

1 **Title page**

2 Autophagy Decreases Alveolar Epithelial Cell Injury by Suppressing the NF- κ B
3 Signaling Pathway and Regulating the Release of Inflammatory Mediators

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24 **Abstract**

25 To research the impact of autophagy on alveolar epithelial cell inflammation and
26 its possible mechanism in early stages of hypoxia, we established a cell
27 hypoxia-reoxygenation model and orthotopic left lung ischemia-reperfusion model.
28 Rat alveolar epithelial cells stably expressing GFP-LC3 were treated with an
29 autophagy inhibitor (3-methyladenine, 3-MA) or autophagy promoter (rapamycin),
30 followed by hypoxia-reoxygenation treatment at 2, 4 and 6h in vitro. In vivo,
31 twenty-four male Sprague-Dawley rats were randomly divided into four groups
32 (model group: no blocking of hilum in the left lung; control group: blocking of hilum
33 in the left lung for 1h with DMSO lavage; 3-MA group: blocking of hilum in the left
34 lung for 1h with 100ml/kg of 3-MA (5 μ mol/L) solution lavage; rapamycin group:
35 blocking of hilum in the left lung for 1h with 100ml/kg of rapamycin (250nmol/L)
36 solution lavage) to establish an orthotopic left lung ischemia model. This study
37 demonstrated that rapamycin significantly suppressed the NF- κ B signaling pathway,
38 restrained the expression of pro-inflammatory factors. A contrary result was
39 confirmed by 3-MA pretreatment. These findings indicate that autophagy reduces
40 ischemia-reperfusion injury by repressing inflammatory signaling pathways in the
41 early stage of hypoxia in vitro and in vivo. This could be a new protective method for
42 lung ischemia-reperfusion injury.

43 **Key words**

44 Inflammation; Autophagy; Hypoxia-reoxygenation; Ischaemia-reperfusion; Alveolar
45 epithelial cell

47 **Introduction**

48 Ischemia–reperfusion (I/R) inflammatory injury, which is characterized by free
49 radical reaction, intracellular calcium overload and leukocyte activation, is a major
50 predisposing factor for lung failure and sudden death in lung transplant operations.
51 However, although intensive investigations of I/R injury in recent decades have
52 promoted the identification of a series of cellular pathologies and improved the
53 operation and survival rate of lung transplantation, many of the mechanisms have not
54 been clarified. Therefore, a better understanding of the pathogenesis of I/R
55 inflammatory injury and the identification of novel therapeutic methods are greatly
56 needed.

57 I/R injury during lung transplantation involves the induction of genes associated
58 with a number of cellular functions, including apoptosis, inflammation, and oxidative
59 stress(1-3). After pretreatment with I/R, alveolar epithelial cells release inflammatory
60 mediators such as reactive oxygen species (ROS), nitric oxide (NO), tumor necrosis
61 factor α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-10 (IL-10)(4-9). Nuclear
62 factor kappa B (NF- κ B) is a transcription factor that is widely known to be associated
63 with inflammatory responses following ischemia(10, 11). In the early stage of I/R, the
64 activation of I κ B kinase beta (IKK β), the most important kinase upstream of NF- κ B,
65 results in the phosphorylation and proteolysis of I κ B α , which promote the expression
66 of pro-inflammatory cytokines such as TNF- α and IL-1 β (12). The positive feedback
67 cascade in I/R leads to an excessive inflammatory response in the lung, which is the
68 main cause of early complications in patients after lung transplantation. Therefore,

69 blocking the NF- κ B signaling pathway is an effective strategy for reducing
70 inflammatory injury during lung I/R(13, 14).

71 TNF- α , IL-1 β , ICAM-1 and MCP-1 are downstream effects of the NF- κ B
72 signaling pathway, which are pro-inflammatory cytokines and can be measured to
73 assess NF- κ B activity(14-16). IL-10 is an anti-inflammatory cytokine that has a
74 crucial role in preventing inflammatory and immune response(17, 18). In the present
75 study, these inflammatory mediators were detected to verify inflammation activity.

76 Autophagy is an intracellular self-digesting pathway that delivers cytoplasmic
77 constituents into the lysosome(19). Autophagy controls the turnover of proteins and
78 organelles within cells to help in survival and longevity of cells in metabolic stress(20,
79 21). Early research indicated that autophagy could be induced by different conditions,
80 including nutrient deprivation/starvation, oxidative stress, hypoxia, and
81 chemotherapeutic drugs(3, 22-24). Autophagy also plays an important role in innate
82 and adaptive immunity and can be regulated by different cytokines, such as TGF- β or
83 IL-6(25-28).

84 Autophagy, inflammatory cytokines and NF- κ B signaling pathways are all
85 involved in lung I/R inflammatory injury, but few researchers have determined its
86 regulatory mechanism. The purpose of this study is to research the impact of
87 autophagy on alveolar epithelial cell inflammatory injury in the early stage of hypoxia
88 in vitro and in vivo and characterize its mechanism. Using an autophagy inhibitor
89 (3-methyladenine, 3-MA) and autophagy promoter (rapamycin) to regulate autophagy
90 levels, we demonstrate that exogenously enhancing autophagy significantly decreases

91 alveolar epithelial cell inflammatory injury by blocking the NF- κ B signaling pathway,
92 attenuating pro-inflammatory cytokine expression and increasing anti-inflammatory
93 cytokine expression. These new findings could be a new protective method in lung
94 ischemia-reperfusion inflammatory injury.

95 **Results**

96 **Effect of 3-MA and rapamycin on GFP-LC3/CCL149 cell viability**

97 The effect of different concentrations of autophagy inhibitor 3-MA and
98 autophagy promoter rapamycin on GFP-LC3/CCL149 cell viability was detected by
99 MTT assay. As shown in **Fig. 1C**, the cell inhibition rates were 14.3, 18.2 and 48.7%
100 for 3-MA at 5, 10 and 15 μ mol/L, respectively. The cell inhibition rates were 2.3, 2.5
101 and 2.4% for rapamycin at 150, 200 and 250nmol/L, respectively. Therefore, 5 μ mol/L
102 3-MA and 250nmol/L rapamycin were chosen for further experiments.

103 **Fluorescence microscopy observation**

104 The effect of 3-MA and rapamycin on autophagy formation in
105 GFP-LC3/CCL149 cells was evaluated by observing autophagosomes under
106 fluorescence microscopy following H/R treatment for 2, 4 and 6h (**Fig. 1A**). Green
107 fluorescence indicated that GFP-LC3/CCL149 cells were successfully constructed.
108 The cellular surface areas of GFP(+) cells were measured by immune staining after
109 pretreating with DMSO, 3-MA (5 μ mol/L) and rapamycin (250nmol/L) followed by
110 H/R treatment for 0, 2, 4 and 6h (**Fig. 1B**). Quantitative results of the green cell
111 surface area of GFP (+) cells indicated that 3-MA decreased the expression of
112 autophagy marker protein LC3. In contrast, rapamycin promoted the expression of

113 autophagy marker protein LC3.

114 **Autophagy is inhibited by 3-MA and strengthened by rapamycin**

115 To research the impact of 3-MA and rapamycin on autophagy, we observed the
116 formation of autophagosomes under transmission electron microscope in
117 GFP-LC3/CCL149 cells after pretreatment with DMSO, 3-MA (5 μ mol/L) and
118 rapamycin (250nmol/L) followed by H/R treatment for 0, 2, 4 and 6h. As shown in
119 **Fig. 2**, autophagy activity was obviously enhanced in cells pretreated with rapamycin.

120 Autophagosomes are indicated by arrows. We further measured the expression of
121 autophagy-related gene LC3-II/I and Beclin1 in indicated groups. Western blotting
122 results showed the protein levels of LC3-II/I and Beclin1 in GFP-LC3/CCL149 cells
123 **(Fig. 3A)**. The protein levels of LC3-II/I and Beclin1 were quantified and analyzed in
124 the indicated groups**(Fig. 3B, C)**.The protein levels of GFP-LC3 and Beclin1 in the
125 3-MA group were significantly lower than in the control group, and those in the
126 rapamycin group were significantly higher than in the control group.

127 To further verified the effect of 3-MA and rapamycin on autophagy, we used
128 flow cytometry assay to assess LC3-II in GFP-LC3/CCL149 cells after pretreatment
129 with DMSO, 3-MA (5 μ mol/L) and rapamycin (250nmol/L) followed by H/R
130 treatment for 0, 2, 4 and 6h. As shown in **Fig. 3D**, the trend of the results was
131 consistent with that of western blot assay and transmission electron microscope assay.
132 The percentage of cells with endogenous LC3 in the rapamycin pretreated group was
133 significantly increased compared to that of the DMSO group and 3-MA group.

134 **NF- κ B was repressed by an autophagy promoter and enhanced by an autophagy**

135 **inhibitor at an early stage of GFP-LC3/CCL149 cell H/R**

136 To research the impact of autophagy on inflammation at early stages of H/R, we
137 measured the NF- κ B signaling pathway by immunohistochemistry in
138 GFP-LC3/CCL149 cells after pretreatment with DMSO, 3-MA (5 μ mol/L) and
139 rapamycin (250nmol/L) followed by H/R treatment for 0, 2, 4 and 6h (**Fig. 4A**).
140 Immunohistochemical analysis revealed that NF- κ B integrated optical density in
141 3-MA group was significantly higher than in the control group and in the rapamycin
142 group was significantly lower than in the control group (**Fig. 4C**). We further
143 measured the protein expression of NF- κ B in the indicated groups (**Fig. 4B**). The
144 protein levels of NF- κ B were quantified and analyzed (**Fig. 4D**). The results
145 suggested that strengthening autophagy suppressed NF- κ B protein expression, which
146 indicated that exogenously enhancing autophagy reduced inflammation injury by
147 suppressing the NF- κ B signaling pathway in alveolar epithelial cell
148 hypoxia-reoxygenation.

149 **I κ B was enhanced by an autophagy promoter and repressed by an autophagy**
150 **inhibitor at an early stage of GFP-LC3/CCL149 cell H/R**

151 To further verify the impact of autophagy on the NF- κ B signaling pathway at an
152 early stage of H/R, we further detected the I κ B expression. We measured I κ B by
153 immunohistochemistry in GFP-LC3/CCL149 cells after pretreatment with DMSO,
154 3-MA (5 μ mol/L) and rapamycin (250nmol/L) followed by H/R treatment for 0, 2, 4
155 and 6h (**Fig. 5A**). I κ B integrated optical density in the 3-MA group was significantly
156 lower than in the control group and that in the rapamycin group was significantly

157 higher than in the control group (**Fig. 5C**). We further measured the protein
158 expression of I κ B in the indicated groups (**Fig. 5B**). The protein levels of I κ B were
159 quantified and analyzed (**Fig. 5D**). The results suggested that strengthening autophagy
160 increased I κ B protein expression, which indicated that exogenously enhancing
161 autophagy reduced inflammation injury by increasing I κ B expression in alveolar
162 epithelial cell hypoxia-reoxygenation.

163 **Effect of autophagy on inflammatory factors at an early stage of**
164 **GFP-LC3/CCL149 cell H/R**

165 In addition to the NF- κ B signaling pathway, we also examined the influence of
166 autophagy on downstream effects of the NF- κ B signaling pathway in
167 GFP-LC3/CCL149 cells after pretreatment with DMSO, 3-MA (5 μ mol/L) and
168 rapamycin (250nmol/L) followed by H/R treatment for 0, 2, 4 and 6h. After
169 determining the concentration of each cellular factor using a spectrophotometer (**Fig.**
170 **6A, C, E, G, I**), we measured pro-inflammatory factors TNF- α and IL-1 β , MCP-1,
171 ICAM-1 and anti-inflammatory cytokine IL-10 by ELISA kits (**Fig. 6B, D, F, H, J**).
172 The result indicated that enhancing autophagy restrained pro-inflammatory factor
173 expression and increased anti-inflammatory cytokine expression.

174 **Autophagy activity was inhibited by 3-MA and strengthened by rapamycin in**
175 **alveolar epithelial cells in rat lung I/R**

176 To further illuminate whether autophagy activity is regulated by 3-MA and
177 rapamycin in alveolar epithelial cells in rat lung I/R, we lavaged rat lungs with DMSO,
178 3-MA or rapamycin during lung ischemia for 1h and then reperfusion for 2h. The

179 model group was not treated with ischemia. Subsequently, we observed the formation
180 of autophagosomes under a transmission electron microscope (**Fig. 7A**) and detected
181 the expression of autophagy-related gene GFP-LC3 and Beclin1 by Western blotting
182 (**Fig. 7B**). The protein levels of GFP-LC3 and Beclin1 were quantified and analyzed
183 in the indicated groups (**Fig. 7C, D**). The protein levels of GFP-LC3 and Beclin1 in the
184 3-MA group were significantly lower than in the control group, and those in the
185 rapamycin group were significantly higher than in the control group.

186 **The NF- κ B signaling pathway was restrained by autophagy in alveolar epithelial** 187 **cell in rat lung I/R**

188 To gain insight into the effect of autophagy on the NF- κ B signaling pathway in
189 rat lung I/R, we lavaged rat lungs with autophagy promotor (rapamycin) or inhibitor
190 (3-MA) during lung ischemia for 1h and then reperfused for 2h. The model group was
191 not treated with ischemia. NF- κ B and I κ B were measured by immunohistochemistry
192 (**Fig. 8A**). Immunohistochemical analysis revealed that strengthening autophagy
193 suppressed NF- κ B protein expression and increased I κ B protein expression (**Fig. 8B**).
194 Western blotting results showed the protein levels of NF- κ B and I κ B in lung tissues
195 from Lewis rats after pretreatment with I/R (**Fig. 8C**). The protein levels of NF- κ B
196 and I κ B were quantified and analyzed in the indicated groups (**Fig. 8D, E**). These
197 results further revealed that exogenously enhancing autophagy reduced inflammation
198 injury by suppressing the NF- κ B signaling pathway in alveolar epithelial cells in rat
199 lung I/R.

200 **Effect of autophagy on inflammatory factors in alveolar epithelial cells in rat**

201 **lung I/R**

202 To obtain further knowledge regarding the mechanisms of autophagy reducing
203 alveolar epithelial cell inflammation injury in oxygen deficiency, we detected
204 inflammatory factors in rat lung I/R, which were lavaged with DMSO, 3-MA or
205 rapamycin during lung ischemia for 1h and then reperfusion for 2h. The
206 pro-inflammatory factors TNF- α and IL-1 β , MCP-1 and ICAM-1 in the 3-MA group
207 were significantly higher than in the control group, and those in the rapamycin group
208 were significantly lower than in the control group (**Fig. 9A, C, D**). In contrast,
209 anti-inflammatory factor IL-10 showed an increasing trend with enhanced autophagy
210 levels (**Fig. 9B**). The result indicated that enhancing autophagy can lessen
211 inflammatory injury by restraining the expression of pro-inflammatory factors and
212 increasing anti-inflammatory cytokine expression.

213 **Discussion**

214 Lung ischemia reperfusion (I/R) is a frequent event in clinic processes, inducing
215 distant organ dysfunction, especially lung transplantation or acute pulmonary tissue
216 injury. The release of pro-inflammatory cytokines during I/R is one of the most
217 important factors that lead to lung failure(29). Our team has proved that exogenously
218 enhancing autophagy decreased alveolar macrophages apoptosis by attenuating
219 endoplasmic reticulum stress and oxidative stress in hypoxia-reoxygenation or
220 ischemia-reperfusion injury(30). In this study, we established a cell model of alveolar
221 epithelial cell hypoxia-reoxygenation (H/R) and a rat model of focal lung I/R. For the
222 first time, the present study reveals that rapamycin decreases alveolar epithelial cell

223 inflammatory injury by blocking the NF- κ B signaling pathway, attenuating the
224 expression of pro-inflammatory cytokines TNF- α , IL-1 β , ICAM-1 and MCP-1 and
225 increasing the expression of anti-inflammatory cytokine IL-10. Based on various in
226 vivo and in vitro tissue ischemia and cell hypoxia models, we clearly identified that
227 exogenously enhancing autophagy as a positive regulator of alveolar epithelial cells
228 and lungs responding to oxygen deficiency via blockade of the NF- κ B signaling
229 pathway attenuate pro-inflammatory cytokine expression and increase
230 anti-inflammatory cytokine expression.

231 It has been confirmed that inflammation is an important component of lung I/R.
232 Previous studies demonstrated that inflammatory cytokine infiltration into the lungs
233 during I/R injury participates in the pathogenesis of acute lung failure, especially in
234 patients after lung transplantation. Previous studies have demonstrated that the NF- κ B
235 pathway involved in lung disease is induced by I/R(13, 15, 16). NF- κ B is combined
236 with the inhibitory unit inhibitory κ B (I κ B) and is located in the cytoplasm. When I κ B
237 is phosphorylated by its kinase I κ B kinase (IKK), NF- κ B could trigger multiple
238 downstream effects including activation of pro-inflammatory cytokines (TNF- α and
239 IL-1 β), ICAM-1 and MCP-1 accumulation, and the infiltration of immune cells in
240 ischemic tissues(31-35). It is also reported that IL-10 can block transepithelial
241 migration of neutrophils(36, 37), which is tightly related to inflammatory and
242 autoimmune pathologies(17). Therefore, inhibiting the inflammatory response is an
243 effective therapeutic method to improve lung I/R injury.

244 To the best of our knowledge, the present study is the first to show that

245 exogenously enhancing autophagy markedly stimulates the expression of
246 anti-inflammatory cytokine IL-10 at an early stage of hypoxia in vitro and in vivo.
247 Furthermore, the NF- κ B signaling pathway and its downstream effects on expression
248 were significantly inhibited by rapamycin under conditions of oxygen deficit. The
249 current results suggested that an autophagy promoter could be a new protective
250 method in lung inflammatory injury induced by ischemia-reperfusion.

251 **Materials and Methods**

252 **Cell culture**

253 For the in vitro studies, the alveolar epithelial cell line CCL149 (ATCC,
254 Manassas, VA, USA, #CCL149) was chosen as the cell model. The cells were
255 maintained in F-12K medium (ATCC, Manassas, VA, USA) supplemented with 20%
256 fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified
257 5% CO₂ atmosphere. Additionally, 10% heat-inactivated fetal calf serum was
258 contained in the medium. When the cells reached 80% confluence, they were digested
259 with 0.25% trypsin.

260 **Constructing a stable GFP-LC3/CCL149 cell line**

261 Briefly, the GFP-LC3 plasmid (Addgene, Cambridge, MA, USA) was
262 transfected into CCL149 cells by applying Lipofectamine 2000 reagent (Invitrogen,
263 Carlsbad, CA, USA). The experiment was conducted in accordance with the
264 instructions. Twenty-four hours later, the cells were transferred to culture in F-12K
265 medium containing 300 μ g/ml of G418 (Invitrogen, Carlsbad, CA, USA). After 2
266 weeks of expansion, the CCL149 cells were observed under a fluorescence

267 microscope (Olympus, Japan), and the strong green fluorescent colonies were selected
268 as stable GFP-LC3/CCL149 cells and cultured in medium containing 100 μ g/ml of
269 G418 and 10% FBS for further experiments in the study.

270 **MTT assay**

271 The general viability of the cells was measured using an MTT assay(38). The
272 percentage of cell viability inhibition was calculated as: cell viability = [OD (treated)
273 –OD (control)]/OD (control) \times 100.

274 **Animal models and procedures**

275 All the animal experimental protocols were approved by the Animal Care and
276 Use Committee of Renmin Hospital of Wuhan University and were conducted in
277 accordance with the National Institutes of Health (NIH) Guide for the Care and Use of
278 Laboratory Animals

279 Male Sprague-Dawley (SD) rats (8weeks old, 250-300g) were fed a standard diet
280 and maintained in a controlled environment of the animal center. In brief, rats were
281 anesthetized by an intraperitoneal injection of 10% chloral hydrate (300mg/kg body
282 weight) and placed in a supine position. The animals were then intubated for artificial
283 ventilation with oxygen using a small animal breathing machine (5 ml tidal volume,
284 frequency of 70 per min) and electrocardiograph monitoring. Thoracotomy was
285 performed at the anterior lateral side of the left fourth intercostal. The muscular layer
286 and pleura were gently dissected to expose the heart and lung. Then, the hilum of the
287 left lung was dissociated, and an artery clamp was used to pass through the hilum of
288 the lung from the upper right to the lower left. The whole clamped left hilum was

289 clearly exposed by slightly stirring up the clamp. Twenty-four SD rats were randomly
290 divided into four groups (5 rats/group) as follows: (1) model group: no blocking of
291 hilum in the left lung; control group: blocking of hilum in the left lung for 1h with
292 DMSO lavage and then reperfusion for 2h; 3-MA group: blocking of hilum in the
293 left lung for 1h with 100ml/kg of 3-MA (5 μ mol/L) solution lavage and then
294 reperfusion for 2h; (4) rapamycin group: blocking of hilum in the left lung for 1h with
295 100ml/kg of rapamycin (250nmol/L) solution lavage and then reperfusion for 2h. Rats
296 in the four groups were sacrificed after the experiment. The left lung tissue of the rats
297 was dissected for further analysis.

298 **Immunofluorescence analysis**

299 The cell surface area of GFP-LC3/CCL149 cells was assessed by
300 immunofluorescent staining. Briefly, after the hypoxia-reoxygenation (H/R) for 0, 2,
301 4 or 6 h, the cells were subsequently fixed with 4% paraformaldehyde (Sigma, USA,
302 #158127), permeabilized with 0.1% Triton X-100/BS for 45 min and then stained with
303 β -actin (1:100 dilution), followed by a fluorescent secondary antibody. The surface
304 areas were measured using Image-Pro Plus6.0 software. Images were captured using a
305 special fluorescence microscope (Olympus, Japan).

306 **Transmission electron microscope**

307 In vitro, cells were fixed with 2.5% glutaraldehyde at 4°C overnight after the
308 H/R treatment for 2, 4 or 6h and then fixed with 1% osmic acid. After being
309 dehydrated with a graded series of ethanol (50, 70, 80, 95, and 100%; each for 15 min)
310 and acetone (twice; each for 15 min), the cells were embedded in epoxide resin.

311 Ultra-thin sections were generated using an ultra-microtome (LKB-V, Bromma,
312 Sweden) followed by staining with uranyl acetate and lead citrate. Then, sections
313 were observed and photographed under a transmission electron microscope (H-600,
314 Hitachi, Tokyo, Japan).

315 In vivo, paraffin-embedded lungs were cut transversely into 0.1 μ m sections.
316 Then, the sections were observed and photographed under a transmission electron
317 microscope (H-600, Hitachi, Tokyo, Japan).

318 **Flow cytometry**

319 GFP-LC3/CCL149 cells were harvested with DMSO, 3-MA or rapamycin, and
320 washed with PBS containing 0.05% saponin. For intracellular staining of endogenous
321 LC3, CCL149 cells were harvested with trypsin, rinsed with culture medium and PBS,
322 and rinsed with PBS containing 0.05% saponin. Cells were then incubated with mouse
323 anti-LC3 primary antibody (Abcam, Ab290) for 20 minutes, rinsed with PBS,
324 incubated with goat antimouse secondary antibody conjugated to R-Phycoerythrin
325 (BosterBiotech, BA1060) for 20 minutes, and rinsed twice with PBS. More than
326 30,000 events were captured for every analysis. Fluorescence activated cell sorter data
327 were collected using a fluorescence activated cell sorter Calibur flow cytometer
328 (Becton Dickinson) with Cell Quest Pro software. This method was previously
329 published in ref(39).

330 **Immunohistochemical analysis**

331 For immunohistochemistry in vitro, GFP-LC3/CCL149 cells growing on glass
332 cover slips were fixed for 15min with 4% paraformaldehyde. After being incubated

333 with 0.5% Triton X-100/PBS solution for 30 min and washed with PBS three times,
334 the GFP-LC3/CCL149 cells were blocked with 3% hydrogen peroxide for 15 min and
335 subsequently incubated overnight at 4 °C with the primary antibodies. Binding was
336 visualized with the appropriate peroxidase-conjugated secondary antibodies (AR1022,
337 ZSGB-BIO) for 20-30 min at 37 °C.

338 For immunohistochemistry *in vivo*, paraffin-embedded lungs were cut
339 transversely into 5µm sections. Following a 5min high-pressure antigen retrieval
340 process in 0.1mol/L citrate buffer with a pH of 6.0, the lung sections were blocked
341 with 3% hydrogen peroxide for 15 min and subsequently incubated overnight at 4 °C
342 with the primary antibodies. Binding was visualized with the appropriate
343 peroxidase-conjugated secondary antibodies (AR1022, ZSGB-BIO) for 20-30 min at
344 37 °C.

345 **Western blotting analysis**

346 Total proteins were extracted from GFP-LC3/CCL149 cells and rat lung tissues
347 in lysis buffer. The protein concentrations were determined using a Pierce
348 Bicinchoninic Acid Protein Assay kit (Biyuntian, Shanghai, China, #P0010). Fifty
349 micrograms of protein was subjected to SDS–polyacrylamide gel electrophoresis
350 (12%PAGE; Amresco) and transferred to a polyvinylidene fluoride membrane
351 (Millipore) followed by incubation overnight at 4 °C with the following primary
352 antibodies: LC3 (Abcam, Cambridge, United Kingdom, #ab62341; 1:200 dilution),
353 Beclin-1 (Santa Cruz, CA, USA, #sc-11427; 1:200 dilution), NF-κB (Bioworld, CA,
354 USA, #BS1257; 1:600 dilution), and IκB-α (Bioworld, CA, USA, #BS3601; 1:600

355 dilution). After incubation with peroxidase-conjugated secondary antibodies (BA1060,
356 at 1:50,000 dilution), the bands were visualized using Bio-Rad Chemi Doc™ XRS+
357 (Bio-Rad). Protein expression levels were normalized to the corresponding β -actin
358 levels.

359 **ELISA measurements of cytokines in rat lung tissues and GFP-LC3/CCL149** 360 **cells**

361 Rat lung tissues were washed and then homogenized on ice with normal saline.
362 Homogenates from rat lung tissues or GFP-LC3/CCL149 cell culture supernatants
363 were centrifuged at 12000rpm for 10min at 4 C, and the supernatants (100 μ L) were
364 used for analysis. The levels of tumor necrosis factor- α (TNF- α), interleukin-1 β
365 (IL-1 β), interleukin-10 (IL-6), macrophage chemoattractant protein-1 (MCP-1) and
366 intercellular adhesion molecule-1 (ICAM-1) were measured by enzyme-linked
367 immunosorbent assay (ELISA) kits (Elabscience, USA) in triplicate according to the
368 manufacturer's recommended protocol.

369 **Statistical Analysis**

370 All data are presented as the mean \pm s.d. from at least three independent
371 experiments. Student's two-tailed *t*-test was used to compare the means of two-group
372 samples. Two-way analysis of variance (ANOVA) was applied for the comparison of
373 multiple groups in different H/R times. A one-way analysis of variance (ANOVA) was
374 applied to determine the significant effect of 3-MA or rapamycin on studied rats
375 pretreated with lung I/R followed by the least significant difference (equal variances
376 assumed) or Tamhane's T2 (equal variances not assumed) tests. All statistical analyses

377 were performed using the *Graph Pad Prism5* software. *P* values less than 0.05 were
378 considered significant. No statistical method was used to predetermine sample size.
379 Randomization and a blinding strategy were used whenever possible.

380 **Competing interests**

381 The authors declare no conflict of interest.

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534

535 **Figure legends**

536 **Fig. 1.** Effect of 3-MA and rapamycin on autophagy formation and
537 GFP-LC3/CCL149 cell viability.

538 (A) Representative fluorescence microscopic images of GFP-LC3/CCL149 cells
539 pretreated with 3-MA or rapamycin followed by H/R treatment for 0, 2, 4 and 6h.

540 Scale bars, 40 μ m.

541 (B) Quantitative results of the green cell surface area of LC3-GFP/CCL149 cells
542 followed by H/R treatment for 0, 2, 4 and 6h in response to DMSO, 3-MA or
543 rapamycin.

544 (C) Impact of 3-MA and rapamycin on LC3-GFP/CCL149 cell viability. The cells are
545 treated with different concentrations of 3-MA (5, 10 and 15 μ mol/L) and rapamycin
546 (150, 200 and 250 nmol/L) for 48h. The control cells are treated with an equal volume
547 of DMSO. MTT assays are used to measure cell viability. The cell inhibition rate (%)
548 is calculated by dividing control values. * $p \geq 0.05$ compared to control at 0h; # $p < 0.05$
549 compared to control at 2, 4 and 6h.

550 **Fig. 2.** Transmission electron microscope evaluating the effect of 3-MA and
551 rapamycin on autophagosomes in alveolar epithelial cells treated with H/R. The cells
552 are pretreated with 3-MA (5 μ mol/L) or rapamycin (250nmol/L) followed by H/R
553 treatment for 0, 2, 4 and 6h. Scale bars, 500nm. The cell ultrastructure is observed
554 under a transmission electron microscope. Arrowheads point to intracellular
555 autophagy.

556 **Fig. 3.** Effect of 3-MA and rapamycin on expression of LC3-II/I and Beclin1 in

557 alveolar epithelial cells treated with H/R.

558 **(A)** Western blots showing the protein of LC3-II/I and Beclin1 in LC3-GFP/CCL149
559 cells pretreated with DMSO, 3-MA and rapamycin followed by H/R treatment for 0, 2,
560 4 and 6 h.

561 **(B, C)** The protein levels of GFP-LC3(b) and Beclin1(c) in LC3-GFP/CCL149 cells
562 were quantified and analyzed in the indicated groups. * $p \geq 0.05$ compared to control at
563 0h; # $p < 0.05$ compared to control at 2, 4 and 6h.

564 **(D)** Flow cytometry was used to measure flux of endogenous LC3 protein. Pretreated
565 with rapamycin exerted a higher level of EGFP-LC3-II-containing autophagosomes.

566 **Fig. 4.** Enhanced autophagy decreases H/R-induced expression of NF- κ B in alveolar
567 epithelial cells treated with H/R.

568 **(A)** Representative images of immunohistochemistry with anti-NF- κ B antibody in
569 LC3-GFP/CCL149 cells pretreated with DMSO, 3-MA and rapamycin followed by
570 H/R treatment for 0, 2, 4 and 6h(n=5/group; scale bar, 30 μ m).

571 **(B)** Western blots showing the protein expression of NF- κ B in the indicated groups.

572 **(C)** Immunohistochemistry analysis of the protein expressions of NF- κ B in
573 LC3-GFP/CCL149 cells in the indicated groups.

574 **(D)** The protein levels of NF- κ B in LC3-GFP/CCL149 cells were quantified and
575 analyzed. * $p \geq 0.05$ compared to control at 0h; # $p < 0.05$ compared to control at 2,4 and
576 6h.

577 **Fig. 5.** Enhanced autophagy increases anti-inflammatory factor expression of I κ B in
578 alveolar epithelial cells treated with H/R.

579 (A) Representative images of immunohistochemistry with anti-I κ B antibody in
580 LC3-GFP/CCL149 cells pretreated with DMSO, 3-MA and rapamycin followed by
581 H/R treatment for 0, 2, 4 and 6h(n=5/group; scale bar, 30 μ m).

582 (B) Western blots showing the protein expression of I κ B in the indicated groups.

583 (C) Immunohistochemistry analysis of the protein expression of I κ B in
584 LC3-GFP/CCL149 cells in the indicated groups.

585 (D) The protein levels of I κ B in LC3-GFP/CCL149 cells were quantified and
586 analyzed. * $p \geq 0.05$ compared to control at 0h; # $p < 0.05$ compared to control at 2,4 and
587 6h.

588 **Fig. 6.** Enhanced autophagy suppresses H/R-induced pro-inflammatory cytokine
589 expression of TNF- α , IL-1 β , MCP-1 and ICAM-1 and increases anti-inflammatory
590 factor expression of IL-10 in alveolar epithelial cells treated with H/R.

591 (A, C, E, G, I) Determining the concentration of TNF- α , IL-1 β , IL-10, MCP-1 and
592 ICAM-1 protein concentration in each sample.

593 (B, D, F, H, J) ELISA measurement of serum TNF- α , IL-1 β , IL-10, MCP-1 and
594 ICAM-1 levels in LC3-GFP/CCL149 cells pretreated with DMSO, 3-MA and
595 rapamycin followed by H/R treatment for 0, 2, 4 and 6h(n=5). The results are
596 analyzed in the indicated groups. * $p \geq 0.05$ compared to control at 0h; # $p < 0.05$
597 compared to control at 2, 4 and 6h.

598 **Fig. 7.** Effect of 3-MA and rapamycin on autophagosome and expression of LC3 and
599 Beclin1 in rat lungs treated with I/R.

600 (A) Transmission electron microscope image showing autophagosomes in lung tissues

601 from Lewis rats after the rats were lavaged with DMSO, 3-MA and rapamycin in lung
602 ischemia for 2h and reperfusion for 2h. The model group was not treated with
603 ischemia. Arrowheads point to autophagosomes.

604 **(B)** Western blots showing the protein levels of LC3 and Beclin 1 in lung tissues from
605 Lewis rats after the rats were lavaged with DMSO, 3-MA and rapamycin in lung
606 ischemia for 2h and reperfusion for 2h. The model group was not treated with
607 ischemia.

608 **(C, D)** The protein levels of LC3 and Beclin 1 in Lewis rats were quantified and
609 analyzed in the indicated groups. # $p < 0.05$ compared to control.

610 **Fig. 8.** Enhanced autophagy blocks the NF- κ B signaling pathway by inhibiting NF- κ B
611 expression and increasing I κ B expression in rat lungs treated with I/R.

612 **(A)** Representative images of immunohistochemical staining of a normal rat lung
613 section (model group) and lung sections lavaged with DMSO (control group), 3-MA
614 (3-MA group) and rapamycin (rapamycin) with an antibody against NF- κ B or I κ B;
615 scale bar, 30 μ m.

616 **(B)** Immunohistochemistry analysis of the protein expression of NF- κ B and I κ B in rat
617 lungs in the indicated groups.

618 **(C)** Western blots showing the protein levels of NF- κ B and I κ B in lung tissues from
619 Lewis rats after the rats were lavaged with DMSO, 3-MA and rapamycin in lung
620 ischemia for 2h and reperfusion for 2h. The model group was not treated with
621 ischemia.

622 **(D, E)** The protein levels of NF- κ B and I κ B in Lewis rats were quantified and

623 analyzed in the indicated groups. # $p < 0.05$ compared to control.

624 **Fig. 9.** Enhanced autophagy suppresses H/R-induced pro-inflammatory cytokine
625 expression of TNF- α , IL-1 β , MCP-1 and ICAM-1 and increases anti-inflammatory
626 factor expression of IL-10 in rat lungs treated with I/R.

627 **(A)** ELISA measurement of serum IL-1 β and TNF- α levels in rat lungs of control,
628 3-MA and rapamycin groups (n=5). The results are analyzed in the indicated groups.

629 **(B)** ELISA measurement of serum IL-10 level in rat lungs of control, 3-MA and
630 rapamycin groups (n=5). The results are analyzed in the indicated groups.

631 **(C)** ELISA measurement of serum MCP-1 levels in rat lungs of control, 3-MA and
632 rapamycin groups (n=5). The results are analyzed in the indicated groups.

633 **(D)** ELISA measurement of serum ICAM-1 levels in rat lungs of control, 3-MA and
634 rapamycin groups (n=5). The results are analyzed in the indicated groups. # $p < 0.05$
635 compared to control.

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