Int J Clin Exp Pathol 2016;9(3):3624-3629
www.ijcep.com /ISSN:1936-2625/IJCEP0020452

Original Article

Impact of autophagy on LMPs-induced airway inflammation in C57/BL6 mice

Gang Chen, Yongfang Xu, Changqing Xu

Department of Respiration, Affiliated Hospital Hangzhou Normal University, 126 Wen Zhou Road, Hangzhou 310015, China

Received November 24, 2015; Accepted January 23, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Objectives: The impacts of LMPs and autophagy on the development of chronic obstructive pulmonary disease and the corresponding mechanism were investigated with the constructed C57/BL6 mouse airway inflammation model. Methods: LMPs were produced by human T lymphocyte leukemia cell line 6T-CEM stimulated by actinomycin D and identified by flow cytometry, and then used to induce C57/BL6 mice in various concentrations and induction times. The optimum induction conditions were determined via the detection of IL-1β and IL-18 in bronchoalveolar lavage fluid of the mice, and the inflammation response was confirmed via HE staining on the lung tissues, whereby the C57/BL6 mouse airway inflammation model was constructed. The impacts of LMPs and autophagy on the development of COPD and the corresponding mechanism were investigated through the detection of the mRNA and protein expression of TLR4, PI3K, AKT, LC-3, NLRP3, caspase-1, etc. in the lung tissues of the mice. Results: The C57/BL6 mouse airway inflammation model was successfully constructed with the optimum induction concentration of LMPs of 50 μg/ml and the optimum induction time of 24 h. In the lung tissues of the mice, the mRNA and protein expressions of TLR4, PI3K, AKT, NLRP3 and caspase-1 were up-regulated to varying degrees except LC-3. Conclusions: NLRP3 is a key protein in LMPs-induced airway inflammation. Autophagy negatively regulates the activation of NLRP3 inflammasomes.

Keywords: Chronic obstructive pulmonary disease, lymphocyte micro-particles, airway inflammation, autophagy

Introduction

Chronic obstructive pulmonary disease (COPD) is featured by airway limitation and a progressive course, and the patients suffer the gradual loss of working and self-care ability and the eventual death [1, 2]. The exact pathogenesis of COPD is uncertain, and multiple mechanisms are involved, such as oxidative stress, inflammatory response, apoptosis, etc. [3-5]. Recently, many studies have indicated involvement of autophagy in the occurrence and development of COPD. Evidence shows enhanced autophagy in COPD patients and mouse lung tissues exposed to tobacco or tobacco extracts [6, 7]. Autophagosomes are formed by to-be-degraded products wrapped by monolayer or double-layer membrane, and then digested and degraded by multiple enzymes in the lysosome for cellular metabolism and organelle renewal, significant for the maintenance of cellular homeostasis. This process is called autophagy. The normal growth mechanism of cells will be disrupted by autophagy disorders.

Lymphocyte micro-particles (LMPs) are vesicle particles of 0.05-1.0 μm in diameter detached from the serosa of lymphocytes via activation or apoptosis [8], which can trigger the biological activities of the target cells via interaction. Recently, LMPs have been found to possess multiple biological activities, playing a significant role in chronic inflammation, commanding extensive attention [9, 10]. LMPs were first found to be pathologically enhanced in bronchoalveolar lavage fluid of COPD patients in the early stage of our research, and the enhancement was positively correlated with disease severity. Moreover, LMPs could induce the release of IL-1β, IL-6, IL-18, etc. from human bronchial epithelial cells in vitro, and promote apoptosis of epithelial cells.

LMPs were speculated to be closely correlated to airway inflammation based on our finding. Therefore, the impacts of LMPs and autophagy on the development of COPD and the corresponding mechanism were investigated with

Table 1. Primers for fluorescent quantitative PCR

Primers	Sequence (5'-3')
TLR4	F: GCTTCAGGCAGGCAGTATCA
TLR4	R: TGCAGTTGTCTAATCGGAACG
PI3K	F: GTAAGAGGACTGGCTGTGACCC
PI3K	R: GTTGTGTCCTGGAACACGTTTC
AKT	F: GCTGCTCAGGTCTTTCTTTTATG
AKT	R: CAAGTGCTGTCTGATTCCAATG
LC-3	F: CATCAGTGTGTCAGTGGTCAGTG
LC-3	R: CTCTTTCTCACCCAGTCCTCA
NLRP3	F: CTACTCTATCAAGGACAGCAACGC
NLRP3	R: CAGCWAAGGGAAGTCATGGG
caspase-1	F: CACGCTCTTCTGCCTGCTG
caspase-1	R: GGCTTGTCACTCGGGGTTC
GAPDH	F: GAGACCTTCAACACCCCAGC
GAPDH	R: ATGTCACGCACGATTTCCC

the C57/BL6 mouse airway inflammation model constructed by LMPs induction, in order to provide new perspectives into the relationship between LMPs and airway inflammation and to reveal new targets for clinical prevention and treatment.

Materials and methods

LMPs preparation

Human T lymphocyte leukemia cell line (6T-CEM, ATCC CCL-119) (cell bank of Chinese Academy of Sciences, Shanghai) was cultured in MEM (Gibco) complete medium containing 1% antibiotic (Penicillin/Streptomycin, Gibco), 10% FBS (Gibco) and incubated at 37°C in 5% CO_2 and saturation humidity. Actinomycin D (Sigma Aldrich) with the final concentration of 0.5 μg/ml was added to well-conditioned cells, and then incubation continued for 24 h. The cultural supernatant was collected and subjected to low temperature centrifugation at 12000 g for 50 min. The precipitate was then resuspended and washed with PBS for three times. LMPs were identified with flow cytometry, and the protein concentration was measured by BCA.

Determination of the optimum induction concentration of LMPs

Six-week female SPF C57/BL6 mice (Animal Center of Third Military Medical University) were randomly divided into four groups (six mice in each): normal control group as well as 20 μg/ml LMPs, 50 μg/ml LMPs, and 150 μg/ml LMPs group based on concentration. The mice were narcotized i.p. with 3.5% chloral hydrate (10 ml/kg body weight) and fixed in the supine position. Then 100 μl LMPSs were instilled through the trachea. After 24 h, the mice were sacrificed and the bronchoalveolar lavage fluid (BALF) was collected for cell counting and the detection of IL-1β and IL-18 with Elisa to determine the optimum induction concentration.

Determination of the optimum induction time of LMPs

Six-week female SPF C57/BL6 mice were randomly divided into four groups (six mice in each): normal control group as well as 12 h, 24 h and 48 h group. After each induction time, the mice were sacrificed and the BALF was collected for cell counting and the detection of IL-1β and IL-18 with Elisa to determine the optimum induction time.

HE staining

The mouse lung tissues were collected and fixed with 4% paraformaldehyde. Then they were dehydrated in ascending series of ethanol, cleared in xylene, embedded in paraffin and sectioned. After HE staining, the sections were dehydrated in ascending series of ethanol, cleared in xylene and sealed with neutral balata.

Fluorescent quantitative PCR

TRIzol reagent (1 ml, Qiagen) was added to every 50~100 mg lung tissues. After grinding, the homogenate was centrifuged at 12000 g at 4°C for 10 min to remove undissolved matters. Then the total RNA was extracted for reverse transcription and SYBR green fluorescent quantitative PCR (Eppendorf) to detect the mRNA levels of TLR4, PI3K, AKT, LC-3, NLRP3 and caspase-1. Primers are shown in **Table 1**. PCR was performed under the following conditions: 94°C for 4 min; 94°C for 20 s, 60°C for 30 s, 72°C for 30 s, 35 circles. Three repetitions were set for each sample.

Western blot

The mouse lung tissues were cut into 1 mm 2 pieces and a proper volume of RIPA lysis buffer

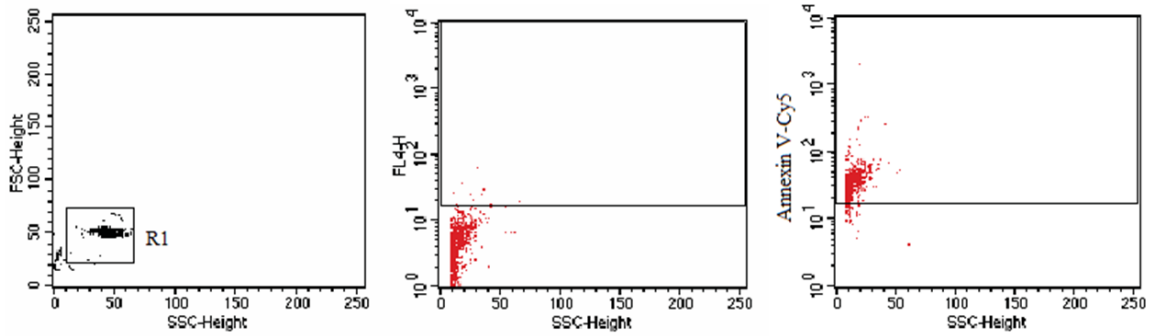

Figure 1. LMPs detected by FCM.

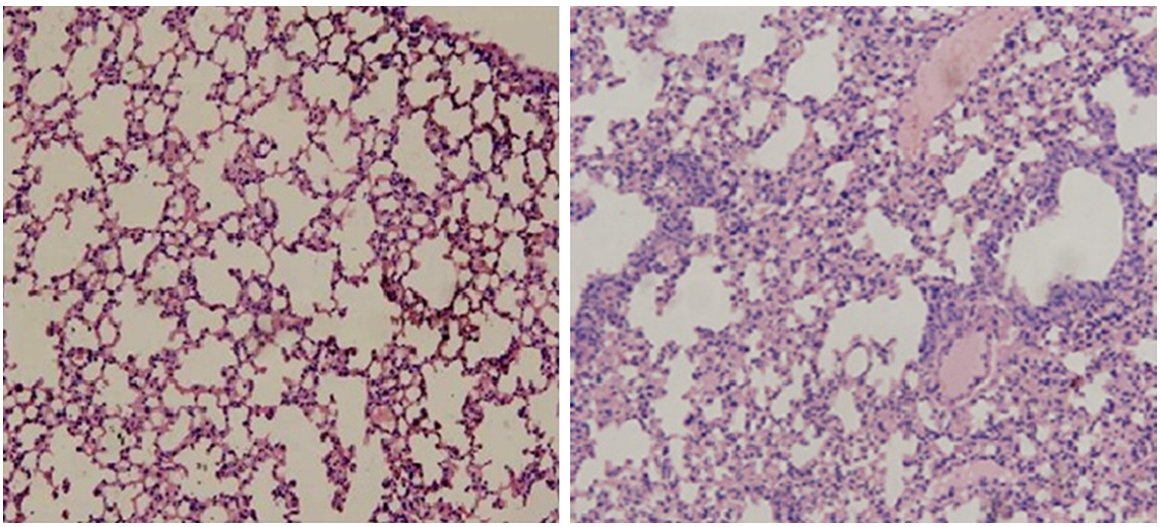

Figure 2. HE staining of the mouse lung tissues treated by LMPs.

was added to grind. The lysate was then centrifuged at 12000 g at 4°C for 5 min and the supernatant was collected for SDS-PAGE and Western blot. After scanning, the gray values of the target bands were analyzed with the UVP gel image processing system Labworks 4.6.

Statistics

Repetitions were set for each trial. The statistical analysis was performed with SPSS 10.0. The sample mean comparison was conducted with *t* test. $P<0.05$ was considered significant.

Results

LMPs identification

Actinomycin D was added to the 6T-CEM cells after three passages and LMPs were released. With the 1 μm beads as the standard, the FCM result (**Figure 1**) showed the prepared LMPs with a diameter less than 1 μm and showed a positive rate of 95.83%. The protein concentration of the acquired LMPs was 240.5±50.25 mg/ml by BCA.

LMPs-induced mouse airway inflammation model screening

Treated by LMPs of different concentrations, the number of BALF cells was increased accordingly compared with the controls as shown in **Figure 2**, and the number of the 20 μg/ml LMPs group was slightly higher, with the number of the 50 μg/ml and 150 μg/ml LMPs group higher and nearly stable. The change of IL-1β and IL-18 was consistent with the cell counting (**Table 2**). Therefore, 50 μg/ml LMPs was chosen as the optimum induction concentration.

Treated by 50 μg/ml LMPs, the number of BALF cells was increased according to the induction time compared with the controls (**Table 3**), and the number of the 12 h group was slightly high-

Table 2. BALF detection 24 h after treated by LMPs of different concentrations

Group	Cell number in BALF (×10^6/L)	IL-1β (pg/ml)	IL-18 (pg/ml)
Control	118.82±1.45	1.07±0.22	36.33±2.55
20 μg/ml	172.13±2.79	1.18±0.03	122.13±2.48
50 μg/ml	226.35±3.14	39.34±0.32	570.88±2.44
150 μg/ml	224.06±8.10	39.53±0.62	581.23±2.45

Table 3. BALF detection after treated by 50 μg/ml LMPs for varying periods

Group	Cell number in BALF (×10^6/L)	IL-1β (pg/ml)	IL-18 (pg/ml)
Control	120.02±2.33	1.04±0.14	37.67±1.88
12 h	173.79±4.11	25.78±0.14	425.87±5.14
24 h	247.11±3.88	39.27±0.12	578.08±2.19
48 h	240.12±3.15	39.67±0.41	583.14±2.11

er, with the numbers of the 24 h and 48 h group higher and nearly stable. The change of IL-1β and IL-18 was consistent with the cell counting (**Table 3**). Therefore, 24 h was chosen as the optimum induction concentration.

Establishment of the LMPs-induced mouse airway inflammation model

The LMPs-induced mouse airway inflammation model was established with the optimum induction concentration and time: six-week female SPF C57/BL6 mice were randomly divided into two groups (10 for each): normal control group and LMPs induction group. The BALF of each group was collected after 24 hour-induction with 50 μg/ml LMPs for cell counting, detection of IL-1β and IL-18 by ELISA, detection of phagocytosis, etc. (**Table 4**). According to the HE staining result, the control group showed lung tissues of a reticulate structure, continuous and integral pulmonary epithelial cells, mesenchyme without swell or breakage, no hyperemia or inflammatory cell infiltration in pulmonary alveoli, and after 24 hour-induction with 50 μg/ml LMPs, the pulmonary lymphocytes remarkably aggregated with partial fibrotic lumen, indicating that LMPs could probably cause airway inflammation.

Changes of the expression levels of relevant factors

The solubility curve of the fluorescent quantitative PCR indicated good specificity of TLR4, PI3K, AKT, LC-3, NLRP3, caspase-1 and GAPDH primers. And the standard curve showed that the amplification efficiency of each PCR system was higher than 95%. The relative quantification result showed mRNA up-regulation of TLR4, PI3K, AKT, NLRP3 and caspase-1 to varying degrees except for LC3 in the lung tissues with statistical significance (**Table 5**), which was further corroborated by Western blot on the protein level (**Figure 3**).

Discussion

Micro-particles, as the vesicle particles detached from cells after activation, injury or apoptosis, display multiple biological activities such as signal transduction, angiogenesis, engaging in the immune response, tissue repair, etc., which are closely related to disease occurrence and development [11, 12]. Circulating micro-particles have been recently found to significantly increase in the alveolus edema tissues and BALF of patients with lung injury, acute respiratory distress syndrome and COPD [13, 14]. The relationship between LMPs and airway inflammation has been rarely reported both at home and abroad by far. Autophagy, featured by the formation of autophagosomes, autolysosomes, etc., is a physiological process widely possessed by eukaryocytes, in which to-be-degraded intracellular proteins and organelles are transferred to the lysosome for metabolism, organelle renewal and cellular homeostasis maintenance. Many researches have verified the significant role of autophagy in COPD and acute lung injury [15, 16]. The regulation of multiple genes is involved in the occurrence and development of autophagy. The Class III PI3K (Vps34)-Beclin-1 complex is closely related to trigger of autophagy, and the extension of autophagosomes relies on the combination of Atg5 and Atg12 as well as the formation of LC3-II-PE [17-19].

To better understand how LMPs induced airway inflammation in vivo, the induction concentration and time of LMPs were optimized, and the C57/BL6 mouse airway inflammation model was constructed based on cell counting and IL-1β and IL-18 detection in BALF. After the administration of LMPs to mouse airway, the cells in BALF were remarkably increased with an enhanced phagocytic ability, indicating the

Table 4. BALF detection 24 h after treated by 50 μg/ml LMPs

Group	Cell number in BALF ($\times 10^6$/L)	IL-1β (pg/ml)	IL-18 (pg/ml)	Phagocyte number in BALF ($\times 10^6$/L)
Control	121.33±2.12	1.54±0.23	29.17±2.58	22.67±2.58
Experimental group	250.09±2.36	45.43±0.44	640.17±8.52	45.22±4.87

Table 5. Relative quantification result of the mRNA levels of relevant factors in the lung tissues treated with LMPs

Group	TLR4	PI3K	AKT	LC-3	NLRP3	caspase-1
Control	4.606	3.874	4.879	2.846	3.535	2.902
Experimental group	2.108	1.451	-1.951	4.905	1.672	1.454

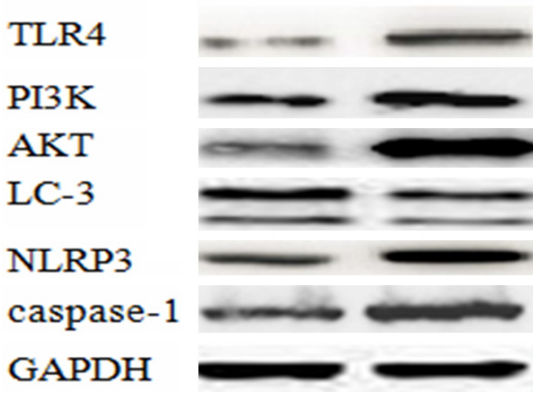

Figure 3. Western blot on the relevant factors in the lung tissues treated with LMPs.

aggregation of inflammatory cells in the airway. The activated phagocytes reached the respiratory tract and changed the microenvironment, and then the activated inflammatory cells released abundant cytokines and inflammatory factors, causing airway inflammatory. Via detecting the mRNA and protein levels of several important inflammatory factors and autophagy-related factors in the mouse lung tissues, it was showed that TLR4, PI3K, AKT, NLRP3 and caspase-1 except LC3 was up-regulated to varying degrees, indicating that LMPs could inhibit the expression of autophagy proteins, up-regulate the expression of TLR4, activate the PI3K/AKT pathway and the NLRP3 inflammasome, further promote the maturation and release of IL-1β and IL-18 and subsequently release abundant inflammatory factors and mediators to cause airway inflammation. LMPs abundance has been clinically found to be closely related to the severity of COPD. Therefore, NLRP3 is a pivotal protein in LMPs-induced airway inflammation, and autophagy negatively regulates the activation of NLRP3 inflammasomes.

In this study, the C57/BL6 mouse airway inflammation model was constructed, and the impacts of LMPs and autophagy on the development of COPD and the corresponding mechanism were investigated, partially laying the foundation and providing new perspectives for the relationship between LMPs and airway inflammation, revealing new targets for the clinical prevention and treatment of airway inflammation, especially COPD and asthma.

Acknowledgements

This work supported by (1) Pharmaceutical and health care and science and technology project, zhejiang province (number: 2014kyb196); (2) Medical of health department general project, zhejiang province (no.: 201466009); (3) The development of science and technology plan projects, hangzhou (no.: 20130633b12).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Changqing Xu, Department of Respiration, Affiliated Hospital, School of Medicine, Hangzhou Normal University, 126 Wen Zhou Road, Hangzhou 310015, China. E-mail: cute1998@163.com

References

[1] National Heart, Lung and Blood Institute. What is COPD? U.S. National Institutes of Health 2010.

[2] Nathell L, Nathell M, Malmberg P, Larsson K. COPD diagnosis related to different guidelines and spirometry techniques. Respir Res 2007; 8: 89.

[3] Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, Zielinski J; Global

Initiative for Chronic Obstructive Lung Disease. Global initiative for chronic obstructive lung disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 2007; 176: 532-555.

[4] Barnes PJ. Mediators of chronic obstructive pulmonary diseases. Pharmacological Review 2004; 56: 515-548.

[5] Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 and 2030. PLoS Med 2006; 3: e442.

[6] VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. Cardiovasc Res 2003; 59: 277-287.

[7] Miyazaki Y, Nomura S, Miyake T, Kagawa H, Kitada C, Taniguchi H, Komiyama Y, Fujimura Y, Ikeda Y, Fukuhara S. High shear stress can initiate both platelet aggregation and shedding of procoagulant containing nmicroparticle. Blood 1996; 88: 3456-3464.

[8] Miyoshi H, Umeshita K, Sakon M, Imajoh-Ohmi S, Fujitani K, Gotoh M, Oiki E, Kambayashi J, Monden M. Calpain activation in plasma membrane bleb formation during tert-butyl hydroperoxide-induced rat hepatocyte injury. Gastroenterology 1996; 110: 1897-1904.

[9] Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol 2001; 3: 339-345.

[10] Mallat Z, Tedgui A. Current perspective on the role of apoptosis in atherothrombotic disease. Circ Res 2001; 88: 998-1003.

[11] Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. Atherosclerosis 2001; 158: 277-287.

[12] Joop K, Berckmans RJ, Nieuwland R, Berkhout J, Romijn FP, Hack CE, Sturk A. Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. Thromb Haemost 2001; 85: 810-820.

[13] VanWijk MJ, Boer K, Berckmans RJ, Meijers JC, van der Post JA, Sturk A, VanBavel E, Nieuwland R. Enhanced coagulation activation in pre-eclampsia: the role of APC resistance, microparticles and other plasma constituents. Thromb Haemost 2002; 88: 415-420.

[14] VanWijk MJ, Nieuwland R, Boer K, van der Post JA, VanBavel E, Sturk A. Microparticle subpopulations are increased in pre-eclampsia: possible involvement in vascular dysfunction? Am J Obstet Gynecol 2002; 187: 450-456.

[15] Nomura S, Imamura A, Okuno M, Kamiyama Y, Fujimura Y, Ikeda Y, Fukuhara S. Platelet-derived microparticle in patients with arteriosclerosis obliterans. Enchancement of high shear-induced microparticel generations by cytokines. Thromb Res 2000; 98: 257-268.

[16] Yang C, Mwaikambo BR, Zhu T, et al. Lymphocytic microparticles inhibits angiogenesis by stimulating oxidative stress and negatively regulating VEGF-induced pathways. Am J Physiol Regul Integr Comp Physiol 2008; 294: R467-476.

[17] van Rijt LS, Kuipers H, Vos N, Hijdra D, Hoogsteden HC, Lambrecht BN. A rapid flow cytometric method for determining the cellular comparison of bronchoalveolar lavage fluid cells in mouse models of asthma. J Immunological Methods 2004; 288: 111-121.

[18] Ho YH, Cai DT, Wang CC, Huang D, Wong SH. Vesicle-associated membrane protein-8/endobrevin negatively regulates phagocytosis of bacteria in dendritic cells. J Immunol 2008; 180: 3148-3157.

[19] Ong E, Gao XP, Predescu D, Broman M, Malik AB. Role of phosphatidylinositol 3-kinase-γ in mediating lung neutrophil sequestration and vascular injury induced by E. coli sepsis. Am J Physiol Lung Cell Mol Physiol 2005; 289: L1094-L1103.