UNIVERSITI PUTRA MALAYSIA

INVESTIGATING THE ROLE OF AUTOPHAGY IN REGULATING INNATE CYTOKINE RESPONSE OF HUMAN LUNG EPITHELIAL CELLS TO RESPIRATORY SYNCYTIAL VIRUS

NUR AMIERA FATIN BINTI AZMAN

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By

NUR AMIERA FATIN BINTI AZMAN

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NUR AMIERA FATIN BINTI AZMAN

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Chair : Saila binti Ismail, PhD
Faculty : Biotechnology and Biomolecular Sciences

Human respiratory syncytial virus (RSV) is one of the leading causes of childhood acute lower respiratory tract infection in Malaysia. It is responsible for significant morbidity and mortality among children, the elderly and individuals with chronic respiratory illnesses worldwide. Despite years of effort, currently there are neither licensed vaccines nor specific antiviral drugs against RSV. The severity of RSV-acquired diseases is predominantly caused by an overexuberant inflammatory response to the virus. Thus, a complete understanding of all the mechanisms that regulate cytokine production during RSV infection is crucial to further refine the therapeutic strategies to alleviate the excessive RSV-induced inflammatory response. Autophagy has recently been linked to the regulation of host cytokine responses to several viruses, including the vesicular stomatitis virus and the human immunodeficiency virus. In vivo studies using mouse model have shown that inhibiting autophagy attenuates the production of RSV-induced cytokines. However, the involvement of autophagy in the innate cytokine response of RSV-infected human cells has not been reported. Lung epithelial cells are known to be the main site of RSV infection and replication. Therefore, the main aim of this study was to determine the potential role of autophagy in regulating the production of RSV-induced innate cytokine C-X-C motif ligand 8 (CXCL8) and C-C motif ligand 5 (CCL5) production in lung epithelial BEAS-2B cells using both pharmacological inhibitors and short-interfering RNA knockdown approaches. It was found that RSV infection induced autophagy in BEAS-2B cells, as measured by CytoID® Autophagy Kit-based fluorescence microscopy and flow cytometry analyses. Inhibition of autophagy was performed using both pharmacological inhibitors and short-interfering RNA knockdown approaches. To confirm that autophagy inhibition does not affect cell viability, lactate dehydrogenase (LDH) assay was conducted. It was observed that inhibition of autophagy by the pharmacological inhibitors SAR405 and chloroquine (CQ); and siRNA-mediated knockdown of the autophagy protein Beclin-1 (Bec-1) did not kill the BEAS-2B cells.

Importantly, in contrast to the previous studies using mouse models, this study demonstrated that pharmacological inhibition of autophagy with SAR405 or CQ had no effect on RSV-induced CXCL8 and CCL5 production, as quantified by ELISA analysis. This was corroborated by a similar result obtained in Bec-1-defecient BEAS-2B cells. Further investigation on the involvement of autophagy in mediating the replication of RSV in BEAS-2B cells was also performed in the present study. Surprisingly, while autophagy has been found to have no effect on cytokine responses, this study showed that inhibiting autophagy with CQ or knocking down the Bec-1 protein resulted in lower expression of RSV fusion (F) protein gene in BEAS-2B cells, implying that autophagy may be involved in the regulation of RSV replication in BEAS-2B cells. In short, although autophagy inhibition may not be an effective approach in reducing RSV-induced airway inflammation, the findings from this study suggest that it may be a critical mechanism for controlling RSV replication in human lung epithelial cells.

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PENYIASATAN PERANAN AUTOFAGI DALAM MENGAWAL TINDAK BALAS SITOKIN SEMULA JADI SEL EPITELIUM PARU-PARU MANUSIA TERHADAP VIRUS PERNAFASAN SINSITIUM

Oleh

NUR AMIERA FATIN BINTI AZMAN

Julai 2021

Pengerusi : Saila binti Ismail, PhD
Fakulti : Bioteknologi dan Sains Biomolekul

Virus pernafasan sinsitium manusia (RSV) merupakan salah satu faktor utama jangkitan saluran pernafasan bawah akut dalam kalangan kanak-kanak di Malaysia. Virus ini boleh menyebabkan morbiditi dan kematian dalam kalangan kanak-kanak, orang tua serta individu yang mempunyai penyakit pernafasan kronik di seluruh dunia. Walaupun banyak kajian telah dijalankan, vaksin berlesen dan ubat antivirus yang spesifik terhadap jangkitan RSV masih belum ditemui. Menurut kajian lepas, tahap keterukan penyakit perolehan RSV adalah disebabkan oleh tindak balas keradangan berlebihan terhadap RSV. Oleh itu, pemahaman yang menyeluruh mengenai mekanisma pengawalan penghasilan sitokin aruhan RSV adalah penting bagi mencari strategi terapeutik yang efektif bagi merencat tindak balas keradangan berlebihan terhadap RSV. Kebelakangan ini, autofagi dilaporkan terlibat dalam pengawalan tindak balas sitokin perumah terhadap pelbagai virus seperti virus stomatitis vesikel dan virus kurang imun manusia. Kajian in-vivo menggunakan model tikus telah menunjukkan keupayaan autofagi dalam merencat penghasilan sitokin aruhan RSV. Walau bagaimanapun, tiada sebarang kajian tentang penglibatan autofagi dalam tindak balas sitokin inat aruhan RSV yang pernah dilaporkan dengan menggunakan sel manusia. Sel epitelium paru-paru manusia merupakan pusat utama jangkitan dan replikasi RSV. Maka, objektif utama kajian ini adalah untuk mengesan fungsi autofagi dalam kawalan penghasilan sitokin inat C-X-C motif ligan 8 (CXCL8) dan C-C motif ligan 5 (CCL5) aruhan RSV di dalam sel BEAS-2B menggunakan perencat farmakologi dan siRNA. Jangkitan RSV telah didapati mengaruh autofagi di dalam sel BEAS-2B berdasarkan analisis mikroskopi pendarfluor dan sitometri aliran berasaskan kit CytoID® Autophagy. Perencatan autofagi dilakukan dengan menggunakan kaedah perencat farmakologi dan kaedah penyahfungsian gen oleh siRNA. Selain itu, asai laktat dehidrogenase (LDH) juga dilakukan bagi memastikan perencatan autofagi tidak mempengaruhi kebolehhidupan sel BEAS-2B. Hasil

kajian menunjukkan perencatan autofagi oleh perencat farmakologi SAR405 dan penyingkiran protein autofagi, Beclin 1 (Bec-1) tidak menyebabkan kematian sel BEAS-2B yang signifikan. Walau bagaimanapun, perencatan autofagi oleh perencat farmakologi SAR405 dan chloroquine (CQ) tidak memberi sebarang kesan terhadap penghasilan CXCL8 and CCL5 aruhan RSV berdasarkan analisis ELISA, dimana ianya berbeza dengan kajian lepas yang menggunakan model tikus. Hasil kajian ini disokong oleh hasil yang diperolehi dengan mengunakan sel BEAS-2B yang kekurangan protein Bec-1. Selanjutnya, kajian tentang penglibatan autofagi dalam mengawal atur replikasi RSV juga dilakukan dan didapati, walaupun perencatan autofagi gagal mengawal tindak balas sitokin aruhan RSV, perencatan autofagi menggunakan CQ dan penyahaktifan protein Bec-1 telah berjaya mengurangkan pengekspresan protein taupan (F) RSV di dalam sel BEAS-2B. Hasil kajian ini menunjukkan autofagi memainkan peranan yang penting dalam mengawal atur replikasi RSV di dalam sel BEAS-2B. Kesimpulannya, walaupun autofagi tidak berupaya untuk merencat keradangan saluran udara aruhan RSV, hasil kajian ini memberikan pemahaman asas mengenai mekanisma autofagi yang penting dalam mengawal atur replikasi RSV di dalam sel epitelium paru-paru manusia.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Saila binti Ismail, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman**)**

Khatijah binti Mohd Yusoff, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Zamberi bin Sekawi, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Chia Suet Lin, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 November 2021

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Signature: _____
Name of Member of Supervisory Committee: Khatijah binti Mohd Yusoff

Signature: _____
Name of Member of Supervisory Committee: Zamberi bin Sekawi

Signature: _____
Name of Member of Supervisory Committee: Chia Suet Lin

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LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
°C	degree Celsius
%	percentage
~	approximately
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
ALRI	acute lower respiratory infection
ANOVA	analysis of variance
ATCC	American Type Culture Collection
ATG	autophagy-related gene
Bec-1	Beclin-1
bp	base pair
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
CCL	chemokine ligand
cm	centimetre
CO_2	carbon dioxide
CPE	cytopathic effect
CQ	chloroquine
CXCL	chemokine (C-X-C Motif) ligand

Da	Dalton
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell
dH_20	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
g	gravity force
h	hour
h.p.i.	hours post infection
HCl	hydrochloric acid
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HRP	Horse Radish Peroxidase
IFN	interferon
IL	interleukin
kb	kilobase
kDA	kilodaltons
L	litre
LDH	lactate dehydrogenase
M	molar
mA	milliamps

MFI	mean fluorescence intensity
min	minute
mg	milligram
mL	millilitre
mm	millimetre
mM	millimolar
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	sodium chloride
ng	nanogram
NTC	no template control
OD	optical density
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
PRR	pattern recognition receptor
RLR	retinoic acid inducible gene-I (RIG-I)-like receptor
rpm	revolutions per minute
RSV	respiratory syncytial virus
s	seconds
scr	non-targeting scrambled control siRNA
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA	short interfering RNA
Ta	annealing temperature
TLR	toll-like receptor
Tm	melting temperature
TNF	tumor necrosis factor
ULK1	uncoordinated-51-like autophagy activating kinase 1
utx	untransfected
V	volt
VPS34	vacuolar protein sorting 34
v/v	volume per volume
W	Watts
w/v	weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background

Respiratory syncytial virus (RSV) is a negative-sense single-stranded RNA virus which belongs to *Pneumoviridae* family (Rima et al., 2017; Tripp, 2004). It is one of the most prevalent respiratory viruses that causes childhood acute lower respiratory infection (ALRI) in Malaysia, and is also causes significant morbidity and mortality among children all over the world (Rahman et al., 2014; Weinberg, 2017). RSV can cause a wide range of respiratory symptoms such as cough, difficulty in breathing, bronchiolitis and pneumonia (Eiland, 2009). In 2015, it was estimated that RSV-associated ALRI caused a total of 76,612 deaths amongst children younger than 5 years in 195 countries including Malaysia (Troeger et al., 2017). Furthermore, childhood RSV hospitalisation has been linked to a higher risk of developing asthma and chronic wheezing later in life. (Henderson et al., 2005; Sigurs et al., 2005). The severity of RSV-associated illnesses is known to be higher among infants and young kids than adults due to their immature immune system and lack of protective antibodies (Paes et al., 2011).

Despite years of effort, to this date, there are neither licensed active prophylactic vaccines nor specific antiviral therapies against RSV (Noor & Krilov, 2018; Rezaee et al., 2017). This is thought to be due to the intricate nature of the host and viral factors involved in disease pathogenesis, as well as the fact that natural infection offers only minimal protection against reinfection and illness (Carvajal et al., 2019; Graham, 2011). Currently, besides supportive care, Ribavirin and Palivizumab are the only licensed antiviral and prophylactic treatments to control RSV infection (Russell et al., 2017). However, the use of these medications are only prescribe to high-risk infants owing to inconvenient way of drug administration, high cost, toxicity and limited effectivity (Heylen et al., 2017). Therefore, the development of effective vaccines and antiviral therapeutics against RSV are urgently required.

RSV infection begins with the entry of RSV into the host through eye, nose and mouth, followed by the spread of the virus to the lower respiratory tract (Eiland, 2009). Lung epithelial cells have been shown to be the main site of RSV replication (Nuriev & Johansson, 2019). The innate immune response of lung epithelial cells is initiated by RSV infection through identification of viral pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) such as toll-like receptor (TLR) 3 and TLR7. This recognition will ultimately contribute to the development of innate proinflammatory cytokines and type I interferons (IFNs) such as chemokine ligand 5 (CCL5/RANTES) as well as chemokine (C-X-C motif) ligand 8 (CXCL8) by the infected cells (Kim & Lee, 2014; Russell et al., 2017). CXCL8 is a known to be

a good neutrophil chemoattractant, whereas CCL5 is well-known for its eosinophil chemoattractant activity (C. Liu et al., 2018). However, excessive level of CXCL8 has been shown to contribute to the severity of RSV infection by mediating the infiltration of neutrophils that can lead to acute inflammation of the infected airway (Russell et al., 2017). Thus, in order to improve the therapeutic strategies for reducing excessive RSV-induced airway inflammation, a thorough understanding of all of the mechanisms that are involved in the control of innate cytokine production is crucial.

Recently, there is increasing evidence that the transmission of cytosolic viral replication intermediates to endosomal TLRs is aided by a self-digesting mechanism, namely autophagy. Autophagy is a cellular mechanism that uses a lysosomal degradation pathway to dispose of defective organelles, denatured proteins, and invading microorganisms. (Qian et al., 2017). Aside from preserving and sustaining cell homeostasis, autophagy has recently been attributed to the transmission of cytosolic PAMPs from several viruses to endosomal TLRs, resulting in the development of proinflammatory and antiviral cytokines. (Richetta & Faure, 2013; Yordy et al., 2013). Interestingly, autophagy has also been discovered to regulate replication of several viruses, including the poliovirus, hepatitis C virus (HCV), and human immunodeficiency virus 1 (HIV-1) (Choi et al., 2018).

The role of autophagy in mediating the innate cytokine response has been reported by previous studies using mouse models. These *in-vivo* and *in-vitro* studies using mice cells demonstrated that inhibition of autophagy suppresses RSV-induced proinflammatory and antiviral cytokines (Morris et al., 2011; Owczarczyk et al., 2015; Pokharel et al., 2016; Michelle Reed et al., 2013). However, the involvement of autophagy in the innate immune response of RSV-infected human cells has not been reported. Since lung epithelial cells are widely acknowledged primary target of RSV infection and replication, the primary purpose of this experiment was to explore the role of autophagy in controlling innate cytokine CXCL8 and CCL5 production in human lung epithelial cells during RSV infection.

1.2 Problem statement and hypothesis

Although autophagy has been shown to be responsible for the overly exuberant immune response in RSV-infected murine cells, its role in regulating the innate cytokine response in human cells remains unknown. It was hypothesised in this study that inhibiting autophagy reduces innate cytokine production in RSV-infected human lung epithelial cells.

1.3 Objectives

The main objective of this research was to determine whether autophagy had a potential role in modulating the production of RSV-induced CXCL8 and CCL5 in human lung epithelial cells.

The specific objectives were:

1. To determine the capability of RSV to stimulate autophagy pathway in human lung epithelial cells.
2. To examine the effect of autophagy inhibition on innate cytokines CXCL8 and CCL5 production in RSV-infected lung epithelial cells using both pharmacological inhibitors and short-interfering RNA knockdown approaches.
3. To elucidate the involvement of autophagy in RSV replication.

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