COPD-Related Modification to the Airway Epithelium Permits Intracellular Residence of Nontypeable Haemophilus influenzae and May Be Potentiated by Macrolide Arrest of Autophagy

Introduction: COPD is an inflammatory airway pathology associated with recurrent infection by nontypeable Haemophilus influenzae (NTHi) that is not effectively managed by macrolide antibiotic therapy. We hypothesised that NTHi is able to reside intracellularly within COPD-derived airway epithelial cells (AEC), and that the factors contained in cigarette smoke when coupled with exposure to erythromycin or azithromycin arrest autophagy, the principal mechanism responsible for clearing intracellular bacteria (called “xenophagy”).

Methods: Cultures of bronchial airway epithelial cells derived from control and COPD patients were differentiated at an air-liquid interface and exposed to macrolide antibiotics, 10% cigarette smoke-extract (CSE) and NTHi. Markers of autophagic flux and intracellular NTHi were assessed using Western blot analysis and transmission electron microscopy.

Results: AEC treated with macrolide antibiotics or 10% CSE exhibited a block in autophagic flux as evidenced by a concomitant increase in LC3-II and Sequestosome abundance (vs control; both \( P < 0.01 \)). While control AEC showed no clear evidence of intracellular NTHi, COPD-derived cultures exhibited abundant NTHi within the cytoplasm. Further, intracellular NTHi that were encapsulated within vesicles propagated from the apical epithelial layer to the basal cell layer.

Discussion: Taken together, our findings indicate that COPD, cigarette smoke and macrolide antibiotics potentiate the susceptibility to persistent intracellular NTHi. A major mechanism for this is arresting normal autophagic flux in airway epithelial cells. Hence, structural modifications that mitigate this off-target effect of macrolides have significant potential to clear intracellular NTHi and thereby reduce the influence of this pathogen in the airways afflicted by COPD.

Keywords: antibiotic resistance, azithromycin, intracellular bacteria, macrolide, xenophagy
individuals. Hence, disease-related modifications during COPD produce conditions that strongly favour NTHi infection. Growing evidence for an intracellular aspect to NTHi’s pathogenicity,\(^3\) aligns with the pathobiology of COPD, whereby protracted activation and injury of airway epithelial cells (AEC) allows the accumulation of intrinsic defects that promote bacterial invasion and an opportunity to exploit the intracellular environment.

Autophagy is a fundamental intracellular process whereby the autophagosome encapsulates potentially harmful cytoplasmic components and is co-digested with the cargo upon fusion with a lysosome, thus releasing biosynthetic intermediates for metabolic processes (the entire process is termed autophagic flux). This is an essential cellular response to cell stress such as starvation, oxidative stress and inflammation.\(^4\) Defective autophagic flux has been reported in several chronic lung diseases,\(^5\) although its role in COPD has not been well defined. However, important for COPD, autophagy is the primary mechanism used to clear invading bacteria, a process called xenophagy.

Xenophagy is a selective form of autophagy that rapidly digests intracellular microbes. Autophagy and xenophagy share molecular mechanisms including mTOR (primary inhibitor of initiation), AuTophaGy (ATG) related complexes that are essential for initiation of auto/xenophagic flux, ATG5, −8 and −16L1 for membrane assembly and maturation, the cargo chaperones Sequestosome, Optineurin, and NDP52, and Light Chain 3-II (LC3-II) which is essential throughout auto/xenophagic flux, LAMP2, RAB7, RAB9, and PtdIns3KC3 (many functions including lysosome formation) and IFN-γ, TLR-3/4, ICAM1, and NOD2 (microbe-host cell interactions).\(^8\)−\(^10\) However, clinically important pathogens have evolved mechanisms allowing them to evade, block and usurp the xenophagic apparatus as an essential aspect of their virulence.\(^11\) Some bacteria that infect the airways evade or usurp the xenophagic machinery by secreting inhibitory factors (eg *Listeria*),\(^1\) preventing autophagosome-lysosome fusion (eg *Legionella*), or upregulating and recruiting LC3 to facilitate their survival and replication (eg *S. aureus*).\(^10\)\(^12\) One study found NTHi can exist within late endosomes,\(^13\) and more recently NTHi was found to promote the generation of *de novo* autophagic complexes in Hep-2 cells.\(^14\)

The prevailing consensus is that autophagic flux is blocked during COPD, which is evidenced by an accumulation of autophagosomes and the inhibition of autophagic survival processes that closely corresponds with COPD-related deficits at the epithelium, including nutrient depletion, fragility, and unscheduled senescence.\(^1\)\(^15\) Further, exogenous exposures associated with COPD, including cigarette smoke and corticosteroids, are known to block autophagic flux,\(^16\) and there is a well-described defect in mitophagy (autophagic clearance of defective mitochondria) that aligns with these observations.\(^17\) Hence, in a scenario for COPD in which xenophagic flux is similarly inhibited, an increase in defective xenophagic apparatus could serve to enhance NTHi’s entry into fragile AEC and provide a niche for intracellular persistence. This is consistent with the mechanism employed by *S aureus* which usurps the xenophagic machinery, where it evades intracellular antimicrobial surveillance and remains metabolically active.\(^11\)\(^12\)

Hence, there is converging evidence that intrinsic defects and exogenous exposures synonymous with COPD potentiate host factors which may restrict xenophagic clearance of NTHi, enabling intracellular residence. Yet, there is a surprising paucity of data describing how the usually innocuous NTHi becomes an influential pathogenic vector in the context of disease, let alone COPD. We report our observations for NTHi within COPD-derived AEC unhindered by autophagic activity, and show AEC exposed to macrolide antibiotics and the factors contained in cigarette smoke exhibit a block in autophagic flux far beyond either treatment in isolation.

**Methods**

**COPD and COPD-Related Exposure Modelling of the Human Airway Epithelium**

We employed an air–liquid interface (ALI) model to approximate normal AEC-NTHi interactions and responses to exogenous exposures. This contrasts with prior studies examining NTHi infection, which have primarily utilised undifferentiated and submerged cell line systems, without a disease component or exposure, and which have yielded limited insights. Bronchial brushing for ALI culture samples were approved by the Royal Adelaide Hospital Human Research Ethics (Protocol #R20020811), and all participants provided written informed consent. Cultures were derived from controls (n=5 never smokers, one female, 40 years ±22.3 SD, FEV1/FVC 88.6% ±7) and COPD participants (n=3, one female, 63 years ±4.1 SD, FEV1/FVC 53.1% ±5).

Basal progenitor AEC stem cells collected from bronchial brushing samples were propagated at an ALI as previously described. Briefly, airway stem cells were expanded in T25
culture flasks, and then transferred to transwells and allowed
to propagate to confluence (4–6 days). Thereafter, the apical
media was removed and the basal chamber media was
replaced with differentiation media containing retinoic acid (24–28 days). Cultures were used as experimental models when the mucociliary phenotype was evident (eg Figure 2A–C), via routine light microscopy and when elec-
trical impediment of the epithelial barrier exceeded 500 Ω
cm². ALI cultures were exposed to a clinical isolate of NTHi
(24 h; MOI 50),18 cigarette smoke extract (CSE; as pre-
viously described),15 in addition to azithromycin, erythromy-
cin and bafilomycin for 24 h (Merck KGaA Inc, Darmstadt,
Germany).

Western Blot Protein Analysis
Protein was isolated from differentiated AEC within the
transwells in situ, and Western blot analysis was performed
as previously described,19 with the exception of the
Rubicon antibody (Cell Signaling Technology Inc.,
Danvers, MA, USA). Densitometry of histogram analyses
was performed using Multi Gauge software (V3.1; Fujiﬁlm,
Tokyo, Japan). Density scores were analysed using a random
effects gamma (log link) mixed-model regression to allow
for correlated treatment responses within each culture. Results
were normalised to both β-actin and the biological control
and expressed as relative abundance. The statistical analysis
was performed using R statistical software (release 3.2.3) for
n=4 control cultures.

Electron Microscopy
High-resolution host-microbe and microbe-autophagic
apparatus interactions were assessed in situ with pre-
viously described methods using transmissio
n electron
microscopy (TEM),15 and scanning electron microscopy
(SEM).20 For both, the ﬁxative agents were applied
directly to cells on the transwell at the end of the exposure
period to maintain cell morphology and preserve host-
microbe interactions. The transwell membranes were then
excised from the transwell frame using a scalpel and
embedded/mounted to the TEM mould and SEM peg
(respectively), for analysis. TEM and SEM analyses were
performed for control (n=3) and COPD (n=3) derived
AEC ALI cultures.

Results and Discussion
First, we asked whether COPD-derived AEC exhibit pro-
tracted (24 h) intracellular survival of NTHi when com-
pared to cultures derived from control participants. Control
AEC cultured with NTHi exhibit strong activation of micro-
brial clearance processes as evidenced by abundant vacu-
olated structures consistent with lysosomes and auto-
phagosomes (Figure 1). The relative efficacy of this
response meant we were unable to identify intracellular
NTHi with certainty, with the exception of irregularly
shaped electron dense structures matching the size of
NTHi (approximately 500 nm) within double membrane
envelopes (Figure 1B), and digestion products within auto-
lysosomes (Figure 1C and D). However, we cannot rule out
that intracellular NTHi was effectively cleared by a related
microbial degradative pathway before the 24 h observation
interval, and which may be determined by blocking autoph-
agy (eg ATG5 knockout) in control cells using a time
course infection protocol. Further, an apparent lack of free
NTHi within the cytosol in control AEC may also be
explained by effective mucociliary (Figure 2A and B),
barrier and defensin activity, and that stem cells from the
relatively older COPD participants (albeit non-signiﬁcant
vs controls), may contribute to intracellular infectivity.

In contrast, COPD-derived AEC were frequently observed
interacting with NTHi via villi-like cytoplasmic projections
(SEM, Figure 2C, D, and H), that appear to encapsulate
and internalise NTHi (as previously described).21 NTHi were
observed in COPD-derived AEC, either free in the cytoplasm
or encapsulated within membrane-bound vesicles, possibly
endosomes, autophagosome and/or LAPosomes (vesicles
of the LC3-Associated Phagocytosis (LAP) non-canonical autoph-
agy pathway,22 Figure 2E–G). These observations in COPD-
derived AEC support findings for intracellular NTHi identi-
fied by Woo et al in the context of otitis media.23 While it is unclear
whether an endocytic process is a requisite for NTHi internalisation, we frequently observed an interruption in a NTHi-
containing vesicle (NTH-CV), and NTHi in proximity to
empty vesicles (Figure 2E–G and I–K), indicating a potential
mechanism of vesicle escape. Indeed, in Figure 2F and K,
NTHi appears to push out from the NTHi-CV, perhaps as part
of a replicative process, as evidenced by its elongated, sig-
moid, and lobular shape. Clustering depicted in Figure 2G
is also suggestive of cell division, and aligns with the observation
of an intracellular bacterial community that was recently
applied to NTHi in a clinical model of otitis media.24
Interestingly, their close proximity to the transwell membrane
(Figure 2F and G) suggests a mode of propagation from the
apical to the basal progenitor cell population, which differenti-
ate to generate the three-dimensional epithelial architecture
that deﬁnes the ALI culture model. Given basal AEC remain
viable for an extended period, the ability to traverse the
The COPD airway is exposed to exogenous factors that modulate autophagy, such as cigarette smoke and corticosteroids,\textsuperscript{15,16} and which may also reprogram progenitor AEC towards chronic disease.\textsuperscript{25} Although macro-lide therapies are relatively efficacious for the clinical signs of NTHi infection, there is a high rate of reinfection in COPD, and there are macrolide-resistant strains. Further, macrolides are known to modulate autophagy. Rapamycin and baflomycin have well established pro- and anti-autophagic effects (respectively); however, less is known about the macrolides indicated for respiratory infections. Indeed, of the two studies which examine azithromycin in secondary-cell models of airway disease, one reports an increase in autophagic flux in smooth muscle compartment may partly explain the clinical persistence of NTHi within the COPD airways, whereby exacerbating factors lead to epithelial fragility and senescence that allows a reservoir of NTHi to express virulence factors after a period of host-cell adaption.\textsuperscript{11} Importantly, we were unable to definitively resolve NTHi within a double membrane bilayer (the diagnostic feature of an autophagosome; eg Figure 1B and D), and NTHi-CVs were not observed interacting with lysosomes, even when in proximity to autophagic activity (Figure 2F). As autophagy/xenophagy is usually an efficient process,\textsuperscript{11} these observations suggest that the inhibition of autophagic flux in the context of COPD may be a primary disease-related phenomena that permits intracellular residence, and resistance to clinical interventions.

Figure 1 Control-derived airway epithelial cells co-cultured with NTHi exhibit frequent autophagic events, and that may clear invading bacteria before they can be clearly identified within the cytoplasm. Control human air–liquid interface cultures exhibiting mucociliary differentiation and three-dimensional growth characteristics were assessed. (A) An airway epithelial cell (AEC) exhibits active degradative processes after 24 h co-culture with NTHi, and which may represent the final stages of clearing intracellular NTHi. Extracellular NTHi is also observed above the apical aspect of the cell (white arrow). (B) Multiple double membrane structures (white arrows), indicative of autophagosomes containing a circular cargo of similar morphology and size (approx. 500 nm) as NTHi, but which lack the distinct morphology observed for NTHi identified within COPD-derived AEC. Also shown is a double membrane phagophore (double white arrows). (C) Another example of an AEC which exhibits degradative activity. Here autolysosomes are observed at the final stage of a degradative process (white box). (D) The autolysosomes are magnified in D (two are indicated with single white arrows). Further in D, a nearby autophagosome (double white arrow) is also resolved. Data is representative of n=3 control AEC donor cultures.
cells for asthma, and the other a decrease in flux and elevated bacterial load in macrophages in the context of cystic fibrosis.27

Hence, we asked whether azithromycin or erythromycin, with or without CSE, can elicit a reduction in autophagy in primary differentiated AEC. We observed a restriction in autophagic flux for either macrolide or CSE-exposed AEC as evidenced by a significant increase in the essential autophagy protein LC3-II (all P<0.05), concomitant with an increase in the autophagy receptor, Sequestosome, which is normally co-degraded with the cargo it chaperones to the autophagosome (Figure 2L–M, and see Figure 3). While the macrolide-induced elevation in Sequestosome did not reach significance, a reduction of this autophagy receptor would be expected with a significant increase in LC3-II (which should also be degraded during autophagy) if autophagic activity is uninhibited. As expected, CSE elicited a moderate blockade of autophagic flux, with concomitant and significant increases in both LC3-II and Sequestosome. In contrast, the combination of macrolide/CSE resulted in an appreciable block in autophagy, with a significant increase in both LC3-II and Sequestosome for each exposure (consistent with a log-additive effect). The block in autophagy was most pronounced for 50 mg/mL azithromycin/CSE (LC3-II +38.3-fold, CI95% [18.99–77.06], P<0.001; Sequestosome +3.3-fold, CI95% [1.65–6.68], P=0.001), compared to that of bafilomycin (10 nM; LC3-II +22.3-fold, CI95% [11.09–45.00], P=0.001; Sequestosome +4.9-fold, CI95% [2.43–9.86], P<0.001), which is a potent inhibitor of autophagy and a positive control (Figure 2L–M). Conversely, while Rubicon was also

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**Figure 2** COPD-derived airway epithelial cells and COPD-related exposures exhibit intracellular *N. Haemophilus* and arrested autophagic flux, respectively. Control and COPD human air-liquid interface cultures exhibiting mucociliary differentiation and three-dimensional growth characteristics were assessed. (A–K) Transmission and scanning electron micrographs (TEM/SEM, respectively) of differentiated cultures infected with *N. Haemophilus*. (A and B) Control cells possess abundant cilia (C), villi (V) and apical-stalateral interaction via tight junctions (TJ). Intracellular *N. Haemophilus* was not observed within AEC after 24 h co-culture (electron dense puncta are cytosolic granules, lysosomes and mitochondria), perhaps due to exclusion from the villi by mucociliary activity/interactions. C and D. COPD-derived cultures associate with *N. Haemophilus* via exposed villi that appear to approximate towards the bacteria (C, dashed box; that is enlarged in H (SEM)), and that are resolved with TEM (D) as membrane projections and pits that interact with *N. Haemophilus*. (E) COPD-derived cultures exhibit *N. Haemophilus* in the cytoplasm (white arrows), that interact with vesicular structures (dashed box). Magnified images for D (I and J) show the NTHi cell wall remains defined while within vesicles. (F) NTHi (white arrows) within proximity to the transwell membrane (TWM) indicates a mode to passage from liquid interface cultures exhibiting mucociliary differentiation and three-dimensional growth characteristics were assessed. (J and K) and (Poh et al B – K fl – K fl.) Western blot analysis for canonical (LC3-I/II and Sequestosome) and non-canonical (Rubicon) autophagy factors demonstrate a block in autophagic flux after exposure to 10% cigarette smoke extract (CSE), and the macrolides azithromycin (Az, μg/mL) and erythromycin (Ery, μg/mL). Co-exposure with CSE and either macrolide produces a striking block in flux as evidenced by an increase in LC3-III concomitant with elevation of the autophagy receptor Sequestosome which is normally co-degraded with the cargo during unrestricted autophagy. Exposure to bafilomycin (10 nM) was included as a positive control for the blockade of autophagic flux. Rubicon, an essential component for LC3-Associated Phagocytosis and a negative regulator of canonical autophagy, is moderately upregulated by CSE. (M) Quantitative analysis of protein expression confirms a strong increase in LC3-II and Sequestosome with the combination treatments, that approximates the effect observed for bafilomycin for the Az/CSE exposure. Protein expression is expressed as relative abundance and normalised to the untreated sample and β-actin. Intervals are 95% CI, and results are significant if the confidence intervals do not cross 1, and significant between exposures when CI do not overlap. Exposure period was 24 h for n=4 primary cultures from control individuals.

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significantly increased following CSE exposure (P<0.05), its abundance was not substantively influenced by either macrolide, suggesting distinct regulation of LAP vs canonical autophagy in the context of these exposures. LAP is known to suppress canonical autophagy and favour internalisation and infectivity of a number of respiratory pathogens,\textsuperscript{22} which is consistent with our observations.

**Conclusion**

For the most part, current therapeutic options for COPD cannot reverse disease progression after smoking cessation, and it is of concern that some therapeutics may contribute to this phenomenon. Here we show that differentiated cultures of COPD-derive primary AEC permit intracellular residence of NTHi (vs control), and that the macrolide antibiotics erythromycin and azithromycin elicit a potent block in autophagic flux, particularly as a co-exposure with CSE (summarised in Figure 3). Taken together, our findings raise questions regarding the use of macrolide antibiotics to manage NTHi in current smokers, particularly as the epithelium in these individuals are already accumulating defects that potentiate autophagic insufficiency. An essential next question is whether there exists a causative link consistent with COPD, and COPD-related exposures that arrest autophagic flux, as primary factors that effect a block in xenophagic clearance of intracellular NTHi. So while modification of the antibiotic properties of macrolides to curtail the emergence of resistant strains is a high priority,
the additional therapeutic challenge is to address the chemical properties of macrolides that produce off-target effects that limit autophagy.

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Disclosure

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