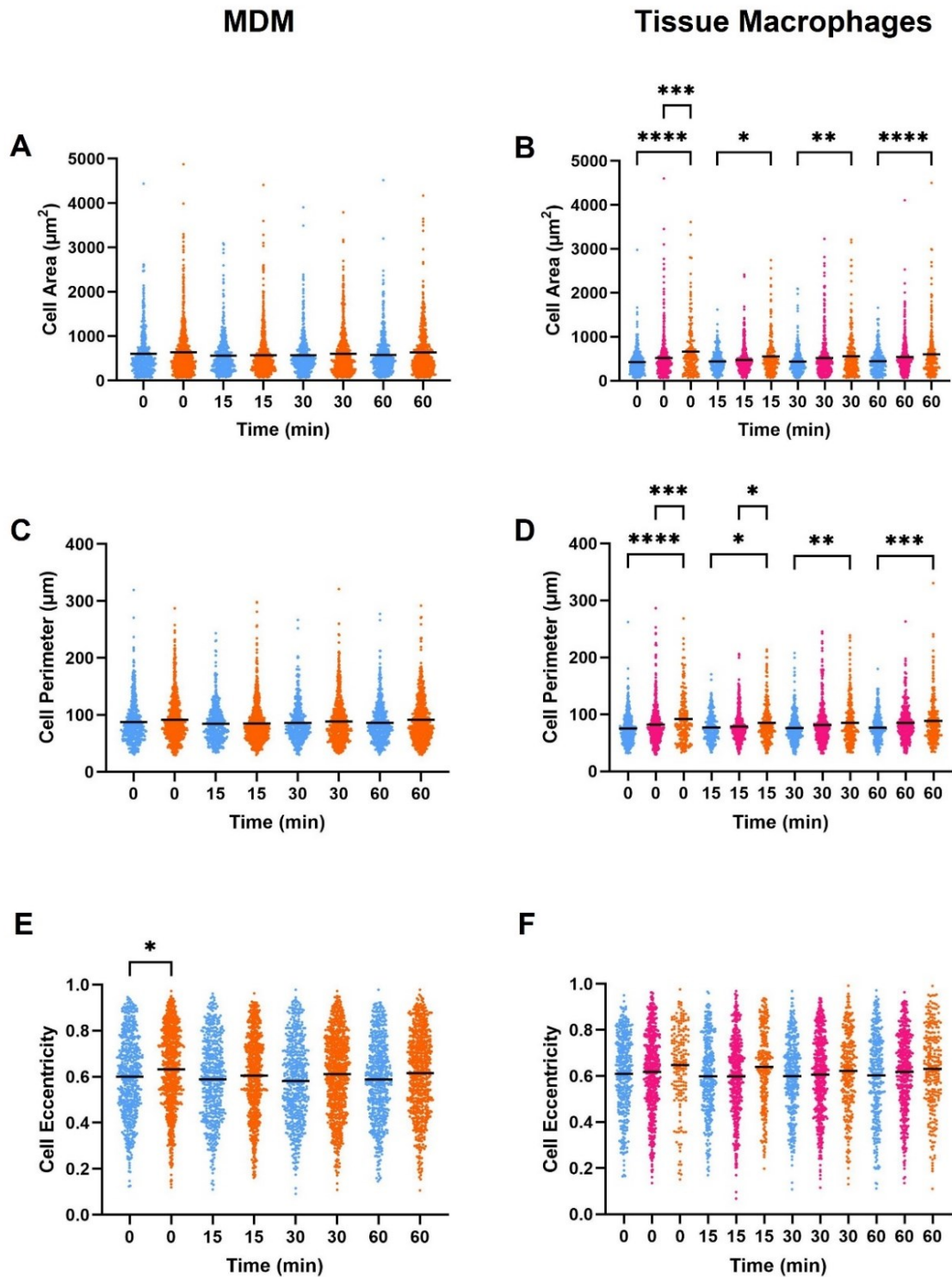


Figure 5.2. Comparison of macrophage cell morphology with subjects pooled into groups. MDM from non-smokers (■, n = 7) and COPD (■, n = 9) subjects were differentiated in the presence of GM-CSF for 12 days (A, C and E) or tissue macrophages from non-smokers (■, n = 4), ex-smokers (■, n = 4) and COPD (■, n = 3) subjects were isolated from resected lung tissue (B, D and F). Cells were incubated with *H. influenzae* for up to 60 min. Data displayed showing cell area (A-B), cell perimeter (C-D) and cell eccentricity (E-F). Data presented as individual points and mean. Data were analysed using a 2-way ANOVA with Sidak's multiple comparisons test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

5.3.2 Relationship of cell morphology and phagocytosis

It is possible that larger cells may phagocytose more bacteria or that cell shape may be an indicator of the capacity of a cell to clear bacteria. To test this, correlations were performed to determine whether phagocytosis by MDM or tissue macrophages, as measured after 60 min exposure to *H. influenzae*, was directly related to cell morphology.

5.3.2.1 Cell area

There was no correlation between cell area of MDM and phagocytosis as measured by the percentage of cells positive for bacteria or for the number of bacteria engulfed for the different subject groups independently or combined (Fig. 5.3 A). Similarly, there was no correlation between cell area and phagocytosis for tissue macrophages when analysing groups independently or together (Fig. 5.3 B).

5.3.2.2 Cell perimeter

As seen for cell area, there were no significant correlations between MDM or tissue macrophage phagocytosis and cell perimeter irrespective of analysing subject groups together or independently (Figs. 5.3 C and D).

5.3.2.3 Cell eccentricity

As seen for other measures of cell morphology, there were no significant correlations between MDM or tissue macrophage phagocytosis and cell shape as measured by eccentricity when analysing subject groups together or independently (Figs. 5.3 E and F).

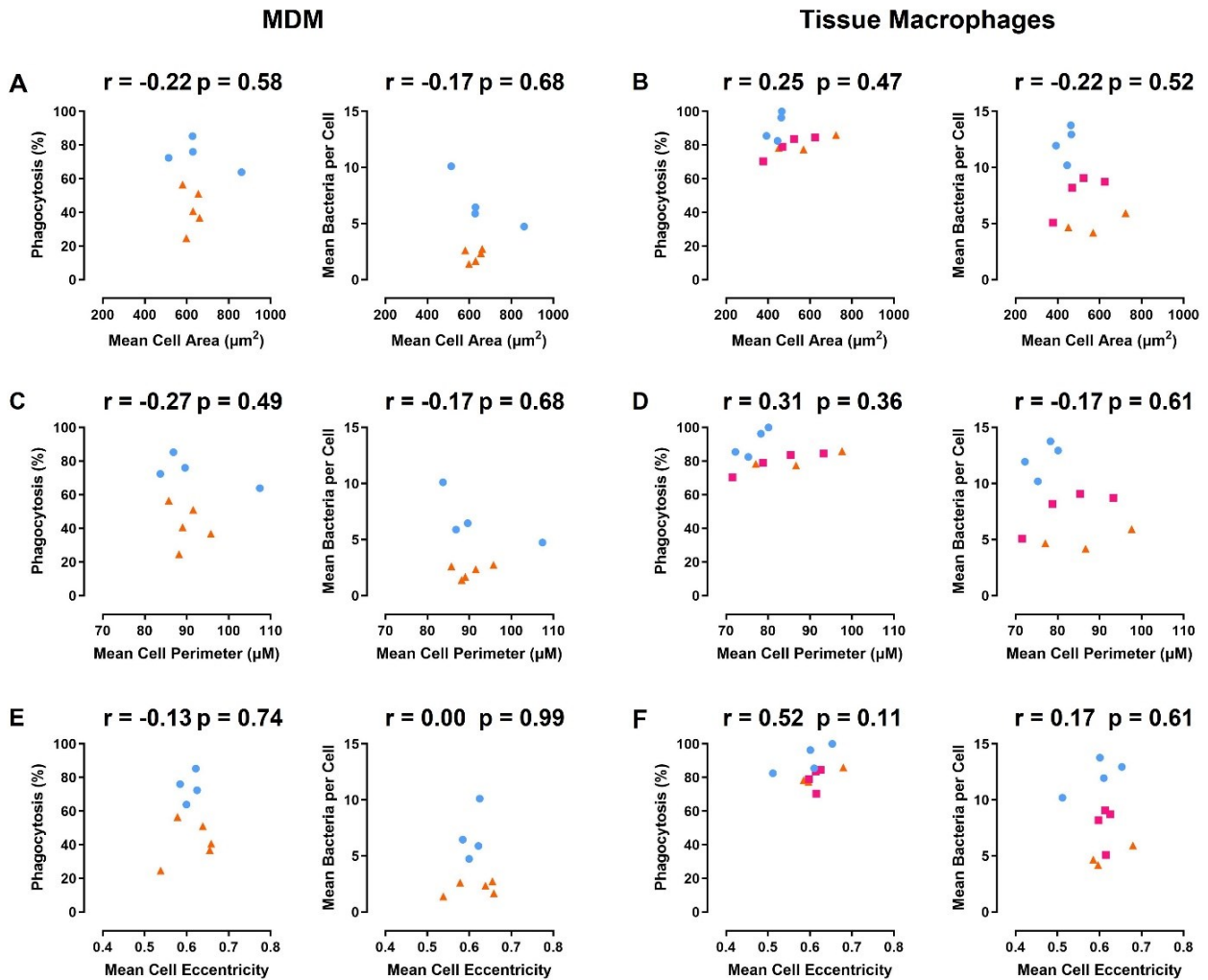


Figure 5.3. Correlations between measurements of cell morphology and phagocytosis of *H. influenzae* by MDM and tissue macrophages. MDM from non-smokers (●, $n = 4$) and COPD (▲, $n = 5$) subjects were differentiated in the presence of GM-CSF for 12 days (A, C and E) or tissue macrophages from non- smokers (●, $n = 4$), ex-smokers (■, $n = 4$) and COPD (▲, $n = 3$) subjects were isolated from resected lung tissue (B, D and F). Cells were incubated with *H. influenzae* for 60 min, with phagocytosis quantified as percentage of cells positive for bacteria and mean bacteria per cell. Data presented as mean, with spearman rank correlation coefficients (r) and associated p value of phagocytosis against mean cell area (A-B), mean cell perimeter (C-D) and mean cell eccentricity (E-F).

5.3.3 Comparison of cell size between MDM and tissue macrophages

Although there did not appear to be a relationship between cell size, shape and phagocytosis, tissue macrophages appear to phagocytose more bacteria than MDM (Fig. 4.5). It is possible that since tissue macrophages are larger than MDM and this larger area could account for increased phagocytosis. Therefore, cell morphology was directly compared between the MDM and tissue macrophages. Tissue macrophages from non-smokers were smaller than MDM at baseline and did not change following exposure to bacteria (Fig. 5.4 A). There was no difference in size between tissue macrophages and MDM from COPD subjects.

When analysing all cells pooled together for each subject group, a similar pattern was observed with tissue macrophages from non-smokers being smaller than MDM at baseline and following exposure to bacteria (Fig. 5.4 B). Therefore, the increase in phagocytosis in tissue macrophages is not due to increased size when compared to MDM.

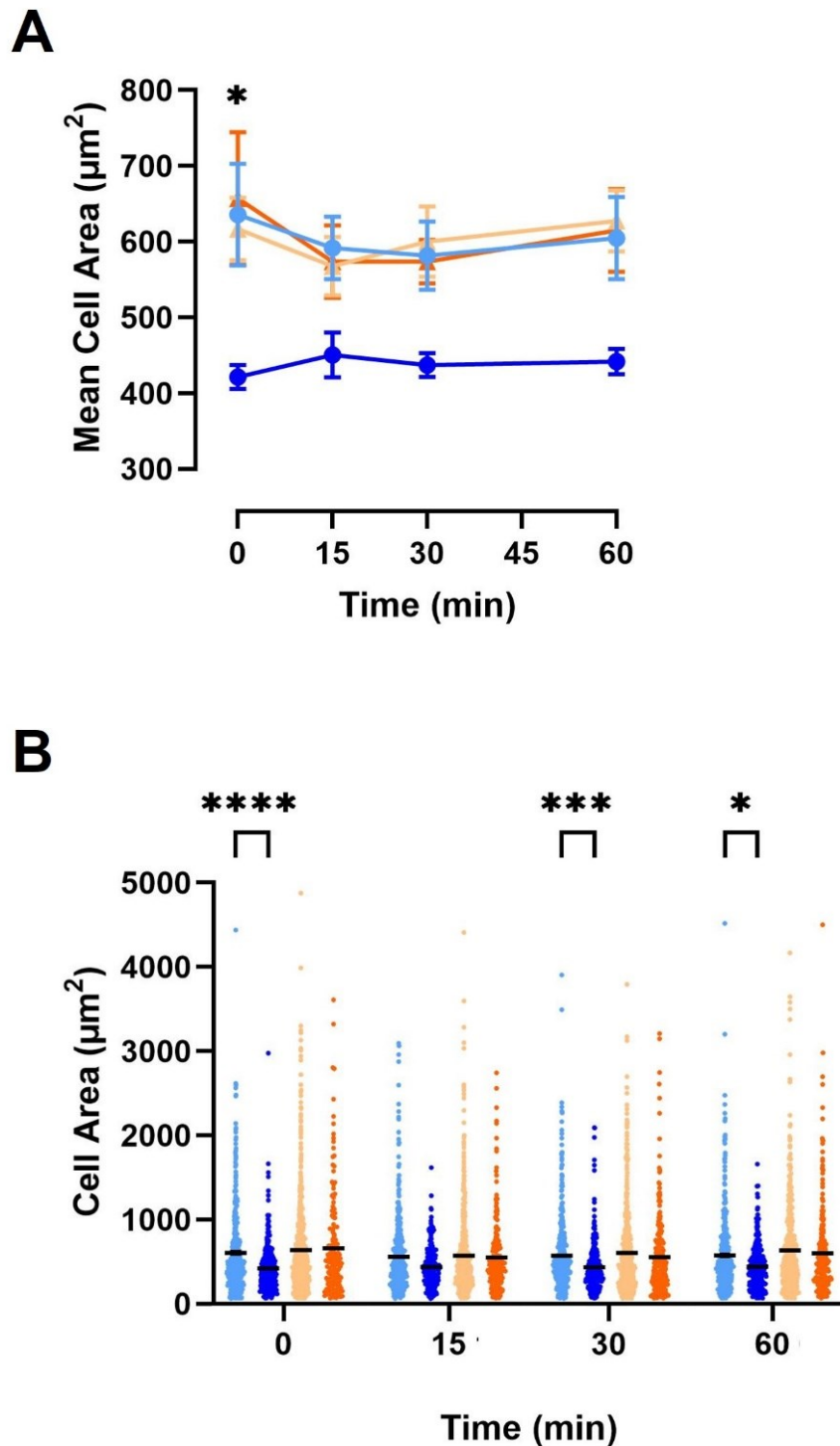


Figure 5.4. Comparison of cell size between MDM and tissue macrophages when undergoing phagocytosis of *H. influenzae* across subject groups. MDM from non-smokers (■, n = 4) and COPD (■, n = 5) were compared to tissue macrophages from non-smoker (■, n = 4) and COPD (■, n = 3) subjects. Cells were incubated with *H. influenzae* for up to 60 min. Phagocytosis was measured with 100 cells per time point analysed for each subject in sample groups. Data presented as mean ± SEM (A) or individual points (B). Data were analysed by Kruskal-Wallis test with Dunn's multiple comparisons test. * p<0.05, *** p<0.001, **** p<0.0001.

5.3.4 Analysis of cytoskeleton proteins in MDM and tissue macrophages by microscopy and image analysis

Dynamic changes in the cytoskeleton are required for phagocytosis to occur. A macrophage must recognise prey, move towards it, engulf it, and transport the particle to lysosomes for destruction. These processes require precise remodelling of the cytoskeletal network and involve changes in the actin microfilaments and the microtubules. Cell imaging allows for concomitant measurements of cytoskeletal components alongside measurements of phagocytosis. Therefore, analyses were performed to examine the changes in the cytoskeleton during phagocytosis and whether these were altered in cells from COPD patients that might account for reduced phagocytosis.

5.3.4.1 Comparison of microtubule network length in both MDM and tissue macrophages from non-smokers, smokers and COPD subjects

Firstly, the microtubule network was analysed in MDM from COPD subjects and controls, by measuring the total length of microtubules within cells, both at baseline and following exposure to *H. influenzae*. Mean microtubule network length was $315 \pm 96 \mu\text{m}$ in non-smokers and $304 \pm 48 \mu\text{m}$ in COPD, with no significant difference between these at baseline. Incubation with *H. influenzae* for up to 60 min did not significantly alter total microtubule length in MDM from either subject group (Fig. 5.5 A).

In contrast, COPD tissue macrophages contained a larger microtubule network, $368 \pm 80 \mu\text{m}$, compared with cells from non-smokers at baseline, $125 \pm 25 \mu\text{m}$ (Fig 5.5 B). Additionally, COPD macrophages displayed an increase in size of the microtubule network at 30 min (NS: $137 \pm 24 \mu\text{m}$, COPD: $258 \pm 23 \mu\text{m}$) and 60 min (NS: $157 \pm 27 \mu\text{m}$, COPD: $284 \pm 25 \mu\text{m}$) following

incubation with *H. influenzae* compared to non-smokers, and an increase in length at all timepoints when compared to smokers.

Further analysis was performed on the dataset where single macrophages from each subject were pooled. Using this analysis there was a significant difference in microtubule length between non-smoker and COPD MDM at baseline, with COPD MDM displaying a larger microtubule network when measuring total length of microtubules. This increase in the COPD microtubule network was sustained following exposure to bacteria for 30 min and 60 min (Fig. 5.5 C). A similar pattern could be seen in the pooled tissue macrophages, where again COPD macrophages displayed a significantly larger microtubule network, when assessing total length at baseline, which was sustained following exposure to bacteria when compared to macrophages from both non-smokers and ex-smokers (Fig. 5.5 D).

5.3.4.2 Comparison of microtubule network within subject groups

In the pooled data set, when analysing total microtubule network length within each subject group, there were no differences in non-smoker MDM when incubated with *H. influenzae* for up to 60 min (Fig. 5.5 E). However, in contrast when COPD MDM were incubated with bacteria, there was a significant decrease in tubulin fibre length after 15 min incubation, which then returned to baseline over the course of the 60 min time course (Fig. 5.5 E).

This was also observed in tissue macrophages, with no difference in tubulin network length observed in non-smokers, but COPD had a decrease after 15 min followed by a normalisation back to baseline by 60 min (Fig. 5.5 F). This was also observed to a lesser extent in ex-smoker tissue macrophages and is suggestive of microtubule remodelling during phagocytosis.

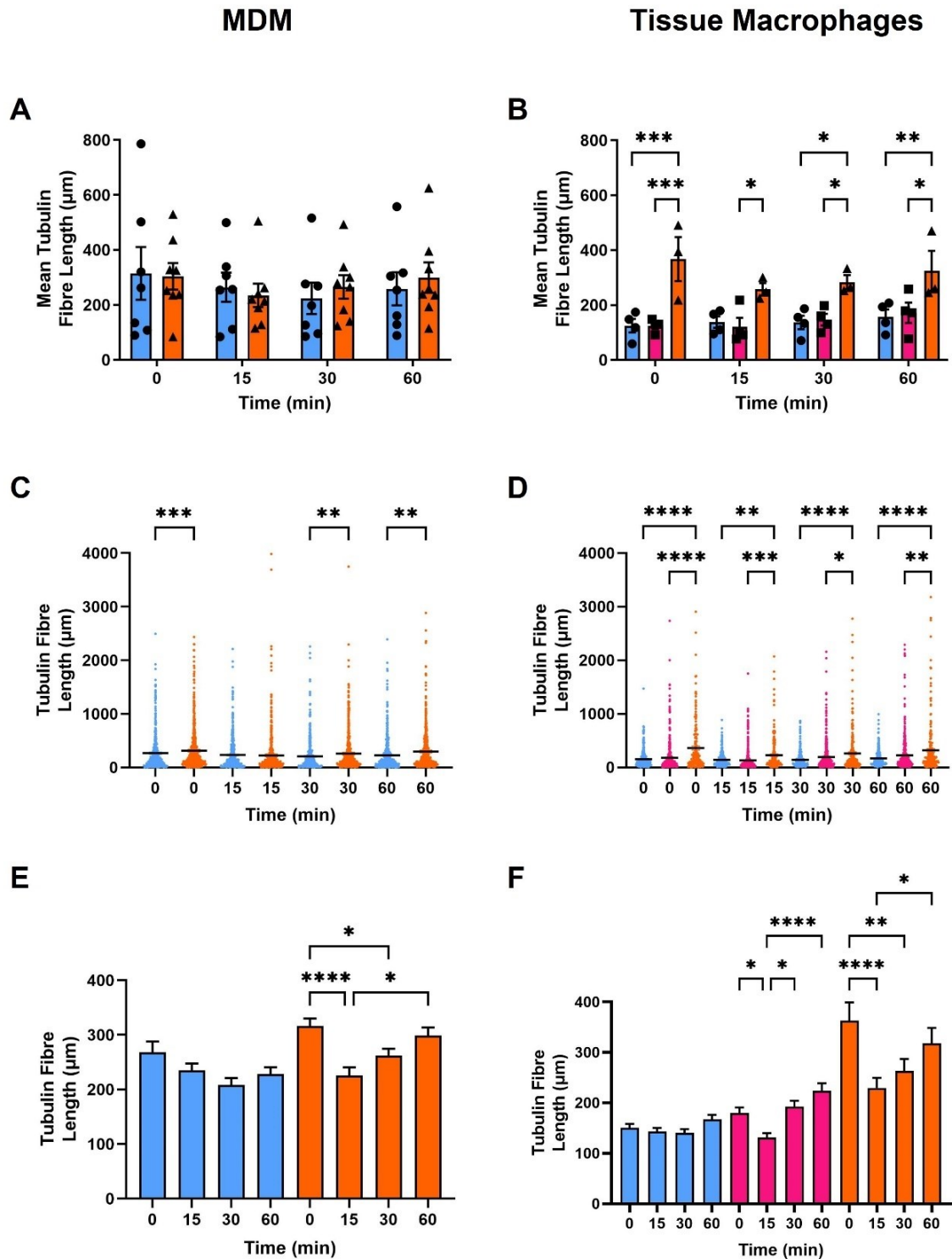


Figure 5.5. Comparison of microtubule network length across subject groups. MDM from non-smokers (■, n = 7) and COPD (■, n = 9) subjects were differentiated in the presence of GM-CSF for 12 days (A, C, E and G) or tissue macrophages from non-smokers (■, n = 4), ex-smokers (■, n = 4) and COPD (■, n = 3) subjects were isolated from resected lung tissue (B, D, F and H). Cells were incubated with *H. influenzae* for up to 60 min and stained for α-tubulin. 100 cells per time point were analysed for each subject in sample groups. Data presented as mean ± SEM (A-B, E-F) or individual points (C-D). Data were analysed using a Kruskal Wallis test with Dunn's multiple comparisons test. * p<0.05, ** p<0.01 *** p<0.001, **** p<0.0001

5.3.4.3 Comparison of tubulin branch-point density in MDM and tissue macrophages from non-smokers, smokers and COPD subjects

As there are differences in total microtubule length between non-smoker and COPD macrophages, further analysis was performed to investigate the nature of microtubule network. Tubulin fibre branch-point density was quantified as the number of points where microtubules intersect within the cell. However, there were no significant differences in tubulin fibre branch-point density between subject groups at baseline or at any timepoint following incubation with *H. influenzae* for MDM (Fig. 5.6 A) or tissue macrophages (Fig. 5.6 B).

Pooled single cell analysis suggested there was a significant difference in microtubule branch-point density, with COPD MDM showing increased branch-point density at baseline, and after 30 min exposure to bacteria (Fig. 5.6 C). In tissue macrophages, there were no differences in branch points between non-smokers and COPD subjects at any timepoints, however ex-smokers showed reduced branch-point density compared to COPD at baseline, as well as compared to non-smokers and COPD at 15-min incubation with *H. influenzae* (Fig. 5.6 D).

5.3.4.4 Comparison of microtubule branching within subject groups

In the pooled data set, when analysing tubulin branch-point density within each subject group, there was a reduction in branch density in non-smokers MDM when incubated with *H. influenzae* for 30 min compared to baseline (Fig. 5.6 E), with no differences observed in non-smoker tissue macrophages. Both COPD MDM and tissue macrophages (Fig. 5.6 F) showed a decrease in branch point density when incubated with bacteria for up to 30 min, which returned to baseline by the 60 min incubation.

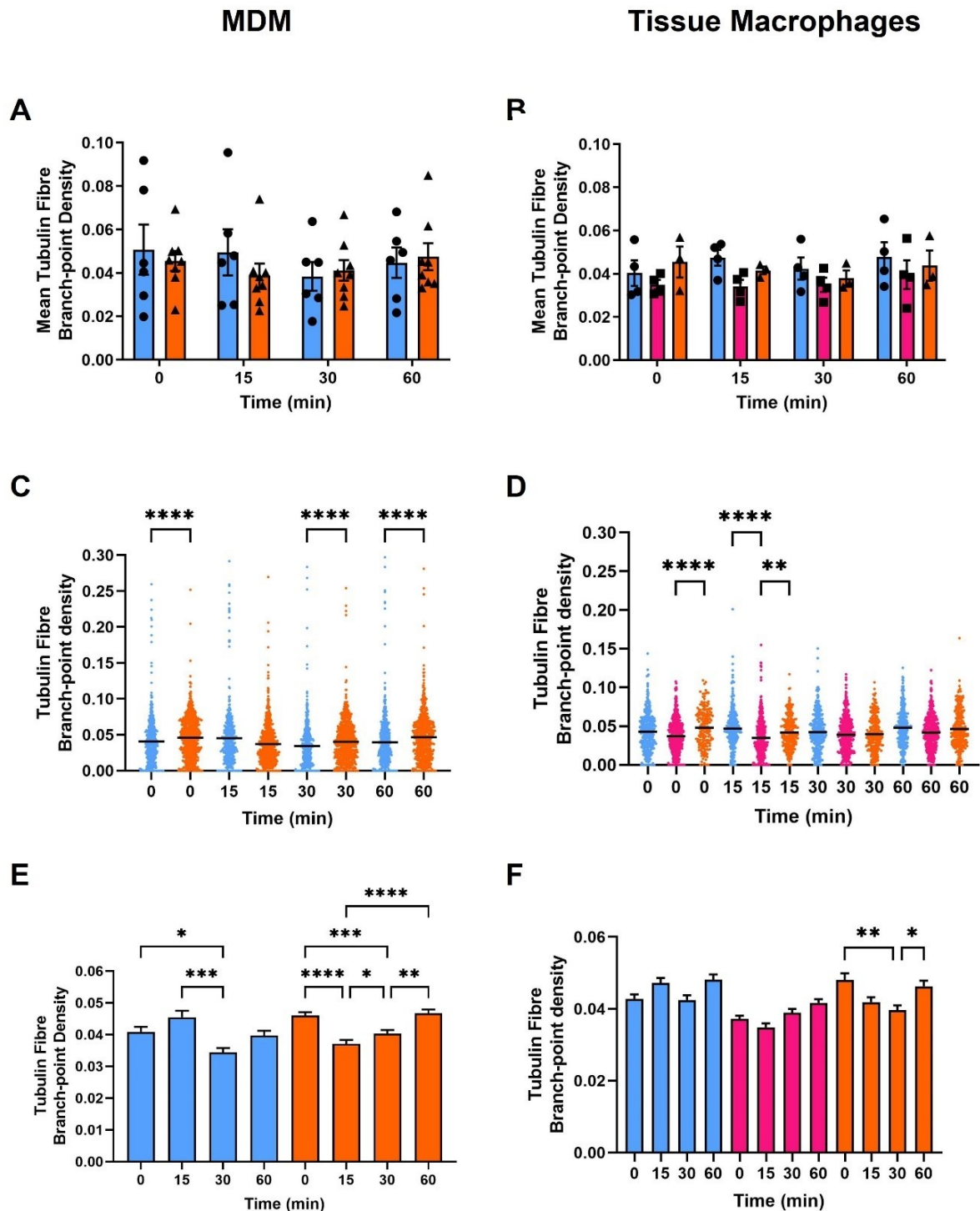


Figure 5.6. Comparison of tubulin branching across subject groups. MDM from non-smokers (■, n = 7) and COPD (■, n = 9) subjects were differentiated in the presence of GM-CSF for 12 days (A, C, E and G) or tissue macrophages from non-smokers (■, n = 4), ex-smokers (■, n = 4) and COPD (■, n = 3) subjects were isolated from resected lung tissue (B, D, F and H). Cells were incubated with *H. influenzae* for up to 60 min and stained for α -tubulin. 100 cells per time point were analysed for each subject in sample groups. Data presented as mean $\pm$ SEM (A-B, E-F) or individual points (C-D). Data were analysed using a Kruskal Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$

5.3.4.5 Comparison of actin punctate structure density in MDM and tissue macrophages from non-smokers, smokers and COPD subjects

Having demonstrated differences in the microtubular network in COPD macrophages, it was important to understand whether similar changes also occurred within the actin network. The punctate actin structures (Fig. 4.1 and 4.3) were investigated to determine if there were any differences between COPD and controls in both MDM and tissue macrophages.

In MDM, there were no differences in mean punctate density of actin between non-smoker and COPD at baseline. However, following 15 min incubation with *H. influenzae*, COPD MDM displayed reduced punctate density compared with controls, but this difference was not sustained at longer time points (Fig. 5.7 A). This contrasted with tissue macrophages where no differences were observed at baseline or with incubation with bacteria (Fig. 5.7 B).

When analysis was performed with all cells pooled into their respective subject groups, there was no difference in punctate density at baseline between COPD and non-smokers in both MDM (Fig. 5.7 C) or tissue macrophages (Fig. 5.7 D). COPD MDM had reduced punctate density at 15-, 30- and 60-min time points when incubated with *H. influenzae* when compared to non-smokers (Fig. 5.7 C). This was also the case for COPD tissue macrophages, which displayed a reduction in punctate density compared to non-smokers and ex-smokers at all timepoints incubated with *H. influenzae* (Fig. 5.7 D).

5.3.4.6 Comparison of punctate density within subject groups

In the pooled data set, when analysing punctate density within each subject group, in non-smokers MDM there was an increase in punctate structures after 30- and 60-min incubation

with *H. influenzae*, whereas COPD MDM showed a reduction in punctate structures after a 15 min incubation (Fig. 5.7 E). The same pattern was seen in tissue macrophages, with non-smokers displaying increase punctate structures upon incubation with bacteria, and ex-smokers and COPD both showing a reduction after 15 min (Fig. 5.7 F).

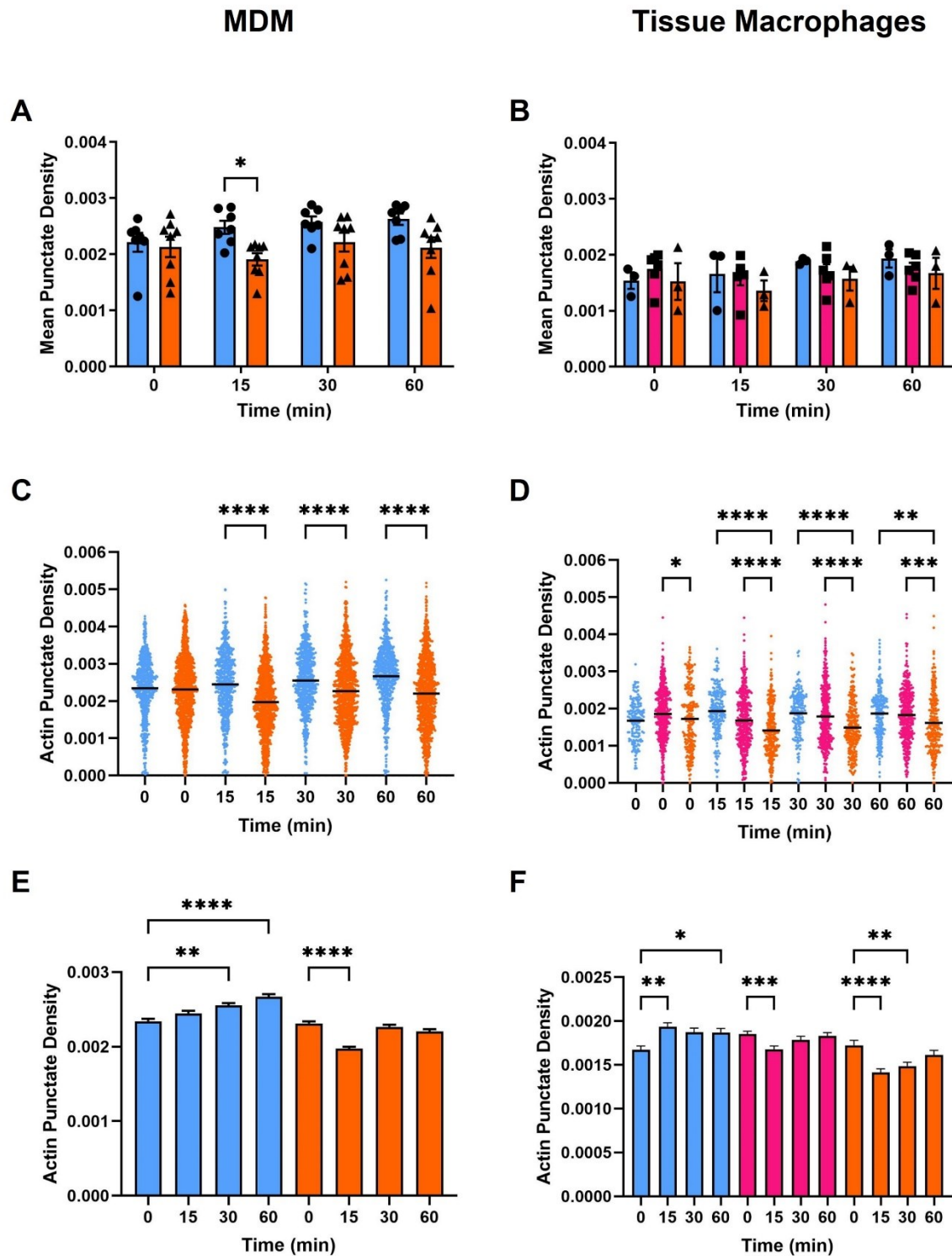


Figure 5.7. Comparison of actin punctate density across subject groups. MDM from non-smokers (■, n = 7) and COPD (■, n = 9) subjects were differentiated in the presence of GM-CSF for 12 days (A, C, E and G) or tissue macrophages from non-smokers (■, n = 4), ex-smokers (■, n = 4) and COPD (■, n = 3) subjects were isolated from resected lung tissue (B, D, F and H). Cells were incubated with *H. influenzae* for up to 60 min and stained for F-actin. 100 cells per time point were analysed for each subject, with data presented as mean $\pm$ SEM (A-B, E-F) or individual points (C-D). Data analysed using Kruskal Wallis test with Dunn's multiple comparisons. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, **** $p < 0.0001$.

5.3.4.7 Comparison of actin punctate distance in MDM and tissue macrophages from non-smokers, smokers and COPD subjects

As there was a difference in punctate density between non-smoker and COPD macrophages, further analysis was performed to confirm the reduction observed in COPD. The distance between punctate structures was investigated, as this can give further insight into the distribution of punctate structures within the cell area. The distance between punctate structures was similar in MDM at baseline, but significantly increased in COPD MDM compared to non-smoker following exposure to bacteria for 15 and 60 min (Fig. 5.8 A), with no differences observed in tissue macrophages (Fig. 5.8 B).

When analysis was performed with all cells pooled into their respective subject groups, there was no difference in distance between punctate structures between non-smoker and COPD MDM at baseline, however upon incubation with *H. influenzae*, COPD MDM showed increased punctate distance at 15-, 30- and 60-min time points when compared to non-smokers (Fig. 5.8 C). Analysis for tissue macrophages also showed COPD cells had significantly increased punctate distance compared to non-smokers when incubated with bacteria for 15- and 30-mins but had normalised by 60 min (Fig. 5.8 D).

5.3.4.8 Comparison of actin punctate distance within subject groups

In the pooled data set, when analysing punctate distance within each subject group, there was no differences in non-smokers or COPD MDM when incubated with *H. influenzae* for up to 60 min (Fig. 5.8 E). However, in non-smoker tissue macrophages there was a decrease in punctate distance after 15 min incubation with bacteria, whereas in contrast both ex-smoker and COPD tissue macrophages displayed increased distance between punctate structures after 15 min incubation with bacteria (Fig. 5.8 F).

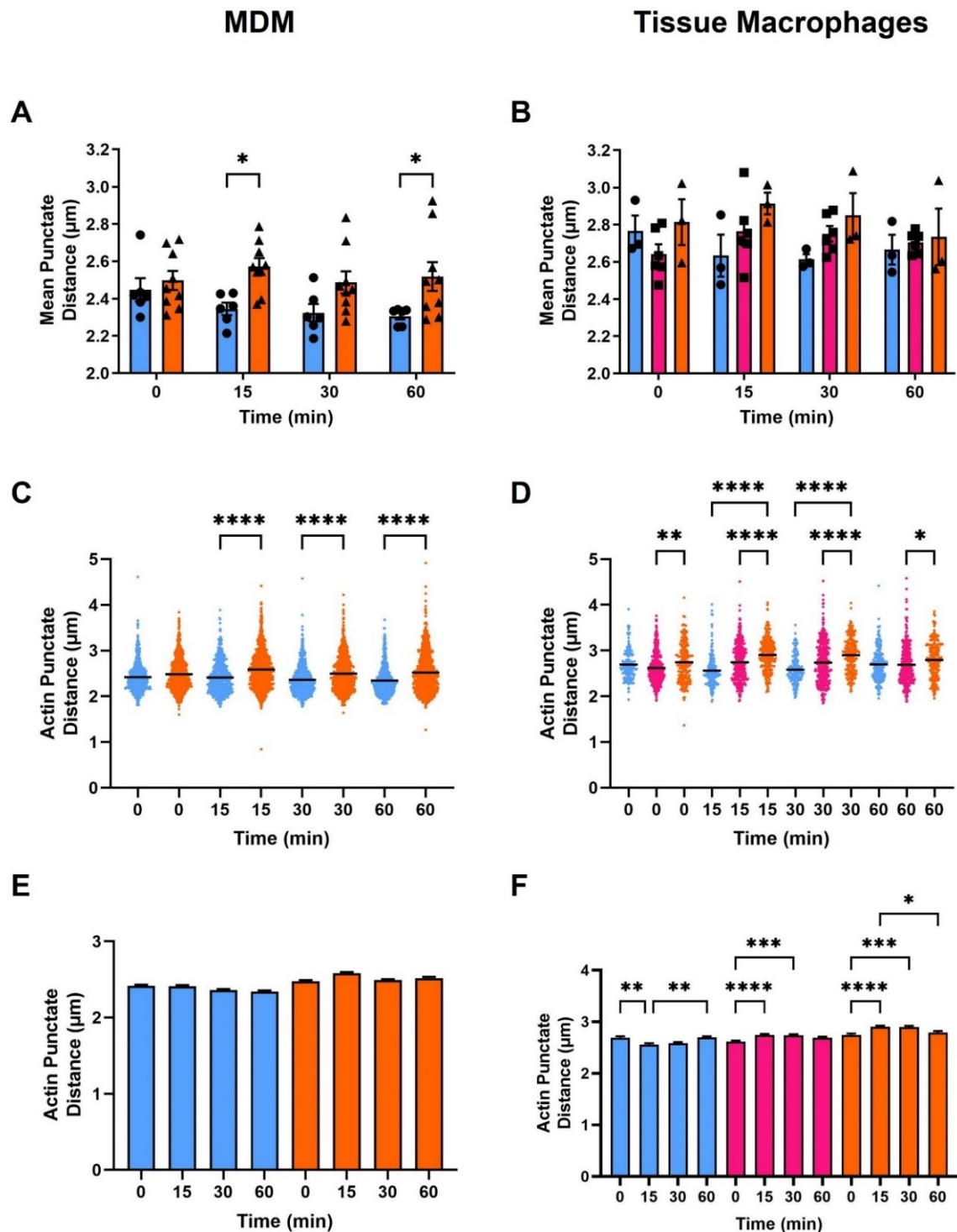


Figure 5.8. Comparison of actin punctate distance across subject groups. MDM from non-smokers (■, n = 7) and COPD (■, n = 9) subjects were differentiated in the presence of GM-CSF for 12 days (A, C, E and G) or tissue macrophages from non-smokers (■, n = 4), ex-smokers (■, n = 4) and COPD (■, n = 3) subjects were isolated from resected lung tissue (B, D, F and H). Cells were incubated with *H. influenzae* for up to 60 min and stained for F-actin. 100 cells per time point were analysed for each subject, with data presented as mean $\pm$ SEM (A-B, E-F) or individual points (C-D). Data analysed using Kruskal Wallis test with Dunn's multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

5.3.5 Relationship of cytoskeleton proteins and phagocytosis

To determine if MDM or tissue macrophage phagocytosis was directly related to the cytoskeleton, correlations were calculated between phagocytosis and cytoskeletal properties.

5.3.5.1 Microtubule network length

There were no correlations between tubulin fibre length and phagocytosis of MDM from non-smoker or COPD subjects either independently or together, for both percentage of MDM positive for *H. influenzae* (phagocytosis %) or the mean number of *H. influenzae* internalised by MDM (Fig. 5.9 A). Similarly, there were no correlations for tissue macrophages and these (Fig. 5.9 B).

5.3.5.2 Microtubule branch-point density

There were no correlations between tubulin fibre branch-point density and phagocytosis of MDM from non-smokers or COPD subjects either independently or together for both percentage of MDM positive for *H. influenzae* (phagocytosis %) or the mean number of *H. influenzae* internalised by MDM (Fig. 5.9 C).

When analysing for tissue macrophages, there was no significant correlations when analysing groups independently, however when analysing the groups together there was a significant positive correlation ($r = 0.7$, $p = 0.02$) for phagocytosis as a percentage of macrophages positive for *H. influenzae* (phagocytosis %) (Fig. 5.9 D).

5.3.5.3 Actin punctate density

There were no correlations between actin punctate density and phagocytosis of MDM from non-smoker or COPD subjects either independently or together, for both percentage of MDM positive for *H. influenzae* (phagocytosis %) or the mean number of *H. influenzae* internalised by MDM (Fig. 5.9 E).

When analysing for tissue macrophages there was no significant correlations when analysing groups independently, however when analysing the groups together there was a significant positive correlation ($r = 0.65$, $p = 0.05$) for phagocytosis as a percentage of macrophages positive for *H. influenzae* (phagocytosis %) (Fig. 5.9 F).

5.3.5.4 Actin punctate distance

There were no correlations between actin punctate distance and phagocytosis of MDM from non-smoker or COPD subjects either independently or together, for both percentage of MDM positive for *H. influenzae* (phagocytosis %) or the mean number of *H. influenzae* internalised by MDM (Fig. 5.9 G). Similarly, there were no correlations for tissue macrophages and these (Fig. 5.9 H).

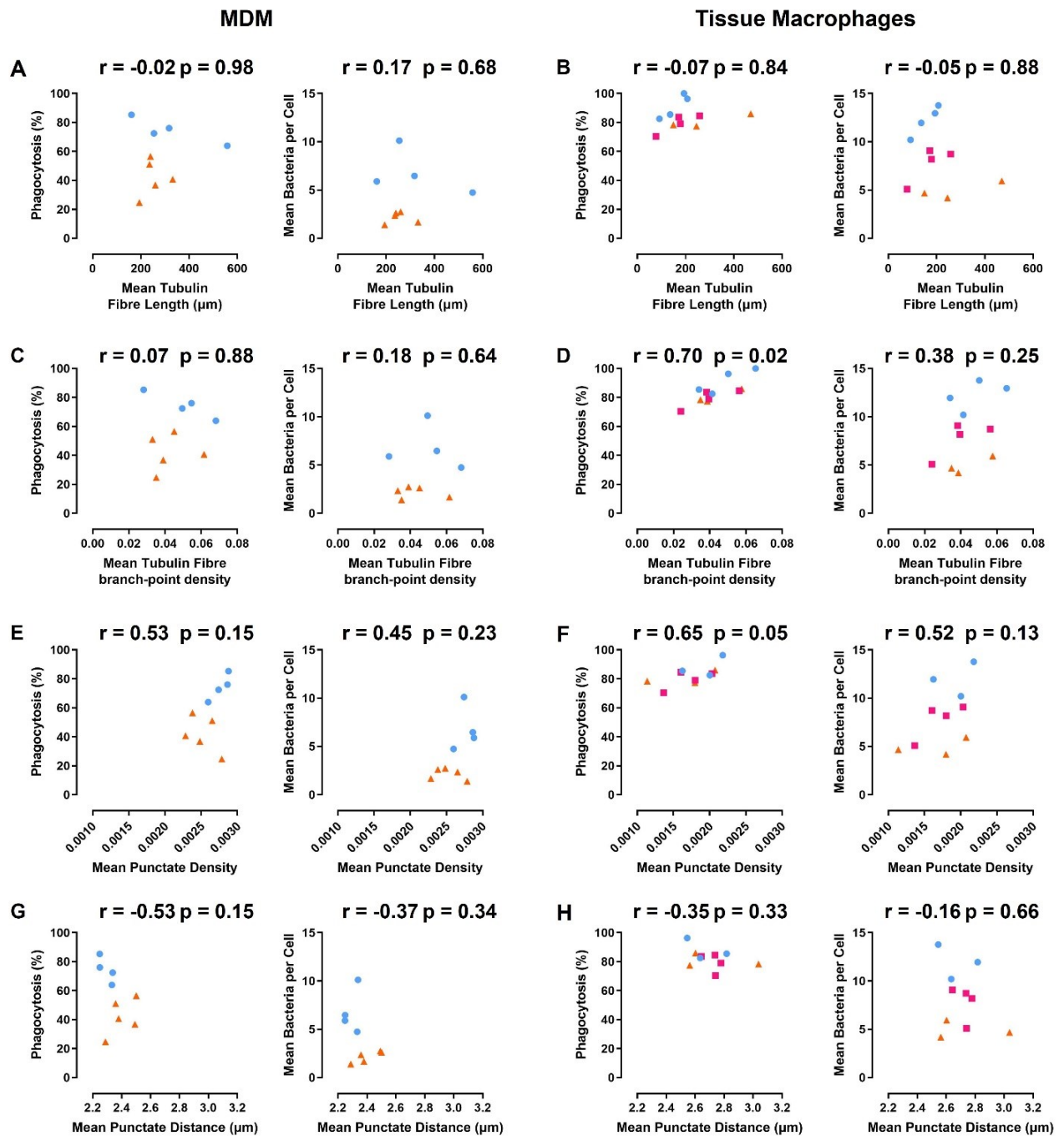


Figure 5.9. Correlation between cytoskeletal properties and measurements of phagocytosis of *H. influenzae* by MDM and tissue macrophages. MDM from non-smokers (●, $n = 4$) and COPD (▲, $n = 5$) subjects were differentiated in the presence of GM-CSF for 12 days (A, C, E and G) or tissue macrophages from non- smokers (●, $n = 4$), ex-smokers (■, $n = 4$) and COPD (▲, $n = 3$) subjects were isolated from resected lung tissue (B, D, F and H). Cells were incubated with *H. influenzae* for 60 min, with phagocytosis quantified as percentage of cells positive for bacteria and mean bacteria per cell. Data presented as Spearman rank correlation coefficients (r) and associated p value of phagocytosis against mean tubulin fibre length (A-B), mean tubulin branch-point density (C-D), mean actin punctate density (E-F) and mean punctate distance (G-H).

5.4 Discussion

This chapter focuses on morphometry of macrophages between at baseline and undergoing phagocytosis. Single cell analysis was performed on MDM and tissue macrophages from non-smokers, smokers and COPD subjects and, examined measures of cell morphology including cell size as determined by the area and perimeter, as well as shape factors like roundness, as measured by eccentricity. The cytoskeleton was also investigated, looking specifically at aspects of tubulin and actin morphology, including total fibre length and branching density of the microtubule network, and density and distance of the punctate structures observed in macrophages stained for actin.

There were no significant morphological differences between non-smoker and COPD MDM, but this contrasted with tissue macrophages, where COPD macrophages were significantly larger in both area and perimeter when compared to non-smoker cells. Differences observed in the morphology of MDM and tissue macrophages between non-smokers and COPD maybe due to potential differences in the lineages or population subsets from the lung or circulating blood. Another reason why COPD tissue macrophages are larger compared to healthy cells, could be due to uptake of particles from cigarette smoke or other airborne pollutants that contribute to oxidative stress, mitochondrial dysfunction and reduced phagocytosis associated with the disease (Bhargava et al., 2018; Tejwani et al., 2021; Xia, Kovochich & Nel, 2007).

When analysing the morphology of MDM and tissue macrophages from non-smoker and COPD subjects together, it revealed that non-smoker tissue macrophages were significantly smaller when compared to all other groups and that there was no size difference between non-smoker MDM, COPD MDM and COPD tissue macrophages. This highlights differences

between the two macrophage models, which can only be identified when experiments have been performed using both models and are directly compared. Non-smoker tissue macrophages were smaller compared to non-smoker MDM, and this could reflect that they come from different endogenous pools. Whereas COPD tissue macrophages being of similar size to their respective MDM, which could reflect the population of macrophages found within the lungs of COPD patients are predominately from a circulatory monocyte-derived population instead of a tissue resident population. This might partially explain the differences in phagocytic capabilities between subject groups, as tissue macrophages phagocytosed more bacteria compared to MDM in Chapter 4. Since COPD tissue macrophages morphologically resemble MDM, this could indicate an increased blood-derived population over tissue resident macrophages, and this more immature population could result in the reduced phagocytosis when compared to non-smoker tissue macrophages.

Macrophages display high plasticity, with the ability to alter between pro- and anti-inflammatory phenotypes based on their microenvironment (Locati, Curtale & Mantovani, 2020). As such, with macrophages located in every tissue, macrophage populations are heterogeneous, with the largest examples found in the liver and lungs (Laskin, Weinberger & Laskin, 2001). Different subpopulations of macrophages based on size, assessed by flow cytometry, can be identified from COPD patients, with alveolar macrophages having a greater population of 'large' macrophages when compared to interstitial macrophages (Dewhurst et al., 2017). This contrasts with other studies that showed that COPD macrophages were smaller when compared to cells from non-smokers (Frankenberger et al., 2004). However, this was a study examining CD14⁺ macrophages from induced sputum and assessing forward scatter by flow cytometry (Frankenberger et al., 2004). Work shown in this chapter has quantitative evidence using microscopy and cell size measurements that COPD tissue

macrophages display a larger morphology in both area and perimeter compared to non-smokers at baseline, with this difference in size being partially maintained during incubation with *H. influenzae*. These differences could be due to the methodologies used for the measurement of cell size. This thesis has used microscopy compared to the measurement of FSC in flow cytometry used by Frankenberger *et al.* It is possible that macrophages on a microscopy slide are 'flatter' or more spread out when compared to cells in suspension for flow cytometry. The use of FSC in flow cytometry is often used to determine the relative cell size (Picot et al., 2012) and is used for doublet discrimination and selection of relevant populations. However the use of FSC is not well-defined for analysis of cell morphology, as differences in the refractive index, viability and membrane permeability of the cell all can affect the FSC parameter (Givan, 2011), and therefore interpretation of data when being used as an absolute for cell size. Another explanation between the discrepancy in macrophage size between these studies is that different compartments are being sampled, with macrophages being isolated from lung tissue being different to macrophages from the airways isolated by sputum.

Upon incubation with bacteria for 15 min, the size difference between non-smoker and COPD macrophages is lost, showing that COPD macrophages alter in size upon exposure to bacteria. They then return to baseline at 60 min following incubation with bacteria where the size difference in macrophages between subjects is restored. This shows a discrete change in cell size during the process of phagocytosis in COPD tissue macrophages and may be indicative of a difference in cytoskeletal remodelling in COPD macrophages. However, this is not seen in MDM that also show the phagocytic defect therefore is unlikely to contribute to the phagocytic dysfunction seen in COPD macrophages irrespective of origin.

Some studies investigating cell eccentricity have shown that cell shape can have effects on activation of growth factor pathways through induction of mitogen-activated protein kinase 1 (MAPK1) (Rangamani et al., 2013) and effect local responses to stimuli (Haftbaradaran Esfahani & Knöll, 2020). Importantly, the cell eccentricity is shown to have an impact on the microtubule network, including polymerisation and depolymerisation of microtubules (Gomez et al., 2016; Płochocka et al., 2021).

More detailed analysis of the cytoskeletal network showed that COPD tissue macrophages had a significantly larger microtubule network compared to cells from non-smokers and ex-smokers, when assessing the total microtubule length within each cell, which has not been reported before in the literature. This may be associated with the larger cell area of COPD tissue macrophages. Single cell analysis also showed differences in total microtubule length between non-smokers and COPD for both MDM and tissue macrophages, with again COPD cells having a larger total tubulin fibre network when compared to non-smokers. As there was no difference in cell size between non-smoker and COPD MDM, the differences observed in the total microtubule length cannot be attributed to a difference in cell size. This would suggest that COPD macrophages found in the lung or blood-derived have an alteration in tubulin, or a dysfunction in their ability to rearrange tubulin via depolymerisation. Previous work has shown that COPD MDM have reduced acetylated tubulin, likely leading to an instable microtubule network, and that treatment with a microtubule stabiliser resulted in increased bacterial phagocytosis of *H. influenzae* (Thomas, 2013)

The imaging performed in this study focused on live cells with an emphasis on fast imaging, where two-dimensional (2D) measurements were acquired and analysed by looking at a single focal plane. Therefore, there is the caveat that cell height and three-dimensional (3D) cell

volume were not measured in this model, as the acquisition time of 3D z-stacks is significantly increased compared to 2D snapshots shown in this chapter. Acquisition of 3D cell volumes has advantages of gaining increased spatial information regarding the cell, however the spatial resolution and imaging quality can be greatly affected by factors such as cell movement and thermal drift, due to the cells being live and the increased acquisition time.

Actin imaged in both MDM and tissue macrophages showed many punctate structures, likely to be podosomes, which have a predominant actin structure in primary macrophages and play a role in cell mobility and macrophage migration (Linder et al., 2000). Podosomes in macrophages contain actin regulatory proteins including WASP and the Arp2/3 complex, and genetic defects seen in Wiskott-Aldrich syndrome include reduced chemotaxis of macrophages (Linder et al., 1999). Within this study, the podosome number in MDM and tissue macrophages was reduced in COPD, as seen been the significant decrease in actin punctate density and increased distance between punctate structures, when compared to non-smoker macrophages. This observation would suggest a dysregulation in actin structure in COPD macrophages, with a reduction in podosomes resulting in a compromised immune response to pathogens or migration.

Overall, the morphology of the cytoskeleton network in both COPD MDM and tissue macrophages are consistently different compared to non-smoker cells. However, this analysis does not explain if these differences result in the reduced phagocytosis observed, nor does this explain if the cytoskeleton is an underlying problem in COPD or a downstream effect caused by defective mitochondrial function.

Chapter 6: Comparison of macrophage mitochondrial morphology and function between non-smoker and COPD subjects

This chapter outlines the use of fluorescence techniques applied to study mitochondrial function in macrophages and compare between subject groups in relation to phagocytosis.

6.1 Introduction

The data presented in the previous chapters has demonstrated dysfunctional macrophages from patients with COPD with respect to phagocytic capacity. There were also some changes in cytoskeletal indices that were associated with this defect. Phagocytosis is an active process requiring ATP to drive cytoskeletal rearrangement to regulate particle engulfment and subsequent destruction inside the macrophage (Kuiper et al., 2008), as such the mitochondria are of interest, with defects in these being implicated in a number of lung diseases including asthma and COPD (Ogger & Byrne, 2021). There are key mitochondrial differences in COPD macrophages, including reduced mitochondrial membrane potential (Belchamber et al., 2019), increased mROS at baseline with no increase when incubated with bacteria, which has been linked to the impaired bacterial phagocytosis (Bewley et al., 2017).

Mitochondrial dysfunction observed in COPD can be attributed, at least in part, to cigarette smoke exposure, with lymphocytes presenting with increased levels of mROS and expression of pro-inflammatory cytokines, as well as displaying a depolarised mitochondrial membrane, oxidative DNA damage and activation of the PI3K pathway (Bhargava et al., 2018). Defective mitochondrial function may also be due to impaired mitophagy, reflecting the impaired autophagy associated with cellular senescence in COPD cells (Barnes, Baker & Donnelly, 2022). COPD alveolar macrophages have lower rates of mitochondrial respiration with a

reduced compensatory capacity mechanism through glycolysis when compared to smokers without COPD (O'Beirne et al., 2020), meaning mitochondrial dysfunction is not purely a smoking related pathology.

Alterations in mitochondria can be hypothesised as the cause or a contributing factor for the reduction in phagocytic capabilities observed in COPD macrophages. Reduced mitochondrial activity could result in a reduction of ATP production within these macrophages, which may account for reduced phagocytosis as the macrophages do not have the required amount of energy to be motile, detect and engulf pathogens. Additionally, a reduction in ATP production in COPD macrophages could produce a dysregulation of cytoskeletal proteins, such as tubulin and actin.

Due to the known mitochondrial dysfunction in COPD, mitochondrial antioxidants have been explored as potential therapeutics as they scavenge mROS and normalise mitochondrial membrane potential (Wiegman et al., 2015). There are a number of mitochondrial antioxidants, two potent forms include SkQ1 (Visomitin) and pyrroloquinoline quinone (PQQ), however their use has not been explored in COPD macrophages to improve defective phagocytosis.

SkQ1 is a mitochondrial antioxidant which consists of decyl-triphenylphosphonium cation (TPP+) conjugated with plastoquinone (Agapova et al., 2008; Titova et al., 2018). It is cell permeable, with high penetration and accumulation in the mitochondrial membrane to exhibit potent antioxidant effects (Antonenko et al., 2008; Bazhin et al., 2016). SkQ1 reduces oxidative stress, improves wound healing (Demyanenko et al., 2015), and reduces inflammation in bone marrow-derived macrophages (Friedman et al., 2018) and endothelial cells (Zakharova et al., 2017). PQQ is a member of the quinoprotein dehydrogenases with

potent antioxidant ability to scavenge free radicals (Anthony, 2001; Jonscher, Chohanadisai & Rucker, 2021). Use in dietary supplements have shown PQQ to have effects at reducing damage by oxidative stress in liver disease, inflammation and neurologic conditions (Jonscher & Rucker, 2019; Stites, Mitchell & Rucker, 2000). Additionally, a mitochondrial antioxidant (mitoQ) has been shown to reduce oxidative stress in airway smooth muscle of mice exposed to ozone, resulting in a repolarisation of the mitochondria membrane and a reduction in mROS (Wiegman et al., 2015). Therefore, it was hypothesised that the use of mitochondrial antioxidants might have a positive effect on COPD macrophages, where disruption to the mitochondria could explain the defective phagocytosis, and by regulating mitochondrial function, phagocytosis could potentially be improved.

This chapter investigates mitochondrial function in live macrophages undergoing phagocytosis, using microscopy and image analysis, as such the use of cell permeable dyes were utilised, as shown in previous chapters. MitoTracker is a cationic dye capable of diffusing through the membrane of live cells and due to its positive charge will accumulate in mitochondria, which under healthy conditions are negatively charged or 'polarised'. Dysfunctional mitochondria will display a depolarised phenotype, whereby they are more positively charged and therefore unable to produce ATP through the electron transport chain. The MitoTracker dye functions in a polarisation-dependent manner, and only accumulates in polarised mitochondria (Clutton et al., 2019; Kholmukhamedov, Schwartz & Lemasters, 2013). As a reduction in mitochondrial membrane potential or depolarisation is a sign of inactive mitochondria or apoptotic cells, the dye fails to be sequestered in the mitochondrial membrane (Elmore et al., 2004). COPD is associated with a depolarised mitochondrial phenotype, therefore the MitoTracker dye can be used as a measurement of polarised mitochondria but does not assess total mitochondrial mass.

The JC-1 dye is a cationic dye, which can be used to measure the polarisation state of mitochondria. The dye is excited with at 514nm but will emit fluorescence at two different wavelengths based upon the membrane potential of the mitochondria; emission at 529nm (green) will occur in depolarised mitochondria and 590nm (red) emission will occur in polarised mitochondria. By measuring the fluorescence intensity from these two channels, the ratio between red and green can be calculated, which indicates the polarisation state of the mitochondria within each individual macrophage.

MitoSox is a cell permeable dye, which is specific for mROS. Measurement of fluorescence intensity allows the relative amount of mROS production to be compared during phagocytosis and between cells from different subjects. The use of these mitochondrial dyes will give significant information regarding mitochondrial morphology and function in COPD macrophages.

6.1.1 Hypothesis

Macrophages from COPD subjects that exhibit reduced macrophage phagocytosis will display dysfunctional mitochondrial function, with both reduced mitochondria membrane potential and higher mitochondrial reactive oxygen species. This mitochondrial dysfunction can be partially restored with treatment by mitochondrial antioxidants, to reduce abnormal mROS levels and produce an increase in phagocytosis of *H. influenzae*.

6.1.2 Aims

To use microscopy, image analysis and bacterial quantification to assess mitochondrial function between subject groups, and to investigate if mitochondrial function can be modulated with the addition of mitochondrial antioxidants, SkQ1 and PQQ.

6.2 Methods

6.2.1 Subject selection

All subjects were recruited under inclusion/exclusion criteria with written, informed consent (described in 2.2.3). All non-smoker and COPD subjects had demographic and lung function information taken at the time of sampling.

6.2.2 Isolation and culture of MDM

MDM were isolated from non-smoker and COPD subjects (described in 2.2.3.1). Monocytes were plated at 1×10^6 cells/well into 8-well chamber slides, pre-treated with poly-L-lysine and washed three times with PBS.

6.2.3 Measurement of phagocytosis and cell parameters by microscopy

MDM were incubated with heat killed *H. influenzae* fluorescently labelled with AF405 (described in 2.2.6.3). Cells were imaged by confocal microscopy (2.2.17) and saved as Tiff, with single cell analysis performed.

6.2.4 Measurement of mitochondria morphology and function by microscopy

MDM morphology was assessed with three mitochondrial dyes in independent experiments: MitoTracker, MitoSox and JC-1. MDM were labelled using a mitochondria dye (described in 2.2.10), before being washed in media to remove excess stain. Cells were then imaged live by confocal microscopy.

6.2.5 Pre-incubation of MDM with mitochondrial antioxidants

MDM were pre-incubated with specific mitochondrial antioxidants, SkQ1 or PQQ, for 24h at a concentration of 50 nM or 500 nM (described in 2.2.11), prior to exposure to *H. influenzae*

and mitochondrial staining. Cells were washed to remove unbound bacteria and were imaged live by confocal microscopy.

6.2.6 Statistical analysis

All data analyses were performed using GraphPad Prism software version 9.3.1. All data are expressed as mean $\pm$ SEM. For correlation analysis, Spearman's rank correlation coefficient was performed with all r and p values stated. For grouped analysis, 2-way ANOVA with Sidak's multiple comparisons test were performed, with p value stated.

6.3 Results

6.3.1 Subject demographics for MDM model

Blood was collected from non-smoker and COPD subjects to generate MDM as previously described. There was no significant difference in age between subject groups for experiments performed on MDM (Table 6.1). There was a significant difference in smoking history between non-smoker and COPD subjects, as non-smoker subjects had no smoking history as part of the inclusion criteria. COPD subjects had significantly lower lung function compared to non-smokers, with a significant reduction in FEV₁, FEV₁ predicted % and FEV₁: FVC ratio.

	MDM	
	Non-smoker N = 10	COPD N = 14
Age	59.17 ± 2.50	64.71 ± 2.62
Gender (M:F)	5:5	8:6
Smoking History (Pack years)	0.00 ± 0.00	46.53 ± 9.36 ****
FEV ₁ (L)	3.44 ± 0.52	1.64 ± 0.18 **
FEV ₁ % predicted	123.4 ± 10.32	61.29 ± 5.53 ***
FVC (L)	4.54 ± 0.71	3.14 ± 0.20
FEV ₁ :FVC ratio	0.76 ± 0.02	0.52 ± 0.04 ***

Table 6.1. Subject demographics for macrophages isolated from blood samples (MDM) between subject groups. Differences between non-smoker and COPD groups analysed by Mann-Whitney test, ** p < 0.01, *** p < 0.001, **** p < 0.0001

6.3.2 Analysis of mitochondria in MDM

6.3.2.1 Visualisation of mitochondria in MDM

Having previously shown that microscopy can be used to measure phagocytosis (chapter 4) and measurements of cell morphology (chapter 5) in MDM, it was then used to investigate mitochondrial function in MDM. Laser scanning confocal microscopy was performed on MDM exposed to *H. influenzae* for up to 60 min and stained with the MitoTracker dye to assess the numbers and morphology of mitochondria present, with a minimum of 100 cells imaged per timepoint, per sample. Representative images for MDM stained with MitoTracker for non-smokers and COPD (Fig. 6.1) also show bacteria within the cell volume.

From these images, the mitochondrial mass of individual cells can be represented by the fluorescence intensity of MitoTracker. Additionally, structural and shape differences of the mitochondria can be assessed by textural analysis of the mitochondria in each macrophage, allowing comparisons between MDM from non-smokers and COPD subjects to investigate any morphological differences in the mitochondria down to a single pixel distance (0.18 μ m).

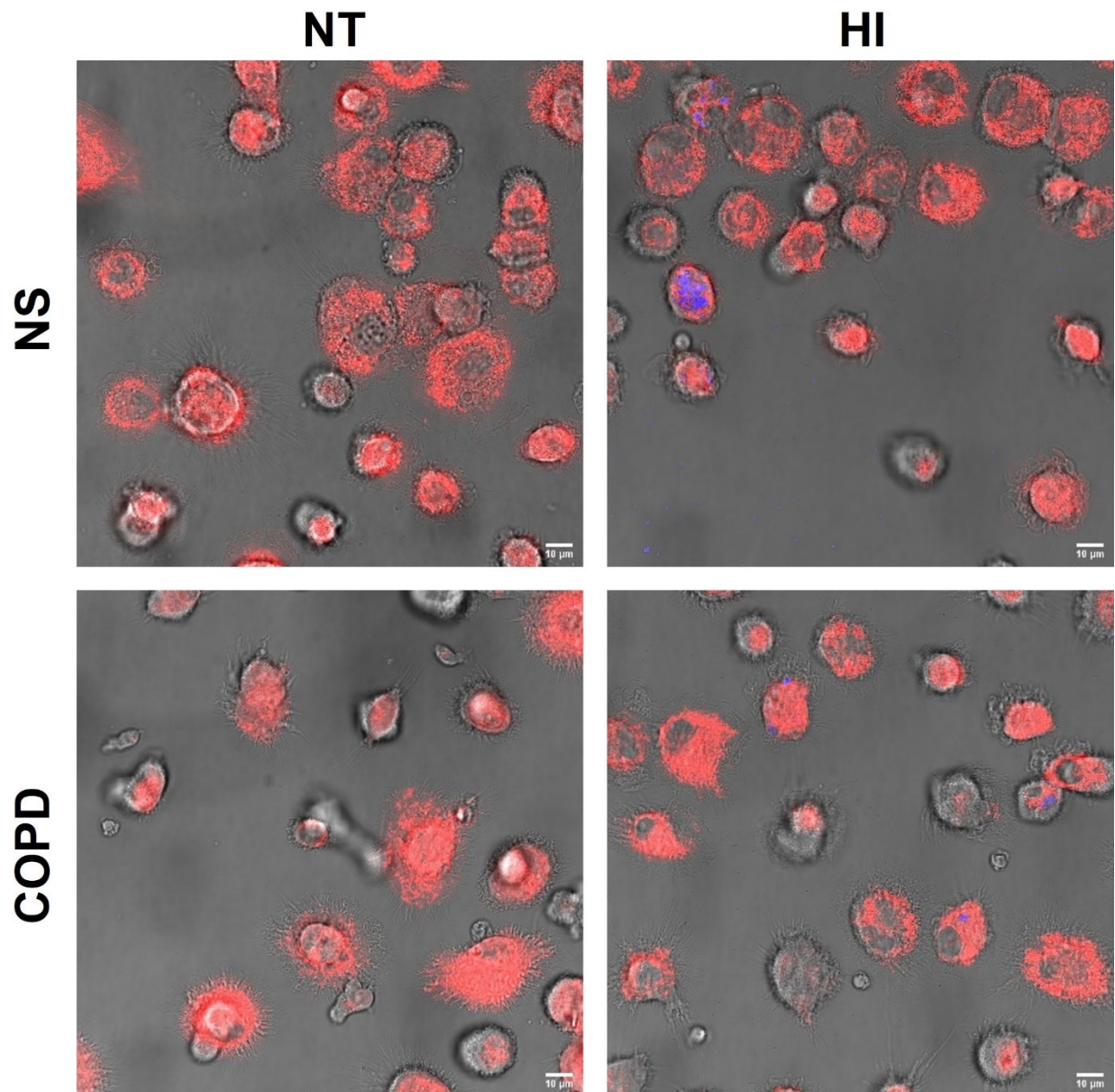


Figure 6.1. Confocal imaging of concurrent phagocytosis and mitochondria in MDM. MDM from non-smokers (NS) and COPD subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* fluorescently labelled with Alexa fluor® 405 (blue) for up to 60 min. Cells were stained for mitochondria with MitoTracker red dye. Representative images selected and 10µm scale shown. Red: mitochondria, Blue: *H. influenzae*.

6.3.4.2 Comparison of mitochondria in MDM between non-smoker and COPD subjects

The total fluorescence intensity is the sum of the intensity of every pixel, therefore is not affected by cell size or shape (Kelley & Paschal, 2019), but can be altered by differences in mitochondrial number within the cell. Whereas mean fluorescence intensity is the total intensity of every pixel divided by the area in pixels, and therefore is proportional to cell size and shape (Kelley & Paschal, 2019; Shiha et al., 2021), but is not affected by mitochondrial number. Both these measurements can be used to give important information into mitochondrial differences in cells.

There were no differences in number of polarised mitochondria in MDM from non-smokers and COPD subjects, with or without exposure to *H. influenzae* (Fig. 6.2 A) when assessing the mean fluorescence intensity from each subject. However, when using image analysis as shown in 5.3.1.2, by examining single cells from several subjects in a pooled data set, COPD MDM had significantly fewer polarised mitochondria at baseline compared to cells from non-smokers (mean MitoTracker intensity NS: 9055 ± 172.6 , COPD: 7568 ± 96.41 . $p < 0.0001$), and this persisted upon exposure to bacteria for up to 30 min, with the differences observed being lost by 60 min exposure (Fig. 6.2 B).

When assessing total MitoTracker fluorescence intensity between non-smoker and COPD MDM, there was no difference at baseline, but with incubation of *H. influenzae* a difference was observed after 30 min exposure, with COPD MDM displaying significantly lower intensity compared to non-smokers, but this difference was not seen at the 60 min incubation time point (Fig. 6.2 C). When assessing all cells pooled into their respective groups, COPD showed reduced total mitochondria fluorescence intensity at baseline and at all timepoint following exposure to bacteria (Fig. 4.2 D).

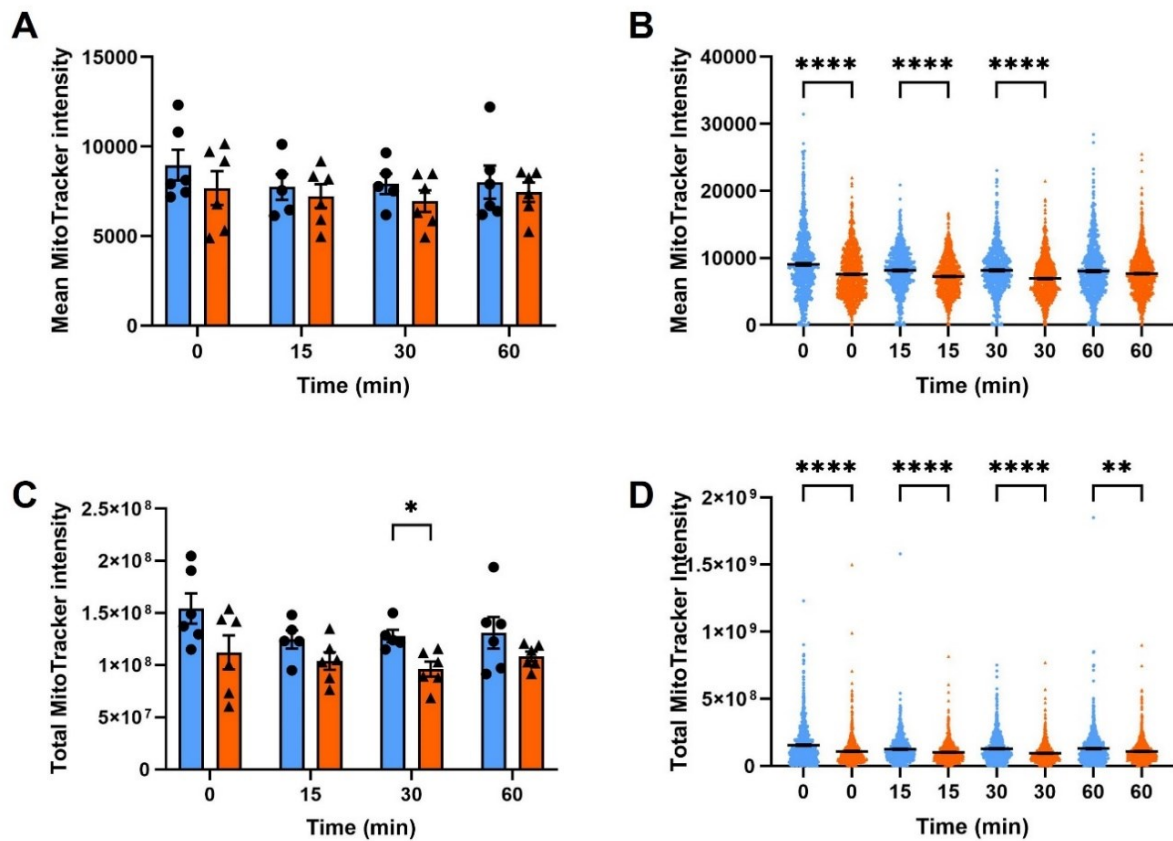


Figure 6.2. Measurement of mitochondria by imaging analysis across subject groups. MDM from non-smokers (■, n = 6) and COPD (■, n = 6) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* for up to 60 min and stained with the MitoTracker Red dye. For analysis 100 cells per time point analysed for each subject in sample groups. Data displayed showing mean MitoTracker fluorescence intensity (A-B) and total MitoTracker fluorescence intensity (C-D), with data presented as mean $\pm$ SEM (A and C) and individual points and mean (B and D). Mean data were analysed using a 2-way ANOVA with Sidak's multiple comparisons test and pooled data was analysed using a Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

6.3.4.3 Comparison of mitochondrial morphology between non-smoker and COPD subjects

Image analysis was performed on the macrophages stained with MitoTracker to compare various aspects of the mitochondrial morphology between non-smoker and COPD subjects. Magnified ROI displaying mitochondria from individual macrophage cells from non-smoker (Fig. 6.3 A-B) and COPD (Fig. 6.3 C-D) subjects are shown in Fig 6.3.

Image analysis techniques were performed to assess the texture of mitochondrial staining. Differences can be assessed as Gaussian gradient modulus which assesses the roughness of the mitochondrial over two set scale values. Gradient 1 (G1) measures the textural differences observed in the images of mitochondria over a 1-pixel range, equal to differences over 0.18 μ m, and so is an assessment of fine detail. The second measurement, gradient 2 (G2), shows differences over a 6-pixel range or 1.08 μ m, which can be termed as coarse differences. The final analysis performed was assessment of zero crossing of linear density (LD), which determines how coarse and dense a signal is, where a lower LD score indicates a coarser texture and denser signal.

Visual analysis of the confocal images of mitochondria with MDM shows a more tubular structure to the non-smoker mitochondria (Fig. 6.3 A-B), when compared to COPD cells where the mitochondria have a more circular appearance (Fig. 6.3 C-D), which could be classified as a more fragmented appearance (Huang & Frohman, 2012).

Confocal imaging showed no mitochondrial morphological differences between non-smoker and COPD MDM, when assessing mean G1 (Fig. 6.4 A). When all cells were pooled from subjects within groups and compared, there was again no difference in fine mitochondrial morphology at baseline and this continued upon incubation with bacteria until 60 min exposure, by which time the mitochondrial morphology was significantly different, with COPD

displaying a higher G1 score compared to non-smokers, this would indicate that the COPD mitochondria are displaying a rougher or less smooth phenotype in their staining (Fig. 6.4 B).

When analysing mean G2, there was again no significant differences between non-smoker and COPD MDM mitochondria (Fig. 6.4 C). However, when assessing data from all cells pooled into their respective subject groups, there was a difference at baseline between non-smoker and COPD mitochondria, with COPD displaying a lower G2 score, which was also seen at 30 min exposure to bacteria, this reduction in G2 would indicate COPD mitochondria are smoother over a 1.08 μm range (Fig. 6.4 D). However, as mitochondria are between approximately 0.5 - 1 μm in diameter (Alberts et al., 2002c), this size of scale may not be analysing single mitochondria, and could be representing clusters of mitochondria.

Finally, when analysing mean linear density, there was again no significant differences between mitochondria from non-smoker and COPD groups (Fig. 6.4 E). When assessing pooled data from the subject groups, there again was no difference at baseline between non-smoker and COPD. However, upon exposure to bacteria there was a significant difference in LD score, with the COPD subject cells increasing in LD score, which would indicate a less coarse texture and reduced density, suggesting a decompaction of the mitochondrial aggregates, which again is likely to be characteristic of mitochondrial fragmentation (Fig. 6.4 F). This reduction of mitochondrial density occurring in COPD cells upon incubation with bacteria and was consistent throughout the exposure to *H. influenzae* up to 60 min timepoint tested.

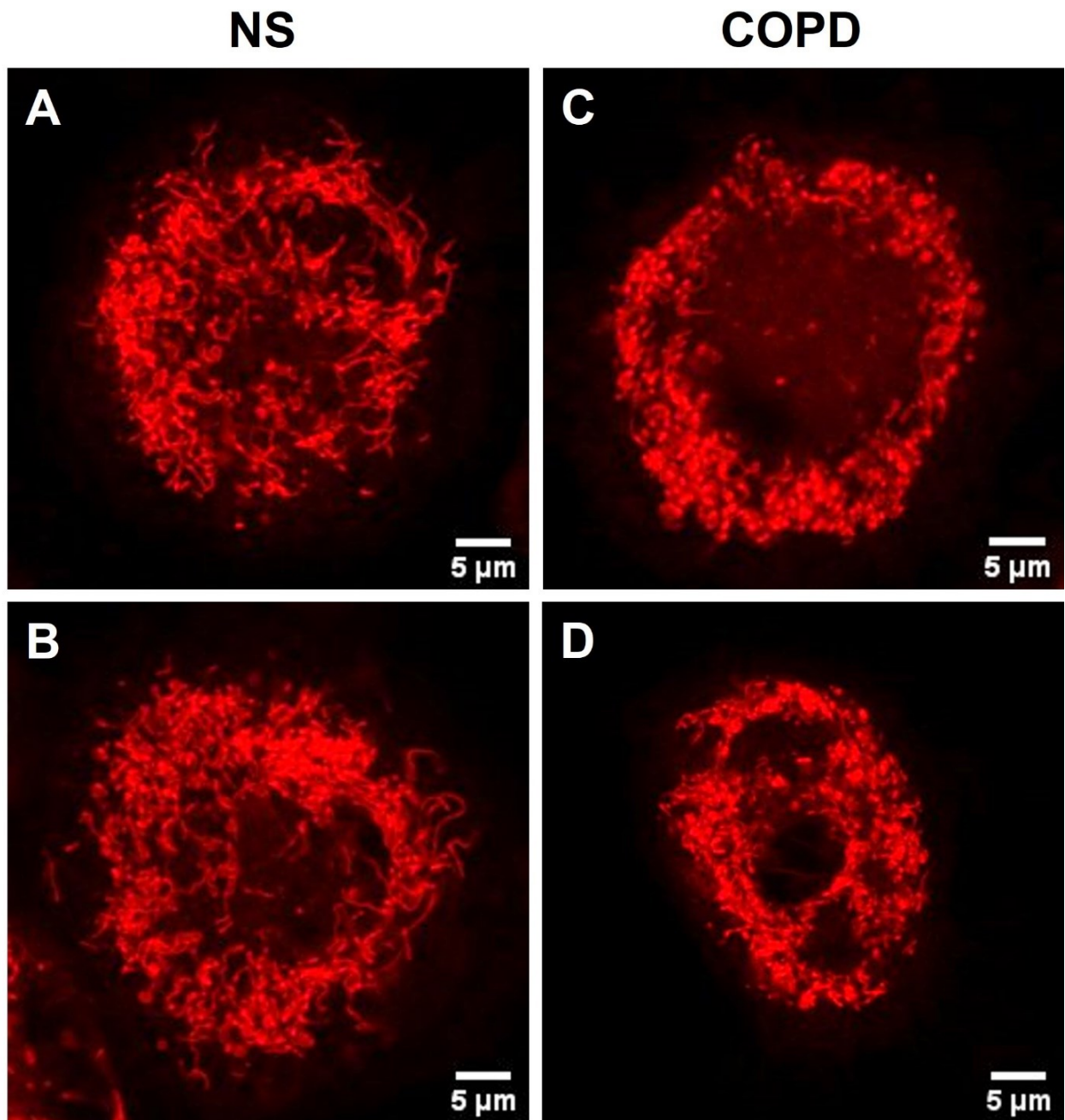


Figure 6.3. Confocal imaging of mitochondria in MDM from non-smoker and COPD subjects. MDM from non-smokers (NS) (A-B) and COPD subjects (C-D) were differentiated in the presence of GM-CSF for 12 days. Cells were stained for mitochondria with MitoTracker red dye. Representative images selected of magnified ROI to identify a single cell, 5 μ m scale shown. Red: mitochondria.

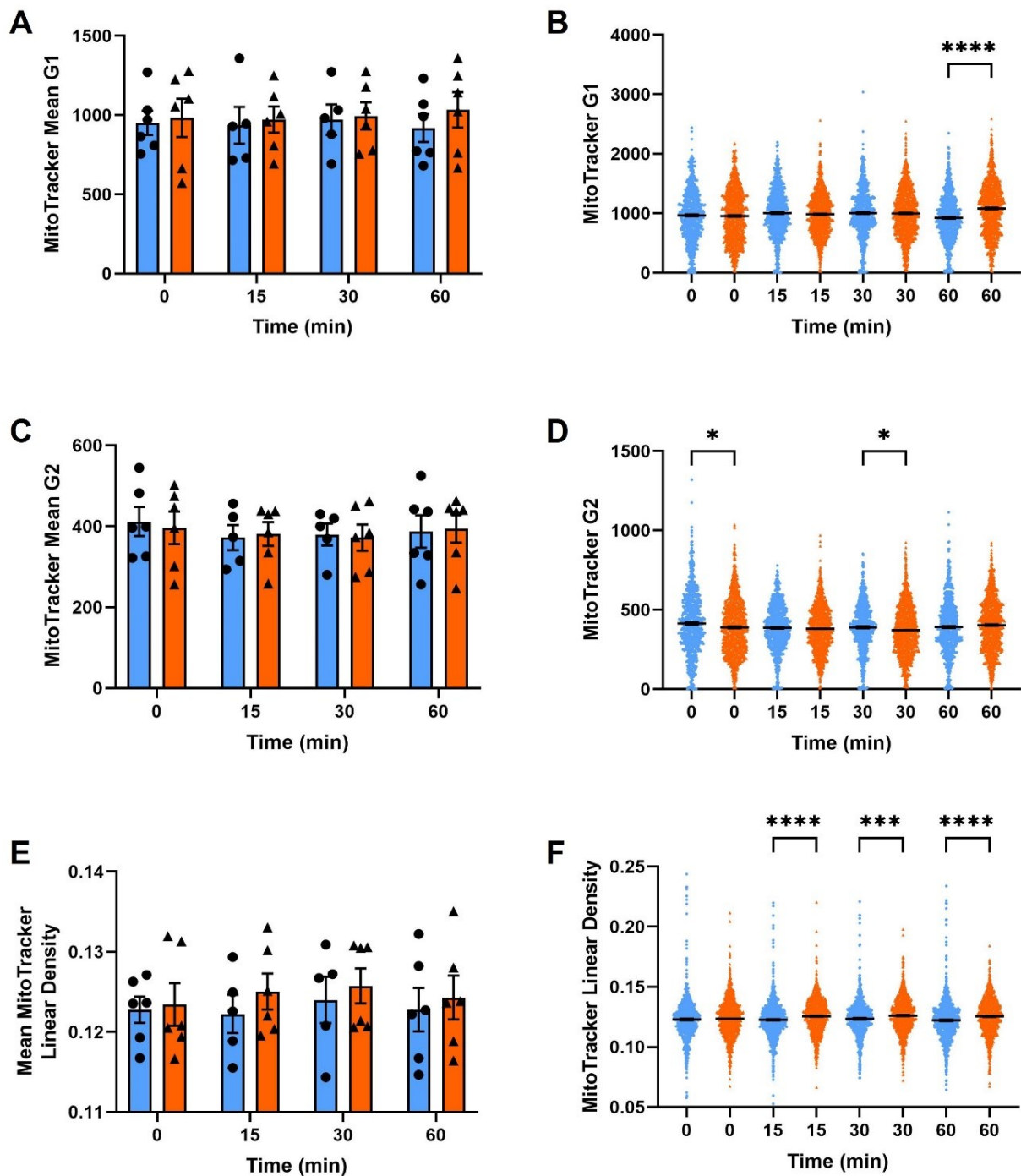


Figure 6.4. Measurement of mitochondrial morphology by imaging analysis across subject groups. MDM from non-smokers (■, n = 6) and COPD (■, n = 6) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* for up to 60 min and stained with the MitoTracker Red dye. For analysis 100 cells per time point analysed for each subject in sample groups. Data displayed showing mean MitoTracker G1 (A-B), mean MitoTracker G2 (C-D) and mean MitoTracker linear density (E-F), with data presented as mean $\pm$ SEM (A, C and E) and individual points and mean (B, D and F). Mean data were analysed using a 2-way ANOVA with Sidak's multiple comparisons test and pooled data was analysed using a Kruskal-Wallis test with Dunn's multiple comparisons test. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

6.3.4.4 Visualisation of mitochondrial membrane potential in MDM

A reported dysfunction in COPD macrophages is a reduced mitochondrial membrane potential (Belchamber et al., 2019), to assess this confocal microscopy was performed on non-smoker and COPD MDM exposed to *H. influenzae* for up to 60 min and stained with JC-1 dye to assess mitochondrial membrane potential, with a minimum of 100 cells imaged per timepoint, per sample.

Representative images of MDM stained with JC-1 (Fig. 6.5) show cells with no bacteria, non-treated (NT) or incubated with *H. influenzae* (HI), which are located within the cell volume. Individual cells can be shown within a magnified ROI (Fig. 6.6), from which the mean fluorescence intensity of the red and green channel was calculated, with fluorescence emitted from the dye depending on mitochondrial polarisation state, green emission represents depolarised, and red emission is polarised mitochondria. The red:green ratio can be quantified for each cell to give the cells mitochondrial membrane potential. Using this technique, the relationship between mitochondrial polarization and phagocytosis can be measured in the same cell.

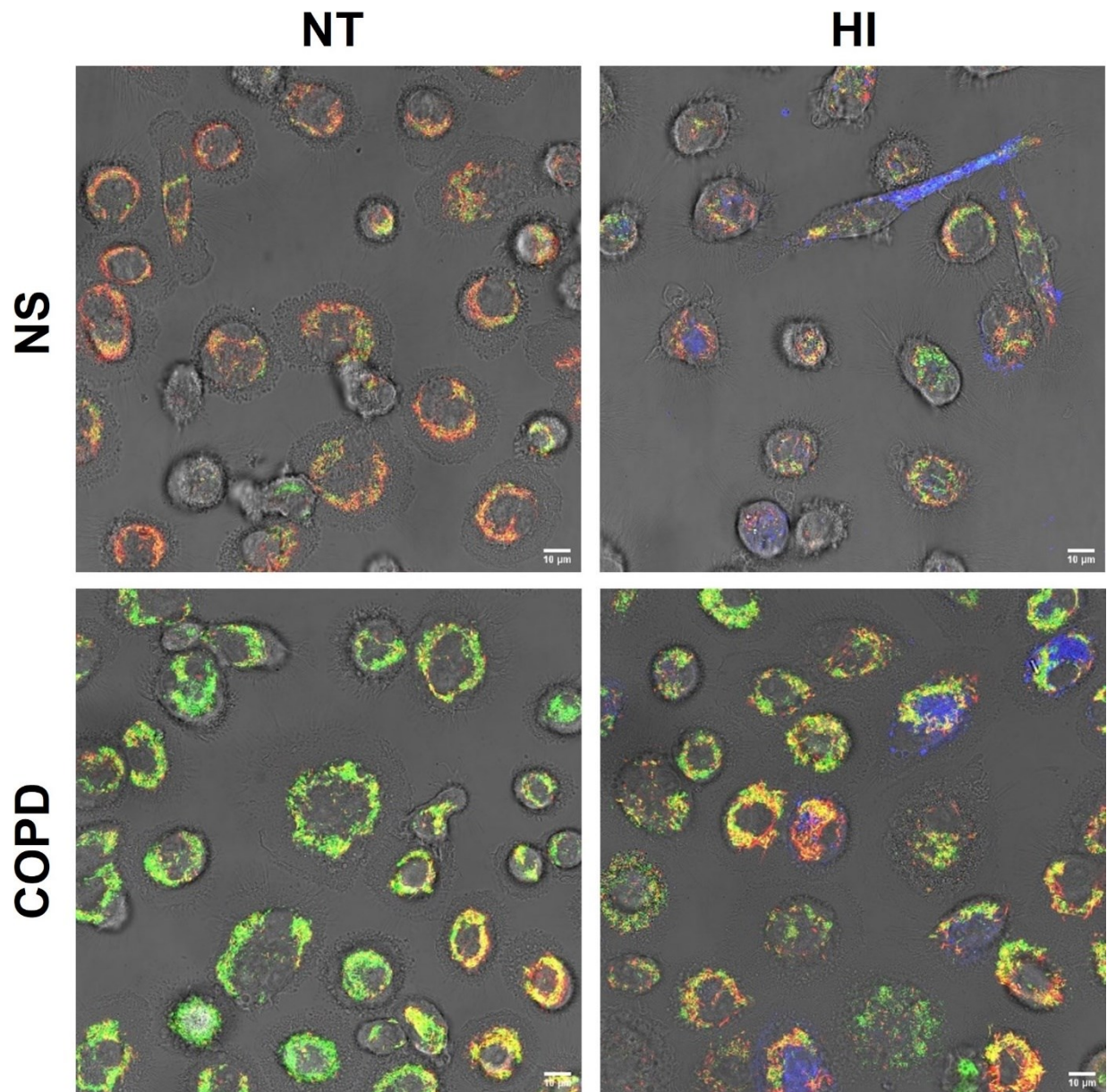


Figure 6.5. Confocal imaging of concurrent phagocytosis and mitochondria polarisation in MDM. MDM from non-smokers (NS) and COPD subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* fluorescently labelled with Alexa fluor® 405 for up to 60 min. Cells were stained for mitochondrial membrane potential with JC-1 dye. Phagocytosis was measured by confocal microscopy. Representative images selected and 10µm scale shown. Red: polarised mitochondria, Green: depolarised mitochondria, Blue: *H. influenzae*.

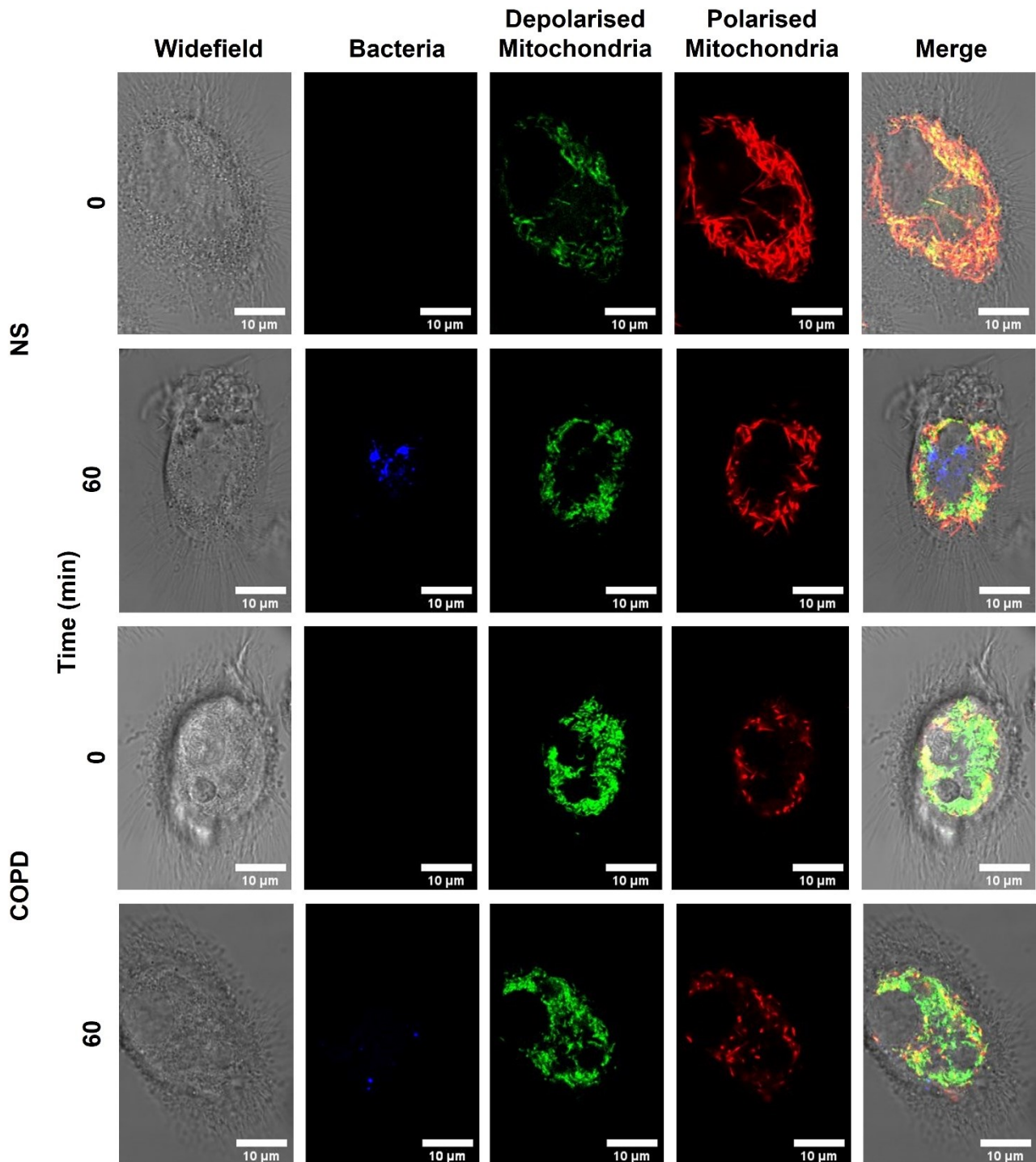


Figure 6.6. Confocal imaging of mitochondria polarisation in single MDM. MDM from non-smokers (NS) and COPD subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* fluorescently labelled with Alexa fluor® 405 for up to 60 min. Cells were stained for mitochondrial membrane potential with JC-1 dye. Phagocytosis was measured by confocal microscopy. Representative images selected and 10µm scale shown. Red: polarised mitochondria, Green: depolarised mitochondria, Blue: *H. influenzae*.

6.3.4.5 Comparison of mitochondrial membrane potential between MDM and COPD subjects

When comparing the mitochondrial membrane potential of MDM from non-smokers and COPD subjects, there was a difference at baseline, with COPD MDM having significantly decreased membrane potential as assessed by red to green fluorescence ratio (0.76 ± 0.05) compared to MDM from non-smokers (1.53 ± 0.10) ($p < 0.0001$) (Fig. 6.7 A). This significant reduction in membrane potential observed in COPD MDM remained after incubation with bacteria, at both a 15 min and 60 min incubation ($p < 0.0001$).

When assessing the mitochondrial membrane potential of the non-smoker and COPD groups upon incubation with bacteria, there was a significant decrease seen in both groups upon addition of *H. influenzae* to the MDM, which could be observed after 15 min and continued to decrease to the 60 min incubation ($p < 0.0001$) (Fig. 6.7 B). However, the level of the depolarisation of the mitochondria membrane in COPD MDM was lesser when compared to the non-smoker level of depolarisation.

Further analysis was performed separating MDM based on the presence of bacteria within the cell (eater) or no bacteria present (non-eater). This analysis showed that after 15 min exposure to bacteria, in non-smoker MDM, cells that had phagocytosed their mitochondria were polarised compared to cells that did not phagocytose, with no differences was observed in COPD MDM (Fig. 6.8 A). With continued incubation with bacteria up to 60 min, this difference in polarisation between eaters and non-eaters was lost by non-smoker MDM, with again no differences observed in COPD (Fig. 6.8 B).

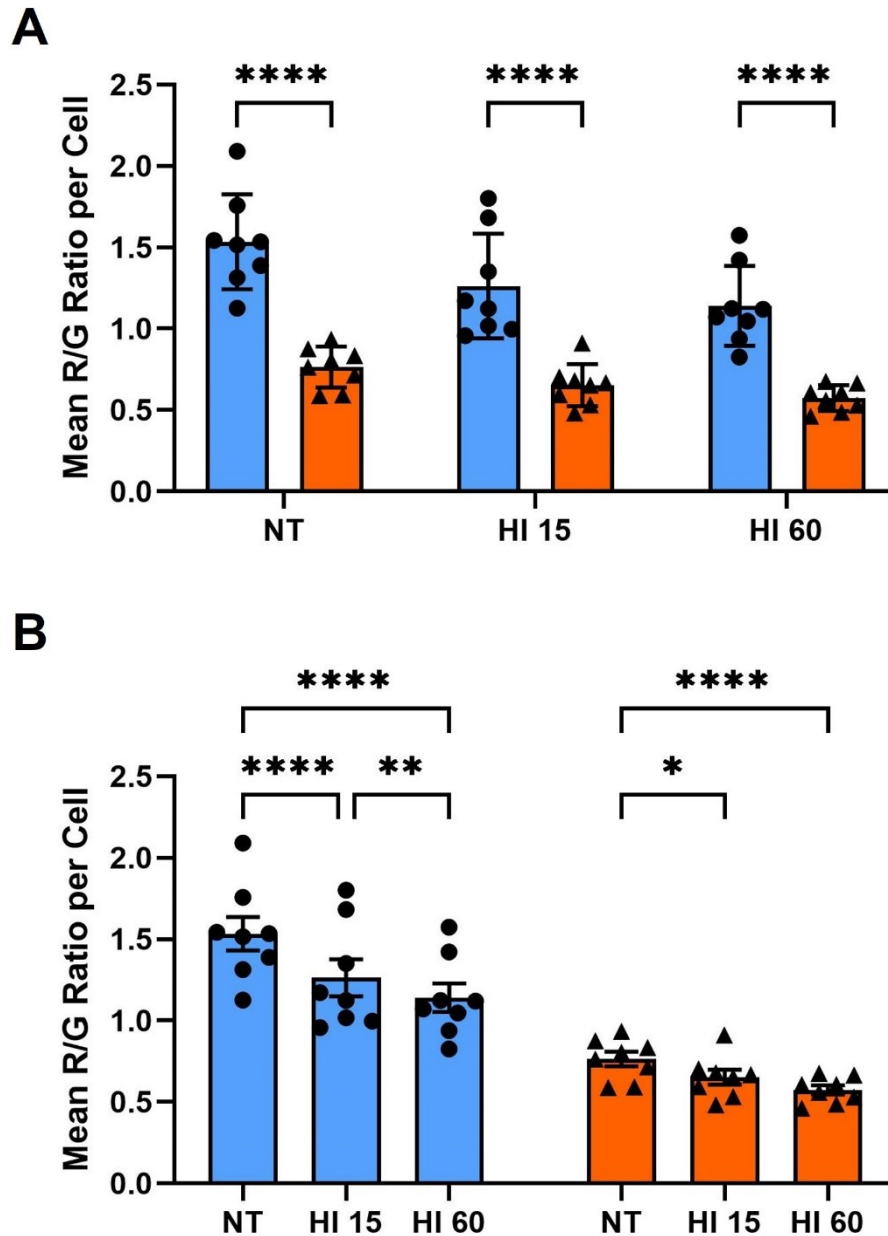


Figure 6.7. Measurement of mitochondrial membrane potential by imaging analysis across subject groups. MDM from non-smokers (■, n = 8) and COPD (■, n = 8) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* for 60 min and stained with the JC-1 dye. For analysis 100 cells per time point analysed for each subject in sample groups. Data presented as mean $\pm$ SEM, with comparison shown between subject groups (A) and comparison within groups (B). Data were analysed using a 2-way ANOVA with Sidak's multiple comparisons test. **** $p < 0.0001$.

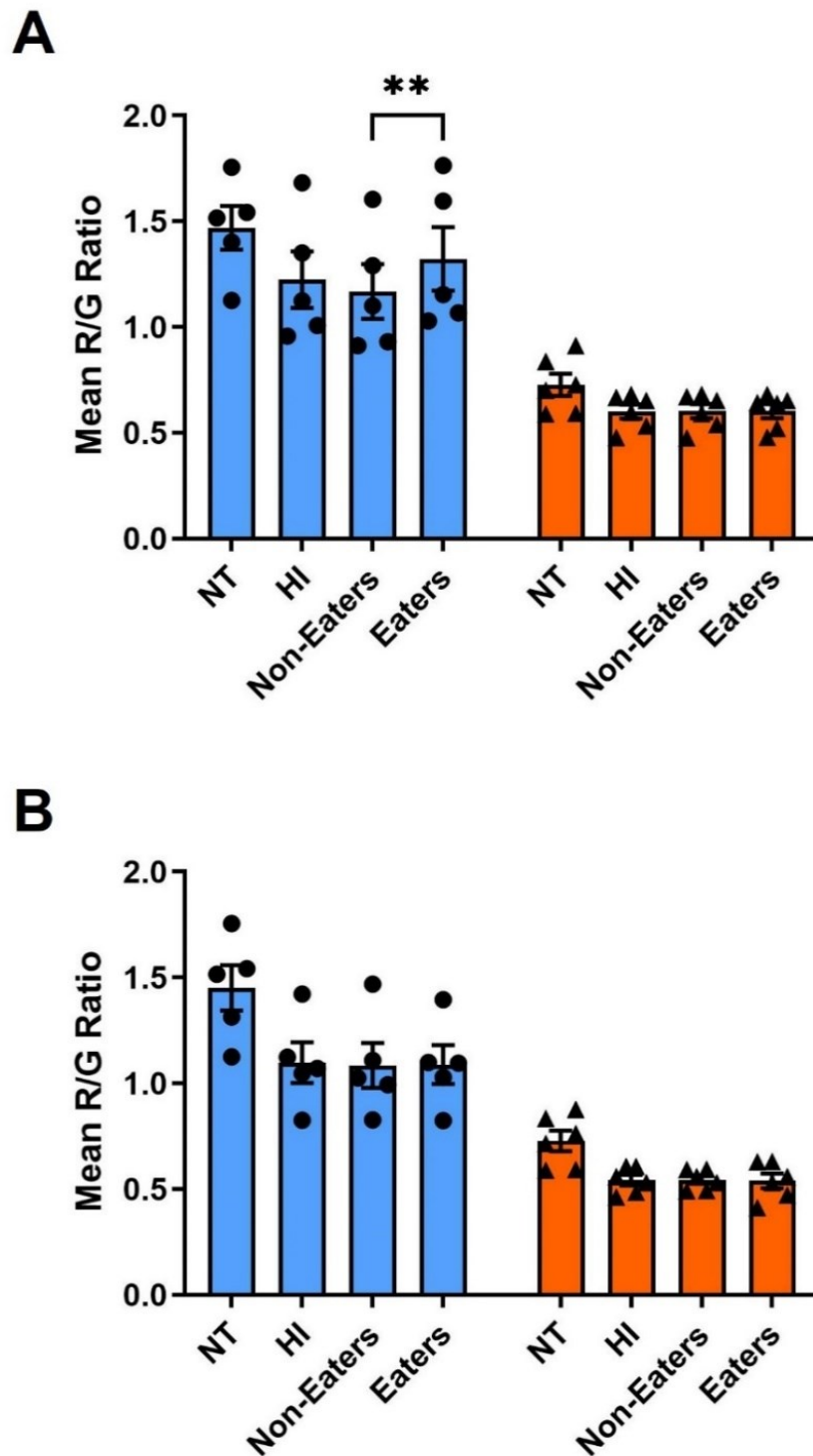


Figure 6.8. Measurement of mitochondrial membrane potential by imaging analysis within subject groups. MDM from non-smokers (■, n = 5) and COPD (■, n = 6) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* for up to 60 min and stained with the JC-1 dye. For analysis 100 cells per time point analysed for each subject in sample groups, with groups further divided by presence of bacteria (eaters) or no bacteria present (non-eaters). Data presented for 15 min HI incubation (A) and 60 min HI incubation (B) as mean $\pm$ SEM. Data were analysed using a 2-way ANOVA with Sidak's multiple comparisons test. ** $p < 0.01$.

6.3.4.6 Comparison of mitochondrial membrane potential and phagocytosis

To determine if MDM phagocytosis was directly related to mitochondrial membrane potential, correlations were calculated between bacterial uptake and the red:green ratio of JC1 staining as used to determine the mitochondrial membrane potential.

When analysing the groups independently, there was a significant negative correlation for between mitochondrial polarisation and phagocytosis in MDM from non-smokers ($r = -0.22$, $p < 0.0001$), but not for cells from COPD subjects (Fig. 6.9). This relationship was maintained when both groups were analysed together ($r = -0.07$, $p = 0.02$). This suggests that increased phagocytosis correlates with a depolarisation of the mitochondrial membrane.

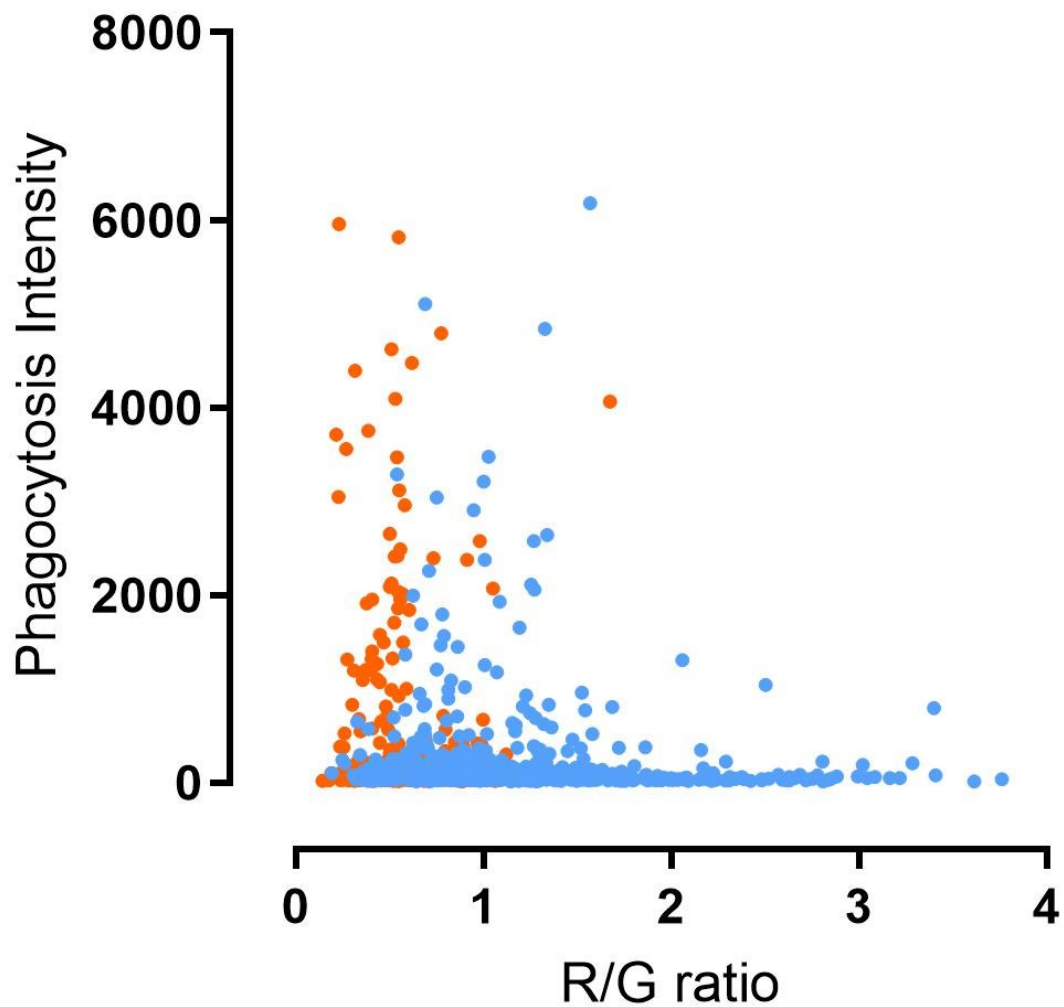


Figure 6.9. Comparison of mitochondrial membrane potential and phagocytosis of *H. influenzae* by MDM across subject groups. MDM from non-smokers (■) and COPD (■) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* for up to 60 min. Data displayed as individual points showing red:green ratio and fluorescence intensity in the bacterial channel. Data presented as individual points, N = 4-5.

6.3.4.7 Visualisation of mitochondrial reactive oxygen species in MDM

Having shown a relationship between mitochondrial membrane potential and phagocytosis, whether this was also accompanied with a change in mitochondrial ROS was also investigated. MDM were exposed to bacteria and stained with MitoSox to assess mitochondrial reactive oxygen species, with a minimum of 100 cells imaged per timepoint, per sample.

Representative images for non-smoker and COPD MDM stained with MitoSox, and either non-treated (NT) or incubated with bacteria (HI), show bacteria within the cell volume (Fig. 6.10). *H. influenzae* were labelled with AF 405 showing in blue, with MitoSox staining showing in red and brightfield showing the cell membrane and cytoplasm in grey. Individual cells can be shown within a magnified ROI (Fig. 6.11), from these images the fluorescence intensity for MitoSox can be calculated giving a relative amount of mROS for each cell.

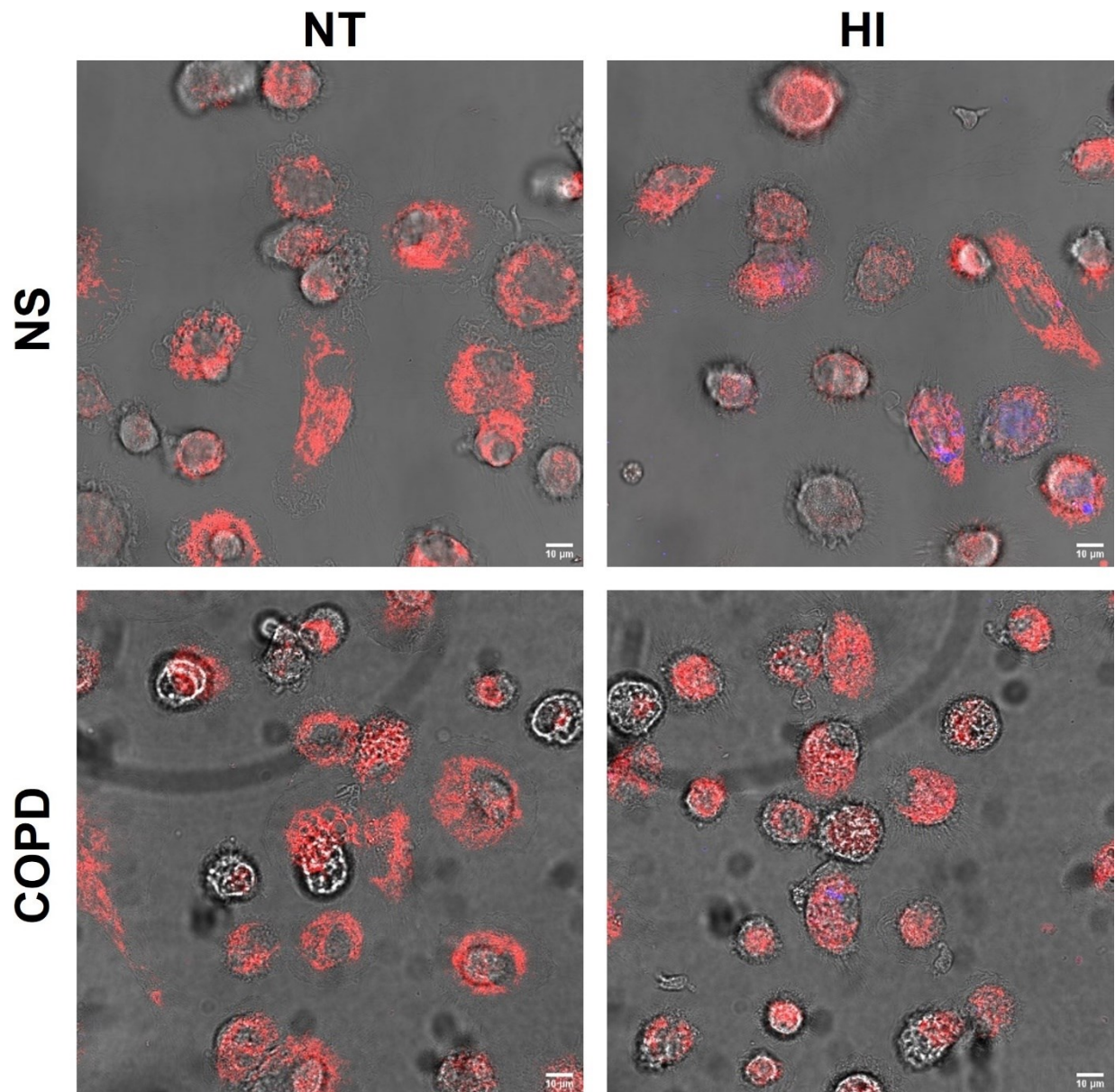


Figure 6.10. Confocal imaging of concurrent phagocytosis and mitochondria reactive oxygen species in MDM. MDM from non-smokers (NS) and COPD subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* fluorescently labelled with Alexa fluor® 405 for up to 60 min. Cells were stained for mROS with MitoSox dye. Phagocytosis was measured by confocal microscopy. Representative images selected and 10µm scale shown. Red: mROS, Blue: *H. influenzae*.

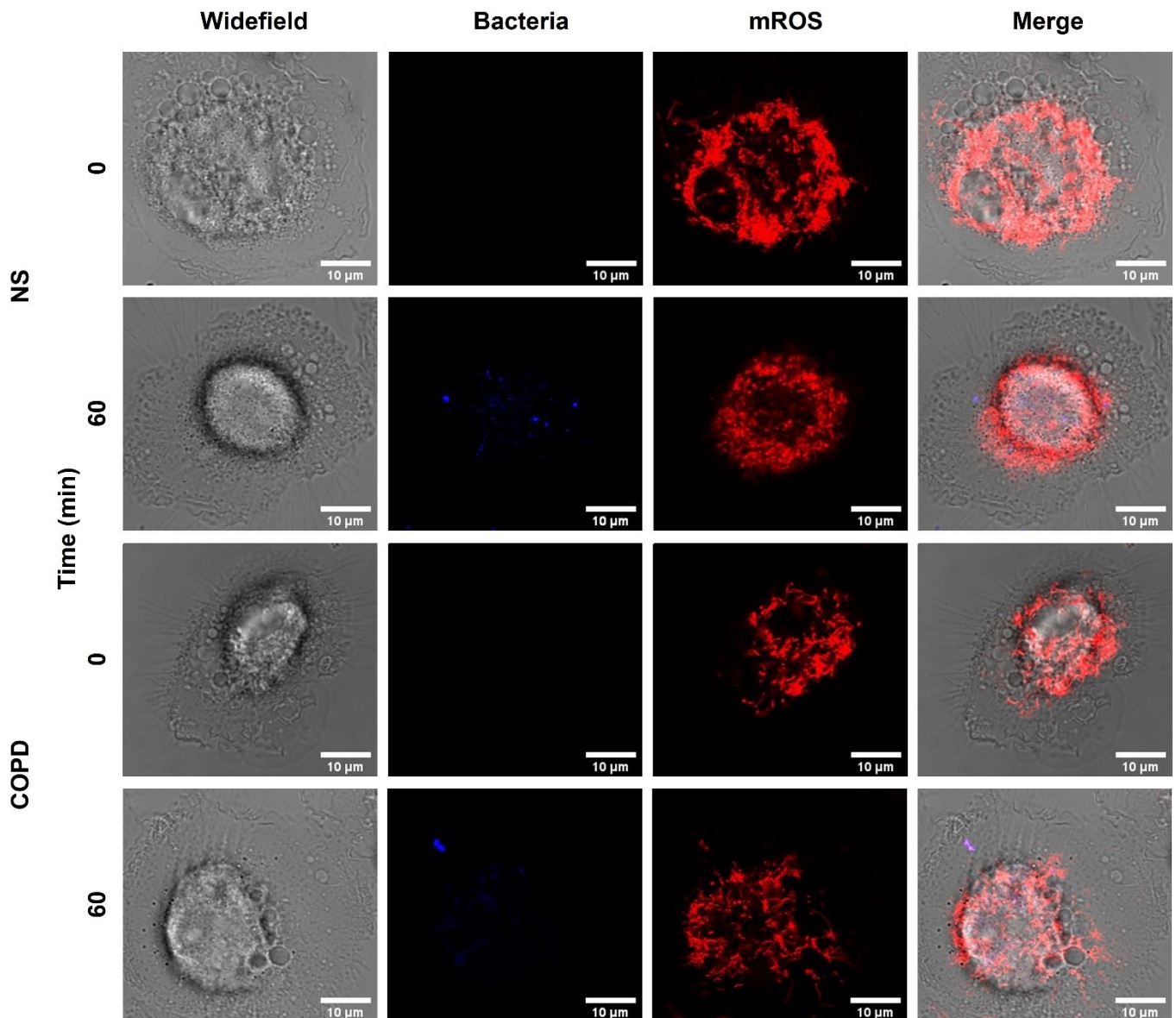


Figure 6.11. Confocal images of single cells displaying concurrent phagocytosis and mitochondria reactive oxygen species in MDM. MDM from non-smokers (NS) and COPD subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* fluorescently labelled with Alexa fluor® 405 for up to 60 min. Cells were stained for mROS with MitoSox dye. Phagocytosis was measured by confocal microscopy. Representative images selected and 10μm scale shown. Red: mROS, Blue: *H. influenzae*.

6.3.4.8 Comparison of mROS in MDM between non-smokers and COPD subjects

There was no difference in mROS measurements between MDM from non-smokers and COPD at baseline or following incubation with *H. influenzae* (Fig. 6.12 A). Analysis was also performed to determine the mROS intensity of each MDM based on the presence of bacteria or not, with MDM that contained at least a single bacterium considered 'eaters' and no bacteria present considered 'non-eaters'.

When this analysis was performed, there was a significant difference in mROS intensity between non-eaters and eaters, with eaters displaying significantly increased level of mROS compared to non-eaters in MDM from both non-smokers ($p < 0.05$) and COPD ($p < 0.01$) (Fig. 6.12 B). This showed that COPD MDM had a greater difference in mROS intensity between eaters and non-eaters compared to non-smokers. There was also a significant decrease in mROS intensity in COPD MDM when comparing the non-eating to the baseline MDM ($p < 0.05$).

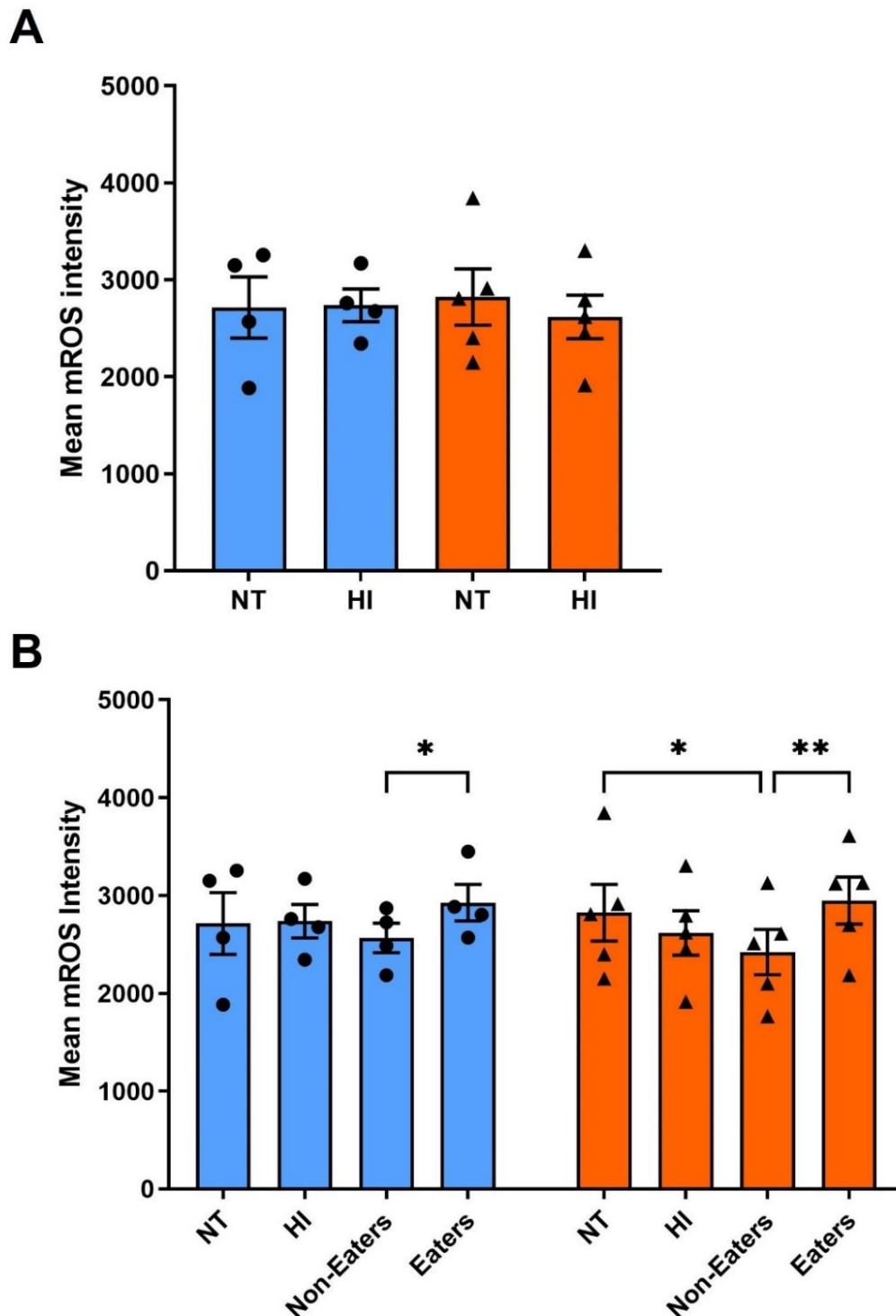


Figure 6.12. Comparison of mitochondrial reactive oxygen species between eating and non-eating MDM across subject groups. MDM from non-smokers (■, n = 4) and COPD (■, n = 5) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* for up to 60 min and stained with MitoSox. For analysis 100 cells per time point analysed for each subject in sample groups. Data presented as mean $\pm$ SEM. Data were analysed using a 2-way ANOVA with Sidak's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.3.4.9 Comparison of mitochondrial reactive oxygen species and phagocytosis

To determine if MDM phagocytosis was related to mROS, correlations were calculated and showed a significant positive correlation for MDM from both non-smokers ($r = 0.33$, $p < 0.0001$) and COPD patients ($r = 0.33$, $p < 0.0001$). When analysed together there was also a significant positive correlation ($r = 0.27$, $p < 0.0001$) (Fig. 6.13).

For both non-smokers and COPD subjects, phagocytosis was weakly correlated with mROS intensity, therefore macrophages with more internalised bacteria may have a higher production of mROS.

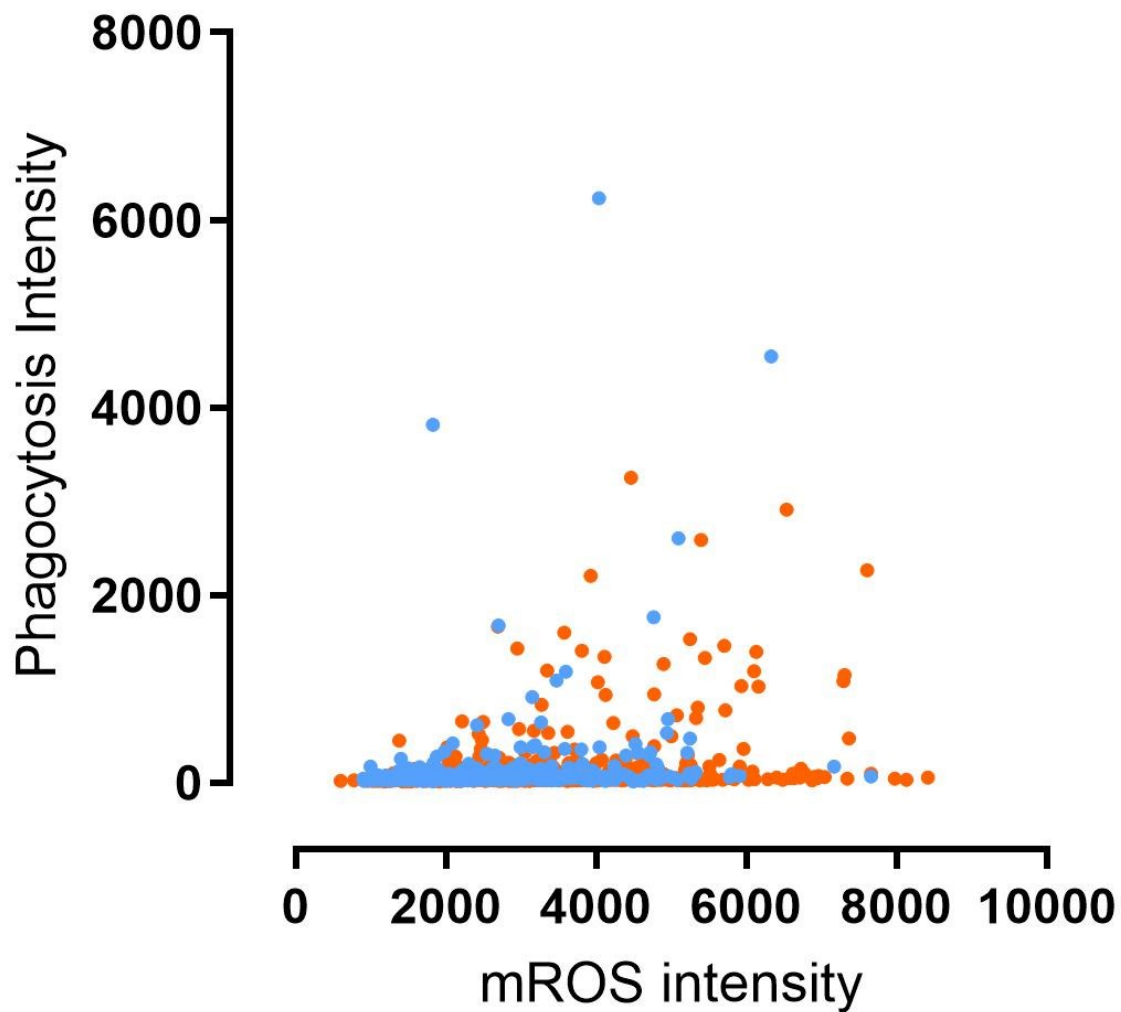


Figure 6.13. Comparison of mitochondrial reactive oxygen species and phagocytosis of *H. influenzae* by MDM across subject groups. MDM from non-smokers (■) and COPD (■) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were incubated with *H. influenzae* for up to 60 min. Data displayed as individual points showing fluorescence intensity of mROS and fluorescence intensity in the bacterial channel. Data presented as individual points, N = 4-5.

6.3.5 Effects of mitochondrial antioxidants on phagocytosis, mROS and membrane potential

Previous studies have shown mitochondrial dysfunction in oxidative stress models of COPD, including reduced mitochondrial potential, which could be modulated with mitochondrial antioxidants (Wiegman et al., 2015). As reduced mitochondrial membrane potential was observed in COPD macrophages, the use of mitochondrial antioxidants was explored

To determine if mitochondrial antioxidants have any effect on mitochondrial function, COPD MDM were pre-incubated with SkQ1 or PQQ for 24h, prior to exposure with *H. influenzae* for 60 min. Live confocal was performed measuring mROS, membrane potential and phagocytosis by image analysis. Preliminary experiments showed no difference in mROS, membrane potential or phagocytosis after 1h pre-incubation with SkQ1 or PQQ.

Image analysis of COPD MDM incubated with either PQQ or SkQ1 for 24h showed no significant change in mitochondrial membrane potential compared to untreated MDM (Fig. 6.14 A-D). There was also no difference in mROS intensity in COPD MDM incubated with mitochondrial antioxidants compared to untreated MDM (Fig 6.14 E-H).

However, exposure to mitochondrial antioxidants for 24h resulted in increased phagocytosis, between the untreated MDM ($33.11 \pm 6.12\%$) and MDM incubated with 50 nM SkQ1 ($46.83 \pm 8.96\%$, $p < 0.01$) (Fig. 6.14 I) and 500 nM SkQ1 ($45.83 \pm 8.29\%$, $p < 0.01$) (Fig. 6.14 J). A similar increase was observed in MDM pre-incubated with 50 nM PQQ ($54.12 \pm 5.90\%$, $p < 0.05$) (Fig. 6.14 K), however no significance was reached in MDM treated with 500 nM PQQ ($52.4 \pm 6.9\%$) (Fig. 6.14 L). Therefore, phagocytosis was increased by incubation with mitochondrial antioxidants without any subsequent changes to mROS or $\Delta\Psi_m$ in COPD MDM.

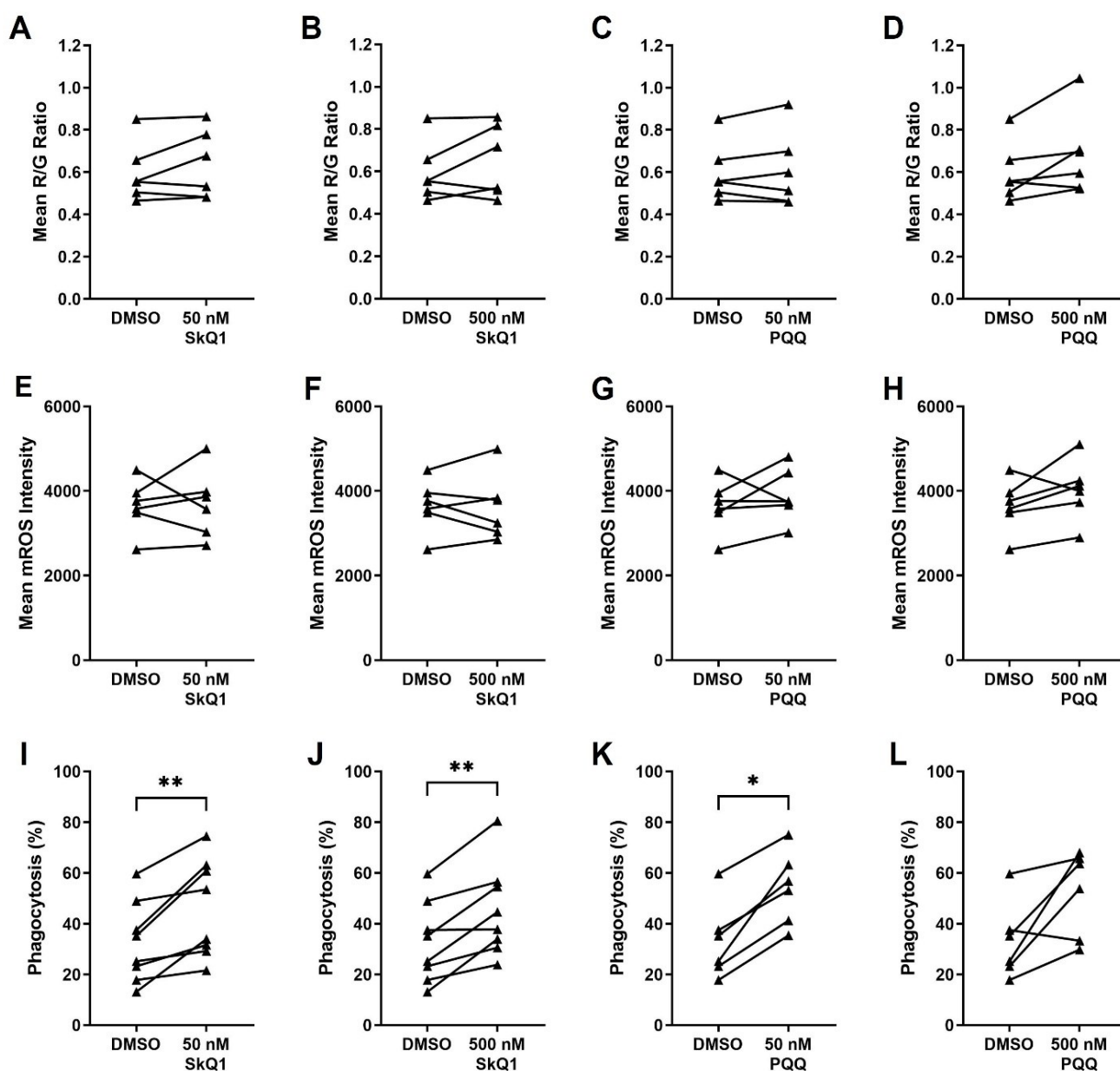


Figure 6.14. Effect of SkQ1 and PQQ on phagocytosis, mROS and $\Delta\Psi_m$ in COPD MDM. MDM from COPD (n = 6-8) subjects were differentiated in the presence of GM-CSF for 12 days. Cells were pre-incubated with SkQ1 or PQQ for 24h prior to incubation with *H. influenzae* for 60 min. Cells were stained with JC-1 or MitoSOX and imaged live by confocal microscopy to quantify $\Delta\Psi_m$ (A-D), mROS (E-H) and phagocytosis (I-L). For analysis 100 cells per time point analysed for each subject in sample groups. Data presented as mean and analysed using a Wilcoxon test, * p<0.05, ** p<0.01.

6.4 Discussion

This chapter investigates the mechanism of defective phagocytosis in COPD macrophages using MDM, examining the role of mitochondrial function. The mitochondrial mass of polarised mitochondria was significantly reduced in COPD MDM when compared to cells from non-smokers, however this study did not look at total mitochondrial number, therefore it is possible that the size of mitochondria could be altered in COPD cells. The MitoTracker dye used in this experiment is dependent on mitochondrial polarisation, and therefore this result likely reflects the more depolarised state of mitochondria in COPD macrophages and may indicate a reduction in functioning mitochondria, with COPD macrophages potentially having mitochondria that could be characterised as ‘damaged’. Other studies assessed the quantity of mitochondria in COPD using mtDNA copy number, which is controversial (Riou et al., 2020), with some data showing reduced mtDNA copy number in blood leukocytes from COPD patients (Liu et al., 2015), which would be in keeping with data from this thesis as MDM are derived from circulating cells. However, other studies have shown an increase in mtDNA copy number in COPD when analysing cell free mitochondria from exhaled breath condensate (Carpagnano et al., 2016), urine (Zhang et al., 2020b), and plasma (Bulgakova et al., 2021; Zhang et al., 2021), which suggests the release of mtDNA from within the cell and is likely to suggest mitochondrial and cellular damage. The increase in mtDNA copy number may be a compensatory mechanism in response to increased oxidative stress (Riou et al., 2020; Wallace, 1992). The decrease in polarised mitochondria in COPD macrophages, shown in this thesis, does not necessarily dispute this, as this quantification of mitochondria does not assess total mitochondria. COPD macrophages could have more mitochondria, but if they are more depolarised, they were not quantified using this dye and are potentially dysfunctional.

When both COPD and non-smoker MDMs were incubated with bacteria, there was a reduction in the polarised mitochondria observed, however this reduction occurs at an earlier time point and was a more significant reduction in COPD MDM, followed by a return to baseline, which was not seen in non-smokers at the timepoints explored in this project. These differences in mitochondria seen by staining with MitoTracker upon incubation with bacteria could be linked with depolarisation of the mitochondrial membrane.

When investigating morphology of the mitochondria within the subject groups, significant differences were observable by visual analysis of textures at a scale of less than 1 μ m. In COPD MDM, mitochondria had increased roughness when assessing fine texture differences (G1) at a scale of 0.18 μ m after 60 min incubation with *H. influenzae*. These differences in texture do not necessarily relate to dysfunction but highlight differences in the appearance and morphology of mitochondria in macrophages from COPD subjects and non-smokers. There is data to suggest that mitochondrial shape is involved in processes such as calcium signalling and cell death, and has even been implicated in neurogenerative disease (Campello & Scorrano, 2010). Therefore, the textural differences observed in this thesis could be linked to damaged mitochondria and impaired autophagy.

Additionally, the differences in the linear density (LD) of mitochondria between non-smokers and COPD, showed that upon incubation with bacteria there was a significant increase in the LD score for COPD MDM when compared to the non-smokers. This increase in LD, showed that COPD mitochondria produced a morphological change that did not occur in non-smokers. Non-smoker mitochondria remained compacted throughout the experiment including during incubation of bacteria, compared to COPD where upon incubation with bacteria the LD-score increased revealing a decompressing or de-aggregation of the mitochondria (Kapetanovic et

al., 2020). This increase in LD-score likely relates to an fragmented phenotype of the mitochondria in COPD, as observed in cells exposed to cigarette smoke (Aravamudan et al., 2014; Hara et al., 2013). Increased fragmentation as a marker for mitochondrial damage could be an indicator of defective mitophagy, which is known to occur in a number of cell types in COPD (Barnes, Baker & Donnelly, 2022), which also agrees with the decreased membrane potential and link to defective phagocytosis in COPD macrophages (Belchamber et al., 2019).

Mitochondrial membrane potential is significantly depolarised in bronchial biopsies and airway smooth muscle from COPD patients when compared with controls (Haji et al., 2020; Wiegman et al., 2015). This mitochondrial dysfunction is not just restricted to the lung, as reductions in mitochondrial polarisation are also observed in MDM isolated from circulating blood (Belchamber et al., 2019), which was also seen in this study. This implies that the mitochondrial damage within COPD is not localised to the airways, and there is a systemic defect in the mitochondria in multiple cell types. Mitochondrial damage and depolarisation of the membrane may attenuate mROS production (Boukhenouna et al., 2018), however in COPD macrophages the depolarisation is significant and prolonged, as shown in both alveolar macrophages and MDM (Belchamber et al., 2019). This depolarisation is likely associated with mitochondrial dysfunction, oxidative stress and excessive mROS production, and related to chronic inflammation observed in COPD cells including macrophages (Bewley et al., 2017), airway epithelial cells (Hara et al., 2013; Hoffmann et al., 2013) and airway smooth muscle (Haji et al., 2020).

The current scope of mitochondrial involvement in impaired bacterial phagocytosis in COPD is unclear. Alveolar macrophages in COPD display increased mROS production at baseline with no increase upon bacterial incubation when compared to cells from non-smokers (Bewley et

al., 2017). However, in a separate study, COPD MDM did not differ in mROS at baseline compared to MDM from non-smokers, but after 4h incubation with *H. influenzae* COPD showed a significant increase in mROS production compared to non-treatment, but this was not seen in cells from controls (Belchamber et al., 2019). With two different accounts of mROS involvement in phagocytosis within COPD macrophages, it was important to investigate this further to clarify baseline mROS in MDM between groups, and then observe production upon incubation of bacteria. We found no differences in mROS between groups at baseline or during incubation with bacteria up to 60 min. However, when mROS intensity was correlated with fluorescence in the bacterial channel, both non-smoker and COPD MDM there was a weak relationship between internalisation of bacteria and mROS production. This is in accordance with data shown in this thesis, where single cell analysis of MDM separated by whether they had internalised any bacteria (eaters) or failed to internalise any bacteria (non-eaters), which showed fluorescence intensity of mROS was significantly increased in macrophages positive for internalised bacteria compared to cells which have no internalised bacteria. These data confirm the importance of mROS for bacterial killing (Cohen et al., 2018). However, data from this thesis seems to dispute the impact of mROS levels in COPD.

Depolarisation of the mitochondrial membrane in COPD macrophages in agreement with data from previous studies suggesting increased mROS production (Bewley et al., 2017), therefore it was hypothesised that the use of mitochondrial specific antioxidants could be used to reduce excessive mROS production and reverse the depolarised state of the mitochondrial membrane. Pre-incubation of COPD MDM with specific potent mitochondrial antioxidants, SkQ1 or PQQ for 24h, had no effect on mitochondrial membrane potential or mROS, however increased phagocytosis of *H. influenzae* by up to 60%. This shows promise for the use of these mitochondrial antioxidants as a therapeutic option in COPD and highlights mitochondrial

dysfunction in COPD as a factor in defective phagocytosis, even though the mechanism was not revealed in this thesis. This could be because the timepoint explored in this thesis missed any mechanistic changes involving mitochondria membrane potential and mROS after incubation with mitochondrial antioxidants. Additionally, ATP production was not measured as part of this thesis, but an increase in the metabolic production of energy could explain the increase in phagocytosis observed. Mitochondrial antioxidants such as MitoQ increase mitochondrial membrane potential and a reduction in mROS (Wiegman et al., 2015), and are capable of restoring intracellular ATP levels such as under glucotoxic conditions in pancreatic cells (Lim et al., 2011), so there is evidence that these antioxidants do affect the mitochondrial markers investigated in this thesis.

Chapter 7: General Discussion

7.1 Summary of main findings

The main hypothesis of this thesis was that high resolution microscopy of live macrophages would determine differences in cellular morphology and the cytoskeletal network, caused by dysfunctional mitochondria, resulting in the defective phagocytosis observed in COPD, and that this system could be used to screen potential correctors to improve phagocytosis. Overall, the work in this thesis has shown that microscopy is a useful technique in the analysis of individual cells when attempting to understand differences in structural proteins, such as tubulin and actin. It has shown clear differences in both the actin and microtubule network, as well as mitochondria function, in COPD macrophages when compared to cells from non-smokers. This technique can be used to assess functional differences in the cells when mitochondrial antioxidants were examined to determine potential efficacy in increasing phagocytosis.

The intent of this thesis was to develop a methodology to perform live super-resolved imaging on macrophages undergoing phagocytosis, to investigate defective phagocytosis in COPD. The purpose of performing live imaging of primary macrophages, below the limit of diffraction, was to observe spatial resolution and to elucidate mechanistic defects in phagocytosis observed in COPD, with the potential use of the methodology as a drug discovery tool used for screening novel therapeutics. However, super-resolved microscopy on live primary macrophages was not possible. Instead, the use of the cell permeable silicon rhodamine dyes as seen in chapter 3, proved very useful in the progression of the thesis towards the investigation of cytoskeleton restructuring in macrophages during phagocytosis.

The use of widefield epi-fluorescence microscopy with post-acquisition deconvolution allowed images to be acquired that were not affected by cell movement or thermal drift, and image quality allowed sufficient resolution to perform bacterial quantification. Using these imaging techniques, the work performed in this thesis revealed differences in cell morphology between non-smoker and COPD MDM and tissue macrophages, as well as differences in aspects of the cytoskeletal whilst undergoing phagocytosis.

Analysis of cell size between subject groups shown in chapter 5, revealed that the tissue macrophage population isolated from COPD lungs are larger from those isolated from non-smokers. This could indicate that they are from different endogenous pools, and likely the COPD population may not be from a tissue resident population, but instead could be mostly comprised from more MDM population that have migrated to the lung due to chronic cigarette exposure and other chemical components. A study performing analysis of lung macrophage populations separated by density, showed differences in expression of cell surface markers, release of MMP-9 and an insensitivity to glucocorticosteroids between non-smoker and COPD macrophages (Chana et al., 2014). This study shows that density or size of macrophages can greatly influence the functional ability of the cell, however density was not determined to be a marker of age or lineage.

These findings are in keeping with previous data showing increased expression of CD14 positive cells in BAL from smokers with or without COPD in comparison to never-smokers, as CD14 is highly expressed in peripheral blood monocytes compared to mature alveolar macrophages (Hodge et al., 2007). If the COPD macrophage population in the lung is comprised of less mature macrophages, they may exhibit different polarisation states reflected by a more inflammatory phenotype (Bazzan et al., 2017; Chana et al., 2014;

Yamasaki & van Eeden, 2018). Patients with COPD have more phagocytic cells in the lung, these macrophages are likely due to migration from the bone marrow and may be younger than tissue resident macrophages. Tissue resident macrophages are a self-sustaining population, established during development (Wynn, Chawla & Pollard, 2013; Yona et al., 2013), and are differentiated due to the local microenvironment of the lung (Martin et al., 2021). However under inflammatory conditions and repeated infection this population is depleted leading to the need for this population to be replenished with MDM recruited from peripheral blood (Martin et al., 2021). In this thesis, comparative analysis of MDM and tissue macrophages has revealed that COPD tissue macrophages are of similar size to both non-smoker and COPD MDM, when analysing area and perimeter. Whereas non-smoker tissue macrophages which were significantly smaller when compared to MDM (chapter 5). This gives credence to the hypothesis that chronic inflammation in the COPD lung has depleted the tissue macrophage population, which has then been replenished with a monocyte-derived macrophage population. Additionally, with tissue macrophages shown to phagocytose more bacteria when compared to MDM (chapter 4), this population of macrophages in the lungs of COPD subjects morphologically resembling MDM could go some way to explain the reduction in phagocytosis observed in COPD. This adds to the current evidence that different phenotypic populations of macrophages in the lungs of COPD patients contributes to the excessive inflammatory response in COPD (Chana et al., 2014; Eapen et al., 2017) and may account for the defective phagocytosis involved.

In chapter 5, using a single cell analytical microscopy approach, significant differences in cytoskeletal proteins; actin and tubulin were observed in macrophages from non-smokers and COPD subjects. COPD macrophages have significantly larger microtubule network when analysing the total length of tubulin fibres, and an increase in microtubule branch-point

density when compared to non-smoker macrophages suggesting that for the tubulin network within COPD macrophages is less able to dynamically rearrange. As this is required to traffic the phagosome inside cells, there may be a failure to internalise bacteria efficiently. Depolymerisation of tubulin with nocodazole results in affected cell motility, cell growth and reduced phagocytosis (Gilberti & Knecht, 2015; Kovács & Csaba, 2006) suggesting that a functioning microtubule network is needed for phagocytosis. Differences in tubulin branch-point density and tubulin fibre length could point to reduced cytoskeleton rearrangement due to defective microtubule stability. Microtubule stability is essential for cell migration (Wittmann & Waterman-Storer, 2001) and interaction with actin filaments (Waterman-Storer & Salmon, 1999), therefore if COPD macrophages have altered tubulin stability this may lead to defective mobility and impaired production of pseudopodia capable of particle engulfment. Additionally, inhibition of the microtubules can affect cell morphology, where depolymerisation prevents the organisation of the actin filaments, resulting in a reduction or alterations in the formation of the cellular protrusions, lamellipodium (Kim & Petroll, 2007; Kwan & Kirschner, 2005), again producing alterations in cell motility and migration (Liao, Nagasaki & Gundersen, 1995; Schliwa et al., 1999), which could account for the changes in cell morphology explored in this thesis.

Tubulin stability seems to be an important factor regarding immune responses in COPD, with the acetylation of tubulin being a marker for microtubule stability (Janke & Bulinski, 2011; Wloga & Gaertig, 2010). The acetylation of α -tubulin is a post-translational modification produced by α -tubulin acetyltransferase 1 (α TAT1) (Li & Yang, 2015; Shah et al., 2018), and is increased in response to cellular stress by the activation of AMP kinase by mROS (Mackeh et

al., 2014), which could explain increased microtubule stability resulting in the cytoskeletal differences observed in this thesis.

Tubulin deacetylation is known to be caused by histone deacetylases (HDAC), which are specifically localised with the microtubule network (Hubbert et al., 2002), and is are key regulators of the dynamic remodelling of the microtubule network (Matsuyama et al., 2002). Over expression of HDAC6 is shown to result in increased cell motility, due to a decrease in microtubule stability (Hubbert et al., 2002; Palazzo, Ackerman & Gundersen, 2003), whereas deletion of HDAC6 results in tubulin hyperacetylation, which affects immune cells and their responses in mice (Zhang et al., 2008). Sirtuins (SIRT) are a family deacetylase which play a key role in COPD pathogenesis (Zhang et al., 2022), with SIRT1 shown to be significantly decreased in COPD lungs and macrophage samples (Rajendrasozhan et al., 2008; Nakamaru et al., 2009) and in serum (Yanagisawa et al., 2017). The role of SIRT2, an α -tubulin deacetylase (North et al., 2003), in COPD is currently unknown, although GWAS has identified SNPs within genes encoding SIRT2 to be significantly associated with COPD (Bakke et al., 2011) and a study using a murine macrophage model has shown SIRT2 controls tubulin deacetylation to a greater extent than HDAC6 (Misawa et al., 2013). COPD MDM have been shown to display reduced levels of acetylated tubulin compared with non-smoker subjects, however no difference in either HDAC6 or SIRT2 expression levels were discovered that could possible explain this decreased acetylation of tubulin (Thomas, 2013). This reduction of acetylated tubulin could be reversed with the use of a microtubule stabiliser, which additionally resulted in an increase in phagocytic capabilities of COPD MDM (Thomas, 2013). Thus giving further evidence that microtubule stability is a key contributor to the defective phagocytosis observed in COPD MDM.

Actin was primarily located in punctate structures, known as podosomes. The amount and distribution of these were different between macrophages from non-smoker and COPD subjects, with significantly less actin punctate structures in COPD macrophages when compared to non-smokers. Podosomes are important in actin reorganisation involved in cell movement towards the leading edge (Evans et al., 2003) and are involved in membrane ruffling (Ridley et al., 1992), which could have a significant bearing on phagocytosis in macrophages. Podosomes are controlled by Ras homolog (Rho) family of small GTPases, predominately Rac1 (Ridley et al., 1992), and involve microtubules to facilitate assembly of actin filaments and arrangement of the cytoskeleton (Elbaum et al., 1999; Linder et al., 1999). Inhibition of microtubules by taxols results in reduced formation of podosomes in macrophages, effecting cell movement and growth (Evans et al., 2003). The reduction in the number of these podosome structures in COPD macrophages observed in this thesis, could be linked to the defective nature of the microtubules also observed in these cells, and could therefore result in reduced motility and less cytoskeletal rearrangement in response to a bacterial stimulus, potentially explaining the reduction of internalised bacteria during phagocytosis.

The interaction between the microtubule network and actin filaments is regulated by Rho small GTPases including Rac1 (Wittmann & Waterman-Storer, 2001), which are also important in the process of phagocytosis in alveolar macrophages (Bewley et al., 2018). Rac1 regulates dynamic actin rearrangement during Fcγ receptor-mediated phagocytosis, including the formation of the cellular protrusions and membrane ruffling during engulfment (Castellano, Montcourrier & Chavrier, 2000), therefore is key to phagosome production in macrophages (Jubrail, Kurian & Niedergang, 2017). Microtubule depolymerisation is also controlled by activation of Rac1 (Wittmann & Waterman-Storer, 2001), however data exploring this in

COPD alveolar macrophages shows that Rac1 expression is unaltered during exposure to a bacterial stimulus such as *S. pneumoniae* (Bewley et al., 2018).

As phagocytosis requires energy produced by the mitochondria in the form of ATP, to undergo dynamic restructuring of the cytoskeletal to internalise bacteria (DeWane, Salvi & DeMali, 2021), differences observed in the structure and localisation of cytoskeletal proteins highlighted in chapter 5 of this thesis could be due to differences in the mitochondria in COPD macrophages. The number of 'active' mitochondria, as indicated by membrane potential, was reduced in COPD macrophages, in agreement with the current literature regarding dysfunctional mitochondria in COPD cells (Birch, Barnes & Passos, 2018; Jiang, Wang & Hu, 2017), decreased functional mitochondria in airway epithelial cells (Prakash, Pabelick & Sieck, 2017), and a reduced mitochondrial DNA copy number in circulating leukocytes (Liu et al., 2015) in these patients. There is information to suggest that total mitochondria number is increased in COPD (Barnes, 2020; Cloonan & Choi, 2016), but this thesis did not perform an assessment of total mitochondrial mass.

Analysis of mitochondrial morphology in macrophages explored in chapter 6, showed a decompaction of the staining of mitochondrial aggregates in COPD cells when compared to non-smokers, similar to that seen in airway epithelial cells (Hoffmann et al., 2013) and airway smooth muscle cells (Aravamudan et al., 2014) exposed to cigarette smoke. This increased mitochondrial fragmentation in COPD macrophages could indicate impaired mitophagy in these cells (Zorov et al., 2019). Impaired mitophagy has been reported in airway fibroblasts and epithelial cells resulting in an accumulation of damaged mitochondria, associated with cellular senescence following chronic cigarette smoke exposure (Ahmad et al., 2015).

COPD macrophages in this study showed a highly depolarised mitochondrial membrane potential, which is a precursor for mitophagy, (Twig & Shirihai, 2011), and is complimentary to the increased fragmentation of the mitochondria. The enlarged size of COPD tissue macrophages could also indicate that they contain particulates from cigarette smoke and other pollutants (Lugg et al., 2022), as seen with smoker alveolar macrophages displaying enlarged size and increased autofluorescent bodies when compared to cells from non-smokers (Pankow et al., 1995) and COPD alveolar macrophages displaying an increased black carbon content, resulting in increased risk and severity of exacerbations (Tejwani et al., 2021).

COPD MDM showed depolarisation of the mitochondria membrane when compared to non-smoker cells. Upon exposure to bacteria, non-smoker, and COPD MDM both produced a depolarisation of the mitochondrial membrane, which was maintained during exposure to bacteria, and hence showed that the bacterial stimulus and potentially the process of phagocytosis results in a depolarisation of the mitochondrial membrane. However, as the COPD MDM were already significantly depolarised at baseline, when they were incubated with bacteria, the degree of depolarisation was lessened. This constant depolarisation could potentially result in differences in the production of ATP through the electron transport chain (Zorova et al., 2018), as well as resulting in cell death (Lyamzaev et al., 2018). COPD is known to present with differences in the metabolic pathways producing ATP in a number of cell types including immune cells (Agarwal et al., 2019; Kao et al., 2012), and as such this could represent a shift from oxidative phosphorylation towards glycolysis. If COPD macrophages are limited in their ability to produce sufficient ATP during exposure to bacteria, this could explain the cytoskeletal differences, where rearrangement and polymerisation requires ATP, and this could explain defective phagocytosis.

COPD alveolar macrophages produce excessive mROS compared to cells from non-smokers, with no increase in mROS upon exposure to bacterial stimulus (Bewley et al., 2017). Similar work on MDM showed no differences in baseline mROS but showed a marked increase in mROS generation after incubation with bacteria (Belchamber et al., 2019). However, work in this thesis examining mROS in MDM by microscopy, could not verify this, but instead showed no difference in mROS at baseline between macrophages from non-smokers and COPD subjects, as well as no significant difference upon incubation with *H. influenzae*. Both non-smoker and COPD showed increased mROS production in MDM which had internalised at least a single bacterium when compared to cells that were unable to phagocytose, showing potential mROS involvement in bactericidal killing in human macrophages (Ariffin et al., 2016; West et al., 2011). This discrepancy with Belchamber *et al.*, could be explained by the use of different techniques, with variable sensitivity, as well as differences in timepoints. Under normal physiological conditions, depolarisation of the mitochondrial membrane reduces mROS with the potential of anti-inflammatory effects (Vyssokikh et al., 2020), however prolonged depolarisation seen in COPD macrophages clearly goes beyond this, with mitochondrial dysfunction associated with a number of pathologies including neurodegenerative disease (Wang et al., 2019), atherosclerosis (Victor et al., 2009), diabetes (Ma, Zhao & Turk, 2012) and cancer (Pokrzywinski et al., 2016).

As visual differences in mitochondria morphology and mitochondrial polarisation in COPD macrophages compared to non-smoker cells could result in abnormal ATP generation, the mitochondria provide an interesting target for therapeutic intervention in an attempt to increase bacterial phagocytosis in COPD MDM. Mitochondrial antioxidants have may normalise mitochondrial membrane potential and reduce mROS after induction of oxidative stress (Wiegman et al., 2015). As shown in this thesis, mitochondrial membrane potential in

COPD MDM is highly depolarised, and excessive mROS production is observed in other COPD studies (Belchamber et al., 2019; Wiegman et al., 2020). Mitochondrial antioxidants such as SkQ1, prevent mitochondrial fragmentation caused by oxidative stress (Plotnikov et al., 2008). COPD MDM pre-incubated with nanomolar concentrations of these mitochondrial antioxidants increased phagocytosis, however there was no effect on mROS production or mitochondrial membrane potential, when measured by microscopy and image analysis. The increase in phagocytosis by COPD MDM *in vitro* after incubation with mitochondrial antioxidants is a significant advancement in understanding the defective phagocytosis in COPD macrophages. It potentially reveals a partial mechanism in the defective phagocytosis, which appears to be unrelated to the depolarisation of the mitochondrial membrane but could be related to the preventing mitochondrial fragmentation (Plotnikov et al., 2008).

7.2 Conclusions

Overall, this thesis has provided further evidence that macrophages from COPD subjects are different to those from non-smokers, both in morphology as well as key cytoskeletal proteins: tubulin and actin. There are also significant mitochondrial differences observed in COPD macrophages, where significant depolarisation of the mitochondrial membrane resulted in reduced capacity to depolarise when exposed to a bacterial stimulus. Mitochondria morphology was also different, displaying a more compact texture, which could be attributed to the reduced number of active mitochondria available or be related to a more fragmented phenotype of cells undergoing mitophagy.

These differences in COPD macrophages could be contributing factors to the defective phagocytosis observed in the disease, as differences in the cytoskeleton and mitochondria

could directly affects the ability to internalise pathogens. Although it is unknown if any of these individual differences are the primary cause of the impact on phagocytosis in COPD macrophages.

The differences seen in this thesis provide additional evidence that COPD produces systemic differences in the immune system, as work carried out in this thesis was performed primarily on MDM. Therefore, differences seen in macrophages between subject groups, including phagocytosis, cell size, tubulin structure, actin density, active mitochondrial number, and mitochondria membrane potential, cannot necessarily be attributed to direct cigarette smoke exposure in the lung or the chronic inflammation that occurs.

The use of microscopy and image analysis to investigate differences in cell morphology, the cytoskeleton and mitochondria has proved useful at adding additional layers of information that cannot be seen when performing bulk analysis. The data provided in this thesis has identified the cytoskeletal and mitochondria as a critical component in the defective phagocytosis observed in COPD macrophages, with potential therapeutic options available in the form of mitochondria antioxidant intervention.

7.3 Future Work

7.3.1 Determine ATP production in macrophages undergoing phagocytosis and compare between COPD and non-smoker subjects

This project used microscopy to image mitochondria within macrophages undergoing phagocytosis to investigate differences in mitochondrial functions between subject groups. However, ATP production from macrophages was not directly investigated, which would be

an interesting addition alongside this data. Identifying any differences in ATP production from between subject groups could be correlated with differences in the cytoskeleton, including actin podosome number which could reflect potential differences in cellular motility resulting in defective phagocytosis. Current unpublished data from Dr J. Baker, shows that COPD macrophages produce less ATP and that there is no shift from oxidative phosphorylation to glycolysis as seen in other inflammatory settings (Kelly & O'Neill, 2015).

This would also be of use when assessing COPD macrophages pre-incubated with mitochondrial antioxidants, to see if this increases ATP production which could explain the increased phagocytosis observed in this thesis, as no differences were detected in mROS production or mitochondrial membrane potential.

7.3.2 Examine effects of mitochondrial antioxidants

There is further work that could be explored to refine the concentration of mitochondrial antioxidants and the length of incubation require to produce a maximal increase in phagocytosis. It is also interesting to extend the period of incubation with bacteria, to understand if there is a plateau of phagocytosis, as in seen in tissue macrophages undergoing phagocytosis, as seen in chapter 4. A further experiment to this, is to investigate the length of time macrophages retain an increased phagocytic capability after incubated with antioxidants and prior to exposure to bacteria.

Further work needs to be performed to explain the potential mechanism producing the increase in phagocytosis after incubation with mitochondrial antioxidants. As no differences were seen in mROS or mitochondrial membrane potential in this dataset when analysed by microscopy. This could be due to the timepoints selected for this study missing any changes to these markers for mitochondrial function, or that these are entirely unaffected in

macrophages and that the mitochondrial antioxidants are having off target effects. Further experiments should be performed to confirm that these mitochondrial antioxidants have no effect on mitochondrial functional, or to understand what underlying mechanism is being affected to cause the increase in phagocytosis observed. Additionally, the differences in the cytoskeleton outlined in this thesis including podosome density, microtubule length and branching, should be investigated with the use of mitochondrial antioxidants, to see if these produce any differences in COPD macrophages which can explain the increased phagocytosis.

7.3.3 Examine effect of prolonged exposure to bacteria or LPS on macrophage morphology

This project primarily explored the early initiation of phagocytosis in macrophages upon exposure to bacteria for up to 60 min. However, experiments that track phagocytosis over a longer period would be interesting, to investigate if the changes observed in this thesis reflect initial bacterial incubation, or whether these are maintained once the bacterial stimulus is removed. An additional experiment would be to perform these experiments using LPS instead of bacteria stimuli, to understand if the differences observed are due to the bacteria and potential binding, or the initiation of inflammation with addition of LPS. LPS interacts with cell surface receptors including CD14 and TLR4 which activates pro-inflammatory pathways to produce inflammatory mediators including TNF- α , IL-1, IL-6 (Fang et al., 2004; Meng & Lowell, 1997), as well as increase chemotaxis activity and phagocytic capabilities (Wu, Chen & Chen, 2009). LPS stimulation also affects mitochondria in macrophages, inducing stimulation of mROS signalling and release (Emre et al., 2007; Wang et al., 2021), as well as increasing mitochondrial number through stimulation of mitochondrial fission by dynamin-related protein 1 (Drp1) GTPase (Kapetanovic et al., 2020). This could result in differences in the cytoskeleton, which would be interesting to investigate in COPD macrophages.

7.3.4 Examine effects of different bacterial species on macrophage cytoskeletal dynamics

The experiments in this thesis focused on the use of the gram-negative bacterium, *H. influenzae*, as it is a common respiratory pathogen associated with bacterial colonisation and inflammation in COPD (Singh, 2015). However, COPD patients are not only colonised by *H. influenzae* and so solely focusing on a single gram-negative bacterium is a limitation in this study. Another common respiratory pathogen often associated with respiratory infections in COPD subjects is *S. pneumoniae*, which is a gram-positive bacterium, explored in other studies investigating defective phagocytosis in COPD, often in tandem with *H. influenzae* (Bewley et al., 2018). The investigation of this bacterium would be beneficial to this study, as it can give additional insight into any specific differences between the two bacterial groups, and any potential differences that this causes in macrophage morphology, cytoskeletal involvement, and mitochondrial interactions in the process of phagocytosis, due to different phagocytic receptor activation. As different types of bacteria have different compositions which are recognised by different TLRs, with lipoteichoic acid (LTA) of gram-positive bacteria being recognised by TLR2, compared to LPS on gram-negative bacteria recognised by TLR4 (Fu & Harrison, 2021; Ninkovic et al., 2016). Differential bacterial binding to TLR leads to activation of different pathways, and therefore could produce differences in both morphology and phagocytosis.

7.3.5 Use super-resolved microscopy to explore differences in the cytoskeleton and mitochondria

Initially work in this thesis aimed to use automated super-resolved microscopy techniques to investigate the cytoskeleton and mitochondria in macrophages undergoing phagocytosis between non-smoker and COPD subjects. However, due to limitations and the technology still

being under development, this was not performed as part of this thesis. However, the use of super-resolved microscopy, such as STORM, to produce images below the diffraction limit of resolution, would give a greater insight into the differences uncovered in this thesis. With a particular interest into mitochondrial morphology differences, due to mitochondria size being considerably below the diffraction limit.

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