


Inhibition of LC3-associated phagocytosis in COPD and in response to cigarette smoke

Patrick F. Asare , Hai B. Tran, Plinio R. Hurtado, Griffith B. Perkins, Phan Nguyen, Hubertus Jersmann, Eugene Roscioli and Sandra Hodge

Ther Adv Respir Dis

2021, Vol. 15: 1–15

DOI: 10.1177/
17534666211039769

© The Author(s), 2021.

Article reuse guidelines:
sagepub.com/journals-
permissions

Abstract

Introduction/Rationale: In chronic obstructive pulmonary disease (COPD), defective macrophage phagocytic clearance of cells undergoing apoptosis by efferocytosis may lead to secondary necrosis of the uncleared cells and contribute to airway inflammation. The precise mechanisms for this phenomenon remain unknown. LC3-associated phagocytosis (LAP) is indispensable for effective efferocytosis. We hypothesized that cigarette smoke inhibits the regulators of LAP pathway, potentially contributing to the chronic airways inflammation associated with COPD.

Methods: Bronchoalveolar (BAL)-derived alveolar macrophages, lung tissue macrophages obtained from lung resection surgery, and monocyte-derived macrophages (MDM) were prepared from COPD patients and control participants. Lung/airway samples from mice chronically exposed to cigarette smoke were also investigated. Differentiated THP-1 cells were exposed to cigarette smoke extract (CSE). The LAP pathway including Rubicon, as an essential regulator of LAP, efferocytosis and inflammation was examined using western blot, ELISA, flow cytometry, and/or immunofluorescence.

Results: Rubicon was significantly depleted in COPD alveolar macrophages compared with non-COPD control macrophages. Rubicon protein in alveolar macrophages of cigarette smoke-exposed mice and cigarette smoke-exposed MDM and THP-1 was decreased with a concomitant impairment of efferocytosis. We also noted increased expression of LC3 which is critical for LAP pathway in COPD and THP-1 macrophages. Furthermore, THP-1 macrophages exposed to cigarette smoke extract exhibited higher levels of other key components of LAP pathway including Atg5 and TIM-4. There was a strong positive correlation between Rubicon protein expression and efferocytosis.

Conclusion: LAP is a requisite for effective efferocytosis and an appropriate inflammatory response, which is impaired by Rubicon deficiency. Our findings suggest dysregulated LAP due to reduced Rubicon as a result of CSE exposure. This phenomenon could lead to a failure of macrophages to effectively process phagosomes containing apoptotic cells during efferocytosis. Restoring Rubicon protein expression has unrecognized therapeutic potential in the context of disease-related modifications caused by exposure to cigarette smoke.

Keywords: cigarette smoke extract, efferocytosis, inflammation, LC3-associated phagocytosis, Rubicon

Received: 12 January 2021; revised manuscript accepted: 20 July 2021.

Introduction

The burden of chronic obstructive pulmonary disease (COPD) is a major global health issue, and is set to become the third leading cause of death worldwide.¹ The consumption of cigarettes is a primary factor causing the sustained inflammation observed in COPD which continues even after the cessation of smoking.^{2–4} Inflammation drives

COPD pathogenesis and the destruction of lung tissues.^{5,6} Defective phagocytic clearance of apoptotic cells (efferocytosis) can perpetuate the unrestricted airway inflammation that participates in the development and progression of COPD.

The clearance of potentially harmful cells and debris by efferocytosis is a primary process that

Correspondence to:

Sandra Hodge
Department of Thoracic
Medicine, Faculty of Health
and Medical Science, The
University of Adelaide,
Adelaide, SA 5005,
Australia
sandra.hodge@adelaide.edu.au

School of Medicine, The
University of Adelaide,
Adelaide, SA, Australia

Department of Thoracic
Medicine, Royal Adelaide
Hospital, Adelaide, SA,
Australia

Patrick F. Asare
Hai B. Tran
Department of Thoracic
Medicine, School of
Medicine, The University
of Adelaide, Adelaide, SA,
Australia

Phan Nguyen
Hubertus Jersmann
Department of Thoracic
Medicine, Royal Adelaide
Hospital, Adelaide, SA,
Australia

Eugene Roscioli
Department of Thoracic
Medicine, School of
Medicine, The University
of Adelaide, Adelaide, SA,
Australia

Royal Adelaide Hospital,
Adelaide, SA, Australia

Plinio R. Hurtado
Department of Renal
Medicine, School of
Medicine, The University
of Adelaide, Adelaide, SA,
Australia

Royal Adelaide Hospital,
Adelaide, SA, Australia

Griffith B. Perkins
Department of Molecular
and Cellular Biology,
School of Biological
Sciences, The University
of Adelaide, Adelaide, SA,
Australia

prevents inflammation and inappropriate immune activation.⁷ Cigarette smoking disrupts this homeostatic process which can lead to the release and protracted exposure of harmful intracellular contents of apoptotic cells into pulmonary micro-environment.^{8,9} Hence, potentiating the efferocytic capacity of airway macrophages and other immune cells is important to maintain normal function of lung tissues. Evidence of this is the increased expression of phagocytic receptors such as MERTK in alveolar macrophages of COPD patients, linked to an increase in cellular turnover and/or efferocytosis demand.¹⁰ Nonetheless, efferocytosis is dysregulated in airways of COPD patients and is considered a homeostatic process that needs to be restored to prevent disease progression.¹¹ The precise reasons underpinning dysregulated efferocytosis in COPD and cigarette smokers remain unclear and studies that elucidate the mechanisms involved have significant promise to inform new therapeutic interventions.

Efficient efferocytosis has recently been shown to rely upon LC3-associated phagocytosis (LAP).¹² LAP involves trafficking LC3 to phagosomes for the formation of a single membrane vesicle known as LAPosome.¹³ In general, lysosomal processing and the removal of apoptotic cells is optimized with the activation/involvement of the LAP pathway.^{12,14} To initiate LAP during efferocytosis, Rubicon (RUN domain Beclin-1 interacting cysteine-rich domain containing) is engaged by phagosomes to favour LC3 recruitment to phagosome membranes.¹⁵ Hence, LAP is normally stimulated in Rubicon-sufficient phagocytes to modulate anti-inflammatory consequences of efferocytosis. In line with this, Martinez and colleagues¹² reported that an abundance of Rubicon enhances efferocytosis and the synthesis (or secretion) of anti-inflammatory mediators such as IL-10, IL-4, and IL-13 while limiting the release of pro-inflammatory cytokines including IL-1 β , TNF- α , and IL-6. Conversely, Rubicon-deficient cells failed to recruit LC3 to the phagosome, leading to a failure of lysosomal acidification and a subsequent accumulation of apoptotic cells.^{12,15} Rubicon can also regulate inflammation independent of LAP by interacting with CARD9 to inhibit TNF- α production. Given these findings for Rubicon, it can be considered a major homeostatic regulator that acts as a sentinel for appropriate inflammatory responses.

Hence, there is significant evidence that Rubicon is a primary regulator of cellular clearance

mechanisms, and therefore, its normal levels in the cell are consistent with effective efferocytosis.¹⁴ This suggests that dysregulation of the mechanisms responsible for the expression of Rubicon can have detrimental consequences in diseases where sustained immune activation and uncontrolled inflammation play a major role, as observed in the airways exposed to cigarette smoke and for COPD. Hallmarks of Rubicon deficiency include protracted inflammation and circulating autoantibodies caused by the leakage of uncleared apoptotic, both of which are clinical characteristics of COPD.^{16,17} It is not yet completely understood how alveolar macrophages of COPD patients regulate the abundance of Rubicon or whether a reduction in Rubicon is linked to the accumulation of apoptotic cells observed in airways of COPD patients.¹⁶ The capacity for airway macrophages to express Rubicon, especially during their responses to unscheduled apoptosis driven by cigarette smoking remains undefined.

We hypothesized that a reduction of Rubicon is linked with the defective efferocytosis in COPD and is potentiated as a response to exposure to cigarette smoke. Here, we comprehensively identify a reduction in Rubicon abundance *in vivo* using alveolar macrophages from COPD patients and a mouse cigarette-exposure model, and *in vitro* using THP-1 macrophages and blood monocyte derived macrophages (MDM) exposed to cigarette smoke extract (CSE). Furthermore, we elucidated a relationship between Rubicon expression, efferocytosis and inflammation after cigarette smoke exposure. Our finding for Rubicon point towards the dysregulation of efferocytosis due to LAP-insufficiency as a phenomenon that contributes to the pathogenesis of COPD.

Results

Macrophages in COPD and in models of exposure to cigarette smoke have reduced protein expression of Rubicon

THP-1 macrophages and MDM obtained from healthy donors were exposed to 10% CSE for 24 h. We observed a decrease in Rubicon expression in both cell types compared to air-treated control macrophages for Western blot analysis (Figure 1(a), $p < 0.0001$; Figure 1(b), $p = 0.0022$). We also identified a significant reduction of Rubicon in alveolar macrophages

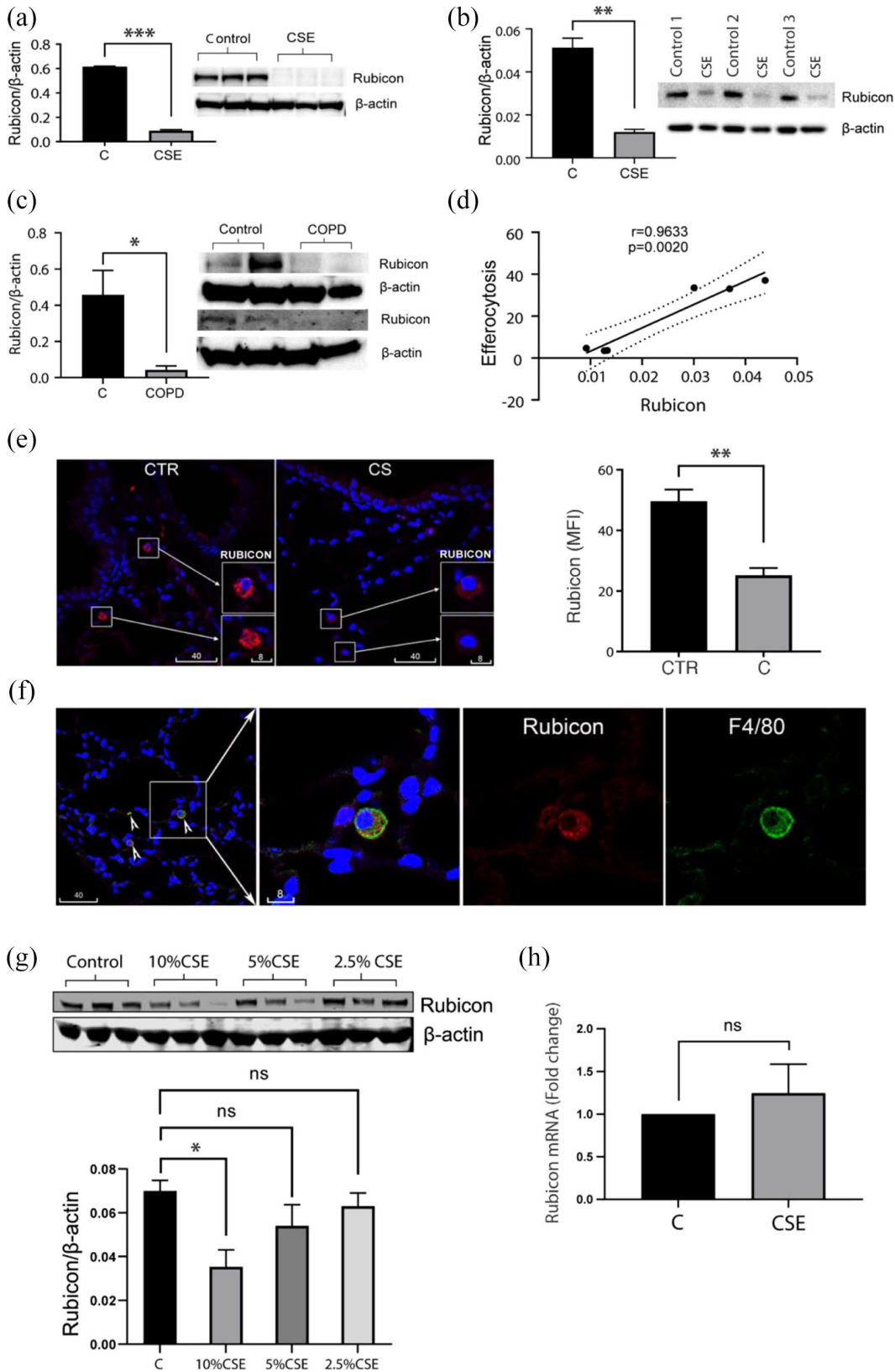


Figure 1. BAL macrophages of COPD patients and macrophages exposed to CSE exhibit a deficiency in Rubicon expression (a) Protein analysis of Rubicon protein expression; Control (C) vs cigarette smoke extract

Figure 1. (Continued)

(CSE). CSE reduced Rubicon expression in blood monocyte-derived macrophages **** $p < 0.0001$; mean \pm SEM, $n = 3$. 'Control' represents different donors' macrophages which were not exposed to cigarette smoke extract, while 'CSE' represents different donors' macrophages exposed to cigarette smoke extract (b) Western blots (and quantitative data) of Rubicon protein expression; Control (C) vs Cigarette Smoke Extract (CSE). CSE reduced Rubicon protein expression in THP-1 macrophages. ** $p = 0.0022$; mean \pm SEM, $n = 6$. (c) Western blots (and quantitative data) of Rubicon protein expression in BAL-derived macrophages, non-COPD (CTR) vs COPD. Rubicon is significantly downregulated in COPD alveolar macrophages * $p = 0.0286$. Mean \pm SEM, $n = 4$ for each group. (d) Correlation between Rubicon protein expression and % efferocytosis. Data show a significant correlation between efferocytosis and protein expression of Rubicon ($p = 0.002$, $r = 0.9633$). (e) Mean fluorescence intensity of Rubicon is decreased in alveolar macrophages of cigarette smoke treated mice. Data are expressed as mean \pm SEM, $n = 6$; ** $p = 0.0022$. (f) Immunolocalization of Rubicon in mouse lung. Mouse lung tissue sections were co-labelled for Rubicon (red, AF594), macrophage marker F4/80 (pseudogreen, AF647), and DAPI (pseudoblue). Yellow colour (arrowheads) indicates Rubicon colocalization with F4/80. High magnification of the boxed area depicts a representative alveolar macrophage distinctive from pneumocytes by large cytoplasm and localisation within the air space of an alveolus. Scale bars are micrometres. (g) Protein analysis of Rubicon protein expression; Control (C) vs different concentrations of CSE (10% CSE, 5% CSE and 2.5% CSE). CSE reduced Rubicon expression in dose-dependent manner, * $p = 0.0382$ for C vs 10% CSE; $p = \text{mean} \pm \text{SEM}$, $n = 3$. (h) *Rubicon* gene transcription is unaffected by CSE, $p > 0.999$, $n = 4$ for each group.

derived from COPD participants compared to non-COPD subjects (Figure 1(c), $p = 0.0286$). Further correlative analysis demonstrated that the reduction in Rubicon protein expression positively correlated with the percentage of macrophages efferocytosing apoptotic cells (Figure 1(d), $p = 0.002$, $r = 0.9633$). Evaluating the expression levels of Rubicon in alveolar macrophages of cigarette smoke-exposed mice, we showed that the alveolar macrophages exhibited a similarly decreased abundance of Rubicon vs alveolar macrophages from the air-exposed control group (Figure 1(e), $p = 0.0022$). Examining the dose-dependent effect of cigarette smoke on Rubicon protein expression, we showed that cigarette smoke reduces Rubicon protein expression in a dose-dependent manner (Figure 1(g), $p = 0.0382$ for C vs 10% CSE; $p = 0.3388$ for C vs 5% CSE; $p > 0.999$ for C vs 2.5% CSE). We further examined the effect of CSE on *Rubicon* gene expression in THP-1 macrophages. The outcome of the study shows that the factors in cigarette smoke have no significant effect on *Rubicon* gene expression (Figure 1(h), $p > 0.999$).

CSE induces apoptosis in THP-1 macrophages

We next investigated the effects of 10% CSE extract on expression levels of cleaved PARP and caspase 3 using western blot analysis. Increased cell death in CSE-treated macrophages was evident by a significant increase in the cleavage of PARP and caspase 3 after 24 h of CSE treatment

when compared to the control group (Figure 2(a), $p = 0.0087$; Figure 2(b), $p = 0.0003$). In line with this *in vitro* data, immunostaining of lung sections from cigarette smoke-exposed mice vs controls demonstrated increased expression of PARP (Figure 2(c), $p = 0.0087$) in alveolar macrophages. In addition, CSE-treated macrophages showed higher expression levels of TIM-4 (Figure 2(d), $p = 0.0158$), a key phagocytic receptor required for removing apoptotic cells.

BAL macrophages and cigarette smoke-exposed THP-1 macrophages exhibit features that characterize defective LAP and autophagy

Immunofluorescence revealed increased LC3 in macrophages from COPD patients compared with control participants (Figure 3(a), $p = 0.0118$). Western blot analysis showed higher total LC3, LC3-II and LC3-I expressions and a nonsignificant increase of LC3-II/LC3-I in THP-1 macrophages exposed to 10% CSE for 24 h compared to the air treated control THP-1 macrophages (Figure 3(b); $p = 0.0022$, $p = 0.0006$; $p = 0.0022$, $p = 0.0649$, respectively). We also noted a significant increase in P62 protein abundance in CSE-treated THP-1 macrophages compared to control (Figure 3(c), $p = 0.0022$). We further examined the effect of cigarette smoke extract on expression of Atg5 and NOX2 which are critical for efferocytosis and LC3 trafficking to phagosome membranes using western blot. Data analysis showed significantly higher Atg5 protein expression (Figure 3(c), $p = 0.0159$) and nonsignificantly

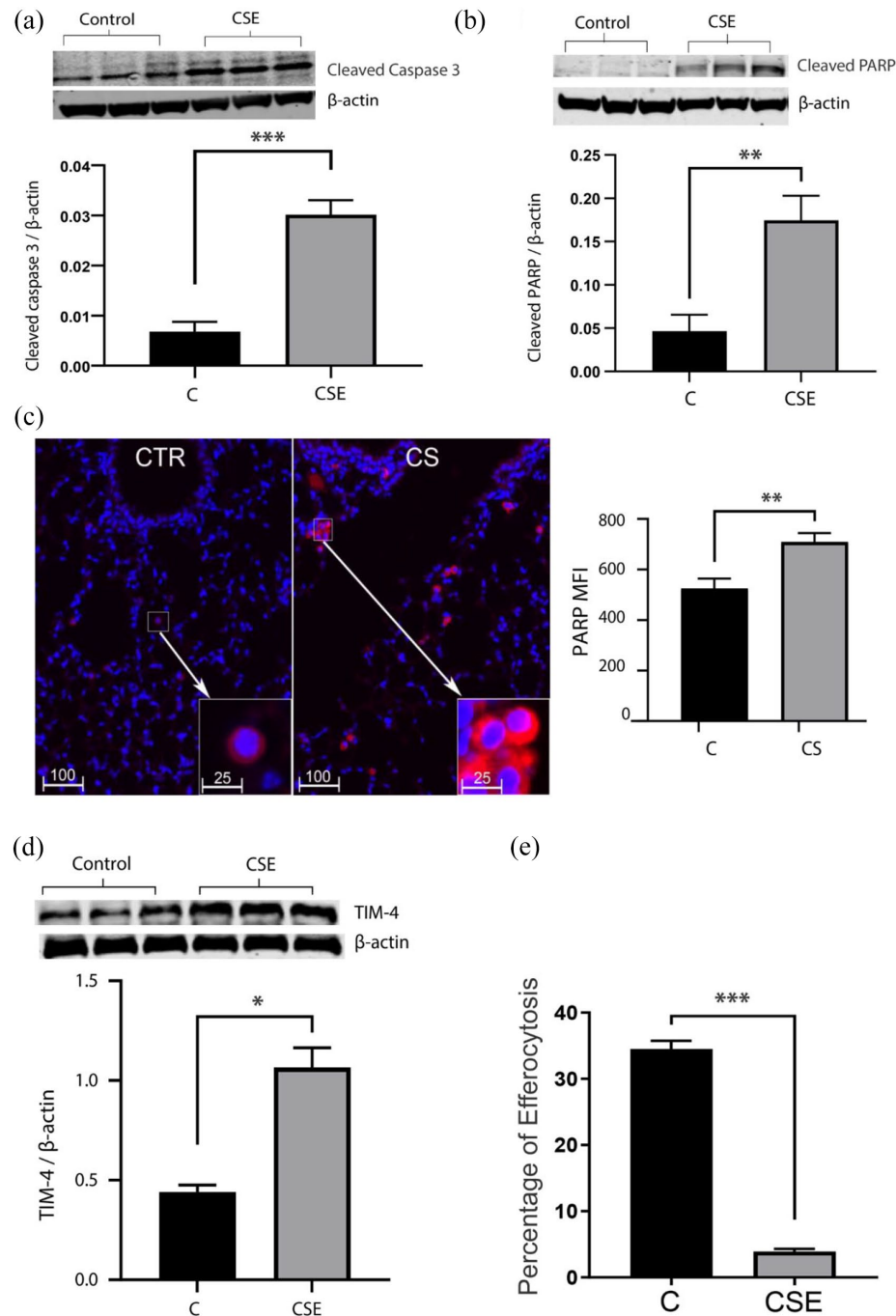


Figure 2. Exposure to cigarette smoke induces apoptosis: (a) Western blots (and quantitative data) of PARP protein expression; Control (C) vs Cigarette smoke extract (CSE). The expression of cleaved PARP in macrophages increased after 24 h of CSE treatment. $**p = 0.0087$. Data are expressed as mean \pm SEM, $n = 6$. (b) Western blots (and quantitative data) of cleaved caspase 3 protein expression; Control (C) vs CSE. Induction of apoptosis by CSE was evidenced by a significant increase in the expression of cleaved caspase 3 in THP-1 differentiated macrophages after 24 h of CSE treatment. $***p = 0.0003$. Data are expressed as mean \pm SEM, $n = 3$. (c) Representative immunostaining (and quantitative data) of PARP protein expression in alveolar macrophages of mice; Control (CTR) vs CS. $**p = 0.0087$. Data are expressed as mean \pm SEM, $n = 6$ animals per group. (d) Western blots (and quantitative data) of phosphatidylserine receptor, TIM-4 protein expression; Control (C) vs CSE. The expression of TIM-4 protein in macrophage increased after 24 h of CSE treatment. $*p = 0.0158$. Data are expressed as mean \pm SEM, $n = 3$. (e) Representative image showing a decline in efferocytosis after 24 h of CSE treatment. Control (C) vs CSE. $***p = 0.0003$. Data are expressed as mean \pm SEM, $n = 3$.

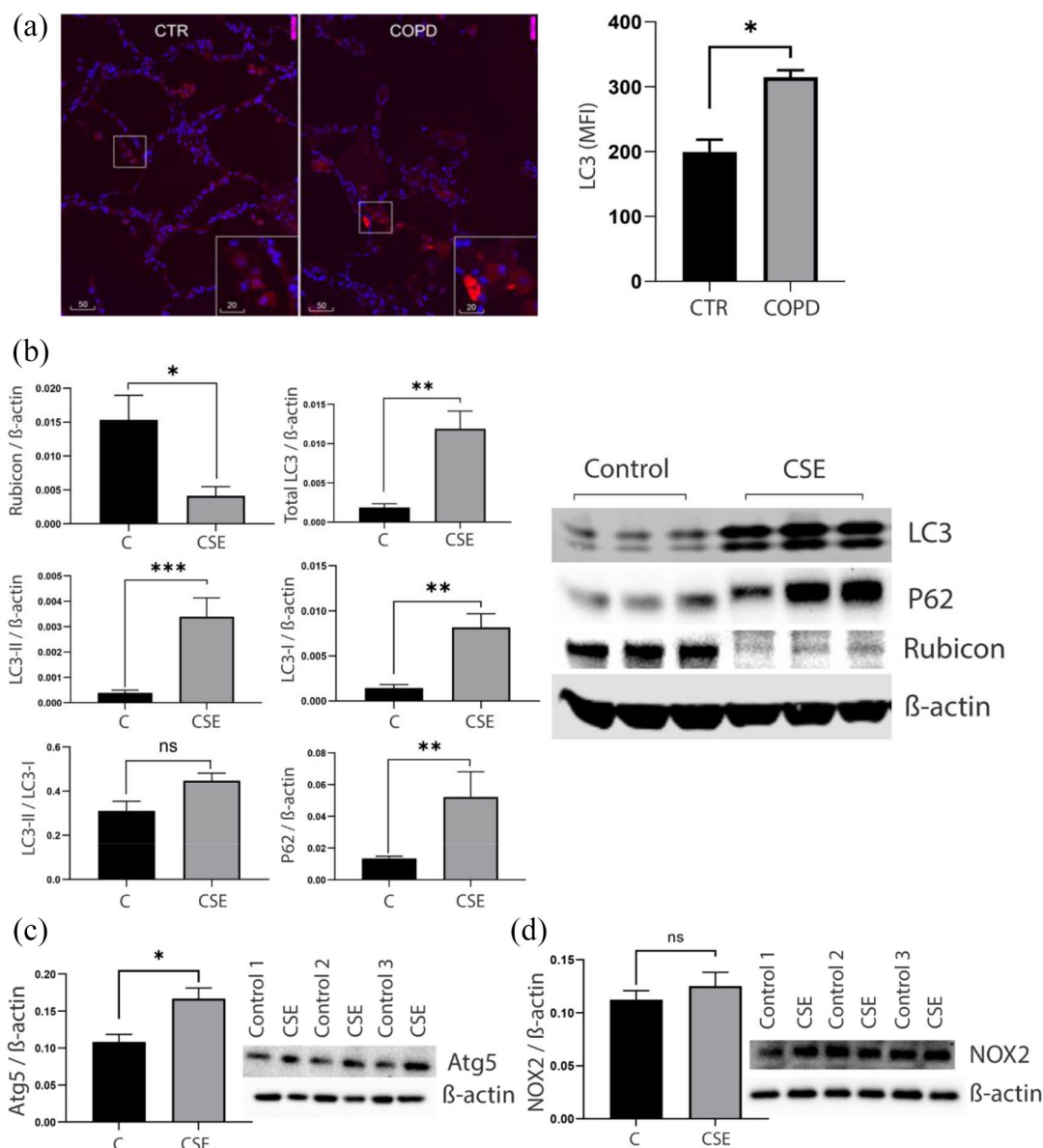


Figure 3. COPD lung macrophages exhibit features of defective LAP. (a) Representative immunofluorescence images of LC3 in lung macrophages from lobectomy biopsies of a non-COPD (CTR) vs COPD patient. The fluorescence intensity of LC3 in COPD lung macrophages is higher compared to those in control participants. $*p = 0.0118$, mean \pm SEM, $n = 3$ in each group. (b) Western blots (and quantitative data) of LC3-II and P62 protein and Rubicon expression; Control (C) vs cigarette smoke extract (CSE). The expression level of P62 significantly increased in CSE treated THP-1 macrophages compared to control. $**p = 0.0022$; mean \pm SEM, $n = 6$. 'Control' represents macrophages that were not exposed to cigarette smoke extract. The expression level of total LC3, LC3-I and LC3-II but not LC3-II/LC3-I significantly increased in CSE treated THP-1 macrophages compared to control ($**p = 0.0022$, $***p = 0.0006$; $p = 0.0022$, $p = 0.0649$, respectively; mean \pm SEM, $n = 6$). 'Control' represents macrophages that were not exposed to cigarette smoke extract. Rubicon protein was significantly reduced in CSE treated macrophages compared to control; $*p = 0.0152$; mean \pm SEM, $n = 6$. 'CSE' represents macrophages exposed to CSE on different days. (c) Western blots (and quantitative data) of Atg5 protein expression; Control (C) vs cigarette smoke extract (CSE)-exposed THP-1 macrophages. The expression was significantly elevated after 24 h of CSE treatment. $*p = 0.0159$; mean \pm SEM, $n = 6$. (d) Western blots (and quantitative data) of NOX2 protein expression; Control (C) vs cigarette smoke extract (CSE). The expression of NOX2 nonsignificantly increased in THP-1 macrophages after 24 h of CSE treatment; $p = 0.6991$, mean \pm SEM, $n = 6$.

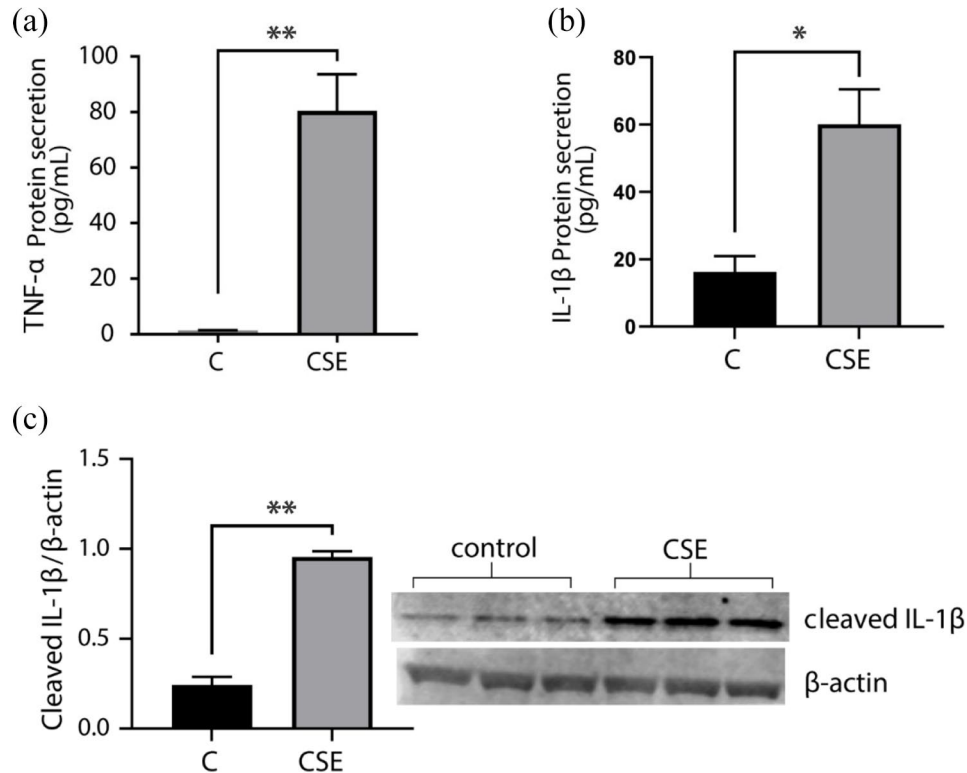


Figure 4. Cigarette smoke extract exposure increases pro-inflammatory marker levels. (a) ELISA results of increased secretion levels of TNF- α in THP-1 macrophages exposed to CSE for 24 h; Control (C) vs Cigarette smoke extract (CSE). Data are expressed as mean \pm SEM, $n = 3$. $**p = 0.0039$. (b) ELISA results of increased secretion levels of IL-1 β in THP-1 macrophages exposed to CSE for 24 h; Control (C) vs cigarette smoke extract (CSE). Data are expressed as mean \pm SEM, $n = 5$. $*p = 0.0159$. (c) Western blot results showing higher expression of intracellular IL-1 β in CSE exposed THP-1 macrophages after cigarette smoke exposure. Data are expressed as mean \pm SEM, $n = 3$; $***p = 0.0004$.

increased NOX2 levels (Figure 3(d), $p = 0.6991$) in THP-1 macrophages exposed to 10% CSE for 24 h compared to the air treated control THP-1 macrophages. Nonetheless, CSE-treated macrophages were defective in their capacity to efferocytose apoptotic cells compared with the control group (Figure 3(e), $p = 0.0003$).

CSE increases the intracellular expression and secretion of pro-inflammatory mediators

The pro-inflammatory mediator TNF- α was significantly increased in THP-1 macrophages exposed to 10% CSE for 24 h compared to air-treated controls (Figure 4(a), $p = 0.0159$). Consistent with previous studies,¹⁸ intracellular and extracellular IL-1 β was observed in control THP-1 macrophages but about four times higher in THP-1 macrophages exposed to cigarette smoke extract (Figure 4(b) and (c); $n = 3$;

$***p = 0.0004$ and $n = 5$; $*p = 0.0159$, respectively). Anti-inflammatory mediators such as IL-10 and IL-4 were all below detection levels (data not shown).

Discussion

The findings of the present study suggest a deficiency of Rubicon in BAL macrophages of COPD patients and in response to cigarette smoke exposure. The observation that exposure to cigarette smoke has no effect on *Rubicon* gene transcription suggests that Rubicon deficiency in COPD/response to cigarette smoke exposure may be resulted from a protein degradation pathway. Further observation of positive correlation between Rubicon and efferocytosis led us to speculate that downregulation of Rubicon may contribute, at least in part, to the impaired efferocytosis that is often observed in association with COPD

and cigarette smoke exposure. Accumulation of apoptotic cells in alveolar macrophages and epithelial cells of cigarette smoke exposed animals has been well documented.^{19,20} The persistence of apoptotic debris is implicated in the pathogenesis of inflammatory diseases and exacerbation of COPD.^{8,21,22} Our findings support a role for dysregulated LAP as one mechanism for these effects and as a potential therapeutic target.

Apoptotic cells can help direct their own clearance by presenting cell surface signals that are recognized by phagocytic receptors which can trigger LAP.¹⁴ In agreement with previous reports, we noted increased cell death in CSE-treated THP-1 macrophages as evidenced by activation of PARP and caspase 3.^{19,23–26} TIM4 is a phagocytic receptor that participates in cargo recognition during LAP by binding to surface phosphatidylserine that is flipped to the outer membrane of apoptotic cells.²⁷ Therefore, our findings of a concomitant increase of TIM-4 in CSE-treated THP-1 macrophages suggests a mechanism for promoting LAP and efferocytosis as a compensation for the reduction of other phagocytic receptors.^{10,28,29} However, the increased expression of LC3-II, which should be cleared with the cargo when LAP is effective, may point to potential defects in degradative flux and is consistent with the absence of Rubicon. Indeed, Cunha and colleagues³⁰ have shown that TIM-4 and Rubicon co-operate in LAP to promote efferocytosis. In our subsequent studies, we will experimentally restore Rubicon in macrophages exposed to cigarette smoke to identify a functional link as this relates to LAP-associated clearance of apoptotic debris for COPD.

Increases in Atg5 and NOX2 expression are permissive of LC3-phagosome interactions, and subsequent biosynthesis of the LAPosome. However, cells deficient in Rubicon exhibit phagosomes that incorporate Atg5 and NOX2 to enhance LC3-II recruitment that initiates canonical autophagy instead of LAP,^{31,32} thereby reducing the frequency of lysosomes-phagosomes interactions.⁸ This adds weight to a previous report of higher levels of LC3 recruited to autophagosome membranes in alveolar macrophages of cigarette smokers and COPD patients.³³ Thus, while further work is needed to delineate between LAP and canonical autophagy in this scenario, Rubicon depletion as a result of cigarette smoke exposure may cause a potential switch of LC3 fusion from phagosome to

autophagosome membranes. This is consistent with the report by Yamamuro and colleagues³⁴ that aging in adipocytes leads to loss of Rubicon and autophagy activation. The excessive autophagy due to Rubicon deficiency is associated with metabolic disorders in adipocytes as well as kidney proximal tubular epithelial cells.^{34,35} These reports demonstrate a potential crosstalk between the decline of Rubicon and metabolic disorders which is commonly observed in COPD.³⁶ However, the concomitant accumulation of LC3-II and P62 levels in CSE treated THP-1 macrophages shows that exposure to cigarette smoke impairs autophagy despite the loss of Rubicon. This further suggests that cigarette smoke exposure impairs both LAP and autophagy, two reciprocal but distinct phenomena essential for several biological activities. Nonetheless, these findings highlight an underappreciated interplay between Rubicon and cellular metabolism. However, incompletely understood is whether the defective efferocytosis at least in part due to Rubicon depletion could contribute to metabolic disorders in COPD. Therefore, the outcome of the present study further opens up new avenue to delineate the potential association between Rubicon deficiency and metabolic disorders in COPD, especially as recent studies provided a link between efferocytosis and phagocyte metabolism.^{37,38}

In the absence of Rubicon or LAP, toxic intracellular contents often leak out of uncleared dying cells to cause inflammatory responses including the production of TNF- α and IL-1 β .^{12,39,40} While abundance of Rubicon prevents inflammatory responses, Rubicon depleted mice produce markedly higher amount of TNF- α and IL-1 β .^{41–43} This is consistent with the observation of defective efferocytosis and a concurrent increase in TNF- α and IL-1 β in CSE-treated THP-1 macrophages. Recent reports show that Rubicon can also regulate TNF- α production through LAP independent pathways.⁴¹ This underscores the physiological function of Rubicon and suggests that Rubicon deficiency may at least in part be causative to altering wide array of inflammation marker levels perpetuated by cigarette smoking. Hence, the outcome of this study highlights the need to further elucidate the link between Rubicon and inflammation in the context of COPD and response to cigarette smoke exposure. Our study characterizes Rubicon deficiency as a possible contributory factor for inflammation-related effects of cigarette smoke. Particularly important is that Rubicon expression

is required for phagocytosis of pathogens and the defence against *Salmonella*, and *Streptococcus pneumoniae* infections.^{42,44} Therefore, future studies that examine the association between Rubicon depletion and dysregulated phagocytosis by cigarette smoke in other cell types such as dendritic cells could provide extended information for therapeutic targets. Moreover, this study provides the basis for future studies to elucidate the causative association between Rubicon deregulation and COPD exacerbation.

Limitations of the study

Future studies will address these limitations of the study: (1) The need to irreversibly overexpress or knockout Rubicon to examine whether Rubicon deficiency is central to the injurious outcome of cigarette smoking and/or COPD/emphysema; (2) Evaluation of how CSE alters the protein expression of Rubicon (whether by inducing the degradation of the protein through proteasome-ubiquitin or lysosomal degradation pathway); and (3) Delineation between autophagy and LAP in the context of COPD.

Concluding remarks

This study addresses the mechanisms of defective efferocytosis in lung and airway macrophages in COPD and in response to cigarette smoke. For the first time, deregulation of the LAP pathway in COPD and in response to cigarette smoke exposure was identified. Decreased expression of the key LAP mediator, Rubicon, was shown to correlate with both reduced efferocytosis and increased inflammation. We conclude that the impaired efferocytosis that we have previously reported in COPD results at least in part from deficiency of the LAP pathway, potentially leading to an accumulation of apoptotic cells and subsequently, release of inflammatory mediators. It is likely that Rubicon deficiency is involved in the deleterious consequences of cigarette consumption and that restoring Rubicon protein levels could be an efficient therapeutic strategy to potentiate efferocytosis and reduce the airways inflammation in COPD patients and cigarette smokers.

Methods

Preparation of cigarette smoke extracts. Cigarette smoke extract (CSE) which is known to dysregulate macrophage function was prepared as a single

stock of 100% CSE. This was prepared as previously reported⁴⁵ and was used to prepare 10% CSE throughout the study. Briefly, we bubbled the cigarette smoke from four 1R5 F research-reference filtered cigarettes containing 1.67 mg of tar and 0.16 mg of nicotine (The Tobacco Research Institute, University of Kentucky, Lexington, KY) through 40 mL RPMI 1640 medium containing 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and with 2 mM L-glutamine (all Thermo Fisher Scientific, MA, USA) using a vacuum pump. This was performed under 5 min per cigarette. The 100% cigarette smoke extracts were then aliquoted and stored at -80°C after adjusting the pH.

Preparation of cell cultures

THP-1 monocyte cell line was obtained from American Type Culture Collection, Manassas, VA, USA. The cell line was cultured at $37^{\circ}\text{C}/5\% \text{CO}_2$ in RPMI 1640 medium containing 10% FBS, penicillin/streptomycin, 2 mM L-glutamine, and 0.05 mM β -mercaptoethanol (Sigma-Aldrich, MO, USA). To differentiate the monocytic cell line into macrophages, we seeded the cells at a density of 5×10^5 cells/mL in culture medium containing 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 72 h, as published previously.⁴⁶ The 16HBE14o- airway epithelial cell line was obtained from Dr Dieter C. Gruenert (University of California, San Francisco, USA) as a generous gift. 16HBE14o- cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, 2 mM L-glutamine and penicillin/streptomycin under humidified $37^{\circ}\text{C}/5\% \text{CO}_2$ conditions. All cell culture materials were obtained from Thermo Fisher Scientific unless specifically indicated.

Subject population

We specifically recruited COPD and never-smoker control subjects to participate in this study. Subjects with other respiratory diseases such as lung cancer and those within 6 weeks of exacerbation COPD were excluded from the study. Ethics approval was obtained from Central Adelaide Local Health Network Human Research Ethics Committee (CALHN HREC) with ethic number 12978. We excluded patients with FEV₁ below 1.4 L from bronchoscopy due to ethical reasons. We obtained written informed consent from all volunteers after obtaining ethics approval

Table 1. Demographic details of COPD patients and control subjects.

	Control	COPD
Number of volunteers	4	4
Age (years)±SEM	52.25 ± 8.26	71.5 ± 6.59
Male	2	2
Female	2	2
Never smokers	4	0
Current smokers	0	2
ex-smokers	0	2
FEV1%PRED	100 ± 6.11	76 ± 1.35
FVC%PRED	94.33 ± 1.86	91.5 ± 1.55
FEV1/FVC% PRED	83.33 ± 5.04	61.75 ± 1.97

Data are presented as mean ± SEM.

from the Royal Adelaide Hospital. GOLD criteria with clinical correlation was used to confirm COPD diagnosis. Bronchoalveolar lavage (BAL) was obtained from a cohort of these participants for examination of Rubicon expression (four subjects with COPD and four never smoker control subjects; Table 1).

Bronchoscopy procedure

As we have previously reported,⁴⁷ BAL was obtained through bronchoscopy. Briefly, 50 mL aliquot of sterile normal saline was instilled into the airways with a syringe then aspirated using low suction at room temperature. Two additional 50 mL aliquots of saline were instilled and aspirated in the same way. The first aspirated BAL specimen for each collection from an individual patient was excluded and was processed for microbiological testing to avoid contaminated airway mucus. The second and third aliquots were collected, kept on ice, and processed within 1 h of collection.

Preparation of monocyte derived macrophage (MDM)

Adult controls were recruited from our volunteer database, were nonsmokers had no history of respiratory or allergic disease. Written informed consent was obtained from healthy

subjects after the invitation to participate in the study. The study protocol was approved by the Royal Adelaide Hospital Research Committee (#020811d). All research procedures were in accordance with the relevant rules and regulations. For monocytes isolation, Lithium-Heparin tubes (Greiner Bio One, Austria) were used to collect whole blood. Blood (1 volume) was diluted with 2 volumes of plain RPMI 1640 medium. Diluted blood was layered over Lymphoprep™ (STEMCELL Technologies, BC, Canada) and centrifuged at 800×g for 25 min with acceleration but no brake. Peripheral blood mononuclear cells (PBMC) layer were isolated according to the manufacturer's instructions. To derive macrophages from monocytes, PBMC were seeded into plates at $1.4 \times 10^6/\text{mL}$ in plain RPMI 1640 medium at 37°C/5% CO₂ for 90 min to allow monocytes to attach. Unattached cells were aspirated. Attached were washed three times with PBS to remove all unattached cells cultured in RPMI 1640 medium containing 2 mM L-glutamine, 10% FBS, penicillin/gentamicin and 20 ng/mL macrophage colony-stimulating factor (M-CSF, Life Sciences) for 12 days with full media changes at 4 and 8 days.

Lung tissues samples

Cohort of patients undergoing lobectomy at the Department of Cardiothoracic Surgery, RAH were recruited and written informed consent was obtained. Lung tissues from these subjects were obtained as previously described.^{48,49} Biopsies were collected from nontumour ('normal') areas well away from the cancer (approximately 5 mm × 5 mm in size). A 'Medimachine' tissue disaggregator (BD) was applied to prepare single cell suspensions from lung tissue as previously described.^{48,50} Samples were categorized as 'Control' (nontumour area from patients with cancer/no COPD) or 'COPD' (nontumour area from patients with cancer + COPD).

Efferocytosis assay

The efferocytic capacity of THP-1 differentiated macrophages co-cultured with smoke extracts or control media was performed as previously reported.¹¹ Briefly, apoptotic 16HBE14o-bronchial epithelial cells were stained with Phrodo Green from Thermo Fisher Scientific. The cells were co-cultured with THP-1 macrophages, at

5:1 ratio, respectively, for 90 min. The cells were washed three times with PBS before lifting them into FACS tubes. The cells were finally analysed by flow cytometry on a FACSCanto II (BD Biosciences, San Diego, USA) to determine the percentage of viable macrophages efferocytosing apoptotic 16HBE14o-bronchial epithelial cells. Procedures for gating have been previously published.^{9,47,51}

Quantitative immunofluorescence analysis of protein expression and localisation in human lung alveolar macrophages and in lung tissues from mice exposed to cigarette smoke

Mouse lung tissue paraffin blocks were stored from our previous study of chronic exposure to cigarette smoke.⁵² Mice were exposed to cigarette smoke for 6 weeks, sufficient to induce inflammatory changes but not emphysema or small airway remodelling.⁵³ Paraffin sections from multiple animals were mounted on tissue arrays for batch analysis. Immunofluorescence staining of mouse lung sections was carried following a protocol adapted from our previous studies.⁵⁴ Primary antibodies were rabbit anti-LC3A from Novus (Centennial, CO, USA, NB100-2331; 1:100) and rabbit anti-Rubicon from Abcam (Cambridge, UK; ab92388; 1:200). Secondary fragment antibody was a donkey IgG F(ab')₂ conjugated with AF594 (Jackson ImmunoResearch, West Grove, PA, USA; 1:200). F4/80, rat monoclonal antibody clone CI: A3-1 from Abcam (Cambridge, UK, ab6640, 1:25), detected by donkey IgG F(ab')₂ anti-rat IgG AF647 (Jackson ImmunoResearch, West Grove, PA, USA; 1:200). Quantitative immunofluorescence was carried out as previously described.⁵⁵ Briefly, multiple images were captured using a conventional fluorescence microscope for LC3 (IX73; Olympus Australia, Notting Hill, VIC, Australia), or a confocal system for Rubicon (FV3000; Olympus Corporation, Shinzuku, Tokyo, Japan). Alveolar macrophages were defined according to their morphology and localisation.⁵⁴ Mean fluorescence intensity was measured using the ImageJ software (NIH, Bethesda, MA, USA). Alveolar macrophages were differentiated from alveolar pneumocytes according to their localisation within alveoli air spaces and their large cytoplasm. Under fluorescence microscopy alveolar macrophages revealed frequent fusiforms, presence of internalized apoptotic bodies (dull DAPI + particles) and increased autofluorescence.

Immunofluorescence and confocal microscopy

The fluorescence intensity of Rubicon, apoptotic marker, poly (ADP-ribose; PAR, a polymer formed by active PARP (poly (ADP-ribose) polymerase) and the autophagy marker, LC3 were assessed in THP-1 macrophages exposed to 10% CSE. Briefly, cells were fixed with 2.5% formalin in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS, pre-blocked with serum-free protein blocker (Dako, Glostrup, Denmark), incubated overnight at 4°C with primary antibodies and 1 h at room temperature with secondary antibodies. Primary antibodies were mouse monoclonal anti-PAR (1/20; Enzo Life Sciences, NY, USA) and anti-Rubicon from Abcam (Cambridge, UK; ab92388; 1:200) and Primary antibodies were rabbit anti-LC3A from Novus (Centennial, CO, USA, NB100-2331; 1:100). All secondary antibodies were donkey IgG F(ab')₂ fragments with Alexa Fluor (AF) conjugates from Jackson ImmunoResearch (West Grove, PA, USA), anti-rabbit IgG (AF594 or AF647), and anti-mouse IgG (AF647). Images were captured on a LSM700 confocal microscope (Carl Zeiss Australia, NSW, Australia). For quantitative analysis, 10 serial images at a 20× objective were captured from each well of an eight-well chamber slide in a blinded manner by focusing on the DAPI channel. Measurement of mean fluorescence intensity (MFI) or percentage of brightly fluorescent cells was determined by ImageJ morphometric software (NIH, Bethesda, MA, USA).

Cytometric bead array (CBA)

Supernatants collected from THP-1 macrophage cells exposed to cigarette smoke extracts or air control for 24 h were assessed with a human inflammatory cytokine CBA kit (BD Biosciences), according to manufacturer instructions. Cytokines, TNF- α , IL-10, and IL-4 were measured on a FACSCanto II and analysed with FCAP Array software (BD Biosciences).

Western blot analysis

For western blot analysis of Rubicon (D9 F7) rabbit mAb (Cell Signalling Technology, USA), LC3 (4105) rabbit mAb (Cell Signalling Technology, USA), Atg5 (ab108327) rabbit pAb (abcam), cleaved caspase 3 (D175) rabbit mAb (Cell Signalling Technology, USA), poly-ADP ribose polymerase (PARP) cleavage (Cell

Signalling Technology, USA), NOX2/gp91phox (ab80508) rabbit pAb (abcam), TIM-4 (ab47637) rabbit pAb (abcam), SQSTM1/p62 (D5E2) Rabbit mAb #8025 (Cell Signalling Technology, USA), and β -actin mouse mAb (Sigma Aldrich, St. Louis, MO, USA; A1798; 1:4000). THP-1 cells were lysed using M-PER mammalian cell protein lysis reagent with PMSF protease inhibitor (Sigma Aldrich). Protein samples were quantified using BCA protein assay (CA, USA). Protein concentration of 10 μ g was electrophoresed on 4–12% gradient Bis-Tris gels before being transferred to nitrocellulose membrane. Membranes were blocked in 5% skim milk (Fonterra, NZ) or 5% bovine serum albumin (BSA) before incubating with primary antibodies overnight. Membranes were incubated with corresponding secondary antibodies and washed three times with TBST and then probed with Bio-Rad software (CA, USA).

Real-time reverse transcription (RT-PCR)

Total RNA extraction from THP-1 macrophages was performed using the RNeasy Mini kit (QIAGEN, Venlo, Netherlands). Then, 1 μ g of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN, Venlo, Netherlands) to synthesize complementary DNA. The *TaqMan* gene expression assay was used to analyse Rubicon mRNA expression (human KIAA0226 or Rubicon; Hs00943570_m1; Thermo Fisher Scientific, Waltham, MA, USA). *Rubicon* gene expression levels were normalized to GAPDH (Hs02758991_g1) and HPRT (Hs 99999901) all from Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Data were analysed using Graphpad prism software (GraphPad, La Jolla, USA). Results are reported as mean \pm SEM unless otherwise indicated. Analysis was performed using the nonparametric Mann Whitney test (for n values more than 3), Welch test (for n values of 3) and Kruskal–Wallis test for more than two different experimental groups. A value of $p < 0.05$ was considered statistically significant. Correlation analysis of Rubicon protein expression and efferocytosis was performed using Pearson's correlation coefficient with significance set at $p < 0.05$.

Acknowledgements

We are grateful for the expertise and support of the clinical staff of the Royal Adelaide Hospital and Queen Elizabeth Hospital Thoracic Units and the participants enrolled into this study who generously provided their valuable time and samples. We would also like to thank Suzanne Maiolo and Rhys Hamon for their technical assistance. Eugene Roscioli received grants from the Royal Adelaide Research Committee during the conduct of this study.

Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Adelaide Scholarship International, Royal Adelaide Hospital Research Fund, South Australia Health Services Charitable Gifts Board, and the Rebecca L Cooper Medical Research Foundation. The authors received no financial support for this project.

ORCID iD

Patrick F. Asare  <https://orcid.org/0000-0001-6389-4978>

References

1. Salvi SS, Manap R and Beasley R. Understanding the true burden of COPD: the epidemiological challenges. *Prim Care Respir J* 2012; 21: 249–251.
2. Laniado-Laborin R. Smoking and chronic obstructive pulmonary disease (COPD). Parallel epidemics of the 21 century. *Int J Environ Res Public Health* 2009; 6: 209–224.
3. Rutgers SR, Postma DS, ten Hacken NH, *et al.* Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000; 55: 12–18.
4. Willemsse BWM, ten Hacken NH, Rutgers B, *et al.* Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J* 2005; 26: 835–845.
5. Hogg JC, Chu F, Utokaparch S, *et al.* The nature of small-airway obstruction in chronic obstructive pulmonary disease. *New Eng J Med* 2004; 350: 2645–2653.

6. King PT. Inflammation in chronic obstructive pulmonary disease and its role in cardiovascular disease and lung cancer. *Clin Transl Med* 2015; 4: 68.
7. Zhang W, Zhao J, Wang R, *et al.* Macrophages reprogram after ischemic stroke and promote efferocytosis and inflammation resolution in the mouse brain. *CNS Neurosci Ther* 2019; 25: 1329–1342.
8. Subramaniam R, Mukherjee S, Chen H, *et al.* Restoring cigarette smoke-induced impairment of efferocytosis in alveolar macrophages. *Mucosal Immunol* 2016; 9: 873–883.
9. Hodge S, Matthews G, Mukaro V, *et al.* Cigarette smoke-induced changes to alveolar macrophage phenotype and function are improved by treatment with procysteine. *Am J Respir Cell Mol Biol* 2011; 44: 673–681.
10. Kazeros A, Harvey B-G, Carolan BJ, *et al.* Overexpression of apoptotic cell removal receptor MERTK in alveolar macrophages of cigarette smokers. *Am J Respir Cell Mol Biol* 2008; 39: 747–757.
11. Hodge S, Hodge G, Scicchitano R, *et al.* Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol* 2003; 81: 289–296.
12. Martinez J, Cunha LD, Park S, *et al.* Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. *Nature* 2016; 533: 115–119.
13. Sanjuan MA, Dillon CP, Tait SWG, *et al.* Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* 2007; 450: 1253–1257.
14. Martinez J, Almendinger J, Oberst A, *et al.* Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proc Nat Acad Sci* 2011; 108: 17396–17401.
15. Martinez J, Malireddi RKS, Lu Q, *et al.* Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. *Nat Cell Biol* 2015; 17: 893–906.
16. Hodge S, Hodge G, Holmes M, *et al.* Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. *Eur Respir J* 2005; 25: 447–454.
17. Hu J-Y, Liu B-B, Du Y-P, *et al.* Increased circulating $\beta(2)$ -adrenergic receptor autoantibodies are associated with smoking-related emphysema. *Sci Rep* 2017; 7: 43962.
18. Xu J, Xu F and Lin Y. Cigarette smoke synergizes lipopolysaccharide-induced interleukin- 1β and tumor necrosis factor- α secretion from macrophages via substance P-mediated nuclear factor- κB activation. *Am J Respir Cell Molec Biol* 2011; 44: 302–308.
19. Aoshiba K, Tamaoki J and Nagai A. Acute cigarette smoke exposure induces apoptosis of alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 2001; 281: L1392–L1401.
20. Sakhatskyy P, Gabino Miranda GA, Newton J, *et al.* Cigarette smoke-induced lung endothelial apoptosis and emphysema are associated with impairment of FAK and eIF2 α . *Microvasc Res* 2014; 94: 80–89.
21. Clark H, Palaniyar N, Strong P, *et al.* Surfactant protein D reduces alveolar macrophage apoptosis in vivo. *J Immunol* 2002; 169: 2892.
22. Morimoto K, Janssen WJ, Fessler MB, *et al.* Lovastatin enhances clearance of apoptotic cells (efferocytosis) with implications for chronic obstructive pulmonary disease. *J Immunol* 2006; 176: 7657–7665.
23. Li M, Yu D, Williams KJ, *et al.* Tobacco smoke induces the generation of procoagulant microvesicles from human monocytes/macrophages. *Arterioscler Thromb Vasc Biol* 2010; 30: 1818–1824.
24. Lim HJ, Park JH, Jo C, *et al.* Cigarette smoke extracts and cadmium induce COX-2 expression through γ -secretase-mediated p38 MAPK activation in C6 astroglia cells. *PLoS ONE* 2019; 14: e0212749.
25. Comer DM, Kidney JC, Ennis M, *et al.* Airway epithelial cell apoptosis and inflammation in COPD, smokers and nonsmokers. *Eur Respir J* 2013; 41: 1058–1067.
26. Park J-W, Kim HP, Lee S-J, *et al.* Protein Kinase $C\alpha$ and ζ differentially regulate death-inducing signaling complex formation in cigarette smoke extract-induced apoptosis. *J Immunol* 2008; 180: 4668–4678.
27. Miyanishi M, Tada K, Koike M, *et al.* Identification of Tim4 as a phosphatidylserine receptor. *Nature* 2007; 450: 435–439.

28. Wang Y, Luo G, Chen J, *et al.* Cigarette smoke attenuates phagocytic ability of macrophages through down-regulating Milk fat globule-EGF factor 8 (MFG-E8) expressions. *Sci Rep* 2017; 7: 42642.
29. McCubbrey AL and Curtis JL. Efferocytosis and lung disease. *Chest* 2013; 143: 1750–1757.
30. Cunha LD, Yang M, Carter R, *et al.* LC3-associated phagocytosis in myeloid cells promotes tumor immune tolerance. *Cell* 2018; 175: 429.e16–441.e16.
31. Matsunaga K, Saitoh T, Tabata K, *et al.* Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol* 2009; 11: 385–396.
32. Boyle KB and Randow F. Rubicon swaps autophagy for LAP. *Nat Cell Biol* 2015; 17: 843–845.
33. Monick MM, Powers LS, Walters K, *et al.* Identification of an autophagy defect in smokers' alveolar macrophages. *J Immunol* 2010; 185: 5425–5435.
34. Yamamuro T, Kawabata T, Fukuhara A, *et al.* Age-dependent loss of adipose Rubicon promotes metabolic disorders via excess autophagy. *Nat Commun* 2020; 11: 4150.
35. Matsuda J, Takahashi A, Takabatake Y, *et al.* Metabolic effects of RUBCN/Rubicon deficiency in kidney proximal tubular epithelial cells. *Autophagy* 2020; 16: 1889–1904.
36. Uzokov J, Alyavi A and Karimov B. Prevalence of metabolic syndrome in patients with chronic obstructive pulmonary disease. *Eur Respir J* 2016; 48: PA3776.
37. Zhang S, Weinberg S, DeBerge M, *et al.* Efferocytosis fuels requirements of fatty acid oxidation and the electron transport chain to polarize macrophages for tissue repair. *Cell Metab* 2019; 29: 443.e5–456.e5.
38. Han CZ and Ravichandran KS. Metabolic connections during apoptotic cell engulfment. *Cell* 2011; 147: 1442–1445.
39. Wong SW, Sil P and Martinez J. Rubicon: LC3-associated phagocytosis and beyond. *FEBS J* 2018; 285: 1379–1388.
40. Fadok VA, Bratton DL, Guthrie L, *et al.* Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J Immunol* 2001; 166: 6847–6854.
41. Yang C-S, Rodgers M, Min C-K, *et al.* The autophagy regulator Rubicon is a feedback inhibitor of CARD9-mediated host innate immunity. *Cell Host Microbe* 2012; 11: 277–289.
42. Inomata M, Xu S, Chandra P, *et al.* Macrophage LC3-associated phagocytosis is an immune defense against *Streptococcus pneumoniae* that diminishes with host aging. *Proc Natl Acad Sci* 2020; 117: 33561–33569.
43. Sil P, Suwanpradid J, Muse G, *et al.* Noncanonical autophagy in dermal dendritic cells mediates immunosuppressive effects of UV exposure. *J Allergy Clin Immunol* 2020; 145: 1389–1405.
44. Masud S, Prajsnar TK, Torraca V, *et al.* Macrophages target *Salmonella* by Lc3-associated phagocytosis in a systemic infection model. *Autophagy* 2019; 15: 796–812.
45. Hodge S, Hodge G, Ahern J, *et al.* Smoking alters alveolar macrophage recognition and phagocytic ability. *Am J Respir Cell Mol Biol* 2007; 37: 748–755.
46. Dehle FC, Mukaro VR, Jurisevic C, *et al.* Defective lung macrophage function in lung cancer ± chronic obstructive pulmonary disease (COPD/emphysema)-mediated by cancer cell production of PGE2. *PLoS ONE* 2013; 8: e61573.
47. Hodge SJ, Hodge GL, Holmes M, *et al.* Flow cytometric characterization of cell populations in bronchoalveolar lavage and bronchial brushings from patients with chronic obstructive pulmonary disease. *Cytometry B Clin Cytom* 2004; 61: 27–34.
48. Soriano C, Mukaro V, Hodge G, *et al.* Increased proteinase inhibitor-9 (PI-9) and reduced granzyme B in lung cancer: mechanism for immune evasion. *Lung Cancer* 2012; 77: 38–45.
49. Cordts F, Pitson S, Tabeling C, *et al.* Expression profile of the sphingosine kinase signalling system in the lung of patients with chronic obstructive pulmonary disease. *Life Sci* 2011; 89: 806–811.
50. Hodge S, Matthews G, Dean MM, *et al.* Therapeutic role for mannose-binding lectin in cigarette smoke-induced lung inflammation? Evidence from a murine model. *Am J Respir Cell Mol Biol* 2010; 42: 235–242.
51. Tran HB, Barnawi J, Ween M, *et al.* Cigarette smoke inhibits efferocytosis via deregulation of sphingosine kinase signaling: reversal with exogenous S1P and the S1P analogue FTY720. *J Leukoc Biol* 2016; 100: 195–202.

52. Tran HB, Jersmann H, Truong TT, *et al.* Disrupted epithelial/macrophage crosstalk via Spinster homologue 2-mediated S1P signaling may drive defective macrophage phagocytic function in COPD. *PLoS ONE* 2017; 12: e0179577.
53. Fricker M, Deane A and Hansbro PM. Animal models of chronic obstructive pulmonary disease. *Exp Opin Drug Disc* 2014; 9: 629–645.
54. Tran HB, Lewis MD, Tan LW, *et al.* Immunolocalization of NLRP3 inflammasome in normal murine airway epithelium and changes following induction of ovalbumin-induced airway inflammation. *J Allergy (Cairo)* 2012; 2012: 819176.
55. Tran HB, Macowan MG, Abdo A, *et al.* Enhanced inflammasome activation and reduced sphingosine-1 phosphate S1P signalling in a respiratory mucoobstructive disease model. *J Inflamm (Lond)* 2020; 17: 16.

Visit SAGE journals online
[journals.sagepub.com/
home/tar](https://journals.sagepub.com/home/tar)

 SAGE journals