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# A novel Fas-binding outer membrane protein and lipopolysaccharide of *Leptospira interrogans* induce macrophage apoptosis through the Fas/FasL-caspase-8/-3 pathway.

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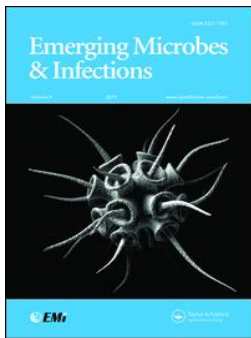
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ARTICLE

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# A novel Fas-binding outer membrane protein and lipopolysaccharide of *Leptospira interrogans* induce macrophage apoptosis through the Fas/FasL-caspase-8/-3 pathway

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## Abstract

*Leptospira interrogans* is the major causative agent of leptospirosis, an emerging, globally spreading zoonotic infectious disease. The pathogen induces macrophage apoptosis, but the molecular basis and mechanism remain unknown. In the present study, we found that *L. interrogans* caused apoptosis of phagocytosis-inhibited macrophages, and the product of the *L. interrogans* LB047 gene (Lep-OMP047) was the unique protein captured by mouse and human Fas proteins. The recombinant expressed Lep-OMP047 (rLep-OMP047) strongly bound mouse and human Fas proteins with equilibrium association constant ( $K_D$ ) values of  $5.20 \times 10^{-6}$  to  $2.84 \times 10^{-9}$  M according to surface plasmon resonance measurement and isothermal titration calorimetry. Flow-cytometric examination showed that 5  $\mu$ g rLep-OMP047 or 1  $\mu$ g lipopolysaccharide of *L. interrogans* (Lep-LPS) caused 43.70% or 21.90% early apoptosis in mouse J774A.1 macrophages and 28.41% or 15.80% for PMA-differentiated human THP-1 macrophages, respectively, but the apoptosis was blocked by Fas-antagonizing IgGs, Fas siRNAs, and caspase-8/-3 inhibitors. Moreover, Lep-OMP047 was significantly upregulated during infection of macrophages. Lep-LPS promoted the expression and cytomembrane translocation of Fas and FasL in macrophages. The JNK and p38 MAPK but not ERK signaling pathways, as well as the transcription factors c-Jun and ATF2 but not CHOP, mediated Lep-LPS-induced Fas/FasL expression and translocation. TLR2 but not TLR4 mediated Lep-LPS-induced JNK/p38 MAPK activation. Therefore, we demonstrated that a novel Fas-binding OMP and LPS of *L. interrogans* induce macrophage apoptosis through the Fas/FasL-caspase-8/-3 pathway.

## Introduction

Leptospirosis is a worldwide-spreading zoonotic disease caused by pathogenic *Leptospira* genospecies<sup>1,2</sup>. The disease has been endemic in Asia, Oceania and South America<sup>2-5</sup>, but in recent years, it has been frequently reported in Europe, North America, and Africa<sup>5-9</sup>, where it is considered an emerging or re-emerging infectious disease<sup>10</sup>.

Leptospirosis is transmitted from host animals to humans by contact with water or wet soil that has been contaminated with pathogenic *Leptospira*-containing

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animal urine<sup>11</sup>. After invading the human body through the skin and mucosa, pathogenic *Leptospira* can promptly enter the blood stream to cause toxic septicemia and then spread into internal organs such as the lungs, liver, and kidneys<sup>11–13</sup>. Leptospirosis patients are clinically characterized by high fever, myalgia, jaundice, superficial lymphadenectasis, and conjunctival hemorrhage, but severe patients can rapidly die due to septic shock, pulmonary diffuse hemorrhage, and renal failure<sup>14,15</sup>.

Professional phagocytes, such as macrophages, play a crucial role in the elimination of pathogens by phagocytosis<sup>16,17</sup>. However, pathogens have also evolved different strategies to resist phagocytosis, including the ability to stimulate apoptosis of macrophages<sup>18,19</sup>. Caspase-mediated cell apoptosis is the main mechanism of apoptosis through cytomembrane-based Fas/FasL-caspase-8/-3 and mitochondrial CytC-caspase-9/-3 pathways<sup>20</sup>. Infection with *Escherichia coli* and *Mycobacterium tuberculosis* causes apoptosis of mouse macrophages through CytC-caspase-9/-3 and Fas/FasL-caspase-8/-3 pathways<sup>21,22</sup>. Our previous studies revealed that pathogenic *Leptospira interrogans* induces the apoptosis of mouse and human macrophages through Fas/FasL-caspase-8/-3 and caspase-independent AIF/EndoG pathways<sup>23,24</sup>. Recently, we observed that *L. interrogans* also caused apoptosis of phagocytosis-inhibited macrophages, implying that some leptospiral surface molecules are involved in the apoptosis. However, the macrophage apoptosis-inducing surface molecules of *L. interrogans* have not been identified yet.

Outer membrane proteins (OMP) and lipopolysaccharide (LPS) are the major surface molecules of Gram-negative prokaryotic microbes, including *Leptospira*<sup>25,26</sup>. In addition to its endotoxicity, *E. coli* LPS promotes exogenous FasL-induced mouse macrophage apoptosis through the JNK/p38 MAPK-signaling pathway-mediated increase of Fas expression and cytomembrane translocation<sup>27,28</sup>. *Pseudomonas aeruginosa* LPS stimulates Fas-dependent mouse macrophage apoptosis<sup>29</sup>. However, the possible role of leptospiral OMP and LPS in the apoptosis of macrophages remains unknown.

*L. interrogans* is the most common causative agent of leptospirosis<sup>1–3</sup>. In China, strains from *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai are responsible for disease in over 60% of leptospirosis patients<sup>3,15</sup>. On the other hand, macrophages but not neutrophils act as the major infiltrating and phagocytotic cells in leptospirosis patients and animals<sup>30,31</sup>. In this study, we identified a novel Fas-binding outer membrane protein of *L. interrogans* as the inducer of human and mouse macrophage apoptosis by activation of the Fas-dependent caspase-8/-3 pathway and *L. interrogans* LPS as a promoter of macrophage apoptosis through JNK/p38 MAPK

signaling-mediated increase of Fas/FasL expression and cytomembrane translocation.

## Results

### *L. interrogans*-induced apoptosis of phagocytosis-inhibited macrophages

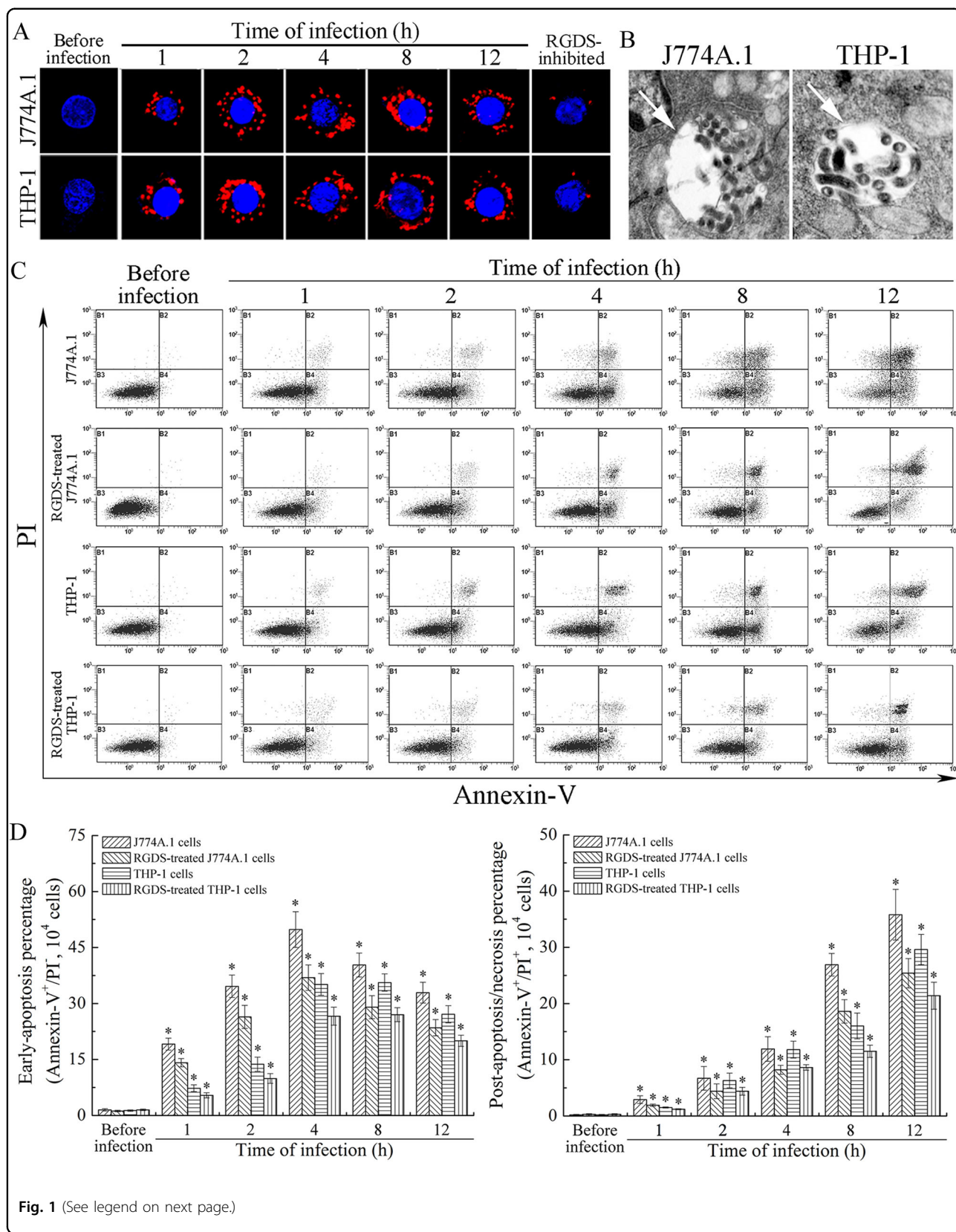
The flow-cytometric examination confirmed that over 90% of THP-1 monocytes were differentiated into CD68<sup>+</sup> macrophages after PMA treatment (Supplementary Figure S1)<sup>32</sup>. The confocal and transmission electron microscopy showed that the J774A.1 and THP-1 macrophages could phagocytose *L. interrogans* strain Lai, but RGDS, a universal integrin-dependent phagocytosis inhibitor<sup>33</sup>, blocked this phagocytosis (Fig. 1a, b). The flow-cytometric examination showed that the two *Leptospira*-infected macrophages exhibited early-apoptosis and post-apoptosis/necrosis features, with 49.77% and 35.63% of maximal early-apoptotic percentages for J774A.1 and THP-1 macrophages at 4 and 8 h post-infection (Fig. 1c, d). However, RGDS only caused a small decrease in the apoptotic percentages. These data suggest that some surface substances of *L. interrogans* may act as an inducer of mouse and human macrophage apoptosis.

### Characterization of Lep-OMPs and Lep-LPS

The OMP extracts of *L. interrogans* strain Lai from aqueous and detergent phases (aLep-OMP and dLep-OMP) presented different SDS-PAGE profiles (Supplementary Figure S2a), but the antibody against rOMP-L1 from *L. interrogans* (rLep-OMP-L1-IgG) bound to both the aLep-OMP and dLep-OMP (Supplementary Figure S2b). Lep-LPS showed up in the gel as heterogeneous macromolecules (Supplementary Figure S2c), as previously reported<sup>34,35</sup>. However, Lep-LPS (0.5 ng) but not a- or dLep-OMPs (100–500 µg) solidified limulus amoebocyte lysates (Supplementary Figure S2d). These data suggest that the a- and dLep-OMP had no LPS contamination and that Lep-LPS possesses endotoxic activity.

### Abilities of a- and dLep-OMP and Lep-LPS to induce macrophage apoptosis

The flow-cytometric examination showed that 200 µg dLep-OMP and 1 µg Lep-LPS induced the apoptosis of J774A.1 and THP-1 macrophages in a concentration-dependent manner (Fig. 2a–d). The maximal early-apoptotic percentages of J774A.1 or THP-1 macrophages were 24.13% or 18.07% at 2 h post treatment of dLep-OMP and 21.90% or 15.80% at 4 h post treatment of Lep-LPS. However, 200 µg aLep-OMP had no macrophage apoptosis-inducing ability (Supplementary Figure S3). These data suggest that some OMP components and LPS of *L. interrogans* can induce mouse and human macrophage apoptosis.



**Fig. 1** (See legend on next page.)

(see figure on previous page)

**Fig. 1 Leptospiral endocytosis and *Leptospira*-induced apoptotic death of macrophages.** **a** Phagocytosis of *L. interrogans* strain Lai by J774A.1 and THP-1 macrophages for the indicated times, determined by confocal microscopy. The blue plaques indicate the nucleus. The red spots indicate the intracellular leptospire. RGDS is an inhibitor of phagocytosis. **b** Phagosomes of *L. interrogans* strain Lai in J774A.1 and THP-1 macrophages, detected by transmission electron microscopy. The arrows indicate the leptospiral phagosomes. **c** Apoptosis of *L. interrogans* strain Lai-infected J774A.1 and THP-1 macrophages for the indicated times, determined by flow cytometry. The annexin V<sup>+</sup>/PI<sup>-</sup> macrophages are early-apoptotic, while the annexin V<sup>+</sup>/PI<sup>+</sup> macrophages are post-apoptotic/necrotic. RGDS is a phagocytosis inhibitor. **d** Statistical summary of the early-apoptotic and post-apoptotic/necrotic percentages in *L. interrogans*-infected J774A.1 and THP-1 macrophages. Statistical data from experiments such as that shown in **c**. Bars show the means  $\pm$  SD of three independent experiments. \*:  $p < 0.05$  vs the early-apoptotic or post-apoptotic/necrotic percentages of the macrophages without infection

### Fas-binding ability of leptospiral LB047 gene product

The co-precipitation test showed that only one band from dLep-OMP was captured by mouse or human Fas protein (Fig. 3a). The NanoLC-LTQ MS/MS identified this band as the product of the *L. interrogans* strain Lai LB047 gene (Lep-OMP047) according to its cleaved peptide sequences (RDGVQTESITYKA and KDGSTVQSEGSYKDDLKT). The western blot assay also confirmed that dLep-OMP but not aLep-OMP contained the Lep-OMP047 (Fig. 3b, c), with a 6.17% relative abundance in dLep-OMP according to the gray scale analysis of immunoblotting signals. The LB047 gene product was annotated as a hypothetical protein (GenBank accession No.: NC\_004343), but our prediction indicated this product as a YwqK-like outer membrane lipoprotein containing a signal peptidase II-cleaved signal peptide sequence (Supplementary Figure S4). YwqK is the antitoxin in the YwqJK toxin-antitoxin module of *Bacillus subtilis*, but its biological function remains unclear<sup>36</sup>. The confocal-microscopic examination showed that Lep-OMP047 was located on the surface of the spirochete and co-localized with Fas in the cytomembrane of mouse and human macrophages (Fig. 3d, e). In particular, the surface plasmon resonance (SPR) measurement revealed equilibrium association constant ( $K_D$ ) values of  $2.84 \times 10^{-9}$  or  $4.41 \times 10^{-9}$  M, respectively, for the recombinant expressed Lep-OMP047 (rLep-OMP047) binding to mouse or human Fas protein, while the isothermal titration calorimetric (ITC) detection showed  $K_D$  values of  $1.08 \times 10^{-6}$  or  $5.20 \times 10^{-6}$  M for rLep-OMP047 binding to mouse or human Fas protein (Fig. 3f, g). SPR and ITC  $K_D$  values lower than  $10^{-6}$  M indicate high affinity of protein-protein binding<sup>37,38</sup>. These data suggest that Lep-OMP047 is a Fas-binding outer membrane lipoprotein of *L. interrogans*.

### rLep-OMP047-induced Fas-caspase-8/-3-dependent macrophage apoptosis

Activated Fas/FasL can induce cell apoptosis through the caspase-8/-3 pathway<sup>20,21</sup>. The flow-cytometric examination showed that rLep-OMP047 (1–10  $\mu$ g) could induce the J774A.1 or THP-1 macrophage apoptosis in a

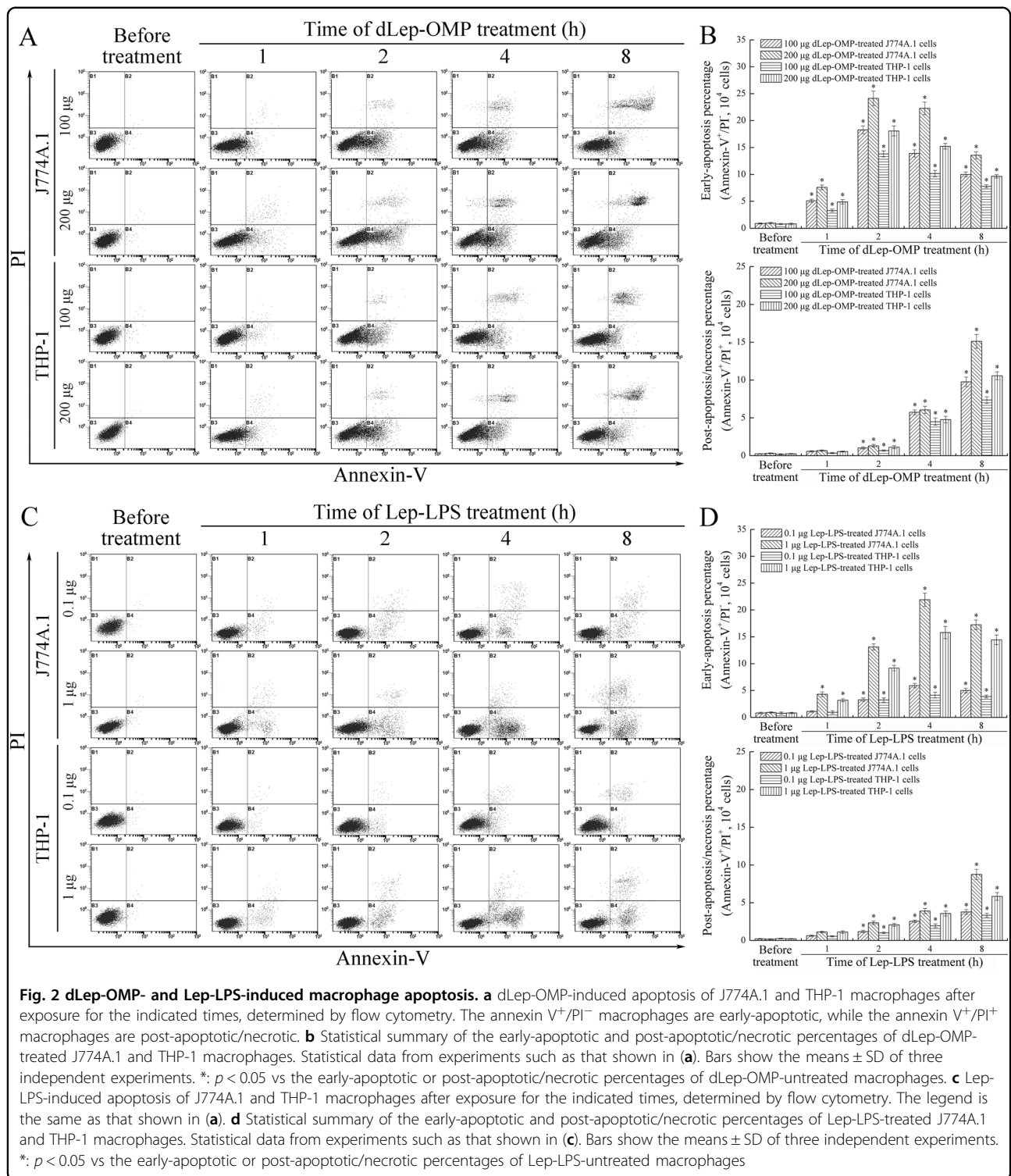
concentration-dependent manner, with the maximal early-apoptotic percentages of 56.61% or 39.55% at 2 h post treatment (Fig. 4a, b). The fluorospectrophotometric examination showed that caspase-8 and -3 but not caspase-9 were activated in the rLep-OMP047-treated macrophages (Fig. 4c). The blockage of Fas in J774A.1 or THP-1 macrophages with Fas-antagonizing IgG and the blockage of rLep-OMP047 with rLep-OMP047-IgG, as well as Fas depletion with siRNA interference and the inhibition of caspase-8 or -3 but not caspase-9, caused a decrease in rLep-OMP047-induced macrophage apoptosis (Fig. 4d). Caspase-8/-3 or caspase-9/-3 mediate cytomembrane Fas/FasL- or mitochondrion-dependent cell apoptosis<sup>20–23</sup>. These data suggest that Lep-OMP047 induces mouse and human macrophage apoptosis through a Fas/FasL-caspase-8/-3-dependent pathway.

### Extensive distribution and increased expression of LB047 gene during infection

The PCR and sequencing data showed that all thirteen tested strains of pathogenic *L. interrogans*, *L. borgpetersenii* and *L. weilii* possessed the LB047 gene, but this gene was not detectable in the two tested strains of saprophytic *L. biflexa* (Fig. 5a). The eight *L. interrogans* strains had high LB047 gene sequence identities (99.1–100%), but much lower identity (82.2–90.2%) compared to the LB047 genes from four *L. borgpetersenii* strains and one *L. weilii* strain (GenBank accession No.: MG557568–MG557580). The Lep-OMP047 mRNA level in *L. interrogans* strain Lai in EMJH or RPMI-1640 medium was relatively low. When the spirochetes were incubated with J774A.1 and THP-1 macrophages, Lep-OMP047 mRNA was significantly increased (Fig. 5b). The western blot assay also confirmed a significant increase in Lep-OMP047 expression during infection (Fig. 5c, d). These data suggest that Lep-OMP047 is involved in infection of host cells by *L. interrogans*.

### Increase in Lep-LPS-induced Fas/FasL expression and translocation

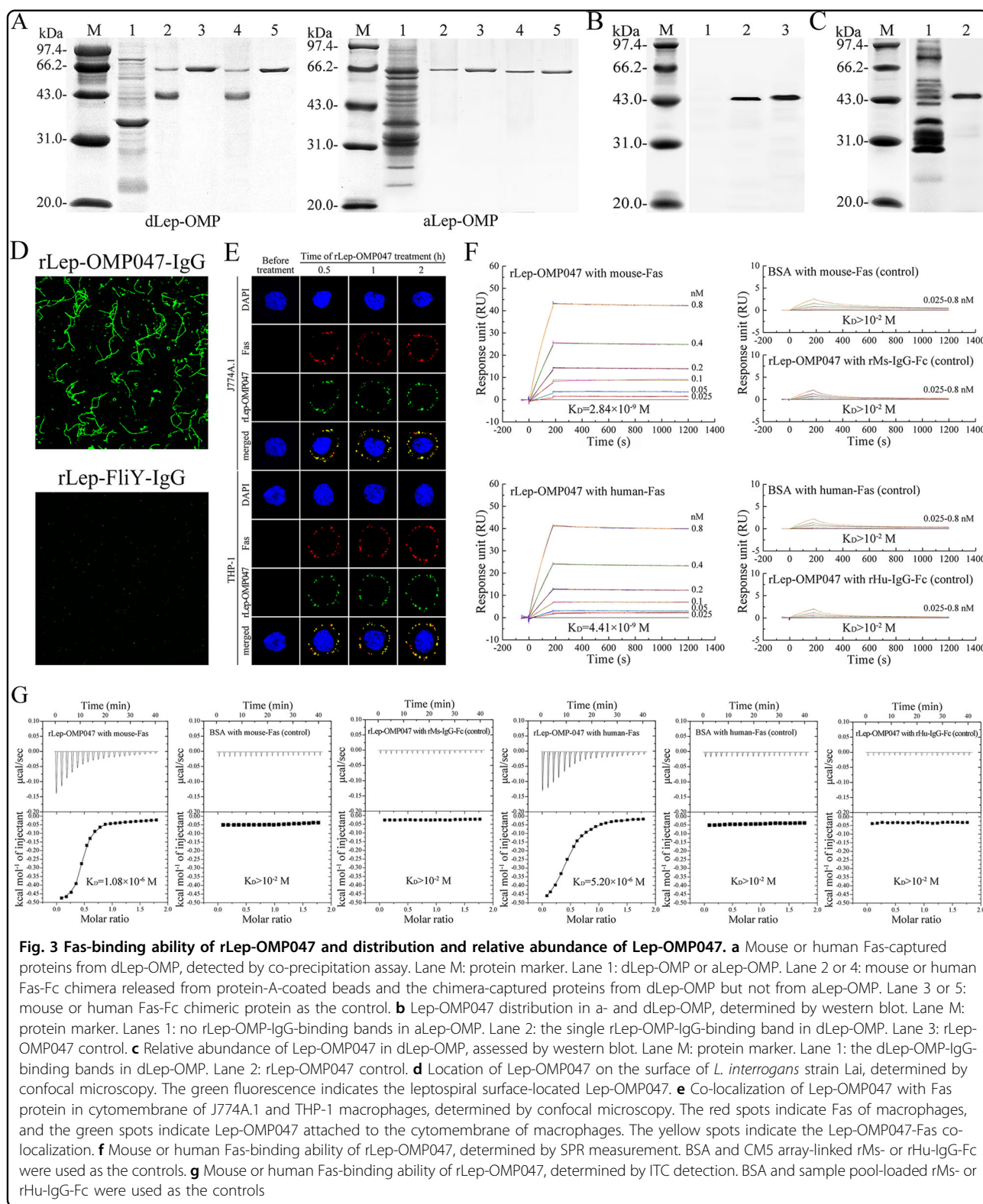
LPS of *E. coli* induces Fas and FasL expression and promotes their cytomembrane translocation<sup>27,28</sup>.



The qRT-PCR, western blot assay and flow-cytometric examination showed that Lep-LPS also significant increased Fas and FasL expression and cytomembrane translocation in J774A.1 and THP-1 macrophages (Fig. 6a–e). However, rLep-OMP047 had no ability to

induce the expression of Fas- and FasL-encoding genes in the two macrophages (Fig. 6a). These data suggest that Lep-LPS is involved in mouse and human macrophage apoptosis by upregulating Fas/FasL expression and cytomembrane translocation.





**Fig. 3** Fas-binding ability of rLep-OMP047 and distribution and relative abundance of Lep-OMP047. **a** Mouse or human Fas-captured proteins from dLep-OMP, detected by co-precipitation assay. Lane M: protein marker. Lane 1: dLep-OMP or aLep-OMP. Lane 2 or 4: mouse or human Fas-Fc chimera released from protein-A-coated beads and the chimera-captured proteins from dLep-OMP but not from aLep-OMP. Lane 3 or 5: mouse or human Fas-Fc chimeric protein as the control. **b** Lep-OMP047 distribution in a- and dLep-OMP, determined by western blot. Lane M: protein marker. Lanes 1: no rLep-OMP-IgG-binding bands in aLep-OMP. Lane 2: the single rLep-OMP-IgG-binding band in dLep-OMP. Lane 3: rLep-OMP047 control. **c** Relative abundance of Lep-OMP047 in dLep-OMP, assessed by western blot. Lane M: protein marker. Lane 1: the dLep-OMP-IgG-binding bands in dLep-OMP. Lane 2: rLep-OMP047 control. **d** Location of Lep-OMP047 on the surface of *L. interrogans* strain Lai, determined by confocal microscopy. The green fluorescence indicates the leptospiral surface-located Lep-OMP047. **e** Co-localization of Lep-OMP047 with Fas protein in cytomembrane of J774A.1 and THP-1 macrophages, determined by confocal microscopy. The red spots indicate Fas of macrophages, and the green spots indicate Lep-OMP047 attached to the cytomembrane of macrophages. The yellow spots indicate the Lep-OMP047-Fas co-localization. **f** Mouse or human Fas-binding ability of rLep-OMP047, determined by SPR measurement. BSA and CM5 array-linked rMs- or rHu-IgG-Fc were used as the controls. **g** Mouse or human Fas-binding ability of rLep-OMP047, determined by ITC detection. BSA and sample pool-loaded rMs- or rHu-IgG-Fc were used as the controls

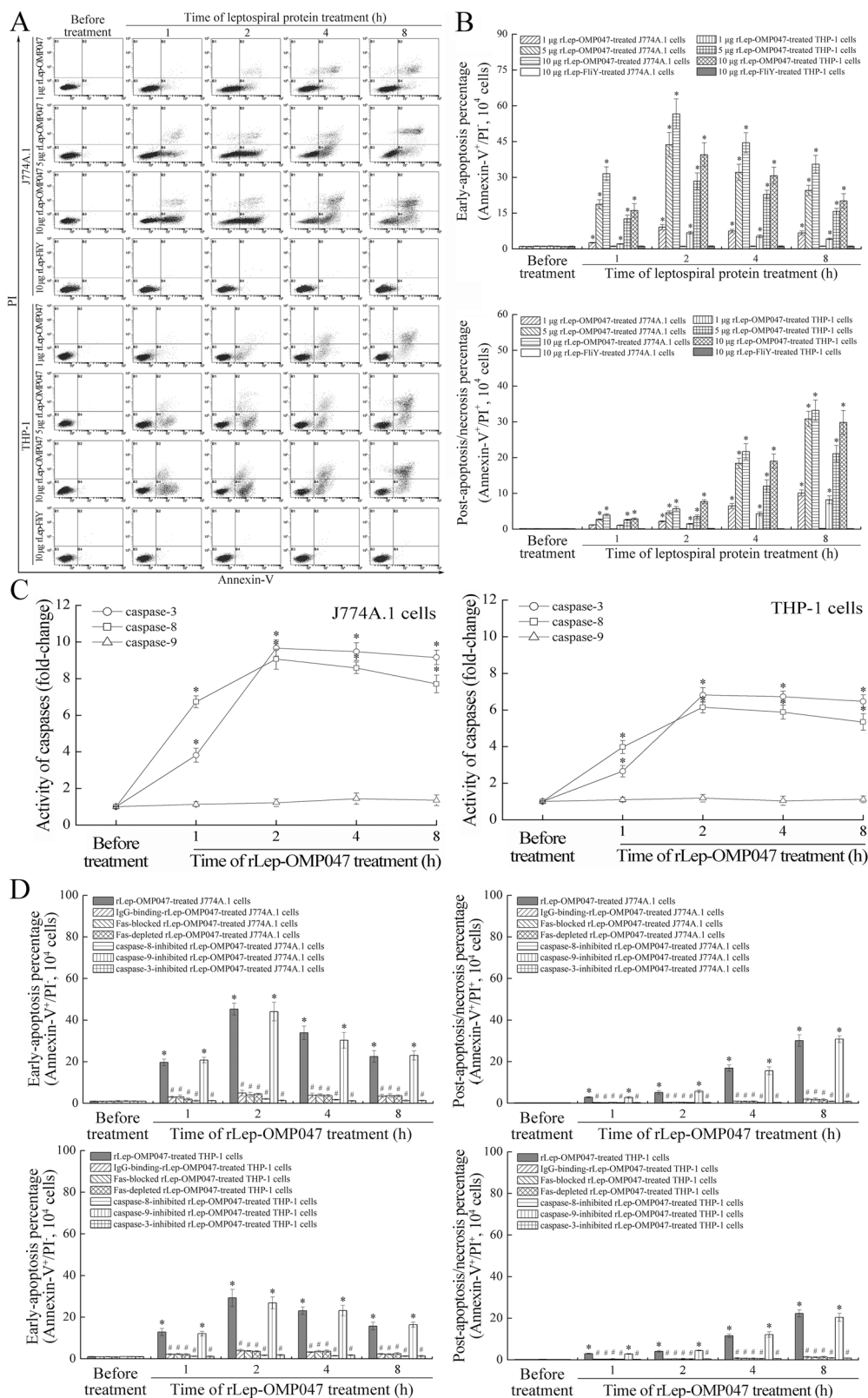


Fig. 4 (See legend on next page.)

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**Fig. 4 rLep-OMP047-induced macrophage apoptosis through Fas-caspase-8/-3 pathway.** **a** rLep-OMP047-induced apoptosis of J774A.1 and THP-1 macrophages after exposure for the indicated times, determined by flow cytometry. The annexin V<sup>+</sup>/PI<sup>-</sup> macrophages are early-apoptotic, while the annexin V<sup>+</sup>/PI<sup>+</sup> macrophages are post-apoptotic/necrotic. rFluY, a recombinant expressed flagellum-associated cytosolic protein from *L. interrogans*, was used as the control. **b** Statistical summary of the early-apoptotic and post-apoptotic/necrotic percentages of rLep-OMP047-treated J774A.1 and THP-1 macrophages. Statistical data from experiments such as that shown in (a). Bars show the means  $\pm$  SD of three independent experiments. \*:  $p < 0.05$  vs the early-apoptotic or post-apoptotic/necrotic percentages of rLep-OMP047-untreated macrophages. **c** Activation of caspase-8/-3 in J774A.1 and THP-1 macrophages treated with rLep-OMP047 for the indicated times, determined by fluorospectrophotometry. Bars show the means  $\pm$  SD of three independent experiments. The fluorescence intensity (FI) values reflecting caspase-8 or caspase-3 activity in rLep-OMP047-untreated macrophages were set as 1.0. \*:  $p < 0.05$  vs the FI values reflecting caspase-8/-3 activity of rLep-OMP047-untreated macrophages. **d** Decrease in rLep-OMP047-induced J774A.1 and THP-1 macrophage apoptosis after Fas-antagonist-IgG/rLep-OMP047-IgG blockage, Fas depletion or caspase-8/-3 inhibition for the indicated times, determined by flow cytometry. Bars show the means  $\pm$  SD of three independent experiments. \*:  $p < 0.05$  vs the early-apoptotic or post-apoptotic/necrotic percentages of rLep-OMP047-untreated macrophages (before treatment). #:  $p < 0.05$  vs the early-apoptotic or post-apoptotic/necrotic percentages of Fas-antagonist-IgG- or rLep-OMP047-IgG-unblocked, Fas-undepleted and caspase-8/-3-uninhibited macrophages

### Lep-LPS-induced Fas/FasL expression through TLR2-JNK/p38 MAPK pathways

*E. coli* LPS induces Fas and FasL expression and cytomembrane translocation through JNK/p38 MAPK signaling pathways<sup>27,28</sup>. The western blot assay showed that the phosphorylation of JNK and p38 MAPK but not ERK in Lep-LPS-treated J774A.1 and THP-1 macrophages was significantly increased (Fig. 7a). However, the confocal-microscopic examination showed that the nuclear translocation of the transcription factors c-Jun and ATF2 but not CHOP, all members of the JNK/p38 MAPK pathways, was notably increased (Fig. 7b). When JNK or p38 MAPK was inhibited, the Lep-LPS-induced Fas and FasL expression and cytomembrane translocation were significantly decreased (Fig. 7c–e). Moreover, TLR2 but not TLR4 depletion decreased JNK and p38 MAPK phosphorylation in Lep-LPS-treated macrophages (Fig. 7f). These data suggest that Lep-LPS induces Fas and FasL expression through TLR2-JNK/p38 MAPK pathways.

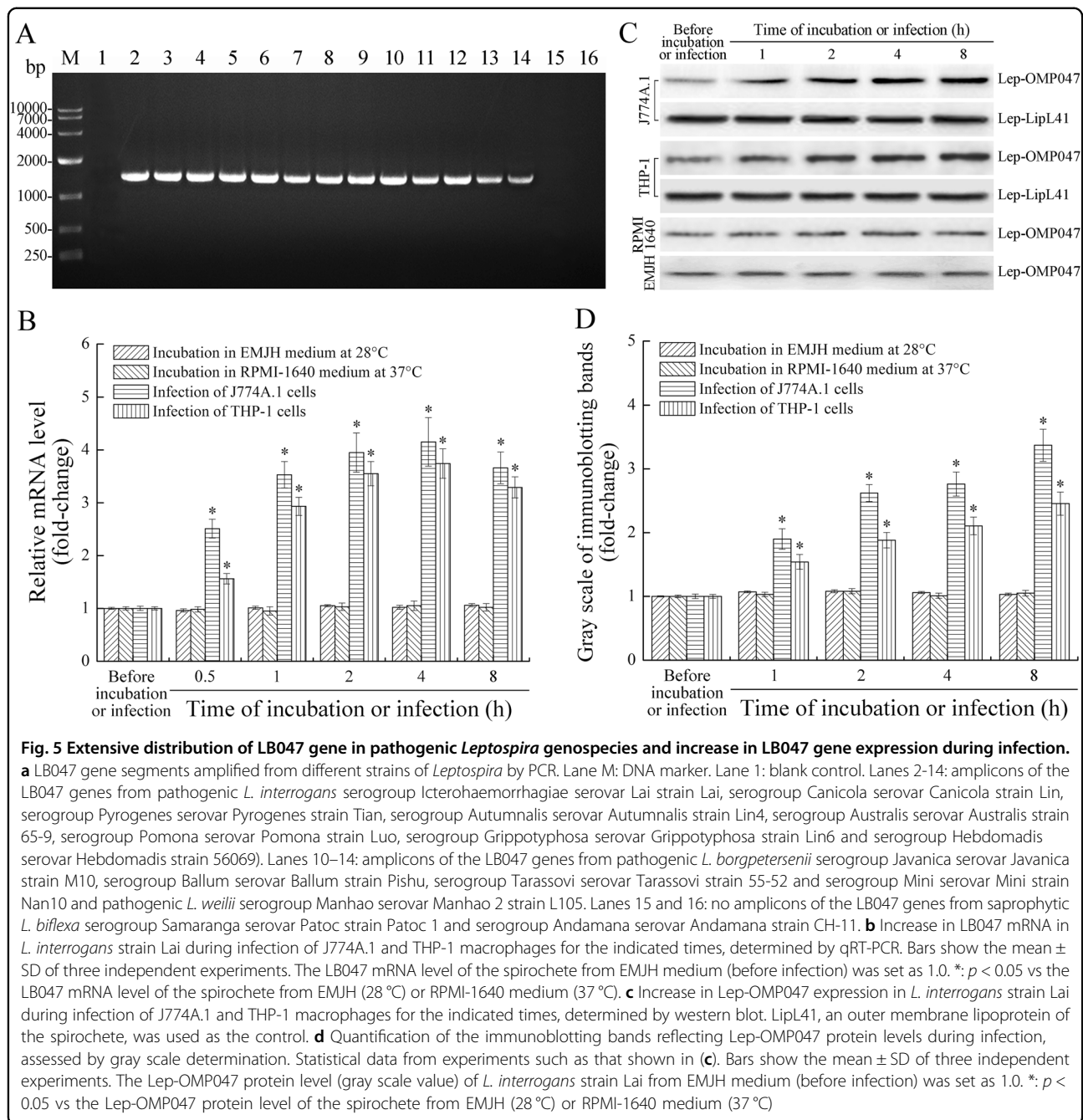
### Discussion

Macrophages and neutrophils play key roles in elimination of invading pathogens, but pathogens also have evasion strategies against phagocytosis by these professional phagocytes, including inducing macrophage apoptosis<sup>18,39</sup>. Leptospirosis is a nonpyrogenic infection, and macrophages but not neutrophils are the major phagocytes involved in elimination of leptospire in vivo<sup>30,31</sup>. Thus, the anti-macrophage ability of *Leptospira* is crucial for its survival in hosts and the pathogenesis of leptospirosis<sup>40,41</sup>.

Until now, except for LPS from a few bacterial species<sup>27–29</sup>, only one study has reported that a lipoprotein of *M. tuberculosis* could induce macrophage apoptosis, but its mechanism remains unknown<sup>42</sup>. No bacterial surface proteins have been confirmed as Fas-binding activators to induce caspase-8/-3-dependent macrophage apoptosis. OMP is a large group of surface proteins in the outer membrane of Gram-negative prokaryotic microbes,

including *Leptospira*<sup>25,26</sup>. The Triton X-114 method is commonly used to extract bacterial OMP, but the extracts only contain most of the OMP components, with a protein component diversity that is due to the differences in the concentration and extraction time of Triton X-114 and other chemical reagents used<sup>43,44</sup>. In the present study, the phagocytosis-inhibited mouse and human macrophages still underwent apoptosis during infection with *L. interrogans*, suggesting that the apoptotic inducers are expressed on the leptospiral surface. Only one OMP (Lep-OMP047) from *L. interrogans* strain Lai was captured by both the mouse and human Fas proteins. SPR and ITC are often used to determine protein–protein binding ability<sup>37,38</sup>. Our SPR and ITC examinations revealed the high-affinity binding between rLep-OMP047 and mouse or human Fas protein. Moreover, rLep-OMP047 did not induce the apoptosis of Fas-blocked or Fas-depleted mouse and human macrophages, while caspase-8 or caspase-3 inhibition also caused a significant decrease in rLep-OMP047-induced macrophage apoptosis. All these data indicate that Lep-OMP047 is a Fas-binding OMP that induces macrophage apoptosis through a Fas-caspase-8/-3-dependent pathway.

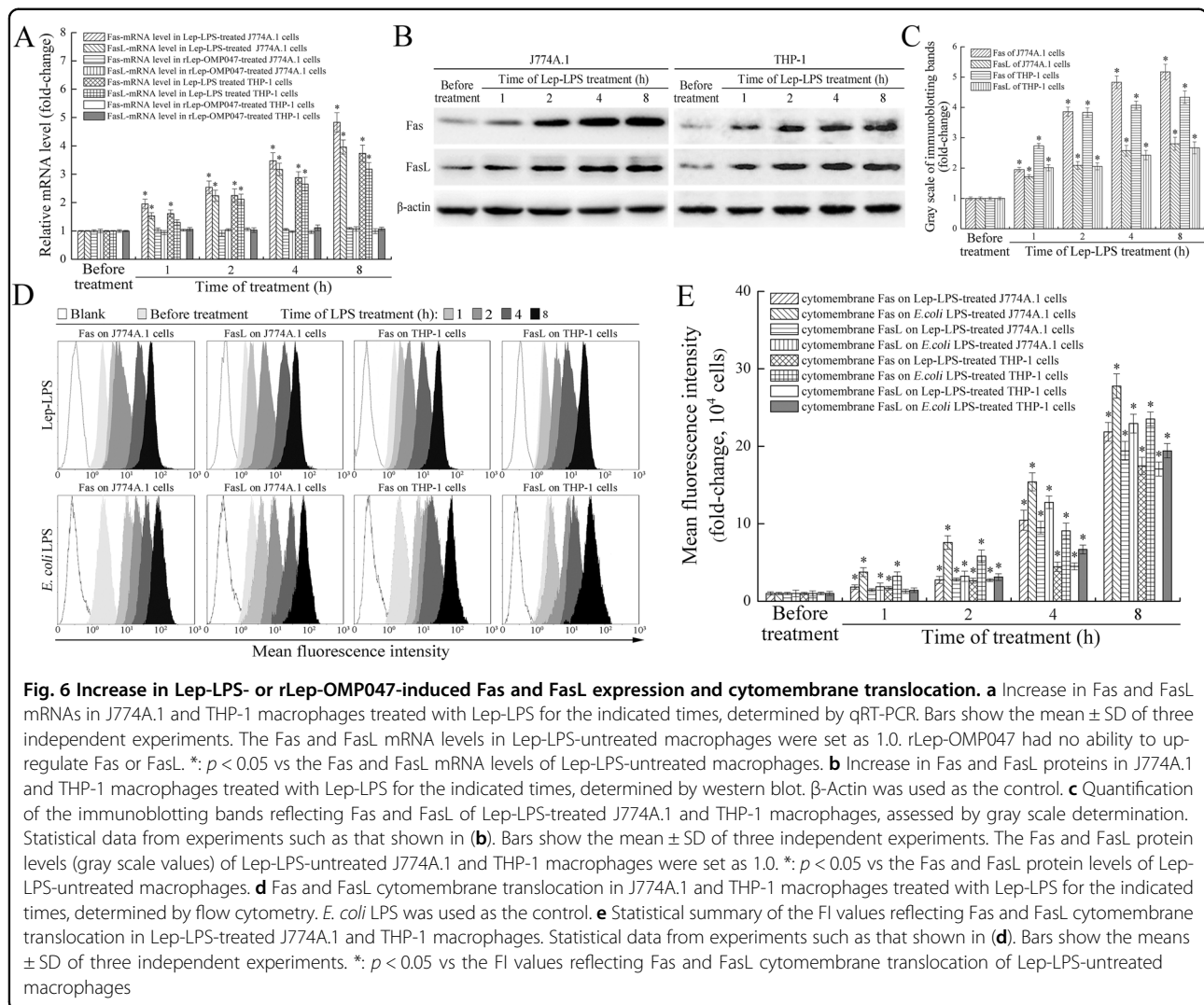
LPS is expressed on the outer membrane of Gram-negative prokaryotic microbes. LPS from several bacteria can induce apoptosis of mouse macrophages through a JNK/p38 MAPK signaling pathway-mediated increase in Fas and FasL expression and cytomembrane translocation<sup>27–29</sup>. However, *E. coli* LPS-activated p38 MAPK upregulates Fas mRNA expression and Fas cytomembrane translocation in mouse vascular endothelial cells, but it only mediates the FasL cytomembrane translocation in mouse T lymphocytes<sup>45</sup>. In the JNK and p38 MAPK signaling pathways, c-Jun or CHOP is the JNK- or p38 MAPK-specific transcription factor, while ATF2 is the co-transcription factor for both of those kinases<sup>46</sup>. Unlike most other bacterial LPSs, TLR2 but not TLR4 of macrophages is responsible for recognizing leptospiral LPS<sup>47,48</sup>. The results of this study show that Lep-LPS



induced the apoptosis of mouse and human macrophages, and JNK and p38 MAPK signaling mediated the Lep-LPS-induced Fas and FasL expression and cytomembrane translocation. However, c-Jun and ATF2, but not CHOP, were involved in the regulation of Fas/FasL expression. Moreover, TLR2 but not TLR4 depletion caused a significant decrease in JNK and p38 MAPK phosphorylation in the Lep-LPS-treated macrophages. All these data indicate that Lep-LPS induces Fas and FasL expression and cytomembrane translocation through the TLR2-JNK/

p38 MAPK signaling pathways to trigger mouse and human macrophage apoptosis.

Compared to the Fas/FasL-caspase-8/-3 pathway-mediated cell apoptosis, bacterial LPS usually induces a lower-level and delayed cell apoptosis due to the greater time required for increasing Fas/FasL expression and cytomembrane translocation<sup>29,49,50</sup>. In the present study, rLep-OMP047 caused earlier and higher maximal early-apoptotic percentages of mouse and human macrophages (43.70% and 28.41% at 2 h post treatment) compared to



Lep-LPS (21.90% and 15.80% at 4 h post treatment). On the other hand, we found that all the tested strains belonging to pathogenic *L. interrogans*, *L. borgpetersenii* and *L. weilii* possessed the LB047 gene, but the tested strains belonging to saprophytic *L. biflexa* had no detectable LB047 gene. Moreover, LB047 gene transcription and protein expression in *L. interrogans* strain Lai during infection of mouse and human macrophages was significantly increased. The higher expression of the *L. interrogans* LB047 gene contributes to the LB047 protein-induced apoptosis of mouse and human macrophages during infection.

## Materials and methods

### Leptospiral strains and cell lines

Thirteen pathogenic *Leptospira* strains and two saprophytic *Leptospira* strains and their cultivation are described in Supplementary Material. J774A.1 macrophages and human THP-1 monocytes were used in this study<sup>24</sup>.

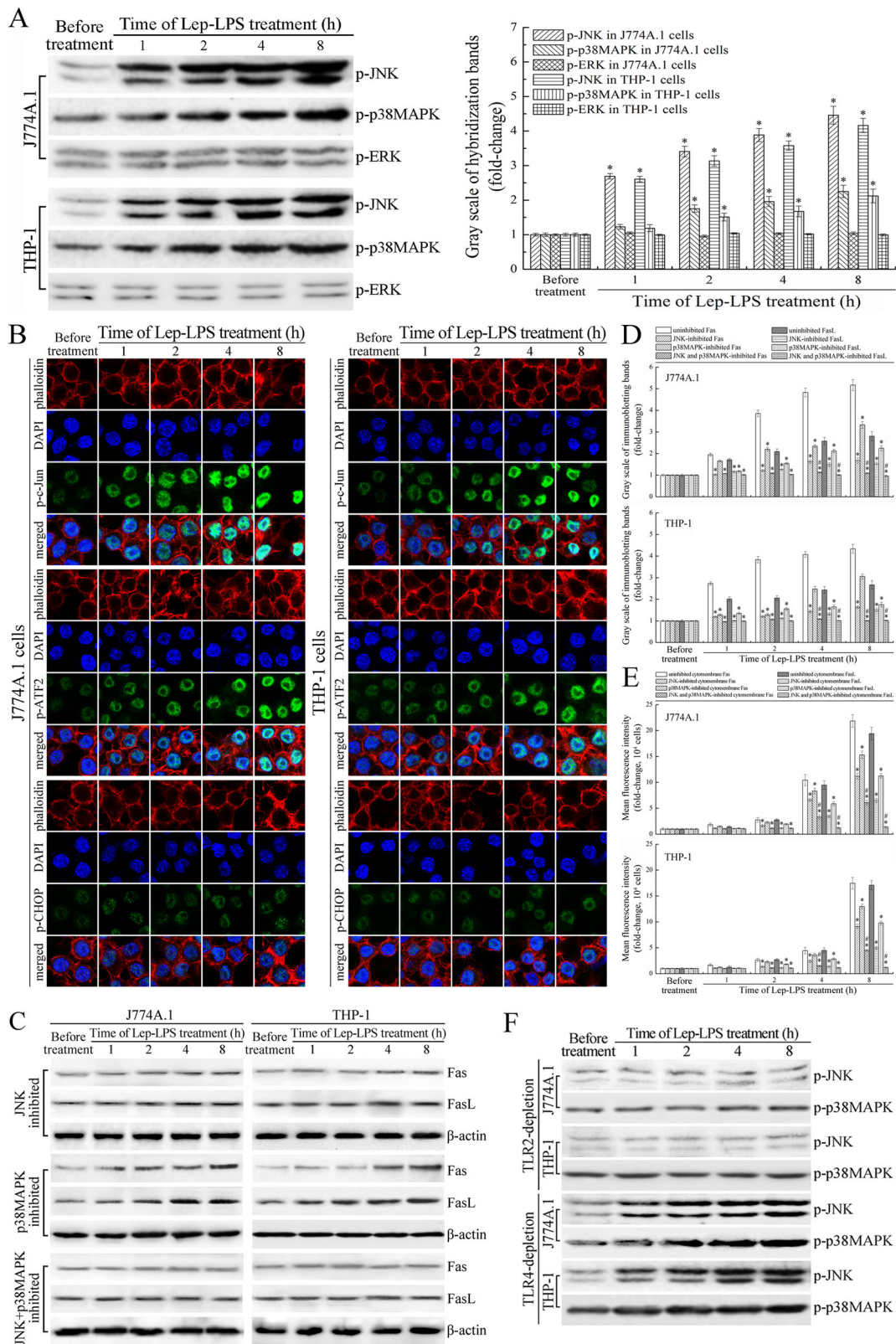
The cell culture, PMA-induced THP-1 cell differentiation and detection of CD68<sup>+</sup> THP-1 macrophages are described in Supplementary Material.

### Primers

The primers used in this study were synthesized by Invitrogen Co., Shanghai, China. The primer sequences are shown in Table 1.

### Macrophage endocytosis test

Freshly cultured *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai was precipitated by a 10,000  $\times$  g centrifugation for 30 min (4  $^{\circ}$ C), and then, the leptospiral pellet was suspended in 2.5% fetal calf serum antibiotic-free RPMI-1640 medium for counting with a Petroff–Hausser chamber under a dark-field microscope<sup>24</sup>. J774A.1 and THP-1 macrophages (10<sup>6</sup> cells per well) were seeded in six-well culture plates for a pre-incubation overnight and then infected with the



**Fig. 7** (See legend on next page.)

(see figure on previous page)

**Fig. 7 Lep-LPS-induced Fas and FasL expression through TLR2-JNK/p38 MAPK pathways.** **a** Increase in JNK and p38 MAPK phosphorylation in J774A.1 and THP-1 macrophages treated with Lep-LPS for the indicated times, detected by western blot. Bars show the mean  $\pm$  SD of three independent experiments. The JNK, p38 MAPK and ERK phosphorylation levels (gray scale values) of Lep-LPS-untreated J774A.1 and THP-1 macrophages were set as 1.0. \*:  $p < 0.05$  vs the JNK and p38 MAPK phosphorylation levels of Lep-LPS-untreated macrophages. **b** Increase in c-Jun and ATF2 nuclear translocation in J774A.1 and THP-1 macrophages treated with Lep-LPS for the indicated times, determined by confocal microscopy. Phalloidin is a fluorescent dye of the cytoskeleton. The blue plaques indicate the nucleus. The green spots indicate the c-Jun or ATF2 that translocated into nuclei. **c** Decrease in Lep-LPS-induced Fas and FasL expression in JNK- and/or p38 MAPK-inhibited J774A.1 and THP-1 macrophages, detected by western blot.  $\beta$ -Actin was used as the control. **d** Quantification of the immunoblotting bands reflecting Fas and FasL in JNK- and/or p38 MAPK-inhibited J774A.1 and THP-1 macrophages, assessed by gray scale determination. Statistical data from experiments such as that shown in (c). Bars show the mean  $\pm$  SD of three independent experiments. The Lep-LPS-induced Fas and FasL expression levels (gray scale values) of JNK- and/or p38 MAPK-uninhibited J774A.1 and THP-1 macrophages were set as 1.0. \*:  $p < 0.05$  vs the Lep-LPS-induced Fas and FasL expression levels of JNK- and/or p38 MAPK-uninhibited macrophages. #:  $p < 0.05$  vs the Lep-LPS-induced Fas and FasL expression levels of JNK- or p38 MAPK-uninhibited macrophages. **e** Decrease in Lep-LPS-induced Fas and FasL cytomembrane translocation in JNK- and/or p38 MAPK-inhibited J774A.1 and THP-1 macrophages, detected by flow cytometry. The mean FI values reflecting the Lep-LPS-induced Fas and FasL cytomembrane translocation levels of JNK- and/or p38 MAPK-uninhibited J774A.1 or THP-1 macrophages were set as 1.0. The other legend is the same as in (d) but for the detection of Fas and FasL cytomembrane translocation. **f** Decrease in Lep-LPS-induced JNK and p38 MAPK phosphorylation in TLR2-depleted J774A.1 and THP-1 macrophages, detected by western blot

**Table 1 Sequences of primers used in this study**

Primer	Sequence (5' to 3')	Purpose	Size (bp)
LB047-1	F: <u>GCGCATATG</u> ( <i>Nde</i> I)AAACGATTTTGTGG R: GCGCTCGAG( <i>Xho</i> I)TTTACAACCTTGCAATTC	Detection and expression of LB047 gene	1317
LB047-2	F: ATGAAACAACCTTTGTTA R: CTTACAAGGAGGACTTTC	Detection of LB047 gene	1323
LB047-3	F: TTCTGCGTGACACTCCTTAC R: CTTCTTCGTCCTCAAACCT	Detection of LB047 mRNA	156
16S-RNA	F: CTTTCGTGCCTCAGCGTCAGT R: CGCAGCCTGCACCTTGAAACTA	Inner reference in LB047 mRNA qRT-PCR	145
Ms-Fas	F: TGTGAACATGGAACCCCTTGA R: TTCAGGGTCATCCTGTCTCC	Detection of J774A.1 Fas mRNA	177
Ms-FasL	F: CATCACAACCACTCCCCTG R: GTTCTGCCAGTTCCTTCTGC	Detection of J774A.1 FasL mRNA	162
Ms-actin	F: AGAGCGGGCCTTGAGAAAAG R: TGGAGAGCCTGGATTGTCTATC	Inner reference in J774A.1 Fas/FasL mRNA qRT-PCR	111
Hu-Fas	F: AGCTTGGTCTAGAGTGAAAA R: GAGGCAGAATCATGAGATAT	Detection of THP-1 Fas mRNA	180
Hu-FasL	F: CACTTTGGGATTCTTCCAT R: GTGAGTTGAGGAGCTACAGA	Detection of THP-1 FasL mRNA	160
Hu-actin	F: ATAGCACAGCCTGGATAGCAACGTAC R: CACCTTCTACAATGAGCTGCGTGTG	Inner reference in THP-1 Fas/FasL mRNA qRT-PCR	158

Underlined areas indicate the sites of endonucleases  
F forward primer, R reverse primer, Ms mouse, Hu human

spirochete at a multiplicity of infection of 100 ( $MOI_{100}$ ) for 1, 2, 4, 8, or 12 h<sup>23,24</sup>. After trypsinization, washing with phosphate-buffered saline (PBS) and centrifugation at  $500 \times g$  for 10 min ( $4^\circ C$ ), the precipitated macrophages

were fixed with 4% paraformaldehyde-PBS for 30 min and then permeabilized with 0.1% Triton X100-PBS for 30 min to allow antibody entry<sup>40</sup>. Using rabbit anti-*L. interrogans* strain Lai-IgG as the primary antibody, Alexa Fluor

594-conjugated goat anti-rabbit-IgG (Invitrogen, Carlsbad, CA, USA) as the secondary antibody and DAPI (Molecular Probes, Eugene, OR, USA) as the cell nucleus dye, the intracellular leptospires were observed under a laser confocal microscope (LSM510-Meta, Zeiss, Germany) (590/617 and 355/460 nm excitation/emission wavelengths for Alexa Fluor 594 and DAPI detection). Moreover, the leptospiral phagosomes were observed under a transmission electron microscope (TECNAI-10, Philips, Holland)<sup>31</sup>.

#### Phagocytosis inhibition test

J774A.1 and THP-1 macrophages were treated with 1 mM RGDS peptide, a universal integrin-dependent phagocytosis inhibitor, at 37 °C for 24 h<sup>33</sup>. According to the results of the macrophage endocytosis test, the RGDS-treated macrophages were infected with *L. interrogans* strain Lai for 8 h, and then, the intracellular leptospires were detected as above.

#### Detection of *Leptospira*-infected macrophage apoptosis

The RGDS-treated or RGDS-untreated J774A.1 and THP-1 macrophages were infected with *L. interrogans* strain Lai at MOI<sub>100</sub> for 1, 2, 4, 8 or 12 h<sup>23,24</sup>. After trypsinization, washing with PBS and centrifugation at 500 × *g* for 10 min (4 °C), the precipitated macrophages were suspended in annexin-binding buffer and then incubated with Alexa Fluor 488-conjugated annexin-V and propidium iodide (PI) at room temperature for 15 min using a cell apoptosis detection kit (Life Technologies, Carlsbad, CA, USA). The stained macrophages were detected using a flow cytometer (type FC500-MCL, Beckman Coulter, Brea, CA, USA) to distinguish the macrophages in early apoptosis (annexin-V<sup>+</sup>/PI<sup>-</sup>) from those in post apoptosis/necrosis (annexin-V<sup>+</sup>/PI<sup>+</sup>).

#### Extraction and identification of leptospiral OMP

Outer membrane protein of *L. interrogans* strain Lai (Lep-OMP) was extracted using the Triton X-114 method as previously described<sup>43,44</sup>. Briefly, freshly cultured *L. interrogans* strain Lai was precipitated by a 10,000 × *g* centrifugation for 30 min (4 °C). The leptospiral pellet was suspended in PBS for counting as above. The leptospiral suspension (5 × 10<sup>8</sup> leptospires/ml) was precipitated by centrifugation and then washed three times with 5 mM MgCl<sub>2</sub>-PBS (pH 7.4), followed by extraction with 1% Triton X-114 (Sigma, St. Louis, MO, USA)-1 mM EDTA-10 mM Tris-HCl (pH 8.0) buffer at 4 °C for 30 min. After a 17,000 × *g* centrifugation for 10 min (4 °C), the supernatant was added to Triton X-114 (final concentration 2%) and then incubated at 37 °C for 10 min. After a 2000 × *g* centrifugation at room temperature for 10 min, Lep-OMP in the aqueous or detergent phase (aLep-OMP or dLep-OMP) was

precipitated with acetone. Possible contaminated leptospiral lipopolysaccharide (Lep-LPS) in a- or dLep-OMP was removed by Detoxi-gel endotoxin-removing column chromatography (Thermo Scientific, Waltham, MA, USA) using pyrogen-free water for elution and then detected using a limulus amoebocyte lysate test kit (Lonza, Switzerland) as previously described<sup>51</sup>. After measurement of protein concentrations of a- and dLep-OMP using a BCA protein assay kit (Thermo Scientific), western blot was used to identify the two OMP extracts using the antibody against rOMP-L1 or rFliY, a recombinant expressed typical OMP component or a flagellum-associated cytosolic protein from *L. interrogans* strain Lai, respectively, as the primary antibody (rLep-OMP-L1-IgG or rLep-FliY-IgG) and HRP-conjugated goat anti-rabbit-IgG (Abcam, Cambridge, MA, USA) as the secondary antibody<sup>52,53</sup>.

#### Extraction and identification of leptospiral LPS

LPS of *L. interrogans* strain Lai (Lep-LPS) was extracted by the phenol-water method as previously described<sup>34,35</sup>. Briefly, freshly cultured *L. interrogans* strain Lai was centrifuged at 10,000 × *g* for 30 min (4 °C). The leptospiral pellet was suspended in PBS for counting as described above. After washing with PBS and centrifugation again, the leptospiral pellet was suspended in Milli-Q water (5 × 10<sup>8</sup> leptospires/ml) for several freeze-thaw cycles and then added to an equal volume of 90% analytical pure phenol (Sigma) to extract Lep-LPS by violent agitation in a 65 °C water bath for 30 min. The extract was separated by a 2500 × *g* centrifugation for 30 min at room temperature to remove the denatured proteins and then dialyzed against Milli-Q water. The dialyzed extract was treated with DNase I and RNase H (Sigma), followed by digestion with proteinase K (TaKaRa, China). The extract was extracted with phenol-water again and then centrifuged to remove the denatured enzymatic proteins and dialyzed as above. The extract was centrifuged at 3000 × *g* for 15 min (4 °C) to remove insoluble substances. The supernatant was centrifuged at 100,000 × *g* for 3 h (4 °C) to precipitate Lep-LPS. This ultracentrifugation step was repeated until the extract showed no absorbance at 260 and 280 nm. Finally, the obtained Lep-LPS was suspended in pyrogen-free water (Sigma) and then lyophilized and weighed. The Lep-LPS was examined by SDS-PAGE after silver staining using *E. coli* O111:B4 LPS (Lonza) as the control<sup>34,35</sup>. The activity of Lep-LPS was determined using a limulus amoebocyte lysate test kit (Lonza)<sup>51</sup>.

#### Determination of aLep-OMP-, dLep-OMP-, or Lep-LPS-induced macrophage apoptosis

J774A.1 and THP-1 macrophages (10<sup>6</sup>) were incubated with 100 or 200 μg a- or dLep-OMP and 0.1 or 1 μg Lep-



LPS at 37 °C for 1, 2, 4, or 8 h. The apoptosis of a- or dLep-OMP- or Lep-LPS-treated macrophages was detected by flow cytometry as above.

#### Capture and identification of Fas-binding Lep-OMP

The Fas-binding Lep-OMP in a- or dLep-OMP was captured by co-precipitation<sup>54</sup>. Briefly, 200 µg of a- or dLep-OMP was mixed with 20 µg mouse or human Fas-Fc chimeric protein (R&D, Minneapolis, MN, USA) in 500 µl PBS for a 2-h incubation in a 90-rpm rotator (4 °C). Each of the mixtures was added to 600 µg protein-A-coated agarose beads (Millipore, Burlington, MA, USA), followed by a 60-min incubation as above. After a 14,000 × *g* centrifugation for 5 min and washing thoroughly with PBS, the precipitated beads were suspended in Laemmli buffer for 5 min in a water bath at 100 °C to release protein-A/Fas-binding proteins. After centrifugation as above, the supernatants were subjected to SDS-PAGE, and the released Fas-binding proteins were then identified with NanoLC-LTQ MS/MS (Thermo Scientific) by the National Laboratory of Biomacromolecules, Chinese Academy of Sciences.

#### Bioinformatic analysis of Fas-binding Lep-OMP047

The NanoLC-MS/MS identified only one Fas-binding Lep-OMP that matched the LB047 gene product of *L. interrogans* strain Lai (Lep-OMP047). This gene was analyzed using TMHMM, SignalP-4.1, LipoP-Servers and NCBI-Batch CD-Search software<sup>55</sup>.

#### Detection of LB047 gene in different leptospiral strains

Detection of the LB047 gene in the fifteen tested leptospiral strains is described in Supplementary Material.

#### Recombinant expression and product extraction of LB047 gene

The recombinant expression and product (rLep-OMP047) extraction of LB047 gene from *L. interrogans* strain Lai in *E. coli* are described in Supplementary Material. Possible contaminated *E. coli* LPS in rLep-OMP047 was removed as described above.

#### Preparation of rLep-OMP047-IgG and a- and dLep-OMP-IgGs

The preparation of rabbit anti-rLep-OMP047-IgG and anti-a- and -dLep-OMP-IgGs is described in Supplementary Material.

#### Determination of distribution and relative abundance of Lep-OMP047

The distribution in a- and dLep-OMP and relative abundance in dLep-OMP of Lep-OMP047 were determined by western blot using rabbit anti-rLep-OMP047-IgG or anti-dLep-OMP-IgG as the primary antibody and

HRP-conjugated goat anti-rabbit-IgG (Abcam) as the secondary antibody. The immunoblotting signals reflecting the relative abundance of Lep-OMP047 were quantified by densitometry (gray scale determination) using an image analyzer (Bio-Rad, Hercules, CA, USA)<sup>24</sup>. In the assay, rLep-OMP047 was used as the control.

#### Detection of Lep-OMP047 location

The smear of *L. interrogans* strain Lai was fixed with 2% paraformaldehyde-5 mM MgCl<sub>2</sub>-PBS for 30 min. Using rabbit anti-rLep-OMP047-IgG as the primary antibody and Alexa Fluor 488-conjugated goat anti-rabbit-IgG (Abcam) as the secondary antibody, Lep-OMP047 location was detected by confocal microscopy as above (495/519 nm excitation/emission wavelengths for Alexa Fluor 488 detection). In the detection, rabbit anti-rLep-FluY-IgG was used as the control<sup>53</sup>.

#### Detection of co-localization between rLep-OMP047 and Fas on macrophages

J774A.1 and THP-1 macrophages (10<sup>6</sup>) were incubated with 5 µg rLep-OMP047 at 37 °C for 0.5, 1 or 2 h. After washing thoroughly with PBS and fixing with paraformaldehyde as above, the co-localization of rLep-OMP047 with Fas on mouse or human macrophages was detected by laser confocal microscopy as above using rabbit anti-rLep-OMP047 IgG, goat anti-mouse-Fas IgG, or goat anti-human-Fas IgG as the primary antibody (R&D), Alexa Fluor 488-conjugated mouse anti-rabbit-IgG or Alexa Fluor 594-conjugated donkey anti-goat-IgG (Invitrogen) as the secondary antibody and DAPI (Molecular Probes) as the nuclear dye. The yellow fluorescence spots indicated the co-localization (yellow) of rLep-OMP047 (green) with mouse or human Fas (red).

#### Determination of rLep-OMP047-Fas binding

The binding of rLep-OMP047 to mouse or human Fas protein (Abcam) was determined by surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC)<sup>37,38</sup>. Briefly, 0.025–0.8 nM rLep-OMP047-0.05% Tween 20-PBS flowed through the surface of a 0.2 nM mouse or human Fas-linked CM5 sensing array (GE, Boston, MA, USA), and the equilibrium association constant (*K<sub>D</sub>*) values reflecting rLep-OMP047-Fas binding were detected using an SPR detector (Type-T200, GE). For ITC detection, 0.1 µM mouse or human Fas-0.05% Tween 20-PBS in sample pool was titrated with 1 µM rLep-OMP047-0.05% Tween 20-PBS in the titration probe, and the *K<sub>D</sub>* values were detected using a microcalorimeter (MicroCal, Northampton, MA, USA). In the detection, bovine serum albumin (BSA, Sigma) in 0.05% Tween 20-PBS was used as the control. In addition, a recombinant mouse or human IgG-Fc fragment (rMs-IgG-Fc or rHu-IgG-Fc, R&D) in 0.05% Tween 20-PBS that

was linked on the CM5 array in SPR and loaded in the sample pool in ITC was also used as a control.

#### Detection of rLep-OMP047-induced macrophage apoptosis

J774A.1 and THP-1 macrophages ( $10^6$ ) were treated with 1, 5, or 10  $\mu\text{g}$  rLep-OMP047 at 37 °C for 1, 2, 4, or 8 h. The rLep-OMP047-induced macrophage apoptosis was detected by flow cytometry as above. In the detection, rFluY from *L. interrogans* strain Lai was used as the control<sup>53</sup>.

#### Antibody blockage and Fas-depletion tests

Cytoplasmic Fas of J774A.1 and THP-1 macrophages ( $10^6$ ) was blocked with rat-15A7 anti-mouse-Fas-antagonist-IgG (Thermo Scientific) or mouse-ZB4 anti-human-Fas-antagonist-IgG (Merck Millipore, Burlington, MA, USA), and rLep-OMP047 (5  $\mu\text{g}$ ) was blocked with rLep-OMP047-IgG at 37 °C for 1 h. The Fas-blocked macrophages were treated with 5  $\mu\text{g}$  rLep-OMP047, while the Fas-unblocked macrophages were treated with IgG-binding rLep-OMP047 for 1, 2, 4, or 8 h. Additionally, Fas-depleted J774A.1 or THP-1 macrophages were generated (shown in Supplementary Material), and the Fas-depleted macrophages were also treated with rLep-OMP047 as above. The macrophage apoptosis was detected by flow cytometry as described above.

#### Detection of rLep-OMP047-induced caspase-3/-8/-9 activation

J774A.1 and THP-1 macrophages ( $10^6$ ) were incubated with 5  $\mu\text{g}$  rLep-OMP047 for 1, 2, 4, or 8 h. The activation of caspase-3/-8/-9 was detected using a caspase assay kit (BioVision, Milpitas, CA, USA) and fluorospectrophotometer (Molecular Devices, San Jose, CA, USA)<sup>23</sup>. The fluorescence intensity (FI) (400/505 nm excitation/emission wavelengths) values reflecting the activity of different caspases hydrolyzing specific fluorescence-labeling substrates were measured for analysis. The details of caspase-3/-8/-9 activation detection are given in Supplementary Material.

#### Caspase inhibition test

J774A.1 and THP-1 macrophages ( $10^6$ ) were treated with 100  $\mu\text{M}$  Z-DEVD-fmk Z-IETD-fmk or Z-LEHD-fmk (BioVision), the caspase-3, -8, or -9 inhibitor, at 37 °C for 1 h<sup>23</sup>, and then incubated with 5  $\mu\text{g}$  rLep-OMP047 for 1, 2, 4 or 8 h. The rLep-OMP047-induced apoptosis of caspase-3/-8/-9-inhibited J774A.1 and THP-1 macrophages was detected by flow cytometry as above.

#### Detection of LB047 gene expression during infection

J774A.1 and THP-1 macrophages ( $10^6$ ) were infected with *L. interrogans* strain Lai at a  $\text{MOI}_{100}$  for 0.5, 1, 2, 4, or

8 h. The mRNA and product of the LB047 gene from the extracellular leptospire were detected by reverse transcription–real-time fluorescence quantitative PCR (qRT-PCR) and western blot<sup>40</sup>. The details of qRT-PCR and western blot are given in Supplementary Material.

#### Detection of Lep-LPS- or rLep-OMP047-induced Fas and FasL expression

J774A.1 and THP-1 macrophages ( $10^6$ ) were treated with 1  $\mu\text{g}$  Lep-LPS or 5  $\mu\text{g}$  rLep-OMP047 for 1, 2, 4, or 8 h. The mRNA and protein levels of Fas and FasL were detected by qRT-PCR and western blot, respectively. The immunoblotting signals were quantified by densitometry (gray scale determination) using an image analyzer (Bio-Rad)<sup>24</sup>. The details of qRT-PCR and western blot assay are given in Supplementary Material.

#### Detection of Lep-LPS-induced Fas and FasL cytoplasmic translocation

J774A.1 and THP-1 macrophages ( $10^6$ ) were treated with 1  $\mu\text{g}$  Lep-LPS for 1, 2, 4, or 8 h and then fixed with 4% paraformaldehyde-PBS at 4 °C overnight. After washing with PBS, the macrophages were stained with PE-conjugated rat anti-mouse-Fas IgG, rat anti-mouse-FasL IgG, mouse anti-human-Fas IgG, or mouse anti-human-FasL IgG (BD Bioscience, San Jose, CA, USA) at room temperature for 15 min in the dark. The PE-stained cytoplasmic Fas or FasL was detected by flow cytometry as above, and the mean FI values reflecting cytoplasmic Fas and FasL levels were quantified for analysis. In the detection, *E. coli* O111:B4 LPS (Lonza) was used as the control.

#### Detection of Lep-LPS-induced MAPK activation

J774A.1 and THP-1 macrophages ( $10^6$ ) were treated with 1  $\mu\text{g}$  Lep-LPS for 1, 2, 4, or 8 h. The macrophages were lysed with 0.05% NaTDC-PBS and then centrifuged at 12,000  $\times g$  for 5 min (4 °C). The supernatants were collected to detect the phosphorylation levels of JNK, p38 MAPK and ERK by western blot using a MAPK-family phosphorylation detection kit (Cell Signaling, Beverly, MA, USA). The immunoblotting signals were quantified by densitometry for analysis as above.

#### Detection of Lep-LPS-induced c-Jun and ATF2 nuclear translocation

J774A.1 and THP-1 macrophages ( $10^6$ ) were treated with 1  $\mu\text{g}$  Lep-LPS for 1, 2, 4, or 8 h. The nuclear translocation of c-Jun, ATF2 and CHOP, the transcription factors of the p38 MAPK and/or JNK signaling pathways, was detected by confocal microscopy as above. The details of confocal-microscopic examination are given in Supplementary Material.

### p38 MAPK and/or JNK inhibition test

J774A.1 and THP-1 macrophages ( $10^6$ ) were treated with 20  $\mu$ M p38 MAPK inhibitor SB203580 and/or JNK inhibitor SP600125 (Tocris Bioscience, Minneapolis, MN, USA) at 37 °C for 1 h<sup>51</sup>, then treated with 1  $\mu$ g Lep-LPS for 1, 2, 4, or 8 h. The Lep-LPS-induced Fas or FasL expression and cytomembrane translocation of p38 MAPK- and/or JNK-inhibited macrophages were detected by western blot and flow cytometry as above.

### Determination of Lep-LPS/TLR2-mediated JNK and p38 MAPK activation

The generation and identification of TLR2- or TLR4-depleted J774A.1 and THP-1 macrophages are described in Supplementary Material. The TLR2/4-deficient macrophages ( $10^6$ ) were treated with 1  $\mu$ g Lep-LPS for 1, 2, 4, or 8 h, and then, the Lep-LPS-induced JNK and p38 MAPK phosphorylation were detected by western blot as above.

### Statistical analysis

Data from a minimum of three experiments were averaged and are presented as the mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to determine significant differences. Statistical significance was defined as  $p < 0.05$ .

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### Author contributions

J.Y., A.H.S., and X.A.L. conceived and designed this study. P.D., S.J.L., K.X.L., and W.L.H. performed the experiments. P.D., S.J.L., and J.Y. analyzed the data. P.D., J.Y., and D.M.O. wrote the article. J.Y. and S.J.L. obtained the funding. All authors reviewed the manuscript.

### Conflict of interest

The authors declare that they have no conflict of interest.

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