

# Molecular mechanism of pyroptosis of mononuclear macrophages induced by pathogenic *E. coli* high pathogenicity island (HPI) in Yunnan Saba pigs

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**Research**

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3 **Yunnan Saba pigs**

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17

## 18 **Abstract**

### 19 Background

20 In this study we evaluated the molecular mechanism by which pyroptosis is induced in  
21 mononuclear macrophages isolated from Saba pigs following infection with pathogenic *E.*  
22 *coli* high pathogenicity island (HPI). Mononuclear macrophages were divided into four  
23 treatment groups: control, Lipopolysaccharide (LPS) + adenosine triphosphate (ATP), HPI  
24 positive (+) strain and HPI negative (-) strain. The mononuclear macrophages and their  
25 culture supernatants were collected at 0.5, 3, 6, 9, 12 and 24 h after infection. DNA changes  
26 were detected by TUNEL staining and the integrity of the cell membrane was evaluated by  
27 propidium iodide (PI) staining. Changes in mRNA expression levels of NLRP3, caspase-1,  
28 IL-1 $\beta$ , and IL-18 gene in mononuclear macrophages were analyzed by quantitative real-time  
29 polymerase chain reaction (RT-PCR) and caspase-1 protein expression was detected by  
30 indirect immunofluorescence. IL-1 $\beta$  and IL-18 concentration in the mononuclear macrophage  
31 culture supernatant were measured by ELISA.

### 32 Results

33 Compared with the control group, TUNEL and PI staining of mononuclear macrophages was  
34 significantly increased following infection with the HPI<sup>+</sup>/HPI<sup>-</sup> strains ( $P < 0.01$  or  $P < 0.05$ ),  
35 with significantly higher levels detected in the HPI<sup>+</sup> group compared with those in the HPI<sup>-</sup>  
36 group ( $P < 0.01$  and  $P < 0.05$ ). Compared with the control group, the expression levels of  
37 NLRP3, caspase-1, IL-1 $\beta$ , and IL-18 in the HPI groups were upregulated after pathogenic *E.*  
38 *coli* infection, with significantly higher levels detected in the HPI<sup>+</sup> group compared with  
39 those in the HPI<sup>-</sup> group ( $P < 0.01$  or  $P < 0.05$ ).

### 40 Conclusions

41 These findings showed that pathogenic *E. coli* HPI infection of Saba pigs results induced  
42 pyroptosis of mononuclear macrophages characterized by increased expression of NLRP3,

43 caspase-1, IL-1 $\beta$  and IL-18 mRNA in mononuclear macrophages, the induction of cell  
44 membrane pore formation, nuclear DNA damage, and the secretion of IL-1 $\beta$  and IL-18 to  
45 enhance the inflammatory response.

46 **Key words:** Pathogenic *E. coli*; HPI; Mononuclear macrophage; Pyroptosis

## 47 **Background**

48 *Escherichia coli* (*E. coli*), which is a typical Gram-negative member of the coliform genus  
49 *Escherichia*, plays an important role in the intestinal symbiosis of warm-blooded animals [1,  
50 2]. Pathogenic *E. coli* strains cause serious harm to human and animal health, often causing  
51 severe diarrhea edema and septicemia [3]. High pathogenicity island (HPI) is an important  
52 factor for the virulence and pathogenicity of *E. coli*, and other highly pathogenic strains. It  
53 was first discovered in *Yersinia* [4] and is present only in virulent strains [5]. HPI has a  
54 functional core region, containing the *irp2-irp1-irp3-irp4-irp5-FyuA* gene axis known as the  
55 *irp2-FyuA* gene cluster, and the *irp2* marker gene [6]. Paauw et al. demonstrated that *Yersinia*  
56 containing HPI was more virulent by studying the iron carrier encoding HPI [7].

57 Inflammatory caspases such as caspase-1 (mouse/human), caspase-4 (human), and  
58 caspase-11 (mouse), contribute to a variety of biological functions [8, 9]. Pyroptosis is a form  
59 of programmed cell death mediated by a caspase-1, which induces cell swelling and is  
60 characterized by the rupture and release of cellular contents leading to an intense  
61 inflammatory reaction that is essential for the control of microbial infections [10].

62 Nucleotide-binding oligomerization domain-like receptor family pyrin  
63 domain-containing 3(NLRP3) inflammasomes are mainly composed of intracellular  
64 pattern-recognition receptor NLRP3, adaptor protein ASC, and pro-caspase-1 [11]. The  
65 NLRP3 inflammasome is involved in the regulation of immune responses, and can be  
66 activated by bacteria, viruses, fungi and apoptosis [12]. NLRP3 inflammasomes activate the  
67 caspase-1 domain. The activated caspase-1 then cleaves inactive pro-interleukin-1 $\beta$   
68 (pro-IL-1 $\beta$ ) and pro-interleukin-18 (pro-IL-18) to generate the active pro-inflammatory  
69 cytokines IL-1 $\beta$  and IL-18 [13].

70 Activated caspase-1 and its precursors cleave gasdermin D (GSDMD) protein to  
71 separate its N-terminal domain from its C-terminal domain, which relieves the inhibition of

72 the N-terminal domain by the C-terminal domain of the GSDMD protein [14]. After cleavage,  
73 the N-terminal domain of the GSDMD protein binds to phosphatidylinositol on the  
74 cytoplasmic membrane, oligomerizes on the cytoplasmic membrane and forms membrane  
75 pores with an inner diameter of 12-14 nm [14]. During pyroptosis, specific pores are formed  
76 on the cell membrane of the target cell, resulting in disruption of the ion concentration  
77 gradient across the cell membrane, and leading to the swelling and disintegration of the cells  
78 [15-17]. Mature IL-1 $\beta$  and IL-18 are then released from the cytoplasm through this cell  
79 membrane pore [14]. Other immune cells are recruited and stimulated by IL-1 $\beta$  and IL-18,  
80 thereby inducing the synthesis of other inflammatory cytokines and enhancing the local and  
81 systemic inflammatory response [18].

82 Several characteristics of pyroptosis appear to overlap with apoptosis, although the  
83 process are distinct. Similar to apoptosis, cells incur DNA damage during pyroptosis [19],  
84 and become positive in the TUNEL assay [20-22]. In contrast, the nuclear morphology of  
85 pyroptotic cells is distinct from that of apoptotic cells [21], and a DNA ladder is not  
86 necessarily observed [22].

87 It has been confirmed that *Shigella flexneri*, *Salmonella*, *Listeria*, *Pseudomonas*  
88 *aeruginosa*, *Francis tularensis*, *Legionella pneumophila* and *Yersinia* induce  
89 caspase-1-dependent pyroptosis in macrophages [18]. During infection, pyroptosis induces  
90 the death of host cells, which is an important process by which the growth and reproduction  
91 of the pathogenic microorganism is limited and the infection is cleared, thus providing  
92 effectively protection of the host. Miao et al. reported that caspase-1 gene knockout mice  
93 were resistant to the death induced by *Salmonella typhimurium*, indicating the importance of  
94 caspase-1 in resisting pathogen invasion [23].

95 Pyroptosis is closely related to the control of various bacterial infectious diseases;  
96 however, its mechanism and regulation mechanism has not been elucidated. Saba pigs are a

97 breed local to Yunnan Province that are reared for their high rate of piglet production and an  
98 excellent meat quality. Saga sows are commonly used in hybrid breeding systems in central  
99 Yunnan Province [24].

100 To elucidate the pathogenic mechanism of *E. coli*, we evaluated the ability of pathogenic  
101 *E. coli* HPI to induce pyroptosis in mononuclear macrophages, and investigated the  
102 underlying mechanism. In addition, we explored the effects of HPI<sup>+</sup> and HPI<sup>-</sup> strains on host  
103 cell infection. This information will provide a better understanding of the mechanism of  
104 pyroptosis-related diseases, and highlight new therapeutic targets for the treatment of related  
105 diseases.

## 106 **Result**

### 107 ***Isolation and identification of pathogenic E. coli HPI***

108 The *E. coli* HPI strains were cultured for 24 h until bright pink round colonies with smooth  
109 and moist surface and flat, neat edges were observed (Fig. 1A). Translucent raised colonies  
110 that were round in shape and with a smooth, moist surface were formed on normal nutrient  
111 agar medium (Fig. 1B). *E. coli* were identified as Gram-positive rod-shaped red cells (Fig.  
112 1C). The presence of the *irp2* gene in the isolates was determined by PCR amplification using  
113 the extracted DNA as a template. In total, 43 of the 96 isolates were *irp2* gene positive  
114 (44.8%) and 53 were negative (55.2%). Representative results for PCR amplification of the  
115 HPI *irp2* gene are shown in Figure 1D.

### 116 ***MTT analysis of cell viability***

117 The IC<sub>50</sub> for LPS was calculated to be approximately 10 µg/ mL according to the Improved  
118 Kou method (Figure 2).

119 ***TUNEL detection***

120 As shown in Figure 3A and B, the IOD values representing the intensity of TUNEL staining  
121 of the experimental groups was significantly higher than that of the control group during the  
122 period from 0.5 to 9 h post-infection ( $P < 0.01$ ). The IOD value of the LPS+ATP group was  
123 significantly higher than that of the other groups after infection ( $P < 0.01$ ). The IOD values in  
124 the HPI treated groups were significantly higher than those in the control group at 0.5 and 3 h  
125 post-infection ( $P < 0.01$ ). As the bacteria multiplied, the IOD value of the HPI<sup>+</sup> group was  
126 obviously different than that of the HPI<sup>-</sup> group at 9 h post-infection ( $P < 0.01$ ).

127 ***PI staining***

128 As shown in Figure 4A and B, the IOD values representing the intensity of PI staining of the  
129 HPI groups were significantly higher than that of the control group during the period of 0.5 to  
130 9 h post-infection ( $P < 0.01$ ). The IOD value of the LPS+ATP group was significantly higher  
131 than those in the other groups after infection ( $P < 0.01$ ). The IOD values of the HPI<sup>+</sup> group  
132 were markedly higher than those of the HPI<sup>-</sup> at different time-points after *E. coli* infection,  
133 with significant differences detected at 0.5 and 9 h ( $P < 0.05$ ) and extremely significant  
134 differences detected at 3 h ( $P < 0.01$ ).

135 ***Effects of HPI infection on the mRNA expression of key pyroptosis genes in mononuclear***  
136 ***macrophages***

137 As shown in Figure 5A, NLRP3 mRNA expression in the experimental groups was  
138 significantly higher than that in the control group at 0.5, 3 and 6 h post-infection ( $P < 0.01$ ).  
139 In addition, NLRP3 mRNA expression in the HPI<sup>+</sup> group was significantly higher than that in  
140 the HPI<sup>-</sup> and control groups at 24 h post-infection ( $P < 0.01$ ).

141 As shown in Figure 5B, the relative expression of caspase-1 mRNA in the experimental

142 groups was higher than that in the control group after *E. coli* infection, with significant  
143 differences between the HPI treatment and control groups at 3, 6, 9, 12 and 24 h ( $P < 0.01$ ).  
144 At each time-point after infection, caspase-1 expression in the HPI<sup>+</sup> group was significantly  
145 higher than that in the HPI<sup>-</sup> group ( $P < 0.01$ ), while the caspase-1 expression level in the HPI<sup>+</sup>  
146 group was significantly lower than in the LPS+APS group at 3 and 6 h ( $P < 0.01$ ).

147 As shown in Figure 5C and D, the relative mRNA expression of IL-1 $\beta$  and IL-18 in the  
148 experimental groups was higher than that in the control group at 6, 9 and 12 h post-infection.  
149 The IL-1 $\beta$  and IL-18 expression levels in the HPI<sup>+</sup> group were significantly different from  
150 those in the control group after infection ( $P < 0.01$  or  $P < 0.05$ ). The IL-1 $\beta$  expression levels  
151 in the HPI<sup>+</sup> group were significantly higher than those in the HPI<sup>-</sup> group and control group at  
152 6 and 9 h post-infection ( $P < 0.01$ ). The IL-18 expression levels in the HPI<sup>+</sup> group were  
153 significantly higher than those in the control group at 6, 9 and 12 h post-infection ( $P < 0.01$ ),  
154 and were significantly higher than those in the HPI<sup>-</sup> at all time-points except 6 h ( $P < 0.01$ ).  
155 Additionally, with increasing time after infection, the relative mRNA expression of IL-1 $\beta$  and  
156 IL-18 gradually increased in the HPI groups.

### 157 ***Immunofluorescence assay (IFA) of caspase-1 protein expression in mononuclear*** 158 ***macrophages***

159 As shown in Figures 6A and B, the IOD values representing the intensity of caspase-1 protein  
160 staining in the experimental groups increased during the period from 0.5 to 9 h post-infection.  
161 However, at 0.5 h post-infection, the IOD values of caspase-1 protein in the experimental  
162 groups were significantly decreased compared with those in the control group ( $P < 0.01$ ),  
163 while those in the HPI<sup>+</sup> group were significantly higher than those in the HPI<sup>-</sup> group ( $P <$   
164  $0.01$ ). At 3 and 9 h post-infection, the IOD values of caspase-1 protein in the experimental  
165 groups were significantly higher than those in the control group ( $P < 0.01$ ). Furthermore, the

166 IOD values of caspase-1 protein in the HPI<sup>+</sup> group were significantly higher than those in the  
167 HPI<sup>-</sup> ( $P < 0.01$ ).

168 ***ELISA analysis of IL-1 $\beta$  and IL-18 levels in mononuclear macrophages after E. coli***  
169 ***infection***

170 The concentrations of IL-1 $\beta$  and IL-18 in the culture supernatants of mononuclear  
171 macrophages were detected by ELISA (Fig. 7A and B). The concentrations both cytokines  
172 increased gradually with time after infection. The IL-1 $\beta$  content in the LPS+ATP group was  
173 significantly higher than that in the control group at 0.5, 3, 6 and 24 h post-infection ( $P <$   
174  $0.01$  or  $P < 0.05$ ). The IL-1 $\beta$  content in the HPI<sup>+</sup> group was significantly higher than that in  
175 the control group at 3, 6, 12 and 24 h post-infection ( $P < 0.05$ ), whereas the difference were  
176 not statistically significant at the other time-points ( $P > 0.05$ ). The IL-1 $\beta$  content in the HPI<sup>-</sup>  
177 group was significantly higher than that in the control group at 3 h post-infection ( $P < 0.05$ ).  
178 The IL-18 content in the control group was significantly lower than those in the experiment  
179 groups at 6 and 24 h post-infection ( $P < 0.01$ ,  $P < 0.05$  or  $P > 0.05$ ). The IL-18 content in the  
180 LPS+ATP group was significantly higher than the other groups at 0.5, 3, 9 and 12 h  
181 post-infection ( $P < 0.05$  or  $P > 0.05$ ). The IL-18 content in the HPI<sup>+</sup> group was significantly  
182 higher than that in the HPI<sup>-</sup> group at 6 h post-infection ( $P < 0.05$ ).

183 **Discussion**

184 Pyroptosis is a form of innate immune defense against intracellular bacteria [23]. This type of  
185 programmed cell death is accompanied by an inflammatory response that induces both  
186 apoptosis and necrosis, which are characterized by DNA and membrane damage. Similar to  
187 apoptosis, the chromatin DNA in pyroptotic cells is damaged and broken, rendering TUNEL  
188 staining-positive [25]. However, in contrast to apoptosis, numerous pores (1–2 nm) are

189 formed on the cell membrane of pyroptotic cells, leading to the release of the cellular  
190 contents and inflammatory factors, such as IL-1 $\beta$ , which further promote the inflammatory  
191 response [26]. Studies have shown that when cells undergo pyroptosis, PI can cross the pores  
192 in the cell membrane to stain the nucleus red [27]. Fink et al. reported that *Salmonella*  
193 infection of mice caused pyroptosis of host macrophages, with the formation of membrane  
194 pores (1.1–2.4 nm) causing cell swelling due to disruption of the osmotic gradient [28]. In  
195 this study, after infection of mononuclear macrophages with pathogenic *E. coli* HPI, the IOD  
196 value of PI staining was significantly increased, with higher values in the HPI<sup>+</sup> treatment  
197 group than that in the HPI<sup>-</sup>. This indicated that *E. coli* HPI infection promoted the formation  
198 of pores in the cell membrane of mononuclear macrophages, and this effect was more marked  
199 in the presence of the HPI *irp2* gene. This is consistent with the observation that the number  
200 of PI-positive macrophages increased with time after infection with listeria [29].

201 Muruve and Lamkanfi observed clear signs of pyroptosis and marked increases in the  
202 relative mRNA levels of NLRP3, caspase-1, IL-1 $\beta$  and IL-18 after LPS/ATP stimulation of  
203 mouse macrophages [30, 31]. Liang reported that the mRNA expression levels of  
204 pyroptosis-related genes (NLRP3, caspase-1, IL-1 $\beta$  and IL-18) were significantly increased in  
205 renal tissues after obstructive nephropathy caused by unilateral ureteral ligation in rats [32].  
206 In this study, after pathogenic *E. coli* infection of mononuclear macrophages in vitro, the  
207 intracellular mRNA expression levels of NLRP3, caspase-1, IL-1 $\beta$  and IL-18 were higher  
208 than those in the control group, with an overall increase in levels observed initially followed  
209 by decreased expression, which was consistent with previous reports. The findings indicate  
210 that *E. coli* activates the pyroptosis signaling pathway, and this effect is promoted more  
211 effectively by *E. coli* HPI. In this study, caspase-1 protein expression varied with the different  
212 treatments. Caspase-1 protein expression in the LPS+ATP group and the HPI groups  
213 increased gradually with time post-infection, which is similar to the pattern of increased

214 caspase-1 protein content in human monocyte macrophages following *Helicobacter pylori*  
215 infection [33]. Furthermore, it has been reported that *Neisseria gonorrhoeae* infection  
216 promoted the activation and secretion of caspase-1 in human monocytes [34]. Our study  
217 confirmed that pathogenic *E. coli* HPI induces pyroptosis in monocyte macrophages, and  
218 caspase-1 protein expression was higher in host cells infected HPI<sup>+</sup> strains containing the HPI  
219 *irp2* marker gene.

220 IL-1 $\beta$  and IL-18 are belong to IL-1 family and are produced by monocyte endothelial  
221 cell fibroblasts and other cell types in response to infection. These cytokines play an  
222 inflammatory role by binding to the corresponding receptors to regulate the release of soluble  
223 antagonists and the expression of precursor enzymes and bait receptors at the transcriptional  
224 level. In addition, they also participate in congenital and adaptive immune effector cells of  
225 the recruitment and activation. IL-1 $\beta$  and IL-18 are synthesized as inactive cytoplasmic  
226 (pro-IL-1 $\beta$  and pro-IL-18) and maturation depends on the caspase-1 activity [35]. The mature  
227 forms of IL-1 $\beta$  and IL-18 are released through the GSDMD channel to perform their  
228 pro-inflammatory functions and cause pyroptosis [30]. Hitzler showed that *H. pylori* infection  
229 of dendritic cells resulted in activation of caspase-1 and induced the maturation and secretion  
230 of IL-1 $\beta$  and IL-18 [36].

231 The results of our study demonstrated that IL-1 $\beta$ /IL-18 levels were significantly elevated  
232 in the culture supernatants of mononuclear macrophages infected with *E.coli* HPI, with  
233 higher levels detected following infection with the strain carrying the *irp2* gene. These  
234 findings provide evidence of the initial activation of the NLRP3/caspase-1 pyroptosis  
235 pathway in cells following HPI infection, which further promoted the secretion of  
236 inflammatory inflammatory factors IL-1 $\beta$  and IL-18. This is consistent with the significant  
237 increase in IL-1 $\beta$  and IL-18 expression after LPS/ATP stimulation of mouse macrophages  
238 reported by Wei [37]. These findings effectively confirm that HPI endows characteristics of

239 strong pathogenicity on *E.coli*, which is closely related to the process of infection.

## 240 **Conclusion**

241 In conclusion, we found that pathogenic *E.coli* HPI infection of Yunnan Saba pigs  
242 upregulated the expression of NLRP3, caspase-1, IL-1 $\beta$  and IL-18 mRNA, and promoted cell  
243 membrane pore formation and nuclear DNA damage in mononuclear macrophages.  
244 Furthermore, we showed that the infection stimulated the release of inflammatory cytokines  
245 IL-1 $\beta$  and IL-18, induce inflammation, and eventually promoted pyroptosis of mononuclear  
246 macrophages. Moreover, the existence of HPI in *E.coli* enhanced the occurrence of pyroptosis  
247 of mononuclear macrophages compared with the effects observed following infection with  
248 the HPI<sup>-</sup> strain. The correlation of *E.coli* HPI infection with the expression of key  
249 pyroptosis-related molecules in monocytes highlights new ideas and directions for further  
250 studies to elucidate the molecular mechanism by which *E.coli* HPI induces pyroptosis in  
251 mononuclear macrophages. Further studies of pyroptosis will contribute to a greater  
252 understanding of the mechanisms of cellular injury and to the development of pharmaceutical  
253 inhibitors of pyroptosis.

## 254 **Materials and methods**

### 255 *Materials and reagents*

256 Mononuclear macrophages were isolated from the peripheral blood of four healthy slag pigs  
257 (2 boars and 2 sows) aged 1–4 months. HPI<sup>+</sup> and HPI<sup>-</sup> strains of pathogenic *E.coli* were  
258 isolated at the laboratory of a pig farm in Chuxiong City, Yunnan Province (China), identified  
259 and preserved by the department of animal pathology (Yunnan Agricultural University) [38].  
260 The HPI gene of *E.coli* was identified by polymerase chain reaction (PCR). HPI<sup>+</sup> and HPI<sup>-</sup>

261 strains have the same serotype (O119) and biochemical characteristics which were tested by  
262 the method reported by Jing et al. [39]. Chloroform and alcohol concentration gradients were  
263 from Beijing Chemical Industry Group Co., Ltd. (Beijing, China).

#### 264 ***PCR detection of HPI irp2 gene and culture of mononuclear macrophages***

265 The *E. coli* HPI strains isolated from Saba pigs were cultured on MacConkey agar medium at  
266 37°C for 24 h. Individual colonies of pathogenic *E. coli* were selected, inoculated and cultured  
267 on Luria-Bertani (LB) agar plates overnight at 37°C. At OD<sub>600</sub> 0.6–0.8, bacterial suspensions  
268 (containing HPI<sup>+</sup>/ HPI<sup>-</sup>), expression of the HPI *irp2* genes was analyzed by PCR using the  
269 *irp2* primers shown in Table 1. The PCR conditions were as follows: 95°C for 5 min; 94°C  
270 for 30 s, 55°C for 30 s, 72°C for 1 min (32 cycles) and one final extension step of 72°C for 10  
271 min. The PCR products were resolved by 1.0% (wt/vol) agarose gel electrophoresis.

272 Mononuclear macrophages were cultured in DMEM containing 10% FBS, penicillin and  
273 streptomycin at 37°C under 5% CO<sub>2</sub>.

#### 274 ***MTT assay of cell viability***

275 Cells in the control and the experimental groups seeded into 96-well plates at 2×10<sup>5</sup>/well with  
276 five duplicate wells for each group. Medium (100 μL) containing different concentrations of  
277 LPS (10<sup>6</sup>, 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10 ng/mL) was added to each well. After 5.5 h, 10 μL ATP  
278 (55 mmol/L) was added. After 24 h, 10 μL MTT reagent (5 mg/mL) was added to each well.  
279 After a further 4 h, the culture supernatant was removed and 100 μL DMSO was added to  
280 each well. The plate was oscillated for 15 min before the absorbance at 490 nm was measured  
281 using an ELx800™ Absorbance Microplate Reader (BioTek Instruments, Winooski, USA).  
282 The IC<sub>50</sub> for LPS was calculated according to the Improved Kou method, where  $\lg IC_{50} =$   
283  $X_m - I(P - (3 - P_m - P_n)/4)$ . X<sub>m</sub>:lg maximum dose; I:lg(maximum dose/ phase dose); P: sum of

284 positive response rates; Pm: maximum positive response rate; Pn: minimum positive response  
285 rate.

### 286 *Mononuclear macrophages infected with E. coli*

287 The mononuclear macrophages were randomly divided into four groups: control, HPI<sup>+</sup>  
288 infection, HPI<sup>-</sup> infection and LPS+ATP, with three replicates for each group. Mononuclear  
289 macrophages were seeded in 96-well plates at  $2 \times 10^5$ /well and infected with HPI<sup>+</sup> strains,  
290 HPI<sup>-</sup> strains, and LPS+ATP by adding 2 mL of cell culture broth containing 1 mL of bacterial  
291 suspension (OD<sub>600</sub> 0.6–0.8). At 0.5, 3, 6, 9, 12 and 24 h post-infection, mononuclear  
292 macrophages and their supernatant were collected for analysis.

### 293 *Real-time polymerase chain reaction (RT-PCR)*

294 Total RNA was extracted, cDNA was synthesized by reverse transcription and stored at  $-20^\circ$   
295 C. RT-PCR reverse transcription kits and SYBR Premix Ex Taq<sup>TM</sup> II were purchased from  
296 TaKaRa Biotechnology Co., Ltd. (Dalian, China). The RT-PCR reaction was carried out  
297 using gene-specific primers for  $\beta$ -actin, NLRP3, caspase-1, IL-1 $\beta$  and IL-18 (Table 1). The  
298 amplification was carried out using the Bio-Rad Cx96 Detection System under the following  
299 reaction conditions:  $95^\circ\text{C}$  30 s;  $95^\circ\text{C}$  5 s, T<sub>m</sub> 47–62 $^\circ\text{C}$  20 s,  $72^\circ\text{C}$  30 s (40 cycles). The  
300 relative mRNA expression for each index was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method.

### 301 *TUNEL detection*

302 Cells were prepared as described in section 2.4. Cells were seeded in 6-well plates  
303 ( $2 \times 10^6$ /well) and fixed with 4% paraformaldehyde on ice for 15 min. The supernatant was  
304 centrifuged and fixed in 70% cold ethanol for 24 h. TUNEL staining was then performed  
305 using TUNEL kits (R&D Systems Co. Ltd., Shanghai, China) according to the

306 manufacturer's instructions.

### 307 ***Propidium iodide (PI) staining***

308 Cells were prepared as described in section 2.4. Cells were seeded in 96-well plates (2×  
309 10<sup>5</sup>/well), with three replicates for each group. The cells were cultured with 100 μL of 6.7  
310 μg/mL PI staining solution at 37°C for 20 min. Mononuclear macrophages were observed  
311 under a light microscope at 200× amplification (Olympus IX73P1F microscope, Japan)

### 312 ***Immunofluorescence assay (IFA) of HPI on caspase-1 protein expression into mononuclear*** 313 ***macrophages***

314 Cells were prepared as described in section 2.4. Cells were fixed, permeabilized and then  
315 incubated overnight at 4°C with anti-caspase-1 (1:50, mouse monoclonal, Santa  
316 Cruz Biotechnology USA). The cells were then incubated in the dark with secondary  
317 detection antibodies before 4', 6-diamidino-2-phenylindole (DAPI) treatment. The IOD  
318 values for caspase-1 protein expression were analyzed using Image Pro-Plus 6.0 software  
319 (Media Cybernetics, Silver Spring, MD, USA).

### 320 ***ELISA***

321 Cells were prepared as described in section 2.4. The supernatants from cells in each group  
322 were collected and the contents of the inflammatory mediators IL-1β and IL-18 were detected  
323 by commercially available Porcine IL-1β and IL-18 ELISA kits (Shanghai Yuanye  
324 Biotechnology Co. Ltd., Shanghai, China) according to the manufacturers' instructions.

### 325 ***Statistical analysis***

326 All data were presented as the mean ± SD (n = 3), and significance of differences between

327 groups were evaluated by ANOVA, followed by the Duncan post-hoc test. Differences were  
328 regarded as significant and highly significant at  $P < 0.05$  and  $P < 0.01$ , respectively.

## 329 **Declarations**

### 330 *Ethics approval and consent to participate*

331 All methods were non-invasive and were approved by the Committee on the Ethics of Animal  
332 Experiments of the Yunnan Agricultural University.

### 333 *Consent for publication*

334 All authors have approved this submitted manuscript in its current form and agreed to  
335 publish.

### 336 *Availability of data and materials*

337 The data sets generated in the current study are available from the corresponding author on  
338 reasonable request.

### 339 *Competing interests*

340 All authors declare that they have no conflict of interests.

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343 31660704 and 31960692).

### 344 *Authors' contributions*

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356 **Contributions**

357 Chunlan Shan and Shushu Miao designed and supervised the study, reviewed and edited the  
358 manuscript. All authors read and approved the final manuscript.

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## 480 **Tables**

481 **Table 1** Specific primers for amplification of target genes and  $\beta$ -actin gene

Name	Sequences	Primer Length	Tm (°C)	Size (bp)
<b><math>\beta</math>-actin</b>	5'-TGCGGGACATCAAGGAGA-3' (F)	18	55	175
AY550069.1	5'-AGGAAGGAGGGCGGAAGAG-3' (R)	20		
<b>NLRP3</b>	5'-TGGATAGCGGCAAGAGT-3' (F)	17	47	145
JQ219660.1	5'-GCAGCCAGTGAGCAGAG-3' (R)	17		
<b>Caspase-1</b>	5'-GCCTTGCCCTCATAATCT-3' (F)	18	60	282
NM_214162.1	5'-ACATCTGGGACTTCTTCG-3' (R)	18		
<b>IL-18</b>	5'-GGATATGCCTGATTCTGACTGTT-3' (F)	19	50	100
AY450287.1	5'-GATGGTTACTGCCAGACCTCTA-3' (R)	19		
<b>IL-1<math>\beta</math></b>	5'-GCAGTGGAGAAGCCGATGA-3' (F)	19	62	223
NM_214055.1	5'-GGTGGAGAGCCTTCAGCAT-3' (R)	19		

482 **Figure captions**

483 **Fig. 1** A: Isolation of pathogenic *E. coli*; B: Purification of pathogenic *E. coli*; C:  
484 Microscopic examination of pathogenic *E. coli* (1,000' magnification) D: The PCR  
485 amplification of HPI *irp2* gene (M: DL2000 Mark; 1–6: Experimental strains; 7: Negative  
486 control)

487 **Fig. 2** The determination of IC50 for LPS

488 **Fig. 3** A: TUNEL staining of mononuclear macrophages at different time-points after *E. coli*  
489 infection (200× magnification; TUNEL staining, green); B: The IOD value of TUNEL in  
490 mononuclear macrophages at 0.5 h, 3 h and 9 h after infection with *E. coli* HPI. \* $P < 0.05$ ,  
491 \*\* $P < 0.01$ .

492 **Fig. 4** A: Propidium iodide (PI) staining of mononuclear macrophages after *E. coli* infection  
493 (200', PI staining, red); B: The IOD value of PI in mononuclear macrophages at 0.5 h, 3 h  
494 and 9 h after infection with *E. coli* HPI. \* $P < 0.05$ , \*\* $P < 0.01$ .

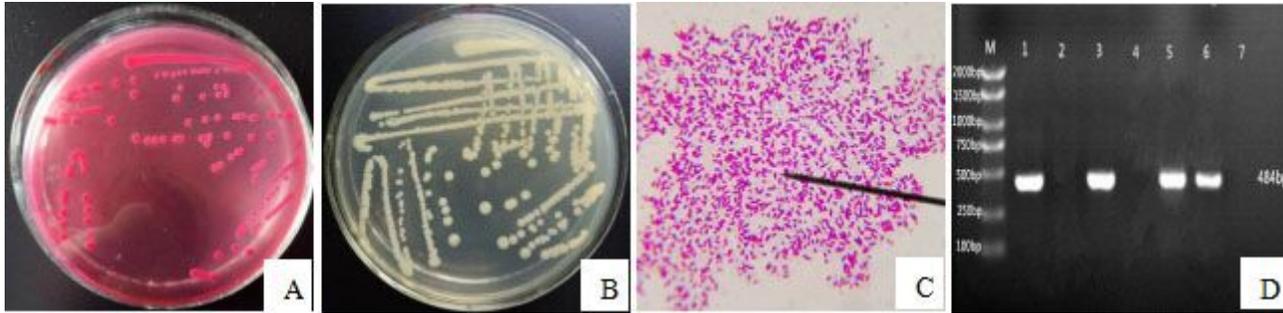
495 **Fig. 5** Relative mRNA expression of NLRP3, caspase-1, IL-1 $\beta$  and IL-18 in mononuclear  
496 macrophages at each time-point after *E. coli* HPI infection. \* $P < 0.05$ , \*\* $P < 0.01$ .

497 **Fig. 6 A:** Caspase-1 protein expression in mononuclear macrophages at different-point after  
498 treatment (200× magnification; caspase-1 staining, green and nuclear staining, blue); B: The  
499 IOD value of caspase-1 protein in mononuclear macrophages at 0.5 h, 3 h and 9 h after  
500 infection with *E. coli* HPI. \* $P < 0.05$ , \*\* $P < 0.01$ .

501 **Fig. 7** The concentration of IL-1 $\beta$  (A) and IL-18 (B) in mononuclear macrophages at 0.5, 3, 6,  
502 9, 12 and 24 h after infection with *E. coli*. \* $P < 0.05$ , \*\* $P < 0.01$ .

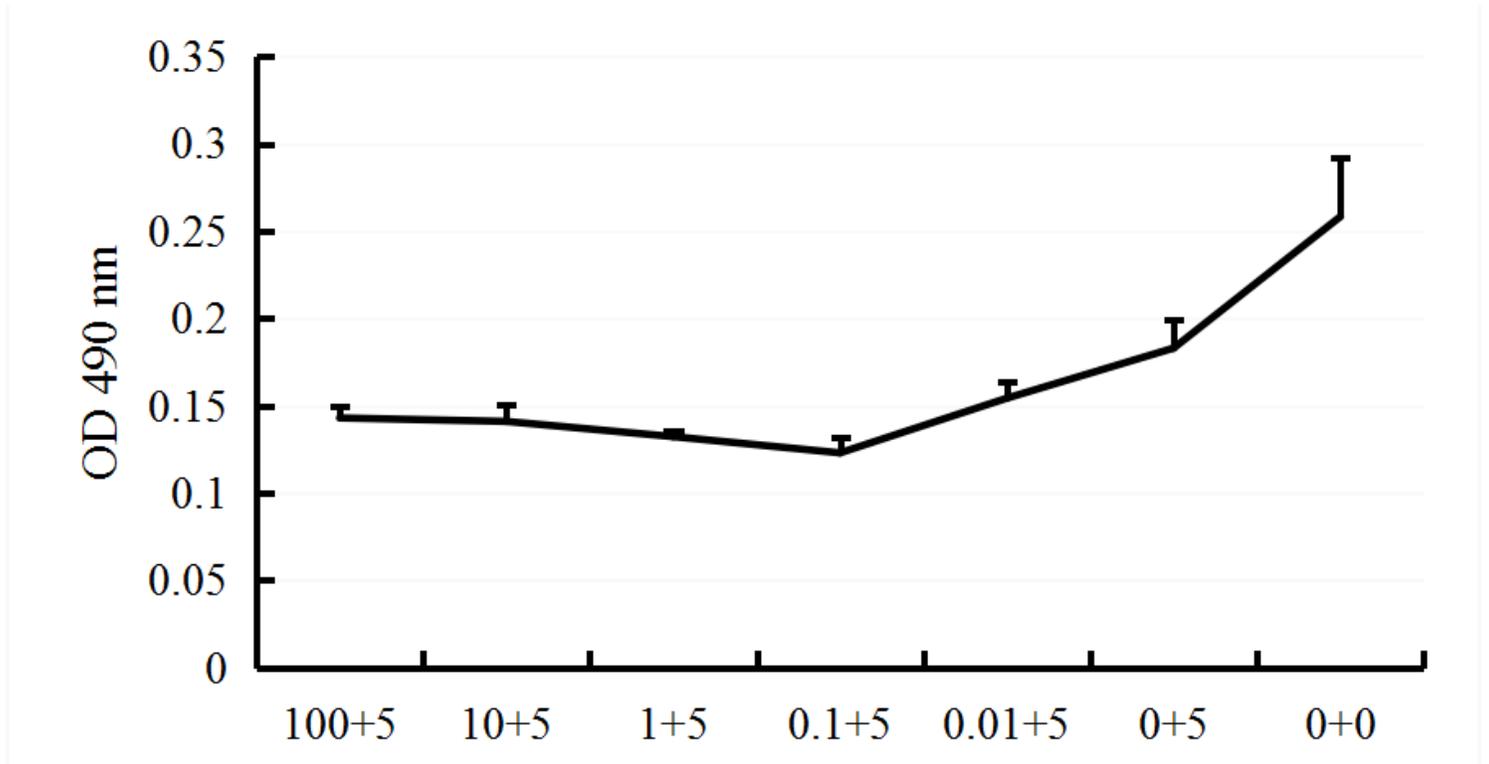
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# Figures



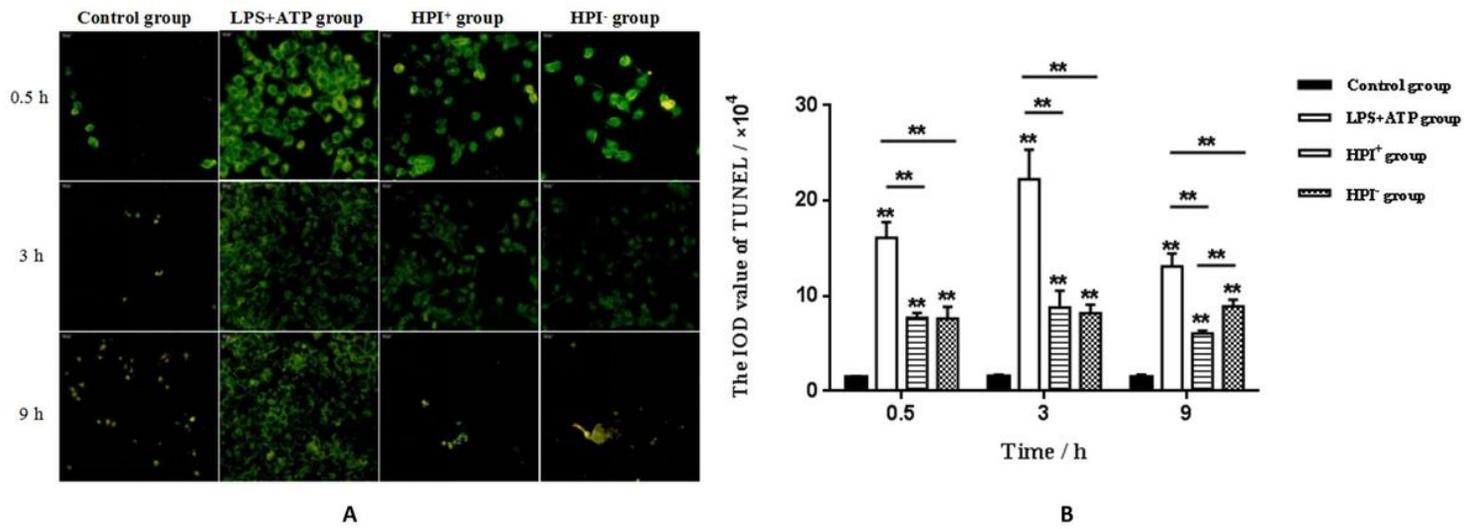
**Figure 1**

A: Isolation of pathogenic *E. coli*; B: Purification of pathogenic *E. coli*; C: Microscopic examination of pathogenic *E. coli* (1,000 $\times$  magnification) D: The PCR amplification of HPI *irp2* gene (M: DL2000 Mark; 1–6: Experimental strains; 7: Negative control)



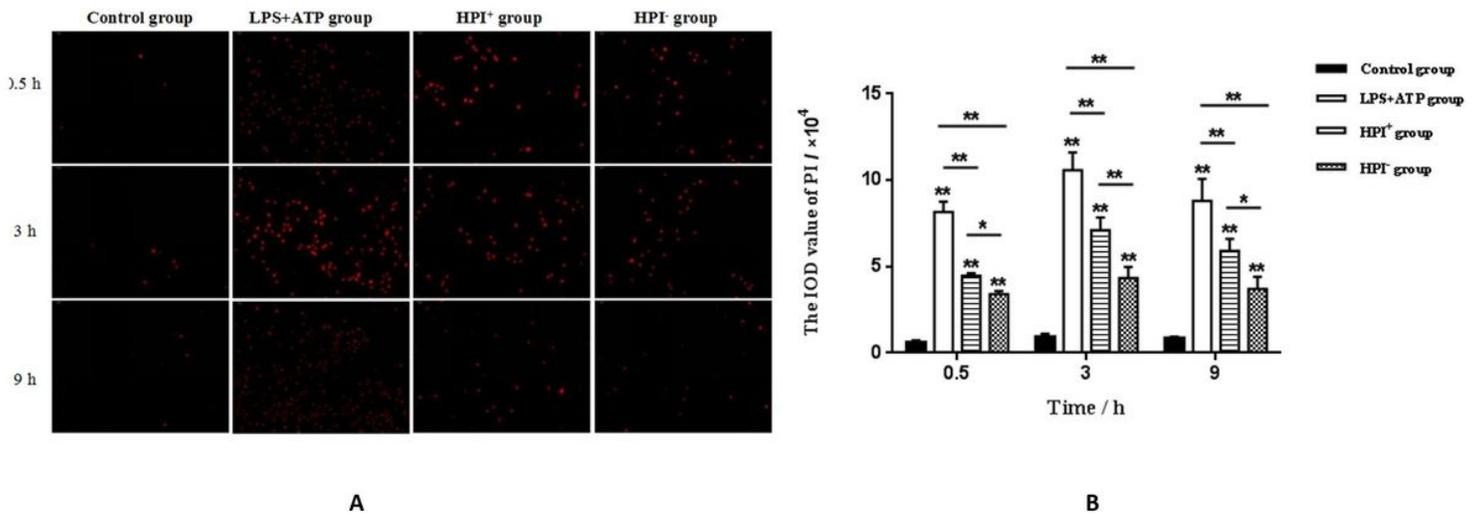
**Figure 2**

The determination of IC50 for LPS



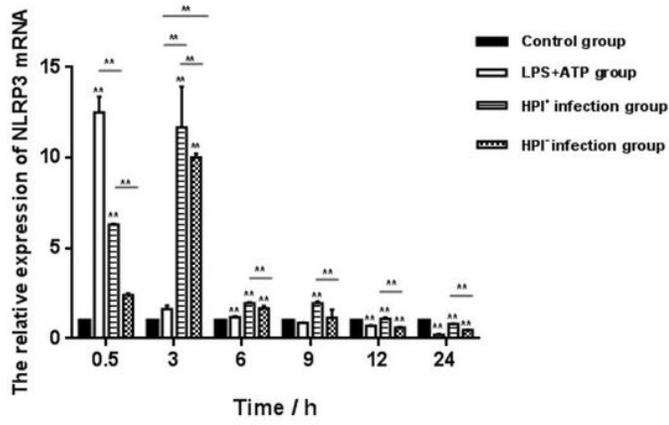
**Figure 3**

A: TUNEL staining of mononuclear macrophages at different time-points after *E. coli* infection (200 $\times$  magnification; TUNEL staining, green); B: The IOD value of TUNEL in mononuclear macrophages at 0.5 h, 3 h and 9 h after infection with *E. coli* HPI. \* $P < 0.05$ , \*\* $P < 0.01$ .

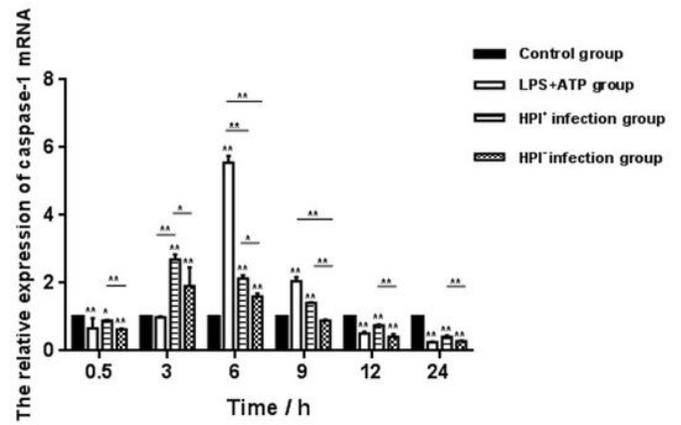


**Figure 4**

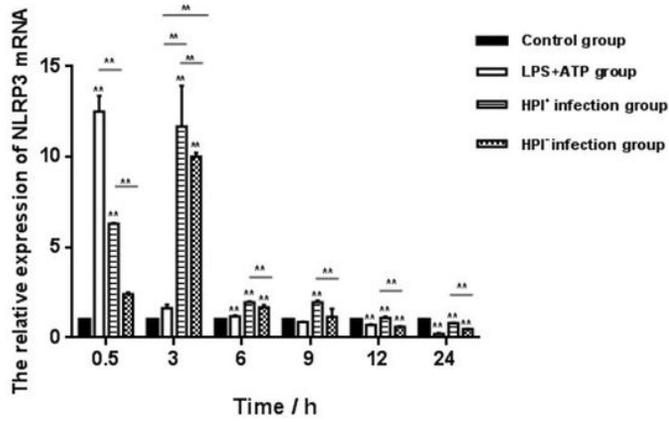
A: Propidium iodide (PI) staining of mononuclear macrophages after *E. coli* infection (200 $\times$ , PI staining, red); B: The IOD value of PI in mononuclear macrophages at 0.5 h, 3 h and 9 h after infection with *E. coli* HPI. \* $P < 0.05$ , \*\* $P < 0.01$ .



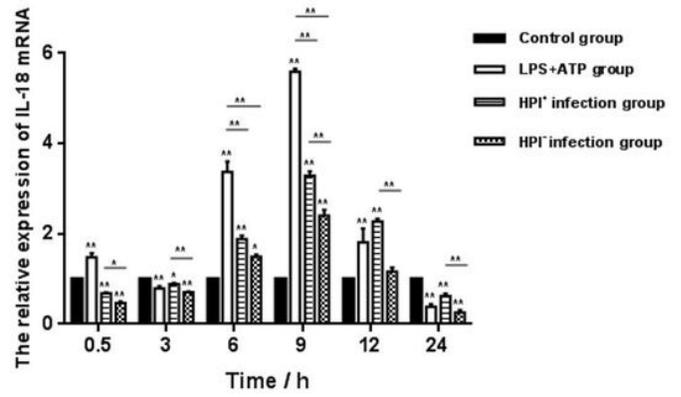
A



B



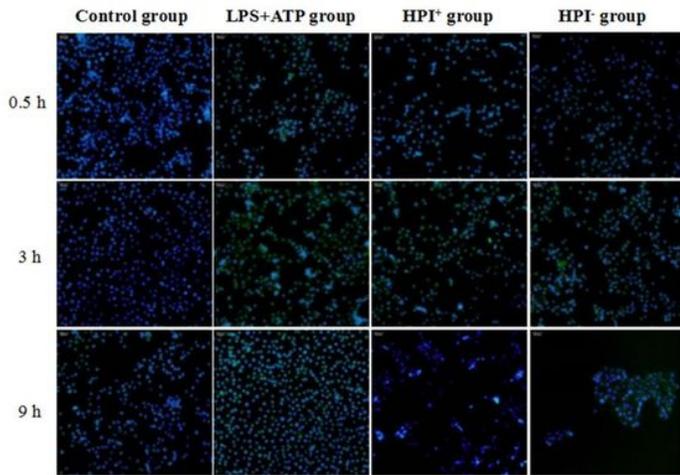
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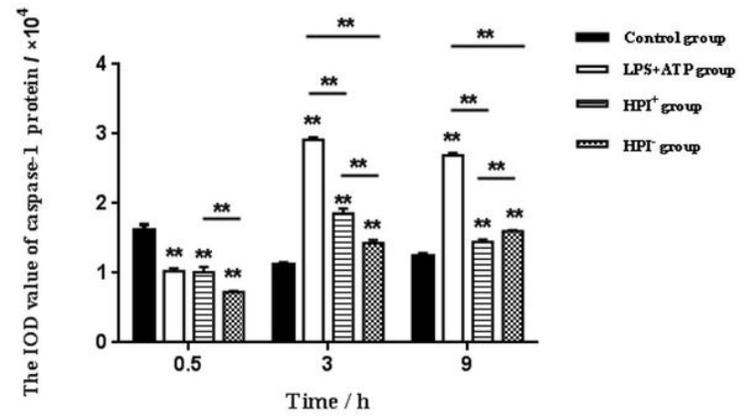
D

Figure 5

Relative mRNA expression of NLRP3, caspase-1, IL-1 $\beta$  and IL-18 in mononuclear macrophages at each time-point after *E. coli* HPI infection. \* $P < 0.05$ , \*\* $P < 0.01$ .



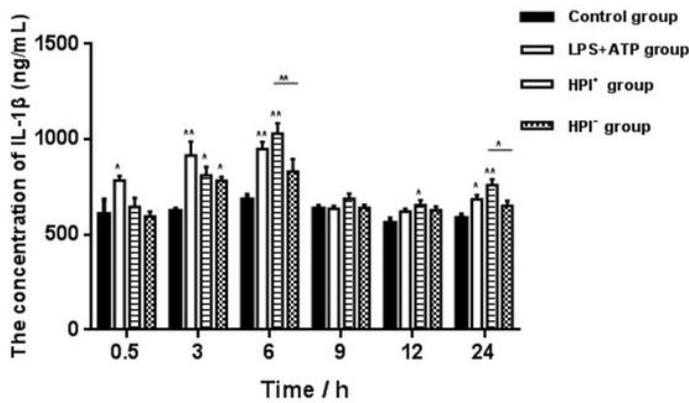
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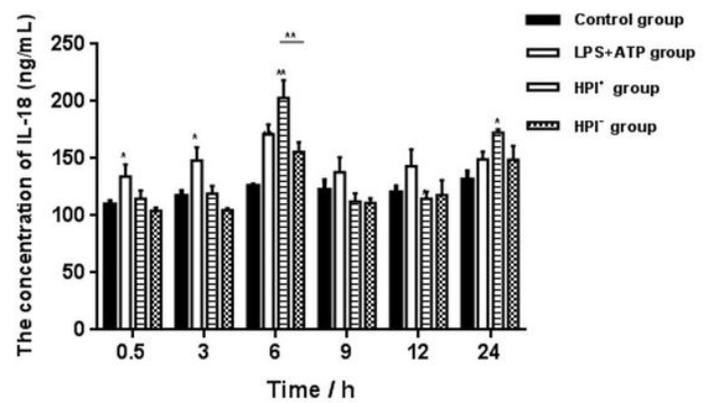
B

**Figure 6**

A: Caspase-1 protein expression in mononuclear macrophages at different-point after treatment (200× magnification; caspase-1 staining, green and nuclear staining, blue); B: The IOD value of caspase-1 protein in mononuclear macrophages at 0.5 h, 3 h and 9 h after infection with *E. coli* HPI. \* $P < 0.05$ , \*\* $P < 0.01$ .



A



B

**Figure 7**

The concentration of IL-1β (A) and IL-18 (B) in mononuclear macrophages at 0.5, 3, 6, 9, 12 and 24 h after infection with *E. coli*. \* $P < 0.05$ , \*\* $P < 0.01$ .

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