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Short Communication

Role of Apolipoprotein A-I in Protecting against Endotoxin Toxicity

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Abstract High density lipoprotein (HDL) binds lipopolysaccharide (LPS or endotoxin) and neutralizes its toxicity. We investigated the function of Apolipoprotein A-I (ApoA-I), a major apolipoprotein in HDL, in this process. Mouse macrophages were incubated with LPS, LPS+ApoA-I, LPS+ApoA-I+LFF (lipoprotein-free plasma fraction d>1.210 g/ml), LPS+HDL, LPS+HDL+LFF, respectively. MTT method was used to detect the mortality of L-929 cells which were attacked by the release-out cytokines in LPS-activated macrophages. It was found that ApoA-I significantly decreased L-929 cells mortality caused by LPS treatment (LPS vs. LPS+ApoA-I, $P<0.05$) and this effect became even more significant when LFF was utilized (LPS vs. LPS+ApoA-I+LFF, $P<0.01$; LPS vs. LPS+HDL+LFF, $P<0.01$). There was no significant difference between LPS+ApoA-I+LFF and LPS+HDL+LFF treatment, indicating that ApoA-I was the main factor. We also investigated *in vivo* effects of ApoA-I on mouse mortality rate and survival time after LPS administration. We found that the mortality in LPS+ApoA-I group (20%) and in LPS+ApoA-I+LFF group (10%) was significantly lower than that in LPS group (80%) ($P<0.05$, $P<0.01$, respectively); the survival time was (43.20 ± 10.13) h in LPS+ApoA-I group and (46.80 ± 3.79) h in LPS+ApoA-I+LFF group, which were significantly longer than that in LPS group (16.25 ± 17.28) h ($P<0.01$). We also carried out *in vitro* binding study to investigate the binding capacity of ApoA-I and ApoA-I+LFF to fluorescence labeled LPS (FITC-LPS). It was shown that both ApoA-I and ApoA-I+LFF could bind with FITC-LPS, however, the binding capacity of ApoA-I+LFF to FITC-LPS (64.47 ± 8.06) was significantly higher than that of ApoA-I alone (24.35 ± 3.70) ($P<0.01$). The results suggest that: (1) ApoA-I has the ability to bind with and protect against LPS; (2) LFF enhances the effect of ApoA-I; (3) ApoA-I is the major contributor for HDL anti-endotoxin function.

Key words Apolipoprotein-I; lipopolysaccharide (LPS); macrophages; FITC-lipopolysaccharide (FITC-LPS); L-929 cells

Lipopolysaccharide (LPS or endotoxin) is released from the outer surface membrane of G^- bacteria. Lipid A is the main component of toxicity in LPS. In human and experimental animals, LPS produces multiple pathophysiological changes, including fever, diarrhea, hypotensive shock, disseminated intravascular coagulation, and multiple organ failure. These changes contribute to the lethal effects of LPS. Endotoxemia is still one of the main concerns in clinic treatment. There is LPS binding protein (LBP) in plasma which binds LPS to form LBP-LPS complex [1]; the complex binds to membrane-associated CD_{14} receptor on monocytes/macrophages and initiates the activation of cytokine release (IL-1, IL-6, TNF-α, etc.) and pathologic response [2]. The LPS-LBP complex binds to plasma lipoproteins, resulting in detoxication of LPS [3–5]. Studies from several laboratories have demonstrated that HDL binds LPS [6] and neutralizes its toxicity [7,8]. Wu *et al.* [9] reported that high-density lipoproteins might possess anti-inflammatory properties and play a crucial role in innate immunity by regulating the inflammatory response as well as reducing the severity of organ injury in animals and patients with septic shock via LPS binding and neutralization. The previous studies in our laboratory showed that HDL was the only component in plasma lipoproteins to bind LPS, VLDL and LDL did not or rarely bind LPS [10]. The mechanism of HDL mediated anti-endotoxin is still unclear. There were several studies indi-

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cated that anti-endotoxin function of HDL was due to the interaction between lipid in LPS and phospholipid in HDL [8,11]. However, there were several other studies showing that ApoA-I, instead of lipid in HDL, might be the major factor [12,13]. ApoA-I, the major protein component of serum HDL, has amphipathic α-helix structure. The lipid bind sites on the amphipathic α-helix may be the domains to bind the lipid A of LPS and neutralize the toxicity of LPS.

In the present study, we investigated the function and mechanism of ApoA-I in protecting against endotoxin toxicity.

Materials and Methods

Materials

Human plasma was purchased from Shanghai Blood Center; LPS (from *E. coli*) was purchased from Sigma; mice were purchased from Laboratory animal Department of Fudan University; Bacillus Calmette-Guerin vaccine (BCG) was purchased from Shanghai Institute of Biological Products (Lot No: 200207001); L-929 cells were obtained from Institute of Biochemistry and Cell Biology (Shanghai, China); CP100MX ultracentrifuge was made by Hitachi Koki Co., Ltd.; BioCAD700E perfusion Chromatography was made by Applied Biosystems Company; LS 55 luminescence spectroscopy was made by Perkin Elmer Ltd.; ultracentrifugation media containing 27 g NaCl, 1.24 g EDTA in 1000 ml H_2O, and KBr was used to adjust the density of the media.

Preparation of HDL and LFF

According to previous method of our laboratory [10], human HDL (d: 1.063–1.210 g/ml)/ and lipoprotein-free plasma fraction (LFF, d>1.210 g/ml) were isolated and collected by ultracentrifugation. After dialyzed extensively and concentrated, the concentration of proteins was measured by the method of Bradford.

Preparation of ApoA-I

HDL was delipidated by regular method. ApoA-I was isolated from the delipidated HDL apolipoproteins (ApoHDL) using anion-exchange column (POROS HQ column) on BIO-CAD workstation. The optimum isolation condition was: elution was achieved using a linear gradient of 0 to 0.8 M NaCl in starting buffer (50 mM Tris-HCl, 6 M Urea, pH 8.2) within 12 min. After each run, the column was washed for 10 min with 2 M NaCl in starting buffer and equilibrated with starting buffer. Then 0.5 mg of protein in starting buffer was applied to HQ column, and separated at a flow rate of 5 ml/min. All samples and buffers were filtered through a 0.22 μm Millipore membrane filter and degassed before use. Peaks were collected by auto-collector. Each peak was identified by SDS-PAGE (6% stacking gel and 15% separating gel).

Protecting role of ApoA-I against cytotoxicity of LPS-activated macrophages

L-929 cells were used as target cells for macrophage cytokine cytotoxicity. Cytotoxicity was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method [14]. 8 mice (20–22 g) were injected with 25 mg/kg starch (i.p.). On the 3rd day of starch injection, the animals were sacrificed and the peritoneal macrophages were harvested by washing with 5 ml PBS (pH 7.4). The macrophages suspension was adjusted to the desired concentration (4×10^6 cells/ml) by RPMI 1640 with 10% FCS, plated in 96 well flat-bottomed microculture plate (0.1 ml/well), and cultured at 37 °C in a 5% CO_2 atmosphere for 4 h. The cultures were washed to remove non-adherent cells, incubated with 0.1 ml of RPMI 1640 for another 16 h.

Exponentially growing L-929 cells were digested by 2.5 g/L trypsin and suspended in RPMI 1640 to a concentration of 5×10^5 cells/ml. 20 μl of L-929 cells were subjected to macrophages and different treatment of LPS at 37 °C for 18 h. The treatments were LPS, LPS+ApoA-I, LPS+ApoA-I+LFF, LPS+HDL, LPS+HDL+LFF, and LPS+LFF, respectively (n=5), with concentration of LPS being 0.2 mg/L, ApoA-I 0.2 g/L, HDL 0.222 g/L, and LFF 0.1 g/L. A control group without LPS was matched to each test group. 20 μl of MTT (5 g/L) were added to each well, incubated at 37 °C for 4 h. 150 μl of DMSO were added to dissolve the formazan. Formazan quantification was performed by measuring the absorbance on an automatic plate reader with a 570 nm test wavelength and a 630 nm reference wavelength. The mortality of L-929 cells was calculated as following [Formula (1)]:

$$\text{Mortality}(\text{Cell}_{\text{L-929}}) = \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100\% \quad (1)$$

In vivo mortality rate and survival time measurements

Mice were injected with BCG solution through the tail vein (dose: 4 mg BCG/20 g mouse weight) on the 10th day prior to the study to improve the sensitivity of the mouse to LPS [15]. 30 mice were randomized to three

groups (n=10), and injected with LPS, LPS+ApoA-I and LPS+ApoA-I+LFF through the tail vein, respectively (doses: LPS 8 mg/kg, ApoA-I 50 mg/kg, LFF 100 mg/kg). The survival time and death rate of mice in each group were observed during 48 h. All mice were kept in a temperature-controlled environment.

In order to evaluate the protective effect of LFF, 20 mice were divided into two groups and injected with LPS and LPS+LFF respectively (n=10), with LPS being 8.4 mg/kg and LFF 100 mg/kg.

In vitro binding of ApoA-I to FITC-LPS

Two experimental groups were ApoA-I+FITC-LPS and ApoA-I+LFF+FITC-LPS (n=3). 96-well plate was coated with 200 μl (100 mg/L) of ApoA-I in 50 mM carbonate buffer (pH 9.6) at 4 °C overnight. After washing, 5 g/L BSA was added to block. 50 μl (25 g/L) of LFF was added if needed and incubated at 37 °C for another 4 h. After washing, 50 μl (2×10^{-4} g/L) of FITC-LPS were added. After 4 h incubation, bound fluorescent strength (FS) was detected (492 nm as excited length, 514 nm as emission length). In control groups, FITC-LPS was replaced by PBS. Bound fluorescent strength was calculated as following [Formula (2)]:

$$FS_{\text{bound}} = FS_{\text{test}} - FS_{\text{control}} \quad (2)$$

In order to observe the direct binding of ApoA-I to LPS further, we use polystyrene bead (3 mm diameter) to substituted 96-well plates, after treated with the same procedure as the ApoA-I+FITC-LPS group, the bound FS on the surface of the bead was observed under fluorescence microscope. The control group was treated in the same procedure (ApoA-I was substituted by BSA).

Statistics

Statistical analysis was performed using Q-test and Student's t-test.

Results

ApoA-I isolation and identification

We purified ApoA-I using perfusion chromatography (POROS HQ column). SDS-PAGE indicated that peak B contained ApoA-I (MW: 28 kD) [Fig. 1(B), lane 2]. ApoA-I in peak B was used in the following experiments.

Protecting role of ApoA-I against cytotoxicity of LPS-activated macrophages

L-929 cells would be killed by the cytokines released from LPS-stimulated macrophages. We found that ApoA-I significantly decreased L-929 cells mortality caused by LPS treatment (LPS vs. LPS+ApoA-I, P<0.05) and this effect became even more significant when LFF was utilized (LPS vs. LPS+ApoA-I+LFF; LPS vs. LPS+HDL+LFF, P<0.01, respectively) (Table 1). There was no difference between LPS+ApoA-I+LFF and LPS+HDL+LFF treatment indicating ApoA-I was the main factor (Table 1).

ApoA-I decreased mortality rate and increased survival rate of mice treated by LPS

In order to confirm the *in vitro* results, the mouse mortality and survival time after challenging with different

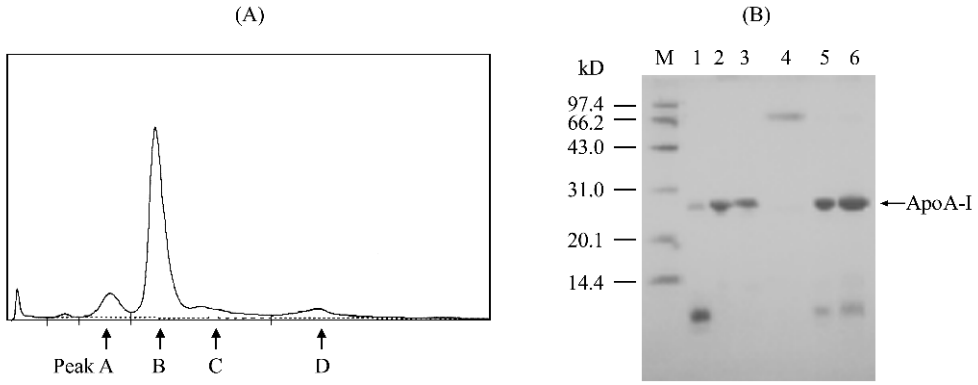

Fig. 1 ApoA-I isolation and identification from perfusion chromatography by SDS-PAGE

(A) ApoA-I isolated from perfusion chromatography, delipidated HDL was loaded on POROS HQ column according to the procedure described in "Materials and Methods". The protein peaks were monitored by A_{280}. (B) Identification of proteins from perfusion chromatography by SDS-PAGE. M, marker; 1, peak A; 2, peak B; 3, peak C; 4, peak D; 5, ApoHDL; 6, HDL.

Table 1 Protecting role of ApoA-I against cytotoxicity of LPS-activated macrophages

Group	Mortality rate of L-929 cells (%)
LPS	4.32 ± 3.71
LPS+ApoA-I	–5.35 ± 2.07*
LPS+ApoA-I+LFF	–8.98 ± 4.81**
LPS+HDL	5.02 ± 3.09
LPS+HDL+LFF	–10.15 ± 4.65**
LPS+LFF	7.32 ± 5.14

*P<0.05, **P<0.01 vs. LPS group, n=5. The minus in the data means that the L-929 cells grow in this experimental condition other than being killed.

conditions of LPS were investigated. As shown in Table 2, the mortality in LPS+ApoA-I (20%) and LPS+ApoA-I+LFF group (10%) was significantly lower than that in LPS group (80%) (P<0.05, P<0.01, respectively), and the survival time in LPS+ApoA-I [(43.20 ± 10.13) h] and LPS+ApoA-I+LFF group [(46.80 ± 3.79) h] was significantly longer than that in LPS group (16.25 ± 17.28) h (P<0.01). LFF itself had no effect on the reduction of toxicity caused by LPS administration (Table 3). These data suggested that ApoA-I had the ability for LPS inactivation, while LFF could only enhance the ability of ApoA-I.

The binding ability of ApoA-I to FITC-LPS

To explore the mechanism for the protecting effect of ApoA-I on LPS induced cellular toxicity, we invested the *in vitro* binding capacity of ApoA-I to LPS. As shown in Table 4, ApoA-I could bind with FITC-LPS (fluorescence labeled LPS) and this bind ability was significantly enhanced while LFF was present (P<0.01). Moreover, we found that ApoA-I coated polystyrene bead can also bind with FITC-LPS (Fig. 2).

Table 2 The effect of ApoA-I on protecting mice from LPS induced death

Group	Death rate (%)	Survival time (h)
LPS	80	16.25 ± 17.28
ApoA-I +LPS	20*	43.20 ± 10.13**
ApoA-I +LFF+LPS	10**	46.80 ± 3.79**

Data are represented as mean ± SD, n=10. *P<0.05, **P<0.01 vs. LPS group.

Table 3 The effect of LFF on protecting mice from LPS induced death

Group	Death rate (%)	Survival time (h)
LPS	100	6.67 ± 2.26
LPS+LFF	100	6.46 ± 3.86

Data are represented as mean ± SD, n=10.

Table 4 The binding ability of ApoA-I to FITC-LPS

Group	FS
ApoA-I	13.77 ± 5.15
ApoA-I+FITC-LPS	38.12 ± 3.70[a]
Δ1	24.35 ± 3.70
ApoA-I+LFF	2.17 ± 1.27
ApoA-I+LFF+FITC-LPS	66.64 ± 8.06[b]
Δ2	64.47 ± 8.06**

Data are represented as mean ± SD, n=3. Δ1, FS(ApoA-I+FITC-LPS)–FS(ApoA-I); Δ2, FS(ApoA-I+LFF+FITC-LPS)–FS(ApoA-I+LFF). [a]P<0.01 vs. ApoA-I group; [b]P<0.01 vs. (ApoA-I+LFF) group; **P<0.01 vs. Δ1.

Discussion

LPS can form complex with LPS-binding protein (LBP) [2]. The binding of the LPS-LBP complex to the CD14 receptor on the surface of the monocytes/macrophages lineage triggers cell responses of production and release of a cascade of cytokines including tumor necrosis factor α (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6), etc. These cytokines mediate the inflammatory response to the G^- bacteria infection and play a direct role in the pathogenesis of endotoxic shock [16]. Imai *et al.* [17] reported that ApoA-I suppressed the TNF-α release in endotoxemia and decreased the mortality rate of rats. L-929 cells were used as target cells for cytokine cytotoxic activity. Because the cytotoxicity of LPS is an integrated result of all released cytokines, we use mortality rate of L-929 cells as an index to judge the release of macrophage cytokines by LPS indirectly. The mortality rate of L-929 cells in LPS+ApoA-I group [(–5.35 ± 2.07)%] demonstrated the detoxification ability of ApoA-I, and ApoA-I could inhibit the release of cytokines from activated macrophages. The mortality rate of L-929 cells in LPS+ApoA-I+LFF [(–8.98 ± 4.81)%] demonstrated that LFF could enhance the detoxicity ability of ApoA-I. The minus of the data means that the L-929 cells grow in this experimental condition other than being killed. It was pos-

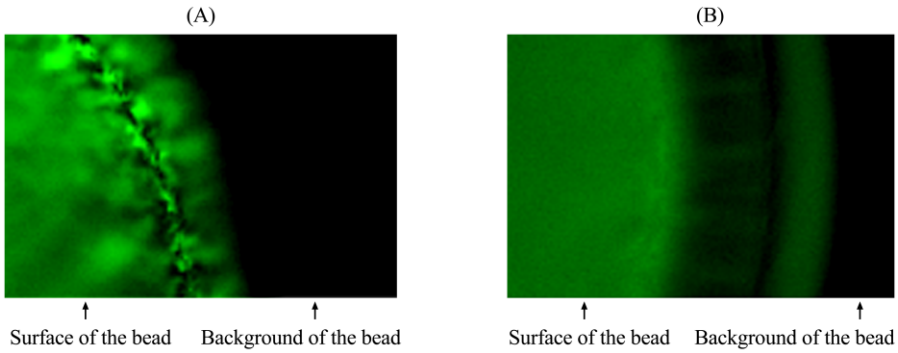

Fig. 2 Fluorescence microphotograph (100×)

(A) The bound FITC-LPS on the surface of 3 mm-diameter bead which was coated by ApoA-I was shown. (B) There was no obvious bound FITC-LPS on the surface of 3 mm-diameter bead which was coated by BSA as shown in the control group.

sible that the cytotoxicity could be decreased because of the detoxification of LPS by ApoA-I. The results of the mortality rate of LPS+HDL+LFF group (–10.15 ± 4.65)% and LPS+ApoA-I+LFF group (–8.98 ± 4.81)% (Table 1) showed that ApoA-I was almost as effective as HDL. We can make the conclusion that ApoA-I is the main component of HDL to protect cells against LPS toxicity.

Mouse had low sensitivity to LPS, and higher dose of LPS than 50 mg/kg was needed to trigger mouse endotoxic shock. We used BCG to sensitize mouse to LPS, so that even 8 mg/kg dose of LPS would mimic endotoxic shock. The results of our previous study [10] and this work (Table 3) showed that LFF alone had no ability to diminish the LPS toxicity. The mouse survival time in LPS+ApoA-I group (43.20 ± 10.13) h, and LPS+ApoA-I+LFF group (46.80 ± 3.79) h were significantly longer than that in LPS group (16.25 ± 17.28) h ($P<0.01$). The mortality rate of mouse in LPS+ApoA-I group (20%, $P<0.05$) and LPS+ApoA-I+LFF group (10%, $P<0.01$) were significantly lower than that in LPS group (80%). Therefore, it is obvious that ApoA-I protects mice against endotoxicity *in vivo*, and LFF enhances this effect.

LBP is a lipid-transfer protein in plasma, it is present in normal serum at <0.5 mg/L in complex form of LBP-HDL, and rises to 50 mg/L 24 hours after induction of an acute phase response. It is a 60-kD glycoprotein of a 50-kD single-chain polypeptide synthesized in hepatocytes. LBP has a binding site for lipid A in LPS, with high affinity to LPS from bacteria. The complex form of LBP-HDL-LPS could neutralize LPS toxicity [18]. During the ultracentrifugation procedure, the LBP associated to HDL would disassociate and fall into LFF fraction (d>1.210 g/ml). Our previous test showed that HDL prepared by PEG deposition method could significantly prolong survival time and decrease death rate of mice which were challenged with LPS, while HDL prepared by ultracentrifugation could not inactivate LPS [10]. If LFF (or LBP) was added, the HDL prepared by ultracentrifugation could show LPS-inactivating capacity. The data in our present cytotoxicity assay also showed that HDL prepared by ultracentrifugation would not decrease the mortality rate of L-929 cells caused by released cytokines from LPS-stimulated macrophages unless LFF was added (Table 1). These results were consistent with those from other laboratories [19].

The present study showed for the first time that different from HDL, ApoA-I purified by ultracentrifugation methods inactivated and bound LPS without LBP (Table 1, 2, and 4, Fig. 2). These results implies that there are at least two kinds of LPS binding site: one is possibly lipid binding domain of hydrophobic regions in ApoA-I amphipathic α-helix structure by which ApoA-I binds LPS (lipid A) directly; another is the LBP binding site in ApoA-I, by which ApoA-I binds LPS indirectly. Recently Massamiri *et al.* [12] reported that at least three regions of ApoA-I located within residues 1–31, 95–164 and 178–200 participated either directly or indirectly in LBP/HDL association. We speculate that HDL can not bind LPS directly because the lipid binding regions of ApoA-I have been bound by lipid component of HDL, so the binding of LPS to HDL needs the participation of LFF. Purified ApoA-I binds LPS directly because the lipid-binding sites on ApoA-I have been exposed. We conclude that ApoA-I can act to protect against endotoxin, and this protection may be augmented by LFF.

References

1 Ulevitch RJ. Recognition of bacterial endotoxins by receptor-dependent mechanisms. Adv Immunol, 1993, 53: 267–289

2 Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS *et al*. Structure and function of lipopolysaccharide binding protein. Science, 1990, 249(4975): 1429–1431
3 de Haas CJ, Poppelier MJ, van Kessel KP, van Strijp JA. Serum amyloid P component prevents high-density lipoprotein-mediated neutralization of lipopolysaccharide. Infect Immun, 2000, 68(9): 4954–4960
4 Levels JH, Abraham PR, van den Ende A, van Deventer SJ. Distribution and kinetics of lipoprotein-bound endotoxin. Infect Immun, 2001, 69(5): 2821–2828
5 Jack RS, Fan X, Bernheiden M, Rune G, Ehlers M, Weber A, Kirsch G *et al*. Lipopolysaccharide-binding protein is required to combat a murine Gram-negative bacterial infection. Nature, 1997, 389(6652): 742–745
6 Brandenburg K, Jurgens G, Andra J, Lindner B, Koch MH, Blume A, Garidel P. Biophysical characterization of the interaction of high-density lipoprotein (HDL) with endotoxins. Eur J Biochem, 2002, 269(23): 5972–5981
7 van Leeuwen HJ, van Beek AP, Dallinga-Thie GM, van Strijp JA, Verhoef J, van Kessel KP. The role of high density lipoprotein in sepsis. Nertherlands J Med, 2001, 59(3): 102–110
8 Levine DM, Parker TS, Donnelly TM, Walsh A, Rubin AL. *In vivo* protection against endotoxin by plasma high density lipoprotein. Proc Natl Acad Sci USA, 1993, 90(24): 12040–12044
9 Wu A, Hinds CJ, Thiemermann C. High-density lipoproteins in sepsis and septic shock: Metabolism, actions, and therapeutic applications. Shock, 2004, 21(3): 210–221
10 Zhang H, Wu MP, Lou B, Chen PF. Role of high density lipoprotein in protecting against endotoxin toxity. Fudan Univ J Med Sci, 2003, 30(5): 474–476
11 Parker TS, Levine DM, Chang JC, Laxer J, Coffin CC, Rubin AL. Reconstituted high-density lipoprotein neutralizes Gram-negative bacterial lipopolysaccharides in human whole blood. Infect Immun, 1995, 63(1): 253–258
12 Massamiri T, Tobias PS, Curtiss LK. Structural determinants for the interaction of lipopolysaccharide binding protein with purified high density lipoproteins: Role of apolipoprotein A-I. J Lipid Res, 1997, 38(3): 516–525
13 Park CT, Wright SD. Plasma lipopolysaccharide-binding protein is found associated with a particle containing apolipoprotein A-I, phospholipid, and factor H-related proteins. J Biol Chem, 1996, 271(30): 18054–18060
14 Ba DN ed. Contemporary Immunological Technology and Application, Beijing: Peking University Medical Press, 1998, 262–263
15 Liu YH, Ding SP, Lin AH, Fang JG, Shi SP. The protective effects of different polar fractions from radix isatids on endotoxin challenged mice. Acta Univ Med Tingji, 2001, 30(3): 272–273
16 Cavaillon JM, Fitting C, Haeffner-Cavaillon N, Kirsch SJ, Warren HS. Cytokine response by monocytes and macrophages to free and lipoprotein-bound lipopolysaccharide. Infect Immun, 1990, 58(7): 2375–2382
17 Imai T, Fujita T, Yamazaki Y. Beneficial effects of apolipoprotein A-I on endotoxemia. Surg Today, 2003, 33(9): 684–687
18 Wurfel MM, Kunitake ST, Lichenstein H, Kane JP, Wright SD. Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in neutralization of LPS. J Exp Med, 1994, 180(3): 1025–1035
19 Flegel WA, Baumstark MW, Weinstock C, Berg A, Northoff H. Prevention of endotoxin-induced monokine release by human low- and high-density lipoproteins and by apolipoprotein A-I. Infect Immun, 1993, 61(12): 5140–5146

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