An investigation of the Rubicon/LC3 associated phagocytosis (LAP) dysregulation as a therapeutic target in chronic obstructive pulmonary diseases (COPD) and in response to cigarette smoke exposure

A thesis presented

by

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“Education is the most powerful weapon which you can use to change the world”.

Nelson Mandela

Thesis supervisor: Prof Sandra Hodge By: Patrick Fordjour Asare

Abstract

Phagocytic clearance of bacteria and apoptotic cells (a process termed efferocytosis) in COPD is critical to protect against microbial infection and lung tissue injury. The compromised phagocytic capacity of alveolar macrophages in COPD allows bacterial colonisation and is postulated to contribute to the disease severity. However, the precise mechanisms that lead to the macrophage phagocytic dysfunction in COPD remains incompletely understood.

LC3-associated phagocytosis (LAP) is a recently discovered cellular event characterised as a critical regulator of effective processing of ingested microbes and apoptotic cells by macrophages. Defective LAP impairs the clearance of pathogens and apoptotic cells by macrophages. Therefore, experiments described in Chapter 2 used novel approaches to measure components of the LAP signalling system including TIM-4, Rubicon, LC3, Atg5, NOX2 in blood monocyte derived macrophages, differentiated THP-1 macrophages and lung tissues of mice exposed to cigarette smoke extract (CSE). Further, the expression of the LAP specific regulator, Rubicon, was examined in bronchoalveolar lavage (BAL)-derived macrophages from COPD patients and healthy controls. The findings of this study showed for the first time that Rubicon/LAP is dysregulated in COPD as a result of CSE exposure. Moreover, the study found that Rubicon inhibition correlated with a defective efferocytosis capacity of alveolar macrophages, confirming a link between LAP inhibition and defective efferocytosis in COPD. Furthermore, this report characterises LAP as a potential therapeutic target for potentiating macrophage efferocytic function in COPD.

Modulation of Rubicon/LAP requires a better understanding of the processes that lead to Rubicon/LAP inhibition in COPD. Hence, Chapter 3 of this thesis addressed the mechanisms of Rubicon inhibition by CSE. It was noted that CSE shortens the half-life of Rubicon protein but does not have significant effects on *Rubicon* mRNA levels. This led to the hypothesis that a protein degradation pathway may contribute to the reduction in Rubicon in macrophages exposed to the factors in cigarette smoke. Further observation that Rubicon degradation could be attenuated by anti-proteases and lysosomal enzyme inhibitors confirmed this hypothesis and demonstrated that lysosomal enzymes may mediate the Rubicon degradation. Moreover, alterations in autophagy or proteasomes did not have significant effects on Rubicon suggesting that Rubicon downregulation by CSE is independent of autophagy or proteasomes.

Overall, the findings of the current study contribute to the concerted efforts of the Hodge lab to delineate the mechanisms associated with chronic infections and defective efferocytosis observed in COPD. Hence, COPD may be improved by LAP targeting therapies that prevent Rubicon degradation and improve bacterial and apoptotic cell clearance. However, recent reports also show that LAP activity can be usurped by tumours to promote their growth and aggressiveness. Careful considerations must be taken when promoting LAP in COPD patients diagnosed with cancer. This is particularly important, as COPD is often associated with increased risk of cancers including lung cancer. To reconcile with this, Chapter 4 discussed the pro-tumour impacts of LAP and highlighted the relationship between LAP and efferocytosis as a cellular event that is usurped by tumours to subvert the host immune defence mechanism.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Acknowledgement

"It is during our darkest moments that we must focus to see the light."

Aristotle

I have always had a strong faith in intelligent work and been highly optimistic for the potential of scientific findings. This is the impetus that created in me, the desire to not only be assiduous but also efficient. I believed that pursuing a PhD in medicine would allow me to obtain the relevant knowledge and skills to be efficient and be part of the solution to some global health problems. However, I was quite pessimistic whether I have a tenacious attitude to overcome the many challenges associated with PhD studies. The belief that 'if I remain focus, I could see the light even when all looks gloomy' motivated me to commit myself to the difficult task that may arise. This would not have been possible without the invaluable supports from my supervisors, family, friends and the researchers in the lung research group that I would like to acknowledge.

Firstly, I would like to thank my supervisor Prof Sandra Hodge for her support and guidance during the past few years of my studies. Prof Hodge provided me with resources and many opportunities that inspired me to become the best version of myself. Her supervision provided me with the opportunity to explore all avenues and improve my research and academic writing skills. Aside being a great supervisor, Prof Hodge gave me the chance to carve a niche to develop myself for a rewarding career post-PhD. I will always be grateful to her for the opportunities she has given me.

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In preparation

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Hai B Tran, Rachel Jakobczak, Abdo Adrian, **Asare PF,** John Beltrame, Sandra Hodge, Peter Zalewski, *Microvascular morphology and functions are mediated by changed immunolocalization of zinc transporters and metallothioneins.*

Conference presentations

Asare PF, E. Roscioli, P.R. Hurtado, H.B. Tran, S. Maiolo, S. Hodge, *The potential dysregulation of LC3-Associated Phagocytosis (LAP) by cigarette smoke in chronic obstructive pulmonary disease (COPD),* presented as an oral presentation at 2019 Thoracic Society of Australia and New Zealand annual scientific meeting, Gold Coast, Queensland, Australia.

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Table of Contents

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of tables

Chapter 2

Chapter 3

Acronyms

AM	Alveolar macrophage	LEU	Leupeptin
µg	Microgram	LLN	Lower limit of normal system
µl	Microliter	LPC	Lysophosphatidylcholine
µM	Micromolar	LXR	Liver X receptor
AEBSF	4-benzenesulfonyl fluoride hydrochloride	MCP	Monocyte chemoattractant protein-1
AMP	Adenosine monophosphate	M-CSF	Macrophage colony-stimulating factor
ATG	Autophagy related	MDM	Blood monocyte derived macrophages
ATP	Adenosine triphosphate	MERTK	Mer proto-oncogene, tyrosine kinase
BAFA1	Bafilomycin A1	MFG	Milk fat globule-EGF factor 8 protein
BAI	Brain-specific angiogenesis inhibitor 1	MFI	Mean fluorescence intensity
BAL	Bronchial alveolar macrophage	MHC	Major histocompatibility complex
BCA	Bicinchoninic acid	mM	Millimolar
BSA	Bovine serum albumin	MMP	Matrix metalloproteinase
BTZ	Bortezomib	mRNA	Messenger ribonucleic acid
C	Control	NADPH	Nicotinamide adenine dinucleotide phosphate
CATBI	Catthepsin B inhibitor	NE	Neutrophil elastase
CBA	Cytometric Bead Array	NET	Neutrophil extracellular traps
CHX	Cyclohexamide	NK	Natural killer
CMA	Chaperone mediated autophagy	nM	Nanomolar
COPD	Chronic obstructive pulmonary disease	NOX	Nicotinamide adenine dinucleotide phosphate oxidase
CQ	Chloroquine	PARP	Poly-ADP ribose polymerase

CRKII	Chicken tumor virus no. 10 (CT10) regulator of kinase II
CS	Cigarette smoke
CSE	Cigarette smoke extract
CT	computed tomography
CTR	Control
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELMO	Engulfment and cell motility protein
EV71	Enterovirus 71
FBS	Foetal bovine serum
FEV	Forced expiratory volume
FIP200	FAK family–interacting protein of 200 kDa
FVC	Forced vital capacity
G2A	G-protein-coupled receptor 132
G3P	Glycerol-3-phosphate
GAS-6	Growth arrest-specific 6
GM-CSF	Granulocyte-macrophage colony-stimulating factor
PBMC	Peripheral blood mononuclear cells
PEPI	Proline endopeptidase inhibitor
PI3KC3	Phosphatidylinositol 3-kinase catalytic subunit type 3
PI3P	Phosphatidylinositol 3-phosphate
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor gamma
PTDSER	Phosphatidylserine
RAP	Rapamycin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse transcription–polymerase chain reaction
Rubicon	RUN domain Beclin-1-interacting and cysteine-rich domain-containing protein
S1P	sphingosine-1-phosphate
SERPINS	Serine peptidase inhibitors
SeV	Sendai virus
SLAM	Signalling lymphocyte-activation molecule)
STING	Stimulator of interferon genes
TAM	Tumour-associated macrophages
TBST	Tris Buffered Saline with Tween

GMP	Guanosine 5′-monophosphate
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GPCR	G-protein-coupled receptors
h	Hours
HBV	Hepatitis B virus
IAV	Influenza A virus
ICAM-1	Intercellular adhesion molecule 1
IF	Immunofluorescence
IFN	Interferon
IL	Interleukin
LAP	Microtubule-associated protein 1A/1B-light chain 3 associated phagocytosis
LC3	Microtubule-associated protein 1A/1B-light chain 3
TGF-β	Transforming growth factor beta
TIL	Tumour infiltrating lymphocytes
TIM	T-cell immunoglobulin and mucin domain family of receptors
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF	Tumour necrosis factor
ULK	Unc51-like kinase 1 complex
UTP	Uridine triphosphate
UV	Ultraviolet
UVRAG	UV radiation resistance associated
VPS	Vacuolar protein sorting
VSV	Vesicular stomatitis viruses

Chapter 1

1. Introduction and literature review

The literature review presented in this chapter is being prepared for publication as *'Novel advances in the mechanisms underlying COPD pathogenesis'* as part of Asare Patrick Fordjour, Hai Bac Tran, Plinio R. Hurtado, Eugene Roscioli, Sandra Hodge, 2021.

1.1. Chronic Obstructive Pulmonary Disease (COPD)

COPD is a leading cause of morbidity and mortality and encompasses a group of respiratory disorders that are characterised by irreversible airflow limitation [1-3]. Clinicians often use the term COPD to describe patients with a low forced expiratory volume (FEV), abnormalities of computed tomography (CT) scans due to structural changes of the lung, and clinical features of chronic lung disease [4, 5]. Coughing, difficulties in breathing and wheezing are some of the major clinical manifestations of COPD [6, 7]. COPD is generally progressive, incurable, and often linked to unrestrained chronic inflammatory responses to noxious particulate matters in the airways.

Later stages of COPD are usually characterised by irreversible structural alterations, while earlier stages (susceptible smokers who do not have COPD as it is currently defined by spirometric criteria) are mostly associated with molecular and cellular changes that often lead to the structural damage [8-10]. Thus, early assessment of structural modifications of the airway, particularly in susceptible individuals, is required to enable optimal responses to therapeutic interventions and potentially prevent airway obstruction.

COPD is most often caused by exposure of the lung to noxious particles/gases, most commonly from cigarette smoke, which causes lung tissue destruction (emphysema) and airflow limitation [11]. Some factors such as co-morbidities, underdiagnoses and lack of efficient therapies contribute to COPD severity and the related mortality [12, 13].

1.1.1. *Classifications of COPD*

Based on the guidelines from Global Initiative for Chronic Obstructive Lung Disease (GOLD) [14] COPD is classified into four main stages of severity, based largely on spirometry determination of airflow limitation by measurement of the forced expiratory volume in 1 second (FEV1; the volume of air that can be forced out of the lung in one second) and the ratio of FEV1/forced vital capacity (FVC). FVC is the total amount of air exhaled during the spirometry test. The classifications are:

1) Mild or GOLD stage 1 for patients with FEV1/FVC < 0.70; FEV1 >80% of predicted normal values. Clinical manifestations may include chronic cough, sputum production and shortness of breath during normal activities, although these symptoms are not always present at this stage and their presence can often be misinterpreted as a sign of aging. Therefore, medical attention may not be sought at this stage, allowing the disease to progress.

2) Moderate or GOLD stage 2 for patients with FEV1/FVC < 0.70; FEV1 79–50% of predicted normal values. This stage is characterised by worsening airflow limitation, with breathlessness typically developing on exertion. Symptoms including tiredness, shortness of breath, sleeping difficulties and cough and sputum production that affect daily activities. The exacerbation of symptoms causes patients to seek medical attention and allows them to become aware of their condition.

3) Severe or GOLD stage 3 for patients with FEV1/FVC < 0.70; FEV1 49–30% of predicted normal. This stage is characterised by deterioration of the condition such that the symptoms are more frequent and have greater impacts on the quality of life. The clinical presentations include worse and frequent cough and mucus production, worsened airflow limitation and frequent breathlessness.

4) Very severe or GOLD stage 4 for FEV1/FVC <0.70; FEV1 <30% of predicted normal. Individuals with respiratory failure are also assigned to this stage regardless of the FEV1 value. Some experts oppose the inclusion of respiratory failure in this category based on a lack of clinical evidence. However, GOLD states there is the need for inclusion as patients' symptoms and complications are the main drivers of patient contact and diagnostic decision. However, GOLD recognises the need for spirometry to be the standard for diagnosing and monitoring the severity and progression of the disease [15, 16].

The recent GOLD guidelines also include stage 0 which is defined by the presence of chronic cough, sputum and breathlessness without airflow obstruction and FEV1/FVC >70% [17]. This permits the identification of susceptible individuals who are likely to develop COPD at a later stage in life. It also permits early intervention before the disease becomes a health crisis. The risk factors include the smoking status and age of the individual.

1.2. Limitations of the GOLD criteria

The GOLD classification has provided an appropriate yardstick for diagnosing and assessing the severity and progression of COPD. It has also promoted COPD research and remarkably enhanced our understanding of the disease. However, age-dependent decline of lung function has led to a debate regarding the accuracy of using present fixed cut-off points for categorising COPD [16, 18, 19]. For example, although both the FEV and FVC values decline with age, FEV declines faster than FVC. This can lead to under-diagnosis in younger adults and over-diagnosis in old individuals using the ration of FEV1/FVC [5].

Hardie *et al* [20] sought to investigate the accuracy of the GOLD criteria using Norwegian residents between 70 and 96 yrs. The findings of this study show that using the GOLD criteria, approximately 35% of the healthy elderly participants in this age group were falsely classified as having at least mild COPD. Cerveri and colleagues [21] also demonstrated that incorporating the age of young adults using the lower limit of normal system (LLN, a spirometry calculation that corrects the variations of individuals), led to identification of airflow obstruction in some individuals that were misidentified using the GOLD criteria. With age, the misidentified young individuals had a significantly higher risk of developing chronic phlegm or cough and a moderate to severe airflow obstruction reminiscent of COPD. This suggests the need for a better system to detect early stage of COPD especially in young adult. However, Güder and colleagues [22] showed that in contrast to the GOLD criteria which over-diagnose COPD in the elderly, the LLN system under-diagnose COPD in the aged. This further shows that the search for a better diagnostic criteria for COPD is not as straightforward as may have previously been envisaged.

Overall, the recent findings show that there is a potential for underdiagnosing young individuals and over-diagnosing older individuals using the current FEV1/FVC value [23]. To reconcile with this, GOLD recommends that FEV1 value rather than the fixed FEV1/FVC value should be the most robust approach for COPD assessment. While there is a concerted effort to discover newer approaches to replace the current guidelines, the FEV1-based system remains beneficial for simplifying and stratifying the disease progression and severity. Nevertheless, it is postulated that incorporating factors such as ethnicity, sex, height and age range of the individual into the reference spirometry equation will remarkably reduce the misdiagnosing and over-diagnosis often caused by the present guidelines [24, 25].

1.3. Mechanisms/pathophysiology of COPD

The precise pathogenic mechanism of COPD remains elusive despite the concerted and decades-long efforts to understand the key processes underlying the development and progression of the disease [26-28]. One key reason for this is that COPD represents a description for several (rather than single) diverse respiratory syndromes. Therefore, there is a significant variation in clinical presentation and progression of the disease. This raises the possibility that several mechanisms contribute to disease pathogenicity [29]. It also highlights the difficulties in searching for therapies that could reduce COPD progression. Interestingly, in spite of the multidimensional nature of COPD, the disease's pathogenic mechanisms are

known to be interrelated and mainly include small airways inflammation (bronchiolitis; airway wall thickening of bronchioles less than 2 mm in diameter), emphysema (destruction of alveoli tissues) and airflow limitation [30].

Chronic inflammation in the airways is a common phenomena that underlies the diverse pathogenicity and clinical presentations of COPD [30-34]. Constant deposition of toxic particulates primarily from cigarette smoke often perpetuate injury to resident cells. This leads to the release of factors such as chemoattractants that cause infiltration of inflammatory cells such as macrophages, cytotoxic T cells and neutrophils into the airways [35-40]. Accumulation of inflammatory cells in the peripheral lung tissues also causes the release of oxidants and proteases that trigger chronic inflammation. Normally, the inflammation is resolved through the body's physiologic and homeostatic mechanisms. However, protracted and unresolved inflammation increases the thickness of small/peripheral airways leading to small airway disease and perpetuates parenchyma destruction. This leads to destruction of alveoli walls and loss of elastic recoil, breathing difficulties and decline of lung function [30, 34]. Hence, the current paradigm is that chronic inflammation in the airways is major contributor to COPD.

1.3.1. *COPD pathogenesis begins from structural changes in the small airway*

As described above, lungs of COPD subjects generally show a combination of bronchiolitis and emphysema associated with chronic inflammation [41, 42]. There has been an interest in determining the sequence of events leading to COPD pathology. Hogg and colleagues [30] demonstrated that the small airway is the major site of initial airflow limitation in COPD. Niewoehner *et al* [43, 44] in a study of lung of young men further postulated that bronchiolitis, a small airway disease characterised by inflammation, precedes emphysematous lung destruction and may account for the physiological abnormalities observed in the airways of smokers. Several follow up studies have also characterised the small airways as the primary site of airflow obstruction in COPD [45, 46]. The occurrence of emphysema in COPD preceding small airway disease predicted a decline in FEV1 [47, 48]. These findings are consistent with the previous reports that small airway disease is a precursor to emphysematous lung destruction [41]. Therefore, COPD pathogenesis begins with small airways disease that can cause abnormal structural changes in the alveoli leading to emphysematous destruction. This suggests that while the appearance of emphysematous destruction is associated with the disease pathogenesis, it is a secondary event contributing to the decline of lung function [49].

1.3.2. *Small airway disease*

Small airway disease is a term describing both non-infectious and infectious conditions affecting the small airways. The small airways lack cartilage and are less than 2 mm in diameter [50, 51]. They comprise airways from the 8th generation to the terminal bronchioles, which divide into respiratory bronchioles. The respiratory bronchioles transition into alveoli ducts, which open into individual alveoli and are critical for gaseous exchange. The small airways have a higher cross-sectional area (180 cm^2) compared to the larger airways or the trachea (2.5 cm^2). This causes the resistance in the small airways to have a lesser impact (less than 10%) on the total airway resistance, although the majority of the lower airway resistance emanates from the small airways [51]. Therefore, while small obstructions in the small airways contribute less to the mechanical property of the normal lung, they have a major effects on ventilation distribution [52]. The small airways are also at a higher risk of collapsing due to little or absence of cartilage. They are also susceptible to obstruction as the internal diameter decreases with increasing generation number. These properties may partially explain why the small airways is the predominant site airway for the airway obstruction that is a cardinal feature of COPD [53, 54].

Several studies have sought to apply markers of dysfunction in the small airway as biomarkers for the early manifestation of COPD [46, 55]. However, this effort has been hampered by the inability of routine pulmonary function test to accurately detect the resistance in the small airways. The observation that it takes approximately 75% obstruction of the small airways for the lung function test to detect any abnormal changes in the airways [50] has led to the characterisation of the small airways as the 'silent zone' [50]. It may explain why COPD is usually diagnosed at a stage when the airways obstruction is already irreversible. Taken together, these findings highlight the urgent need for interventions that particularly target small airway disease to modulate the pathophysiological process of COPD.

1.3.3. *Role of chronic inflammation in COPD pathogenesis/pathology*

Chronic inflammation in the airways is a hallmark of COPD and can be exacerbated by microbial infections including bacterial, fungal and viral infections [31, 56-62]. While acute inflammation is normally self-limiting and necessary for immune responses against foreign particles and infections, uncontrolled chronic inflammation in the lungs coupled with airway remodelling can result in the development of irreversible airflow obstruction [30, 63, 64]. Clinical and experimental studies show that cigarette smoke exposure can also directly lead to

protease-antiprotease imbalance and oxidative stress that can further contribute to changes in structure of the airways associated with increased inflammatory responses [65-68]. The accumulation of inflammatory mucous exudates that thickens the airway walls also contributes to the pathogenic mechanisms of COPD [30, 69-71].

Cigarette smoke also increases the infiltration of inflammatory cells that cause unrestrained inflammation and subsequent destruction of the airways [72, 73]. The inflammatory cells can perpetuate sub-epithelial fibrosis, hyperplasia of smooth muscle cells, metaplasia of goblet cells and structural cell destruction [74]. It is well established that in the airways of COPD patients, inflammatory cells infiltrate into the lung parenchyma and bronchial mucosa [75, 76]. This leads to the release of proteases and inflammatory mediators that can cause airway remodelling and destruction as shown in **Figure 1**. These substances can dysregulate some cellular and physiological functions such as the clearance of pathogens and noxious particles [77-79]. The resultant effect of this is chronic inflammation and inappropriate immune activation that correlates with COPD progression [75].

The small airways is likely to be particularly susceptible to inflammatory insults, highlighted by the observation of a higher number of inflammatory cells leading to airflow limitation in the small airways of smokers and COPD subjects [30, 80]. A further report showed that chronic inflammation orchestrated by harmful particles and gases mainly from cigarette smoke led to narrowing of the small airways [81]. The molecular mechanisms driving the small airway dysfunction in COPD, however, have are not been well defined, indicating an urgent need for further research that precisely underpins the signalling pathways involved. Interestingly, uncontrolled inflammation and unchecked immune activation have also been implicated in the pathogenesis of co-morbidities of COPD including autoimmunity, metabolic disorder, cardiovascular diseases and lung cancer [82-86].

Therefore, resolution of inflammation is one common aim for COPD therapies. Unfortunately, available anti-inflammatory therapies such as corticosteroids, used as first-line treatments are ineffective in completely resolving the airways inflammation in COPD [87, 88]. A better understanding of the role of inflammatory and immune cells that drive airways chronic inflammation and COPD pathogenesis, particularly in the small airways, may facilitate the identification of efficient treatment strategies. Hence, we next discuss the accumulated evidence related to the role and participation of inflammatory cells such as macrophages, neutrophils and dendritic cells in COPD pathogenesis.

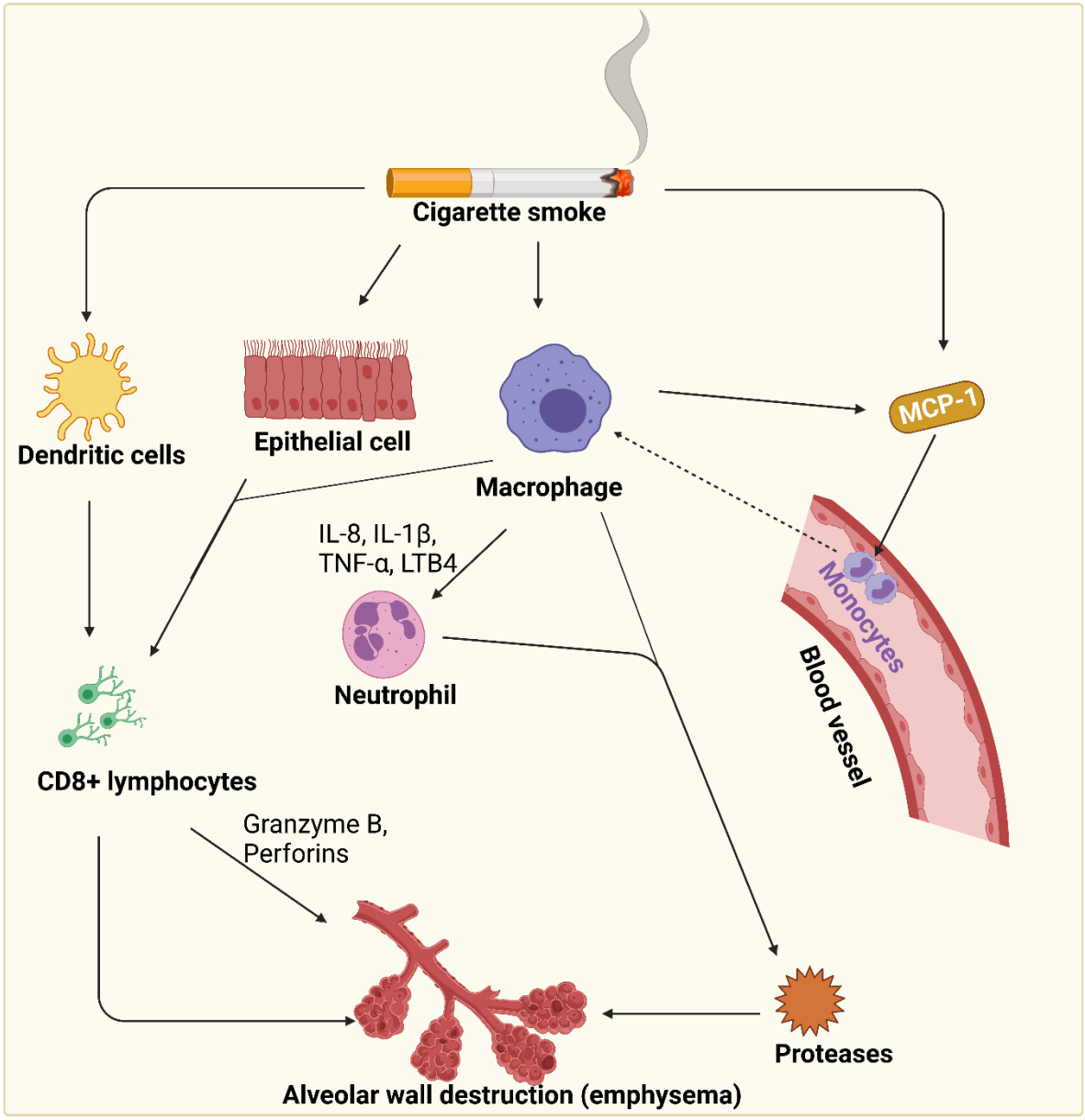

Figure 1: Involvement of inflammatory cells in COPD development in response to cigarette smoking. Cigarette smoking can stimulate macrophages to activate monocyte chemotactic factors such as monocyte chemoattractant protein-1 (MCP-1). Activated MCP-1 increases macrophage infiltration. Macrophages release chemotactic factors such as tumour necrosis factor-α (TNF-α), interleukin (IL)-8, IL-1β and leukotriene B4 (LTB4) to activate and recruit neutrophils into the airway. Macrophages and neutrophils activate proteases that can degrade lung parenchyma connective tissue leading to emphysema. Dendritic cells, macrophages and epithelial cells can stimulate T cells, mostly CD8+ T cells that can release cytotoxic mediators, perforins and granzyme B, and cause alveolar wall destruction (emphysema).

1.4. Dendritic cells

The lung is a portal entry of toxic particulates from the environment. Exposure of the airways to toxic particles and gases especially from cigarette smoke can facilitate the recruitment of dendritic cells in the airways [89]. Dendritic cells have a pivotal role in regulating both innate and adaptive immune responses in the airways. Base on their anatomical localisation, dendritic cells have a unique ability to continually survey and present antigens to both naïve and cytotoxic T cells [90]. Dendritic cells are also integral for regulating both active and tolerogenic immunity making them capable of serving as sentinels in the airways [91-93]. Additionally, dendritic cell activation is critical to activate immune responses against invading pathogens, gases or particles. This function has been investigated in preclinical and clinical studies, providing profound insights for the design of therapies and vaccines [94]. In fact, factors such as nicotine have been shown to compromise the function of dendritic cells system, resulting in reduction of the effectiveness of some vaccines [95]. Hence, dendritic cells are essential for immune response against foreign structures.

Dendritic cells primarily capture foreign invaders and effectively process them into immunogenic peptides [96]. This is a critical process required for subsequent transportation and presentation of the antigen on major histocompatibility complex molecules to T lymphocytes for naïve T cell activation [97]. Two main processes or signals are required for dendritic cell activation of naïve T cells and stimulation of immune responses; 1) Interaction between T cell receptors and peptide-major histocompatibility complex (MHC) to generate signals essential for activating nuclear transcription pathways [98] and 2) Engagement of T cells with dendritic cells through the expression of co-stimulatory molecules such as CD80, CD86, 4-1BBL and OX40L [99-101]. T cells can simultaneously interact with peptide-MHC complex and dendritic cells for activation. Naïve T cell stimulation through T cell receptors in the absence of co-stimulatory molecules expressed on dendritic cells, will lead to T cell anergy with immune tolerance or deletion [99, 102-104].

T cell activation is critical to enhance immune defence capabilities against infections and foreign particles. However, inappropriate and/or unchecked T cell activation can amplify inflammatory responses by producing higher amount of the pro-inflammatory cytokines IFN-γ and TNF-α, which may directly or indirectly influence lung tissue destruction [105-107]. This is consistent with a report of dendritic cell accumulation in association with increased T-cell costimulatory molecules expression and T cell activation in a cigarette smoke exposed lung

tissue [69]. Overall, airways dendritic cells can orchestrate adaptive immune responses including CD8+ T-cell cytotoxicity and T helper (Th1 and Th17) CD4+ T cells that can contribute to the chronic inflammation in the airways [108]. Hence, it is contended that the increased number of airway T cells observed in COPD subjects correlating with the disease's severity may be due to the enhanced recruitment of dendritic cells in the airways perpetuated by cigarette smoke [89, 109].

The multiplicity of roles attributed to dendritic cells in the immune response is exemplified by their ability to also regulate natural killer (NK) cell activation and cytotoxicity, resulting in destruction of lung epithelial cells that can contribute to emphysema [110, 111]. Recent studies have shown NK cell levels to be elevated in the airways of smoker and COPD patients and associated with COPD severity [112, 113]. Dendritic cells can also directly produce pro-fibrogenic cytokines and amplify airway inflammation that together can contribute to airway structure modification and remodelling [111]. Thus, chronic inflammation in the airways can be potentially controlled by regulating the recruitment and function of dendritic cells.

1.4.1. *Controversies regarding the role of dendritic cells in COPD*

There is a debate about the role and function of dendritic cells in COPD and in response to cigarette smoke. Some studies postulate that compromised function of dendritic cells may account for the impaired anti-microbial immune defence capability observed in COPD. However, others associated the activated and differentiated T cells correlating with COPD severity to dendritic cells accumulation in the airways. For example, Liao *et al* [114] showed reduced maturation and infiltration of dendritic cells in airways of COPD patients and in response to cigarette smoke exposure. In line with this observation, Robbins and colleagues [115] reported that cigarette smoke reduces airway dendritic cells and linked this effect to the dysregulated antiviral immune responses observed in COPD and in response to cigarette smoke exposure. However, several other studies reported increased airway infiltration and accumulation of dendritic cells correlating with COPD severity [89, 109, 116]. It has also been proposed that the integral role played by dendritic cells in activating and differentiating T cells makes them potential participants in the inflammatory process of COPD [116]. This is consistent with the report of increased number of both dendritic cells and T cells in the small airways of COPD patients and cigarette smokers [111, 116]. Thus, reconciling the discrepancies regarding the role of dendritic cells in COPD and in response to cigarette smoke

exposure will undoubtedly consolidate current knowledge and unravel new therapeutic strategies.

1.5. Neutrophils

Neutrophils are short-lived leukocytes with a half-life of 8 h [117]. Neutrophils are recruited to the airways to provide innate immune defence against pathogens and toxic stimuli [118]. Chronic exposure to cigarette smoke destroys the defensive structure of the respiratory system comprising the epithelial cells tight junctions and mucociliary carpet to cause epithelial damage [119, 120]. These injuries causes the release of chemotactic mediators to recruit neutrophils in the lung [120, 121]. Considering the immune defence capabilities of neutrophils, it is logical that their accumulation in the airway would protect against bacteria colonisation usually orchestrated by *Streptococcus pneumonia* and/or Nontypeable *Haemophilus influenzae* (NTHi) in COPD [35]. However, the increased neutrophil abundance in the COPD airway correlates with the severity of COPD, suggesting that neutrophil activities in COPD airways are impaired and/or participate in COPD pathogenesis [122]. Moreover, COPD patients who have persistent NTHi infection show significantly higher airway neutrophil counts and inflammation than those without chronic NTHi infection [123, 124]. Neutrophil activation in the airway is a common finding for smokers and COPD patients, and plays a remarkable role in COPD pathogenesis [125], by secreting high levels of pro-inflammatory mediators [126, 127]. Lonergan and colleagues [128] showed higher blood neutrophil counts in COPD and suggested that blood neutrophil count could also be used to identify the frequency of exacerbation and mortality. Several studies have shown that neutrophils in the lung of smokers and COPD subjects have impaired ability to remove bacteria [129, 130]. This permits bacterial persistence, further contributing to airway inflammation and exacerbation of COPD.

Therefore, there is a concerted effort to design therapies to reduce neutrophil numbers to improve inflammation in COPD [131, 132]. However, precautions must also be taken to avoid excessively reducing neutrophil count as this could potentially also compromise immune function.

Neutrophils also activate proteases such as neutrophil elastase, matrix metalloproteinase 9 (MMP-9), MMP-8, proteinase 3 and cathepsin G while inhibiting the activity of anti-proteases [133, 134]. Over-exuberant proteases in the airways cause protease-anti-protease imbalances consequently leading to mucus hypersecretion, thickening and narrowing of the small airways and lung tissue destruction which are peculiar hallmarks of COPD [135]. Therefore, it is of no

surprise that neutrophil activation markers in sputum such as neutrophil extracellular traps (NET), neutrophil elastase (NE) and myeloperoxidase are linked to COPD severity and the rate of lung function deterioration [130, 136-138]. Thus, the immune protective functions of neutrophils are compromised in COPD, providing a detrimental microenvironment for the disease progression and severity.

1.6. Macrophages

Macrophages are mononuclear phagocytes and innate immune cells. These highly specialised cells are responsible for protecting the airways against pathogens and toxic particulate matters. They survey and engulf foreign structures from the extracellular milieu to maintain homeostasis [139, 140]. Two macrophage subsets reside in the lung. 1) Alveolar macrophages which populate the airspaces in close proximity to type I and type II alveolar epithelial cells. Type I alveolar epithelial cells cover approximately 95-98% of the alveolar surface area and are critical for gaseous exchange while type II alveolar epithelial cells cover a relatively small portion of the alveolar surface area, approximately 2-5% and are vital for secreting surfactant and preserving lung fluid balance [141, 142]. Alveolar macrophages originate from fetal monocytes that transform into long-lived macrophages in the first week of developmental stages [143]; 2) Interstitial macrophages which are located in the interstitial space between the alveolar epithelium and the microvascular endothelium where dendritic cells, T cells and B cells are mostly found. Interstitial macrophages are usually derived from blood monocytes during environmental stimuli [142, 144]. For example, in response to infection or noxious stimuli, blood monocytes can infiltrate tissues where they can differentiate into interstitial macrophages or dendritic cells [144].

1.6.1. *Macrophage polarisation in health and diseases*

Macrophages are capable of altering their phenotype and function to provide unique and efficient response to environmental stimuli. Macrophages can be polarised into an alternatively activated macrophages (M2) or classically activated macrophages (M1) which exhibit functionally distinct phenotypes [145]. Both macrophage M1 and M2 phenotypes can be stimulated by cytokines derived from T cells. M1 macrophages can be activated by IFN-γ while M2 macrophages can be stimulated by IL-40 or IL-10. Generally, M1 macrophages can activate pro-inflammatory T cell immune response by polarising T cells to immune reactive T helper 1 (Th1) cells. Conversely, M2 macrophages are usually anti-inflammatory and suppress immune response by switching T cells to immunosuppressive T helper 2 (Th2) cells [146]. Moreover,

M1 macrophages generally produce pro-inflammatory mediators and can participate in antigen presentation. In contrast, M2 macrophages usually produce anti-inflammatory mediators and are inefficient in presenting antigens but vital for the phagocytic removal of apoptotic cells [147]. This may explain why the M1 phenotype is essential for activating immune response against pathogens while M2 macrophages are vital for wound healing and removal of apoptotic cells [148, 149].

Eapen *et al* [150] showed that in a normal non-smoker's lung, the macrophages obtained from bronchoalveolar lavage (BAL) are predominately of the M1 phenotype, while undifferentiated (M0) macrophages are the dominant macrophages residing in small airway tissues (interstitial macrophages). These findings confirm an earlier report from our group [147] who identified a mixed predominately M1 phenotype in alveolar macrophages from COPD patients and healthy smokers compared to control subjects, evident by a heightened ability to produce inflammatory cytokines in response to stimuli (M1) in both groups. In contrast, there was a significant defect in their capacity for efferocytosis (reduced M2 functionality), reduced arginase and reduced expression of M1 markers involved with antigen presentation (MHC Classes I and II).

1.6.2. *Role of macrophages in COPD*

Macrophages are the major type of immune cells in the airway, representing approximately 90% of the immune cells and making them central 'gatekeepers' of the respiratory system [151, 152]. The abundance of macrophages in the lung is critical to facilitate rapid and efficient responses to environmental irritants and microbial invasion. These sentinel functions of airway macrophages could confer protection against the inflammatory responses and lung tissue destruction observed in COPD. However, several studies have implicated defective functions of airway macrophages in COPD pathophysiology [153]. This adds complexities to the roles of macrophages in COPD.

There is an increased number of alveolar macrophages in the airway of smokers and COPD patients compared with normal subjects [62, 154]. These macrophages produce higher amounts of inflammatory mediators and proteases that can cause airway structural alterations and airflow obstruction in COPD [62, 147]. Interestingly, Hogg *et al* [30] showed that the increased production of pro-inflammatory mediators by macrophages from COPD subjects significantly correlate with COPD severity.

The increased airway macrophages in COPD and in response to cigarette smoke exposure is partly attributed to the heightened migration and infiltration of monocyte from the blood stream to lung tissues through the vascular endothelium. Mediators of this process include monocyte chemoattractant chemokines such as MCP-1, which is also highly expressed in BAL of COPD patients [155-157]. It is also possible that accumulation of macrophages in COPD airways is enhanced due to a higher proliferative and survival rate as a compensatory mechanism to increase their defensive capabilities against noxious stimuli.

1.6.3. *Recent advances in macrophage dysfunction in COPD*

Concerns about the association between higher macrophage number and COPD severity have spurred renewed interest to delineate the molecular mechanism underlying macrophage dysregulation in COPD. Reports by our group [158-161] and several others [152, 162, 163] show that alveolar macrophages from cigarette smokers and COPD subjects are defective in their ability to remove bacteria and apoptotic cells compared to those from healthy subjects. The compromised clearance of bacteria is a likely reason for the bacterial colonisation observed in COPD airways [158, 164] showed that one reason may be a result of a compromised phagocytic activity of macrophages. It is postulated that the dysregulated phagocytic removal of pathogens and apoptotic cells also contributes to the COPD progression [162].

This suggests that strategies to modulate phagocytic function of macrophages in COPD could potentially halt disease progression and improve patients' quality of life and that further investigations are warranted to better understand the molecular mechanisms underpinning the defective macrophages phagocytosis in COPD and in response to cigarette smoke. Hence, we next discuss the molecular events and mechanisms underlying macrophage phagocytosis of apoptotic cells and pathogens. Further, we identify molecular targets essential for development and design of appropriate COPD therapies.

1.6.4. *Macrophage phagocytosis of apoptotic cells*

Apoptosis is a homeostatic mechanism that occurs frequently in healthy individuals, spontaneously or in response to oxidative stress and is essential to replace damage or unwanted cells. Airway epithelial and endothelial cells also undergo apoptosis in response to inhaled noxious particles and/or gases [38, 165]. The close proximity of these cells with alveolar macrophages permits rapid phagocytic clearance of the apoptotic cells by the macrophages through a process known as efferocytosis [142]. Efferocytosis is a non-phlogistic phenomenon critical for repairing damaged tissue and releasing anti-inflammatory cytokines to resolve

inflammation [166]. Hence, the relevance of efferocytosis goes beyond ingestion and degradation of dying cells and their intracellular contents. It is also critical for modulating the immune system to resolve inflammation. For instance, impaired efferocytosis allows the apoptotic cells to accumulate, rupture and release their toxic contents including oxidants, noxious enzymes and lysosomal proteases into the tissue microenvironment through a process termed secondary necrosis or post-apoptotic necrosis [167, 168]. This process can stimulate uncontrolled inflammation and inappropriate immune responses that are precursors of lung tissue destruction (emphysema). In line with this, Ramirez *et al* [169] and others [170, 171] demonstrated that removing the efferocytic capabilities of macrophages in mice led to increased number of apoptotic cells, nephritis, dermatitis, uncontrolled inflammation and autoimmune diseases. Therefore, it is reasonable to posit that timely removal of apoptotic cells by macrophages in COPD is a prerequisite for mitigating the progression of several diseases. This also explains why the alterations in macrophage function in COPD influence the severity of the disease, as reported by Eltboli *et al* and others[163] [172], and consistent with reports that cigarette smoke suppresses macrophage efferocytosis capacity [147, 161, 173]. Hodge et al [160] further demonstrated that deliberately manipulating macrophage efferocytic capacity has therapeutic potential in COPD [158]. However, incomplete understanding of the mechanisms underlying the macrophage dysfunction at various stages of efferocytosis limits the search for therapeutic strategies for restoring macrophage function. To this end, we next describe the known pathways that coordinate efficient efferocytosis.

1.7. Stages of efferocytosis

The clearance processes can be grouped into four main stages: (1) The expression of "good-bye" metabolites and chemoattractant "find me" signals by apoptotic cells [174]. These signals permit the infiltration of phagocytes to the site of injury or cell death. Examples of "good-bye" metabolites include adenosine monophosphate (AMP), glycerol-3-phosphate (G3P), spermidine, creatine and guanosine 5′-monophosphate (GMP). Examples of "Find me" signals include lysophosphatidylcholine (LPC), CX3C motif chemokine ligand 1 (CX3CL1 or fractalkine) sphingosine-1-phosphate (S1P), and nucleotides (adenosine triphosphate; ATP and uridine triphosphate; UTP); (2) The release of "eat me" signals such as phosphatidylserine (PtdSer) and calreticulin or ICAM-1 epitopes by the apoptotic cells for identification by the phagocyte. This leads to ingestion of the dying cells that is a fundamental step in the processes regulating phagosome formation. (3) Engagement of some components of autophagy machinery including reactive oxygen species (ROS), NADPH oxidase 2 (NOX2),

Phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3 complex) comprising Vacuolar protein sorting 34 (VPS34), Beclin-1 and ultraviolet radiation resistance associated (UVRAG), autophagy-related (Atg) 3, Atg12-Atg5-Atg16L1, and Atg7 and Rubicon (RUN domain Beclin-1-interacting and cysteine-rich domain-containing protein). This permits the recruitment of lapidated Microtubule-associated protein 1A/1B-light chain 3 (LC3) to the phagosome membrane for LAPosome formation where the ingested apoptotic cells are processed for digestion by lysosomal enzymes. LC3-associtated phagocytosis (LAP) is an under-recognised but essential step required for phagolysosomal maturation and quiescent removal of apoptotic cells as illustrated below in **figure 2**. (4) Post-digestion outcomes include the secretion of anti-inflammatory cytokines, immunologically silent signals and exportation of digested products into the cytosol to influence intracellular metabolic and biosynthetic pathways [175-177].

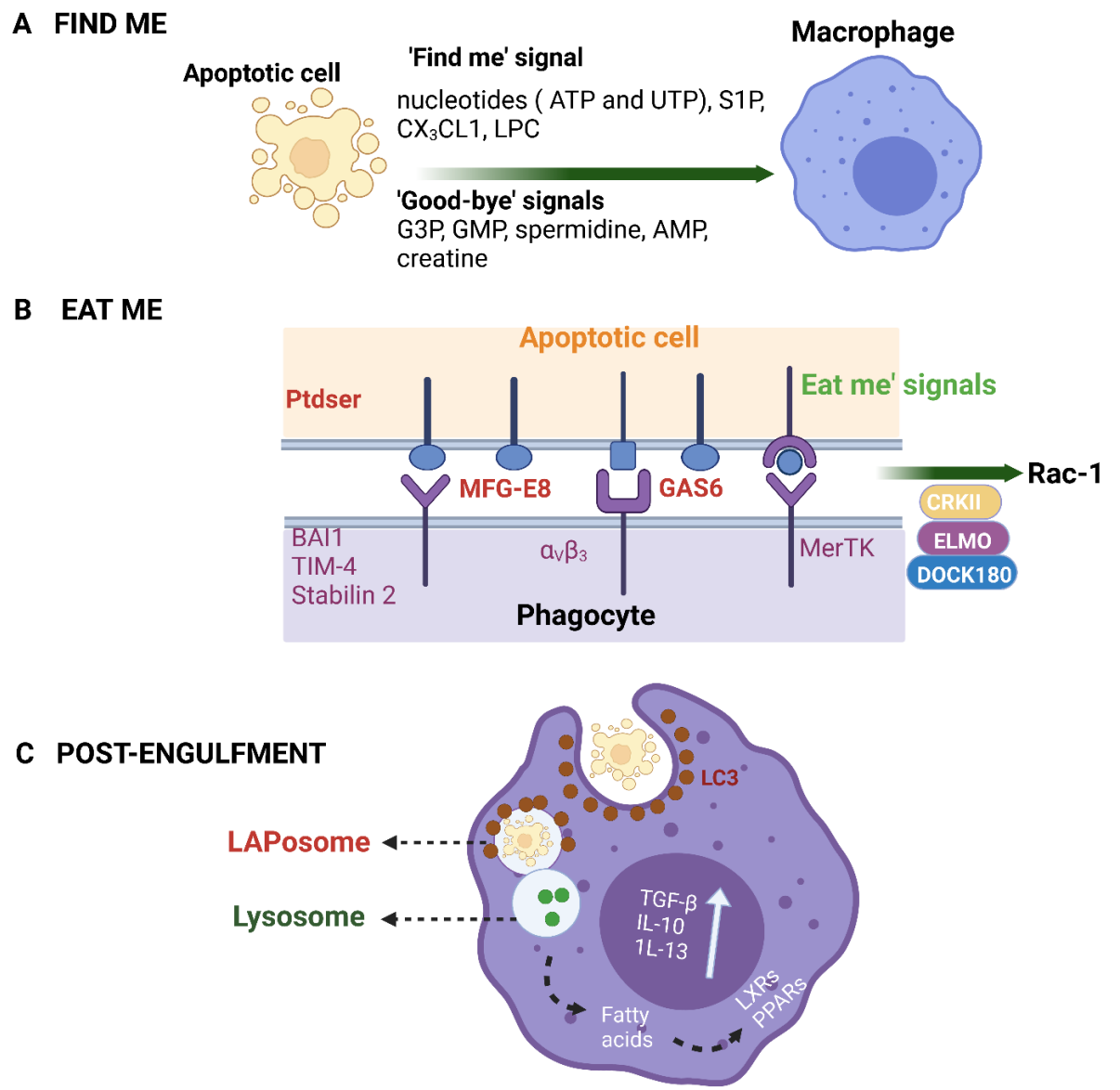

Figure 2: Release of "find me" and "eat me" signals is a requisite for recognition and engulfment necessary for apoptotic cell clearance. (A) "Find me" signals: dying cells express signals that drive phagocytes to the site of apoptotic cell death. These signals include S1P, CX3CL1, LPC and nucleotides (UTP and ATP). Phagocytes use cognate receptors such as S1PRs, G2A, CXCR3 and P2Y2 to find the apoptotic **(B)** "Eat me" signals: the dying cells express "eat me" signals that permit localisation of macrophages to identify and ingest the dying cells using bridging molecules and surface receptors. Ptdser is the main "eat me" signal released by macrophages and is normally recognized by phagocytic receptors such as TIM4, BAI and stabilin 2. Macrophages can also utilise tyrosine kinase receptor including Mertk and avB3 to bind Ptdser indirectly via bridging molecules including Gas-6 and MFG-E8, respectively. **(C)** Phagosome formation process: engulfment receptors are recruited in the

macrophages through the CRKII-ELMO-DOCK180 complex. This triggers Rac-1 signalling pathway is for cytoskeletal rearrangement and phagosome formation. After phagosome formation, LC3 is trafficked to phagosomes (now termed as 'LAPosome'). This enhances phagolysosomal fusion and digestion of the engulfed apoptotic structure. Digested cellular debris release fatty acids to trigger PPARγ and LXR for cholesterol efflux l causes the secretion of anti-inflammatory cytokines including IL-10, TGF-β and IL-13. LPC, lysophosphatidylcholine; C-X3-C Motif Chemokine Ligand 1; S1P, sphingosine-1-phosphate; CX3CL1; ATP, adenosine 5′ triphosphate; UTP, uridine 5′ triphosphate;

BAI, brain-specific angiogenesis inhibitor 1; G2A, G-protein-coupled receptor 132; PtdSer, phosphatidylserine; CXCR3, C-X-C motif chemokine receptor 3; SIPRs, sphingosine-1-phosphate receptors; P2Y2, purinergic receptors; Gas-6, growth arrest-specific 6; MFG-E8, milk fat globule-EGF factor 8 protein; mertk, mer proto-oncogene, tyrosine kinase; avB3, alpha-v beta-3; TIM-4, T cell immunoglobulin mucin receptor-4; AMP, adenosine monophosphate; GMP, 5′-monophosphate; G3P, glycerol-3-phosphate guanosine; CRKII, chicken tumor virus no. 10 (CT10) regulator of kinase II; DOCK, dedicator of cytokinesis; ELMO, engulfment and cell motility protein; LC3, microtubule-associated protein 1A/1B-light chain 3; PPARγ, peroxisome proliferator-activated receptor gamma; LXR, liver X receptor; interleukin (IL)-13, IL-10, and transforming growth factor- beta (TGF-β); *Created with BioRender.

1.8. LC3-associated phagocytosis (LAP)

The process of recruiting LC3 to phagosome membranes is termed LAP, and is a non-canonical form of autophagy. LAP was described about a decade ago when Sanjuan *et al* [178] observed an interaction between the LC3 conjugation system and a single-membrane vesicle which facilitated the recruitment of lysosomal enzymes and enhanced elimination of ingested particles. Since then there has been a growing interest to elucidate the role of LAP in regulating phagosome maturation and lysosomal trafficking. Several studies demonstrated that LAP activity facilitates the physiological function of phagocytes by enhancing the trafficking of lysosomes with phagosomes-containing cargo [179-182]. Impaired LAP can disrupt the physiological functions of tissues by delaying phagolysosomal acidification and the clearance of foreign particles [183, 184]. This shows that the activity of LAP is integral for preserving cellular homeostasis during environmental stress caused by factors such as apoptosis, fungal,

viral and bacterial infection. Hence, impaired LAP is one key pathological process at the crossroads of defective phagocytosis and disease. Whether LAP impairment contributes to the defective removal of microbes and apoptotic cells in COPD remains to be elucidated, and a better understanding of the role of LAP in phagocytosis and its potential implications in COPD could provide a new direction for treatment of the disease.

1.8.1. *Role of LAP in efferocytosis*

Several studies showed that dysregulation of LAP causes defective efferocytosis [179, 185]. This subsequently induces the production of high levels of inflammatory mediators and T cell activation that can lead to a lupus-like autoimmune syndrome. Further, there are reports that activity the of LAP correlates with efficient efferocytosis is consistent with these findings [179]. Overall, these reports are salient evidence demonstrating that LAP is a principal regulator of efferocytosis. Therefore, factors that diminish or compromise LAP can have detrimental implications in diseases where protracted inflammation and sustained immune activation are major determinants, as observed in COPD and upon cigarette smoke exposure [186]. Particularly important is that typical hallmarks of diminished LAP, including the acquisition of necrotic features perpetuated by the leakage of uncleared apoptotic cells, have also been related to COPD pathogenesis and severity [187, 188]. Future studies are required to examine whether LAP, as an essential biological phenomenon that regulates efferocytosis, is insufficient or impaired in COPD and in response to cigarette smoke exposure.

The search for the possible relationship between LAP and COPD has highlighted two potential causes of the defective efferocytosis in COPD: (1) increased rate of apoptosis that overwhelms macrophage clearance capacity. In this context, it is plausible that normal LAP activity is insufficient to execute the clearance of apoptotic cells generated by the spontaneous cell death that often occurs in COPD and, (2) defects in the pathways coordinating the clearance of apoptotic cells by airway macrophages. This raises the possibility that the factors contained in cigarette smoke can also impair the processes that coordinate LAP to dysregulate efferocytosis as described in **figure 3**. Surprisingly, the significance of LAP activity in defective efferocytosis in COPD is yet to be delineated. Incompletely understood also is the ability of airway macrophages to trigger LAP in COPD to clear the apoptotic cells orchestrated by cigarette smoke.

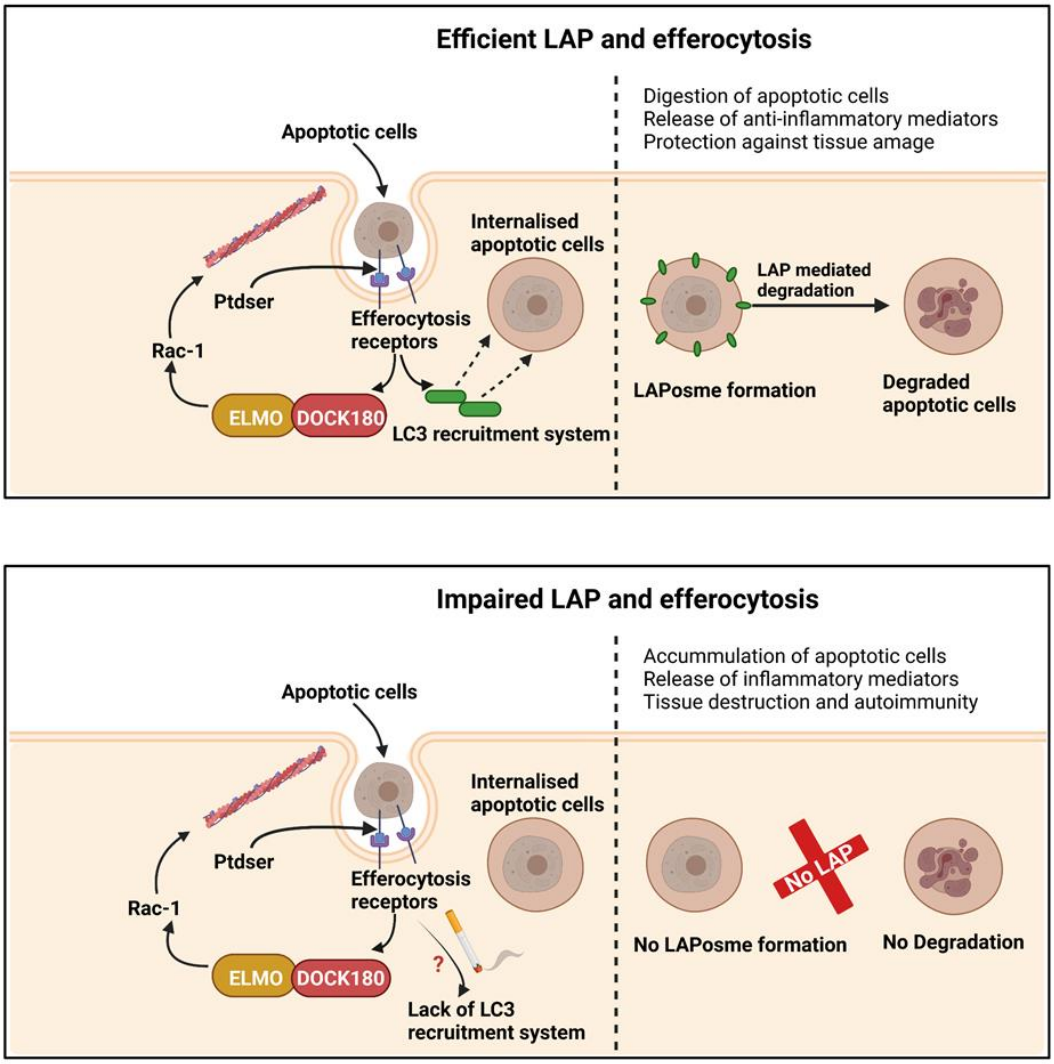

Figure 3: Potential consequences of defective LAP and efferocytosis in COPD. **(A)** Apoptotic cells express PtdSer on their surface, which is recognised by macrophages through the efferocytic receptors. Interaction between efferocytic receptors and PtdSer trigger activation of ELMO, DOCK180 and Rac1 for cytoskeletal arrangement necessary for ingestion and formation of phagosome. Formation of phagosome rapidly triggers LC3-recruitment system to initiate LAP. Sufficient LAP, which suggests that LC3 is localised on phagosome membranes, permits the transfer of lysosomal enzymes from lysosomes to phagosome to degrade the engulfed cargo. This results in production of anti-inflammatory mediators and protection against tissue damage. (B) Deficiencies in the LC3 recruitment system leads to defective LAP and impaired degradation of apoptotic cells. In consequence, apoptotic cells accumulate and leak toxic intracellular content in the tissue microenvironment. This can cause inflammation that could lead to tissue damage. DOCK,

dedicator of cytokinesis; PtdSer, phosphatidylserine; ELMO, engulfment and cell motility protein; LC3, microtubule-associated protein 1A/1B-light chain 3-II; *Created with BioRender

1.8.2. *Clinical relevance of LAP during infection*

Professional phagocytes including macrophages employ phagocytosis to provide immune protection against microbes by engulfing them to form phagosomes [189]. A key process required for this clearance process is the maturation and acidification of the phagosome [190]. This step is critical for the recruitment of lysosomal acid hydrolases and their fusion with the phagosomes and consequently degradation of the microbes. Interestingly, lysosomal tethering and fusion with phagosomes-containing microbes is enhanced in macrophages that are LAP-sufficient, but delayed in the absence of LAP [178]. Deficiency of LAP significantly impairs the anti-microbial function of macrophages even under conditions where lysosomal enzymes activities are preserved [191]. In contrast, LAP sufficient macrophages have rapid phagolysosomal fusion and subsequent clearance of bacteria such as *Aspergillus fumigatus* [181], *Listeria monocytogenes* [191] and *Legionella dumoffii* [192]. The anti-microbial function of LAP is also evident in the lung of experimental mice where age-dependent impairment of LAP heightens bacteria colonisation and uncontrolled inflammatory responses [184]. Thus, the abundance of LAP underlies the anti-microbial potentials of macrophages.

Importantly, some bacteria such as *Mycobacterium tuberculosis* [193] and *Aspergillus fumigatus* have evolved strategies for manipulating LAP and compromising macrophage function to avoid phagocytic clearance. For example, mutations in *Mycobacterium tuberculosis* incorporating CpsA into their cell wall component have been shown to allow them to subvert LAP and microbicidal effects of macrophages [193]. This permits the bacteria to survive and colonise in the host cellular compartment. Conversely, macrophages infected with CpsA-deficient *M. tuberculosis* are able to employ LAP to efficiently execute their micobicidal effects. Moreover, while *Aspergillus fumigatus* can be specifically targeted by LAP for elimination, the fungus can evolve strategies to obviate LAP and sustain their survival. *A. fumigatus* can present melanin on the surface of their cell wall to prevent being targeted and degraded by LAP. However, human or mice macrophages infected with *A. fumigatus* conidia lacking the melanin component on their cell wall have increased LAP and in consequent an enhanced fungicidal activity [194]. Several experimental studies using different mouse models

and microbial infection strategies have demonstrated impaired LAP as a critical contributor to defective phagocytosis by macrophages [195-197]. Thus, the fate and virulence of some microbes are dependent on the abundance and deficiency of LAP in phagocytes as illustrated in **figure 4**.

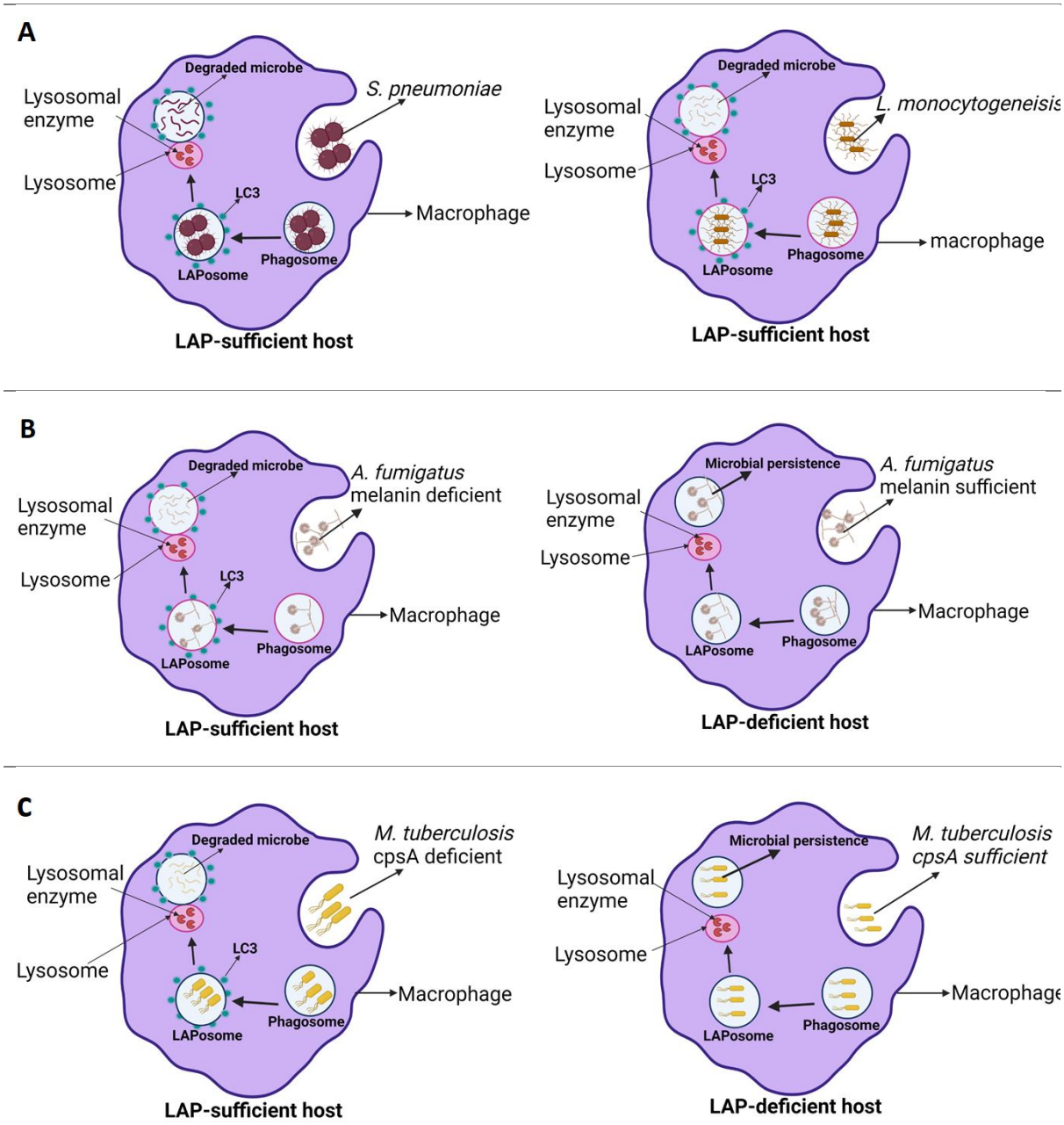

Figure 4: A-C Clearance of pathogens in LAP sufficient and LAP deficient macrophages. (A) LAP sufficient macrophages facilitate the clearance of streptococcus pneumonia and Listeria monocytogenesis. (B) Engulfment of melanin deficient Aspergillus fumigatus trigger LAP for macrophages to protect against fungal infection. However, Aspergillus fumigatus can mutate to incorporate melanin in their cell wall to subvert LAP.

LAP deficiency causes the fungus to survive and elicit detrimental effects. (C) Macrophages employ LAP to execute microbicidal effects on mycobacterium tuberculosis. However, mutation of Mycobacterium tuberculosis incorporating cpsA into the cell wall permits LAP evasion and bacterial survival.

It is now evident that microbial colonisation observed in COPD could be a result of impaired LAP [190, 198]. This heightens the proclivity that sustained microbial infections and colonisation observed in the airways of COPD subjects and in response to cigarette smoke exposure may also relate to lack of LAP. However, the paucity of information linking LAP and COPD adds uncertainty for the precise role of LAP in the diseases pathogenesis and progression and there are no current interventional LAP-targeted strategies for COPD. Therefore, better understanding the signalling pathways that leads to LAP induction and elucidate a role for LAP in COPD may be of significant clinical value. Next, we discuss the coordinating factors involved in LAP and identify molecules that could specifically be targeted to regulate LAP activity.

1.8.3. *Mechanism of LAP induction*

LAP is triggered when phagocytes recognise and ingest foreign structures in a single membrane vesicle called phagosome [198]. As detailed above, host cells recognise the presence of bacteria, fungi and apoptotic cells by expressing phagocytic receptors such as toll-like receptor (TLR), C-type lectin receptor Dectin-1 (also known as CLEC7A) or Dectin-2 and TIM-4, respectively. As a consequence, the cells engulf and internalise the cargo in the phagosome. This is followed by rapid interaction of the phagosome with the PI3KC3 complex (Phosphatidylinositol 3-kinase catalytic subunit type 3). The PI3KC3 complex comprises Vacuolar protein sorting 34 (VPS34), Vacuolar protein sorting 15 (VPS15), Beclin-1, UVRAG and Rubicon. The interaction allows VPS34 to produce phosphatidylinositol 3-phosphate (PI3P) on the phagosome [178, 181, 199]. PI3P thereby interacts with p40phox subunit of the phagocytic NADPH oxidase (NOX) complex. This stabilizes the activity of NOX2 complex and the trafficking of cytosolic NADPH oxidase factors p67phox, p47phox, p22phox and gp91phox (NOX2 isofom in phagocytes) [181, 200, 201]. Subsequently, reactive oxygen species (ROS) are produced in the lumen of the phagosome. ROS generation is crucial for recruiting Atg5-Atg12, Atg3-Atg7 and Atg16 to conjugate phosphatidylethanolamine (PE) to LC3-I. This causes lipidation of LC3-I to LC3-II. LC3-II can be deconjugated to LC3-I by

Atg4. ROS inhibits the de-conjugation activity of Atg4 to preserve LC3-II generation on the surface of the phagosome. The LC3-conjugated phagosome (known as LAPosome) allows the transfer of acid hydrolases from the lysosome to the phagosomal lumen to digest the engulfed structures [179, 198, 202].

Noteworthy, the interaction between Rubicon and the phagosome leads to the generation of PI3P and ROS [181, 203]. ROS production is a requisite for LAP [181, 190, 199]. ROS abundance in the phagosomal lumen is reduced in the absence of Rubicon or if PI3P fails to bind to NOX2. This will lead to LAP impairment because LC3-II will fail to associate with the phagosome. For example, deliberately deleting Rubicon in mice or cells destabilises NOX2 and subsequently reduces ROS. Moreover, Rubicon deficiency causes PI3P to recruit LC3-II to a double membrane vesicle to trigger canonical autophagy instead of LAP [149, 204, 205]. Hence, Rubicon is specific for LAP. This shows that Rubicon can be specifically targeted to inhibit LAP without adversely interfering with the physiological functions of canonical autophagy. It also confirms the current understanding that Rubicon inhibition represents LAP impairment [206].

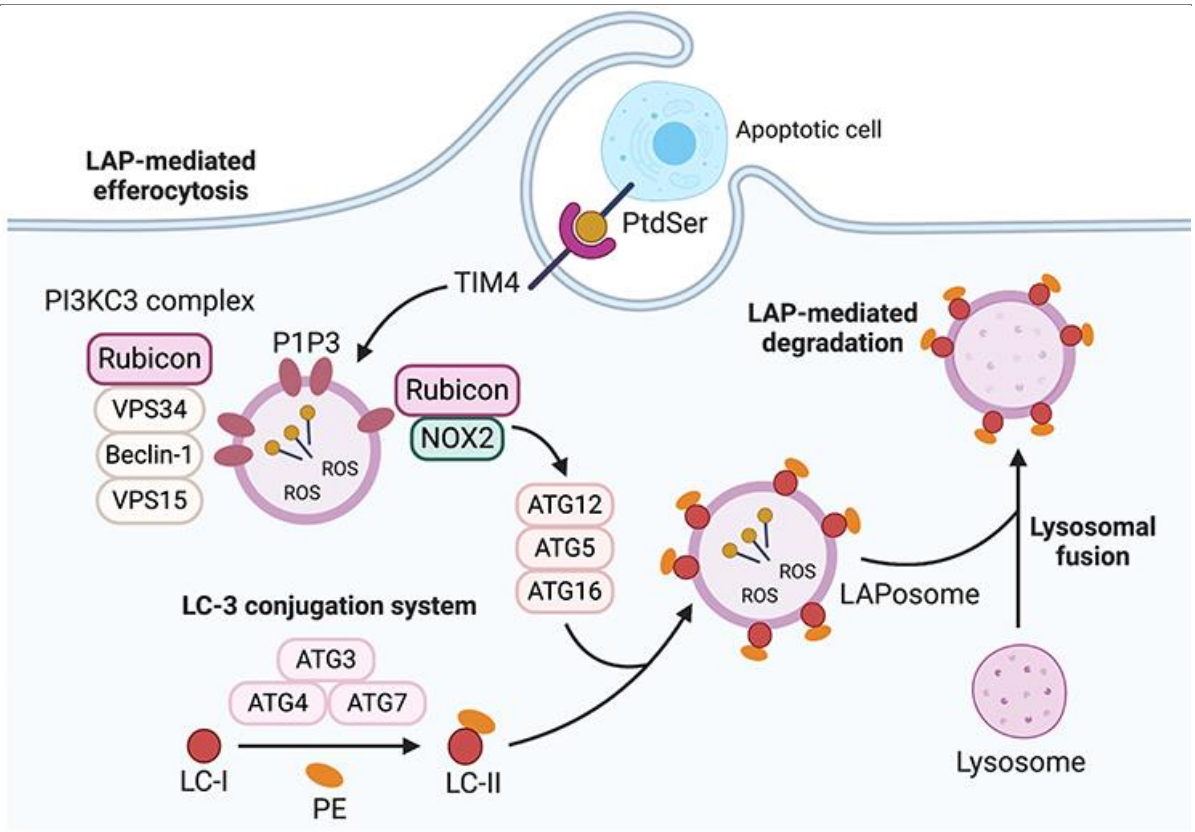

Figure 5: LC3-Associated phagocytosis (LAP). LAP coordinating activity is initiated after phagosome formation to finalise the digestion process of the engulfed particle. Phagocytes

recognise the foreign particle through the recognition receptors including TLR and TIM-4 to internalise the particle to form phagosome. To trigger LAP, the phagosome interacts with the components of PI3KC3 complex including beclin-1, Rubicon, vps15 and vps34, which permits the recruitment of PI3P to the phagosome. This preserves the NOX2 activity for continuous production of ROS, which is essential for recruiting LC3-II to the phagosome (now termed as LAPosome). Lysosomes then localise and fuse with the LAPosome for LAPosome maturation and effective degradation of the engulfed particle. PI3KC3 complex (Phosphatidylinositol 3-kinase catalytic subunit type 3); PI3P (phosphatidylinositol 3-phosphate); VPS15 (Vacuolar protein sorting 15); VPS34 (Vacuolar protein sorting 34); TIM-4 (T-cell immunoglobulin, and mucin domain family of receptor 4); Rubicon (Run domain beclin-1-interacting and cysteine-rich Domain-containing protein); LC3-II (microtubule-associated protein 1A/1B-light chain 3-II); NOX2 (NADPH oxidase 2) and ; ROS (reactive oxygen species). Created with BioRender.

1.8.4. *The current understanding relating to LAP and co-morbidities of COPD*

It is estimated that more than 50% of COPD patients develop co-morbidities such as metabolic disorders, autoimmune diseases and lung cancer [207], that are major causes of increased mortality rate in COPD [208]. However, the causal relationship between COPD and these co-morbidities are less well defined. This caused the American Thoracic Society/European Respiratory Society (ATS/ERS) to issue a Research Statement emphasising the need to delineate the missing link between COPD and the co-morbidities [209]. It is now evident that uncontrolled pulmonary and systemic inflammation is a risk factor associated with these phenomena [208, 210]. Therefore, processes such as LAP that can control inflammation may have significant impacts on some of the co-morbidities of COPD. Already, some components of LAP have been implicated in the pathogenesis of metabolic disorders and cancer [185, 211]. Hence, the next two paragraphs briefly discuss the potential relationship between LAP and metabolic disorder and cancer.

Metabolic disorders. Metabolic disorders are an independent risk factor for cardiovascular diseases and mortality in COPD [212, 213]. Marquis and colleagues [214] showed that metabolic disorders are two times more likely to occur in patients with COPD compared to normal individuals. Inflammation in the systemic circulation and adipose tissue is considered a major cause of metabolic disorder in COPD [215-217]. This reveals a potential role of LAP

in the pathogenesis of metabolic syndrome in COPD especially as Yamamuro *et al* [211] linked the loss of Rubicon (A specific regulator of LAP) during aging in adipose tissues to metabolic syndrome in mice. A separate study by Kuk et al [218] showed that metabolic syndrome correlates with age of individuals and suggested that older people are more susceptible to develop this syndrome compared to control. Therefore, it is plausible that a diminished LAP in COPD, which is usually a disease of the elderly, may influence the co-morbidity of metabolic syndrome.

Lung cancer. Several studies showed that COPD is a major risk factor for lung cancer [219, 220]. It is estimated that COPD patients are five times higher to develop lung cancer compared to non-COPD control subjects. Approximately, 1% of COPD patients develop lung cancer every year [220]. Studies demonstrated that deoxyribonucleic acid (DNA) damage caused by inflammation is one of the major causes of lung cancer. Normally, DNA damage can be repaired by the body's homeostatic mechanism. However, COPD patients fail to repair the DNA damage, which can lead to an increased susceptibility to several types of cancer including lung cancer [221]. This demonstrates that resolving inflammation in COPD to alleviate protracted DNA damage has the potential to prevent lung cancer as a co-morbidity. Therefore, it is tempting to posit that the anti-inflammatory impact of LAP has the propensity to protect against the initiation of cancer. However, Cunha et al [185] have recently shown that the anti-inflammatory and immune tolerogenic impact of LAP generates a microenvironment supportive of tumour growth and aggressiveness. Therefore, while LAP may protect against tumour development in the early stages, this process can paradoxically favour the growth of an established tumour. Inspired by the dual role of LAP on tumour growth, we provided a comprehensive literature review detailing the reported impacts of LAP in the tumour microenvironment [149].

1.9. Project summary and rationale

COPD is a progressive chronic inflammatory lung disease and the third leading cause of death worldwide. Cigarette smoking contributes to approximately 90% of all COPD events, making it the major risk factor for this disease. Smoking cessation is critical, however, once COPD is established, smoking cessation alone will not completely reverse the abnormal inflammation in the lungs, improve physiological functioning or reduce susceptibility to acute deterioration as a result of new insults such as infection. COPD mostly affects the aging population: although a reduction in the prevalence of smoking has been achieved, the lag time of 20-50 years will

mean that there will be little reduction in incidence in the near future [222]. COPD is currently incurable and existing treatments are largely symptomatic. This has been a major hurdle for clinicians, researchers as well as policymakers. Thus, there is an increasing effort to provide new research and that can provide novel therapeutic options. Impaired phagocytic clearance of pathogens and apoptotic cells (a process termed efferocytosis) by airway macrophages promotes chronic inflammation and bacterial colonisation in the lower airways of cigarette smokers and COPD subjects. This consequently leads to increased oxidative stress and inflammation, which can cause lung tissue destruction. Despite extensive research in the area, the exact mechanisms through which cigarette smoke impairs the clearance of microbes and apoptotic cell is still not well defined. This suggests that the network of molecular signalling involved in this defect is complex and not as straightforward as might be perceived.

Lysosomal processing and phagocytic removal ofpathogens and apoptotic cells is regulated by the activation/involvement of a process termed LC3-associated phagocytosis (LAP). LAP involves trafficking of LC3 to phagosomes for the formation of a single membrane vesicle known as a LAPosome. For example, to initiate LAP during efferocytosis, components of the PI3KC3 complex, particularly Rubicon, are engaged by phagosomes to favour LC3 recruitment to phagosome membranes [223]. Hence, LAP is normally stimulated in Rubicon-sufficient phagocytes to modulate anti-inflammatory consequences of efferocytosis. Rubicon-deficient cells fail to recruit LC3 to the phagosome, leading to a failure of lysosomal acidification and a subsequent accumulation of apoptotic cells and bacterial colonisation [179, 224]. Therefore, the overall hypothesis of this thesis is that defective LAP demonstrated by Rubicon deficiency is a key component underlying the defective phagocytic clearance by alveolar macrophages in COPD.

Finally, the search for the therapeutic interventions that modulate LAP in COPD and its co-morbidities such as lung cancer cancer revealed a potential pro-tumorigenic role of LAP. Considering that tumour development could also be a consequence of chronic infections and inflammation, one would expect the salutary effects of LAP to at least in part include protection against tumour growth and progression. However, in contrast to this, alterations in mice with lung cancer that lead to LAP attrition restrict tumour growth and progression, while LAP-sufficient tumour mice exhibit increased tumour growth and aggressiveness [185]. Moreover, analysis of a publicly available data showed that Rubicon expression correlates with poor prognosis in patients with stomach cancer, breast cancer, endometrial cancer, liver cancer, colorectal cancer, and testicular cancer. These reports point towards LAP as an influential

mediator of tumour progression and are consistent with recent findings that higher apoptotic tumour cells indices correlate with tumour aggressiveness [225]. Noteworthy, the clearance of apoptotic cells involves the coordinated activity between the processes of LAP and efferocytosis. This can lead to the production of anti-inflammatory and immunetolerogenic signals that can be employed by live tumour cells to subvert anti-tumour immunity. As a consequence, this favours tumour proliferation and aggressiveness suggesting that LAP and efferocytosis can unwittingly enhance tumour growth and survival. In line with this, recent reports show that the tumour microenvironment with unscheduled apoptosis exhibits an immune suppressive niche that favours evasion of immune defence mechanism, aggressive cancers and metastatic spread. Therefore, the activity of LAP, which is normally utilised to remove apoptotic cells and avoid damage in normal tissues, tends to be tumorigenic in the tumour microenvironment. This highlights the need for a better understanding of the role of LAP, and its implications in the tumour microenvironment, to advise strategies that could be useful in manipulating LAP in COPD while preventing its potential pro-tumour influence. Therefore, this thesis also highlights the role of LAP and efferocytosis in tumour growth and proliferation.

Aims/objectives

The overall aim of this study was to identify dysregulation of the LAP pathway as a therapeutic target and pathogenic mechanism of COPD.

Using THP-1 macrophage cell lines, blood monocyte derived macrophages (MDM), bronchoalveolar lavage (BAL)-derived alveolar macrophages and lung tissue macrophages from mice and COPD subjects, our objectives were to:

1. Examine the components of the LAP signalling pathway in alveolar and lung tissue macrophages of COPD and in response to cigarette smoke exposure
2. Investigate the molecular basis through which the specific LAP regulator, Rubicon can be regulated in COPD and upon cigarette smoke exposure
3. Delineate the relationship between LAP/Rubicon dysregulation and the defective phagocytic clearance of pathogens and apoptotic cells in COPD and upon cigarette smoke exposure.

References

1. Johns DP, Walters JAE, Walters EH. Diagnosis and early detection of COPD using spirometry. J Thorac Dis. 2014;6(11):1557-69.
2. Barnes PJ. Mediators of Chronic Obstructive Pulmonary Disease. Pharmacological Reviews. 2004;56(4):515.
3. Agustí A, Celli B. Natural history of COPD: gaps and opportunities. ERJ Open Research. 2017;3(4):00117-2017.
4. Celli BR, MacNee W, Agusti A, Anzueto A, Berg B, Buist AS, et al. Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. European Respiratory Journal. 2004;23(6):932-46.
5. Fazleen A, Wilkinson T. Early COPD: current evidence for diagnosis and management. Therapeutic Advances in Respiratory Disease. 2020 2020/01/01;14:1753466620942128.
6. Miravitlles M, Ribera A. Understanding the impact of symptoms on the burden of COPD. Respiratory Research. 2017 2017/04/21;18(1):67.
7. Miravitlles M, Ferrer J, Baró E, Lleonart M, Galera J. Differences between physician and patient in the perception of symptoms and their severity in COPD. Respiratory Medicine. 2013 2013/12/01/;107(12):1977-85.
8. Davidson W, Bai TR. Lung structural changes in chronic obstructive pulmonary diseases. Current drug targets Inflammation and allergy. 2005 Dec;4(6):643-9.
9. Siafakas N, Bizymi N, Mathioudakis A, Corlateanu A. EARLY versus MILD Chronic Obstructive Pulmonary Disease (COPD). Respiratory Medicine. 2018 2018/07/01/;140:127-31.
10. Soriano JB, Polverino F, Cosio BG. What is early COPD and why is it important? European Respiratory Journal. 2018;52(6):1801448.
11. Anthonisen NR, Skeans MA, Wise RA, Manfreda J, Kanner RE, Connett JE. The Effects of a Smoking Cessation Intervention on 14.5-Year Mortality. Annals of Internal Medicine. 2005 2005/02/15;142(4):233-9.
12. Morgan AD, Zakeri R, Quint JK. Defining the relationship between COPD and CVD: what are the implications for clinical practice? Ther Adv Respir Dis. 2018 Jan-Dec;12:1753465817750524-.
13. Johnson KM, Bryan S, Ghanbarian S, Sin DD, Sadatsafavi M. Characterizing undiagnosed chronic obstructive pulmonary disease: a systematic review and meta-analysis. Respiratory Research. 2018 2018/02/07;19(1):26.
14. Global Initiative for Chronic Obstructive Lung Disease (GOLD). Global strategy for the diagnosis m, and prevention of chronic obstructive pulmonary disease. GOLD, 2021. Available at: www.goldcopd.org.
15. Calverley PMA. The GOLD Classification Has Advanced Understanding of COPD. American Journal of Respiratory and Critical Care Medicine. 2004;170(3):211-2.
16. Bailey KL. The importance of the assessment of pulmonary function in COPD. Med Clin North Am. 2012;96(4):745-52.
17. de Marco R, Accordini S, Cerveri I, Corsico A, Sunyer J, Neukirch F, et al. An international survey of chronic obstructive pulmonary disease in young adults according to GOLD stages. Thorax. 2004 Feb;59(2):120-5.
18. Juan Garcia J, Sierra Herranz L, García García S, Carazo Fernández L, Naveiro Rilo JC, Martínez García F, et al. Concordance among GOLD, NICE and LLN criteria in the spirometry diagnosis of airflow obstruction. European Respiratory Journal. 2016;48(suppl 60):PA4625.
19. Vestbo J, Hurd SS, Agustí AG, Jones PW, Vogelmeier C, Anzueto A, et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary

Disease. American Journal of Respiratory and Critical Care Medicine. 2013 2013/02/15;187(4):347-65.
20. Hardie JA, Buist AS, Vollmer WM, Ellingsen I, Bakke PS, Mørkve O. Risk of over-diagnosis of COPD in asymptomatic elderly never-smokers. European Respiratory Journal. 2002;20(5):1117-22.
21. Cerveri I, Corsico AG, Accordini S, Niniano R, Ansaldo E, Antó JM, et al. Underestimation of airflow obstruction among young adults using FEV₁/FVC <70% as a fixed cut-off: a longitudinal evaluation of clinical and functional outcomes. Thorax. 2008;63(12):1040.
22. Güder G, Brenner S, Angermann CE, Ertl G, Held M, Sachs AP, et al. "GOLD or lower limit of normal definition? a comparison with expert-based diagnosis of chronic obstructive pulmonary disease in a prospective cohort-study". Respiratory Research. 2012 2012/02/06;13(1):13.
23. Diab N, Gershon AS, Sin DD, Tan WC, Bourbeau J, Boulet LP, et al. Underdiagnosis and Overdiagnosis of Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med. 2018 Nov 1;198(9):1130-9.
24. Culver BH. Interpretation of spirometry: we can do better than the GOLD standard. Respiratory care. 2006 Jul;51(7):719-21.
25. Aggarwal AN, Gupta D, Agarwal R, Jindal SK. Comparison of the lower confidence limit to the fixed-percentage method for assessing airway obstruction in routine clinical practice. Respiratory care. 2011 Nov;56(11):1778-84.
26. MacNee W. Pathogenesis of chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2005;2(4):258-91.
27. Barnes PJ, Burney PGJ, Silverman EK, Celli BR, Vestbo J, Wedzicha JA, et al. Chronic obstructive pulmonary disease. Nature Reviews Disease Primers. 2015 2015/12/03;1(1):15076.
28. Chung KF, Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. European Respiratory Journal. 2008;31(6):1334.
29. Singh D, Roche N, Halpin D, Agusti A, Wedzicha JA, Martinez FJ. Current Controversies in the Pharmacological Treatment of Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med. 2016 Sep 1;194(5):541-9.
30. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med. 2004 Jun 24;350(26):2645-53.
31. Churg A, Wang RD, Tai H, Wang X, Xie C, Wright JL. Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. Am J Respir Crit Care Med. 2004 Sep 1;170(5):492-8.
32. Stone H, McNab G, Wood AM, Stockley RA, Sapey E. Variability of sputum inflammatory mediators in COPD and α₁-antitrypsin deficiency. European Respiratory Journal. 2012;40(3):561-9.
33. Cosio Piqueras MG, Cosio MG. Disease of the airways in chronic obstructive pulmonary disease. European Respiratory Journal. 2001;18(34 suppl):41s-9s.
34. MacNee W. Pathology, pathogenesis, and pathophysiology. BMJ. 2006;332(7551):1202-4.
35. Beech AS, Lea S, Kolsum U, Wang Z, Miller BE, Donaldson GC, et al. Bacteria and sputum inflammatory cell counts; a COPD cohort analysis. Respiratory Research. 2020 2020/11/01;21(1):289.
36. Ravi AK, Plumb J, Gaskell R, Mason S, Broome CS, Booth G, et al. COPD monocytes demonstrate impaired migratory ability. Respiratory Research. 2017 2017/05/11;18(1):90.

37. Hodge S, Hodge G, Holmes M, Jersmann H, Reynolds PN. Increased CD8 T-cell granzyme B in COPD is suppressed by treatment with low-dose azithromycin. Respirology. 2015;20(1):95-100.
38. Hodge S, Hodge G, Holmes M, Reynolds PN. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. European Respiratory Journal. 2005;25(3):447-54.
39. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nature Reviews Immunology. 2008 2008/03/01;8(3):183-92.
40. Brusselle GG, Joos GF, Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. The Lancet. 2011 2011/09/10/;378(9795):1015-26.
41. McDonough JE, Yuan R, Suzuki M, Seyednejad N, Elliott WM, Sanchez PG, et al. Small-Airway Obstruction and Emphysema in Chronic Obstructive Pulmonary Disease. New England Journal of Medicine. 2011;365(17):1567-75.
42. Oh JY, Sin DD. Lung inflammation in COPD: why does it matter? F1000 Med Rep. 2012;4:23-.
43. MacNee W, Murchison JT. Small Airway Disease or Emphysema: Which Is More Important in Lung Function and FEV1 Decline? An Old Story with a New Twist. American Journal of Respiratory and Critical Care Medicine. 2016 2016/07/15;194(2):129-30.
44. Niewoehner DE, Kleinerman J, Rice DB. Pathologic changes in the peripheral airways of young cigarette smokers. N Engl J Med. 1974 Oct 10;291(15):755-8.
45. Cosio M, Ghezzo H, Hogg JC, Corbin R, Loveland M, Dosman J, et al. The relations between structural changes in small airways and pulmonary-function tests. N Engl J Med. 1978 Jun 8;298(23):1277-81.
46. Hogg JC, McDonough JE, Suzuki M. Small airway obstruction in COPD: new insights based on micro-CT imaging and MRI imaging. Chest. 2013;143(5):1436-43.
47. Yuan R, Hogg JC, Paré PD, Sin DD, Wong JC, Nakano Y, et al. Prediction of the rate of decline in FEV₁ in smokers using quantitative computed tomography. Thorax. 2009;64(11):944-9.
48. Vestbo J, Edwards LD, Scanlon PD, Yates JC, Agusti A, Bakke P, et al. Changes in Forced Expiratory Volume in 1 Second over Time in COPD. New England Journal of Medicine. 2011;365(13):1184-92.
49. Hersh CP, Make BJ, Lynch DA, Barr RG, Bowler RP, Calverley PMA, et al. Non-emphysematous chronic obstructive pulmonary disease is associated with diabetes mellitus. BMC Pulmonary Medicine. 2014 2014/10/24;14(1):164.
50. Burgel P-R, Bergeron A, de Blic J, Bonniaud P, Bourdin A, Chanez P, et al. Small airways diseases, excluding asthma and COPD: an overview. European Respiratory Review. 2013;22(128):131-47.
51. Hogg JC, Paré PD, Hackett T-L. The Contribution of Small Airway Obstruction to the Pathogenesis of Chronic Obstructive Pulmonary Disease. Physiol Rev. 2017;97(2):529-52.
52. MACKLEM PT. The Physiology of Small Airways. American Journal of Respiratory and Critical Care Medicine. 1998;157(5):S181-S3.
53. Crisafulli E, Pisi R, Aiello M, Vigna M, Tzani P, Torres A, et al. Prevalence of Small-Airway Dysfunction among COPD Patients with Different GOLD Stages and Its Role in the Impact of Disease. Respiration. 2017;93(1):32-41.
54. Higham A, Quinn AM, Cançado JED, Singh D. The pathology of small airways disease in COPD: historical aspects and future directions. Respiratory Research. 2019 2019/03/04;20(1):49.
55. Yip KP, Stockley RA, Sapey E. Catching "Early" COPD - The Diagnostic Conundrum. Int J Chron Obstruct Pulmon Dis. 2021;16:957-68.

56. Churg A, Zhou S, Wang X, Wang R, Wright JL. The role of interleukin-1beta in murine cigarette smoke-induced emphysema and small airway remodeling. American journal of respiratory cell and molecular biology. 2009 Apr;40(4):482-90.
57. Pauwels NS, Bracke KR, Dupont LL, Van Pottelberge GR, Provoost S, Vanden Berghe T, et al. Role of IL-1α and the Nlrp3/caspase-1/IL-1β axis in cigarette smoke-induced pulmonary inflammation and COPD. The European respiratory journal. 2011 Nov;38(5):1019-28.
58. Huvenne W, Lanckacker EA, Krysko O, Bracke KR, Demoor T, Hellings PW, et al. Exacerbation of cigarette smoke-induced pulmonary inflammation by Staphylococcus aureus enterotoxin B in mice. Respir Res. 2011 May 27;12(1):69.
59. Bracke KR, Verhamme FM, Seys LJ, Bantsimba-Malanda C, Cunoosamy DM, Herbst R, et al. Role of CXCL13 in cigarette smoke-induced lymphoid follicle formation and chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2013 Aug 1;188(3):343-55.
60. Shapiro SD. End-Stage Chronic Obstructive Pulmonary Disease. American Journal of Respiratory and Critical Care Medicine. 2001;164(3):339-40.
61. Zhu J, Mallia P, Footitt J, Qiu Y, Message SD, Kebadze T, et al. Bronchial mucosal inflammation and illness severity in response to experimental rhinovirus infection in COPD. The Journal of allergy and clinical immunology. 2020 Oct;146(4):840-50.e7.
62. Isajevs S, Taivans I, Svirina D, Strazda G, Kopeika U. Patterns of inflammatory responses in large and small airways in smokers with and without chronic obstructive pulmonary disease. Respiration. 2011;81(5):362-71.
63. King PT. Inflammation in chronic obstructive pulmonary disease and its role in cardiovascular disease and lung cancer. Clin Transl Med. 2015;4(1):68-.
64. Di Stefano A, Turato G, Maestrelli P, Mapp CE, Ruggieri MP, Roggeri A, et al. Airflow limitation in chronic bronchitis is associated with T-lymphocyte and macrophage infiltration of the bronchial mucosa. Am J Respir Crit Care Med. 1996 Feb;153(2):629-32.
65. Fischer BM, Voynow JA, Ghio AJ. COPD: balancing oxidants and antioxidants. Int J Chron Obstruct Pulmon Dis. 2015;10:261-76.
66. Rahman I, Morrison D, Donaldson K, MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. Am J Respir Crit Care Med. 1996 Oct;154(4 Pt 1):1055-60.
67. Täger M, Piecyk A, Köhnlein T, Thiel U, Ansorge S, Welte T. Evidence of a defective thiol status of alveolar macrophages from COPD patients and smokers. Chronic obstructive pulmonary disease. Free radical biology & medicine. 2000 Dec;29(11):1160-5.
68. Wong ET, Szostak J, Titz B, Lee T, Wong SK, Lavrynenko O, et al. A 6-month inhalation toxicology study in Apoe(-/-) mice demonstrates substantially lower effects of e-vapor aerosol compared with cigarette smoke in the respiratory tract. Archives of toxicology. 2021 May;95(5):1805-29.
69. D'hulst AI, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. European Respiratory Journal. 2005;26(2):204-13.
70. Donohue KM, Hoffman EA, Baumhauer H, Guo J, Budoff M, Austin JHM, et al. Cigarette smoking and airway wall thickness on CT scan in a multi-ethnic cohort: the MESA Lung Study. Respiratory medicine. 2012;106(12):1655-64.
71. Amin K, Ekberg-Jansson A, Löfdahl C-G, Venge P. Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: a biopsy study. Thorax. 2003;58(2):135-42.
72. Tanino M, Betsuyaku T, Takeyabu K, Tanino Y, Yamaguchi E, Miyamoto K, et al. Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. Thorax. 2002;57(5):405-11.

73. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Bristot L, Capelli A, et al. Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD. Thorax. 2009;64(11):968-75.
74. Wang Y, Xu J, Meng Y, Adcock IM, Yao X. Role of inflammatory cells in airway remodeling in COPD. Int J Chron Obstruct Pulmon Dis. 2018;13:3341-8.
75. Rutgers SR, Postma DS, ten Hacken NHT, Kauffman HF, van der Mark TW, Koëter GH, et al. Ongoing airway inflammation in patients with COPD who do not currently smoke. Thorax. 2000;55(1):12-8.
76. Gamble E, Grootendorst DC, Hattotuwa K, O'Shaughnessy T, Ram FSF, Qiu Y, et al. Airway mucosal inflammation in COPD is similar in smokers and ex-smokers: a pooled analysis. European Respiratory Journal. 2007;30(3):467-71.
77. Lee K-H, Lee J, Jeong J, Woo J, Lee C-H, Yoo C-G. Cigarette smoke extract enhances neutrophil elastase-induced IL-8 production via proteinase-activated receptor-2 upregulation in human bronchial epithelial cells. Experimental & Molecular Medicine. 2018 2018/07/01;50(7):1-9.
78. Guiedem E, Pefura-Yone EW, Ikomey GM, Nkenfou CN, Mesembe M, Yivala MM, et al. Cytokine profile in the sputum of subjects with post-tuberculosis airflow obstruction and in those with tobacco related chronic obstructive pulmonary disease. BMC Immunol. 2020 Oct 1;21(1):52.
79. Lugade AA, Bogner PN, Thatcher TH, Sime PJ, Phipps RP, Thanavala Y. Cigarette smoke exposure exacerbates lung inflammation and compromises immunity to bacterial infection. Journal of immunology (Baltimore, Md : 1950). 2014;192(11):5226-35.
80. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. Lancet (London, England). 2004 Aug 21-27;364(9435):709-21.
81. Barnes PJ. Small Airways in COPD. New England Journal of Medicine. 2004;350(26):2635-7.
82. Perticone M, Maio R, Caroleo B, Suraci E, Corrao S, Sesti G, et al. COPD significantly increases cerebral and cardiovascular events in hypertensives. Scientific Reports. 2021 2021/04/12;11(1):7884.
83. Park HY, Kang D, Shin SH, Yoo K-H, Rhee CK, Suh GY, et al. Chronic obstructive pulmonary disease and lung cancer incidence in never smokers: a cohort study. Thorax. 2020;75(6):506-9.
84. Ogawa K, Koh Y, Kaneda H, Izumi M, Matsumoto Y, Sawa K, et al. Can smoking duration alone replace pack-years to predict the risk of smoking-related oncogenic mutations in non-small cell lung cancer? A cross-sectional study in Japan. BMJ Open. 2020;10(9):e035615.
85. Zhou J-S, Li Z-Y, Xu X-C, Zhao Y, Wang Y, Chen H-P, et al. Cigarette smoke-initiated autoimmunity facilitates sensitisation to elastin-induced COPD-like pathologies in mice. European Respiratory Journal. 2020:2000404.
86. Sinden NJ, Stockley RA. Systemic inflammation and comorbidity in COPD: a result of 'overspill' of inflammatory mediators from the lungs? Review of the evidence. Thorax. 2010;65(10):930-6.
87. Hodge G, Roscioli E, Jersmann H, Tran HB, Holmes M, Reynolds PN, et al. Steroid resistance in COPD is associated with impaired molecular chaperone Hsp90 expression by pro-inflammatory lymphocytes. Respiratory research. 2016;17(1):135-.
88. Sun Y, Wang K, Li M-X, He W, Chang J-R, Liao C-C, et al. Metabolic changes of H2S in smokers and patients of COPD which might involve in inflammation, oxidative stress and steroid sensitivity. Scientific Reports. 2015 2015/10/12;5(1):14971.

89. Vassallo R, Walters PR, Lamont J, Kottom TJ, Yi ES, Limper AH. Cigarette smoke promotes dendritic cell accumulation in COPD; a Lung Tissue Research Consortium study. Respiratory Research. 2010 2010/04/26;11(1):45.
90. Roy RM, Klein BS. Dendritic cells in antifungal immunity and vaccine design. Cell host & microbe. 2012;11(5):436-46.
91. Condon TV, Sawyer RT, Fenton MJ, Riches DWH. Lung dendritic cells at the innate-adaptive immune interface. Journal of leukocyte biology. 2011;90(5):883-95.
92. Guery L, Hugues S. Tolerogenic and Activatory Plasmacytoid Dendritic Cells in Autoimmunity. Frontiers in Immunology. [Review]. 2013 2013-March-06;4(59).
93. Price JD, Tarbell KV. The Role of Dendritic Cell Subsets and Innate Immunity in the Pathogenesis of Type 1 Diabetes and Other Autoimmune Diseases. Frontiers in Immunology. [Review]. 2015 2015-June-15;6(288).
94. Wei F-Q, Sun W, Wong T-S, Gao W, Wen Y-H, Wei J-W, et al. Eliciting cytotoxic T lymphocytes against human laryngeal cancer-derived antigens: evaluation of dendritic cells pulsed with a heat-treated tumor lysate and other antigen-loading strategies for dendritic-cell-based vaccination. J Exp Clin Cancer Res. 2016;35:18-.
95. Nouri-Shirazi M, Guinet E. Exposure to Nicotine Adversely Affects the Dendritic Cell System and Compromises Host Response to Vaccination. The Journal of Immunology. 2012;188(5):2359-70.
96. Mbongue J, Nicholas D, Firek A, Langridge W. The role of dendritic cells in tissue-specific autoimmunity. J Immunol Res. 2014;2014:857143-.
97. Fu C, Peng P, Loschko J, Feng L, Pham P, Cui W, et al. Plasmacytoid dendritic cells cross-prime naive CD8 T cells by transferring antigen to conventional dendritic cells through exosomes. Proceedings of the National Academy of Sciences. 2020;117(38):23730-41.
98. Rossy J, Owen DM, Williamson DJ, Yang Z, Gaus K. Conformational states of the kinase Lck regulate clustering in early T cell signaling. Nature immunology. 2013 Jan;14(1):82-9.
99. Huang G, Wang Y, Vogel P, Kanneganti T-D, Otsu K, Chi H. Signaling via the kinase p38α programs dendritic cells to drive TH17 differentiation and autoimmune inflammation. Nature immunology. 2012;13(2):152-61.
100. Airoldi I, Lualdi S, Bruno S, Raffaghello L, Occhino M, Gambini C, et al. Expression of costimulatory molecules in human neuroblastoma. Evidence that CD40+ neuroblastoma cells undergo apoptosis following interaction with CD40L. British Journal of Cancer. 2003 2003/05/01;88(10):1527-36.
101. Barrios CS, Johnson BD, D Henderson J, Jr., Fink JN, Kelly KJ, Kurup VP. The costimulatory molecules CD80, CD86 and OX40L are up-regulated in Aspergillus fumigatus sensitized mice. Clin Exp Immunol. 2005;142(2):242-50.
102. Tai Y, Wang Q, Korner H, Zhang L, Wei W. Molecular Mechanisms of T Cells Activation by Dendritic Cells in Autoimmune Diseases. Front Pharmacol. 2018;9:642-.
103. Magee CN, Boenisch O, Najafian N. The role of costimulatory molecules in directing the functional differentiation of alloreactive T helper cells. Am J Transplant. 2012;12(10):2588-600.
104. Oriss TB, Ostroukhova M, Seguin-Devaux C, Dixon-McCarthy B, Stolz DB, Watkins SC, et al. Dynamics of Dendritic Cell Phenotype and Interactions with CD4⁺ T Cells in Airway Inflammation and Tolerance. The Journal of Immunology. 2005;174(2):854-63.
105. Williams M, Todd I, Fairclough LC. The role of CD8 + T lymphocytes in chronic obstructive pulmonary disease: a systematic review. Inflammation Research. 2021 2021/01/01;70(1):11-8.

106. Maeno T, Houghton AM, Quintero PA, Grumelli S, Owen CA, Shapiro SD. CD8+ T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice. Journal of immunology (Baltimore, Md : 1950). 2007 Jun 15;178(12):8090-6.
107. Bracke KR, D'hulst AI, Maes T, Moerloose KB, Demedts IK, Lebecque S, et al. Cigarette Smoke-Induced Pulmonary Inflammation and Emphysema Are Attenuated in CCR6-Deficient Mice. The Journal of Immunology. 2006;177(7):4350-9.
108. Kumar S, Jeong Y, Ashraf MU, Bae Y-S. Dendritic Cell-Mediated Th2 Immunity and Immune Disorders. Int J Mol Sci. 2019;20(9):2159.
109. Demedts IK, Bracke KR, Van Pottelberge G, Testelmans D, Verleden GM, Vermassen FE, et al. Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2007 May 15;175(10):998-1005.
110. Olloquequi J, Montes JF, Prats A, Rodríguez E, Montero MA, García-Valero J, et al. Significant increase of CD57⁺ cells in pulmonary lymphoid follicles of COPD patients. European Respiratory Journal. 2011;37(2):289-98.
111. Freeman CM, Curtis JL. Lung Dendritic Cells: Shaping Immune Responses throughout Chronic Obstructive Pulmonary Disease Progression. American journal of respiratory cell and molecular biology. 2017;56(2):152-9.
112. Osterburg AR, Lach L, Panos RJ, Borchers MT. Unique natural killer cell subpopulations are associated with exacerbation risk in chronic obstructive pulmonary disease. Scientific Reports. 2020 2020/01/27;10(1):1238.
113. Eriksson Ström J, Pourazar J, Linder R, Blomberg A, Lindberg A, Bucht A, et al. Cytotoxic lymphocytes in COPD airways: increased NK cells associated with disease, iNKT and NKT-like cells with current smoking. Respir Res. 2018 Dec 7;19(1):244.
114. Liao S-X, Ding T, Rao X-M, Sun D-S, Sun P-P, Wang Y-J, et al. Cigarette smoke affects dendritic cell maturation in the small airways of patients with chronic obstructive pulmonary disease. Mol Med Rep. 2015;11(1):219-25.
115. Robbins CS, Dawe DE, Goncharova SI, Pouladi MA, Drannik AG, Swirski FK, et al. Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness. American journal of respiratory cell and molecular biology. 2004 Feb;30(2):202-11.
116. Freeman CM, Martinez FJ, Han MK, Ames TM, Chensue SW, Todt JC, et al. Lung dendritic cell expression of maturation molecules increases with worsening chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine. 2009;180(12):1179-88.
117. Jasper AE, McIver WJ, Sapey E, Walton GM. Understanding the role of neutrophils in chronic inflammatory airway disease. F1000Res. 2019;8:F1000 Faculty Rev-557.
118. Giacalone VD, Margaroli C, Mall MA, Tirouvanziam R. Neutrophil Adaptations upon Recruitment to the Lung: New Concepts and Implications for Homeostasis and Disease. Int J Mol Sci. 2020;21(3):851.
119. Guiedem E, Ikomey GM, Nkenfou C, Walter P-YE, Mesembe M, Chegou NN, et al. Chronic obstructive pulmonary disease (COPD): neutrophils, macrophages and lymphocytes in patients with anterior tuberculosis compared to tobacco related COPD. BMC Res Notes. 2018;11(1):192-.
120. Rovina N, Koutsoukou A, Koulouris NG. Inflammation and immune response in COPD: where do we stand? Mediators of inflammation. 2013;2013:413735-.
121. Stockley RA, Mannino D, Barnes PJ. Burden and pathogenesis of chronic obstructive pulmonary disease. Proc Am Thorac Soc. 2009 Sep 15;6(6):524-6.

122. O'Donnell RA, Peebles C, Ward JA, Daraker A, Angco G, Broberg P, et al. Relationship between peripheral airway dysfunction, airway obstruction, and neutrophilic inflammation in COPD. Thorax. 2004 Oct;59(10):837-42.
123. Ganesan S, Comstock AT, Kinker B, Mancuso P, Beck JM, Sajjan US. Combined exposure to cigarette smoke and nontypeable Haemophilus influenzae drives development of a COPD phenotype in mice. Respiratory Research. 2014 2014/02/04;15(1):11.
124. Sethi S, Maloney J, Grove L, Wrona C, Berenson CS. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2006 May 1;173(9):991-8.
125. Battaglia S, Mauad T, van Schadewijk AM, Vignola AM, Rabe KF, Bellia V, et al. Differential distribution of inflammatory cells in large and small airways in smokers. J Clin Pathol. 2007;60(8):907-11.
126. Sapey E, Stockley JA, Greenwood H, Ahmad A, Bayley D, Lord JM, et al. Behavioral and structural differences in migrating peripheral neutrophils from patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2011 May 1;183(9):1176-86.
127. Gompertz S, O'Brien C, Bayley DL, Hill SL, Stockley RA. Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. The European respiratory journal. 2001 Jun;17(6):1112-9.
128. Lonergan M, Dicker AJ, Crichton ML, Keir HR, Van Dyke MK, Mullerova H, et al. Blood neutrophil counts are associated with exacerbation frequency and mortality in COPD. Respiratory Research. 2020 2020/07/01;21(1):166.
129. Chien J, Hwang JH, Nilaad S, Masso-Silva JA, Jeong Ahn S, McEachern EK, et al. Cigarette Smoke Exposure Promotes Virulence of Pseudomonas aeruginosa and Induces Resistance to Neutrophil Killing. Infect Immun. 2020 Oct 19;88(11).
130. Dicker AJ, Crichton ML, Pumphrey EG, Cassidy AJ, Suarez-Cuartin G, Sibila O, et al. Neutrophil extracellular traps are associated with disease severity and microbiota diversity in patients with chronic obstructive pulmonary disease. Journal of Allergy and Clinical Immunology. 2018 2018/01/01/;141(1):117-27.
131. Rennard SI, Dale DC, Donohue JF, Kanniess F, Magnussen H, Sutherland ER, et al. CXCR2 Antagonist MK-7123. A Phase 2 Proof-of-Concept Trial for Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med. 2015 May 1;191(9):1001-11.
132. Singh D. Chronic Obstructive Pulmonary Disease, Neutrophils and Bacterial Infection: A Complex Web Involving IL-17 and IL-22 Unravels. EBioMedicine. 2015;2(11):1580-1.
133. Su Y-C, Jalalvand F, Thegerström J, Riesbeck K. The Interplay Between Immune Response and Bacterial Infection in COPD: Focus Upon Non-typeable Haemophilus influenzae. Frontiers in immunology. 2018;9:2530-.
134. Overbeek SA, Braber S, Koelink PJ, Henricks PA, Mortaz E, LoTam Loi AT, et al. Cigarette smoke-induced collagen destruction; key to chronic neutrophilic airway inflammation? PLoS One. 2013;8(1):e55612.
135. Dey T, Kalita J, Weldon S, Taggart CC. Proteases and Their Inhibitors in Chronic Obstructive Pulmonary Disease. J Clin Med. 2018;7(9):244.
136. Thulborn SJ, Mistry V, Brightling CE, Moffitt KL, Ribeiro D, Bafadhel M. Neutrophil elastase as a biomarker for bacterial infection in COPD. Respiratory Research. 2019 2019/07/30;20(1):170.
137. Grabcanovic-Musija F, Obermayer A, Stoiber W, Krautgartner W-D, Steinbacher P, Winterberg N, et al. Neutrophil extracellular trap (NET) formation characterises stable and exacerbated COPD and correlates with airflow limitation. Respiratory Research. 2015 2015/05/22;16(1):59.

138. Park HY, Man SFP, Tashkin D, Wise RA, Connett JE, Anthonisen NA, et al. The relation of serum myeloperoxidase to disease progression and mortality in patients with chronic obstructive pulmonary disease (COPD). PloS one. 2013;8(4):e61315-e.
139. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. Int J Mol Sci. 2017;19(1):92.
140. Nagre N, Cong X, Pearson AC, Zhao X. Alveolar Macrophage Phagocytosis and Bacteria Clearance in Mice. Journal of visualized experiments : JoVE. 2019 Mar 2(145).
141. Gonzalez RF, Dobbs LG. Isolation and culture of alveolar epithelial Type I and Type II cells from rat lungs. Methods in molecular biology (Clifton, NJ). 2013;945:145-59.
142. Hu G, Christman JW. Editorial: Alveolar Macrophages in Lung Inflammation and Resolution. Frontiers in Immunology. [Editorial]. 2019 2019-September-24;10(2275).
143. Guilliams M, De Kleer I, Henri S, Post S, Vanhoutte L, De Prijck S, et al. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. J Exp Med. 2013 Sep 23;210(10):1977-92.
144. Landsman L, Jung S. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. Journal of immunology (Baltimore, Md : 1950). 2007 Sep 15;179(6):3488-94.
145. Misharin AV, Budinger GRS, Perlman H. The Lung Macrophage: A Jack of All Trades. American Journal of Respiratory and Critical Care Medicine. 2011;184(5):497-8.
146. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. Journal of immunology (Baltimore, Md : 1950). 2000 Jun 15;164(12):6166-73.
147. Hodge S, Matthews G, Mukaro V, Ahern J, Shivam A, Hodge G, et al. Cigarette smoke-induced changes to alveolar macrophage phenotype and function are improved by treatment with procysteine. American journal of respiratory cell and molecular biology. 2011 May;44(5):673-81.
148. Ley K. M1 Means Kill; M2 Means Heal. The Journal of Immunology. 2017;199(7):2191-3.
149. Asare PF, Roscioli E, Hurtado PR, Tran HB, Mah CY, Hodge S. LC3-Associated Phagocytosis (LAP): A Potentially Influential Mediator of Efferocytosis-Related Tumor Progression and Aggressiveness. Front Oncol. [Review]. 2020 2020-August-05;10(1298).
150. Eapen MS, Hansbro PM, McAlinden K, Kim RY, Ward C, Hackett T-L, et al. Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease (COPD). Scientific reports. 2017;7(1):13392-.
151. Liegeois M, Legrand C, Desmet CJ, Marichal T, Bureau F. The interstitial macrophage: A long-neglected piece in the puzzle of lung immunity. Cellular Immunology. 2018 2018/08/01/;330:91-6.
152. Berenson CS, Garlipp MA, Grove LJ, Maloney J, Sethi S. Impaired phagocytosis of nontypeable Haemophilus influenzae by human alveolar macrophages in chronic obstructive pulmonary disease. The Journal of infectious diseases. 2006 Nov 15;194(10):1375-84.
153. Finkelstein R, Fraser RS, Ghezzo H, Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. Am J Respir Crit Care Med. 1995 Nov;152(5 Pt 1):1666-72.
154. Hiemstra PS. Altered Macrophage Function in Chronic Obstructive Pulmonary Disease. Annals of the American Thoracic Society. 2013;10(Supplement):S180-S5.
155. Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellularmechanisms. European Respiratory Journal. 2003;22(4):672-88.
156. Traves SL, Culpitt SV, Russell RE, Barnes PJ, Donnelly LE. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. Thorax. 2002 Jul;57(7):590-5.

157. Brozyna S, Ahern J, Hodge G, Nairn J, Holmes M, Reynolds PN, et al. Chemotactic mediators of Th1 T-cell trafficking in smokers and COPD patients. Copd. 2009 Feb;6(1):4-16.
158. Hodge S, Reynolds PN. Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects. Respirology. 2012 Jul;17(5):802-7.
159. Hodge S, Hodge G, Jersmann H, Matthews G, Ahern J, Holmes M, et al. Azithromycin improves macrophage phagocytic function and expression of mannose receptor in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2008 Jul 15;178(2):139-48.
160. Hodge S, Hodge G, Brozyna S, Jersmann H, Holmes M, Reynolds PN. Azithromycin increases phagocytosis of apoptotic bronchial epithelial cells by alveolar macrophages. The European respiratory journal. 2006 Sep;28(3):486-95.
161. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. Immunology and cell biology. 2003 Aug;81(4):289-96.
162. Berenson CS, Kruzel RL, Eberhardt E, Dolnick R, Minderman H, Wallace PK, et al. Impaired innate immune alveolar macrophage response and the predilection for COPD exacerbations. Thorax. 2014;69(9):811-8.
163. Eltboli O, Bafadhel M, Hollins F, Wright A, Hargadon B, Kulkarni N, et al. COPD exacerbation severity and frequency is associated with impaired macrophage efferocytosis of eosinophils. BMC Pulmonary Medicine. 2014 2014/07/09;14(1):112.
164. Taylor AE, Finney-Hayward TK, Quint JK, Thomas CMR, Tudhope SJ, Wedzicha JA, et al. Defective macrophage phagocytosis of bacteria in COPD. European Respiratory Journal. 2010;35(5):1039-47.
165. Aoshiba K, Yokohori N, Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. American journal of respiratory cell and molecular biology. 2003 May;28(5):555-62.
166. Bosurgi L, Cao YG, Cabeza-Cabrerizo M, Tucci A, Hughes LD, Kong Y, et al. Macrophage function in tissue repair and remodeling requires IL-4 or IL-13 with apoptotic cells. Science. 2017;356(6342):1072-6.
167. Szondy Z, Garabuczi E, Joós G, Tsay GJ, Sarang Z. Impaired clearance of apoptotic cells in chronic inflammatory diseases: therapeutic implications. Frontiers in immunology. 2014;5:354-.
168. Hodge S, Hodge G, Holmes M, Reynolds PN. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. The European respiratory journal. 2005 Mar;25(3):447-54.
169. Ramirez-Ortiz ZG, Pendergraft WF, 3rd, Prasad A, Byrne MH, Iram T, Blanchette CJ, et al. The scavenger receptor SCARF1 mediates the clearance of apoptotic cells and prevents autoimmunity. Nature immunology. 2013 Sep;14(9):917-26.
170. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, et al. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. Science. 2004 May 21;304(5674):1147-50.
171. Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, et al. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. 2001 May 10;411(6834):207-11.
172. Belchamber KBR, Donnelly LE. Macrophage Dysfunction in Respiratory Disease. Results and problems in cell differentiation. 2017;62:299-313.
173. Richens TR, Linderman DJ, Horstmann SA, Lambert C, Xiao Y-Q, Keith RL, et al. Cigarette smoke impairs clearance of apoptotic cells through oxidant-dependent activation of RhoA. American journal of respiratory and critical care medicine. 2009;179(11):1011-21.

174. Medina CB, Mehrotra P, Arandjelovic S, Perry JSA, Guo Y, Morioka S, et al. Metabolites released from apoptotic cells act as tissue messengers. Nature. 2020 2020/04/01;580(7801):130-5.
175. Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, et al. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature. 2010 2010/10/01;467(7317):863-7.
176. Roszer T, Menéndez-Gutiérrez MP, Lefterova MI, Alameda D, Núñez V, Lazar MA, et al. Autoimmune kidney disease and impaired engulfment of apoptotic cells in mice with macrophage peroxisome proliferator-activated receptor gamma or retinoid X receptor alpha deficiency. Journal of immunology (Baltimore, Md : 1950). 2011 Jan 1;186(1):621-31.
177. Yurdagul A, Jr., Subramanian M, Wang X, Crown SB, Ilkayeva OR, Darville L, et al. Macrophage Metabolism of Apoptotic Cell-Derived Arginine Promotes Continual Efferocytosis and Resolution of Injury. Cell Metab. 2020 Mar 3;31(3):518-33.e10.
178. Sanjuan MA, Dillon CP, Tait SWG, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature. 2007 2007/12/01;450(7173):1253-7.
179. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc Natl Acad Sci U S A. 2011 Oct 18;108(42):17396-401.
180. Muniz-Feliciano L, Doggett TA, Zhou Z, Ferguson TA. RUBCN/rubicon and EGFR regulate lysosomal degradative processes in the retinal pigment epithelium (RPE) of the eye. Autophagy. [Article]. 2017;13(12):2072-85.
181. Martinez J, Malireddi RKS, Lu Q, Cunha LD, Pelletier S, Gingras S, et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nature Cell Biology. [Article]. 2015;17(7):893-906.
182. Sanjuan MA, Milasta S, Green DR. Toll-like receptor signaling in the lysosomal pathways. Immunological Reviews. 2009;227(1):203-20.
183. Wan J, Weiss E, Ben Mkaddem S, Mabire M, Choinier P-M, Picq O, et al. LC3-associated phagocytosis protects against inflammation and liver fibrosis via immunoreceptor inhibitory signaling. Science Translational Medicine. 2020;12(539):eaaw8523.
184. Inomata M, Xu S, Chandra P, Meydani SN, Takemura G, Philips JA, et al. Macrophage LC3-associated phagocytosis is an immune defense against Streptococcus pneumoniae that diminishes with host aging. Proceedings of the National Academy of Sciences. 2020;117(52):33561-9.
185. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford JC, et al. LC3-Associated Phagocytosis in Myeloid Cells Promotes Tumor Immune Tolerance. Cell. 2018 Oct 4;175(2):429-41.e16.
186. Singh S, Verma SK, Kumar S, Ahmad MK, Nischal A, Singh SK, et al. Correlation of severity of chronic obstructive pulmonary disease with potential biomarkers. Immunology Letters. 2018 2018/04/01/;196:1-10.
187. Toorn Mvd, Slebos D-J, Bruin HGd, Leuvenink HG, Bakker SJL, Gans ROB, et al. Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2007;292(5):L1211-L8.
188. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. Nature. 2016 May 5;533(7601):115-9.
189. Uribe-Querol E, Rosales C. Control of Phagocytosis by Microbial Pathogens. Frontiers in Immunology. [Review]. 2017 2017-October-24;8(1368).

190. Herb M, Gluschko A, Schramm M. LC3-associated phagocytosis - The highway to hell for phagocytosed microbes. Seminars in Cell & Developmental Biology. 2020 2020/05/01/;101:68-76.
191. Gluschko A, Herb M, Wiegmann K, Krut O, Neiss WF, Utermöhlen O, et al. The β(2) Integrin Mac-1 Induces Protective LC3-Associated Phagocytosis of Listeria monocytogenes. Cell Host Microbe. 2018 Mar 14;23(3):324-37.e5.
192. Hubber A, Kubori T, Coban C, Matsuzawa T, Ogawa M, Kawabata T, et al. Bacterial secretion system skews the fate of Legionella-containing vacuoles towards LC3-associated phagocytosis. Scientific Reports. 2017 2017/03/20;7(1):44795.
193. Köster S, Klevorn T, Papavinasasundaram K, Sassetti CM, Portal-Celhay C, Philips JA. Consequence of enhanced LC3-trafficking for a live, attenuated M. tuberculosis vaccine. Vaccine. [Article]. 2018;36(7):939-44.
194. Akoumianaki T, Kyrmizi I, Valsecchi I, Gresnigt MS, Samonis G, Drakos E, et al. Aspergillus Cell Wall Melanin Blocks LC3-Associated Phagocytosis to Promote Pathogenicity. Cell Host Microbe. 2016 Jan 13;19(1):79-90.
195. Andrianaki AM, Kyrmizi I, Thanopoulou K, Baldin C, Drakos E, Soliman SSM, et al. Iron restriction inside macrophages regulates pulmonary host defense against Rhizopus species. Nature Communications. [Article]. 2018;9(1).
196. Tam JM, Mansour MK, Acharya M, Sokolovska A, Timmons AK, Lacy-Hulbert A, et al. The role of autophagy-related proteins in Candida albicans infections. Pathogens. [Review]. 2016;5(2).
197. Tam JM, Mansour MK, Khan NS, Seward M, Puranam S, Tanne A, et al. Dectin-1-dependent LC3 recruitment to phagosomes enhances fungicidal activity in macrophages. The Journal of infectious diseases. 2014;210(11):1844-54.
198. Schille S, Crauwels P, Bohn R, Bagola K, Walther P, van Zandbergen G. LC3-associated phagocytosis in microbial pathogenesis. International Journal of Medical Microbiology. [Review]. 2018;308(1):228-36.
199. Ellson CD, Anderson KE, Morgan G, Chilvers ER, Lipp P, Stephens LR, et al. Phosphatidylinositol 3-phosphate is generated in phagosomal membranes. Current biology : CB. 2001 Oct 16;11(20):1631-5.
200. Huang J, Canadien V, Lam GY, Steinberg BE, Dinauer MC, Magalhaes MAO, et al. Activation of antibacterial autophagy by NADPH oxidases. Proceedings of the National Academy of Sciences. 2009;106(15):6226-31.
201. Lam GY, Huang J, Brumell JH. The many roles of NOX2 NADPH oxidase-derived ROS in immunity. Semin Immunopathol. 2010 Dec;32(4):415-30.
202. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. The international journal of biochemistry & cell biology. 2004 Dec;36(12):2503-18.
203. Heckmann BL, Boada-Romero E, Cunha LD, Magne J, Green DR. LC3-Associated Phagocytosis and Inflammation. J Mol Biol. 2017 Nov 24;429(23):3561-76.
204. Boyle KB, Randow F. Rubicon swaps autophagy for LAP. Nature Cell Biology. 2015 2015/07/01;17(7):843-5.
205. Sun Q, Zhang J, Fan W, Wong KN, Ding X, Chen S, et al. The RUN domain of rubicon is important for hVps34 binding, lipid kinase inhibition, and autophagy suppression. J Biol Chem. 2011;286(1):185-91.
206. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. Nature. 2016 2016/05/01;533(7601):115-9.
207. van Manen JG, Bindels PJ, CJ IJ, van der Zee JS, Bottema BJ, Schadé E. Prevalence of comorbidity in patients with a chronic airway obstruction and controls over the age of 40. Journal of clinical epidemiology. 2001 Mar;54(3):287-93.

208. Sin DD, Anthonisen NR, Soriano JB, Agusti AG. Mortality in COPD: role of comorbidities. European Respiratory Journal. 2006;28(6):1245-57.
209. Celli BR, Decramer M, Wedzicha JA, Wilson KC, Agustí A, Criner GJ, et al. An Official American Thoracic Society/European Respiratory Society Statement: Research questions in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2015 Apr 1;191(7):e4-e27.
210. Fabbri LM, Luppi F, Beghé B, Rabe KF. Complex chronic comorbidities of COPD. European Respiratory Journal. 2008;31(1):204-12.
211. Yamamuro T, Kawabata T, Fukuhara A, Saita S, Nakamura S, Takeshita H, et al. Age-dependent loss of adipose Rubicon promotes metabolic disorders via excess autophagy. Nature Communications. 2020 2020/08/18;11(1):4150.
212. Kim S-H, Park J-H, Lee J-K, Heo EY, Kim DK, Chung HS. Chronic obstructive pulmonary disease is independently associated with hypertension in men: A survey design analysis using nationwide survey data. Medicine. 2017;96(19).
213. Naik D, Joshi A, Paul TV, Thomas N. Chronic obstructive pulmonary disease and the metabolic syndrome: Consequences of a dual threat. Indian J Endocrinol Metab. 2014;18(5):608-16.
214. Marquis K, Maltais F, Duguay V, Bezeau AM, LeBlanc P, Jobin J, et al. The metabolic syndrome in patients with chronic obstructive pulmonary disease. Journal of cardiopulmonary rehabilitation. 2005 Jul-Aug;25(4):226-32; discussion 33-4.
215. Clini E, Crisafulli E, Radaeli A, Malerba M. COPD and the metabolic syndrome: an intriguing association. Internal and Emergency Medicine. 2013 2013/06/01;8(4):283-9.
216. Tkacova R. Systemic inflammation in chronic obstructive pulmonary disease: may adipose tissue play a role? Review of the literature and future perspectives. Mediators Inflamm. 2010;2010:585989.
217. Breyer M-K, Rutten EPA, Locantore NW, Watkins ML, Miller BE, Wouters EFM, et al. Dysregulated adipokine metabolism in chronic obstructive pulmonary disease. European Journal of Clinical Investigation. 2012;42(9):983-91.
218. Kuk JL, Ardern CI. Age and sex differences in the clustering of metabolic syndrome factors: association with mortality risk. Diabetes Care. 2010;33(11):2457-61.
219. Sekine Y, Katsura H, Koh E, Hiroshima K, Fujisawa T. Early detection of COPD is important for lung cancer surveillance. European Respiratory Journal. 2012;39(5):1230.
220. van Gestel YRBM, Hoeks SE, Sin DD, Hüzeir V, Stam H, Mertens FW, et al. COPD and cancer mortality: the influence of statins. Thorax. 2009;64(11):963-7.
221. Sauler M, Lamontagne M, Finnemore E, Herazo-Maya JD, Tedrow J, Zhang X, et al. The DNA repair transcriptome in severe COPD. The European respiratory journal. 2018;52(4):1701994.
222. Kim E-J, Yoon S-J, Kim Y-E, Go D-S, Jung Y. Effects of Aging and Smoking Duration on Cigarette Smoke-Induced COPD Severity. J Korean Med Sci. 2018;34(Suppl 1):e90-e.
223. Martinez J, Malireddi RKS, Lu Q, Cunha LD, Pelletier S, Gingras S, et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nature cell biology. 2015;17(7):893-906.
224. Masud S, van der Burg L, Storm L, Prajsnar TK, Meijer AH. Rubicon-Dependent Lc3 Recruitment to Salmonella-Containing Phagosomes Is a Host Defense Mechanism Triggered Independently From Major Bacterial Virulence Factors. Frontiers in Cellular and Infection Microbiology. [Original Research]. 2019 2019-August-02;9(279).
225. Ford Catriona A, Petrova S, Pound John D, Voss Jorine JLP, Melville L, Paterson M, et al. Oncogenic Properties of Apoptotic Tumor Cells in Aggressive B Cell Lymphoma. Current Biology. 2015 2015/03/02/;25(5):577-88.

Chapter 2

2. Inhibition of LC3-associated phagocytosis (LAP) in COPD and in response to cigarette smoke

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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. Further, 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 unrecognised therapeutic potential in the context of disease-related modifications caused by exposure to cigarette smoke.

Key words: Cigarette smoke extract; LC3-associated phagocytosis, Rubicon, Inflammation, Efferocytosis

2.1. 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 that 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 prevents inflammation and inappropriate immune activation [7]. Cigarette smoking disrupts this homeostatic process that can lead to the release and protracted exposure of harmful intracellular contents of apoptotic cells into pulmonary microenvironment [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 optimised 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 et al [12] 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-1β, 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 [18]. The capacity for airway macrophages to express Rubicon, especially during their responses to unscheduled apoptosis driven by cigarette smoking remains undefined.

We hypothesised 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 *in vitro* using THP-1 macrophages and blood monocyte derived macrophages (MDM) exposed to cigarette smoke extract (CSE). Further, 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.

2.2. Method

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 reported [19] and was used to prepare 10% CSE throughout the study. Briefly, we bubbled the cigarette smoke from four 1R5F 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 °C/5% CO2 in RPMI 1640 medium containing 10% FBS, penicillin/streptomycin, 2 mM L-glutamine, and 0.05 mM ß-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 [20]. 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 °C/5% CO2 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 FEV_1 below 1.4 L from bronchoscopy due to ethical reasons. We obtained written informed consent from all volunteers after obtaining ethics approval from the Royal Adelaide Hospital. GOLD criteria with clinical correlation was use to confirm COPD diagnosis. Bronchoalveolar lavage (BAL) was obtained from a cohort of these

participants for examination of Rubicon expression (4 subjects with COPD and 4 never-smoker control subjects).

Table 1. Demographic details of COPD patients and control subjects

	Controls	COPD
Number of volunteers	4	4
Age(yrs)±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

Table 1: Demographic details of COPD patients and control subjects. Data are presented as mean±SEM

Bronchoscopy Procedure

As we have previously reported [21], 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 one hour of collection.

Preparation of Monocyte Derived Macrophage (MDM)

Adult controls were recruited from our volunteer database, were non-smokers 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^{TM} (STEMCELL Technologies, BC, Canada) and centrifuged at 800xg for 25 min with acceleration but no brake. Peripheral blood mononuclear cells (PBMC) layer were isolated according to the manufactures instructions. To derive macrophages from monocytes, PBMC were seeded into plates at 1.4×10^6/mL in plain RPMI 1640 medium at 37 °C/5% CO_2 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 tissue from these subjects were obtained as previously described [22, 23]. Biopsies were collected from non-tumour ('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 [22, 24]. Samples were categorized as 'Control' (non-cancer area from patients with cancer/no COPD) or 'COPD' (non-cancer 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 efferccytosisng apoptotic 16HBE14o- bronchial epithelial cells. Procedures for gating have been previously published [9, 21, 25].

Quantitative immunofluorescence analysis of protein expression and localization 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 [26]. Mice were exposed to cigarette smoke for 6 weeks, sufficient to induce inflammatory changes but not emphysema or small airway remodelling [27]. 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 [28]. 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 [29]. 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 localization [28]. Mean fluorescence intensity was measured using the ImageJ software (NIH, Bethesda, MA, USA). Alveolar macrophages were differentiated from alveolar pneumocytes according to their localization 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 8-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-a, 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 (D9F7) 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 μg 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, 1μg 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 SEM unless otherwise indicated. Analysis was performed using the non-parametric 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 $p<0.05$.

2.3. 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 1A,** $p<0.0001$; **1B,** $p=0.0022$). We also identified a significant reduction of Rubicon in alveolar macrophages derived from COPD participants compared to non-COPD subjects (**Figure 1C,** $p=0.0286$). Further correlative analysis demonstrated that the reduction in Rubicon protein expression positively correlated with the percentage of macrophages efferocytosing apoptotic cells (**Figure 1D**, $p=0.002$, $r=0.9633$). 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 1E**, $p=0.0022$). 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 1G,** 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 H, p>0.999).

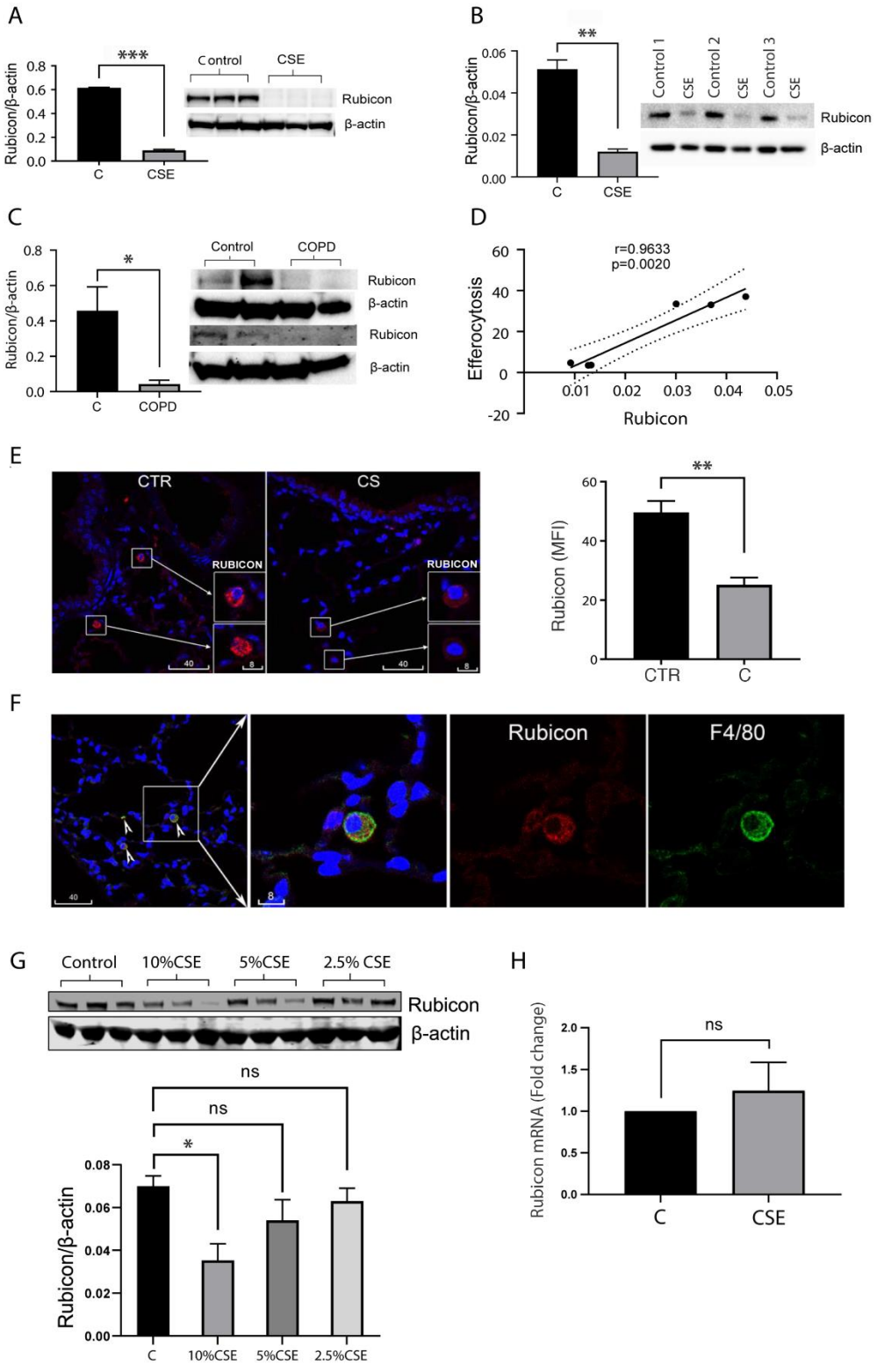

Figure 1: BAL macrophages of COPD patients and macrophages exposed to CSE exhibit a deficiency in Rubicon expression.

(A) Protein analysis of Rubicon protein expression; Control (C) vs cigarette smoke extract (CSE). CSE reduced Rubicon expression in blood monocyte-derived macrophages ****p<0.0001; mean ± 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 ± 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 ± SEM, n=4 for each group. **(D)** Correlation between Rubicon protein expression and % efferocytosis. Data shows 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 ± 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 localization 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= mean ± SEM, n=3. **(H)** Rubicon gene transcription is unaffected by CSE p>0.999, n=4 for each group).

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 **(Figures 2A,** p=0.0087**; 2B,** 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 2C,** p=0.0087**)** in alveolar macrophages. In addition, CSE-treated macrophages showed higher expression levels of TIM-4 (**Figure 2D,** p=0.0158**)**, a key phagocytic receptor required for removing apoptotic cells. Nonetheless, CSE-treated macrophages were defective in their capacity to efferocytose apoptotic cells compared with the control group **(Figure 2E** p=0.0003**)**.

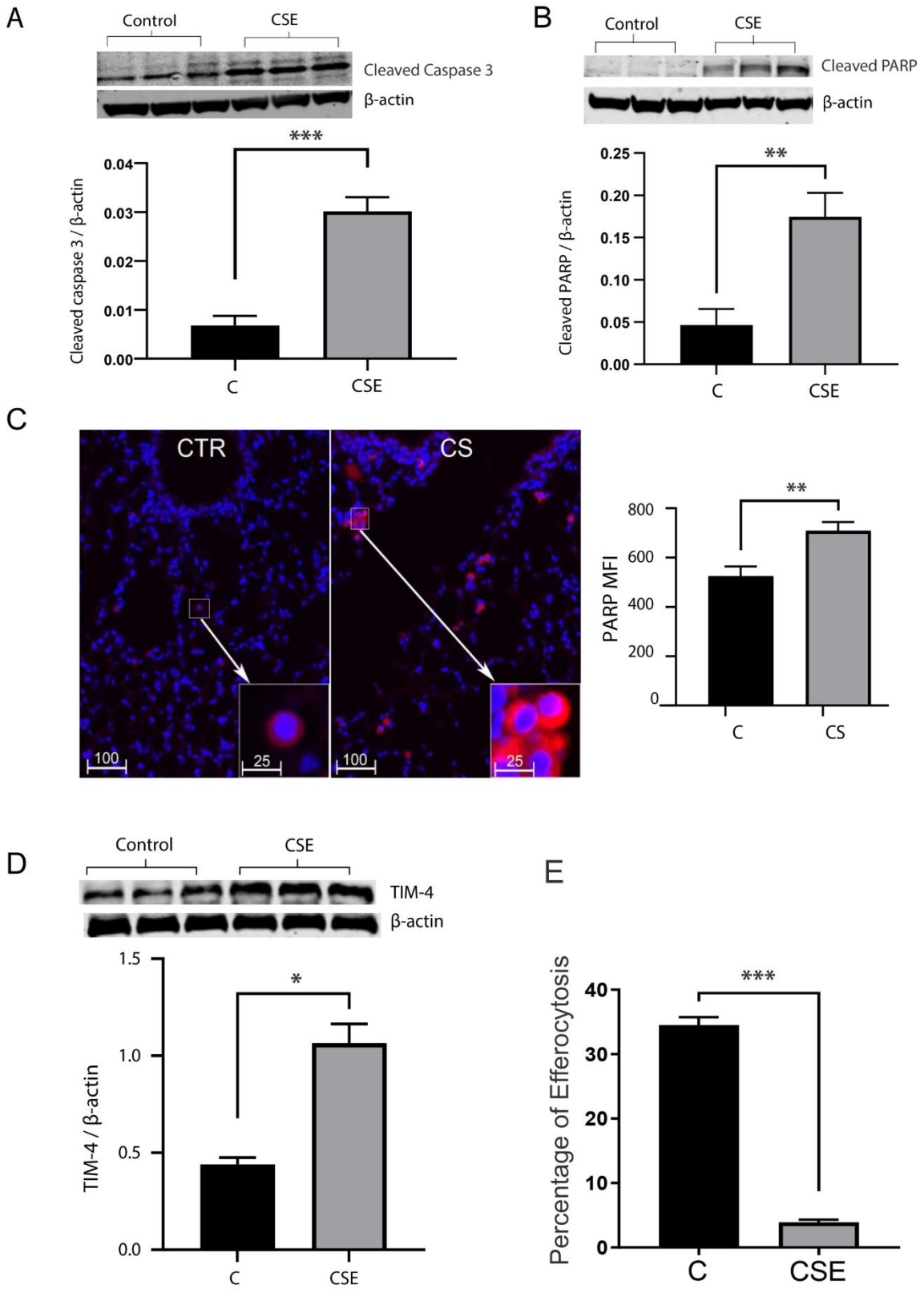

Figure 2: Exposure to cigarette smoke induces apoptosis

(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 ± 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 ± 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 ± 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 ± 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 ± SEM, n=3.

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

Immunofluorescence revealed increased LC3 in macrophages from COPD patients compared with control participants (**Figure 3A**, p=0.0118). Western blot analysis showed higher total LC3, LC3-II and LC3-I expressions and a non-significant 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 3B**; 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 3B**; 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 3C**; p=0.0159 and non-significantly increased NOX2 levels (**Figure 3D**; p=0.6991) in THP-1 macrophages exposed to 10% CSE for 24 h compared to the air treated control THP-1 macrophages.

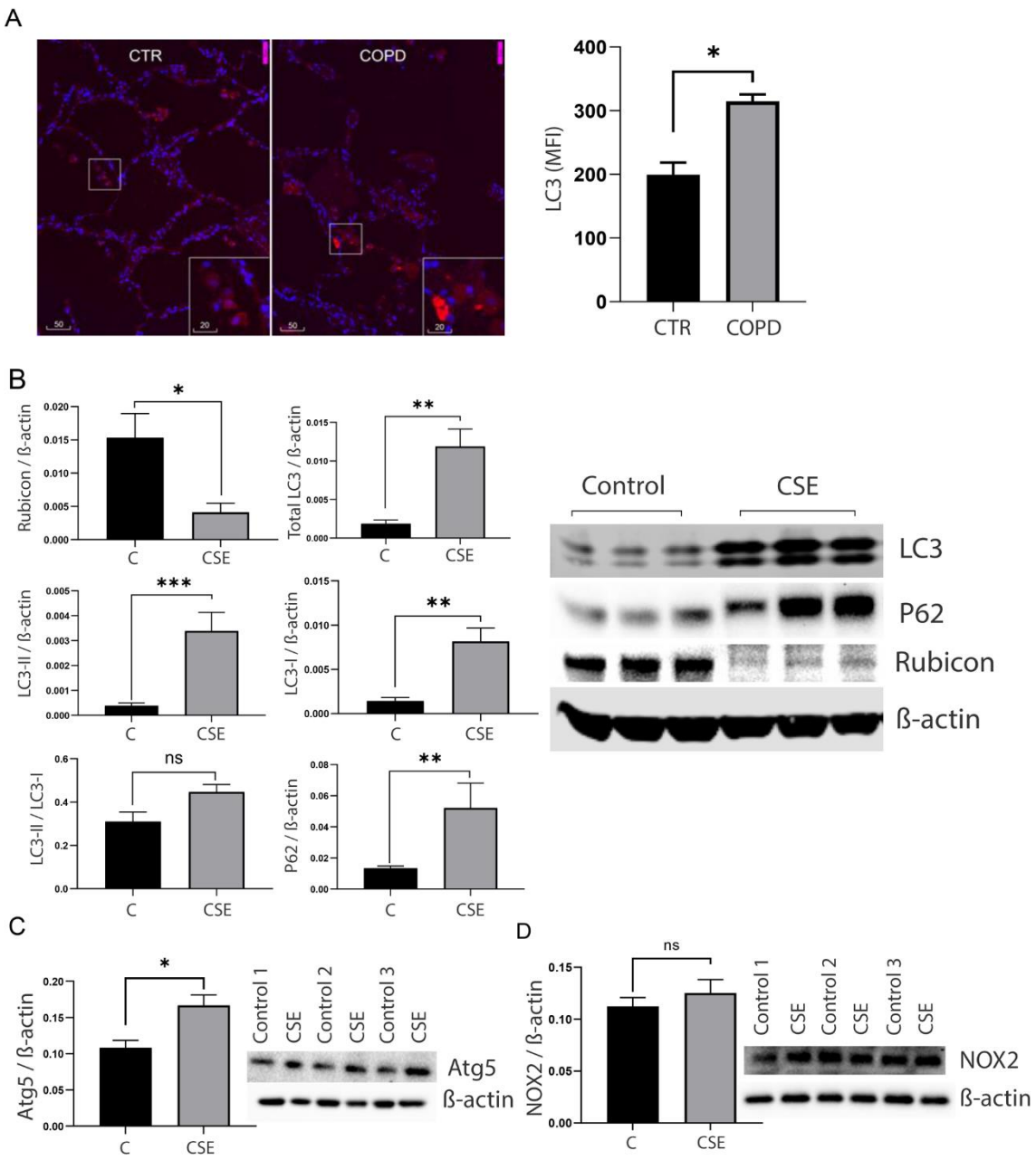

Figure 3: COPD lung macrophages exhibit features of defective LAP

(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 ± 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 ± 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 ± 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 ± 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 ± SEM, n=6. **(D)** Western blots (and quantitative data) of NOX2 protein expression; Control (C) vs cigarette smoke extract (CSE). The expression of NOX2 non-significantly increased in THP-1 macrophages after 24 h of CSE treatment; p=0.6991, mean ± SEM, n=6.

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**s **4A;** p=0.0159**).** Consistent with previous studies [30], intracellular and extracellular IL-1β was observed in control THP-1 macrophages but about four times higher in THP-1 macrophages exposed to cigarette smoke extract (**Figures B and C;** n=3; ***p=0.0004 and n=5; *p=0.0159 respectively). Anti-inflammatory mediators such as IL-10 and 1L-4 were all below detection levels (data not shown).

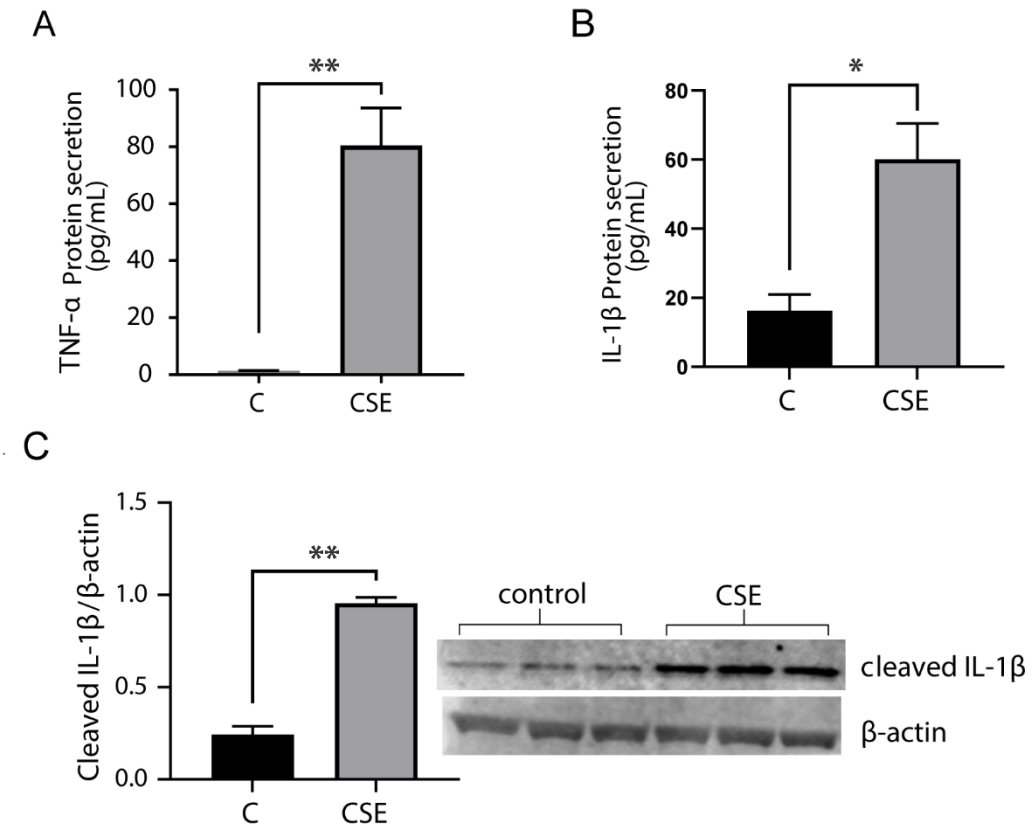

Figure 4: Cigarette smoke extract exposure increases pro-inflammatory marker levels

(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 ± SEM, n=3. **p=0.0039. **(B)** ELISA results of increased secretion levels of IL-1β in THP-1 macrophages exposed to CSE for 24 h; Control (C) vs cigarette smoke extract (CSE). Data are expressed as mean ± SEM, n=5. *p=0.0159. **(C)** Western blot results showing higher expression of intracellular IL-1β in CSE exposed THP-1 macrophages after cigarette smoke exposure. Data are expressed as mean ± SEM, n=3; ***p=0.0004.

2.4. 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 [31, 32]. The persistence of apoptotic debris is implicated in the pathogenesis of inflammatory diseases and exacerbation of COPD [8, 33, 34]. 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 recognised 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 [31, 35-38]**.** 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 [39]. 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, 40, 41]. 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 colleagues have shown that TIM-4 and Rubicon co-operate in LAP to promote efferocytosis [42]. 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 [43, 44], 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 [45]. 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 colleagues [46] 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 [46, 47]. These reports demonstrate a potential crosstalk between the decline of Rubicon and metabolic disorders which is commonly observed in COPD [48]. 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 [49, 50].

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-1β [12, 51, 52]. While abundance of Rubicon prevents inflammatory responses, Rubicon depleted mice produce markedly higher amount of TNF-α and IL-1β [53-55]. This is consistent with the observation of defective efferocytosis and a concurrent increase in TNF-α and IL-1β in CSE-treated THP-1 macrophages**.** Recent reports show that Rubicon can also regulate TNF-α production through LAP independent pathways [53]. 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 characterises 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 *pneumonia* infections [54, 56]. 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.

Conflict of interest: Authors have no conflict of interests to declare

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References

1. Salvi SS, Manap R, Beasley R. Understanding the true burden of COPD: the epidemiological challenges. Primary Care Respiratory Journal. 2012 2012/09/01;21(3):249-51.
2. Laniado-Laborín R. Smoking and chronic obstructive pulmonary disease (COPD). Parallel epidemics of the 21 century. Int J Environ Res Public Health. 2009;6(1):209-24.
3. Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, van Der Mark TW, Koëter GH, et al. Ongoing airway inflammation in patients with COPD who do not currently smoke. Thorax. 2000;55(1):12-8.
4. Willemse BWM, ten Hacken NHT, Rutgers B, Lesman-Leegte IGAT, Postma DS, Timens W. Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. European Respiratory Journal. 2005;26(5):835-45.
5. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med. 2004 Jun 24;350(26):2645-53.
6. King PT. Inflammation in chronic obstructive pulmonary disease and its role in cardiovascular disease and lung cancer. Clin Transl Med. 2015;4(1):68-.
7. Zhang W, Zhao J, Wang R, Jiang M, Ye Q, Smith AD, et al. Macrophages reprogram after ischemic stroke and promote efferocytosis and inflammation resolution in the mouse brain. CNS Neuroscience & Therapeutics. 2019;25(12):1329-42.
8. Subramaniam R, Mukherjee S, Chen H, Keshava S, Neuenschwander P, Shams H. Restoring cigarette smoke-induced impairment of efferocytosis in alveolar macrophages. Mucosal Immunology. 2016 2016/07/01;9(4):873-83.
9. Hodge S, Matthews G, Mukaro V, Ahern J, Shivam A, Hodge G, et al. Cigarette smoke-induced changes to alveolar macrophage phenotype and function are improved by treatment with procysteine. American journal of respiratory cell and molecular biology. 2011 May;44(5):673-81.
10. Kazeros A, Harvey B-G, Carolan BJ, Vanni H, Krause A, Crystal RG. Overexpression of apoptotic cell removal receptor MERTK in alveolar macrophages of cigarette smokers. American journal of respiratory cell and molecular biology. 2008;39(6):747-57.
11. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. Immunology and cell biology. 2003 Aug;81(4):289-96.
12. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. Nature. 2016 2016/05/01;533(7601):115-9.
13. Sanjuan MA, Dillon CP, Tait SWG, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature. 2007 2007/12/01;450(7173):1253-7.
14. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proceedings of the National Academy of Sciences. 2011;108(42):17396-401.
15. Martinez J, Malireddi RKS, Lu Q, Cunha LD, Pelletier S, Gingras S, et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nat Cell Biol. 2015;17(7):893-906.
16. Hodge S, Hodge G, Holmes M, Reynolds PN. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. European Respiratory Journal. 2005;25(3):447.

17. Hu J-Y, Liu B-B, Du Y-P, Zhang Y, Zhang Y-W, Zhang Y-Y, et al. Increased circulating β(2)-adrenergic receptor autoantibodies are associated with smoking-related emphysema. Sci Rep. 2017;7:43962-.
18. Hodge S, Hodge G, Holmes M, Reynolds PN. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. The European respiratory journal. 2005 Mar;25(3):447-54.
19. Hodge S, Hodge G, Ahern J, Jersmann H, Holmes M, Reynolds PN. Smoking Alters Alveolar Macrophage Recognition and Phagocytic Ability. American journal of respiratory cell and molecular biology. 2007 2007/12/01;37(6):748-55.
20. Dehle FC, Mukaro VR, Jurisevic C, Moffat D, Ahern J, Hodge G, 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(4):e61573-e.
21. Hodge SJ, Hodge GL, Holmes M, Reynolds PN. Flow cytometric characterization of cell populations in bronchoalveolar lavage and bronchial brushings from patients with chronic obstructive pulmonary disease. Cytometry Part B, Clinical cytometry. 2004 Sep;61(1):27-34.
22. Soriano C, Mukaro V, Hodge G, Ahern J, Holmes M, Jersmann H, et al. Increased proteinase inhibitor-9 (PI-9) and reduced granzyme B in lung cancer: mechanism for immune evasion? Lung cancer (Amsterdam, Netherlands). 2012 Jul;77(1):38-45.
23. Cordts F, Pitson S, Tabeling C, Gibbins I, Moffat DF, Jersmann H, et al. Expression profile of the sphingosine kinase signalling system in the lung of patients with chronic obstructive pulmonary disease. Life sciences. 2011 Nov 21;89(21-22):806-11.
24. Hodge S, Matthews G, Dean MM, Ahern J, Djukic M, Hodge G, et al. Therapeutic role for mannose-binding lectin in cigarette smoke-induced lung inflammation? Evidence from a murine model. American journal of respiratory cell and molecular biology. 2010 Feb;42(2):235-42.
25. Tran HB, Barnawi J, Ween M, Hamon R, Roscioli E, Hodge G, et al. Cigarette smoke inhibits efferocytosis via deregulation of sphingosine kinase signaling: reversal with exogenous S1P and the S1P analogue FTY720. Journal of Leukocyte Biology. 2016;100(1):195-202.
26. Tran HB, Jersmann H, Truong TT, Hamon R, Roscioli E, Ween M, 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(11):e0179577.
27. Fricker M, Deane A, Hansbro PM. Animal models of chronic obstructive pulmonary disease. Expert opinion on drug discovery. 2014 Jun;9(6):629-45.
28. Tran HB, Lewis MD, Tan LW, Lester SE, Baker LM, Ng J, 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-.
29. Tran HB, Macowan MG, Abdo A, Donnelley M, Parsons D, Hodge S. Enhanced inflammasome activation and reduced sphingosine-1 phosphate S1P signalling in a respiratory mucoobstructive disease model. Journal of Inflammation. 2020 2020/04/21;17(1):16.
30. Xu J, Xu F, Lin Y. Cigarette Smoke Synergizes Lipopolysaccharide-Induced InterIeukin-1β and Tumor Necrosis Factor–α Secretion from Macrophages via Substance P–Mediated Nuclear Factor–κB Activation. American journal of respiratory cell and molecular biology. 2011 2011/03/01;44(3):302-8.
31. Aoshiba K, Tamaoki J, Nagai A. Acute cigarette smoke exposure induces apoptosis of alveolar macrophages. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2001;281(6):L1392-L401.

32. Sakhatskyy P, Gabino Miranda GA, Newton J, Lee CG, Choudhary G, Vang A, et al. Cigarette smoke-induced lung endothelial apoptosis and emphysema are associated with impairment of FAK and eIF2α. Microvasc Res. 2014;94:80-9.
33. Clark H, Palaniyar N, Strong P, Edmondson J, Hawgood S, Reid KBM. Surfactant Protein D Reduces Alveolar Macrophage Apoptosis In Vivo. The Journal of Immunology. 2002;169(6):2892.
34. Morimoto K, Janssen WJ, Fessler MB, McPhillips KA, Borges VM, Bowler RP, et al. Lovastatin Enhances Clearance of Apoptotic Cells (Efferocytosis) with Implications for Chronic Obstructive Pulmonary Disease. The Journal of Immunology. 2006;176(12):7657-65.
35. Li M, Yu D, Williams KJ, Liu M-L. Tobacco smoke induces the generation of procoagulant microvesicles from human monocytes/macrophages. Arterioscler Thromb Vasc Biol. 2010;30(9):1818-24.
36. Lim HJ, Park JH, Jo C, Yoon K, Koh YH. Cigarette smoke extracts and cadmium induce COX-2 expression through γ-secretase-mediated p38 MAPK activation in C6 astroglia cells. PLoS One. 2019;14(2):e0212749.
37. Comer DM, Kidney JC, Ennis M, Elborn JS. Airway epithelial cell apoptosis and inflammation in COPD, smokers and nonsmokers. European Respiratory Journal. 2013;41(5):1058-67.
38. Park J-W, Kim HP, Lee S-J, Wang X, Wang Y, Ifedigbo E, et al. Protein Kinase Cα and ζ Differentially Regulate Death-Inducing Signaling Complex Formation in Cigarette Smoke Extract-Induced Apoptosis. The Journal of Immunology. 2008;180(7):4668-78.
39. Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. Nature. 2007 2007/11/01;450(7168):435-9.
40. Wang Y, Luo G, Chen J, Jiang R, Zhu J, Hu N, 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-.
41. McCubbrey AL, Curtis JL. Efferocytosis and lung disease. Chest. 2013;143(6):1750-7.
42. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford JC, et al. LC3-Associated Phagocytosis in Myeloid Cells Promotes Tumor Immune Tolerance. Cell. 2018;175(2):429-41.e16.
43. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, et al. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat Cell Biol. 2009 2009/04/01;11(4):385-96.
44. Boyle KB, Randow F. Rubicon swaps autophagy for LAP. Nature Cell Biology. 2015 2015/07/01;17(7):843-5.
45. Monick MM, Powers LS, Walters K, Lovan N, Zhang M, Gerke A, et al. Identification of an autophagy defect in smokers' alveolar macrophages. Journal of immunology (Baltimore, Md : 1950). 2010;185(9):5425-35.
46. Yamamuro T, Kawabata T, Fukuhara A, Saita S, Nakamura S, Takeshita H, et al. Age-dependent loss of adipose Rubicon promotes metabolic disorders via excess autophagy. Nature Communications. 2020 2020/08/18;11(1):4150.
47. Matsuda J, Takahashi A, Takabatake Y, Sakai S, Minami S, Yamamoto T, et al. Metabolic effects of RUBCN/Rubicon deficiency in kidney proximal tubular epithelial cells. Autophagy. 2020 Oct;16(10):1889-904.
48. Uzokov J, Alyavi A, Karimov B. Prevalence of metabolic syndrome in patients with chronic obstructive pulmonary disease. European Respiratory Journal. 2016;48(suppl 60):PA3776.
49. Zhang S, Weinberg S, DeBerge M, Gainullina A, Schipma M, Kinchen JM, et al. Efferocytosis Fuels Requirements of Fatty Acid Oxidation and the Electron Transport Chain

to Polarize Macrophages for Tissue Repair. Cell Metabolism. 2019 2019/02/05/;29(2):443-56.e5.
50. Han CZ, Ravichandran KS. Metabolic connections during apoptotic cell engulfment. Cell. 2011;147(7):1442-5.
51. Wong SW, Sil P, Martinez J. Rubicon: LC3-associated phagocytosis and beyond. The FEBS journal. 2018 Apr;285(8):1379-88.
52. Fadok VA, Bratton DL, Guthrie L, Henson PM. Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. Journal of immunology (Baltimore, Md : 1950). 2001 Jun 1;166(11):6847-54.
53. Yang C-S, Rodgers M, Min C-K, Lee J-S, Kingeter L, Lee J-Y, et al. The autophagy regulator Rubicon is a feedback inhibitor of CARD9-mediated host innate immunity. Cell Host Microbe. 2012;11(3):277-89.
54. Inomata M, Xu S, Chandra P, Meydani SN, Takemura G, Philips JA, et al. Macrophage LC3-associated phagocytosis is an immune defense against Streptococcus pneumoniae that diminishes with host aging. Proceedings of the National Academy of Sciences. 2020;117(52):33561-9.
55. Sil P, Suwanpradid J, Muse G, Gruzdev A, Liu L, Corcoran DL, et al. Noncanonical autophagy in dermal dendritic cells mediates immunosuppressive effects of UV exposure. The Journal of allergy and clinical immunology. 2020 May;145(5):1389-405.
56. Masud S, Prajsnar TK, Torraca V, Lamers GEM, Benning M, Van Der Vaart M, et al. Macrophages target Salmonella by Lc3-associated phagocytosis in a systemic infection model. Autophagy. 2019;15(5):796-812.

Chapter 3

3. Reduction in Rubicon by cigarette smoke is associated with impaired phagocytosis and occurs through lysosomal degradation pathway.

The work presented in this chapter is submitted to ‘European journal of pharmacology” as part of Asare Patrick Fordjour, Plinio R Hurtado, Hai Bac Tran, Eugene Roscioli, Sandra Hodge, 2021.

Statement of Authorship

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Contribution to the Paper	Conceptualization, investigation, methodology, writing-original draft and writing-review and editing
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

Background*.** A common feature of COPD is a defective lung macrophage phagocytic capacity that can contribute to chronic lung inflammation and infection. The precise mechanisms remain incompletely understood, although cigarette smoke is a known contributor. We previously showed deficiency of the LC3 associated phagocytosis (LAP) regulator, Rubicon, in macrophages from COPD subjects and in response to cigarette smoke. The current study investigated the molecular basis through which cigarette smoke extract (CSE) reduces Rubicon in airway macrophages, and the relationship between Rubicon deficiency and CSE-impaired phagocytosis. ***Methodology. Phagocytic capacity of CSE-treated macrophages was measured by flow cytometry, Rubicon expression by Western blot and real time polymerase chain reaction (RT-PCR) and autophagy flux by LC3 and p62 levels. The effect of CSE on Rubicon degradation was determined using cyclohexamide to inhibit Rubicon protein synthesis and monitor its half-life. ***Results*.** Phagocytosis was significantly impaired in CSE-exposed macrophages and strongly correlated with Rubicon expression. CSE impaired autophagy, accelerated Rubicon degradation, and reduced its half-life. Lysosomal protease inhibitors, but not proteasome inhibitors, attenuated this effect. Autophagy induction did not significantly affect Rubicon expression. ***Conclusions*.** CSE degrades Rubicon through lysosomal degradation pathway. Rubicon degradation and/or LAP impairment may contribute to dysregulated phagocytosis perpetuated by CSE.

Key words: LC3-associated phagocytosis (LAP); Rubicon; Phagocytosis; Autophagy; Inflammation

3.1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by chronic inflammation, emphysema, and recurrent chronic infection of the lower airways. Currently, COPD is the third leading cause of death, responsible for approximately 3.22 million of the 55.4 million deaths worldwide in 2019 [1]. Cigarette smoking can destroy lung tissue and impair host defence mechanisms, which often increases susceptibility to bacterial and viral infections [2, 3]. There is a correlation between phagocytic dysfunction of alveolar macrophages and severity of COPD [4].

Alveolar macrophages are critical for host defence against respiratory infections. [5, 6]. The ability of these cells to remove pathogens and dying (apoptotic) cells through phagocytosis helps to attenuate chronic lung inflammation and infections [7]. Phagocytosis is also a necessary cellular event that protects lung tissues against foreign pathogens to preserve lung function; however, in COPD, the capacity of alveolar macrophages to phagocytose apoptotic cells and bacteria is impaired [8, 9]. Thus, a better understanding of the mechanisms involved in phagocytosis, and how these are impaired by cigarette smoke, may identify new therapeutic targets for the treatment of COPD.

LC3-associated phagocytosis (LAP) is an essential step in the phagocytic process, which is required for the effective removal of apoptotic cells and pathogens by phagocytic cells such as macrophages in the airways [10]. LAP enhances microbicidal activity of macrophages by promoting the fusion of lysosomes to phagosome. This results from a cascade of protein interactions that leads to the recruitment of LC3-conjugating system through the interaction between the phagosome and Rubicon [11, 12]. Rubicon is a Run domain Beclin-1-interacting and cysteine-rich domain-containing intracellular protein that is engaged by phagosomes following the recognition and ingestion of pathogens and apoptotic cells by phagocytes [11, 13]. Experimental silencing or age-dependent loss of Rubicon leads to LAP attrition and impairs the clearance of phagosome-containing pathogen by preventing the delivery to lysosome for digestion [14].

The function of Rubicon in LAP and phagocytosis has attracted attention due to the potential clinical relevance in protecting against infection and inflammation [12, 15]. For example, animals deficient in Rubicon exhibit a dysregulation in the clearance of some microbes and a remarkable increase in the production of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) [16]. In contrast, Rubicon overexpression leads to inhibition of

interferon-gamma (IFN-γ) signalling to protect against the establishment of pro-inflammatory state [17].

We have previously shown Rubicon deficiency in macrophages from COPD patients and in response to cigarette smoke exposure [18], suggesting LAP dysregulation and potential contribution to the impaired bacterial clearance and colonisation of the airway in COPD. However, the molecular basis of Rubicon depletion in COPD and upon cigarette smoke exposure remains incompletely understood. To this end, the current study investigated the mechanism by which Rubicon deficiency occurs in COPD and in response to cigarette smoke exposure. We determined the correlation between Rubicon deficiency and phagocytosis and the effect of cigarette smoke extract on Rubicon gene expression. Further, we investigated the impact of regulatory factors such as autophagy, proteasome and lysosomal enzymes on Rubicon protein expression in macrophages exposed to cigarette smoke extract. The findings of this study show that Rubicon degradation by cigarette smoke results from lysosomal enzymes activity independent of the autophagy pathway. Therefore, we provide mechanistic insights for restoring Rubicon in macrophages, with potential clinical relevance in COPD patients where microbial burden and exacerbation of inflammation is frequently observed [19].

3.2. Methodology

Preparation of cigarette smoke extract. Cigarette smoke extract (CSE), prepared as a single stock of 100% CSE, was used to make 10% CSE (a concentration that we showed inhibits macrophage phagocytic function) as previously described [20, 21]. Briefly, we bubbled the cigarette smoke from four 1R5F 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 2 mM L-glutamine (all Thermo Fisher Scientific, MA, USA) using a vacuum pump. This was performed under 5 min per cigarette. The 100% CSE was filtered, the pH was adjusted and aliquots were maintained at −80 °C.

Cell cultures and exposures. A THP-1 monocyte cell line, obtained from American Type Culture Collection, Manassas, VA, USA, was cultured at 37 °C/5% CO2 in RPMI 1640 medium containing 10% FBS, penicillin/streptomycin, 2 mM L-glutamine, and 0.05 mM ß-mercaptoethanol (Sigma-Aldrich, MO, USA). The monocytic cell line was seeded 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 for differentiation into macrophages, as published previously [22]. The cells were treated with or without CSE and Cyclohexamide (CHX, 50 µg/ml), leupeptin (Leu, 40 µM), chloroquine hydrosulphate (CHQ, 50 µM), 4-benzenesulfonyl fluoride hydrochloride (AEBSF, 2.5 mM), MG-132 (5 µM), prolyl endopeptidase inhibitor (S 17092), cathepsin B inhibitor (CA-074), rapamycin (rap, 100 nM) or bafilomycin A1 (BAFA1, 50 nM) all from Sigma-Aldrich and bortezomib (PS-341, 1 µM) (Selleckchem Chemicals, Pittsburgh, USA).

Subject population

We specifically recruited COPD and non-smoker control subjects to participate in this study. Subjects with other respiratory diseases such as lung cancer and those within 6 weeks of a COPD exacerbation were excluded from the study. Ethics approval was obtained from the Royal Adelaide Hospital Human Research Ethics Committee (Ethics numbers 12978 and #020811d) and written informed consent was obtained from all subjects. GOLD criteria with clinical correlation was use to confirm COPD diagnosis. Adult controls were recruited from our volunteer database and were non-smokers with no history of respiratory or allergic disease. Blood was collected from non-smoker control subjects for preparation of monocyte-derived macrophages (we have previously shown these to be excellent surrogates for alveolar

macrophages when assessing phagocytic functions) [20]. Bronchoalveolar lavage (BAL) was obtained from a cohort of 3 subjects in each group for examination of Rubicon expression.

Table 1. Demographic details of COPD patients and control subjects

	Controls	COPD
Number of volunteers	3	3
Age(yrs)±SEM	52.9±6.24	67.0±5.132
Male	2	2
Female	1	1
Never smokers	3	0
Current smokers	0	2
Ex-smokers	0	1
FEV1%PRED	96.33±1.543	81.0±4.410
FVC%PRED	92.67±0.8819	94.33±2.186
FEV1/FVC% PRED)	81.33±1.930	66.66±1.453

Table 1: Demographic details of COPD patients and control subjects. Data are presented as mean±SEM.

Bronchoscopy Procedure

BAL was obtained at bronchoscopy from COPD and non-COPD subjects. Briefly, a 50 mL aliquot of sterile normal saline was instilled into the airways with a syringe then aspirated using low suction at room temperature (previously reported [23]). 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 Blood Monocyte Derived Macrophage (MDM)

For monocyte isolation, whole blood collected into Lithium-Heparin tubes (Greiner Bio One, Vienna, Austria) was diluted with 2 volumes of plain RPMI 1640 medium. Diluted blood was layered over Lymphoprep™ (STEMCELL Technologies, BC, Canada) and centrifuged at

800xg for 25 min with acceleration but no brake. Peripheral blood mononuclear cells (PBMC) were isolated according to the manufacture's instructions. To derive macrophages from monocytes, PBMC were seeded into plates at 1.4×10^6/mL in plain RPMI 1640 medium at 37 °C/5% CO_2 for 90 min to allow monocytes to attach. Unattached cells were aspirated and adherent cells were washed three times with PBS and cultured in RPMI 1640 medium containing 2 mM L-glutamine, 10% FBS, penicillin/gentamicin and 20 ng/mL macrophage colony-stimulating factor (GM-CSF, Peprotech, Cranbury, New Jersey, USA) for 12 days with media changes at days 4 and 8.

Phagocytosis assay

The phagocytosis capacity of THP-1 differentiated macrophages co-cultured with CSE or control media was performed as previously reported [9]. Briefly, Non-typeable *Haemophilus influenzae* (NTHi), applied as phagocytic targets, were stained with Phrodo Red from Thermo Fisher Scientific. The bacteria were co-cultured with THP-1 macrophages, at 100:1 ratio 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 phagocytosing NTHi. Procedures for gating have been previously published [20, 23, 24].

Western blot analysis

For western blot analysis of Rubicon (D9F7) rabbit mAb (Cell Signalling Technology, Danvers, Massachusetts USA), LC3 (4105) rabbit mAb (Cell Signalling Technology, Danvers, Massachusetts, USA), Atg7 (ab52472) rabbit pAb (Abcam, Waltham, Massachusetts, USA), Becin-1 (G-11; SC-48381) rabbit mAb (Santa Cruz Biotechnology, Dallas, Texas, USA), SQSTM1/p62 (D5E2) Rabbit mAb #8025 (Cell Signalling Technology, Danvers, Massachusetts, USA), hsp70 (D69) rabbit mAb (Cell Signalling Technology Danvers, Massachusetts, USA), hsp90 AC88] (ab13492) Mouse mAb (Abcam, Waltham, Massachusetts, USA), Cathepsin B (#219408) Rabbit pAb, (Calbiochem, La Jolla, California, USA) TIM-4 (ab47637) rabbit pAb (Abcam, Waltham, Massachusetts, USA),) and β-actin mouse mAb (Sigma Aldrich; A1798; St. Louis, MO, USA). THP-1 cells were lysed using M-PER mammalian cell protein lysis reagent with Halt protease inhibitor (company) and PMSF protease inhibitor (Sigma Aldrich). Protein samples were quantified using BCA protein assay (Thermo Fisher). Protein amount of 10 μg was electrophoresed in 4–12% gradient Bis-Tris gels before being transferred to nitrocellulose membrane. Membranes were blocked in 5% skim

milk (Fonterra, Auckland, NZ) or 5% bovine serum albumin (BSA, Sigma Aldrich) before incubating with primary antibodies overnight at 4 ^{o}C. Membranes were incubated with corresponding secondary antibodies and washed three times with Tris Buffered Saline with Tween (TBST) and then probed with Bio-Rad software (CA, USA).

Real-time reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA extraction from THP-1 macrophages was performed according to the manufacturer's instruction using the RNeasy Mini kit (QIAGEN, Venlo, Netherlands). Then, 1 μg of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN) 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). Rubicon gene expression levels were normalized to GAPDH (Hs02758991_g1) and HPRT (Hs 99999901) all from Thermo Fisher Scientific.

Immunofluorescence and confocal microscopy

Immunofluorescence was carried out as previously described [18]. Briefly cells on chamberslides were incubated overnight at 4^{o}C with primary antibodies then 40 minutes at ambient temperature with secondary antibodies, multiple washes in Tween-20/PBS applied after each incubation. Immunofluorescence was photographed from multiple optical fields using an Olympus confocal system FV3000, mean fluorescence intensities measured using the ImageJ software as described [18].

Statistical analysis

Data were analysed using GraphPad prism software (GraphPad, La Jolla, USA). Results are reported as mean±SEM unless otherwise indicated. Analysis was performed using the non-parametric Mann Whitney test (for comparison between two groups with n values more than 3), Welch test (for comparison between two groups with n values of 3) and one-way Dunnett's post hoc test for more than two different experimental groups. $P < 0.05$ was considered statistically significant. Correlation analysis of Rubicon protein expression and phagocytosis was performed using Pearson's correlation coefficient with significance set at $p<0.05$.

3.3. Results

Reduction in Rubicon protein levels by CSE correlates with impaired phagocytosis

We evaluated the effect of CSE on the phagocytic capacity of THP-1 macrophages using flow cytometry. We found that CSE inhibits the ability of the macrophages to phagocytose NTHi compared with untreated control cells **(Figure 1A, **p=0.0079),** corroborating our previous reports [8]. Further, we demonstrated a reduction of Rubicon protein in CSE-treated THP-1 macrophages compared to control cells (**Figure 1B, *p=0.0265).** We also observed a positive correlation between Rubicon protein expression and phagocytosis of NTHi by THP-1 macrophages **(Figure 1C, *p=0.0139, r=0.9022, r^2=0.8140)**. To determine the molecular basis of Rubicon reduction induced by CSE, we first investigated the effects of CSE on mRNA levels of Rubicon in THP-1 differentiated and COPD alveolar macrophages from in comparison to non-COPD control macrophages. We found that CSE did not have a significant effect on Rubicon gene transcription compared to untreated control THP-1 macrophages confirming our earlier observation [18] **(Figure 1D, p=0.2077).** However, Rubicon mRNA levels in macrophages from COPD subjects were significantly lower (about 2-fold reduction) compared to the control non-COPD subjects **(Figure 1E, *p=0.0495)**. While the effect size difference is modest, it indicates that Rubicon gene transcription in alveolar macrophage tends to reduce in COPD. Hence, while both Rubicon gene and protein levels are reduced in COPD alveolar macrophages, CSE exposure leads to the reduction of Rubicon protein but not gene levels in macrophages.

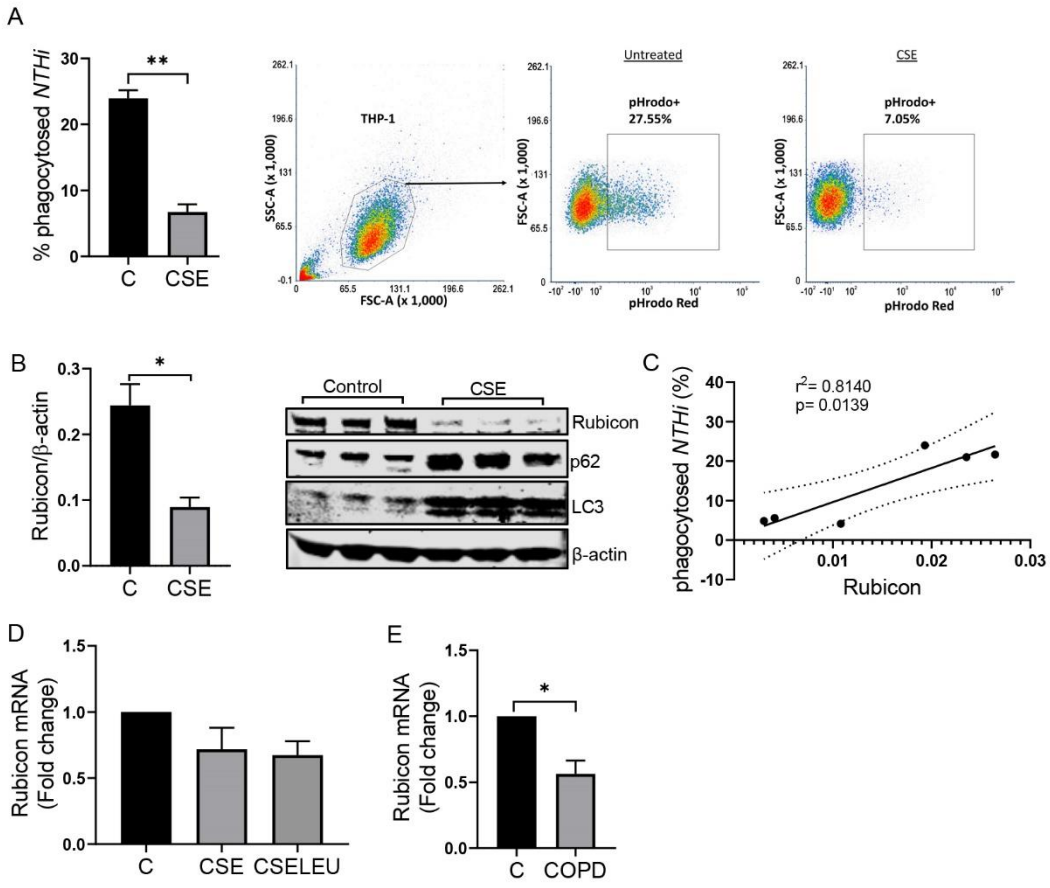


Figure 1: Reduction in Rubicon protein levels by CSE correlates with impaired phagocytosis.

(A) Representative results showing a decline in phagocytosis after 24 h of CSE exposure. Control (C) vs CSE. **p=0.0079. Data are expressed as mean ± SEM, n=5. **(B)** Quantification of Rubicon protein expression. CSE reduced Rubicon protein compared to the control untreated THP-1 macrophages. *p=0.0265; mean ± SEM, n=3. 'C' represents control cells while CSE represents cells exposed to cigarette smoke extract. **(C)** Correlation between Rubicon protein expression and % phagocytosis. Data shows a significant positive correlation between phagocytosis and protein expression of Rubicon (n=6, p=0.0139, r=0.9022, r^2=0.8140). **(D)** Rubicon gene transcription is unaffected by cigarette smoke extract (CSE) and leupeptin (Leu) compared to control (C) untreated THP-1 macrophages; p=0.2077 and p=0.1433 for C vs CSE and C vs CSE+Leu respectively, n=3 for each group. **(E)** Representative results showing a decrease in Rubicon gene transcription in COPD alveolar macrophages compared to non-COPD control subjects (C). *p=0.0495. Data are expressed as mean ± SEM, n=3 for each group.

Steady state turnover of Rubicon protein occurs through the lysosomal degradation pathway

To delineate the molecular basis of Rubicon protein reduction, we investigated whether CSE influences the abundance of Rubicon protein through protein degradation pathways. This led us to determine the Rubicon half-life in THP-1 macrophages using cyclohexamide chase assay as previously described [25]. We found that Rubicon has a half-life of ~8-10 h in this cell model **(Figure 2A).** Interestingly, it was observed that CSE accelerates Rubicon degradation, and reduces its half-life to ~3 h **(Figure 2B).** This led us to reason that the reduction in Rubicon protein in CSE exposed macrophages is due to Rubicon degradation. Therefore, we assessed the impacts of interventions that inhibit the two main protein degradation pathways, proteasomes and lysosomal proteases on Rubicon protein degradation. To evaluate the role of proteasomes, the cells were treated with MG-132, a selective 26S proteasome inhibitor in the presence of the protein synthesis inhibitor, cyclohexamide . We found that proteasome inhibition did not delay the normal degradation of Rubicon. Rather, it reduced it from ~9 h to ~4 h (**Figure 2C).** Next, we examined the role of lysosomal proteases on Rubicon protein degradation by treating the cells with leupeptin, a broad-spectrum lysosomal proteases inhibitor in the presence of cylohexamide. We found that lysosomal protease delayed the degradation of Rubicon demonstrating the involvement of proteolytic enzymes if Rubicon degradation (**Figure 2D).**

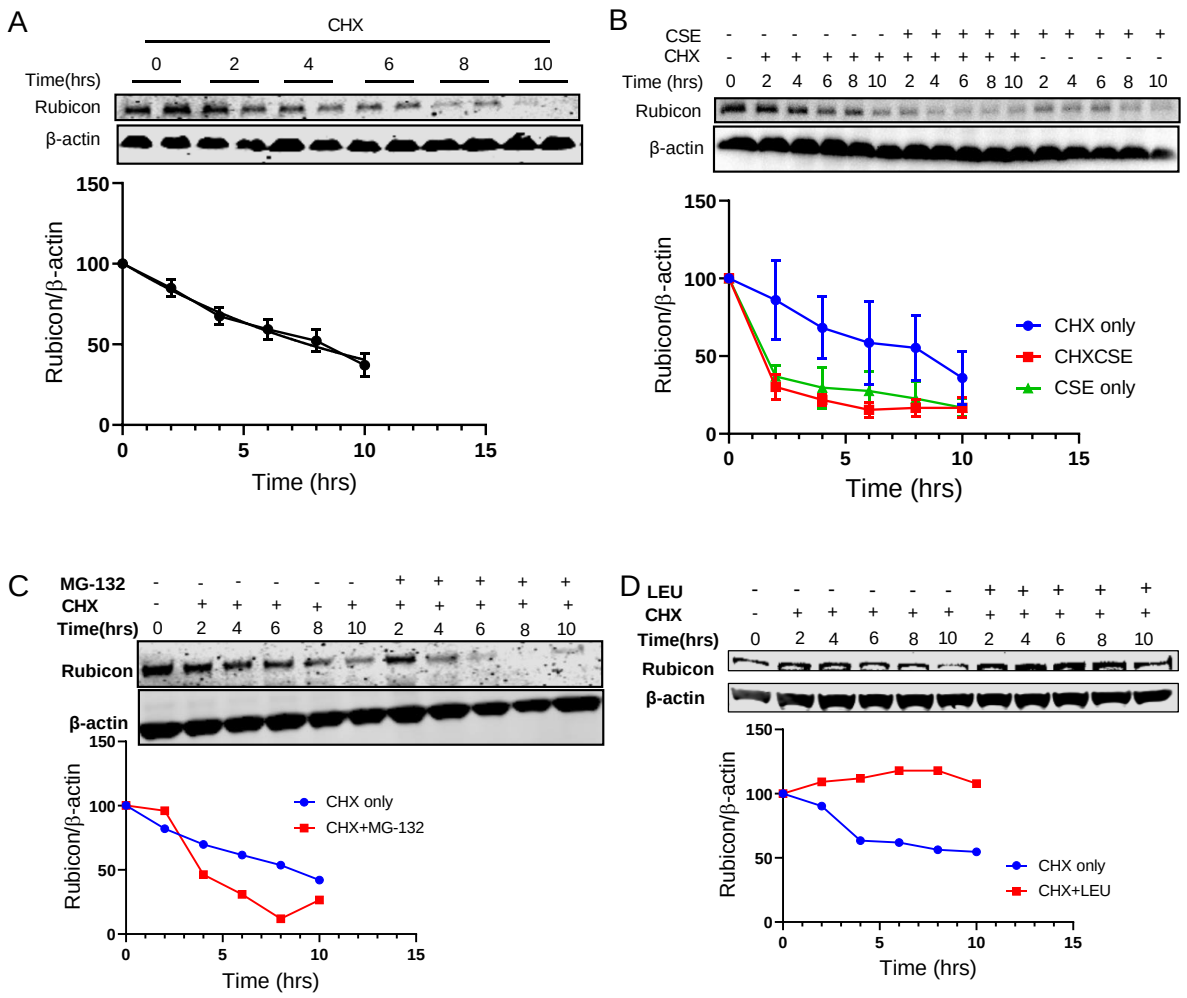


Figure 2: Steady state turnover of Rubicon protein occurs through the lysosomal degradation pathway.

(A, B, C, D) Western blot analyses were carried out using cell lysates of macrophages treated with cyclohexamide (CHX). Band intensities of Rubicon were quantified and normalized to those of β-actin that served as control. **(A)** Densitometric plots of Rubicon protein decay versus time of CHX exposure in THP-1 macrophages. Rubicon decayed with a half-life of 8-10 h; n= 7, mean ± SEM. **(B)** Densitometric plots of Rubicon protein decay versus time of CHX only, CHX+CSE and CSE only from exposure of THP-1 differentiated macrophages. Rubicon decayed with a half-life of -108 h in CHX only treated cells but the degradation was accelerated upon CSE exposure resulting in a shortened half-life of ~2 h. Rubicon in CSE only treated cells also reduced with time and had a half-life of ~3 h. Data presented as mean ± SEM of three independent experiments. **(C)** Densitometric plots of Rubicon protein decay versus time of CHX only and CHX+MG-132 exposure in THP-1. While Rubicon decayed with a half-life of ~9 h after inhibition of protein synthesis, treatment with the

combination of CHX and MG-132 shortened the half-life to ~ 4 h. **(D)** Representative results of Rubicon protein decay versus time of CHX only and CHX+leupeptin exposure in THP-1. The half-life of Rubicon obtained in THP-1 macrophages after CHX only treatment was ~10 h. However, Rubicon protein degradation was delayed in cells treated with both CHX and leupeptin.

CSE-induced degradation of Rubicon is proteasome-independent

The above data demonstrated that degradation of Rubicon under steady state occurs via the lysosomal and not proteasomal pathway. However, it has been reported that CSE induces the degradation of a number of proteins via the ubiquitin-proteasome system (UPS). This led us to examine whether the proteasome might be involved in CSE-mediated degradation of Rubicon using the proteasome inhibitors MG-132 and bortezomib in monocyte-derived macrophages.

We first treated blood MDM with MG-132 at concentrations of 1µM, 5 µM, 10µM and 20µM for 24 h. Similar to in THP-1 macrophages, CSE significantly reduced Rubicon protein levels in MDMs by a factor of three **(Figure 3A; **p=0.0025).** Addition of the proteasome inhibitor MG-132 did not reverse the effect of CSE on Rubicon**.** In fact, we unexpectedly found that MG-132 treatment resulted in a further 2-fold reduction in Rubicon in CSE-treated MDM **(Figure 3A, ***p=0.0007)**. Similarly, bortezomib further reduced Rubicon levels in CSE-treated cells rather than abrogating its degradation **(Figure 3B, **p=0.0023).** To our surprise, bortezomib treatment also significantly reduced Rubicon expression levels in MDM not exposed to CSE over 24 h **(Figure 3B, *p=0.0182)**.

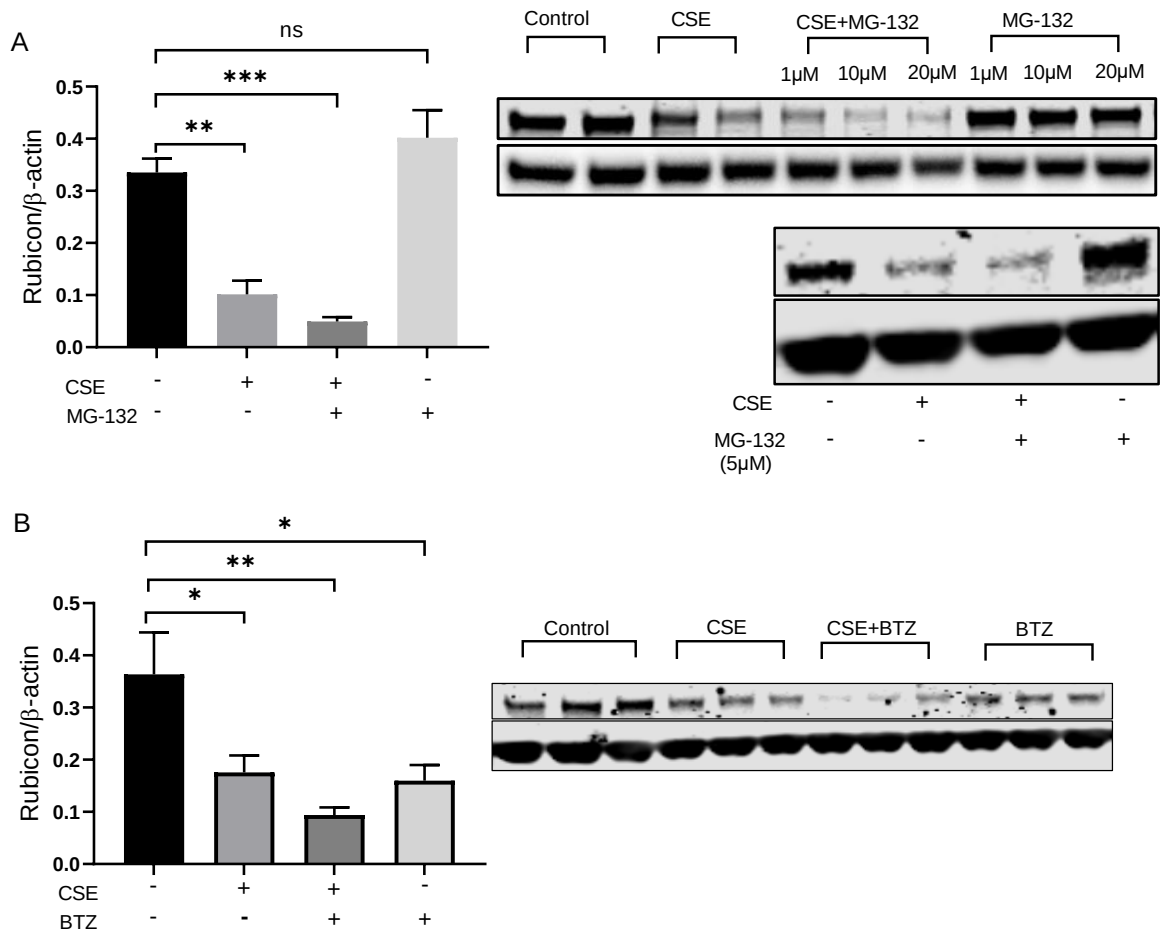


Figure 3: CSE-induced degradation of Rubicon is proteasome-independent.

(A) Western blots (and quantitative data) of Rubicon protein expression; Control vs Cigarette smoke extract (CSE), control vs CSE+MG-132 and C vs MG-132 only. 'Control' represents different donors' macrophages which were not exposed to cigarette smoke extract, 'CSE' represents different donors' macrophages exposed to cigarette smoke extract, CSE+MG-132 depicts different donors' macrophages which were exposed to cigarette smoke extract and MG-132 while MG-132 represents different donors' macrophages which were exposed to MG-132 only. Data are expressed as mean ± SEM, n=6. CSE only treatment significantly reduced the expression of Rubicon in blood monocyte derived macrophages by 3 times, **p=0.0025. Co-treatment of CSE and MG-132 further reduced Rubicon by 6 times compared with the control cells ***p=0.0007. MG-132 only treated cells exhibited non-significant reduction of Rubicon compared to control untreated cells, p=0.3860. **(B)** Western blots (and quantitative data) of Rubicon protein expression in blood monocyte derived macrophages (MDM) in the presence and absence of bortezomib (1 μM for 24 h). Control represents untreated MDM while CSE represents MDM exposed to cigarette smoke extract

only. CSE+BTZ represents MDM treated with CSE and bortezomib. BTZ represents MDM treated with bortezomib only. Data are expressed as mean ± SEM, n=5. CSE only treatment reduced the expression of Rubicon in blood monocyte derived macrophages, *p=0.0294. Rubicon level in MDM treated with a combination of CSE and bortezomib was six times lower compared to control untreated MDM, **p=0.0023. BTZ only treated cells also exhibit reduced Rubicon expression compared to control untreated cells, *p=0.0182.

CSE-induced degradation of Rubicon is mediated by lysosomal proteases

We initially hypothesised that similar to other proteins studied, cigarette smoke decreases Rubicon by inducing its degradation in the proteasome. However, we have now demonstrated that CSE-induced degradation of Rubicon in independent of the proteasome. Additionally, we have demonstrated that the steady state turnover of Rubicon is mediated by lysosomal proteases. We therefore tested whether lysosomal proteases participate in CSE-induced Rubicon degradation. In support of this, we found that the lysosomal protease inhibitor leupeptin prevented CSE-induced reduction of Rubicon as there was no significant difference in Rubicon expression in CSE+leupeptin treated cells in comparison to control cells **(Figure 4A, p=0.5442).**

To confirm the contribution of the lysosome, we measured the effect on CSE-induced Rubicon degradation of chloroquine (CQ) and bafilomycin A1 (BAFA1), two drugs that interfere with lysosome function by altering the pH. Both CQ and BAFA1 prevented CSE-induced reduction in Rubicon, confirming the central role of the lysosome in this process **(Figure 4B, 4C).**

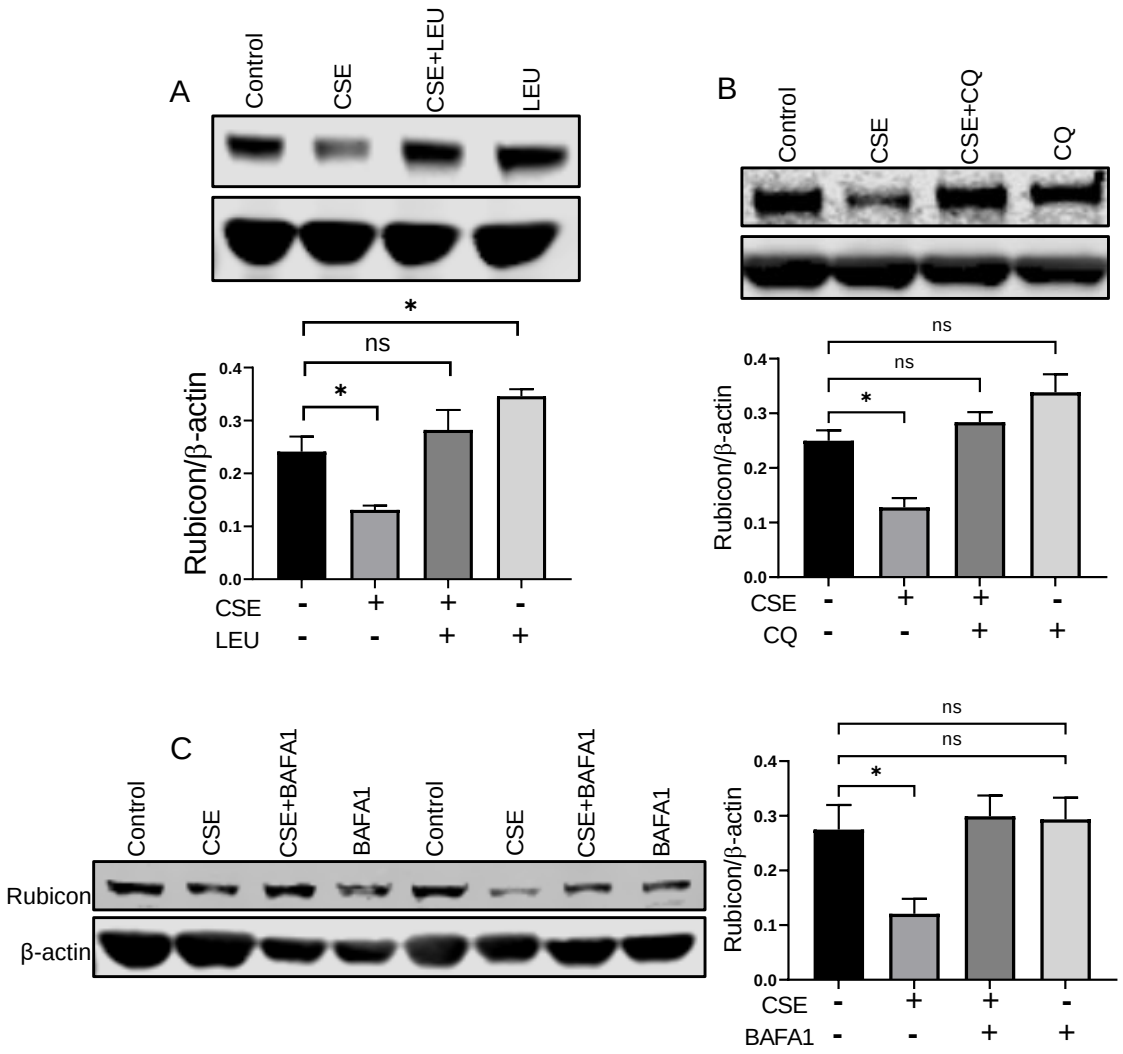


Figure 4: CSE-induced degradation of Rubicon is mediated by lysosomal proteases.

Western blots (and quantitative data) of Rubicon protein expression; Control vs Cigarette smoke extract (CSE), control vs CSE+leupeptin and C vs leupeptin only. control represents untreated THP-1 macrophages while CSE, CSE+leu and Leu represent THP-1 macrophages exposed to cigarette smoke extract only, combination of CSE and leupeptin and leupeptin only respectively. Data are expressed as mean ± SEM, n=3. **(A)** CSE only treatment reduced the expression of Rubicon in THP-1 differentiated macrophages,*p=0.0348 compared to the control cells. Co-treatment of CSE and leupeptin prevented CSE-induced Rubicon deficiency and increased Rubicon to near normal levels compared to control untreated cells. p=0.5442, n=3. leupeptin only treated cells significantly increased Rubicon expression compared to control untreated cells,*p= 0.0440. **(B)** Rubicon reduction is abrogated by the addition of chloroquine (CQ) in CSE treated THP-1 macrophages compared to control untreated cells. p=0.6128; n=3; Data are expressed as mean ± SEM, n=3. **(C)** Loss of

Rubicon protein induced by CSE was attenuated by the treatment of CSE exposed cells to bafilomycin A1 (BAFA1) in THP-1 treated macrophages compared to control untreated cells. p=0.9419; n=3; Data are expressed as mean ± SEM, n=3

Serine proteases attenuate CSE-induced reduction of Rubicon in THP-1 macropahges

The effect of leupeptin indicates that Rubicon is degraded by proteases. Cathepsin-B is a major mediator of lysosome-dependent degradation [26], which depends on lysosomal pH for auto-activation [27, 28]. To investigate its potential involvement, we measured cathepsin-B following CSE treatment of THP-1 cells and found that it became upregulated in CSE-treated cells **(Figure 5A, *p= 0.0310).** However, treatment of cells with CA074, a selective inhibitor of cathepsin-B, did not restore Rubicon in CSE-treated cells **(Figure 5B).**

Serine proteases are the largest group of proteases, making up one third of all proteolytic enzymes in the cell [29]. To test whether serine proteases were responsible for degradation of Rubicon in response to CSE, we used the selective inhibitor 4-benzenesulfonyl fluoride hydrochloride (AEBSF). Inhibition of serine proteases by AEBSF treatment completely abrogated the effect of CSE on Rubicon degradation (**Figure 5C**).

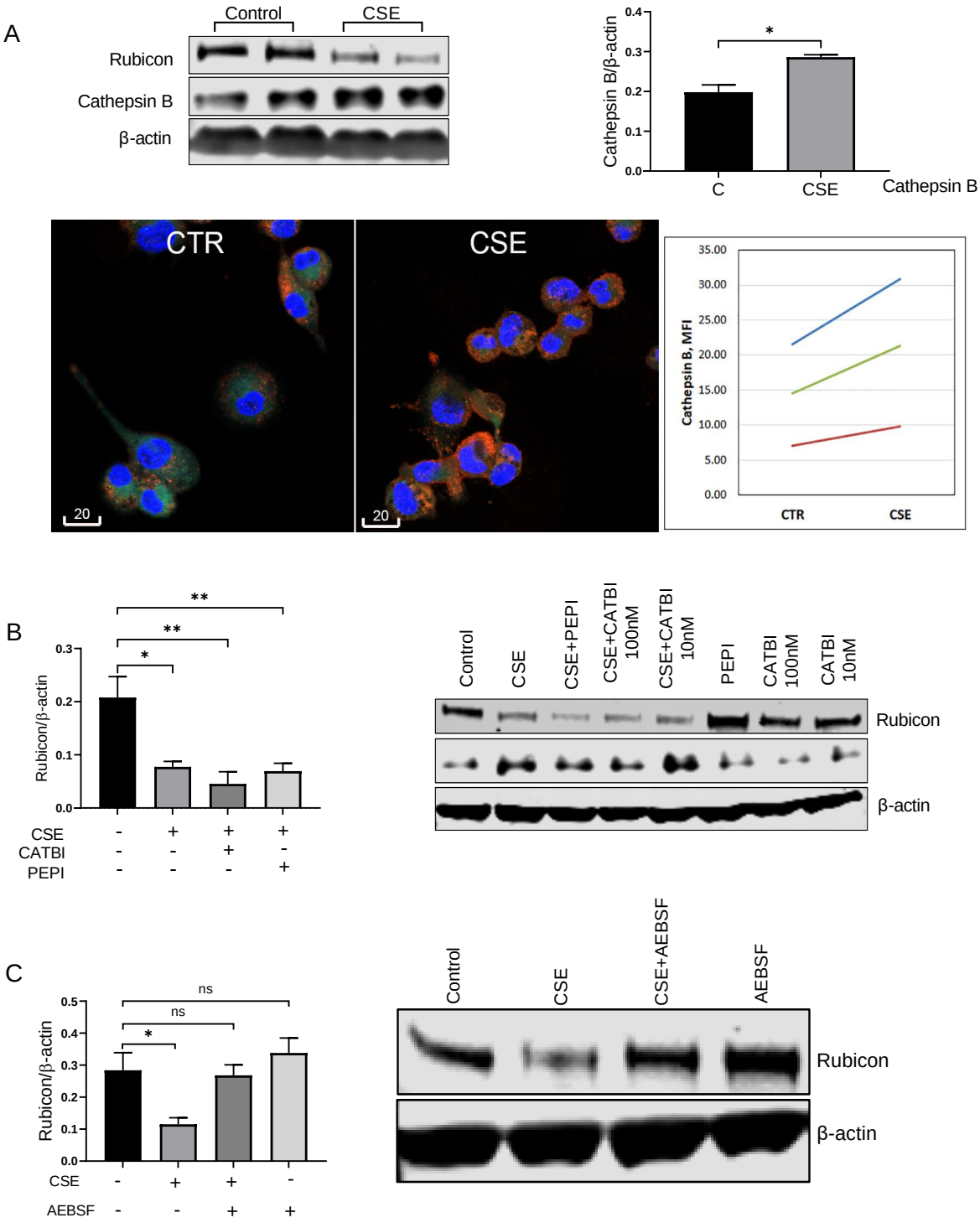


Figure 5: Serine proteases attenuate CSE-induced reduction of Rubicon in THP-1 macropahges.

(A) Western blots (and quantitative data) and immunofluorescence images of cathepsin B protein expression; Control vs CSE. Cathepsin B protein increased in CSE treated macrophages compared to control untreated THP-1 macrophages; *p= 0.0310. Data are expressed as mean ± SEM, n=3. **(B)** Western blots (and quantitative data) of Rubicon protein

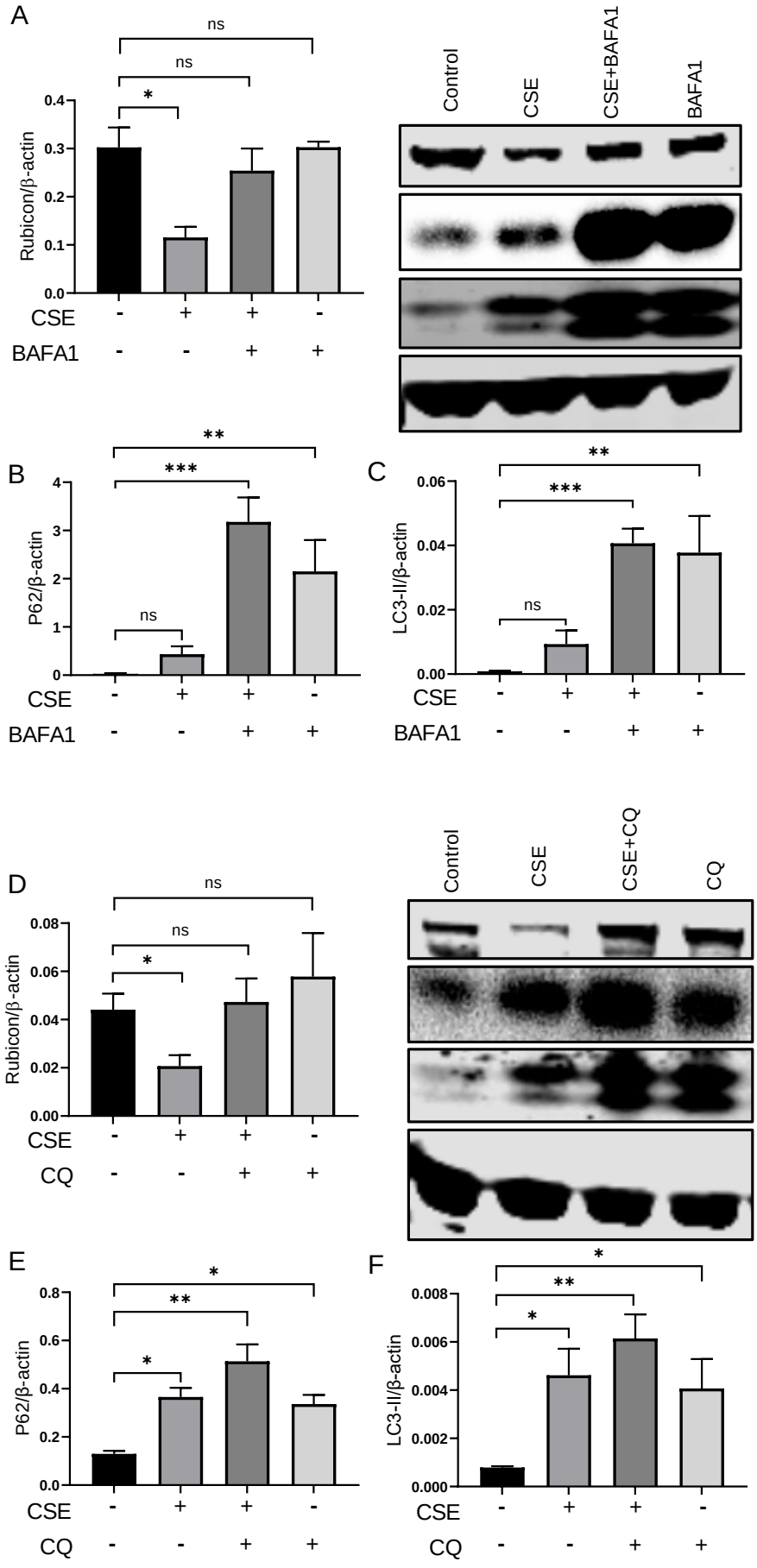
expression; Control vs CSE, C vs CSE+cathepsin B inhibitor (CATBI; 10 and 100 nM). Control represents untreated THP-1 differentiated macrophages, CSE represents THP-1 macrophages exposed to cigarette smoke extract only, CSE+CATBI represents THP-1 macrophages treated with both cigarette smoke extract and cathepsin B inhibitor and CSE+PEPI represents THP-1 macrophages treated with combination of cigarette smoke extract and prolyl endopeptidase inhibitor. Data are expressed as mean ± SEM, n=3. **(C)** Western blots (and quantitative data) of Rubicon protein expression; control vs Cigarette smoke extract (CSE), control vs CSE+AEBSF and control vs AEBSF only. Control represents untreated THP-1 differentiated macrophages, CSE represents THP-1 macrophages exposed to cigarette smoke extract only. CSE+AEBSF represents THP-1 macrophages treated with cigarette smoke extract and 4-benzenesulfonyl fluoride hydrochloride. AEBSF represents THP-1 macrophages treated with 4-benzenesulfonyl fluoride hydrochloride only. Data are expressed as mean ± SEM, n=3. CSE only treatment reduced the expression of Rubicon in THP-1 differentiated macrophages, *p=0.0461 compared to the control. Co-treatment of CSE and AEBSF further prevented CSE-induced downregulation of Rubicon and increased Rubicon to near basal levels compared to control untreated cells, p=0.9849. AEBSF only treated cells showed non-significant increase in Rubicon compared to control untreated cells, p=0.6823.

CSE-induced degradation of Rubicon may not involve autophagy activity

Multiple pathways can lead to lysosomal degradation of cellular proteins; the most common is via the autophagy machinery. Up to this point, we have identified three compounds (leupeptin, BAFA1, CQ) that abrogate the effect of CSE on Rubicon degradation; all of these compounds have been suggested to preferentially affect the autophagy pathway. In **Figures 6B, 6C, 6E and 6F**, we confirm the potent effect of BAFA1 and CQ on the downregulation of the autophagy machinery.

To specifically interrogate the possible involvement of autophagy on Rubicon degradation, we induced autophagy in THP-1 macrophages using the mTOR inhibitor rapamycin **(Figure 6G, 6H and 6I)**. If autophagy is involved in Rubicon degradation, we would expect rapamycin treatment to increase Rubicon degradation in the presence and absence of CSE. Contrary to this, we were unable to demonstrate a significant reduction of Rubicon in CSE+rapamycin treated cells compared with cells treated with CSE alone **(Figure 6G, p=0.7896).** Interestingly,

CSE itself significantly impaired autophagy, as measured by p62 and LC3 accumulation over 24 h **(Figure 6H and 6I)**. This is compelling evidence that CSE-induced degradation of Rubicon does not occur via the autophagy pathway.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CSE - + + -
BAFA1 - - + +
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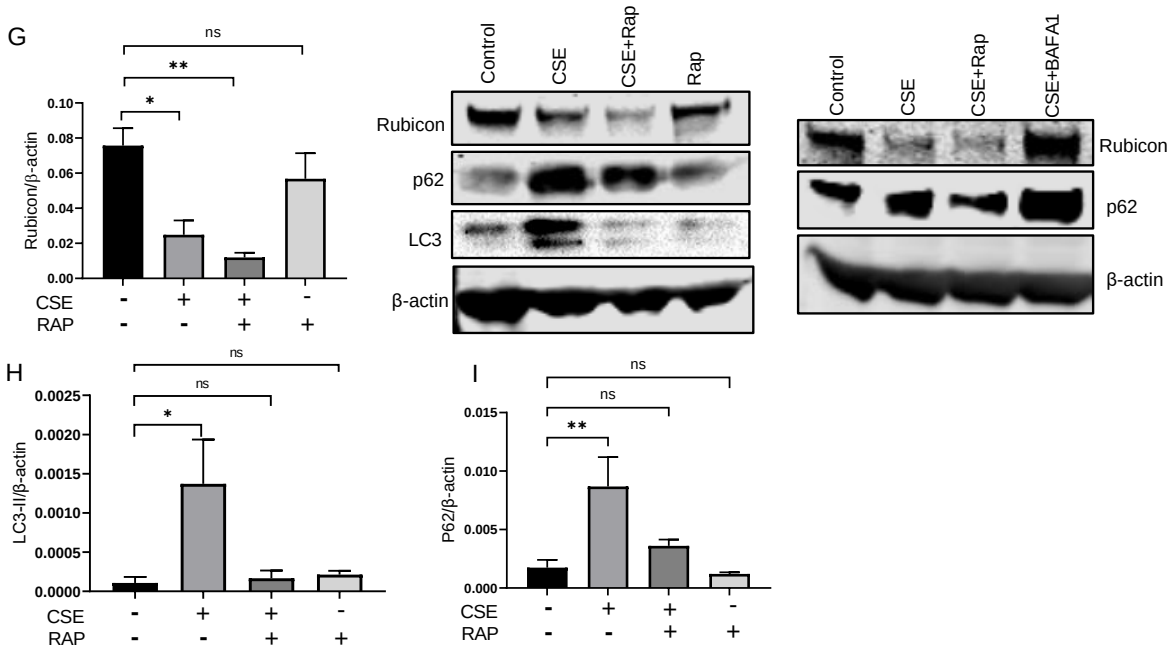


Figure 6: Serine proteases attenuate CSE-induced reduction of Rubicon in THP-1 macropahges.

(A, B, C) Western blots (and quantitative data) of Rubicon, p62 and LC3 protein expression respectively; from CSE treated THP-1 macrophages in the presence and absence of bafilomycin (BAFA1; 50 nM for 24 h). Data are expressed as mean ± SEM, n=3. **(A)** CSE only treatment reduced the expression of Rubicon in THP-1 differentiated macrophages compared to the control, *p=0.0364. Co-treatment of CSE and BAFA1 alleviated CSE-induced reduction in Rubicon and increased Rubicon to near basal levels compared to control untreated cells. p= 0.8960. BAFA1 only treated cells showed no significant effect on Rubicon expression compared to control untreated cells, p>0.9999. Data are expressed as mean ± SEM, n=3. **(B)** CSE only treatment increased the expression of p62 by 15 fold in THP-1 differentiated macrophages compared to the control. Co-treatment of CSE and BAFA1 further increased the accumulation of p62 compared to control untreated cells, ***p=0.0002. BAFA1 only treated cells led to a significant increase in p62 expression compared to control untreated cells, **p=0.0067. Data are expressed as mean ± SEM, n=5. **(C)** CSE only treatment non-significantly increased the expression of LC3-II in THP-1 differentiated macrophages, p=0.6878 compared to the control. Co-treatment of CSE and BAFA1 further increased the accumulation of LC3-II compared to control untreated cells, ***p=0.0009. BAFA1 only treated cells led to a significant increase in LC3-II expression compared to control untreated cells, **p=0.0019. Data are expressed as mean ± SEM, n=6. **(D, E, F)** Representative images of Western blot analysis for Rubicon, p62 and LC3

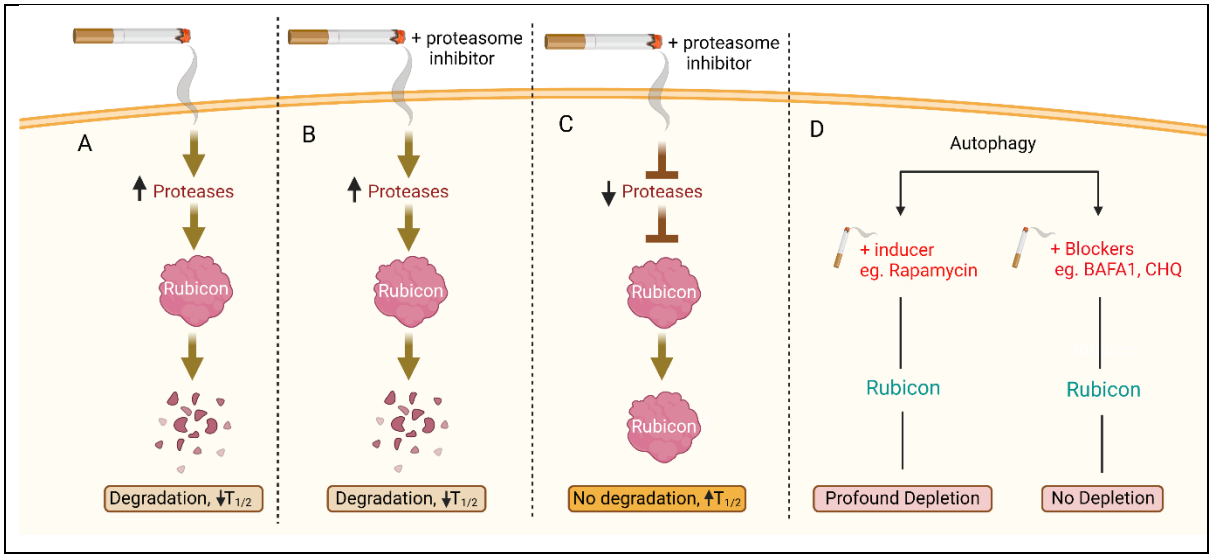
respectively in cell lysates from CSE-exposed THP-1 macrophages in the presence or absence of chloroquine (50 μM, for 24 h). (D) CSE only treatment reduced the expression of Rubicon in THP-1 differentiated macrophages compared to the control, *p=0.0164. Co-treatment of CSE and chloroquine alleviated CSE-induced reduction in Rubicon and increased Rubicon to near normal levels compared to control untreated cells. p=0.6800. Chloroquine only treated cells did not have any significant effect on Rubicon expression compared to control untreated cells, p=0.5920. Data are expressed as mean ± SEM, n=3. (E) CSE only treatment increased the expression of p62 in THP-1 differentiated macrophages,*p=0.0234 compared to the control. Co-treatment of CSE and CQ further increased the accumulation of P62 compared to control untreated cells, **p=0.0013. CQ only treated cells led to a significant increase in P62 expression compared to control untreated cells, *p=0.0446. Data are expressed as mean ± SEM, n=3. (F) CSE only treatment increased the expression of LC3-II in THP-1 differentiated macrophages, *p=0.0212 compared to the control. Co-treatment of CSE and CQ further increased the accumulation of LC3-II compared to control untreated cells, **p= 0.0024. CQ only treated cells led to a significant increase in LC3-II expression compared to control untreated cells, *p=0.0256. Data are expressed as mean ± SEM, n=6. **(G, H, I)** THP-1 differentiated macrophages were treated with CSE in the presence or absence of rapamycin (100 nM for 24 h). This was followed by densitometric analysis of Rubicon, LC3 and p62/SQSTM1 relative to β-actin. **(G)** CSE only treatment reduced the expression of Rubicon in THP-1 differentiated macrophages by 3 times, *p=0.0356. Co-treatment of CSE and rapamycin further reduced Rubicon by 6 times compared with the control cells **p=0.0077. Rapamycin only treated cells non-significantly reduced Rubicon compared to control untreated cells, p=0.7707. Data are expressed as mean ± SEM, n=6. **(H)** CSE only treatment increased the expression of LC3-II in THP-1 differentiated macrophages, *p=0.0375. Co-treatment of CSE and decreased the LC3-II levels near normal values compared with the control cells p=0.9977. Rapamycin only treated cells did not exhibit any significant change in LC3-II expression compared to control untreated cells, p=0.9872. Data are expressed as mean ± SEM, n=3. **(I)** CSE only treatment increased the expression of p62 in THP-1 differentiated macrophages, **p=0.0081. Co-treatment of CSE and decreased the p62 levels near basal values compared with the control cells p=0.6415. Rapamycin only treated cells did not exhibit any significant change in p62 expression compared to control untreated cells, p=0.9839. Data are expressed as mean ± SEM, n=4.

3.3.1. *Results highlights and graphical summary*

Highlights

- ❖ The specific LAP regulator, Rubicon is reduced in macrophages from patients with COPD and by exposure to cigarette smoke
- ❖ Cigarette smoke-induced downregulation of Rubicon correlates with compromised phagocytosis by THP-1 macrophages
- ❖ Cigarette smoke accelerates degradation of Rubicon by lysosomal enzymes including serine proteases
- ❖ Cigarette smoke-induced Rubicon degradation may be independent of autophagy

Figure 7: Graphical summary



3.4. Discussion

The present study shows that exposure of THP-1 macrophages to CSE causes Rubicon deficiency correlating with diminished phagocytosis of Non-typeable *Haemophilus influenzae* (NTHi), a bacterium that is commonly observed in the lungs of COPD patients [4, 19, 30]. This observation is consistent with previous reports that Rubicon deficiency causes defective clearance of pathogens and subsequently increases the production of pro-inflammatory mediators [16, 31]. It also raises the possibility that LAP impairment due to Rubicon deficiency is a key contributory factor for the NTHi colonisation in the airways of COPD patients [14]. Identification of strategies to prevent Rubicon deficiency in COPD and upon cigarette smoke exposure also requires a better understanding of the processes involved in Rubicon depletion.

The aim of the current study was to delineate the molecular basis through which cigarette smoke depletes Rubicon in macrophages. We previously identified a deficiency in Rubicon protein levels in alveolar macrophages from COPD patients. Herein, we demonstrate a corresponding reduction in Rubicon gene transcript in COPD macrophages compared with healthy counterparts. While the deficiency in Rubicon protein was reproduced *in vitro* by the treatment of THP-1 macrophages with cigarette smoke extract, we unexpectedly found no changes in Rubicon gene transcription in THP-1 macrophages exposed to CSE. This led us to hypothesise that cigarette smoke may reduce Rubicon through a protein degradation pathway. Using cyclohexamide to inhibit protein synthesis, we determined the half-life of endogenous Rubicon protein in blood monocyte-derived, and THP-1-derived, macrophages to be 8 to 10 h. Exposure to CSE shortened the half-life of Rubicon to approximately 3 h, confirming our hypothesis that cigarette smoke accelerates Rubicon degradation. This finding is in line with previous studies that have reported cigarette smoke to induce the degradation of proteins via lysosomal and/or proteasomal pathways [32-36].

In investigating the steady state half-life of Rubicon, we found that treatment with the lysosomal protease inhibitor leupeptin, but not with the proteasome inhibitor MG-132, increased the half-life of Rubicon. This suggests that the normal turnover of Rubicon is mediated by the lysosomal, rather than the proteosomal, degradation pathway. Given this, we assessed whether inhibiting the lysosomal degradation pathway could abrogate CSE-induced reduction of Rubicon protein. Consistent with our findings at steady state, we found that inhibiting lysosomal proteases, but not the proteasome, preserved Rubicon levels in CSE-treated macrophages. To further confirm the role of the lysosomal enzymes in this process, we repeated the experiment using two agents that interfere with lysosome function, CQ and

BAFA1. Again, we found that both of these agents abrogated the effect of CSE on Rubicon degradation.

Protease inhibitors have been suggested as a therapeutic strategy for the treatment of COPD. To explore this as a therapeutic target, we investigated further which proteases are mediating the effect of cigarette smoke on Rubicon. We first investigated cathepsin B due to its known involvement in lysosomal degradation and lung tissue destruction [37, 38]. Although cathepsin B was upregulated by cigarette smoke, the selective cathepsin B inhibitor, CA-074 did not prevent CSE-mediated degradation of Rubicon. Another family of proteolytic enzymes, serine proteases, is a major contributor to cellular proteolysis [29]. We examined the effect of the selective serine protease inhibitor, AEBSF on Rubicon expression levels. We observed that the serine protease inhibitor prevented Rubicon degradation in CSE-treated macrophages. Given that serine protease activity is increased in COPD [39], we surmise that serine proteases may contribute to the Rubicon deficiency that we previously observed in airway macrophages of COPD patients [18]. Our findings identify an alternative mechanism by which the use of serine protease inhibitors as therapeutics could lead to improve symptoms in COPD [40] and modulate cigarette smoke-induced release of inflammatory mediators [41]. Moreover, our data provide rationale for future studies to investigate the proteolytic activities of the excessive lysosomal proteases observed in the lung of COPD subjects and cigarette smokers [42]. This is particularly critical as the pathogenic roles of excessive proteases in COPD are less well characterised despite the causal link with the disease pathogenesis.

Having identified the mechanism behind Rubicon degradation, we investigated how cigarette smoke exposure leads to the degradation of Rubicon by serine proteases in the lysosome. Autophagy activity as a major lysosomal degradation pathway is modulated by cigarette smoke [43-45]. Therefore, we examined whether interventions that modulate autophagy activity could affect Rubicon degradation. We began by determining whether CSE has any effect on autophagy. Interestingly we found that CSE potently suppressed LC3-II and p62 in THP-1 macrophages. While this itself suggests that CSE is unlikely to induce degradation of Rubicon via autophagy, we treated macrophages with the mTOR inhibitor rapamycin to try to restore autophagy and measure the effect of this on Rubicon levels. We found that rapamycin was able to prevent CSE suppression of autophagy; however, this did not significantly affect Rubicon depletion. Altogether, these results indicate that CSE-induced degradation of Rubicon occurs is mediated by proteolytic enzyme activity and may not involve the autophagy pathway.

Conclusion and future direction

In conclusion, we found that CSE causes a decrease in Rubicon half-life in macrophages by accelerating its degradation by lysosomal serine proteases. Furthermore, we found that the reduction in Rubicon availability relates to impairment in the capacity of macrophages to phagocytose NTHi, a pathogen commonly associated with lung infection in COPD. Our findings bring new insights into the impaired phagocytic activity described in COPD patients, which is suggested to be a critical contributing factor to infections in these patients. The mechanistic findings presented in this study are highly relevant to identify novel therapeutic approaches aiming to modulate Rubicon and preserve its cellular and biological functions.

Limitation of study

We were unable to identify the specific serine proteases that mediate CSE-induced degradation of Rubicon. Therefore, we recommend future studies that selectively inhibit the various lysosomal proteases to identify the specific proteases responsible for Rubicon breakdown.

Future studies should investigate in detail the clinical relevance and mechanisms associated with the effect of proteasome inhibitors on Rubicon protein expression. This is especially important as proteasome inhibitors such as bortezomib are used in clinical settings to control cancer. Moreover, Rubicon/LAP activity is reported to favour tumour growth and aggressiveness [46, 47].

Similarly, while we ruled out autophagy, we were unable to determine the pathway through which CSE targets Rubicon for lysosomal degradation. Chaperone mediated autophagy (CMA) can also serve as a lysosomal degradation pathway that can degrade proteins with KFERQ motif [48]. While cigarette smoke could stimulate markers for CMA (LAMP2A and hsc70), it is less well understood whether cigarette smoke stimulates CMA to degrade proteins. Thus, although Rubicon lacks the KFERQ motif required for lysosomal degradation through the CMA, future studies that aim to exclude CMA could provide valuable information regarding the breakdown of Rubicon to current literature.

Future studies could identify modulators of Rubicon by elucidating the factors that lead to the reduction of Rubicon transcription in COPD. We also recommend future investigation into the component of cigarette smoke responsible for the effect that we have described.

References

1. 2021 Whs. monitoring health for the SDGs, sustainable development goals. Geneva: World Health Organization; 2021., 2021 Contract No.: Licence: CC BY-NC-SA 3.0 IGO.
2. Mutepe ND, Cockeran R, Steel HC, Theron AJ, Mitchell TJ, Feldman C, et al. Effects of cigarette smoke condensate on pneumococcal biofilm formation and pneumolysin. European Respiratory Journal. 2013;41(2):392-5.
3. Hodge S, Hodge G, Ahern J, Jersmann H, Holmes M, Reynolds PN. Smoking Alters Alveolar Macrophage Recognition and Phagocytic Ability. American Journal of Respiratory Cell and Molecular Biology. 2007 2007/12/01;37(6):748-55.
4. Berenson CS, Kruzel RL, Eberhardt E, Sethi S. Phagocytic dysfunction of human alveolar macrophages and severity of chronic obstructive pulmonary disease. J Infect Dis. 2013;208(12):2036-45.
5. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. Int J Mol Sci. 2017;19(1):92.
6. Phipps JC, Aronoff DM, Curtis JL, Goel D, O'Brien E, Mancuso P. Cigarette smoke exposure impairs pulmonary bacterial clearance and alveolar macrophage complement-mediated phagocytosis of Streptococcus pneumoniae. Infect Immun. 2010 Mar;78(3):1214-20.
7. Knapp S, Leemans JC, Florquin S, Branger J, Maris NA, Pater J, et al. Alveolar macrophages have a protective antiinflammatory role during murine pneumococcal pneumonia. Am J Respir Crit Care Med. 2003 Jan 15;167(2):171-9.
8. Hodge S, Reynolds PN. Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects. Respirology. 2012 Jul;17(5):802-7.
9. Hodge S, Hodge G, Scicchitano R, Reynolds PN, Holmes M. Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. Immunology and cell biology. 2003 Aug;81(4):289-96.
10. Ma J, Becker C, Lowell CA, Underhill DM. Dectin-1-triggered recruitment of light chain 3 protein to phagosomes facilitates major histocompatibility complex class II presentation of fungal-derived antigens. J Biol Chem. 2012 Oct 5;287(41):34149-56.
11. Schille S, Crauwels P, Bohn R, Bagola K, Walther P, van Zandbergen G. LC3-associated phagocytosis in microbial pathogenesis. International Journal of Medical Microbiology. [Review]. 2018;308(1):228-36.
12. Wong SW, Sil P, Martinez J. Rubicon: LC3-associated phagocytosis and beyond. FEBS Journal. [Review]. 2018;285(8):1379-88.
13. Sanjuan MA, Milasta S, Green DR. Toll-like receptor signaling in the lysosomal pathways. Immunological Reviews. 2009;227(1):203-20.
14. Inomata M, Xu S, Chandra P, Meydani SN, Takemura G, Philips JA, et al. Macrophage LC3-associated phagocytosis is an immune defense against Streptococcus pneumoniae that diminishes with host aging. Proceedings of the National Academy of Sciences. 2020;117(52):33561-9.
15. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. Nature. 2016 May 5;533(7601):115-9.
16. Martinez J, Malireddi RKS, Lu Q, Cunha LD, Pelletier S, Gingras S, et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nature Cell Biology. [Article]. 2015;17(7):893-906.
17. Yang C-S, Rodgers M, Min C-K, Lee J-S, Kingeter L, Lee J-Y, et al. The autophagy regulator Rubicon is a feedback inhibitor of CARD9-mediated host innate immunity. Cell host & microbe. 2012;11(3):277-89.

18. Asare PF HBT, Plinio R. Hurtado, Griffith B Perkins, Phan Nguyen, Hubertus Jersmann, Eugene Roscioli, Sandra Hodge, . Inhibition of LC3-associated phagocytosis (LAP) in COPD and in response to cigarette smoke. Therapeutic Advances in Respiratory Disease. 2021;In press.
19. Bouquet J, Tabor DE, Silver JS, Nair V, Tovchigrechko A, Griffin MP, et al. Microbial burden and viral exacerbations in a longitudinal multicenter COPD cohort. Respiratory Research. 2020 2020/03/30;21(1):77.
20. Hodge S, Matthews G, Mukaro V, Ahern J, Shivam A, Hodge G, et al. Cigarette smoke-induced changes to alveolar macrophage phenotype and function are improved by treatment with procysteine. American journal of respiratory cell and molecular biology. 2011 May;44(5):673-81.
21. Hamon R, Tran HB, Roscioli E, Ween M, Jersmann H, Hodge S. Bushfire smoke is pro-inflammatory and suppresses macrophage phagocytic function. Scientific reports. 2018;8(1):13424-.
22. Dehle FC, Mukaro VR, Jurisevic C, Moffat D, Ahern J, Hodge G, 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(4):e61573-e.
23. Hodge SJ, Hodge GL, Holmes M, Reynolds PN. Flow cytometric characterization of cell populations in bronchoalveolar lavage and bronchial brushings from patients with chronic obstructive pulmonary disease. Cytometry Part B, Clinical cytometry. 2004 Sep;61(1):27-34.
24. Tran HB, Barnawi J, Ween M, Hamon R, Roscioli E, Hodge G, et al. Cigarette smoke inhibits efferocytosis via deregulation of sphingosine kinase signaling: reversal with exogenous S1P and the S1P analogue FTY720. Journal of Leukocyte Biology. 2016;100(1):195-202.
25. Kao S-H, Wang W-L, Chen C-Y, Chang Y-L, Wu Y-Y, Wang Y-T, et al. Analysis of Protein Stability by the Cycloheximide Chase Assay. Bio Protoc. 2015;5(1):e1374.
26. Qi X, Man SM, Malireddi RKS, Karki R, Lupfer C, Gurung P, et al. Cathepsin B modulates lysosomal biogenesis and host defense against Francisella novicida infection. J Exp Med. 2016;213(10):2081-97.
27. Giusti I, D'Ascenzo S, Millimaggi D, Taraboletti G, Carta G, Franceschini N, et al. Cathepsin B mediates the pH-dependent proinvasive activity of tumor-shed microvesicles. Neoplasia (New York, NY). 2008;10(5):481-8.
28. Linebaugh BE, Sameni M, Day NA, Sloane BF, Keppler D. Exocytosis of active cathepsin B enzyme activity at pH 7.0, inhibition and molecular mass. European journal of biochemistry. 1999 Aug;264(1):100-9.
29. Yang L, Mei Y, Fang Q, Wang J, Yan Z, Song Q, et al. Identification and characterization of serine protease inhibitors in a parasitic wasp, Pteromalus puparum. Scientific Reports. 2017 2017/11/16;7(1):15755.
30. Atack JM, Murphy TF, Pettigrew MM, Seib KL, Jennings MP. Non-typeable Haemophilus influenzae isolates from patients with chronic obstructive pulmonary disease contain new phase-variable modA methyltransferase alleles controlling phasevarions. Scientific Reports. 2019 2019/11/04;9(1):15963.
31. Masud S, van der Burg L, Storm L, Prajsnar TK, Meijer AH. Rubicon-Dependent Lc3 Recruitment to Salmonella-Containing Phagosomes Is a Host Defense Mechanism Triggered Independently From Major Bacterial Virulence Factors. Frontiers in Cellular and Infection Microbiology. [Original Research]. 2019 2019-August-02;9(279).
32. Panda K, Chattopadhyay R, Chattopadhyay D, Chatterjee IB. Cigarette smoke-induced protein oxidation and proteolysis is exclusively caused by its tar phase: prevention by vitamin C. Toxicology letters. 2001 Aug 6;123(1):21-32.

33. Pandey KC, De S, Mishra PK. Role of Proteases in Chronic Obstructive Pulmonary Disease. Frontiers in Pharmacology. [Review]. 2017 2017-August-08;8(512).
34. Ghosh A, Coakley RD, Ghio AJ, Muhlebach MS, Esther CR, Jr., Alexis NE, et al. Chronic E-Cigarette Use Increases Neutrophil Elastase and Matrix Metalloprotease Levels in the Lung. American journal of respiratory and critical care medicine. 2019;200(11):1392-401.
35. Vlahos R, Bozinovski S, Jones JE, Powell J, Gras J, Lilja A, et al. Differential protease, innate immunity, and NF-κB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2006;290(5):L931-L45.
36. Andrault P-M, Schamberger AC, Chazeirat T, Sizaret D, Renault J, Staab-Weijnitz CA, et al. Cigarette smoke induces overexpression of active human cathepsin S in lungs from current smokers with or without COPD. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2019;317(5):L625-L38.
37. Lesser M, Padilla ML, Cardozo C. Induction of emphysema in hamsters by intratracheal instillation of cathepsin B. The American review of respiratory disease. 1992 Mar;145(3):661-8.
38. McGlinchey RP, Lee JC. Cysteine cathepsins are essential in lysosomal degradation of α-synuclein. Proceedings of the National Academy of Sciences. 2015;112(30):9322-7.
39. Uemasu K, Tanabe N, Tanimura K, Hasegawa K, Mizutani T, Hamakawa Y, et al. Serine Protease Imbalance in the Small Airways and Development of Centrilobular Emphysema in Chronic Obstructive Pulmonary Disease. American journal of respiratory cell and molecular biology. 2020 2020/07/01;63(1):67-78.
40. Tanabe N, Sato A, Mizutani T, Hamakawa Y, Uemasu K, Sato S, et al. Protease anti-protease imbalance and small airways disease in COPD. European Respiratory Journal. 2018;52(suppl 62):PA4256.
41. Numanami H, Koyama S, Nelson D, Hoyt J, Freels JL, Habib M, et al. Serine protease inhibitors modulate smoke-induced chemokine release from human lung fibroblasts. American journal of respiratory cell and molecular biology. 2003;29 5:613-9.
42. Barnes PJ. Mediators of Chronic Obstructive Pulmonary Disease. Pharmacological Reviews. 2004;56(4):515.
43. Kono Y, Colley T, To M, Papaioannou AI, Mercado N, Baker JR, et al. Cigarette smoke-induced impairment of autophagy in macrophages increases galectin-8 and inflammation. Scientific Reports. 2021 2021/01/11;11(1):335.
44. Vij N, Chandramani-Shivalingappa P, Van Westphal C, Hole R, Bodas M. Cigarette smoke-induced autophagy impairment accelerates lung aging, COPD-emphysema exacerbations and pathogenesis. American journal of physiology Cell physiology. 2018 Jan 1;314(1):C73-c87.
45. Eskelinen E-L, Saftig P. Autophagy: A lysosomal degradation pathway with a central role in health and disease. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2009 2009/04/01/;1793(4):664-73.
46. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford JC, et al. LC3-Associated Phagocytosis in Myeloid Cells Promotes Tumor Immune Tolerance. Cell. 2018 Oct 4;175(2):429-41.e16.
47. Asare PF, Roscioli E, Hurtado PR, Tran HB, Mah CY, Hodge S. LC3-Associated Phagocytosis (LAP): A Potentially Influential Mediator of Efferocytosis-Related Tumor Progression and Aggressiveness. Front Oncol. [Review]. 2020 2020-August-05;10(1298).
48. Koga H, Martinez-Vicente M, Macian F, Verkhusha VV, Cuervo AM. A photoconvertible fluorescent reporter to track chaperone-mediated autophagy. Nature Communications. 2011 2011/07/12;2(1):386.

Chapter 4

4. LC3-associated phagocytosis (LAP): a potentially influential mediator of efferocytosis-related tumour progression and aggressiveness

All of the work presented in this chapter was published in *'frontiers in Oncology'* as Patrick F Asare, Eugene Roscioli, Plinio R Hurtado, Hai Bac Tran, Chu Yan Mah, Sandra Hodge, 2020. The complete publication can be found in appendix B of this thesis.

Statement of Authorship

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Overall percentage (%)	85%		
Certification:	This paper reports on review research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Abstract

One aim of cancer therapies is to induce apoptosis of tumour cells. Efficient removal of the apoptotic cells requires coordinated efforts between the processes of efferocytosis and LC3-associated phagocytosis (LAP). However, this activity has also been shown to produce anti-inflammatory and immunosuppressive signals that can be utilised by live tumour cells to evade immune defence mechanisms, resulting in tumour progression and aggressiveness. In the absence of LAP, mice exhibit suppressed tumour growth during efferocytosis, while LAP-sufficient mice show enhanced tumour progression. Little is known about how LAP or its regulators directly affect efferocytosis, tumour growth and treatment responses, and identifying the mechanisms involved has the potential to lead to the discovery of novel approaches to target cancer cells. Also incompletely understood is the direct effect of apoptotic cancer cells on LAP. This is particularly important as induction of apoptosis by current cytotoxic cancer therapies can potentially stimulate LAP following efferocytosis. Herein, we highlight the current understanding of the role of LAP and its relationship with efferocytosis in the tumour microenvironment with a view to presenting novel therapeutic strategies.

Keywords: Efferocytosis; Tumour cell apoptosis; LAP; Tumour immune response; M2 macrophage activation

4.1. Introduction

Induction of apoptotic cell death in tumours serves to promote the body's main defence against cancer. Therapeutic intervention that promotes the apoptotic death of tumour cells restricts their growth, proliferation and survival and is a frontline strategy in anti-tumour therapy. However, apoptotic cells can directly (and indirectly) influence tumour progression and survival. Studies suggest that these effects are due to cellular events that are called in to remove dead or dying cells from the tumour microenvironment. A significant consequence of these events is the production of anti-inflammatory and immunosuppressive signals that then can facilitate the growth and progression of tumour cells [1, 2]. Therefore, a better understanding of the processes that lead to the clearance of apoptotic cells may provide critical clues to help prevent the potential pro-tumour actions of dead or dying cells. Here we discuss two such cellular events; efferocytosis and LC3 Associated Phagocytosis (LAP).

Efferocytosis is a term coined by Peter Henson and colleagues in 2003 to describe the engulfment and clearance of cells undergoing apoptosis by phagocytes [3]. Defects in efferocytosis can result in the rupture of uncleared apoptotic bodies and the subsequent leakage of cytosolic contents onto adjacent cells and exposure of tissues to oxidants, harmful enzymes and factors including caspases and other proteases [4, 5]. This can induce inflammation and tissue damage through a process referred to as secondary necrosis [6, 7]. Less understood is that during efferocytosis, signals from neighbouring cells can initiate particular components of canonical autophagy to engage with the phagosome to increase the efficacy of the digestion of engulfed cellular cargo [8, 9]. When components of autophagy are recruited to cargo-containing single membrane phagosomes to form LAPosome for digestion and clearance, the process is called LAP. Hence, LAP is a form of non-canonical autophagy that represents a hybrid between the processes governing the autophagic clearance of intracellular factors and those that accept extracellular cargos for degradation.

As the name suggests, a distinguishing feature of LAP is the recruitment of microtubule-associated protein light chain 3 (LC3) to the phagosome membrane after internalisation of apoptotic cells or pathogens [10]. In the normal situation, LAP is vital to resolve inflammation, promote wound healing and prevent auto-immunity and immune-mediated tissue damages [11, 12]. Reports by Martinez et al show that dysregulation of LAP impairs efferocytosis, culminating in necrosis of the phagocyte that has engulfed the uncleared cell and uncontrolled inflammation [12]. These findings suggest that LAP may be essential for efferocytosis and preventing the many deleterious consequences of secondary necrosis. In support of this, Cunha

and colleagues demonstrated that defects in LAP restricts tumour immunosuppression afforded by efferocytosis [13]. Hence, these studies provide evidence that LAP may also be an influential contributor to the production of tolerogenic signals that are activated upon engulfment of apoptotic cells during efferocytosis. Martinez et al further showed that in the absence of LAP, phagocytosis of apoptotic bodies still occurs but the vesicles remain in the phagocyte for a prolonged duration or are not digested, and points to a critical role of LAP in finalising the intracellular digestion process [12]. Hence, in the context of cancer, defects in LAP have paradoxically beneficial effects including the activation of tumour infiltrating lymphocytes (TIL), enhanced effector T cells activity and elevated secretion of inflammatory mediators including stimulator of interferon genes (STING), type I interferons (IFNs) and tumour necrosis factor alpha (TNF-α) [13]. Taken together, these data suggest that, 1) LAP is critical for governing the digestion of the engulfed cellular debris and prevention of inflammatory and immune responses, 2) internalisation of dead or dying cells alone is not solely responsible for immunosuppressive activities in the context of clearing apoptotic cells, and 3) LAP activation plays a critical role in immune tolerance. These recent findings of LAP as a concomitant effector of efferocytosis brings new significance to the processes that govern the clearance of apoptotic cells in the tumour microenvironment (TME), where there is frequent instances of programmed cell death. To that end, we discuss recent findings for the LAP signalling network as another mechanism that is hijacked by tumour cells to promote their growth and aggressiveness.

4.2. Overview and molecular mechanism of LAP during phagocytosis of apoptotic cells

Apoptotic cells are cleared in a tightly controlled and coordinated manner, to avoid damaging healthy tissues. The removal processes can be partitioned into four functional stages: 1) The release of chemoattractant ‘find me’ signals [14] and ‘good-bye’ metabolites from cells undergoing programmed cell death. ‘Find me’ signals such as nucleotides (adenosine triphosphate; ATP and uridine triphosphate; UTP), sphingosine-1-phosphate (S1P), CX3C motif chemokine ligand 1 (CX3CL1 or fractalkine) and lysophosphatidylcholine (LPC) and ‘good-bye’ metabolites including glycerol-3-phosphate (G3P), guanosine 5′-monophosphate (GMP), spermidine, adenosine monophosphate (AMP) and creatine [14] are released as demonstrated in **Figure 1a**. This secretory activity promotes the recruitment of phagocytes or neighbouring cells to the area of cell death in the tissue compartment. 2) The expression of ‘eat me’ signals on the surface of apoptotic cells regulates the recognition by the phagocyte, subsequent engulfment of the cellular debris and preparatory steps in the mechanisms

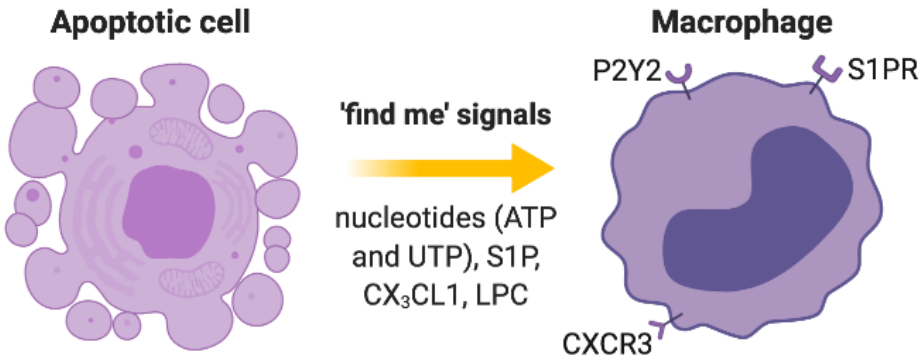
governing phagosome formation (**Figure 1b**). 3) Recruitment of the LC3 conjugation system (**Figure 1c**) to the phagosome including autophagy related proteins and factors (Atg)12-Atg5-Atg16L1, Atg3, Atg7, NADPH oxidase 2 (NOX2), reactive oxygen species (ROS), Phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3 complex) comprising Beclin-1, Vacuolar protein sorting 34 (VPS34) and UV radiation resistance associated (UVRAG) and Rubicon (RUN domain Beclin-1-interacting and cysteine-rich domain-containing protein) for LAPosome formation (**Figure 2**) where the engulfed cellular debris can be digested. 4) Post-digestion activities (**Figure 1c**) including the release of inflammation-resolving cytokines, immune tolerogenic signals and exportation of breakdown products into the cytosol for metabolic recycling that service intracellular biosynthetic pathways [15-18]. This previously under-recognised phenomenon for efferocytosis; localisation of LC3 to phagosome, is now understood to be an essential step for lysosomal trafficking and selective immunologically silent removal of apoptotic cells [9, 12].

Finite steps involved in LAP are now being identified (**Figures 1b and 2**). LAP is initiated upon engulfment of apoptotic cells that have externalised eat me signals such as phosphatidylserine (PtdSer) which binds to T-cell immunoglobulin and mucin domain family of receptors (TIM); TIM-1, TIM-3, and TIM-4 or stabilin-1, stabilin-2 and the GPCR brain angiogenesis inhibitor 1 (BAI1). Alternatively, phagocytes can employ tyrosine kinase receptors (TYRO3, AXL and Mer) also known as TAM receptors to bind to PtdSer indirectly through bridging molecules such as Gas-6 and Protein S. Interaction via these receptors stimulates the CRKII-ELMO-DOCK180 complex within phagocytes to activate the rac-1 signalling pathway (**Figure 1b**) [19-22]. This leads to cytoskeletal rearrangement and internalisation of the apoptotic cell resulting in phagosome formation. Once internalised, the phagosome recruits Rubicon which facilitates the activity of a Class III PI3K complex containing UVRAG, but which lacks Atg14 and Ambra 1 (used in canonical autophagy) [23]. Consequently, phosphatidylinositol 3-phosphate (PI3P) is generated on the fully formed and sealed phagosome during LAP [10, 24]. The timing of PI3P generation during LAP is different from that of canonical autophagy where PI3P is generated in some portions of autophagophore for closure and autophagosome cup formation [25, 26]. This process is necessary to stabilize the NOX2 complex, and thereby sustains the production of ROS which is crucial for recruiting the LC3 conjugation system components such as Atg5, Atg3, Atg12, Atg7 and Atg16. This leads to LC3 lipidation and its localisation to the phagosome, as a necessary prerequisite for the formation of the LAPosome and to promote the fusion of lysosomes (**Figure 2**). The

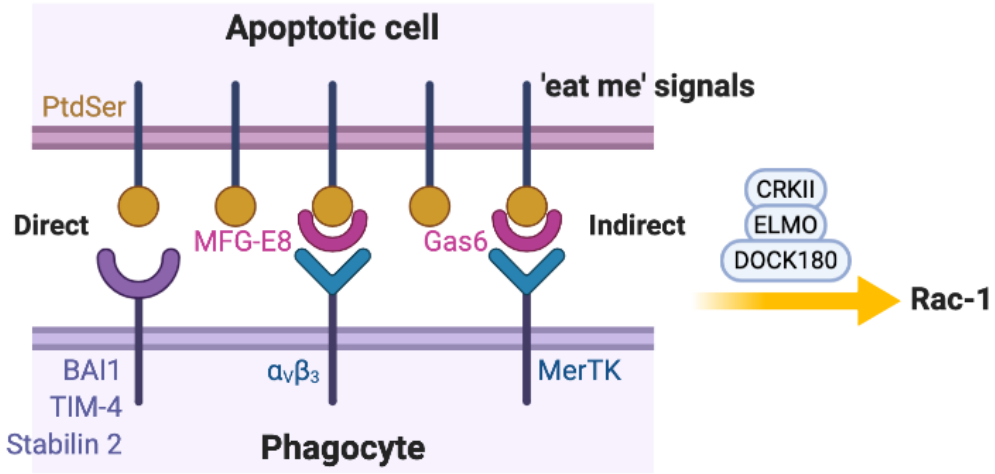
phagosomal content can then be efficiently processed via enzymatic digestion, and hence the immune response is better regulated to protect against autoimmunity and inflammation.

Rubicon plays a critical role as a mediator of LAP. Rubicon (via its binding to NOX2) favours the initiation of LAP-associated non-canonical autophagic activities over canonical autophagy [27], by reducing VPS34 lipid kinase activity, which also promotes the production of PI3P on LAPosomes rather than autophagosome [28-30]. Hence, further requisites for LAP is NOX2 and the production of ROS. The NOX2 subunit p40phox binds to PI3P, effectively sequestering this factor as a consequence of Rubicon activity. If ROS production is dysregulated and p40phox subunit of NOX2 fails to associate with LAPosome, leading to LAP impairment [23]. Hence, Rubicon-dependent production of PI3P is integral for these processes. Further to this, Yang et al recently showed that Rubicon directly associates with the p22phox and gp91phox subunits of NOX2 to stabilize the complex for optimum and continuous production of ROS [31]. Cells or mice altered to lack Rubicon exhibit a destabilised NOX2 which thereby inhibits ROS necessary for LAP. The consequent of this is inefficient recruitment of essential LAP-related factors including ATG5, ATG7 and LC3-II to the membrane of phagosome. Moreover, cells deficient in NOX2 exhibit LAPosomes that incorporate PI3P, and exogenous induction of superoxides that serve to enhance LC3-II recruitment that initiates canonical autophagy instead of LAP [23, 30, 32]. Hence, the proper regulation and expression of Rubicon is an integral part of LAP (i.e. PI3P produced via Rubicon-dependent pathways), and the interaction of Rubicon with NOX2 is a necessary event for this mode of non-canonical autophagy. As a result, Rubicon represents a new and attractive therapeutic target to specifically modulate LAP (vs canonical autophagy) in diseases such as cancer. Considering that Rubicon and/or LAP forms a critical component of efferocytosis, we next highlight correlative studies between tumour progression and efferocytosis.

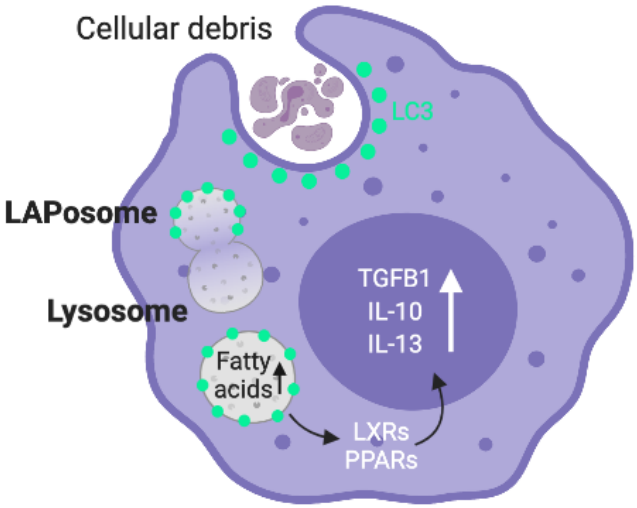
a. FIND ME



b. EAT ME



c. Post-Engulfment



Efferocytosis
Wound Healing
M2-like Macrophages
Immunosuppressive Cytokines

Figure 1: Clearance processes for apoptotic cell by phagocytes rely on 'find me' and 'eat me' signals that signal their internalisation. (A) 'Find me' signal: apoptotic cells release signals that attract phagocytes to the site of programmed cell death. These signals include nucleotides (UTP and ATP), S1P, CX3CL1 and LPC. Phagocytes recognise the "find me" signals using cognate receptors such as P2Y2, S1PRs, CXCR3 and G2A. **(B)** 'Eat me' signal: the apoptotic cells express 'eat me' signals that allow phagocytes to recruit surface receptors and bridging molecules to identify and engulf apoptotic cells. PtdSer is a primary 'eat me' signal expressed by phagocytes and is directly recognised by phagocytic receptors including BAI, TIM4 and stabilin 2. Phagocytes can employ avB3 and tyrosine kinase receptor such as Mertk bind to PtdSer indirectly through bridging molecules such as MFG-E8 and Gas-6 respectively. **(C)** The engulfment process: after recruitment of engulfment receptors via the CRKII-ELMO-DOCK180 complex within phagocytes, rac-1 signalling pathway is activated for phagosome formation. Once the phagosome is formed, LC3 is recruited to phagosomes (now the LAPosome) leading to lysosome-mediated digestion of the internalised apoptotic body. Degraded products release fatty acids that stimulate LXR and PPARγ for cholesterol efflux leading to the production of anti-inflammatory cytokines such as TGF-β, IL-10 and IL-13. Abbreviations: UTP, uridine 5' triphosphate; ATP, adenosine 5' triphosphate; S1P, sphingosine-1-phosphate; CX3CL1, C-X3-C Motif Chemokine Ligand 1; LPC, lysophosphatidylcholine; P2Y2, purinergic receptors; SIPRs, sphingosine-1-phosphate receptors; CXCR3, C-X-C motif chemokine receptor 3; G2A, G-protein-coupled receptor; PtdSer, phosphatidylserine; BAI, brain-specific angiogenesis inhibitor 1; TIM-4, T cell immunoglobulin mucin receptor-4; avB3, alpha-v beta-3; mertk, mer proto-oncogene, tyrosine kinase; MFG-E8, milk fat globule-EGF factor 8 protein; Gas-6, growth arrest-specific 6; ELMO, engulfment and cell motility protein; DOCK, dedicator of cytokinesis; CRKII, chicken tumour virus no. 10 (CT10) regulator of kinase II; G3P, glycerol-3-phosphate guanosine; GMP, 5′-monophosphate, AMP, adenosine monophosphate; LC3, microtubule-associated protein 1A/1B-light chain 3; LXR, liver X receptor; PPARγ, peroxisome proliferator-activated receptor gamma; TGF-β, transforming growth beta; IL-10, interleukin 10; IL-13, interleukin-13.

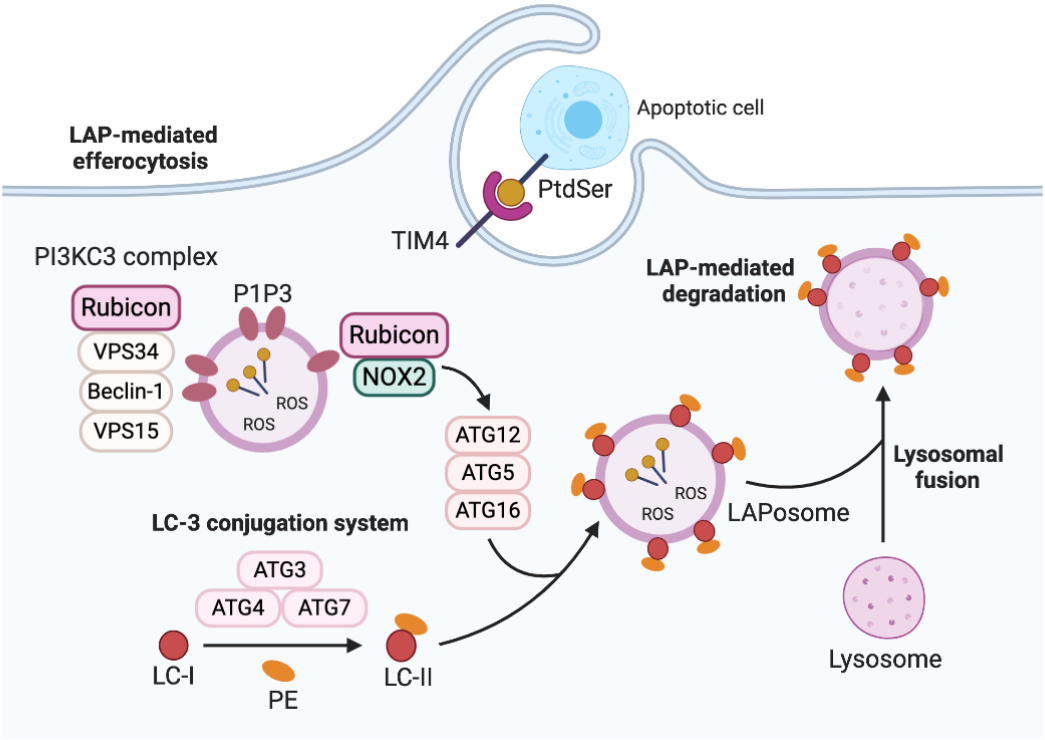


Figure 2: LC3-Associated phagocytosis (LAP). LAP recruitment process is triggered for degradation of the phagocytosed cargo following phagosome formation. The cargo is recognised by cell recognition receptors such as TIM-4 which causes the cargo to be engulfed in a single membrane phagosome called LAPosome. This process is initiated by recruitment of PI3KC3 complex consisting of Rubicon, vps34, beclin-1 and vps15 which enable PI3P to be localized to the LAPosomes. This stabilises the NOX2 complex to produce ROS which is necessary to recruit LC3-II to the phagosome (LAPosome). The LAPosome then fuses with lysosomes to mature and to effective e digest the cargo. Abbreviations: TIM-4, T-cell immunoglobulin and mucin domain family of receptors; Phosphatidylinositol 3-kinase catalytic subunit type 3; Rubicon, Run domain beclin-1-interacting and cysteine-rich Domain-containing protein; VPS34, Vacuolar protein sorting 34 ; VPS15; vacuolar protein sorting 15; PI3P, phosphatidylinositol 3-phosphate, NOX2; NADPH oxidase 2; ROS, reactive oxygen species, LC3-II, microtubule-associated protein 1A/1B-light chain 3-II. "created with bioreneder".

4.3. Efferocytosis in the TME promotes tumour progression and metastatic potential

Women diagnosed with breast cancer within 5 years of postpartum (which is characterised by massive cell death) have higher mortality rates, compared with nulliparous women (where cell

death is relatively low) diagnosed with breast cancer [33-35]. Additionally, breast cancer cells transplanted into involuting postpartum mammary glands grow and invade more rapidly than do cells in the mammary glands of a nulliparous host, even when corrected for age and histological grade [36, 37]. This raises the possibility that conditions in the postpartum breast increase the aggressiveness of established tumours. Recently, massive cell death and the subsequent efferocytosis in postpartum involution have been identified as factors that are associated with the pro-tumoral and metastatic features of breast cancers in parous women [38]. This is based on the observation that increased levels of efferocytosis occurs during postpartum involution and facilitates influx of wound-healing macrophages that are supportive of tumour progression and metastasis [38, 39].

Postpartum involution links the growth and metastasis of tumours to efferocytosis [38]. This is consistent with the observation that efferocytosis receptors TIM-4 and MerTK are elevated in various cancer cells which is reported to correlate with disease severity [40, 41]. In addition, a scenario in which cancer cells express MFG-E8 [42], NOX2 [43], PtdSer [21], in proximity to M2 macrophages [44] correlates with tumour aggressiveness. These observations suggest that phagocyte-homing in response to debris generated by tumour cells contributes to tumour-cell proliferation and may be heightened by the cytotoxic effects of anti-tumour therapies [45]. Further, the promotion of tumour cell death by radiotherapy and chemotherapy can also lead to increased expression of efferocytic receptors such as Mertk [46] and TIM-4 [47]. These findings suggest that targeting efferocytosis activity in the surrounding tumour microenvironment may mitigate this phenomenon and heighten the efficacy of conventional anti-tumour therapies.

Efferocytosis of dead or dying tumour cells is a rapid process involving engulfment receptors required for efficient clearance of the cellular debris. Several studies have shown that blocking the incorporation of apoptotic cells by inhibiting engulfment receptors such as TIM-4, MFG-E8, MerTK and its ligands including Gas6 and protein S, is an effective strategy for the treatment of tumours [42, 48-50]. An emerging area of study is the identification of targets critical for the degradation process of engulfed cargoes during efferocytosis. Internalisation of apoptotic tumour cells per se, of course, does not influence tumour progression. Immune tolerogenic signals released by the digested apoptotic cells also contribute to a mechanism whereby tumours are able to evade clearance by lymphocytes and monocytes [13]. LAP is a key mechanism involved in the final stages of clearing apoptotic tumour cells [13]. LAP activity in dendritic cells and macrophages effectively clears cellular debris leading to the

secretion of anti-inflammatory and immunosuppressive mediators [12, 51]. Moreover, elevated expression of efferocytosis receptors by tumour-derived apoptotic cells, in the absence of LAP, can enhance tumour-progression. Hence, a more complete understanding of LAP in the phagocytes that home to the tumour microenvironment may offer new strategies for the management of cancer

4.4. The role of LAP in tumour progression

During efferocytosis, LAP facilitates the fusion of phagosome to lysosomes to enhance hydrolytic digestion and elimination of apoptotic cell constituents [23]. Once apoptotic cells are digested in phagolysosomes, phagocytes become burdened with macromolecular digest components that they then either use in biosynthetic processes or efflux to the extracellular environment. An overload of cholesterol from degraded apoptotic cells stimulates members of the peroxisome proliferator-activated receptor gamma (PPARγ) and liver X receptor (LXR) families of nuclear receptors as illustrated in **Figure 1** . These nuclear receptors inhibit pro-inflammatory cytokines and drive the polarisation of M2-like phenotype which can mediate the production of anti-inflammatory cytokines. This is one important mechanism that reduces inflammatory signals following the removal apoptotic cells [52, 53], with immunogenic consequences including the suppression of tumour immune responses and increased resistance to immunotherapies.

LAP activity in dendritic cells and macrophages enhances phagocytic removal of pathogens and cells undergoing programmed cell death [9, 51]. This is evidenced in LAP-deficient dendritic cells that exhibit impaired efferocytosis and elevated expression of major histocompatibility complex class I [51] that is needed for antigen presentation and anti-tumour immunity [51, 54]. Moreover, macrophages and mice altered to obviate LAP, internalise and accumulate undigested apoptotic cells. These cells exhibit STING-dependent IFN responses, M1 polarisation, pro-inflammatory mediators, granzyme B and enhanced anti-tumour immune response [13, 26, 55, 56]. Further, DNA from apoptotic cells can activate STING and mediate immune recognition of tumour cells [57] which can also lead to interferonopathy and autoimmunity [58]. This suggests that while LAP-dependent degradation of the DNA of engulfed apoptotic tumour cells counters the induction of inflammation and autoimmunity, it can also suppress anti-tumour immune responses. Hence, it follows that internalisation of apoptotic material per se, does not lead to M2 polarisation and immunosuppression. Rather it is the digested and degraded products of apoptotic cells that induce immune tolerogenic signals utilised by tumour cells to promote their growth and progression during efferocytosis. As

illustrated in **Figure 3**, LAP facilitates digestion and removal of apoptotic tumour cells upon phagosomal engulfment that has unfortunate consequence of switching anti-tumour M1 macrophage phenotype to a pro-tumour M2 phenotype, leading to tumour progression and severity [13]. This indicates that LAP participates in the regulatory events that form a complex network of interactions that collectively enhance anti-inflammatory and pro-tumorigenic effects of apoptotic cells and efferocytosis. Therefore, targeting LAP provides the attractive possibility of attenuating tumour progression and potentially serves as a target that could be therapeutically modulated to help prevent tumour aggressiveness.

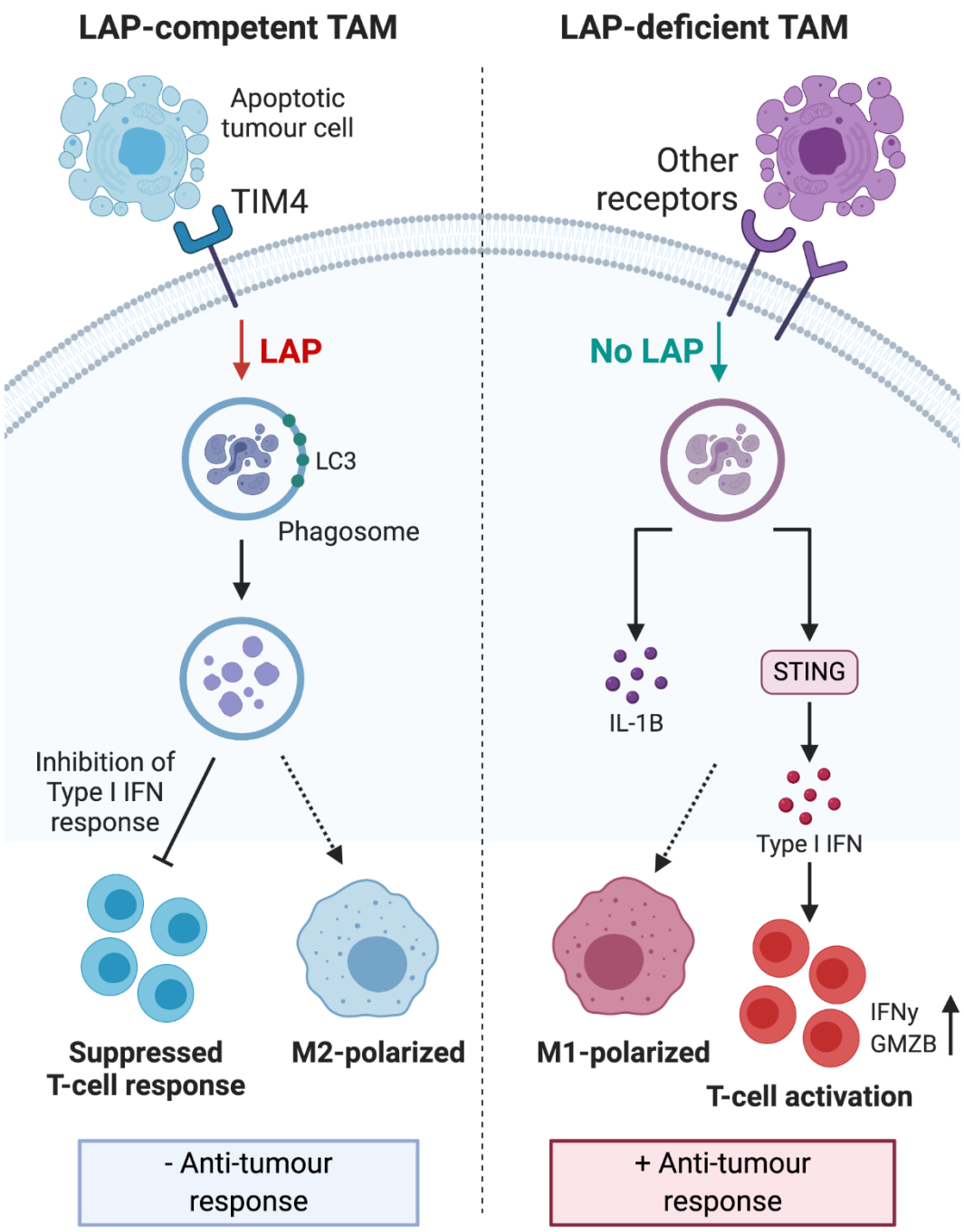


Figure 3: Engulfment of apoptotic tumour cells induces LAP to inhibit type I IFN response and polarise tumour associated macrophages toward the immunosuppressive M2 phenotype. This process leads to suppressed TIL function, reduced anti-tumour response and ultimately, sustained tumour survival. LAP deficiency leads to polarization of TAMs toward the pro-inflammatory M1 phenotype and stimulation of a STING-dependent type I IFN response, which enhances TIL function and expression of IFNγ to inhibit tumour growth. Taken together, these mechanisms suggest that targeting the LAP pathway may have

therapeutic potential. Abbreviations: IFN, interferon; TIL, tumour-infiltrating lymphocytes; STING, stimulator of interferon genes; IFNγ, interferon gamma.

4.5. LAP contributes to tumour progression by promoting M2 macrophage polarization

Macrophages are immune cells that are widespread throughout the body. They exhibit diverse functions including the regulation of inflammation, homeostasis and tumour immunity. They are generally categorised into classically activated or M1 macrophages and alternatively activated or M2 macrophages. M1 macrophages are activated by Th1 lymphocytes, interferon-γ (IFN-γ), TNF-α and natural killer cells (NK), while M2 macrophages are induced by cytokine signatures presented by Th2 cells such as interleukin-13 (IL-13), IL-10 and IL-4. Tumour-associated macrophages (TAMs) are usually triggered by factors that polarise macrophages to M2 phenotype and are often infiltrated into tumour sites to suppress the cytotoxic function of anti-tumour immune cells [44, 59, 60]. This leads to dysregulation of tumour immune response contributing to the immunosuppressive tumour microenvironment. Indeed, observations from in vitro studies and clinical findings from cancer patients show that increased TAM infiltration correlates with poor prognosis [44]. Accordingly, mitigating TAM infiltration, by blocking M2 activation or stimulating a pro-inflammatory M1 phenotype in TAMs, reduces tumour growth and metastasis [60-62].

Importantly, M2 macrophages promote tumour aggressiveness and progression. This phenomenon is exhibited through three mechanisms. Firstly, rapidly proliferating tumour cells that outpace the rate of blood supply need to establish new blood vessels through a process known as angiogenesis. M2 macrophages produce growth factors such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) to promote vascularization and allow highly proliferative tumours to obtain adequate blood supply [63-66]. This enhances metastatic spread as it provides the principal route by which tumour cells exit the primary tumour site and enter the circulation. Secondly, secretion of matrix metalloproteases by tumour-infiltrating M2 macrophages has been shown to contribute to the degradation of the Extra Cellular Matrix (ECM) which facilitates the invasion and spread of cancer cells to distant sites within the body and secondary metastasis [67]. This phenomenon has been effectively described in human tongue, squamous cell and colorectal cancer [68, 69]**.** Thirdly, the production of anti-inflammatory cytokines and chemokines can also shift the immune response towards an immunotolerogenic phenotype, which is unable to sustain cell-mediated tumour immunity [70-

72]. This antagonises activities of effector T cells and enhances tumour growth and survival, as they are spared from immune destruction. For these reasons, inhibition of M2 macrophages has shown therapeutic efficacy in the management of several tumours [60, 73, 74], and provides evidence that the characterisation of factors that fine-tune M2 macrophage polarisation can provide novel therapeutic options for promoting tumour immunity.

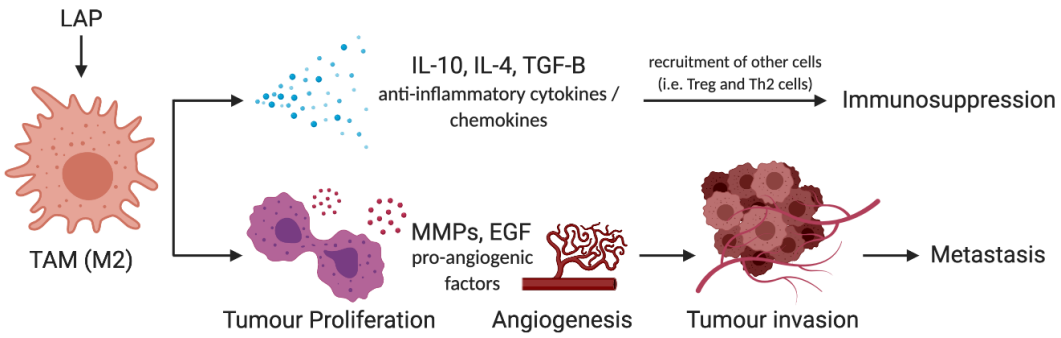


Figure 4: Schema of mechanism by which LAP could facilitate metastatic potential of tumours via M2 polarisation. M2 promotes tumour progression through IL-10, IL-4 and TGF-β-mediated immunosuppression. They also trigger pro-survival signals such as MMPs and EGF to amplify metastatic potential of tumours by facilitating the invasion and spread of cancer cells to distant sites within the body. Abbreviations: IL-10, interleukin-10; IL-4, interleukin-4; TGF-β, transforming growth factor-beta; MMP, matrix metallopeptidases; EGF, epidermal growth factor.

LAP has been identified as a new mechanism that participates in M2 polarisation, and promotes an immunosuppressive environment that favours the tumour growth [13, 62, 75]. Therefore, the involvement of LAP in macrophage function warrants further elucidation. This is evidenced by pro-tumour events that are established by LAP related to macrophage function. For example, 1) markers of tolerance and the polarisation of macrophages to M2 phenotype is observed in LAP-sufficient tumour animal models [13, 76-78], 2) the M2 phenotype correlates with poor prognosis and promotes tumour progression [2, 79], and 3) LAP-sufficiency in tumour mouse models has been shown to accumulate M2 macrophages that support the pro-tumorigenic effects of TAMs [13]. Therefore, M2 macrophage accumulation in tumours after chemotherapeutic treatment [80, 81] may be linked to LAP when it is called to enhance the efferocytosis of the apoptotic tumour cells. This causes the reduction of cytotoxic T cell activity

to limit tumour immune responses and enhance the cancer cell proliferation [13, 82]. Further, Cunha and colleagues showed that genetic or pharmacological inhibition of LAP overcomes some of the pro-tumour effects of TAMs e.g. by increasing CD8+ T cells function and reducing M2 macrophage production [13]. These observations point towards an unrecognised target to combat the progression of tumours.

4.6. Characterisation of LAP as a distinct pathway reveals a new therapeutic approach to control tumour growth

A current aim for cancer therapy is to target autophagy in the tumour microenvironment [83, 84]. For example, the translocation of LC3-II to membranous structures, considered to be a hallmark for autophagy, has been associated with tumour progression [85]. Further, inhibition of LC3 recruitment can potentiate anti-tumour immune response and reduce tumour growth [86, 87]. Recent discovery that LC3 is recruited to phagosome membranes via LAP provides evidence that at least some pro-tumour functions of LC3 recruitment may be specifically linked to LAP [10, 13]. Indeed, pro-tumoral qualities of some autophagy components have been associated with LC3 recruitment to phagosome membranes via LAP. Noteworthy, translocation of LC3 to phagosomes is a reliable marker for LAP, that distinguishes it from processes related to canonical autophagy [23]. Also, while LAP involves the recruitment of LC3 to single phagosome membranes [10, 88], autophagy utilises LC3-enriched double membrane autophagosomes [89]. Hence, delineating between these two processes can be used to identify unrecognised roles and contribution of LAP in tumour progression.

A critical distinction between LAP and autophagy is that while LAP requires Beclin-1, PI3KC3 complex, Atg5 and Atg7 for recruitment of LC3 to membranous structures, unlike autophagy, LAP does not require Unc51-like kinase 1 complex (ULK-1) or FAK family–interacting protein of 200 kDa (FIP200) [90-93]. Moreover, while both LAP and autophagy require mediators such as SLAM (signalling lymphocyte-activation molecule) receptors to interact with the PI3KC3 complex to regulate phagosome and autophagosome maturation respectively, unlike autophagy, LAP employs Rubicon-containing PI3KC3 and does not require Atg14 or ULK-1 to mediate LC3 recruitment process [23, 27]. In fact, inhibiting Rubicon specifically block LAP without inhibiting canonical autophagy process.

Moreover, even though VPS34 activity is required for both autophagy and LAP, VPS34 produced in the absence of Rubicon triggers autophagy instead of LAP [23, 32]. Conversely, Rubicon swaps VPS34 activity on autophagosome for LAPosme [23, 27, 94]. This again characterises Rubicon as an indispensable and specific component of LAP. These

characterisations of LAP as a distinct mechanism provides a new avenue to regulate tumour growth without interfering with canonical autophagy processes.

4.7. Targeting core components of LAP in tumours

Given that LAP elicits pro-tumour effects in the context of cancer, cancer cells expression of factors that lead to these phenomena may have significant therapeutic value. The expression of Rubicon is elevated in cancer and is strikingly associated with poor prognosis in patients with breast cancer, endometrial cancer, testicular cancer, liver cancer, colorectal cancer and stomach cancer as shown in **Figure 5** [95]. This clear link between the abundance of Rubicon and poor prognosis of cancer (**Figure 5**), calls for a better understanding of the events associated with the pro-tumour effects of Rubicon to inform prognostic and therapeutic investigations. What is certain is that Rubicon is associated with LAP and is a requisite member of the LAP pathway [28]. Further, Rubicon is upregulated following internalisation of apoptotic cells to recruit LC3-conjugation system for LAP initiation and subsequent degradation of apoptotic cells [23]. This supports the view that Rubicon expression forms a critical component of the processes required to remove apoptotic cells. In fact, inhibition of Rubicon in preclinical settings enhances immune activation and restricts tumour growth by ablating LAP-mediated apoptotic cell removal. Conversely, Rubicon expression increases tumour progression and limits immune responses through LAP induction [13]. This suggests that LAP may be an influential pathway through which Rubicon mediates tumour progression and decreases survival rate in some cancer patients.

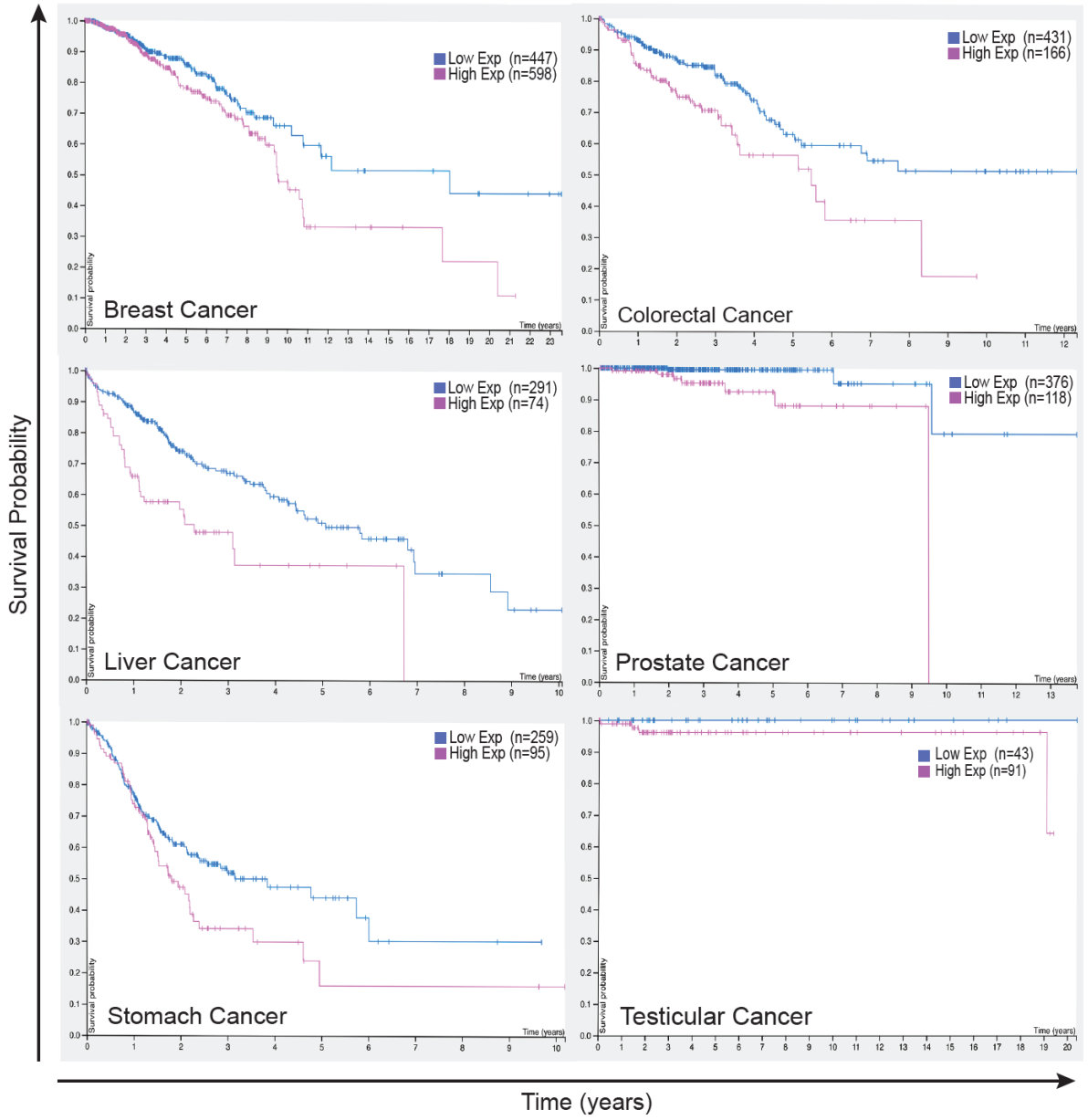


Figure 5: Rubicon expression as a potential prognostic marker that has the power to predict survival outcome of some cancer patients. Representative images of Rubicon expression and survival rate of patients with breast cancer, colorectal cancer, liver cancer, prostate cancer, stomach cancer and testicular cancer. Cancer patients overexpressing Rubicon have lower survival rate compared with patients with lower Rubicon expression. This demonstrates the need for further studies to establish prognostic values of Rubicon in different types of cancers. Credit: Human Protein Atlas, www.proteinatlas.org/humancell [95]. Image available at the following URL :v19.proteinatlas.org/humancell.

PtdSer is another integral component of LAP induction and efferocytosis [9, 96]. It is usually expressed on the surface of apoptotic cells to allow phagocytes to remove the dead or dying cells through efferocytosis and LAP [21, 97] It can be predicted that its inhibition may ablate LAP and therefore many pro-tumorigenic consequences as shown in **Figure 6**. For example, pre-clinical models of B16F10 melanoma demonstrates that inhibiting PtdSer receptor, TIM-4 is an effective approach to reduce LAP and tumour growth [13]. Indeed, expression of PtdSer on the surface of apoptotic cell is an early event during apoptosis and is markedly increased during chemotherapeutic treatment of tumours [98]. PtdSer is also overexpressed in different cancer types such as glioblastoma, breast cancer and astrocytoma [99, 100]. Its inhibition could lead to LAP impairment and subsequently result in a decrease in M2-like tumour associated macrophages and a switch in the expression of immunosuppressive cytokines to immunostimulatory cytokines [98]. Indeed, Bavituximab which blocks PtdSer is providing beneficial outcomes for some cancers in phase II clinical trials [101, 102].

Moreover, several clinical studies are in progress to design therapies that may have the potential to prevent the impact of LAP in the tumour microenvironment. These include Blocking TGF-β signalling to inhibit the growth and metastasis of orthotopic mammary carcinoma [103]. TGF-β is usually upregulated and is integral for tumour immunosuppressive effects of LAP [13, 26, 82], suggesting that targeting factors involved in the initiation of LAP as well as its resultant effects could be an effective strategy for tumour destruction.

IL-10, a further anti-inflammatory cytokine, is produced by both M2 macrophages [104] and by the tumour cells [105, 106]. IL-10 can promote tumour growth and the clinical utility of inhibiting this effect is widely reported [107-109]. However, the precise mechanism that informs IL-10 overexpression and its pro-tumoral qualities remains to be completely elucidated. Data from experiment settings link overexpression of IL-10 to the LAP pathway and implicate this association in LAP-mediated tumour progression [13]. This underscores the role of LAP plays in IL-10 expression and provides a potential target to regulating the pro-tumour cytokine in tumour cells. Noteworthy, other biological events may also contribute to IL-10 mediated tumour progression. A better understanding of LAP as an influential driver in this regard is crucial to discover a novel approach to block the immunosuppressive and pro-tumour effects of IL-10 for effective clinical management of tumours.

V-ATPase activity has recently been shown to be required for LAP, and inhibiting V-ATPase following apoptotic cell death prevents LAP by blocking lipidation of LC3 [110, 111]. This provides a further potentially novel therapeutic target for regulating LAP. Additionally, studies

by Fletcher et al suggest that WD repeat-containing C-terminal domain of ATG 16L1 inhibition arrests LAP [112]. Blunting WD repeat-containing C-terminal domain (WD 40 CTD) of ATG 16L1 blocks LC3 recruitment to endolysosomal membranes during LAP without affecting canonical autophagy [112, 113].

The mode of cell death has a significant impact on the immune tolerogenic effects of LAP. Non-apoptotic cell death such as necrotic and necroptotic cells can also induce LAP to complete the efferocytosis process [9]. In contrast to apoptotic cells, necrotic and necroptotic cells induce inflammatory and immunostimulatory cytokines [114, 115] and can switch macrophages from pro-tumoral M2 phenotype to anti-tumoral and immunostimulatory M1 phenotype [116, 117]. This is because the extra cholesterol produced after the degradation of necrotic cells does not stimulate cholesterol efflux pathway [118] which is required for the release of anti-inflammatory cytokines essential for M2 phenotype switching [119, 120]. This provides another promising approach for circumventing the pro-tumoral effects of LAP. Noteworthy, induction of non-apoptotic modalities of cell death such as necrosis or necroptosis as a new therapeutic direction to potentiate anti-tumour immunity has been suggested as a promising alternative [117, 121-123]. Therefore, further studies in this regard are warranted especially as the effectiveness of non-apoptotic form of tumour cell death has not been explicitly elucidated.

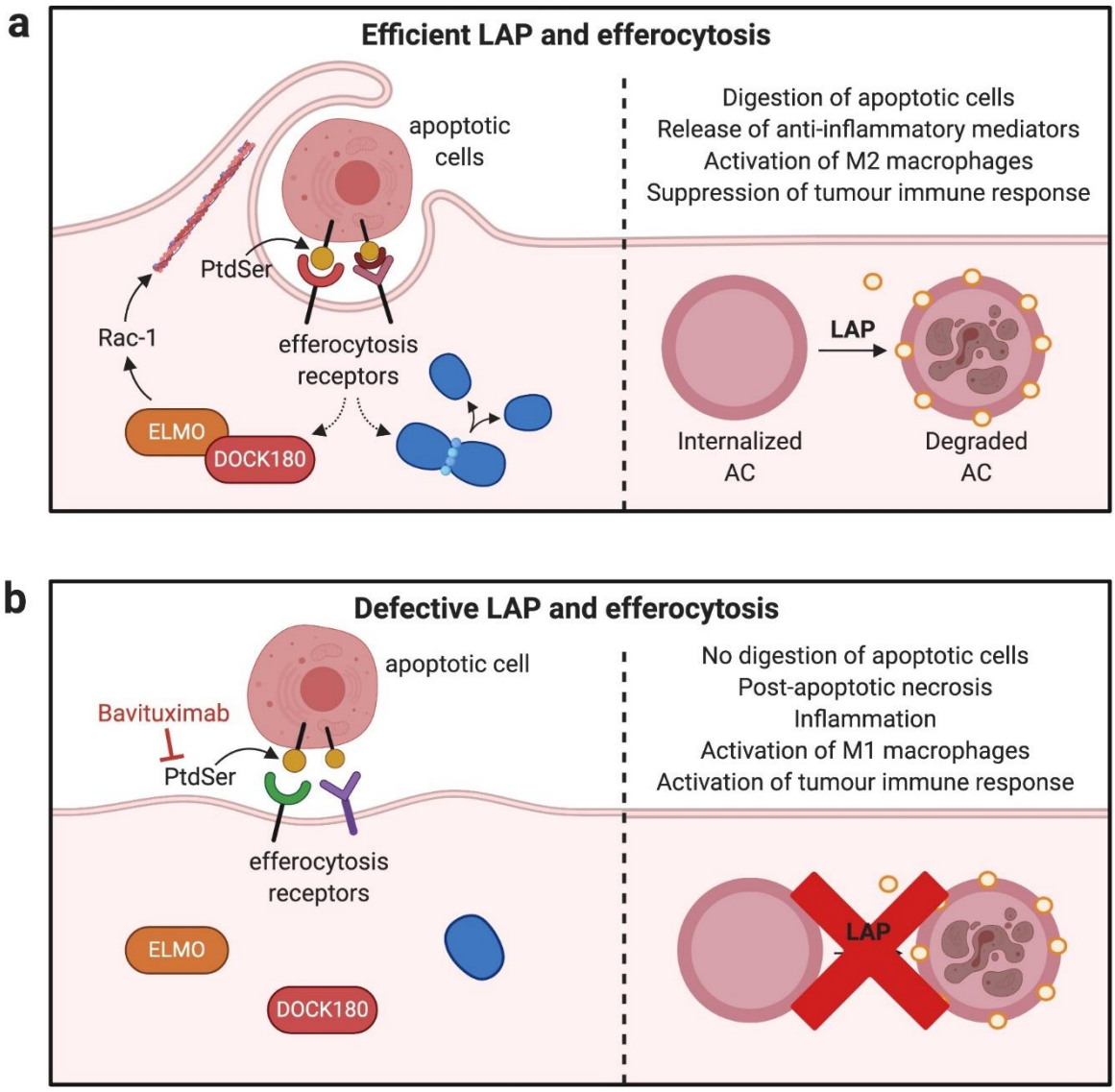


Figure 6: Consequences of inhibiting factors involved in efferocytosis and LAP. (A) Macrophages interact with PtdSer externalized on apoptotic cells through efferocytic receptors on the surface of phagocytes. Many PtdSer receptors stimulate interactions to activate Rac1 and cytoskeletal arrangement for phagosome formation and internalisation. Once apoptotic cells are internalised in the phagosome, LC3-recruitment process through LAP is triggered to fuse LC3 to phagosomes membranes, which facilitates phagolysosomal fusion and subsequent digestion apoptotic cell. This results in production of M2 macrophages and suppression of tumour immune activation. (B) Inhibition of PtdSer leads to defects in the phagosome formation by preventing the cytoskeletal rearrangement through Rac-1 and ELMO–DOCK180 interactions. This leads to inefficient clearance of apoptotic cells and polarisation of M1 macrophages to release mediators that potentiate tumour

immune responses. Abbreviations: PtdSer, phosphatidylserine; LC3, microtubule-associated protein 1A/1B-light chain 3-II; ELMO, engulfment and cell motility protein; DOCK, dedicator of cytokinesis.

4.8. Concluding remarks

A common effect of anticancer therapies is to induce apoptosis of cancer cells. However, there is a surprising paucity of information that considers the complex processes that are responsible for clearing the cellular debris. In line with this, there is emerging evidence that implicates the non-canonical autophagy pathway, LAP, as an essential component involved in the coordinated clearance of apoptotic cancer cells. However, unlike canonical autophagy and phagocytosis, LAP-mediated efferocytosis elicits an anti-inflammatory response in the surrounding environment that may have significant implications for the evasion of immune surveillance by cells involved in tumorigenesis. Indeed, it has been shown that mice altered to abrogate LAP as a part of the cell's clearance response exhibit a reduction in tumour growth, while the restoration of LAP influences tumour progression. Further, cancer tissues overexpression of Rubicon predicts adverse overall survival in many cancer patients [95]. These findings have several clinical implications: (1) Rubicon expression in tumour tissues comprising infiltrated TAMs and other phagocytic cells can lead to modulation of LAP pathway in the TME [13]. This implies that LAP can specifically be targeted in the TME to restrict tumour growth. (2) Rubicon expression could be a prognostic marker in different types of cancers and (3) Therapies that could inhibit Rubicon expression could be incorporated into clinical trials as drugs that regulate LAP during phagocytosis of apoptotic tumour cells. However, to the best of our knowledge, there is no drug at present that could specifically target Rubicon. Therefore, these findings point to a need to clearly determine how LAP is regulated during the efferocytosis of apoptotic tumour cells and whether alterations implicit with uncontrolled carcinogenesis favour the survival of cells that employ LAP to establish tumours of clinical importance. If so, perhaps a more immediate priority is to identify those therapeutic options which unwittingly potentiate cancer by overbalancing the recruitment of phagocytes and/or that directly promote LAP in cancer cells themselves. Given that LAP functions to regulate inflammation and undesired immune responses, inhibition of LAP while restricting tumour growth has the potential to result in autoimmunity and chronic inflammation [12]. This provides a worthwhile consideration when inhibiting LAP to control tumour progression. Herein, we discuss the current understanding of LAP during the development of cancer, its

relationship with efferocytosis in the tumour microenvironment, and suggest strategies that may inform current and novel therapeutic approaches.

Declaration of conflict interest

The authors declare no conflict of interests.

References

1. Kumar S, Calianese D, Birge RB. Efferocytosis of dying cells differentially modulate immunological outcomes in tumor microenvironment. Immunol Rev. 2017 Nov;280(1):149-64.
2. Jones JD, Sinder BP, Paige D, Soki FN, Koh AJ, Thiele S, et al. Trabectedin Reduces Skeletal Prostate Cancer Tumor Size in Association with Effects on M2 Macrophages and Efferocytosis. Neoplasia. 2019 2019/02/01/;21(2):172-84.
3. deCathelineau AM, Henson PM. The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. Essays in biochemistry. 2003;39:105-17.
4. Sarode GS. Efferocytosis in oral squamous cell carcinoma. J Oral Maxillofac Pathol. 2016 May-Aug;20(2):170-2.
5. Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. Chest. 2006 Jun;129(6):1673-82.
6. Proto JD, Doran AC, Gusarova G, Yurdagul A, Jr., Sozen E, Subramanian M, et al. Regulatory T Cells Promote Macrophage Efferocytosis during Inflammation Resolution. Immunity. 2018 Oct 16;49(4):666-77.e6.
7. Tao H, Yancey PG, Babaev VR, Blakemore JL, Zhang Y, Ding L, et al. Macrophage SR-BI mediates efferocytosis via Src/PI3K/Rac1 signaling and reduces atherosclerotic lesion necrosis. J Lipid Res. 2015;56(8):1449-60.
8. Green DR, Oguin TH, Martinez J. The clearance of dying cells: table for two. Cell Death & Differentiation. 2016 2016/06/01;23(6):915-26.
9. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc Natl Acad Sci U S A. 2011 Oct 18;108(42):17396-401.
10. Sanjuan MA, Dillon CP, Tait SWG, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature. 2007 2007/12/01;450(7173):1253-7.
11. Heckmann BL, Boada-Romero E, Cunha LD, Magne J, Green DR. LC3-Associated Phagocytosis and Inflammation. Journal of Molecular Biology. [Review]. 2017;429(23):3561-76.
12. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. Nature. 2016 May 5;533(7601):115-9.
13. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford JC, et al. LC3-Associated Phagocytosis in Myeloid Cells Promotes Tumor Immune Tolerance. Cell. 2018 Oct 4;175(2):429-41.e16.
14. Medina CB, Mehrotra P, Arandjelovic S, Perry JSA, Guo Y, Morioka S, et al. Metabolites released from apoptotic cells act as tissue messengers. Nature. 2020 2020/04/01;580(7801):130-5.
15. Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, et al. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature. 2010 2010/10/01;467(7317):863-7.
16. Roszer T, Menéndez-Gutiérrez MP, Lefterova MI, Alameda D, Núñez V, Lazar MA, et al. Autoimmune kidney disease and impaired engulfment of apoptotic cells in mice with macrophage peroxisome proliferator-activated receptor gamma or retinoid X receptor alpha deficiency. Journal of immunology (Baltimore, Md : 1950). 2011 Jan 1;186(1):621-31.

17. Yurdagul A, Jr., Subramanian M, Wang X, Crown SB, Ilkayeva OR, Darville L, et al. Macrophage Metabolism of Apoptotic Cell-Derived Arginine Promotes Continual Efferocytosis and Resolution of Injury. Cell Metab. 2020 Mar 3;31(3):518-33.e10.
18. Zhang S, Weinberg S, DeBerge M, Gainullina A, Schipma M, Kinchen JM, et al. Efferocytosis Fuels Requirements of Fatty Acid Oxidation and the Electron Transport Chain to Polarize Macrophages for Tissue Repair. Cell Metabolism. 2019 2019/02/05/;29(2):443-56.e5.
19. Gumienny TL, Brugnera E, Tosello-Trampont AC, Kinchen JM, Haney LB, Nishiwaki K, et al. CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. Cell. 2001 Oct 5;107(1):27-41.
20. Grabiec AM, Denny N, Doherty JA, Happonen KE, Hankinson J, Connolly E, et al. Diminished airway macrophage expression of the Axl receptor tyrosine kinase is associated with defective efferocytosis in asthma. The Journal of allergy and clinical immunology. 2017 Oct;140(4):1144-6.e4.
21. Nishi C, Yanagihashi Y, Segawa K, Nagata S. MERTK tyrosine kinase receptor together with TIM4 phosphatidylserine receptor mediates distinct signal transduction pathways for efferocytosis and cell proliferation. Journal of Biological Chemistry. 2019 March 7, 2019.
22. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature. 2007 Nov 15;450(7168):430-4.
23. Martinez J, Malireddi RKS, Lu Q, Cunha LD, Pelletier S, Gingras S, et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nature Cell Biology. [Article]. 2015;17(7):893-906.
24. Ellson CD, Anderson KE, Morgan G, Chilvers ER, Lipp P, Stephens LR, et al. Phosphatidylinositol 3-phosphate is generated in phagosomal membranes. Current biology : CB. 2001 Oct 16;11(20):1631-5.
25. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol. 2008 Aug 25;182(4):685-701.
26. Heckmann BL, Boada-Romero E, Cunha LD, Magne J, Green DR. LC3-Associated Phagocytosis and Inflammation. J Mol Biol. 2017 Nov 24;429(23):3561-76.
27. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, et al. Two Beclin 1-binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat Cell Biol. 2009 Apr;11(4):385-96.
28. Wong SW, Sil P, Martinez J. Rubicon: LC3-associated phagocytosis and beyond. FEBS Journal. [Review]. 2018;285(8):1379-88.
29. Masud S, van der Burg L, Storm L, Prajsnar TK, Meijer AH. Rubicon-Dependent Lc3 Recruitment to Salmonella-Containing Phagosomes Is a Host Defense Mechanism Triggered Independently From Major Bacterial Virulence Factors. Frontiers in Cellular and Infection Microbiology. [Original Research]. 2019 2019-August-02;9(279).
30. Boyle KB, Randow F. Rubicon swaps autophagy for LAP. Nature Cell Biology. 2015 2015/07/01;17(7):843-5.
31. Yang C-S, Lee J-S, Rodgers M, Min C-K, Lee J-Y, Kim HJ, et al. Autophagy protein Rubicon mediates phagocytic NADPH oxidase activation in response to microbial infection or TLR stimulation. Cell host & microbe. 2012;11(3):264-76.
32. Sun Q, Zhang J, Fan W, Wong KN, Ding X, Chen S, et al. The RUN domain of rubicon is important for hVps34 binding, lipid kinase inhibition, and autophagy suppression. J Biol Chem. 2011;286(1):185-91.

33. Callihan EB, Gao D, Jindal S, Lyons TR, Manthey E, Edgerton S, et al. Postpartum diagnosis demonstrates a high risk for metastasis and merits an expanded definition of pregnancy-associated breast cancer. Breast Cancer Res Treat. 2013;138(2):549-59.
34. Van den Rul N, Han SN, Van Calsteren K, Neven P, Amant F. Postpartum breast cancer behaves differently. Facts Views Vis Obgyn. 2011;3(3):183-8.
35. Goddard ET, Bassale S, Schedin T, Jindal S, Johnston J, Cabral E, et al. Association Between Postpartum Breast Cancer Diagnosis and Metastasis and the Clinical Features Underlying Risk. JAMA Network Open. 2019;2(1):e186997-e.
36. Schedin P, O'Brien J, Rudolph M, Stein T, Borges V. Microenvironment of the involuting mammary gland mediates mammary cancer progression. Journal of mammary gland biology and neoplasia. 2007 Mar;12(1):71-82.
37. Daling JR, Malone KE, Doody DR, Anderson BO, Porter PL. The Relation of Reproductive Factors to Mortality from Breast Cancer. Cancer Epidemiology Biomarkers & Prevention. 2002;11(3):235-41.
38. Stanford JC, Young C, Hicks D, Owens P, Williams A, Vaught DB, et al. Efferocytosis produces a prometastatic landscape during postpartum mammary gland involution. The Journal of Clinical Investigation. 2014 11/03/;124(11):4737-52.
39. Martinson HA, Jindal S, Durand-Rougely C, Borges VF, Schedin P. Wound healing-like immune program facilitates postpartum mammary gland involution and tumor progression. International journal of cancer. 2015;136(8):1803-13.
40. Wang Y, Moncayo G, Morin P, Jr., Xue G, Grzmil M, Lino MM, et al. Mer receptor tyrosine kinase promotes invasion and survival in glioblastoma multiforme. Oncogene. 2013 Feb 14;32(7):872-82.
41. Tan X, Zhang Z, Yao H, Shen L. Tim-4 promotes the growth of colorectal cancer by activating angiogenesis and recruiting tumor-associated macrophages via the PI3K/AKT/mTOR signaling pathway. Cancer letters. 2018 Nov 1;436:119-28.
42. Soki FN, Koh AJ, Jones JD, Kim YW, Dai J, Keller ET, et al. Polarization of prostate cancer-associated macrophages is induced by milk fat globule-EGF factor 8 (MFG-E8)-mediated efferocytosis. J Biol Chem. 2014 Aug 29;289(35):24560-72.
43. Harrison IP, Vinh A, Johnson IRD, Luong R, Drummond GR, Sobey CG, et al. NOX2 oxidase expressed in endosomes promotes cell proliferation and prostate tumour development. Oncotarget. 2018 Oct 23;9(83):35378-93.
44. Jackute J, Zemaitis M, Pranys D, Sitkauskiene B, Miliauskas S, Vaitkiene S, et al. Distribution of M1 and M2 macrophages in tumor islets and stroma in relation to prognosis of non-small cell lung cancer. BMC Immunol. 2018;19(1):3-.
45. Zhou Y, Yao Y, Deng Y, Shao A. Regulation of efferocytosis as a novel cancer therapy. Cell Commun Signal. 2020;18(1):71-.
46. Crittenden MR, Baird J, Friedman D, Savage T, Uhde L, Alice A, et al. Mertk on tumor macrophages is a therapeutic target to prevent tumor recurrence following radiation therapy. Oncotarget. 2016;7(48):78653-66.
47. Baghdadi M, Yoneda A, Yamashina T, Nagao H, Komohara Y, Nagai S, et al. TIM-4 glycoprotein-mediated degradation of dying tumor cells by autophagy leads to reduced antigen presentation and increased immune tolerance. Immunity. 2013 Dec 12;39(6):1070-81.
48. DeRyckere D, Lee-Sherick AB, Huey MG, Hill AA, Tyner JW, Jacobsen KM, et al. UNC2025, a MERTK Small-Molecule Inhibitor, Is Therapeutically Effective Alone and in Combination with Methotrexate in Leukemia Models. Clinical cancer research : an official journal of the American Association for Cancer Research. 2017 Mar 15;23(6):1481-92.
49. Cook RS, Jacobsen KM, Wofford AM, DeRyckere D, Stanford J, Prieto AL, et al. MerTK inhibition in tumor leukocytes decreases tumor growth and metastasis. The Journal of clinical investigation. 2013;123(8):3231-42.

50. Loges S, Schmidt T, Tjwa M, van Geyte K, Lievens D, Lutgens E, et al. Malignant cells fuel tumor growth by educating infiltrating leukocytes to produce the mitogen Gas6. Blood. 2010 Mar 18;115(11):2264-73.
51. Sil P, Suwanpradid J, Muse G, Gruzdev A, Liu L, Corcoran DL, et al. Noncanonical autophagy in dermal dendritic cells mediates immunosuppressive effects of UV exposure. Journal of Allergy and Clinical Immunology. 2020 2020/05/01/;145(5):1389-405.
52. Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nat Med. 2003 Feb;9(2):213-9.
53. Heming M, Gran S, Jauch S-L, Fischer-Riepe L, Russo A, Klotz L, et al. Peroxisome Proliferator-Activated Receptor-γ Modulates the Response of Macrophages to Lipopolysaccharide and Glucocorticoids. Frontiers in Immunology. [Original Research]. 2018 2018-May-08;9(893).
54. Sloan JM, Kershaw MH, Touloukian CE, Lapointe R, Robbins PF, Restifo NP, et al. MHC class I and class II presentation of tumor antigen in retrovirally and adenovirally transduced dendritic cells. Cancer Gene Therapy. 2002 2002/11/01;9(11):946-50.
55. Zhong X, Lee H-N, Kim SH, Park S-A, Kim W, Cha Y-N, et al. Myc-nick promotes efferocytosis through M2 macrophage polarization during resolution of inflammation. The FASEB Journal. 2018;32(10):5312-25.
56. Zhou Y, Fei M, Zhang G, Liang W-C, Lin W, Wu Y, et al. Blockade of the Phagocytic Receptor MerTK on Tumor-Associated Macrophages Enhances P2X7R-Dependent STING Activation by Tumor-Derived cGAMP. Immunity. 2020 2020/02/18/;52(2):357-73.e9.
57. Woo S-R, Fuertes Mercedes B, Corrales L, Spranger S, Furdyna Michael J, Leung Michael YK, et al. STING-Dependent Cytosolic DNA Sensing Mediates Innate Immune Recognition of Immunogenic Tumors. Immunity. 2014 2014/11/20/;41(5):830-42.
58. Ahn J, Gutman D, Saijo S, Barber GN. STING manifests self DNA-dependent inflammatory disease. Proc Natl Acad Sci U S A. 2012 Nov 20;109(47):19386-91.
59. Lin Y, Xu J, Lan H. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. Journal of Hematology & Oncology. 2019 2019/07/12;12(1):76.
60. Ries CH, Cannarile MA, Hoves S, Benz J, Wartha K, Runza V, et al. Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy. Cancer cell. 2014 Jun 16;25(6):846-59.
61. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. Nat Med. 2013 Oct;19(10):1264-72.
62. Yang M, Liu J, Piao C, Shao J, Du J. ICAM-1 suppresses tumor metastasis by inhibiting macrophage M2 polarization through blockade of efferocytosis. Cell Death & Disease. 2015 2015/06/01;6(6):e1780-e.
63. Ke X, Chen C, Song Y, Cai Q, Li J, Tang Y, et al. Hypoxia modifies the polarization of macrophages and their inflammatory microenvironment, and inhibits malignant behavior in cancer cells. Oncol Lett. 2019;18(6):5871-8.
64. Roda JM, Wang Y, Sumner LA, Phillips GS, Marsh CB, Eubank TD. Stabilization of HIF-2α induces sVEGFR-1 production from tumor-associated macrophages and decreases tumor growth in a murine melanoma model. Journal of immunology (Baltimore, Md : 1950). 2012 Sep 15;189(6):3168-77.
65. Huber R, Meier B, Otsuka A, Fenini G, Satoh T, Gehrke S, et al. Tumour hypoxia promotes melanoma growth and metastasis via High Mobility Group Box-1 and M2-like macrophages. Sci Rep. 2016 Jul 18;6:29914.

66. Poh AR, Ernst M. Targeting Macrophages in Cancer: From Bench to Bedside. Front Oncol. 2018;8:49-.
67. Vinnakota K, Zhang Y, Selvanesan BC, Topi G, Salim T, Sand-Dejmek J, et al. M2-like macrophages induce colon cancer cell invasion via matrix metalloproteinases. J Cell Physiol. 2017 Dec;232(12):3468-80.
68. Petruzzi MNMR, Cherubini K, Salum FG, de Figueiredo MAZ. Role of tumour-associated macrophages in oral squamous cells carcinoma progression: an update on current knowledge. Diagn Pathol. 2017;12(1):32-.
69. Chiu KC, Lee CH, Liu SY, Chou YT, Huang RY, Huang SM, et al. Polarization of tumor-associated macrophages and Gas6/Axl signaling in oral squamous cell carcinoma. Oral oncology. 2015 Jul;51(7):683-9.
70. Li Z, Jiang J, Wang Z, Zhang J, Xiao M, Wang C, et al. Endogenous Interleukin-4 Promotes Tumor Development by Increasing Tumor Cell Resistance to Apoptosis. Cancer Res. 2008;68(21):8687-94.
71. Zeni E, Mazzetti L, Miotto D, Lo Cascio N, Maestrelli P, Querzoli P, et al. Macrophage expression of interleukin-10 is a prognostic factor in nonsmall cell lung cancer. European Respiratory Journal. 2007;30(4):627-32.
72. Seifert L, Werba G, Tiwari S, Giao Ly NN, Nguy S, Alothman S, et al. Radiation Therapy Induces Macrophages to Suppress T-Cell Responses Against Pancreatic Tumors in Mice. Gastroenterology. 2016 Jun;150(7):1659-72.e5.
73. Gazzaniga S, Bravo AI, Guglielmotti A, van Rooijen N, Maschi F, Vecchi A, et al. Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. The Journal of investigative dermatology. 2007 Aug;127(8):2031-41.
74. Chanmee T, Ontong P, Konno K, Itano N. Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment. Cancers (Basel). 2014;6(3):1670-90.
75. Martinez J, Verbist K, Wang R, Green DR. The relationship between metabolism and the autophagy machinery during the innate immune response. Cell metabolism. 2013;17(6):895-900.
76. Lee C, Jeong H, Bae Y, Shin K, Kang S, Kim H, et al. Targeting of M2-like tumor-associated macrophages with a melittin-based pro-apoptotic peptide. Journal for ImmunoTherapy of Cancer. 2019 2019/06/07;7(1):147.
77. Vaught DB, Stanford JC, Cook RS. Efferocytosis creates a tumor microenvironment supportive of tumor survival and metastasis. Cancer Cell Microenviron. 2015;2(1):e666.
78. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nature immunology. 2010 Oct;11(10):889-96.
79. Li J, Xie Y, Wang X, Li F, Li S, Li M, et al. Prognostic impact of tumor-associated macrophage infiltration in esophageal cancer: a meta-analysis. Future oncology (London, England). 2019 Jul;15(19):2303-17.
80. Brown JM, Recht L, Strober S. The Promise of Targeting Macrophages in Cancer Therapy. Clinical Cancer Research. 2017;23(13):3241-50.
81. Hughes R, Qian BZ, Rowan C, Muthana M, Keklikoglou I, Olson OC, et al. Perivascular M2 Macrophages Stimulate Tumor Relapse after Chemotherapy. Cancer Res. 2015 Sep 1;75(17):3479-91.
82. Heckmann BL, Green DR. LC3-associated phagocytosis at a glance. Journal of Cell Science. 2019;132(5):jcs222984.
83. Amaravadi RK, Kimmelman AC, Debnath J. Targeting Autophagy in Cancer: Recent Advances and Future Directions. Cancer Discovery. 2019;9(9):1167-81.

84. Garufi A, Pistritto G, Baldari S, Toietta G, Cirone M, D'Orazi G. p53-Dependent PUMA to DRAM antagonistic interplay as a key molecular switch in cell-fate decision in normal/high glucose conditions. J Exp Clin Cancer Res. 2017;36(1):126-.
85. Yoshioka A, Miyata H, Doki Y, Yamasaki M, Sohma I, Gotoh K, et al. LC3, an autophagosome marker, is highly expressed in gastrointestinal cancers. International journal of oncology. 2008 Sep;33(3):461-8.
86. Gong J, Muñoz AR, Chan D, Ghosh R, Kumar AP. STAT3 down regulates LC3 to inhibit autophagy and pancreatic cancer cell growth. Oncotarget. 2014;5(9):2529-41.
87. Lazova R, Camp RL, Klump V, Siddiqui SF, Amaravadi RK, Pawelek JM. Punctate LC3B expression is a common feature of solid tumors and associated with proliferation, metastasis, and poor outcome. Clinical cancer research : an official journal of the American Association for Cancer Research. 2012 Jan 15;18(2):370-9.
88. Gluschko A, Herb M, Wiegmann K, Krut O, Neiss WF, Utermöhlen O, et al. The β(2) Integrin Mac-1 Induces Protective LC3-Associated Phagocytosis of Listeria monocytogenes. Cell Host Microbe. 2018 Mar 14;23(3):324-37.e5.
89. Nguyen TN, Padman BS, Usher J, Oorschot V, Ramm G, Lazarou M. Atg8 family LC3/GABARAP proteins are crucial for autophagosome–lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. Journal of Cell Biology. 2016;215(6):857-74.
90. Fazeli G, Wehman AM. Safely removing cell debris with LC3-associated phagocytosis. Biology of the Cell. [Review]. 2017;109(10):355-63.
91. Kim J-Y, Zhao H, Martinez J, Doggett TA, Kolesnikov AV, Tang PH, et al. Noncanonical autophagy promotes the visual cycle. Cell. 2013;154(2):365-76.
92. Turco E, Witt M, Abert C, Bock-Bierbaum T, Su MY, Trapannone R, et al. FIP200 Claw Domain Binding to p62 Promotes Autophagosome Formation at Ubiquitin Condensates. Molecular cell. 2019 Apr 18;74(2):330-46.e11.
93. Nazio F, Carinci M, Valacca C, Bielli P, Strappazzon F, Antonioli M, et al. Fine-tuning of ULK1 mRNA and protein levels is required for autophagy oscillation. Journal of Cell Biology. 2016;215(6):841-56.
94. Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, et al. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. Nature cell biology. 2009;11(4):468-76.
95. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419.
96. Segawa K, Suzuki J, Nagata S. Constitutive exposure of phosphatidylserine on viable cells. Proceedings of the National Academy of Sciences. 2011;108(48):19246-51.
97. Birge RB, Boeltz S, Kumar S, Carlson J, Wanderley J, Calianese D, et al. Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. Cell Death & Differentiation. 2016 2016/06/01;23(6):962-78.
98. Huang X, Yin Y, Ye D, Brekken R, Thorpe P. Phosphatidylserine-targeting antibody induces M1 macrophage polarization, promotes myeloid derived suppressor cell differentiation and boosts tumor-specific immunity. Journal for ImmunoTherapy of Cancer. 2013 2013/11/07;1(1):P154.
99. Sharma B, Kanwar SS. Phosphatidylserine: A cancer cell targeting biomarker. Semin Cancer Biol. 2018 Oct;52(Pt 1):17-25.
100. Vallabhapurapu SD, Blanco VM, Sulaiman MK, Vallabhapurapu SL, Chu Z, Franco RS, et al. Variation in human cancer cell external phosphatidylserine is regulated by flippase activity and intracellular calcium. Oncotarget. 2015 Oct 27;6(33):34375-88.

101. Jain M, Raizada N, Kuttan R, Shan J, Digumarti R. Phase II study of bavituximab plus paclitaxel and carboplatin in locally advanced or metastatic breast cancer: Interim results. Journal of Clinical Oncology. 2010;28(15_suppl):1062-.
102. Digumarti R, Bapsy PP, Suresh AV, Bhattacharyya GS, Dasappa L, Shan JS, et al. Bavituximab plus paclitaxel and carboplatin for the treatment of advanced non-small-cell lung cancer. Lung Cancer. 2014;86(2):231-6.
103. Nam J-S, Terabe M, Mamura M, Kang M-J, Chae H, Stuelten C, et al. An anti-transforming growth factor beta antibody suppresses metastasis via cooperative effects on multiple cell compartments. Cancer Res. 2008;68(10):3835-43.
104. Montuenga LM, Pio R. Tumour-associated macrophages in nonsmall cell lung cancer: the role of interleukin-10. European Respiratory Journal. 2007;30(4):608-10.
105. Hatanaka H, Abe Y, Kamiya T, Morino F, Nagata J, Tokunaga T, et al. Clinical implications of interleukin (IL)-10 induced by non-small-cell lung cancer. Annals of oncology : official journal of the European Society for Medical Oncology. 2000 Jul;11(7):815-9.
106. Vahl JM, Friedrich J, Mittler S, Trump S, Heim L, Kachler K, et al. Interleukin-10-regulated tumour tolerance in non-small cell lung cancer. British Journal of Cancer. 2017 2017/11/01;117(11):1644-55.
107. Alas S, Emmanouilides C, Bonavida B. Inhibition of interleukin 10 by rituximab results in down-regulation of bcl-2 and sensitization of B-cell non-Hodgkin's lymphoma to apoptosis. Clinical cancer research : an official journal of the American Association for Cancer Research. 2001 Mar;7(3):709-23.
108. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B cell production of IL-10 inhibits lymphoma depletion during CD20 immunotherapy in mice. J Clin Invest. 2011 Nov;121(11):4268-80.
109. García-Hernández ML, Hernández-Pando R, Gariglio P, Berumen J. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. Immunology. 2002;105(2):231-43.
110. Florey O, Gammoh N, Kim SE, Jiang X, Overholtzer M. V-ATPase and osmotic imbalances activate endolysosomal LC3 lipidation. Autophagy. 2015;11(1):88-99.
111. Jacquin E, Leclerc-Mercier S, Judon C, Blanchard E, Fraitag S, Florey O. Pharmacological modulators of autophagy activate a parallel noncanonical pathway driving unconventional LC3 lipidation. Autophagy. 2017;13(5):854-67.
112. Fletcher K, Ulferts R, Jacquin E, Veith T, Gammoh N, Arasteh JM, et al. The WD40 domain of ATG16L1 is required for its non-canonical role in lipidation of LC3 at single membranes. EMBO Journal. [Article]. 2018;37(4).
113. Rai S, Arasteh M, Jefferson M, Pearson T, Wang Y, Zhang W, et al. The ATG5-binding and coiled coil domains of ATG16L1 maintain autophagy and tissue homeostasis in mice independently of the WD domain required for LC3-associated phagocytosis. Autophagy. 2019 Apr;15(4):599-612.
114. Orozco SL, Daniels BP, Yatim N, Messmer MN, Quarato G, Chen-Harris H, et al. RIPK3 Activation Leads to Cytokine Synthesis that Continues after Loss of Cell Membrane Integrity. Cell Reports. 2019 2019/08/27/;28(9):2275-87.e5.
115. Yang Y, Jiang G, Zhang P, Fan J. Programmed cell death and its role in inflammation. Military Medical Research. 2015 2015/05/19;2(1):12.
116. Yang J, Zhao Y, Zhang L, Fan H, Qi C, Zhang K, et al. RIPK3/MLKL-Mediated Neuronal Necroptosis Modulates the M1/M2 Polarization of Microglia/Macrophages in the Ischemic Cortex. Cereb Cortex. 2018;28(7):2622-35.
117. Lecis D, De Cesare M, Perego P, Conti A, Corna E, Drago C, et al. Smac mimetics induce inflammation and necrotic tumour cell death by modulating macrophage activity. Cell Death & Disease. 2013 2013/11/01;4(11):e920-e.

118. Kiss RS, Elliott MR, Ma Z, Marcel YL, Ravichandran KS. Apoptotic cells induce a phosphatidylserine-dependent homeostatic response from phagocytes. Current biology : CB. 2006 Nov 21;16(22):2252-8.
119. A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, et al. Apoptotic Cells Promote Their Own Clearance and Immune Tolerance through Activation of the Nuclear Receptor LXR. Immunity. 2009 2009/08/21/;31(2):245-58.
120. Sag D, Cekic C, Wu R, Linden J, Hedrick CC. The cholesterol transporter ABCG1 links cholesterol homeostasis and tumour immunity. Nature communications. 2015;6:6354-.
121. Wang Y, Hao F, Nan Y, Qu L, Na W, Jia C, et al. PKM2 Inhibitor Shikonin Overcomes the Cisplatin Resistance in Bladder Cancer by Inducing Necroptosis. Int J Biol Sci. 2018;14(13):1883-91.
122. Jin G, Liu Y, Xu P, Jin G. Induction of Necroptosis in Human Breast Cancer Drug-Resistant Cells by SMAC Analog LCL161 After Caspase Inhibition Requires RIP3. Die Pharmazie. 2019 Jun 1;74(6):363-8.
123. Snyder AG, Hubbard NW, Messmer MN, Kofman SB, Hagan CE, Orozco SL, et al. Intratumoral activation of the necroptotic pathway components RIPK1 and RIPK3 potentiates antitumor immunity. Science immunology. 2019 Jun 21;4(36).

Chapter 5

5. Summary and future recommendations

In conclusion, the studies were designed to address the knowledge gap in the mechanisms of defective phagocytic function of airway macrophages in COPD and in response to cigarette smoke, and to advise new therapeutic targets. The data presented in this thesis are the first to describe LC3-associated phagocytosis (LAP) as a factor that contributes to alveolar macrophage phagocytic dysfunction in COPD.

The central findings describe LAP dysregulation in COPD explained by Rubicon degradation and that was caused by the factors present in cigarette smoke. Moreover, the study found that LAP-inhibition correlated with limitations for the phagocytosis of Non-typeable *Haemophilus influenzae* (NTHi) and efferocytosis of apoptotic epithelial cells, which underscore a link for LAP inhibition and defective macrophage phagocytic function in COPD. This finding could serve as the basis for future studies that elucidate the mechanisms underlying the Rubicon-COPD axis and the value of measuring Rubicon expression as a new prognostic marker to predict COPD before it significantly reduces the quality of life.

Furthermore, there was concomitant increase of pro-inflammatory mediators interleukin-1β (IL-1β) and tumour necrosis factor- α (TNF-α) in macrophages exposed to cigarette smoke extract. These findings are consistent with previous reports that LAP deficiency could impair the macrophage clearance system and lead to protracted inflammation and autoimmune diseases [1, 2]. In this regard, similar associations between LAP impairment and the pathogenesis of some diseases including systemic lupus erythematosus and auto-inflammation in mice models have been reported [1, 2].

Hence, the findings of this study identified an undescribed, potential therapeutic target that is relevant to COPD, and other diseases that are potentiated by uncontrolled inflammation and autoimmune-like sequele. Furthermore, the observation that the activity of lysosomal proteases direct Rubicon degradation and/or LAP inhibition aligns with reports that, in COPD, proteases are activated and degrade cellular proteins critical that have important consequence on normal cellular physiological activity [3, 4]. This is in agreement with the concept that protease inhibitors could have therapeutic implications in COPD [3, 4]. Hence, this study provides a new understanding that could serve as a basis for the synthesis of LAP-modulating therapies for the management of COPD.

LAP modulators as a potential therapeutic approach for COPD requires a better understanding of the impacts of dysregulated LAP as this relates to co-morbidities of COPD such as metabolic disorder, autoimmune diseases and cancer. Studies show that LAP and/or Rubicon is essential for metabolism and immunomodulatory activites. This suggests that modulators of LAP could be protective of a wide range of disorders linked with the range of COPD co-morbidities [5]. However, studies in mice in which LAP was manipulated at the genetic level to prevent efferocytosis, produced outcomes that limited tumour growth, while LAP sufficiency contributed to tumour progression [6]. Hence, while LAP sufficiency has the proclivity to protect against some COPD-related diseases, the pro-tumour impact of LAP is likely to have detrimental consequences on patients with COPD and cancer. This shows a potential limitation of LAP targeting therapies in COPD. This understanding prompted me to review the literature regarding the role of LAP during efferocytosis in the tumourigenic microenvironment. The review provided a better understanding of the pro-tumour potentials of LAP and expounded on how tumour cells can leverage LAP for growth and survival. This knowledge is essential to inform LAP modulating therapies as this relates to COPD and cancer, and the relative contribution of LAP for either disease state.

5.1. Future recommendations

The relative mRNA and protein expression of the LAP-specific regulator Rubicon was assessed in human alveolar macrophages obtained from smokers with COPD and healthy non-smoking control subjects. Rubicon protein was reduced in COPD alveolar macrophages and in response to cigarette smoke exposure. While there was a correlation between reduced Rubicon and defective clearance of NTHi and apoptotic epithelial cells, the association between LAP impairment and the clinical phenotypes of COPD such as emphysema and the disease severity is unknown. Sample size was insufficient for correlative assessment of Rubicon expression and COPD severity. Therefore, it will be important for future studies to examine whether LAP and/or Rubicon correlates with lung function of cigarette smokers and COPD patients using large patient samples. Further recommendation is to examine whether interventions that modulate LAP could be harnessed for therapeutic gain by improving phagocytosis and inflammation in COPD.

The finding that Rubicon degradation occurs through lysosomal degradation pathway adds complexity to the development of LAP modulating therapies. This is because lysosomal

enzymes are also required to digest engulfed cargoes and their inhibition could impair macrophage clearance system. Therefore, future studies are required to examine whether the degradation of Rubicon by cigarette smoke is a direct result of a broad biochemical activation of lysosomal enzymes by oxidants present in cigarette smoke or indirectly through an alternative lysosomal degradation pathway such as chaperone mediated autophagy and microautophagy. The outcome of this study would further enhance our understanding of Rubicon degradation, thereby leading to the identification of the specific lysosomal protease responsible for LAP inhibition in COPD.

Dexamethasone is a corticosteroid and anti-inflammatory drug used in the clinical management of COPD. The data presented in the supplementary sheet of this thesis show that dexamethasone prevents the reduction of Rubicon by cigarette smoke extract. However, dexamethasone was unable to improve phagocytosis of NTHi upon cigarette smoke exposure despite Rubicon overexpression. Studies show that dexamethasone inhibits some phagocytic genes to impair phagocytosis [7]. This confirms that modulation of other cellular events in addition to Rubicon/LAP activity may also be required for restoring phagocytosis in COPD and upon cigarette smoke exposure. Nevertheless, this finding raises an important research question of whether the Rubicon overexpression triggered by dexamethasone is a result of secondary effects of the corticosteroid or critical for its biological role in resolving inflammation in diseases. Thus, one recommendation is to employ editing technology directed at determining the role of Rubicon (and/or LAP) in the physiological activity of dexamethasone. This will provide new information about the relevance of Rubicon and/or LAP on the therapeutic effects of dexamethasone.

Undoubtably, studies that apply a gene editing approach have significant promise to extend the understanding of the importance of dysregulated LAP in COPD. One barrier is that macrophages represent a significant challenge for the aspect of these interventions requiring the transfection of exogenous DNA. This is in part because they are professional phagocytes and will clear extracellular particles vs allowing them to incorporate into the genome or transiently, in the cytosol. This may explain the failure of my numerous attempts to examine the impact of Rubicon gene alterations on cigarette smoke-induced defective clearance of bacteria and apoptotic cells in macrophages using nucleofection and lipofectamine assays. Considering that genetic alterations via viral transduction could circumvent the clearance system of macrophages, a recommendation for future studies is the use of viral transduction technique to modify Rubicon gene in macrophages to obtain a successful outcome.

Lastly, the anti-inflammatory and immunosuppressive effects of LAP has been shown to promote lung tumour tumour growth and aggressiveness [6]. Also, available data show that higher Rubicon levels in some cancers including testicuclar and prostate cancers, correlates with higher mortality rates compared to cancer cells expressing lower levels of Rubicon [8]. However, no significant correlation between Rubicon levels and the mortalities associated with lung cancer has been reported [8]. This highlights the incomplete understanding of the impact of LAP/Rubicon on lung cancer progression and aggressiveness. Therefore, it will be logical for future studies to determine the relationship between Rubicon and/or LAP signalling system and COPD-related lung cancer progression. There is also an obvious need to conduct research on smoking associated defects in Rubicon/LAP responses in lung cancer based upon the evidence provided in this thesis.

References

1. Bandyopadhyay U, Overholtzer M. LAP: The protector against autoimmunity. Cell Research. [Article]. 2016;26(8):865-6.
2. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. Nature. [Article]. 2016;533(7601):115-9.
3. Aggarwal AN, Gupta D, Agarwal R, Jindal SK. Comparison of the lower confidence limit to the fixed-percentage method for assessing airway obstruction in routine clinical practice. Respiratory care. 2011 Nov;56(11):1778-84.
4. Yang L, Mei Y, Fang Q, Wang J, Yan Z, Song Q, et al. Identification and characterization of serine protease inhibitors in a parasitic wasp, Pteromalus puparum. Scientific Reports. 2017 2017/11/16;7(1):15755.
5. Yamamuro T, Kawabata T, Fukuhara A, Saita S, Nakamura S, Takeshita H, et al. Age-dependent loss of adipose Rubicon promotes metabolic disorders via excess autophagy. Nature Communications. 2020 2020/08/18;11(1):4150.
6. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford JC, et al. LC3-Associated Phagocytosis in Myeloid Cells Promotes Tumor Immune Tolerance. Cell. 2018 Oct 4;175(2):429-41.e16.
7. Olivares-Morales MJ, De La Fuente MK, Dubois-Camacho K, Parada D, Diaz-Jiménez D, Torres-Riquelme A, et al. Glucocorticoids Impair Phagocytosis and Inflammatory Response Against Crohn's Disease-Associated Adherent-Invasive Escherichia coli. Frontiers in immunology. 2018;9:1026-.
8. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419.

Appendix A

Supplementary results

Dexamethasone upregulates Rubicon protein in a dose-dependent manner

Dexamethasone (DEX) is a corticosteroid reported to promote phagocytosis of bacteria and used in the clinical setting to control inflammation [1]. We treated THP-1 macrophages with different concentrations of dexamethasone in the presence or absence of CSE. We found that dexamethasone increased the expression of Rubicon protein in a dose dependent manner. However, in contrast to the report by Van and colleagues [1] we found that dexamethasone inhibited phagocytosis of NTHi by THP-1 macrophages. This finding is inconsistent with reports that Rubicon overexpression allows efficient clearance of pathogens. However, this is reconciled by the previous finding that dexamethasone decreases some phagocytosis associated genes to inhibit phagocytosis [2]. This suggests that although Rubicon is critical for efficient clearance system for pathogens but its overexpression alone is not enough to remove pathogens in the absence of other key players of phagocytosis.

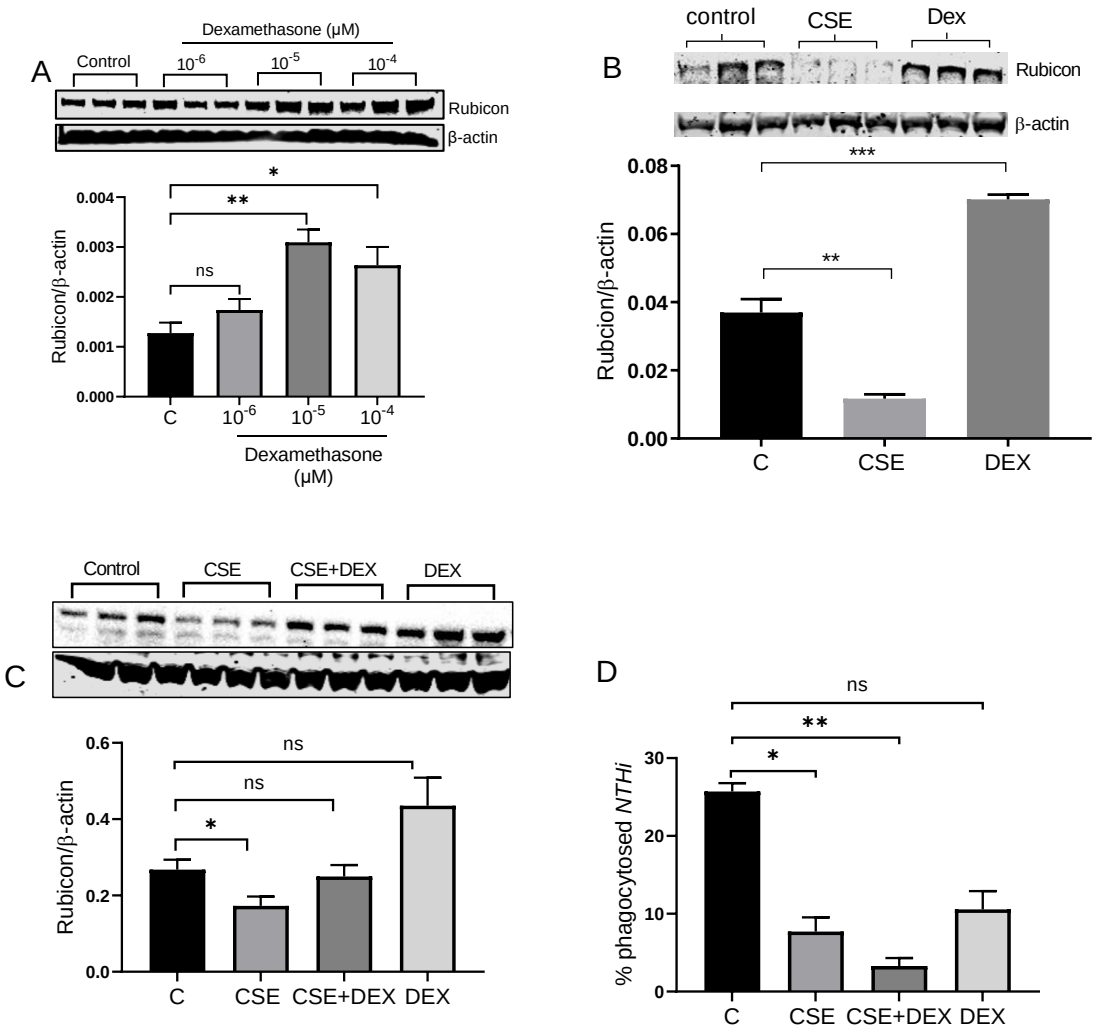


Figure 1: Dexamethasone upregulates Rubicon protein in a dose-dependent manner. Western blot images and quantitative data of Rubicon expression in dexamethasone-treated THP-1 macrophages in the presence or absence of CSE. *P < 0.05, **P < 0.01

Dexamethasone reduces phagocytosis of NTHi by THP-1 macrophages

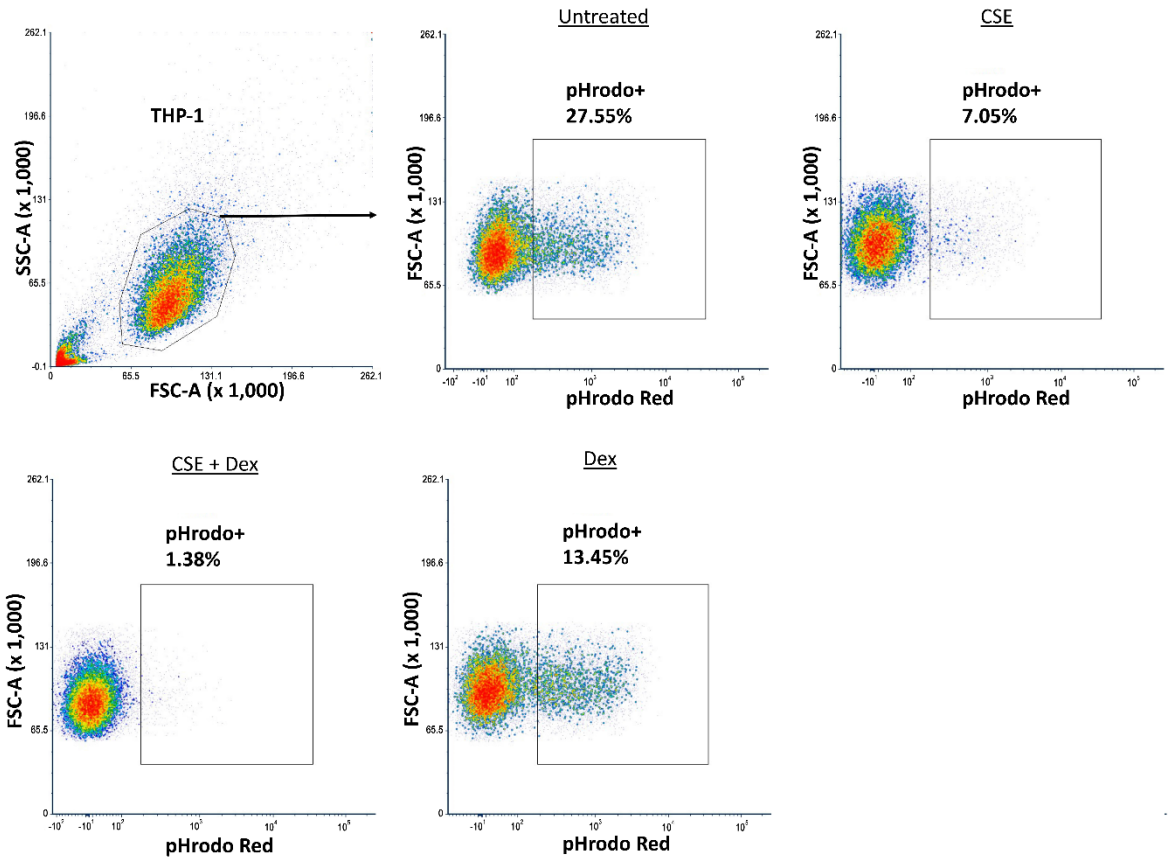


Figure 2: Representative images of phagocytosis of NTHi by untreated and CSE and dexamathosone (Dex) treated THP-1 macrophages

Autophagy protein, Beclin-1 and Atg7 are unaffected by CSE

Although CSE impairs autophagy, we found that autophagy proteins such as Beclin-1 and Atg7 remained unchanged. Moreover, leupeptin prevented the degradation of Rubicon by CSE but did not affect the autophagy flux as both LC3 and p62 remained unchanged compared to control untreated THP-1 cells.

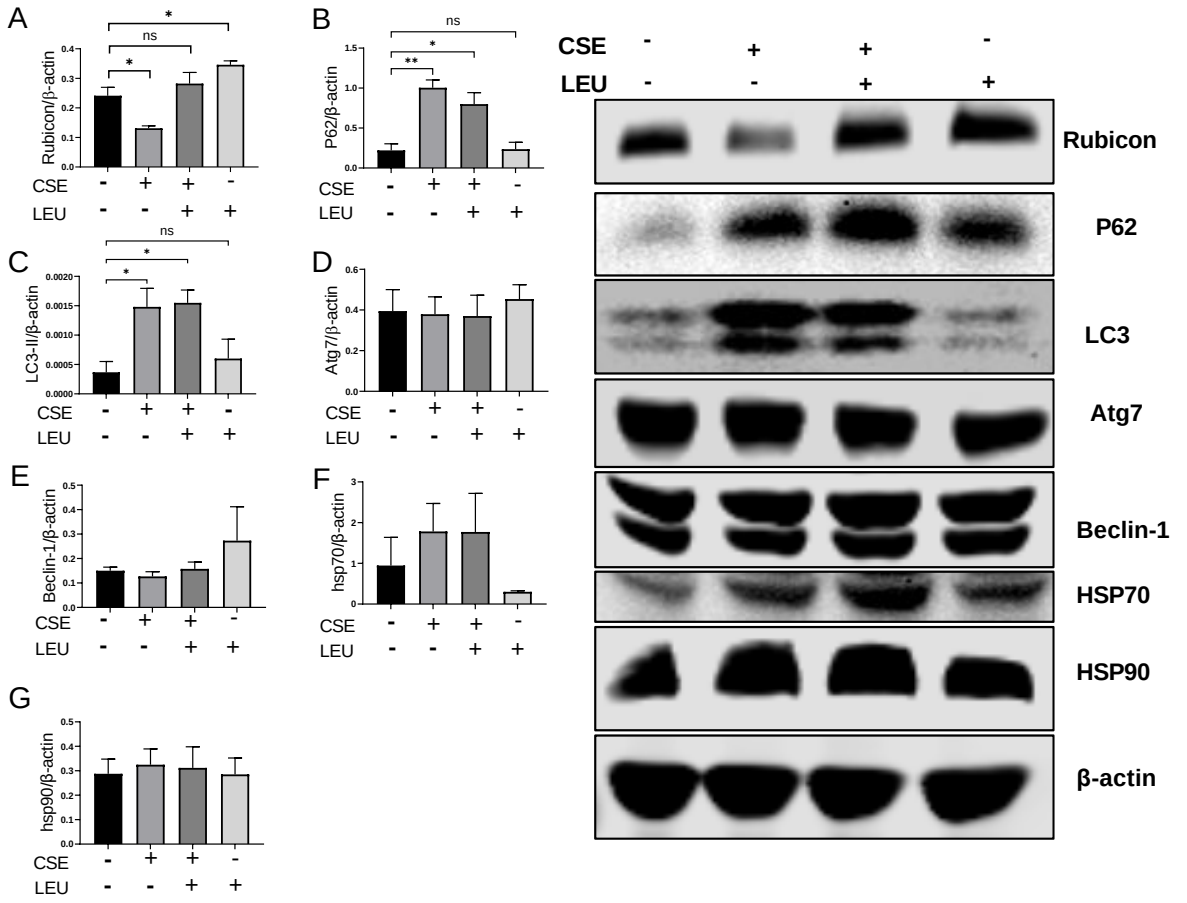
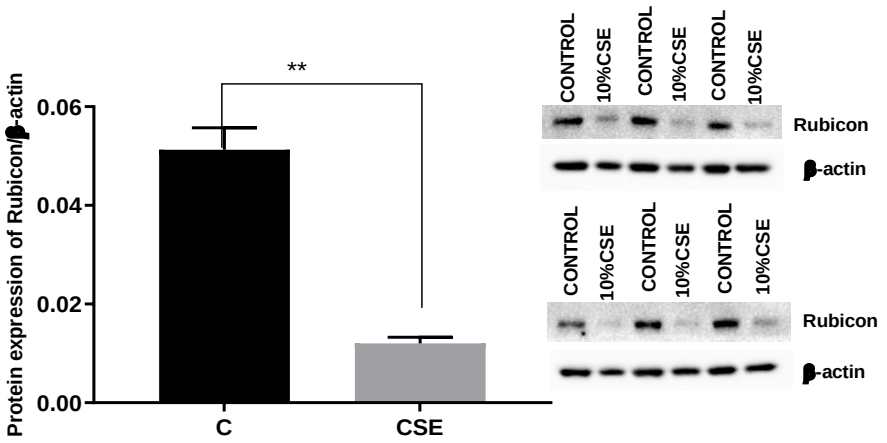
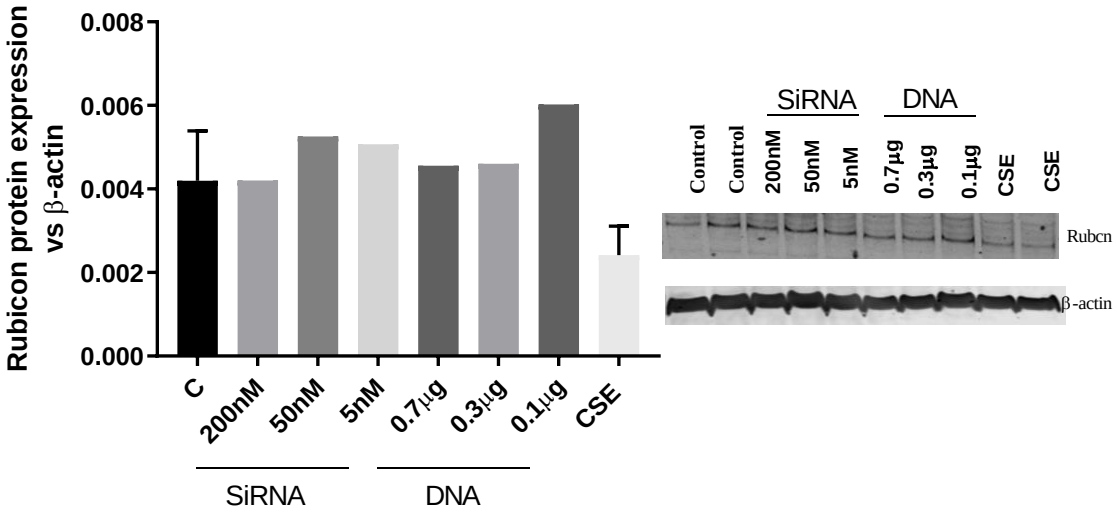


Figure 3: Autophagy protein, Beclin-1 and Atg7 are unaffected by CSE. (A-G) Effects of CSE on Rubicon, p62, LC3-II, Atg7, Beclin-1, hsp70 and hsp90 expression in the presence or absence of leupeptin (n = 3). *P < 0.05, **P < 0.01. CSE; cigarette smoke extract, LEU; leupeptin.

Unsuccessful gene modification of Rubicon using lipofectamine

Similar to the difficulties experienced by other labs working with macrophages, I was unsuccessful in transfecting the macrophages with Rubicon gene as these cells are reported to engulf and digest foreign particles they come into contact with. Gene modification via viral transduction of Rubicon in macrophages is currently ongoing to elucidate in detail the relevance of Rubicon in COPD pathogenesis.


**
Protein expression of Rubicon/β-actin
0.06
0.04
0.02
0.00
C
CSE
CONTROL
10%CSE
CONTROL
10%CSE
CONTROL
10%CSE
Rubicon
β-actin
CONTROL
10%CSE
CONTROL
10%CSE
CONTROL
10%CSE
Rubicon
β-actin


Rubicon protein expression vs β-actin
0.008
0.006
0.004
0.002
0.000
C
200nM
50nM
5nM
0.7μg
0.3μg
0.1μg
CSE
SiRNA
DNA
SiRNA
DNA
Control
Control
200nM
50nM
5nM
0.7μg
0.3μg
0.1μg
CSE
CSE
Rubcn
β-actin

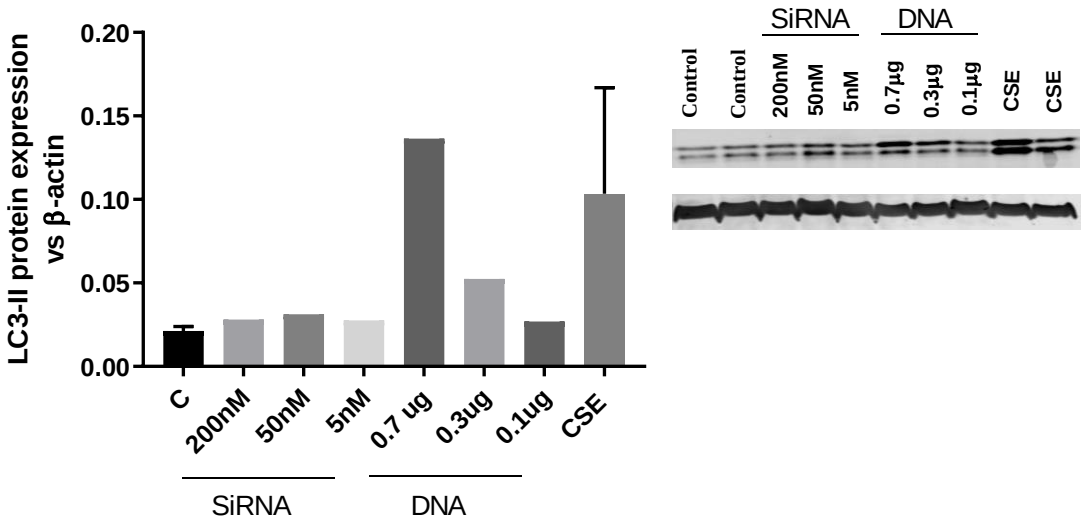
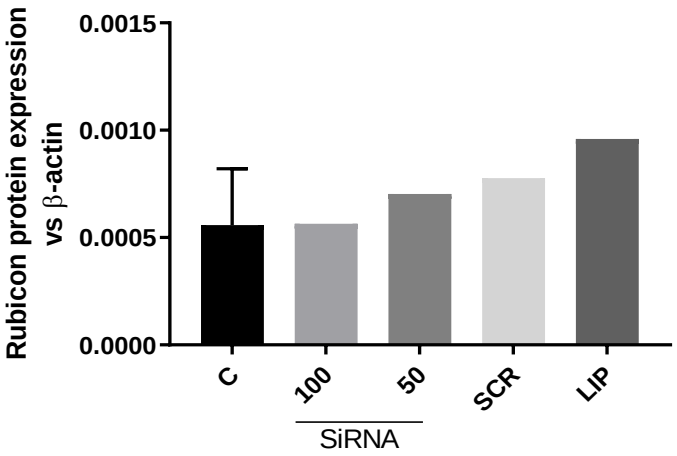

LC3-II protein expression vs β-actin
0.20
0.15
0.10
0.05
0.00
C
200nM
50nM
5nM
0.7 ug
0.3ug
0.1ug
CSE
SiRNA
DNA
SiRNA
DNA
Control
Control
200nM
50nM
5nM
0.7μg
0.3μg
0.1μg
CSE
CSE

Figure 4: Unsuccessful gene modification of Rubicon using lipofectamine. (B,C) Rubicon and LC3 expression after treating cells with lipofectamine containing Rubicon SiRNA or dsDNA for 72 h.


Rubicon protein expression vs β-actin
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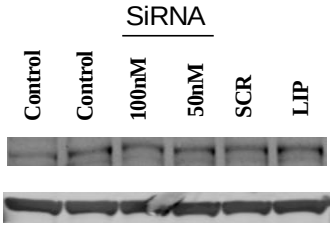
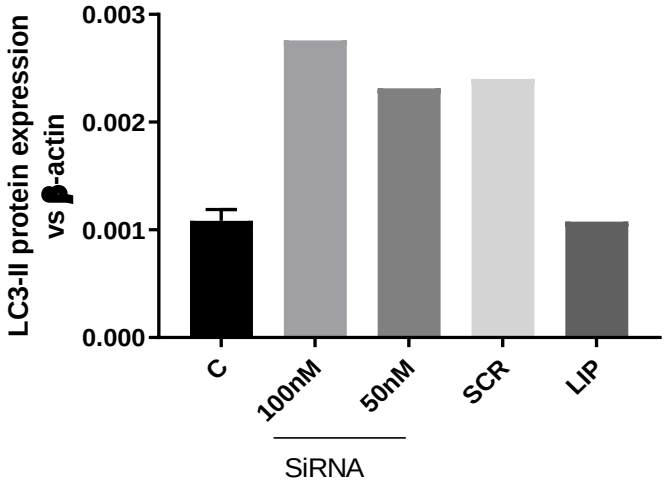
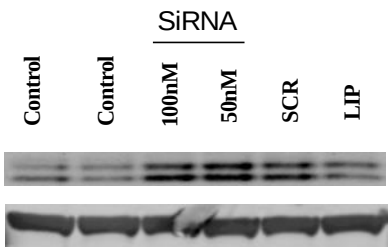
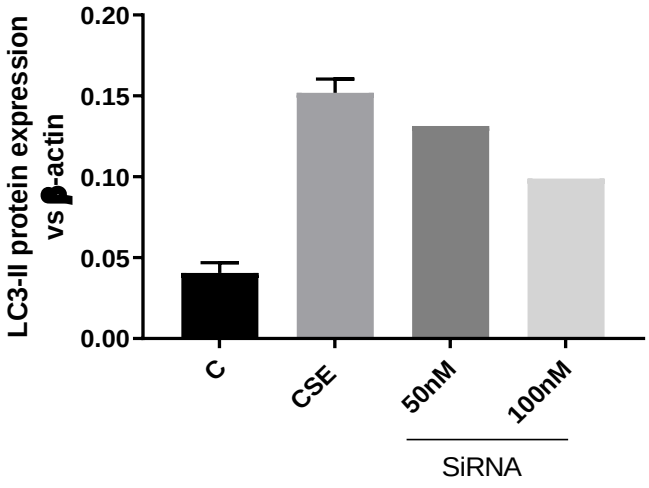
0.0010
0.0005
0.0000
C
100
50
SCR
LIP
SiRNA


SiRNA
Control
Control
100nM
50nM
SCR
LIP


LC3-II protein expression vs β-actin
0.003
0.002
0.001
0.000
C
100nM
50nM
SCR
LIP
SiRNA


SiRNA
Control
Control
100nM
50nM
SCR
LIP


LC3-II protein expression vs β-actin
0.20
0.15
0.10
0.05
0.00
C
CSE
50nM
100nM
SiRNA

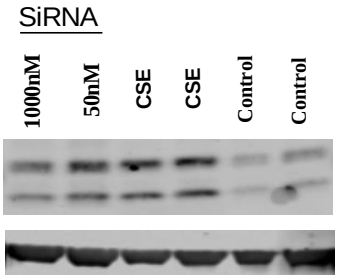

SiRNA
1000nM
50nM
CSE
CSE
Control
Control

Figure 5: Unsuccessful gene modification of Rubicon using lipofectamine. (A-D) Rubicon and LC3 expression after treating cells with lipofectamine containing Rubicon SiRNA or dsDNA for 48 h. SCR; scramble SiRNA, LIP; lipofectamine only.

Viral transduction of GFP in THP-1 macrophages

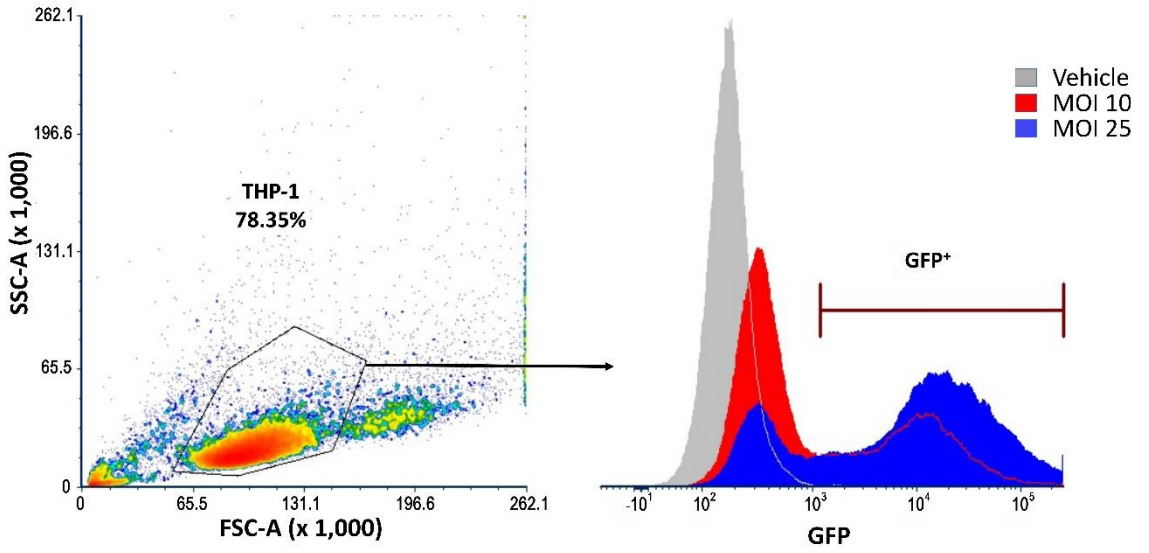


Figure 6: Representative flow cytometry images of transfected GFP using vira transduction technique

References

1. van der Goes A, Hoekstra K, van den Berg TK, Dijkstra CD. Dexamethasone promotes phagocytosis and bacterial killing by human monocytes/macrophages in vitro. Journal of leukocyte biology. 2000 Jun;67(6):801-7.
2. Olivares-Morales MJ, De La Fuente MK, Dubois-Camacho K, Parada D, Diaz-Jiménez D, Torres-Riquelme A, et al. Glucocorticoids Impair Phagocytosis and Inflammatory Response Against Crohn's Disease-Associated Adherent-Invasive Escherichia coli. Frontiers in immunology. 2018;9:1026-.

Appendix B

Publication 1

Therapeutic Advances in Respiratory Disease

Original Research

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

Ther Adv Respir Dis

2021, Vol. 15: 1–15

DOI: 10.1177/17534666211039769

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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

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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

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 microenvironment.[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 colleagues[12] 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-1β, 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 *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

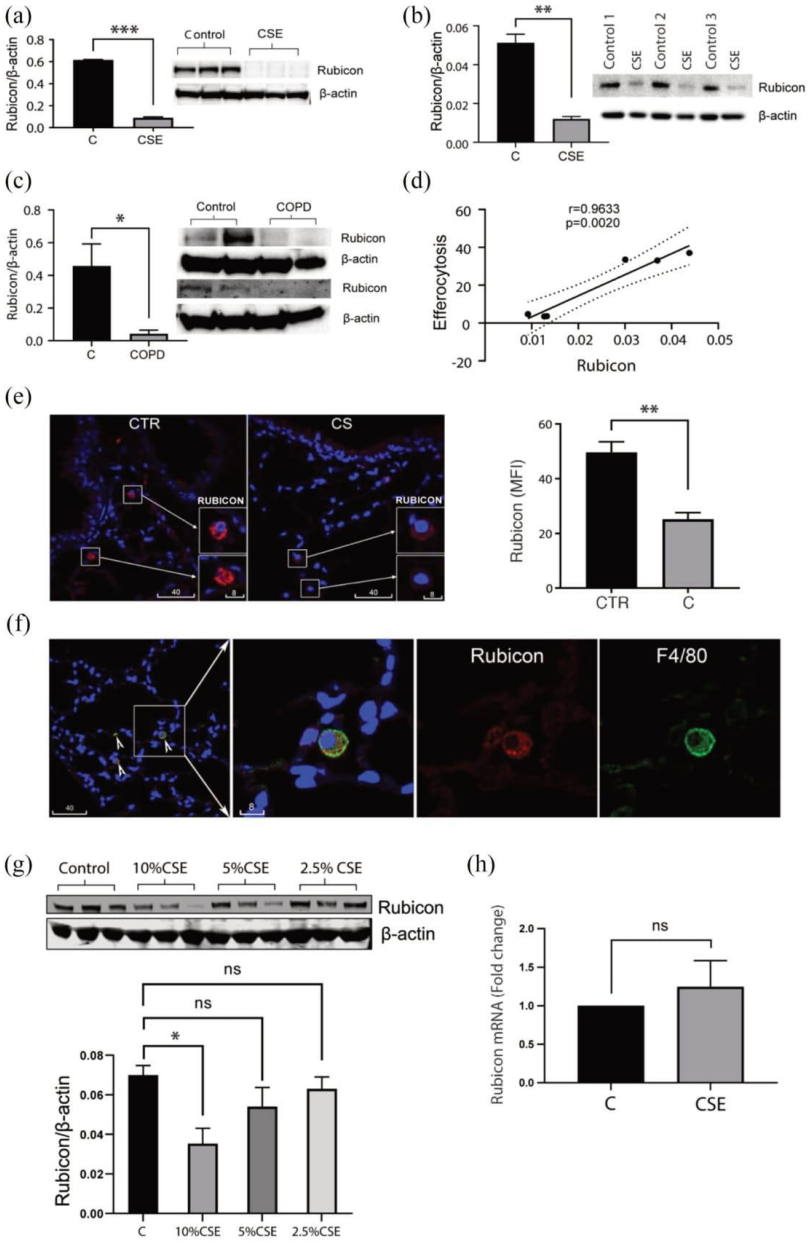

Figure 1. BAL macrophages of COPD patients and macrophages exposed to CSE exhibit a deficiency in Rubicon expression (a) Protein analysis of Rubicon protein expression; Control (C) vs cigarette smoke extract

Figure 1. (Continued)

(CSE). CSE reduced Rubicon expression in blood monocyte-derived macrophages ****$p < 0.0001$; mean ± 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 ± 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 ± 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 ± 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 = mean ± 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

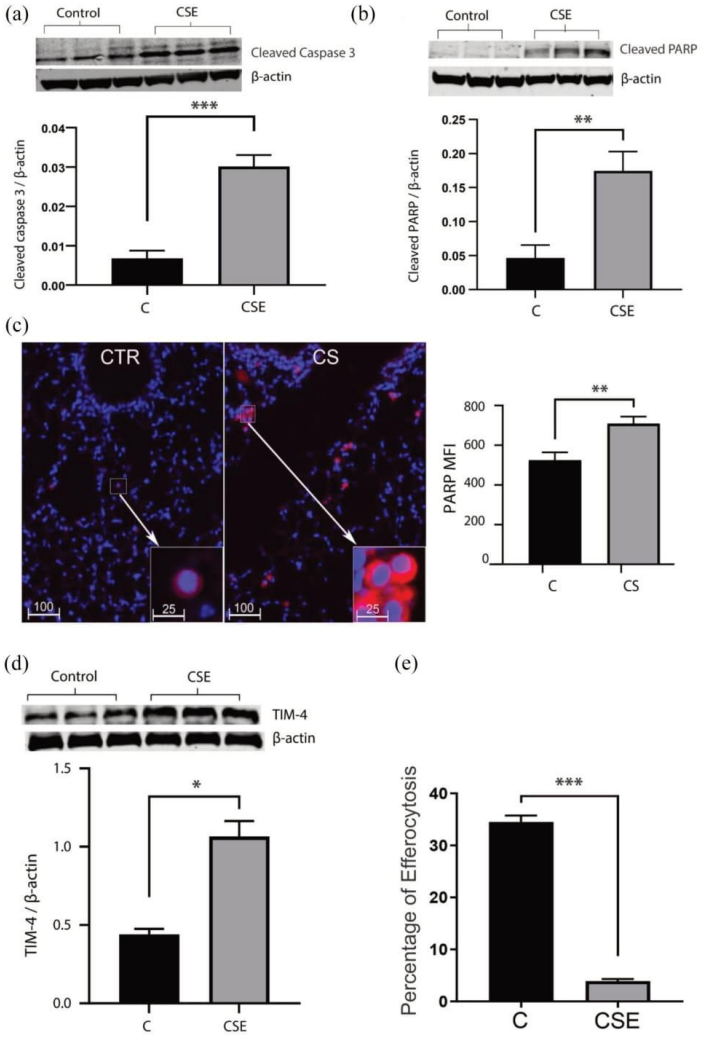

Figure 2. Exposure to cigarette smoke induces apoptosis: (a) Western blots (and quantitative data) of PARP protein expression; Control (C) vs Cigarette smoke extract (CSE). The expression of cleaved PARP in macrophages increased after 24 h of CSE treatment. $^{**}p = 0.0087$. Data are expressed as mean ± 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 ± 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 ± 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 ± 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 ± SEM, $n = 3$.

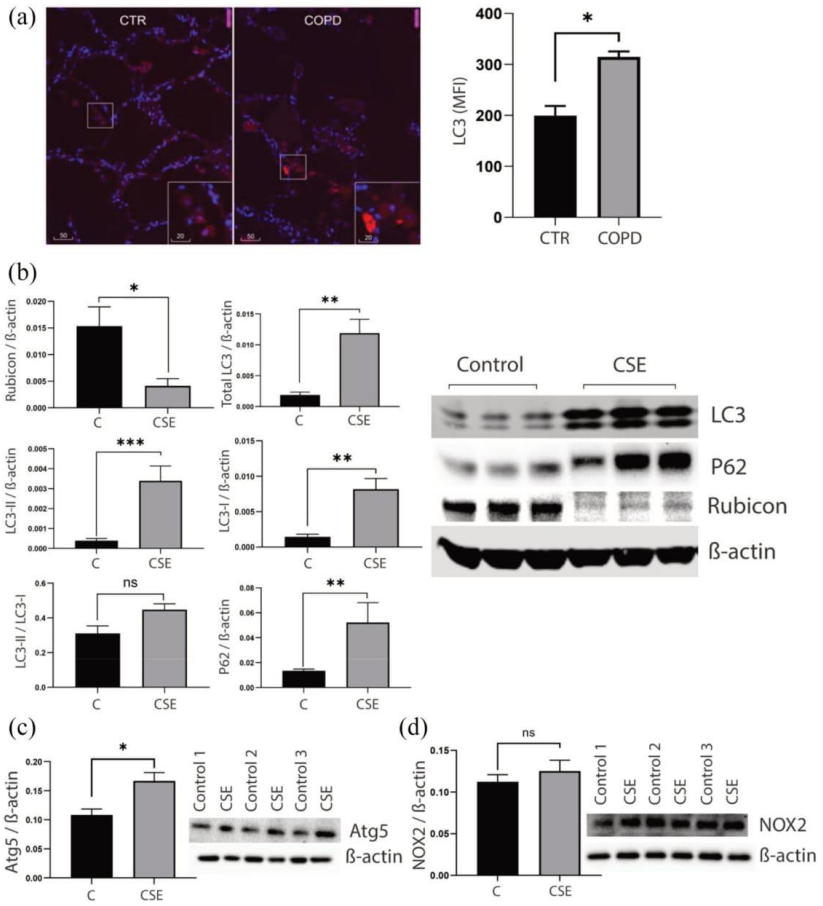

Figure 3. COPD lung macrophages exhibit features of defective LAP. (a) Representative immunofluorescence images of LC3 in lung macrophages from lobectomy biopsies of a non-COPD (CTR) vs COPD patient. The fluorescence intensity of LC3 in COPD lung macrophages is higher compared to those in control participants. *$p = 0.0118$, mean ± 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 ± 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 ± 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 ± 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 ± 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 ± SEM, $n = 6$.

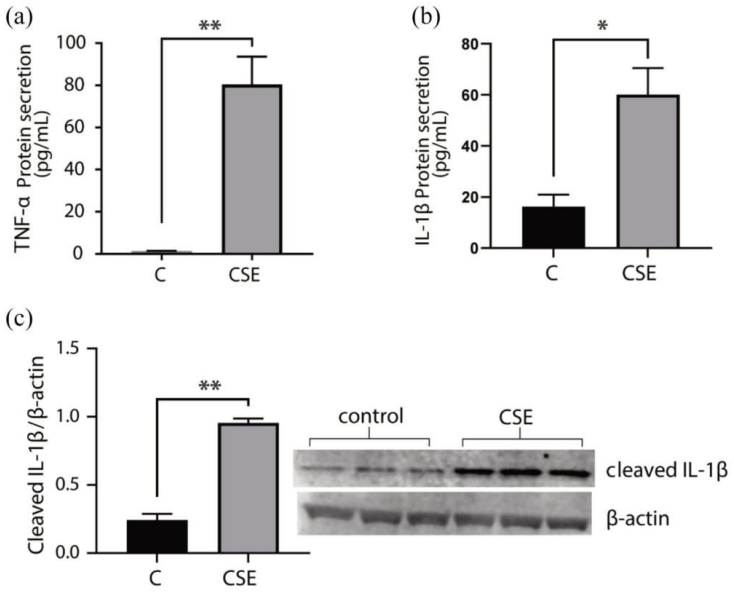

Figure 4. Cigarette smoke extract exposure increases pro-inflammatory marker levels. (a) ELISA results of increased secretion levels of TNF-α in THP-1 macrophages exposed to CSE for 24 h; Control (C) vs Cigarette smoke extract (CSE). Data are expressed as mean ± SEM, $n = 3$. $^{**}p = 0.0039$. (b) ELISA results of increased secretion levels of IL-1β in THP-1 macrophages exposed to CSE for 24 h; Control (C) vs cigarette smoke extract (CSE). Data are expressed as mean ± SEM, $n = 5$. $^{*}p = 0.0159$. (c) Western blot results showing higher expression of intracellular IL-1β in CSE exposed THP-1 macrophages after cigarette smoke exposure. Data are expressed as mean ± 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-1β 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 colleagues[30] 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 colleagues[34] 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-1β.[12,39,40] While abundance of Rubicon prevents inflammatory responses, Rubicon depleted mice produce markedly higher amount of TNF-α and IL-1β.[41–43] This is consistent with the observation of defective efferocytosis and a concurrent increase in TNF-α and IL-1β 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 *pneumonia* 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 reported[45] 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°C/5% CO_2 in RPMI 1640 medium containing 10% FBS, penicillin/streptomycin, 2 mM L-glutamine, and 0.05 mM ß-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°C/5% 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 FEV_1 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 use 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 manufactures instructions. To derive macrophages from monocytes, PBMC were seeded into plates at 1.4×10^6/mL in plain RPMI 1640 medium at 37°C/5% CO_2 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' (noncancer area from patients with cancer/no COPD) or 'COPD' (noncancer 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-a, 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 μg 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 μg 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 ± 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 $p < 0.05$.

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Conflict of interest statement

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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- Laborín 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. Willemse 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 β(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 interieukin-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 eIF2α. *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 Cα 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 Nat 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.

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REVIEW

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LC3-Associated Phagocytosis (LAP): A Potentially Influential Mediator of Efferocytosis-Related Tumor Progression and Aggressiveness

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One aim of cancer therapies is to induce apoptosis of tumor cells. Efficient removal ofthe apoptotic cells requires coordinated efforts between the processes of efferocytosis and LC3-associated phagocytosis (LAP). However, this activity has also been shownto produce anti-inflammatory and immunosuppressive signals that can be utilized by live tumor cells to evade immune defense mechanisms, resulting in tumor progressionand aggressiveness. In the absence of LAP, mice exhibit suppressed tumor growth during efferocytosis, while LAP-sufficient mice show enhanced tumor progression. Little is known about how LAP or its regulators directly affect efferocytosis, tumor growth and treatment responses, and identifying the mechanisms involved has the potential to lead tothe discovery of novel approaches to target cancer cells. Also incompletely understoodis the direct effect of apoptotic cancer cells on LAP. This is particularly important as induction of apoptosis by current cytotoxic cancer therapies can potentially stimulateLAP following efferocytosis. Herein, we highlight the current understanding of the role of LAP and its relationship with efferocytosis in the tumor microenvironment with a view to presenting novel therapeutic strategies.

Keywords: efferocytosis, tumor cell apoptosis, LAP, tumor immune response, M2 macrophage activation

INTRODUCTION

Induction of apoptotic cell death in tumors serves to promote the body's main defense against cancer. Therapeutic intervention that promotes the apoptotic death of tumor cells restricts theirgrowth, proliferation and survival and is a frontline strategy in anti-tumor therapy. However, apoptotic cells can directly (and indirectly) influence tumor progression and survival. Studies suggest that these effects are due to cellular events that are called in to remove dead or dying cellsfrom the tumor microenvironment. A significant consequence of these events is the production of anti-inflammatory and immunosuppressive signals that then can facilitate the growth and progression of tumor cells (1, 2). Therefore, a better understanding of the processes that lead tothe clearance of apoptotic cells may provide critical clues to help prevent the potential pro-tumoractions of dead or dying cells. Here we discuss two such cellular events; efferocytosis and LC3 Associated Phagocytosis (LAP).

Efferocytosis is a term coined by Peter Henson and colleagues in 2003 to describe the engulfment and clearance of cells undergoing apoptosis by phagocytes (3). Defects in efferocytosis can result in the rupture of uncleared apoptotic bodies and the subsequent leakage of cytosolic contents onto adjacentcells and exposure of tissues to oxidants, harmful enzymes and factors including caspases and other proteases (4, 5). This can induce inflammation and tissue damage through a process referred to as secondary necrosis (6, 7). Less understood is that during efferocytosis, signals from neighboring cells can initiate particular components of canonical autophagy to engage with the phagosome to increase the efficacy of the digestion of engulfed cellular cargo (8, 9). When components of autophagy are recruited to cargo-containing single membrane phagosomes to form LAPosome for digestion and clearance, the process is called LAP. Hence, LAP is a form of non-canonical autophagy that represents a hybrid between the processes governing the autophagic clearance of intracellular factors and those that accept extracellular cargos for degradation.

As the name suggests, a distinguishing feature of LAP is the recruitment of microtubule-associated protein light chain 3 (LC3) to the phagosome membrane after internalization of apoptotic cells or pathogens (10). In the normal situation, LAP is vital to resolve inflammation, promote wound healing and prevent auto-immunity and immune-mediated tissue damages (11, 12). Reports by Martinez et al. show that dysregulation of LAP impairs efferocytosis, culminating in necrosis of the phagocyte that has engulfed the uncleared cell and uncontrolled inflammation (12). These findings suggest that LAP may be essential for efferocytosis and preventing the many deleterious consequences of secondary necrosis. In support of this, Cunha and colleagues demonstrated that defects in LAP restricts tumor immunosuppression afforded by efferocytosis (13). Hence, these studies provide evidence that LAP may also be an influential contributor to the production of tolerogenic signals that are activated upon engulfment of apoptotic cells during efferocytosis. Martinez et al. further showed that in the absence of LAP, phagocytosis of apoptotic bodies still occurs but the vesicles remain in the phagocyte for a prolonged duration or are not digested, and points to a critical role of LAP in finalizing the intracellular digestion process (12). Hence, in the context of cancer, defects in LAP have paradoxically beneficial effects including the activation of tumor infiltrating lymphocytes (TIL), enhanced effector T cells activity and elevated secretion of inflammatory mediators including stimulator of interferon genes (STING), type I interferons (IFNs) and tumor necrosis factor alpha (TNF-α) (13). Taken together, these data suggest that, (1) LAP is critical for governing the digestion of the engulfed cellular debris and prevention of inflammatory and immune responses, (2) internalization of dead or dying cells alone is not solely responsible for immunosuppressive activities in the context of clearing apoptotic cells, and (3) LAP activation plays a critical role in immune tolerance. These recent findings of LAP as a concomitant effector of efferocytosis brings new significance to the processes that govern the clearance of apoptotic cells in the tumor microenvironment (TME), where there is frequent instances of programmed cell death. To that end, we discuss recent findings for the LAP signaling network as another mechanism that is hijacked by tumor cells to promote their growth and aggressiveness.

OVERVIEW AND MOLECULAR MECHANISM OF LAP DURING PHAGOCYTOSIS OF APOPTOTIC CELLS

Apoptotic cells are cleared in a tightly controlled and coordinated manner, to avoid damaging healthy tissues. The removal processes can be partitioned into four functional stages: (1) The release of chemoattractant "find me" signals (14) and "good-bye" metabolites from cells undergoing programmed cell death. "Find me" signals such as nucleotides (adenosine triphosphate; ATP and uridine triphosphate; UTP), sphingosine-1-phosphate (S1P), CX3C motif chemokine ligand 1 (CX3CL1 or fractalkine) and lysophosphatidylcholine (LPC) and "good-bye" metabolites including glycerol-3-phosphate (G3P), guanosine 5′-monophosphate (GMP), spermidine, adenosine monophosphate (AMP) and creatine (14) are released as demonstrated in **Figure 1A**. This secretory activity promotes the recruitment of phagocytes or neighboring cells to the area of cell death in the tissue compartment. (2) The expression of"eat me" signals on the surface of apoptotic cells regulates the recognition by the phagocyte, subsequent engulfment of the cellular debris and preparatory steps in the mechanisms governing phagosome formation (**Figure 1B**). (3) Recruitment of the LC3 conjugation system (**Figure 1C**) to the phagosome including autophagy related proteins and factors (Atg)12-Atg5-Atg16L1, Atg3, Atg7, NADPH oxidase 2 (NOX2), reactive oxygen species (ROS), Phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3 complex) comprising Beclin-1, Vacuolar protein sorting 34 (VPS34), and UV radiation resistance associated (UVRAG) and Rubicon (RUN domain Beclin-1- interacting and cysteine-rich domain-containing protein) for LAPosome formation (**Figure 2**) where the engulfed cellular debris can be digested. (4) Post-digestion activities (**Figure 1C**) including the release of inflammation-resolving cytokines, immune tolerogenic signals, and exportation of breakdown products into the cytosol for metabolic recycling that service intracellular biosynthetic pathways (15–18). This previously under-recognized phenomenon for efferocytosis; localization ofLC3 to phagosome, is now understood to be an essential step for lysosomal trafficking and selective immunologically silent removal of apoptotic cells (9, 12).

Finite steps involved in LAP are now being identified (**Figures 1B**, **2**). LAP is initiated upon engulfment of apoptotic cells that have externalized eat me signals such as phosphatidylserine (PtdSer) which binds to T-cell immunoglobulin and mucin domain family of receptors (TIM); TIM-1, TIM-3, and TIM-4 or stabilin-1, stabilin-2, andthe GPCR brain angiogenesis inhibitor 1 (BAI1). Alternatively, phagocytes can employ tyrosine kinase receptors (TYRO3, AXL, and Mer) also known as TAM receptors to bind to PtdSer indirectly through bridging molecules such as Gas-6 and Protein

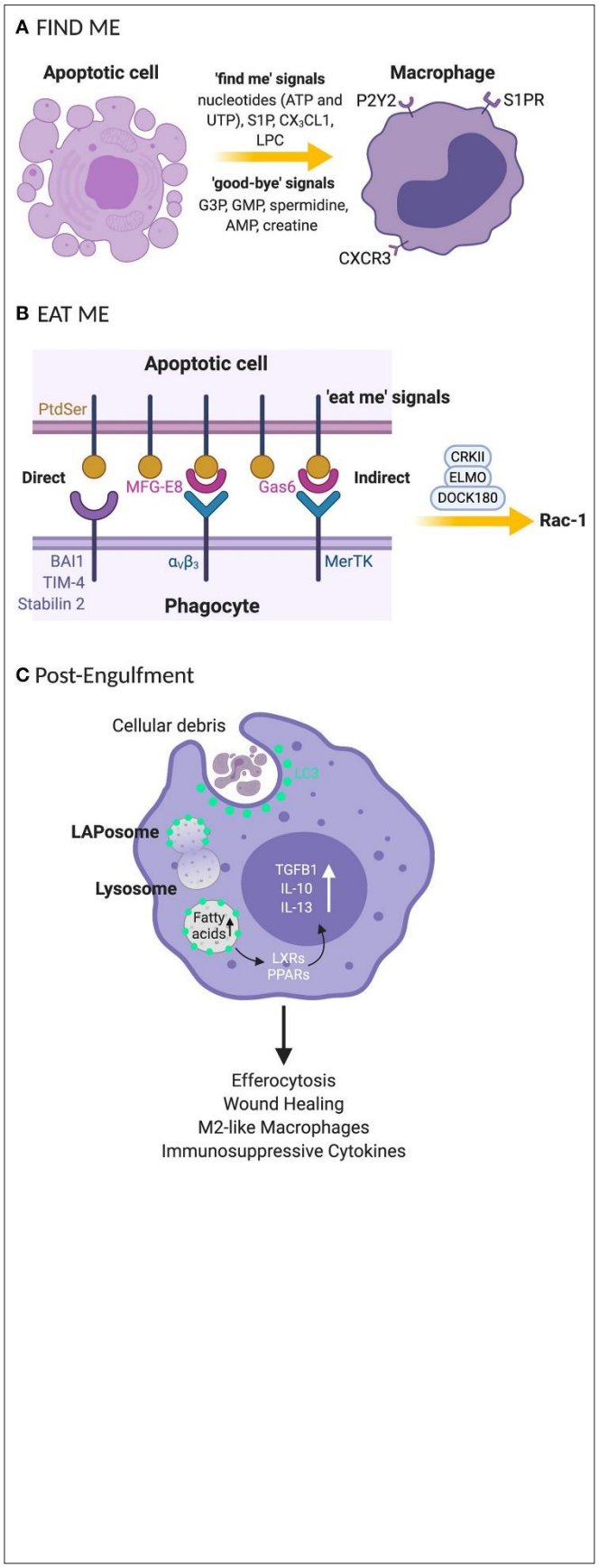
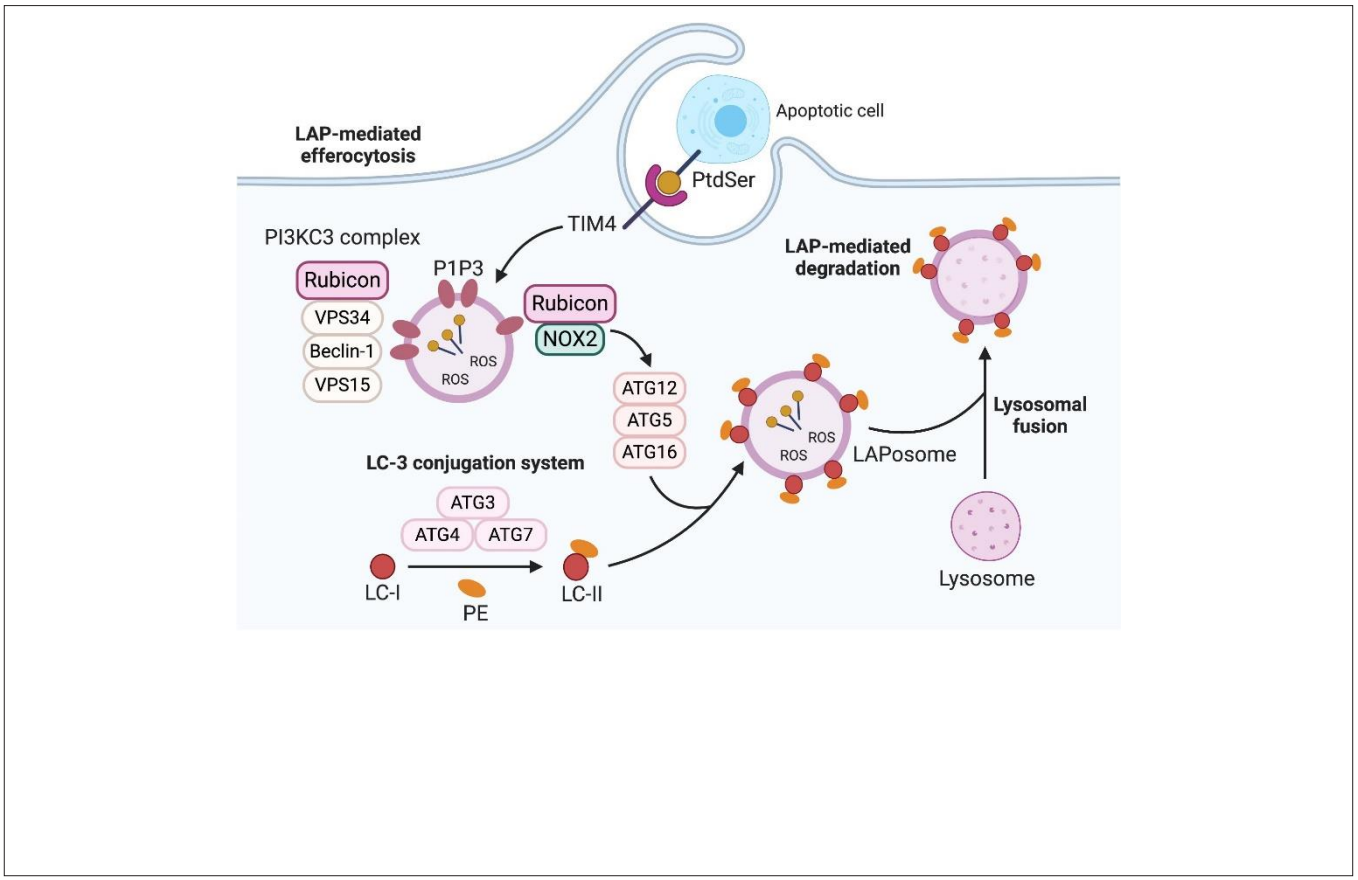


FIGURE 1 | Once the phagosome is formed, LC3 is recruited to phagosomes (now the LAPosome) leading to lysosome-mediated digestion of the internalized apoptotic body. Degraded products release fatty acids that stimulate LXR and PPAR*γ* for cholesterol efflux leading to the production of anti-inflammatory cytokines such as TGF-*β*, IL-10 and IL-13. UTP, uridine 5′ triphosphate; ATP, adenosine 5′ triphosphate; S1P, sphingosine-1-phosphate; CX3CL1, C-X3-C Motif Chemokine Ligand 1; LPC, lysophosphatidylcholine; P2Y2, purinergic receptors; SIPRs, sphingosine-1-phosphate receptors; CXCR3, C-X-C motif chemokine receptor 3; G2A, G-protein-coupled receptor; PtdSer, phosphatidylserine; BAI, brain-specific angiogenesis inhibitor 1; TIM-4, T cell immunoglobulin mucin receptor-4; avB3, alpha-v beta-3; mertk, mer proto-oncogene, tyrosine kinase; MFG-E8, milk fat globule-EGF factor 8 protein; Gas-6, growth arrest-specific 6; ELMO, engulfment and cell motility protein; DOCK, dedicator of cytokinesis; CRKII, chicken tumor virus no. 10 (CT10) regulator of kinase II; G3P, glycerol-3-phosphate guanosine; GMP, 5′-monophosphate; AMP, adenosine monophosphate; LC3, microtubule-associated protein 1A/1B-light chain 3; LXR, liver X receptor; PPAR*γ*, peroxisome proliferator-activated receptor gamma; TGF-*β*, transforming growth beta; IL-10, interleukin 10; IL-13, interleukin-13. *Created

S. Interaction via these receptors stimulates the CRKII-ELMO-DOCK180 complex within phagocytes to activate the rac-1 signaling pathway (**Figure 1B**) (19–22). This leads to cytoskeletal rearrangement and internalization of the apoptotic cell resulting in phagosome formation. Once internalized, the phagosome recruits Rubicon which facilitates the activity of a Class III PI3K complex containing UVRAG, but which lacks Atg14 and Ambra 1 (used in canonical autophagy) (23). Consequently, phosphatidylinositol 3-phosphate (PI3P) is generated on the fully formed and sealed phagosome during LAP (10, 24). The timing of PI3P generation during LAP is different from that of canonical autophagy where PI3P is generated in some portions of autophagophore for closure and autophagosome cup formation (11, 25). This process is necessary to stabilize the NOX2 complex, and thereby sustains the production of ROS which is crucial for recruiting the LC3 conjugation system components such as Atg5, Atg3, Atg12, Atg7, and Atg16. This leads to LC3 lipidation and its localization to the phagosome, as a necessary prerequisite for the formation of the LAPosome and to promote the fusion of lysosomes (**Figure 2**). The phagosomal content can then be efficiently processed via enzymatic digestion, and hence the immune response is better regulated to protect against autoimmunity and inflammation.

Rubicon plays a critical role as a mediator of LAP. Rubicon (via its binding to NOX2) favors the initiation of LAP- associated non-canonical autophagic activities over canonical autophagy (26), by reducing VPS34 lipid kinase activity, which also promotes the production of PI3P on LAPosomes rather than autophagosome (27–29). Hence, further requisites for LAP is NOX2 and the production of ROS. The NOX2 subunit p40phox binds to PI3P, effectively sequestering this factor as a consequence of Rubicon activity. If ROS production is dysregulated and p40phox subunit of NOX2 fails to associate with LAPosome, leading to LAP impairment (23). Hence, Rubicon-dependent production of PI3P is integral for these processes. Further to this, Yang et al. recently showed that Rubicon directly associates with the p22phox and gp91phox subunits of NOX2 to stabilize the complex for optimum and



continuous production of ROS (30). Cells or mice altered to lack Rubicon exhibit a destabilized NOX2 which thereby inhibits ROS necessary for LAP. The consequent of this is inefficient recruitment of essential LAP-related factors including ATG5, ATG7, and LC3-II to the membrane of phagosome. Moreover, cells deficient in NOX2 exhibit LAPosomes that incorporate PI3P, and exogenous induction of superoxides that serve to enhance LC3-II recruitment that initiates canonical autophagy instead of LAP (23, 29, 31). Hence, the proper regulation and expression of Rubicon is an integral part of LAP (i.e., PI3P produced via Rubicon-dependent pathways), and the interaction of Rubicon with NOX2 is a necessary event for this mode of non-canonical autophagy. As a result, Rubicon represents a new and attractive therapeutic target to specifically modulate LAP (vs. canonical autophagy) in diseases such as cancer. Considering that Rubicon and/or LAP forms a critical component of efferocytosis, wenext highlight correlative studies between tumor progression and efferocytosis.

EFFEROCYTOSIS IN THE TME PROMOTESTUMOR PROGRESSION AND METASTATICPOTENTIAL

Women diagnosed with breast cancer within 5 years of postpartum (which is characterized by massive cell death) have higher mortality rates, compared with nulliparous women (where cell death is relatively low) diagnosed with breast cancer (32–34). Additionally, breast cancer cells transplanted into involuting postpartum mammary glands grow and invade more rapidly than do cells in the mammary glands of a nulliparous host, even when corrected for age and histological grade (35, 36). This raises the possibility that conditions in the postpartum breast increase the aggressiveness of established tumors. Recently, massive cell death and the subsequent efferocytosis in postpartum involution have been identified as factors that are associated with the pro-tumoral and metastatic features of breast cancers in parous women (37). This is based on the observation that increased levels of efferocytosis occurs during postpartum involution and facilitates influx of wound-healing macrophages that are supportive of tumor progression and metastasis (37, 38).

Postpartum involution links the growth and metastasisof tumors to efferocytosis (37). This is consistent with the observation that efferocytosis receptors TIM-4 and MerTK are elevated in various cancer cells which is reported to correlate with disease severity (39, 40). In addition, a scenario in which cancer cells express MFG-E8 (41), NOX2 (42), PtdSer (21), in proximity to M2 macrophages (43) correlates with tumor aggressiveness. These observations suggest that phagocyte-homing in response to debris generated by tumor cells contributes to tumor- cell proliferation and may be heightened by the cytotoxic effects of anti-tumor therapies (44). Further, the promotion

of tumor cell death by radiotherapy and chemotherapy can also lead to increased expression of efferocytic receptors such as Mertk (45) and TIM-4 (46). These findings suggest that targeting efferocytosis activity in the surrounding tumor microenvironment may mitigate this phenomenon and heighten the efficacy of conventional anti-tumor therapies.

Efferocytosis of dead or dying tumor cells is a rapid process involving engulfment receptors required for efficient clearance of the cellular debris. Several studies have shown that blocking the incorporation of apoptotic cells by inhibiting engulfment receptors such as TIM-4, MFG-E8, MerTK and its ligands including Gas6 and protein S, is an effective strategy for the treatment of tumors (41, 47–49). An emerging area of study is the identification of targets critical for the degradation process of engulfed cargoes during efferocytosis. Internalization of apoptotic tumor cells *per se*, of course, does not influence tumor progression. Immune tolerogenic signals released by the digested apoptotic cells also contribute to a mechanism whereby tumors are able to evade clearance by lymphocytes and monocytes (13). LAP is a key mechanism involved in the final stages of clearing apoptotic tumor cells (13). LAP activity in dendritic cells and macrophages effectively clears cellular debris leading to the secretion of anti-inflammatory and immunosuppressive mediators (12, 50). Moreover, elevated expression of efferocytosis receptors by tumor-derived apoptotic cells, in the absence of LAP, can enhance tumor-progression. Hence, a more complete understanding of LAP in the phagocytes that home to the tumor microenvironment may offer new strategies for the management of cancer.

THE ROLE OF LAP IN TUMORPROGRESSION

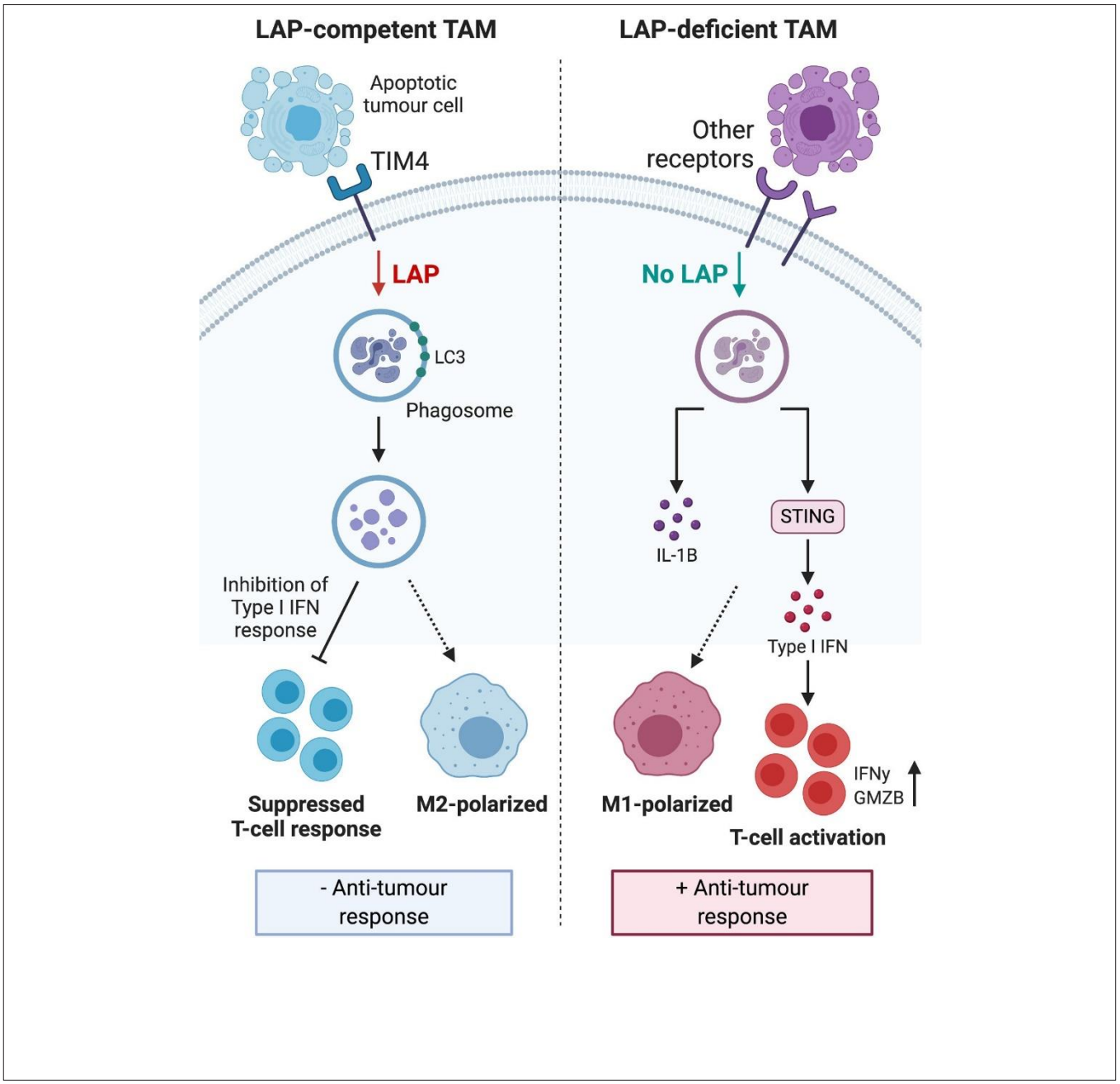
During efferocytosis, LAP facilitates the fusion of phagosome to lysosomes to enhance hydrolytic digestion and elimination of apoptotic cell constituents (23). Once apoptotic cells are digested in phagolysosomes, phagocytes become burdened with macromolecular digest components that they then either use in biosynthetic processes or efflux to the extracellular environment. An overload of cholesterol from degraded apoptotic cells stimulates members of the peroxisome proliferator-activated receptor gamma (PPAR*γ*) and liver X receptor (LXR) families of nuclear receptors as illustrated in **Figure 1**. These nuclear receptors inhibit pro-inflammatory cytokines and drive the polarization of M2-like phenotype which can mediate the production of anti-inflammatory cytokines. This is one important mechanism that reduces inflammatory signals following the removal apoptotic cells (51, 52), with immunogenic consequences including the suppression of tumor immune responses and increased resistance to immunotherapies.

LAP activity in dendritic cells and macrophages enhances phagocytic removal of pathogens and cells undergoing programmed cell death (9, 50). This is evidenced in LAP-deficient dendritic cells that exhibit impaired efferocytosis and elevated expression of major histocompatibility complex class I (50) that is needed for antigen presentation and anti-tumor immunity (50, 53). Moreover, macrophages and mice altered to obviate LAP, internalize and accumulate undigested apoptotic cells. These cells exhibit STING-dependent IFN responses, M1 polarization, pro-inflammatory mediators, granzyme B and enhanced anti-tumor immune response (11, 13, 54, 55). Further, DNA from apoptotic cells can activate STING and mediate immune recognition of tumor cells (56) which can also lead to interferonopathy and autoimmunity (57). This suggests that while LAP-dependent degradation of the DNA of engulfed apoptotic tumor cells counters the induction of inflammation and autoimmunity, it can also suppress anti-tumor immune responses. Hence, it follows that internalization of apoptotic material *per se*, does not lead to M2 polarization and immunosuppression. Rather it is the digested and degraded products of apoptotic cells that induce immune tolerogenic signals utilized by tumor cells to promote their growth and progression during efferocytosis. As illustrated in **Figure 3**, LAP facilitates digestion and removal of apoptotic tumor cells upon phagosomal engulfment that has unfortunate consequence of switching anti-tumor M1 macrophage phenotype to a pro-tumor M2 phenotype, leading to tumor progression and severity (13). This indicates that LAP participates in the regulatory events that form a complex network of interactions that collectively enhance anti-inflammatory and pro-tumorigenic effects of apoptotic cells and efferocytosis. Therefore, targeting LAP provides the attractive possibility of attenuating tumor progression and potentially serves as a target that could be therapeutically modulated to help prevent tumor aggressiveness.

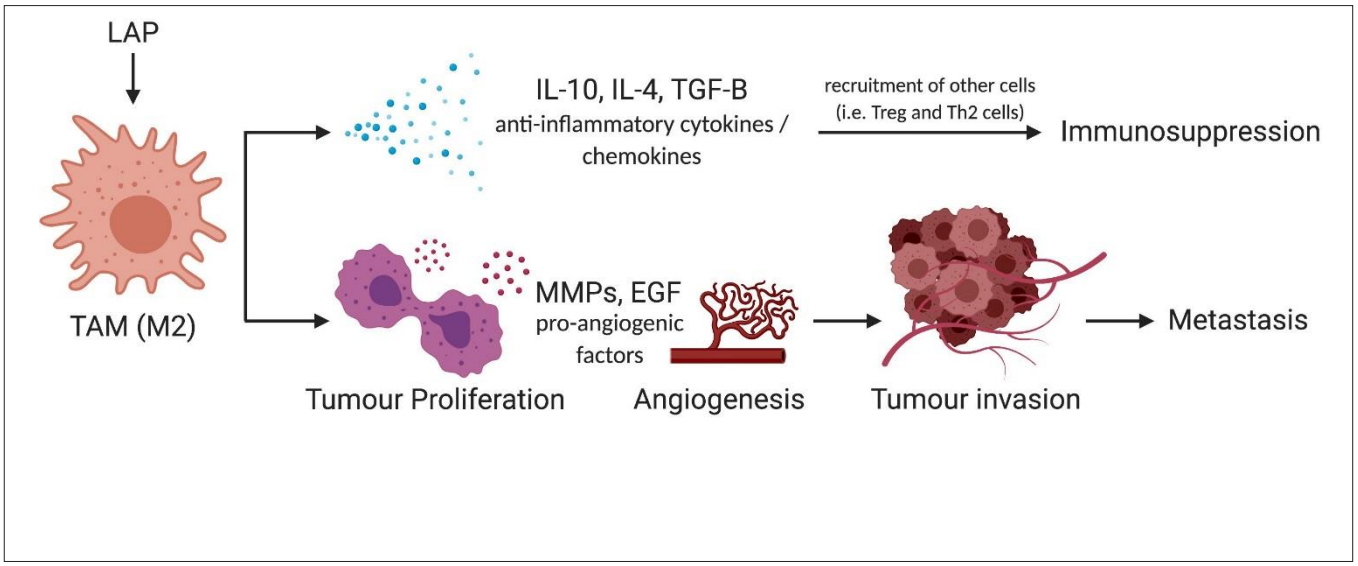
LAP CONTRIBUTES TO TUMOR PROGRESSION BY PROMOTING M2MACROPHAGE POLARIZATION

Macrophages are immune cells that are widespread throughout the body. They exhibit diverse functions including the regulation of inflammation, homeostasis, and tumor immunity. They are generally categorized into classically activated or M1 macrophages and alternatively activated or M2 macrophages. M1 macrophages are activated by Th1 lymphocytes, interferon-*γ* (IFN-*γ*), TNF-*α*, and natural killer cells (NK), while M2 macrophages are induced by cytokine signatures presented by Th2 cells such as interleukin-13 (IL-13), IL-10, and IL-4. Tumor-associated macrophages (TAMs) are usually triggered by factors that polarize macrophages to M2 phenotype and are often infiltrated into tumor sites to suppress the cytotoxic function of anti-tumor immune cells (43, 58, 59). This leads to dysregulation of tumor immune response contributing to the immunosuppressive tumor microenvironment. Indeed, observations from *in vitro* studies and clinical findings from cancer patients show that increased TAM infiltration correlates with poor prognosis (43). Accordingly, mitigating TAM infiltration, by blocking M2 activation or stimulating a pro-inflammatory M1 phenotype in TAMs, reduces tumor growth and metastasis (59–61).

Importantly, M2 macrophages promote tumor aggressiveness and progression. This phenomenon is exhibited through three



mechanisms as shown in **Figure 4**. Firstly, rapidly proliferating tumor cells that outpace the rate of blood supply need to establish new blood vessels through a process known as angiogenesis. M2 macrophages produce growth factors such as Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) to promote vascularization and allow highly proliferative tumors to obtain adequate blood supply (62–65). This enhances metastatic spread as it provides the principal route by which tumor cells exitthe primary tumor site and enter the circulation. Secondly,secretion of matrix metalloproteases by tumor-infiltrating M2 macrophages has been shown to contribute to the degradation of the Extra Cellular Matrix (ECM) which facilitates the invasion and spread of cancer cells to distant sites within the body and secondary metastasis (66). This phenomenon has been effectively described in human tongue, squamous cell, and colorectal cancer (67, 68). Thirdly, the production of anti-inflammatory cytokines and chemokines can also shift the immune response toward an immunotolerogenic phenotype, which is unable to sustain cell-mediated tumor immunity (69–71). This antagonizes activities of effector T cells and enhances tumor growth and survival, as they are spared from immune destruction. For these reasons, inhibition of M2 macrophages has shown therapeutic efficacy in the management of several tumors (59, 72, 73), and provides evidence that the characterization of factors that fine-tune M2



macrophage polarization can provide novel therapeutic options for promoting tumor immunity.

LAP has been identified as a new mechanism that participates in M2 polarization, and promotes an immunosuppressive environment that favors the tumor growth (13, 61, 75). Therefore, the involvement of LAP in macrophage function warrants further elucidation. This is evidenced by pro-tumor events that are established by LAP related to macrophage function. For example, (1) markers of tolerance and the polarization of macrophages to M2 phenotype is observed in LAP-sufficient tumor animal models (13, 76–78), (2) the M2 phenotype correlates with poor prognosis and promotes tumor progression (2, 79), and (3)LAP-sufficiency in tumor mouse models has been shown to accumulate M2 macrophages that support the pro-tumorigenic effects of TAMs (13). Therefore, M2 macrophage accumulation in tumors after chemotherapeutic treatment (80, 81) may be linked to LAP when it is called to enhance the efferocytosis of the apoptotic tumor cells. This causes the reduction of cytotoxic T cell activity to limit tumor immune responses and enhance the cancer cell proliferation (13, 82). Further, Cunha and colleagues showed that genetic or pharmacological inhibition of LAP overcomes some of the pro-tumor effects of TAMs e.g., by increasing CD8+ T cells function and reducing M2 macrophage production (13). These observations point toward an unrecognized target to combat the progression of tumors.

CHARACTERIZATION OF LAP AS A DISTINCT PATHWAY REVEALS A NEW THERAPEUTIC APPROACH TO CONTROLTUMOR GROWTH

A current aim for cancer therapy is to target autophagyin the tumor microenvironment (83, 84). For example, the translocation of LC3-II to membranous structures, considered to be a hallmark for autophagy, has been associated with tumor progression (85). Further, inhibition of LC3 recruitment can potentiate anti-tumor immune response and reduce tumor growth (86, 87). Recent discovery that LC3 is recruited to phagosome membranes via LAP provides evidence that at least some pro-tumor functions of LC3 recruitment may bespecifically linked to LAP (10, 13). Indeed, pro-tumoral qualities of some autophagy components have been associated with LC3 recruitment to phagosome membranes via LAP. Noteworthy, translocation of LC3 to phagosomes is a reliable marker for LAP, that distinguishes it from processes related to canonical autophagy (23). Also, while LAP involves the recruitmentof LC3 to single phagosome membranes (10, 88), autophagy utilizes LC3-enriched double membrane autophagosomes (89). Hence, delineating between these two processes can be used to identify unrecognized roles and contribution of LAP in tumor progression.

A critical distinction between LAP and autophagy is that while LAP requires Beclin-1, PI3KC3 complex, Atg5 and Atg7for recruitment of LC3 to membranous structures, unlike autophagy, LAP does not require Unc51-like kinase 1 complex (ULK-1) or FAK family–interacting protein of 200 kDa (FIP200) (90–93). Moreover, while both LAP and autophagy require mediators such as SLAM (signaling lymphocyte-activation molecule) receptors to interact with the PI3KC3 complexto regulate phagosome and autophagosome maturation, respectively, unlike autophagy, LAP employs Rubicon-containing PI3KC3 and does not require Atg14 or ULK-1to mediate LC3 recruitment process (23, 26). In fact, inhibiting Rubicon specifically block LAP without inhibiting canonical autophagy process.

Moreover, even though VPS34 activity is required for both autophagy and LAP, VPS34 produced in the absence of Rubicon triggers autophagy instead of LAP (23, 31). Conversely, Rubicon swaps VPS34 activity on autophagosome for LAPosme (23, 26, 94). This again characterizes Rubicon as an indispensable and specific component of LAP. These characterisations of LAP as a distinct mechanism provides a new avenue to regulate tumor growth without interfering with canonical autophagy processes.

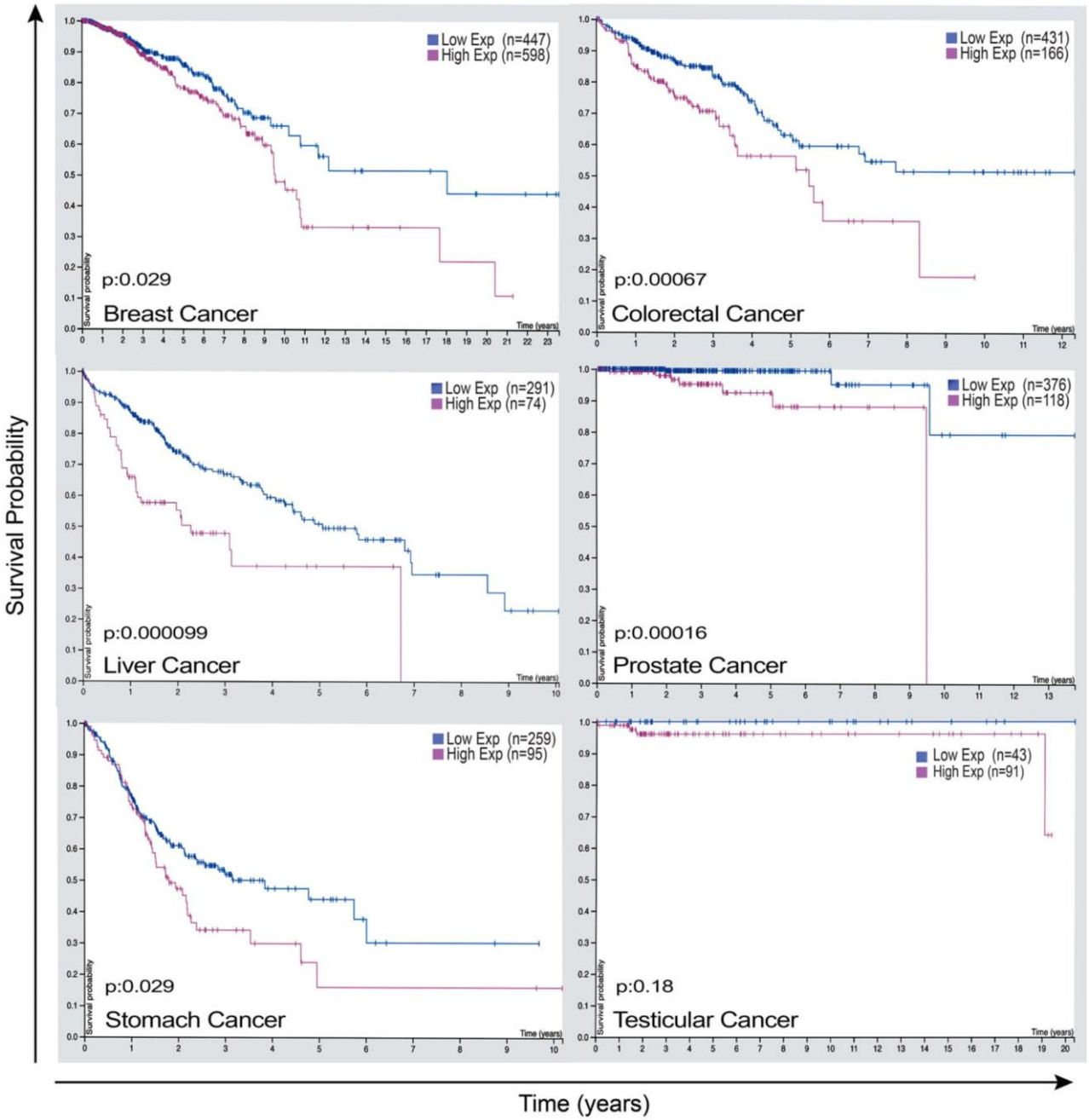


FIGURE 5 | Rubicon expression as a potential prognostic marker that has the power to predict survival outcome of some cancer patients. Representative images of Rubicon expression and survival rate of patients with breast cancer, colorectal cancer, liver cancer, prostate cancer, stomach cancer, and testicular cancer. Cancer patients overexpressing Rubicon have lower survival rate compared with patients with lower Rubicon expression. This demonstrates the need for further studies to establish prognostic values of Rubicon in different types of cancers. Credit: Human Protein Atlas, www.proteinatlas.org/humancell (95). Image available at the following URL: v19.proteinatlas.org/humancell.

TARGETING CORE COMPONENTS OF LAPIN TUMORS

Given that LAP elicits pro-tumor effects in the context of cancer, cancer cells expression of factors that lead to these phenomena may have significant therapeutic value. The expression of Rubicon is elevated in cancer and is strikingly associated with poor prognosis in patients with breast cancer, endometrial cancer, testicular cancer, liver cancer, colorectal cancer, and stomach cancer as shown in **Figure 5** (95). This clear link

between the abundance of Rubicon and poor prognosis of cancer (**Figure 5**), calls for a better understanding of the events associated with the pro-tumor effects of Rubicon to inform prognostic and therapeutic investigations. What is certain is that Rubicon is associated with LAP and is a requisite member of the LAP pathway (27). Further, Rubicon is upregulated following internalization of apoptotic cells to recruit LC3-conjugation system for LAP initiation and subsequent degradation of apoptotic cells (23). This supports the view that Rubicon expression forms a critical component of the processes required to remove apoptotic cells. In fact, inhibition of Rubicon in preclinical settings enhances immune activation and restricts tumor growth by ablating LAP-mediated apoptotic cell removal. Conversely, Rubicon expression increases tumor progression and limits immune responses through LAP induction (13). This suggests that LAP may be an influential pathway through which Rubicon mediates tumor progression and decreases survival rate in some cancer patients.

PtdSer is another integral component of LAP induction and efferocytosis (9, 96). It is usually expressed on the surface of apoptotic cells to allow phagocytes to remove the dead or dying cells through efferocytosis and LAP (21, 97). It can be predicted that its inhibition may ablate LAP and therefore many pro-tumorigenic consequences as shown in **Figure 6**. For example, pre-clinical models of B16F10 melanoma demonstrates that inhibiting PtdSer receptor, TIM-4 is an effective approach to reduce LAP and tumor growth (13). Indeed, expression of PtdSer on the surface of apoptotic cell is an early event during apoptosis and is markedly increased during chemotherapeutic treatment of tumors (98). PtdSer is also overexpressed in different cancer types such as glioblastoma, breast cancer and astrocytoma (99, 100). Its inhibition could lead to LAP impairment and subsequently result in a decrease in M2-like tumor associated macrophages and a switch in the expression of immunosuppressive cytokines to immunostimulatory cytokines (98). Indeed, Bavituximab which blocks PtdSer is providing beneficial outcomes for some cancers in phase II clinical trials (101, 102).

Moreover, several clinical studies are in progress to design therapies that may have the potential to prevent the impact of LAP in the tumor microenvironment. These include Blocking TGF-β signaling to inhibit the growth and metastasis of orthotopic mammary carcinoma (103). TGF-β is usually upregulated and is integral for tumor immunosuppressive effects of LAP (11, 13, 82), suggesting that targeting factors involved inthe initiation of LAP as well as its resultant effects could be an effective strategy for tumor destruction.

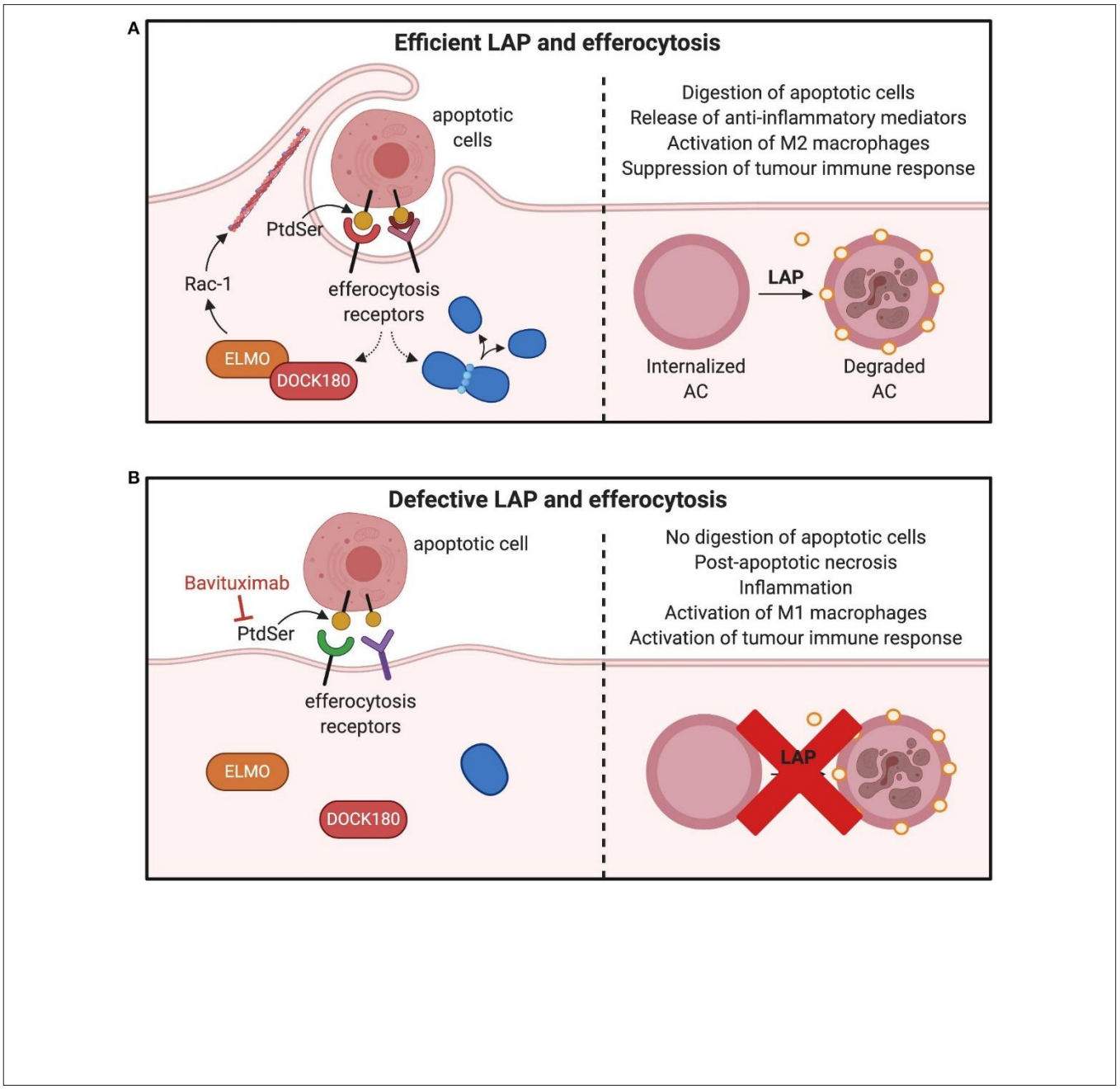
IL-10, a further anti-inflammatory cytokine, is produced by both M2 macrophages (104) and by the tumor cells (105, 106). IL-10 can promote tumor growth and the clinical utility of inhibiting this effect is widely reported (107–109). However, the precise mechanism that informs IL-10 overexpression and its pro-tumoral qualities remains to be completely elucidated. Data from experiment settings link overexpression of IL-10 to the LAP pathway and implicate this association in LAP-mediated tumor progression (13). This underscores the role of LAP plays in IL- 10 expression and provides a potential target to regulating the pro-tumor cytokine in tumor cells. Noteworthy, other biological events may also contribute to IL-10 mediated tumor progression. A better understanding of LAP as an influential driver in this regard is crucial to discover a novel approach to block the immunosuppressive and pro-tumor effects of IL-10 for effective clinical management of tumors.

V-ATPase activity has recently been shown to be required for LAP, and inhibiting V-ATPase following apoptotic cell death prevents LAP by blocking lipidation of LC3 (110, 111). This provides a further potentially novel therapeutic target for regulating LAP. Additionally, studies by Fletcher et al. suggest that WD repeat-containing C-terminal domain of ATG 16L1 inhibition arrests LAP (112). Blunting WD repeat-containing C-terminal domain (WD 40 CTD) of ATG 16L1 blocks LC3 recruitment to endolysosomal membranes during LAP without affecting canonical autophagy (112, 113).

The mode of cell death has a significant impact on the immune tolerogenic effects of LAP. Non-apoptotic cell death such as necrotic and necroptotic cells can also induce LAP to complete the efferocytosis process (9). In contrast to apoptotic cells, necrotic and necroptotic cells induce inflammatory and immunostimulatory cytokines (114, 115) and can switch macrophages from pro-tumoral M2 phenotype to anti-tumoral and immunostimulatory M1 phenotype (116, 117). This is because the extra cholesterol produced after the degradation of necrotic cells does not stimulate cholesterol efflux pathway (118) which is required for the release of anti-inflammatory cytokines essential for M2 phenotype switching (119, 120). This provides another promising approach for circumventing the pro-tumoral effects of LAP. Noteworthy, induction of non-apoptotic modalities of cell death such as necrosis or necroptosis as a new therapeutic direction to potentiate anti-tumor immunity has been suggested as a promising alternative (117, 121–123). Therefore, further studies in this regard are warranted especially as the effectiveness of non-apoptotic form of tumor cell death has not been explicitly elucidated.

CONCLUDING REMARKS

A common effect of anticancer therapies is to induce apoptosis of cancer cells. However, there is a surprising paucity of information that considers the complex processes that are responsible for clearing the cellular debris. In line with this, there is emerging evidence that implicates the non-canonical autophagy pathway, LAP, as an essential component involved in the coordinated clearance of apoptotic cancer cells. However, unlike canonical autophagy and phagocytosis, LAP-mediated efferocytosis elicits an anti-inflammatory response in the surrounding environment that may have significant implications for the evasion of immune surveillance by cells involved in tumorigenesis. Indeed, it has been shown that mice altered to abrogate LAP as a part of the cell's clearance response exhibit a reduction in tumor growth, while the restoration of LAP influences tumor progression. Further, cancer tissues overexpression of Rubicon predicts adverse overall survival in many cancer patients (95). These findings have several clinical implications: (1) Rubicon expression in tumor tissues comprising infiltrated TAMs and



other phagocytic cells can lead to modulation of LAP pathway in the TME (13). This implies that LAP can specifically be targeted in the TME to restrict tumor growth. (2) Rubicon expression could be a prognostic marker in different types of cancers and (3) Therapies that could inhibit Rubicon expression could be incorporated into clinical trials as drugs that regulate LAP during phagocytosis of apoptotic tumor cells. However, to the best of our knowledge, there is no drug at present that could specifically target Rubicon. Therefore, these findings point to a need to clearly determine how LAP is regulated during the efferocytosis of apoptotic tumor cells and whether alterations implicit with uncontrolled carcinogenesis favor the survival of cells that employ LAP to establish tumors of clinical importance. If so, perhaps a more immediate priority is to identify those therapeutic options which unwittingly potentiate cancer by overbalancing the recruitment of phagocytes and/or that directly promote LAP in cancer cells themselves. Given that LAP functionsto regulate inflammation and undesired immune responses, inhibition of LAP while restricting tumor growth has the potential to result in autoimmunity and chronic inflammation (12). This provides a worthwhile consideration when inhibiting LAP to control tumor progression. Herein, we discuss the current

understanding of LAP during the development of cancer, its relationship with efferocytosis in the tumor microenvironment, and suggest strategies that may inform current and novel therapeutic approaches.

AUTHOR CONTRIBUTIONS

PA drafted the manuscript and revised it critically for important intellectual content. ER, PH, HT, and CM revised manuscript critically for important intellectual content. SH revised manuscript critically for important intellectual content and have final approval for submission.

All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Kumar S, Calianese D, Birge RB. Efferocytosis of dying cells differentially modulate immunological outcomes in tumor microenvironment. *Immunol Rev.* (2017) 280:149–64. doi: 10.1111/imr.12587
2. Jones JD, Sinder BP, Paige D, Soki FN, Koh AJ, Thiele S, et al. Trabectedin reduces skeletal prostate cancer tumor size in association witheffects on M2 macrophages and efferocytosis. *Neoplasia.* (2019) 21:172– 84. doi: 10.1016/j.neo.2018.11.003
3. de Cathelineau AM, Henson PM. The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. *Essays Biochem.* (2003) 39:105–17. doi: 10.1042/bse0390105
4. Sarode GS. Efferocytosis in oral squamous cell carcinoma. *J Oral Maxillofac Pathol.* (2016) 20:170–2. doi: 10.4103/0973-029X.185918
5. Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest.* (2006) 129:1673–82. doi: 10.1378/chest.129.6.1673
6. Proto JD, Doran AC, Gusarova G, Yurdagul A Jr, Sozen E, Subramanian M, et al. Regulatory T cells promote macrophage efferocytosis during inflammation resolution. *Immunity.* (2018) 49:666–77.e6. doi: 10.1016/j.immuni.2018.07.015
7. Tao H, Yancey PG, Babaev VR, Blakemore JL, Zhang Y, Ding L, et al. Macrophage SR-BI mediates efferocytosis via Src/PI3K/Rac1 signaling and reduces atherosclerotic lesion necrosis. *J Lipid Res.* (2015) 56:1449– 60. doi: 10.1194/jlr.M056689
8. Green DR, Oguin TH, Martinez J. The clearance of dying cells: table for two. *Cell Death Differ.* (2016) 23:915–26. doi: 10.1038/cdd.2015.172
9. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, et al. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proc Natl Acad Sci USA.* (2011) 108:17396–401. doi: 10.1073/pnas.1113421108
10. Sanjuan MA, Dillon CP, Tait SWG, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature.* (2007) 450:1253–7. doi: 10.1038/nature06421
11. Heckmann BL, Boada-Romero E, Cunha LD, Magne J, Green DR. LC3-associated phagocytosis and inflammation. *J Mol Biol.* (2017) 429:3561– 76. doi: 10.1016/j.jmb.2017.08.012
12. Martinez J, Cunha LD, Park S, Yang M, Lu Q, Orchard R, et al. Noncanonical autophagy inhibits the autoinflammatory, lupus-like response to dying cells. *Nature.* (2016) 533:115–9. doi: 10.1038/nature17950
13. Cunha LD, Yang M, Carter R, Guy C, Harris L, Crawford JC, et al. LC3-associated phagocytosis in myeloid cells promotes tumor immune tolerance. *Cell.* (2018) 175:429–41.e16. doi: 10.1016/j.cell.2018.08.061
14. Medina CB, Mehrotra P, Arandjelovic S, Perry JSA, Guo Y, Morioka S, et al. Metabolites released from apoptotic cells act as tissue messengers. *Nature.* (2020) 580:130–5. doi: 10.1038/s41586-020-2121-3
15. Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, et al. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. *Nature.* (2010) 467:863–7. doi: 10.1038/nature09413
16. Roszer T, Menéndez-Gutiérrez MP, Lefterova MI, Alameda D, Núñez V, Lazar MA, et al. Autoimmune kidney disease and impaired engulfment of apoptotic cells in mice with macrophage peroxisome proliferator-activated receptor gamma or retinoid X receptor alpha deficiency. *J Immunol.* (2011) 186:621–31. doi: 10.4049/jimmunol.1002230
17. Yurdagul A Jr, Subramanian M, Wang X, Crown SB, Ilkayeva OR, Darville L, et al. Macrophage metabolism of apoptotic cell-derived arginine promotes continual efferocytosis and resolution of injury. *Cell Metab.* (2020) 31:518–33.e10. doi: 10.1016/j.cmet.2020.01.001
18. Zhang S, Weinberg S, DeBerge M, Gainullina A, Schipma M, Kinchen JM, et al. Efferocytosis fuels requirements of fatty acid oxidation and the electron transport chain to polarize macrophages for tissue repair. *Cell Metab.* (2019) 29:443–56.e5. doi: 10.1016/j.cmet.2018.12.004
19. Gumienny TL, Brugnera E, Tosello-Trampont AC, Kinchen JM, Haney LB, Nishiwaki K, et al. CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. *Cell.* (2001) 107:27–41. doi: 10.1016/S0092-8674(01)00520-7
20. Grabiec AM, Denny N, Doherty JA, Happonen KE, Hankinson J, Connolly E, et al. Diminished airway macrophage expression of the Axl receptor tyrosine kinase is associated with defective efferocytosis in asthma. *J Allergy Clin Immunol.* (2017) 140:1144–6.e4. doi: 10.1016/j.jaci.2017.03.024
21. Nishi C, Yanagihashi Y, Segawa K, Nagata S. MERTK tyrosine kinase receptor together with TIM4 phosphatidylserine receptor mediates distinct signal transduction pathways for efferocytosis and cell proliferation. *J Biol Chem.* (2019) 294:7221–30. doi: 10.1074/jbc.RA118.006628
22. Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. *Nature.* (2007) 450:430–4. doi: 10.1038/nature06329
23. Martinez J, Malireddi RKS, Lu Q, Cunha LD, Pelletier S, Gingras S, et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. *Nat Cell Biol.* (2015) 17:893–906. doi: 10.1038/ncb3192
24. Ellson CD, Anderson KE, Morgan G, Chilvers ER, Lipp P, Stephens LR, et al. Phosphatidylinositol 3-phosphate is generated in phagosomal membranes. *Curr Biol.* (2001) 11:1631–5. doi: 10.1016/S0960-9822(01)00447-X
25. Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, et al. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol.* (2008) 182:685–701. doi: 10.1083/jcb.200803137
26. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N,et al. Two beclin 1-binding proteins, Atg14L and rubicon, reciprocally regulate autophagy at different stages. *Nat Cell Biol.* (2009) 11:385– 96. doi: 10.1038/ncb1846
27. Wong SW, Sil P, Martinez J. Rubicon: LC3-associated phagocytosis and beyond. *FEBS J.* (2018) 285:1379–88. doi: 10.1111/febs.14354
28. Masud S, van der Burg L, Storm L, Prajsnar TK, Meijer AH. Rubicon-dependent Lc3 recruitment to salmonella-containing phagosomes is a host defense mechanism triggered independently from major

bacterial virulence factors. *Front Cell Infect Microbiol.* (2019) 9:279. doi: 10.3389/fcimb.2019.00279

29. Boyle KB, Randow F. Rubicon swaps autophagy for LAP. *Nat Cell Biol.* (2015) 17:843–5. doi: 10.1038/ncb3197
30. Yang CS, Lee JS, Rodgers M, Min C-K, Lee J-Y, Kim HJ, et al. Autophagy protein rubicon mediates phagocytic NADPH oxidase activation in response to microbial infection or TLR stimulation. *Cell Host Microbe.* (2012) 11:264–76. doi: 10.1016/j.chom.2012.01.018
31. Sun Q, Zhang J, Fan W, Wong KN, Ding X, Chen S, et al. The RUN domain of rubicon is important for hVps34 binding, lipid kinase inhibition, autophagy suppression. *J Biol Chem.* (2011) 286:185–91. doi: 10.1074/jbc.M110.126425
32. Callihan EB, Gao D, Jindal S, Lyons TR, Manthey E, Edgerton S, et al. Postpartum diagnosis demonstrates a high risk for metastasis and merits an expanded definition of pregnancy-associated breast cancer. *Breast Cancer Res Treat.* (2013) 138:549–59. doi: 10.1007/s10549-013-2437-x
33. van den Rul N, Han SN, Van Calsteren K, Neven P, Amant F. Postpartum breast cancer behaves differently. *Facts Views Vis Obgyn.* (2011) 3:183–8.
34. Goddard ET, Bassale S, Schedin T, Jindal S, Johnston J, Cabral E, et al. Association between postpartum breast cancer diagnosis and metastasis and the clinical features underlying risk. *JAMA Netw Open.* (2019) 2:e186997. doi: 10.1001/jamanetworkopen.2018.6997
35. Schedin P, O'Brien J, Rudolph M, Stein T, Borges V. Microenvironment of the involuting mammary gland mediates mammary cancer progression. *J Mammary Gland Biol Neoplasia.* (2007) 12:71– 82. doi: 10.1007/s10911-007-9039-3
36. Daling JR, Malone KE, Doody DR, Anderson BO, Porter PL. The relation of reproductive factors to mortality from breast cancer. *Cancer Epidemiol Biomarkers Prev.* (2002) 11:235–41.
37. Stanford JC, Young C, Hicks D, Owens P, Williams A, VaughtDB, et al. Efferocytosis produces a prometastatic landscape during postpartum mammary gland involution. *J Clin Invest.* (2014) 124:4737–52. doi: 10.1172/JCI76375
38. Martinson HA, Jindal S, Durand-Rougely C, Borges VF, Schedin P. Wound healing-like immune program facilitates postpartum mammary gland involution and tumor progression. *Int J Cancer.* (2015) 136:1803–13. doi: 10.1002/ijc.29181
39. Wang Y, Moncayo G, Morin P Jr, Xue G, Grzmil M, Lino MM, et al. Mer receptor tyrosine kinase promotes invasion and survival in glioblastoma multiforme. *Oncogene.* (2013) 32:872–82. doi: 10.1038/onc.2012.104
40. Tan X, Zhang Z, Yao H, Shen L. Tim-4 promotes the growth of colorectal cancer by activating angiogenesis and recruiting tumor-associated macrophages via the PI3K/AKT/mTOR signaling pathway. *Cancer Lett.* (2018) 436:119–28. doi: 10.1016/j.canlet.2018.08.012
41. Soki FN, Koh AJ, Jones JD, Kim YW, Dai J, Keller ET, et al. Polarization of prostate cancer-associated macrophages is induced by milk fat globule-EGF factor 8 (MFG-E8)-mediated efferocytosis. *J Biol Chem.* (2014) 289:24560–72. doi: 10.1074/jbc.M114.571620
42. Harrison IP, Vinh A, Johnson IRD, Luong R, Drummond GR, SobeyCG, et al. NOX2 oxidase expressed in endosomes promotes cell proliferation and prostate tumour development. *Oncotarget.* (2018) 9:35378– 93. doi: 10.18632/oncotarget.26237
43. Jackute J, Zemaitis M, Pranys D, Sitkauskiene B, Miliauskas S, Vaitkiene S, et al. Distribution of M1 and M2 macrophages in tumor islets and stroma in relation to prognosis of non-small cell lung cancer. *BMC Immunol.* (2018) 19:3. doi: 10.1186/s12865-018-0241-4
44. Zhou Y, Yao Y, Deng Y, Shao A. Regulation of efferocytosis as a novel cancer therapy. *Cell Commun Signal.* (2020) 18:71. doi: 10.1186/s12964-020-00542-9
45. Crittenden MR, Baird J, Friedman D, Savage T, Uhde L, Alice A, et al. Mertk on tumor macrophages is a therapeutic target to prevent tumor recurrence following radiation therapy. *Oncotarget.* (2016) 7:78653–66. doi: 10.18632/oncotarget.11823
46. Baghdadi M, Yoneda A, Yamashina T, Nagao H, Komohara Y, Nagai S,et al. TIM-4 glycoprotein-mediated degradation of dying tumor cells by autophagy leads to reduced antigen presentation and increased immune tolerance. *Immunity.* (2013) 39:1070–81. doi: 10.1016/j.immuni.2013.09.014
47. DeRyckere D, Lee-Sherick AB, Huey MG, Hill AA, Tyner JW, Jacobsen KM, et al. UNC2025, a MERTK small-molecule inhibitor, is therapeutically effective alone and in combination with methotrexate in leukemia models. *Clin Cancer Res.* (2017) 23:1481–92. doi: 10.1158/1078-0432.CCR-16-1330
48. Cook RS, Jacobsen KM, Wofford AM, de Ryckere D, Stanford J, Prieto AL, et al. MerTK inhibition in tumor leukocytes decreases tumor growth and metastasis. *J Clin Invest.* (2013) 123:3231–42. doi: 10.1172/JCI67655
49. Loges S, Schmidt T, Tjwa M, van Geyte K, Lievens D, Lutgens E,et al. Malignant cells fuel tumor growth by educating infiltrating leukocytes to produce the mitogen Gas6. *Blood.* (2010) 115:2264–73. doi: 10.1182/blood-2009-06-228684
50. Sil P, Suwanpradid J, Muse G, Gruzdev A, Liu L, Corcoran DL,et al. Noncanonical autophagy in dermal dendritic cells mediates immunosuppressive effects of UV exposure. *J Allerg Clin Immunol.* (2020) 145:1389–405. doi: 10.1016/j.jaci.2019.11.041
51. oseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med.* (2003) 9:213–9. doi: 10.1038/nm820
52. Heming M, Gran S, Jauch S-L, Fischer-Riepe L, Russo A, Klotz L, et al. Peroxisome proliferator-activated receptor-γ modulates the response of macrophages to lipopolysaccharide and glucocorticoids. *Front Immunol.* (2018) 9:893. doi: 10.3389/fimmu.2018.00893
53. Sloan JM, Kershaw MH, Touloukian CE, Lapointe R, Robbins PF, Restifo NP, et al. MHC class I and class II presentation of tumor antigen in retrovirally and adenovirally transduced dendritic cells. *Cancer Gene Ther.* (2002) 9:946–50. doi: 10.1038/sj.cgt.7700509
54. Zhong X, Lee H-N, Kim SH, Park S-A, Kim W, Cha Y-N,et al. Myc-nick promotes efferocytosis through M2 macrophage polarization during resolution of inflammation. *FASEB J.* (2018) 32:5312–25. doi: 10.1096/fj.201800223R
55. Zhou Y, Fei M, Zhang G, Liang W-C, Lin W, Wu Y, et al. Blockade of the phagocytic receptor MerTK on tumor-associated macrophages enhances P2X7R-dependent STING activation by tumor-derived cGAMP. *Immunity*. (2020) 52:357–73.e9. doi: 10.1016/j.immuni.2020.01.014
56. Woo SR, Fuertes MB, Corrales L, Spranger S, Furdyna MJ, LeungMY, et al. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity*. (2014) 41:830– 42. doi: 10.1016/j.immuni.2014.10.017
57. Ahn J, Gutman D, Saijo S, Barber GN. STING manifests self DNA- dependent inflammatory disease. *Proc Natl Acad Sci USA.* (2012) 109:19386– 91. doi: 10.1073/pnas.1215006109
58. Lin Y, Xu J, Lan H. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J Hematol Oncol.* (2019) 12:76. doi: 10.1186/s13045-019-0760-3
59. Ries CH, Cannarile MA, Hoves S, Benz J, Wartha K, Runza V, et al. Targeting tumor-associated macrophages with anti-CSF-1R antibody reveals a strategy for cancer therapy. *Cancer Cell.* (2014) 25:846– 59. doi: 10.1016/j.ccr.2014.05.016
60. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med.* (2013) 19:1264–72. doi: 10.1038/nm.3337
61. Yang M, Liu J, Piao C, Shao J, Du J. ICAM-1 suppresses tumor metastasis by inhibiting macrophage M2 polarization through blockade of efferocytosis. *Cell Death Dis.* (2015) 6:e1780. doi: 10.1038/cddis.2015.144
62. Ke X, Chen C, Song Y, Cai Q, Li J, Tang Y, et al. Hypoxia modifies the polarization of macrophages and their inflammatory microenvironment, and inhibits malignant behavior in cancer cells. *Oncol Lett.* (2019) 18:5871–8. doi: 10.3892/ol.2019.10956
63. Roda JM, Wang Y, Sumner LA, Phillips GS, Marsh CB, Eubank TD. Stabilization of HIF-2α induces sVEGFR-1 production from tumor- associated macrophages and decreases tumor growth in a murine melanoma model. *J Immunol.* (2012) 189:3168–77. doi: 10.4049/jimmunol.1103817
64. Huber R, Meier B, Otsuka A, Fenini G, Satoh T, Gehrke S, et al. Tumour hypoxia promotes melanoma growth and metastasis via high mobility group box-1 and M2-like macrophages. *Sci Rep.* (2016) 6:29914. doi: 10.1038/srep29914
65. Poh AR, Ernst M. Targeting macrophages in cancer: from bench to bedside. *Front Oncol.* (2018) 8:49. doi: 10.3389/fonc.2018.00049
66. Vinnakota K, Zhang Y, Selvanesan BC, Topi G, Salim T, Sand- Dejmek J, et al. M2-like macrophages induce colon cancer cell

invasion via matrix metalloproteinases. *J Cell Physiol.* (2017) 232:3468–80. doi: 10.1002/jcp.25808

67. Petruzzi MN, Cherubini K, Salum FG, de Figueiredo MA. Role of tumour-associated macrophages in oral squamous cells carcinoma progression: an update on current knowledge. *Diagn Pathol.* (2017) 12:32. doi: 10.1186/s13000-017-0623-6
68. Chiu KC, Lee CH, Liu SY, Chou YT, Huang RY, Huang SM, et al. Polarization of tumor-associated macrophages and gas6/Axl signaling in oral squamous cell carcinoma. *Oral Oncol.* (2015) 51:683–9. doi: 10.1016/j.oraloncology.2015.04.004
69. Li Z, Jiang J, Wang Z, Zhang J, Xiao M, Wang C, et al. Endogenous interleukin-4 promotes tumor development by increasing tumor cell resistance to apoptosis. *Cancer Res.* (2008) 68:8687–94. doi: 10.1158/0008-5472.CAN-08-0449
70. Zeni E, Mazzetti L, Miotto D, Lo Cascio N, Maestrelli P, QuerzoliP, et al. Macrophage expression of interleukin-10 is a prognostic factor in nonsmall cell lung cancer. *Eur Respir J.* (2007) 30:627– 32. doi: 10.1183/09031936.00129306
71. Seifert L, Werba G, Tiwari S, Giao Ly NN, Nguy S, Alothman S, et al. Radiation therapy induces macrophages to suppress T-cell responses against pancreatic tumors in Mice. *Gastroenterology.* (2016) 150:1659–72.e5. doi: 10.1053/j.gastro.2016.02.070
72. Gazzaniga S, Bravo AI, Guglielmotti A, van Rooijen N, Maschi F, Vecchi A, et al. Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. *J Invest Dermatol.* (2007) 127:2031–41. doi: 10.1038/sj.jid.5700827
73. Chanmee T, Ontong P, Konno K, Itano N. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers.* (2014) 6:1670– 90. doi: 10.3390/cancers6031670
74. Wang J, Li D, Cang H, Guo B. Crosstalk between cancer and immune cells: role of tumor-associated macrophages in the tumor microenvironment. *Cancer Med.* (2019) 8:4709–21. doi: 10.1002/cam4.2327
75. Martinez J, Verbist K, Wang R, Green DR. The relationship between metabolism and the autophagy machinery during the innate immune response. *Cell Metab.* (2013) 17:895–900. doi: 10.1016/j.cmet.2013.05.012
76. Lee C, Jeong H, Bae Y, Shin K, Kang S, Kim H, et al. Targeting of M2-like tumor-associated macrophages with a melittin-based pro-apoptotic peptide. *J ImmunoTher Cancer.* (2019) 7:147. doi: 10.1186/s40425-019-0610-4
77. Vaught DB, Stanford JC, Cook RS. Efferocytosis creates a tumor microenvironment supportive of tumor survival and metastasis. *Cancer Cell Microenviron.* (2015) 2:e666. doi: 10.1016/B978-0-12-394447-4.30050-5
78. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol.* (2010) 11:889–96. doi: 10.1038/ni.1937
79. Li J, Xie Y, Wang X, Li F, Li S, Li M, et al. Prognostic impact of tumor-associated macrophage infiltration in esophageal cancer: a meta-analysis. *Future Oncol.* (2019) 15:2303–17. doi: 10.2217/fon-2018-0669
80. Brown JM, Recht L, Strober S. The promise of targetingmacrophages in cancer therapy. *Clin Cancer Res.* (2017) 23:3241–50. doi: 10.1158/1078-0432.CCR-16-3122
81. Hughes R, Qian BZ, Rowan C, Muthana M, Keklikoglou I, Olson OC, et al. Perivascular M2 macrophages stimulate tumor relapse after chemotherapy. *Cancer Res.* (2015) 75:3479–91. doi: 10.1158/0008-5472.CAN-14-3587
82. Heckmann BL, Green DR. LC3-associated phagocytosis at a glance. *J Cell Sci.* (2019) 132:jcs222984. doi: 10.1242/jcs.222984

83. Amaravadi RK, Kimmelman AC, Debnath J. Targeting autophagy in cancer: recent advances and future directions. *Cancer Discov.* (2019) 9:1167– 81. doi: 10.1158/2159-8290.CD-19-0292
84. Garufi A, Pistritto G, Baldari S, Toietta G, Cirone M, D'Orazi G. p53-dependent PUMA to DRAM antagonistic interplay as a key molecular switch in cell-fate decision in normal/high glucose conditions. *J Exp Clin Cancer Res.* (2017) 36:126. doi: 10.1186/s13046-017-0596-z
85. Yoshioka A, Miyata H, Doki Y, Yamasaki M, Sohma I, Gotoh K, et al. LC3, an autophagosome marker, is highly expressed in gastrointestinal cancers. *Int J Oncol.* (2008) 33:461–8. doi: 10.1158/2159-8290.Cd-19-0292
86. Gong J, Muñoz AR, Chan D, Ghosh R, Kumar AP. ASTAT3 down regulates LC3 to inhibit autophagy and pancreatic cancer cell growth. *Oncotarget.* (2014) 5:2529–41. doi: 10.18632/oncotarget.1810
87. Lazova R, Camp RL, Klump V, Siddiqui SF, Amaravadi RK, PawelekJM. Punctate LC3B expression is a common feature of solid tumors and associated with proliferation, metastasis, poor outcome. *Clin Cancer Res.* (2012) 18:370–9. doi: 10.1158/1078-0432.CCR-11-1282
88. Gluschko A, Herb M, Wiegmann K, Krut O, Neiss WF, UtermöhlenO, et al. The *β*(2) integrin Mac-1 induces protective LC3-associated phagocytosis of listeria monocytogenes. *Cell Host Microbe.* (2018) 23:324–37.e5. doi: 10.1016/j.chom.2018.01.018
89. Nguyen TN, Padman BS, Usher J, Oorschot V, Ramm G, Lazarou M. Atg8 family LC3/GABARAP proteins are crucial for autophagosome–lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. *J Cell Biol.* (2016) 215:857–74. doi: 10.1083/jcb.201607039
90. Fazeli G, Wehman AM. Safely removing cell debris with LC3-associated phagocytosis. *Biol Cell.* (2017) 109:355–63. doi: 10.1111/boc.201700028
91. Kim J-Y, Zhao H, Martinez J, Doggett TA, Kolesnikov AV, Tang PH, et al. Noncanonical autophagy promotes the visual cycle. *Cell.* (2013) 154:365–76. doi: 10.1016/j.cell.2013.06.012
92. Turco E, Witt M, Abert C, Bock-Bierbaum T, Su MY, TrapannoneR, et al. FIP200 claw domain binding to p62 promotes autophagosome formation at ubiquitin condensates. *Mol Cell.* (2019) 74:330–46.e11. doi: 10.1016/j.molcel.2019.01.035
93. Nazio F, Carinci M, Valacca C, Bielli P, Strappazzon F, Antonioli M, et al. Fine-tuning of ULK1 mRNA and protein levels is required for autophagy oscillation. *J Cell Biol.* (2016) 215:841–56. doi: 10.1083/jcb.201605089
94. Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, et al. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat Cell Biol.* (2009) 11:468– 76. doi: 10.1038/ncb1854
95. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. *Science.* (2015) 347:1260419. doi: 10.1126/science.1260419
96. Segawa K, Suzuki J, Nagata S. Constitutive exposure of phosphatidylserine on viable cells. *Proc Natl Acad Sci USA.* (2011) 108:19246–51. doi: 10.1073/pnas.1114799108
97. Birge RB, Boeltz S, Kumar S, Carlson J, Wanderley J, Calianese D, et al. Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. *Cell Death Differ.* (2016) 23:962–78. doi: 10.1038/cdd.2016.11
98. Huang X, Yin Y, Ye D, Brekken R. Thorpe phosphatidylserine-targeting antibody induces M1 macrophage polarization, promotes myeloid derived suppressor cell differentiation and boosts tumor-specific immunity. *J ImmunoTher Cancer.* (2013) 1:P154. doi: 10.1186/2051-1426-1-S1-P154
99. Sharma B, Kanwar SS. Phosphatidylserine: a cancer cell targeting biomarker. *Semin Cancer Biol.* (2018) 52(Pt 1):17– 25. doi: 10.1016/j.semcancer.2017.08.012
100. Vallabhapurapu SD, Blanco VM, Sulaiman MK, Vallabhapurapu SL, Chu Z, Franco RS, et al. Variation in human cancer cell external phosphatidylserine is regulated by flippase activity and intracellular calcium. *Oncotarget.* (2015) 6:34375–88. doi: 10.18632/oncotarget.6045
101. Jain M, Raizada N, Kuttan R, Shan J, Digumarti R. Phase II studyof bavituximab plus paclitaxel and carboplatin in locally advanced or metastatic breast cancer: interim results. *J Clin Oncol.* (2010) 28(Suppl. 15):1062. doi: 10.1200/jco.2010.28.15_suppl.1062
102. Digumarti R, Bapsy PP, Suresh AV, Bhattacharyya GS, Dasappa L, Shan JS, et al. Bavituximab plus paclitaxel and carboplatin for the treatment of advanced non-small-cell lung cancer. *Lung Cancer.* (2014) 86:231– 6. doi: 10.1016/j.lungcan.2014.08.010
103. Nam JS, Terabe M, Mamura M, Kang M-J, Chae H, Stuelten C, et al. An anti-transforming growth factor beta antibody suppresses metastasis via cooperative effects on multiple cell compartments. *Cancer Res.* (2008) 68:3835–43. doi: 10.1158/0008-5472.CAN-08-0215
104. Montuenga LM, Pio R. Tumour-associated macrophages in nonsmall cell lung cancer: the role of interleukin-10. *Eur Respir J.* (2007) 30:608– 10. doi: 10.1183/09031936.00091707
105. Hatanaka H, Abe Y, Kamiya T, Morino F, Nagata J, Tokunaga T, et al. Clinical implications of interleukin (IL)-10 induced by non-small- cell lung cancer. *Ann Oncol.* (2000) 11:815–9. doi: 10.1023/A:1008375 208574

106. Vahl JM, Friedrich J, Mittler S, Trump S, Heim L, Kachler K, et al. Interleukin-10-regulated tumour tolerance in non-small cell lung cancer. *BrJ Cancer.* (2017) 117:1644–55. doi: 10.1038/bjc.2017.336
107. Alas S, Emmanouilides C, Bonavida B. Inhibition of interleukin 10 by rituximab results in down-regulation of bcl-2 and sensitization of B-cell non-Hodgkin's lymphoma to apoptosis. *Clin Cancer Res.* (2001) 7:709–23.
108. Horikawa M, Minard-Colin V, Matsushita T, Tedder TF. Regulatory B cell production of IL-10 inhibits lymphoma depletionduring CD20 immunotherapy in mice. *J Clin Invest.* (2011) 121:4268–80. doi: 10.1172/JCI59266
109. García-Hernández ML, Hernández-Pando R, Gariglio P, Berumen J. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and inductionof tumour and vascular cell proliferation. *Immunology.* (2002) 105:231–43. doi: 10.1046/j.1365-2567.2002.01363.x
110. Florey O, Gammoh N, Kim SE, Jiang X, Overholtzer M. V- ATPase and osmotic imbalances activate endolysosomal LC3 lipidation. *Autophagy.* (2015) 11:88–99. doi: 10.4161/15548627.2014.9 84277
111. Jacquin E, Leclerc-Mercier S, Judon C, Blanchard E, Fraitag S, Florey O. Pharmacological modulators of autophagy activate a parallel noncanonicalpathway driving unconventional LC3 lipidation. *Autophagy.* (2017) 13:854–67. doi: 10.1080/15548627.2017.1287653
112. Fletcher K, Ulferts R, Jacquin E, Veith T, Gammoh N, Arasteh JM,et al. The WD40 domain of ATG16L1 is required for its non-canonicalrole in lipidation of LC3 at single membranes. *EMBO J.* (2018) 37:e97840. doi: 10.15252/embj.201797840
113. Rai S, Arasteh M, Jefferson M, Pearson T, Wang Y, Zhang W, et al. The ATG5-binding and coiled coil domains of ATG16L1 maintain autophagy and tissue homeostasis in mice independently of the WD domain required for LC3-associated phagocytosis. *Autophagy.* (2019) 15:599–612. doi: 10.1080/15548627.2018.1534507
114. Orozco SL, Daniels BP, Yatim N, Messmer MN, Quarato G, Chen- Harris H, et al. RIPK3 activation leads to cytokine synthesis that continues after loss of cell membrane integrity. *Cell Rep.* (2019) 28:2275– 87.e5. doi: 10.1016/j.celrep.2019.07.077
115. Yang Y, Jiang G, Zhang P, Fan J. Programmed cell death and its role in inflammation. *Mil Med Res.* (2015) 2:12. doi: 10.1186/s40779-015- 0039-0Yang J, Zhao Y, Zhang L, Fan H, Qi C, Zhang K, et al. RIPK3/MLKL- mediated neuronal necroptosis modulates the M1/M2 polarization of microglia/macrophages in the ischemic cortex. *Cereb Cortex.* (2018) 28:2622–35. doi: 10.1093/cercor/bhy089
116. Lecis D, De Cesare M, Perego P, Conti A, Corna E, Drago C,et al. Smac mimetics induce inflammation and necrotic tumour cell death by modulating macrophage activity. *Cell Death Dis.* (2013) 4:e920. doi: 10.1038/cddis.2013.449
117. Kiss RS, Elliott MR, Ma Z, Marcel YL, Ravichandran KS. Apoptoticcells induce a phosphatidylserine-dependent homeostatic response from phagocytes. *Curr Biol.* (2006) 16:2252–8. doi: 10.1016/j.cub.2006.09.043
118. Gonzalez AN, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, et al. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity.* (2009) 31:245– 58. doi: 10.1016/j.immuni.2009.06.018
119. Sag D, Cekic C, Wu R, Linden J, Hedrick CC. The cholesterol transporter ABCG1 links cholesterol homeostasis and tumour immunity. *Nat Commun.* (2015) 6:6354. doi: 10.1038/ncomms7354
120. Wang Y, Hao F, Nan Y, Qu L, Na W, Jia C, et al. PKM2 inhibitor shikonin overcomes the cisplatin resistance in bladder cancer by inducing necroptosis. *Int J Biol Sci.* (2018) 14:1883–91. doi: 10.7150/ijbs.27854
121. Jin G, Liu Y, Xu P, Jin G. Induction of necroptosis in human breast cancer drug-resistant cells by SMAC analog LCL161 after caspase inhibitionrequires RIP3. *Pharmazie.* (2019) 74:363–8. doi: 10.1691/ph.2019.9307
122. Snyder AG, Hubbard NW, Messmer MN, Kofman SB, Hagan CE, Orozco SL, et al. Intratumoral activation of the necroptotic pathway components RIPK1 and RIPK3 potentiates antitumor immunity. *Sci Immunol.* (2019) 4:eaaw2004. doi: 10.1126/sciimmunol.aaw2004

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