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SIGNALING VIA ITGB1/FAK AND MICROFILAMENT REARRANGEMENT MEDIATES THE INTERNALIZATION OF *LEPTOSPIRA INTERROGANS* IN MOUSE J774A.1 MACROPHAGES

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Abstract - Leptospirosis caused by pathogenic *Leptospira* species is a worldwide zoonotic infectious disease, but the mechanisms of leptospiral internalization remain poorly understood. Here, we report that mouse J774A.1 macrophages expressed integrin-subfamily proteins (ITGB1, ITGB2 and ITGB3). Antibody blockage and siRNA-based knockdown of ITGB1 decreased the internalization of leptospires into mouse J774A.1 macrophage cells. The internalization required focal adhesion kinase (FAK) activation in J774A.1 cells rather than phosphoinositide-3-kinase (PI3K), and microfilament rather than microtubule aggregation during infection. The data indicated that the ITGB1/FAK/microfilament signaling pathway is responsible for leptospiral internalization in mouse macrophages.

Key words: Leptospira interrogans; mouse macrophage cell; internalization; signaling; cytoskeletal rearrangement

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INTRODUCTION

Infection with pathogenic *Leptospira* species causes leptospirosis, a global zoonotic infectious disease (Bharti et al., 2003). Southeast Asia and South America are the long-term epidemic areas of the disease (Romero et al., 2003; Pappas et al., 2008; Zhang et al., 2012). However, in North America and Europe, leptospirosis is considered an emerging infectious disease due to the frequent case reports and several outbreaks in the last ten years (Meites et al., 2004; Ko et al., 2009; Hotez et al., 2011; Lo et al., 2011).

Many animals, including dogs, livestock and rodents, serve as the natural hosts of pathogenic *Leptospira* species (Faine et al., 2009). After being shed in the urine of infected animals, the spirochete survives for long periods of time in moist soil and natural bodies of water (Adler et al., 2010). An individual is infected with the spirochete through contact with leptospire-contaminated soil or water (McBride et al., 2005). After invasion into the human body through the skin and mucosa, the pathogenic leptospires enter the blood stream rapidly and

may cause septicemia, and then pass through small blood vessels to spread into internal organs such as the lungs, liver and kidneys within 3 to 5 days after infection (McBride et al., 2005; Levett et al., 2001). Clinical manifestations of human leptospirosis are characterized by high fever and myalgia in mild cases or hemorrhage, jaundice, renal impairment and septic shock in severe cases. However, the mechanism of internalization of leptospire is poorly understood.

Adherence to host cells is the initial step for microbial pathogens during interaction with host cells (Bhavsar et al., 2007). Several surface proteins such as LigA, LigB, Lsa21, Lsa24 and Lsa63 of *Leptospira* have been confirmed as the adhesins that bind to fibronectin (FN), laminin (LN), collagen I (COL1), COL3 and COL4 molecules in the extracellular matrix (ECM) of different host cells (Barbosa et al., 2006; Choy et al., 2007; Stevenson et al., 2007; Hauk et al., 2008; Atzingen et al., 2008; Vieira et al., 2010). After binding by microbial adhesins, allosteric changes in the ECM molecules enable them to bind to the N-terminus of integrins via their RGD motif. Many extracellular matrix (ECM) proteins contain the tripeptide arginine-glycine-aspartic acid (RGD) as the integrin recognition site; these proteins include fibronectin, laminin and collagens (Secott et al., 2004; Hoffmann et al., 2011; Zhang et al., 2012; Ruoslahti et al., 1987). An integrin molecule is composed of two subunits (α and β), and all the integrins can be classified into β1, β2 and β3 subfamilies according to the identity of their β subunits (ITGB1, ITGB2 and ITGB3) (Takada et al., 2007). Binding of the ECM molecules induces the integrin polymerization that triggers focal adhesion kinase (FAK)- or phosphoinositide-3-kinase (PI3K)-dependent intracellular signaling to mediate the internalization of microbial pathogens (Harburger et al., 2009). For example, group A streptococci bind to the ECM protein fibronectin, which in turn binds to integrins. These interactions then trigger the uptake of streptococci by encapsulating invaginations (Parton et al., 2009).

L. interrogans is the most prevalent pathogenic *Leptospira* species in the world (Ren et al., 2003; Nascimento et al., 2004). In China, about 70% of leptospirosis cases are due to infection with *L. interrogans* serovar Lai (Zhang et al., 2012). In the present study, we characterized integrins expressed by mouse macrophages. The roles of different integrins and intracellular signaling pathways in the internalization of the spirochete were subsequently examined.

MATERIALS AND METHODS

Leptospiral strain and culture

L. interrogans serogroup Icterohaemorrhagiae serovar Lai strain Lai was provided by the National Institute for Control of Pharmaceutical and Biological Products in Beijing, China. The strain was cultivated at 28°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium supplemented with 5% albumin bovine fraction V (Sigma, USA) and 0.05% Tween-80 (Difco, USA) (Hu et al., 2013).

Cell lines and culture

A mouse macrophage-like cell line (J774A.1) was provided by the Cell Bank of the Institute of Cytobiology, Chinese Academy of Science, Shanghai, China. The cells were maintained in RPMI-1640 liquid medium (Gibco, USA), supplemented with 10% fetal calf serum (FCS, Gibco), 100 U ml^{-1} penicillin (Sigma, USA) and 100 μg ml^{-1} streptomycin (Sigma) at 37°C in an atmosphere of 5% CO_2.

Detection of intracellular leptospires by transmission electron microscopy

J774A.1 cells (1×10^6 per well) were seeded in 6-well culture plates (Corning) for a 24-h incubation at 37°C. The cell monolayers were infected with *L. interrogans* strain Lai at a multiplicity of infection (MOI) of 100 (100 leptospires per cell) at 37°C for 1 h (Jin et al., 2009). After washing with PBS and fixation with 2.5% glutaraldehyde-PBS for 2 h, the cell monolayers were scraped from the wells for a 10-min centrifugation at 250×g. The cell pellets were postfixed, dehydrated, embedded in TAAB resin, ultrathin sectioned, and stained as previously described (Zhang

et al., 2012). The intracellular leptospires were observed under a transmission electron microscope (type TECNAI-10, Philips, Holland).

Detection of cellular integrins by flow cytometry

The ITGB1, ITGB2 or ITGB3 expressed by J774A.1 cells were detected as previously described (Gray et al., 2002). Briefly, J774A.1 cell (1×10^6) were blocked with 2% donkey serum-PBS for 15 min at 4°C. Using goat anti-ITGB1, ITGB2 or ITGB3-IgG (Santa Cruz) as the primary antibody, and Alexa Fluor488-conjugated donkey anti-goat-IgG (Invitrogen) as the secondary antibody, the ITGB1, ITGB2 or ITGB3 expressed by the cells was detected using a flow cytometer (type FC500MCL, Beckman, Germany). In the assay, the goat IgG of irrelevant specificity (Santa Cruz) instead of the goat anti-ITGB-IgGs as the primary antibody was used as the isotype controls.

RNA interference

Knockdown of ITGB1, ITGB2 or ITGB3 genes in J774A.1 cells was achieved using a siRNA Transfection Kit (Thermo Scientific) with 5-30 μM Stealth select RNAi™ siRNAs from *[Mus musculus]* databases (Invitrogen) according to the manufacturer's protocols.

Determination of the role of integrins in leptospiral internalization

Wild type J774A.1 cells or ITGB1, ITGB2, ITGB3-knockdown J774A.1 cells (1×10^5 per well) were seeded in 12-well culture plates (Corning) for a 24-h incubation at 37°C. After washing with PBS, each of the wild-type cells was incubated with 30 μg goat anti-ITGB1, ITGB2 or ITGB3-IgG (Santa Cruz) (Wang et al., 2010), for 1 h at 37°C. After washing with PBS again, all the cells were infected with *L. interrogans* strain Lai at an MOI of 100 for 1 h at 37°C (Rejman et al., 2004), then treated with gentamicin and trypsin and centrifuged to detach the extracellular leptospires. The observation of intracellular leptospires was by confocal microscopy. In the detection, J774A.1 cells transfected with non-specific siRNAs (Invitrogen) as well as the goat IgG of irrelevant specificity (Santa Cruz) were used as isotype controls.

Detection of AKT and PI3K phosphorylation in cells during infection

J774A.1 cells (1×10^6 per well) were seeded in 6-well culture plates (Corning) for a 24-h incubation at 37°C. The cell monolayers were washed with PBS, and then infected with *L. interrogans* strain Lai at an MOI of 100 for 0.5 or 1 h at 37°C (Jin et al., 2009). After treatment with gentamicin and trypsin and centrifugation to detach the extracellular leptospires as above, the cells were lysed with RIPA lysis buffer (Millipore). The lysates were centrifuged at 400×g for 10 min (4°C) to remove cell debris, and the supernatants were harvested to measure protein concentration as above. The supernatants were collected to measure protein concentration using a BCA Protein Assay Kit (Thermo Scientific); then 200 ng total proteins each sample were added. After SDS-PAGE and electro-transferring onto PVDF membrane (Millipore), the phosphorylation of AKT or PI3K in the protein samples was detected by Western blot using rabbit anti-total FAK or phospho-FAK-IgG, anti-total PI3K or phospho-PI3K-IgG (Cell Signaling Technology) as the primary antibody, and HRP-conjugated goat anti-rabbit-IgG (Cell Signaling Technology) as the secondary antibody. The immunoblotting signals reflecting phosphorylation levels of FAK and PI3K were quantified by densitometry (gray scale determination) using an Image Analyzer (type Gel Doc XR-T2A, Bio-Rad) (Zhang et al., 2012). In the assay, normal J774A.1 cells without infection served as controls.

Detection of cytoskeletal rearrangement in cells during infection

J774A.1 cells (1×10^5 per well) were seeded in 12-well plates (Corning) for a 24-h incubation at 37°C. After washing with PBS, the cell monolayers were infected with *L. interrogans* strain Lai at an MOI of 100

for 1 or 2 h (Jin et al., 2009). After treatment with gentamycin and trypsin, fixation with paraformaldehyde, permeabilization with Triton X-100 and centrifugation as above, the precipitated cells were incubated with phalloidin-FITC (Sigma) for 40 min to stain cellular microfilaments or with rat anti-tubulin-IgG (Abcam) as the primary antibody and Alexa Fluor488-conjugated donkey anti-rat-IgG (Invitrogen) as the secondary antibody to stain cellular microtubules for 1 h at room temperature. The cells were then incubated with rabbit anti-*L. interrogans* strain Lai-IgG prepared by our laboratory, followed by incubation with Alexa Fluor594-conjugated donkey anti-rabbit-IgG (Invitrogen) for 1 h to stain intracellular leptospires. Finally, the cells were smeared on glass slides and observed under a laser confocal microscope (type FV1000, Olympus). In this assay, the normal J774A.1 cells without infection were used as the controls.

Determination of the roles of AKT and PI3K signaling and cytoskeletal rearrangement in leptospiral internalization

J774A.1 cells (1×10^5 per well) were seeded in 12-well plates (Corning) for a 24-h incubation at 37°C. After washing with PBS, the cell monolayers were treated with 5 μM FAK inhibitor-I (Calbiochem, Germany) (34), 20 μM PI3K inhibitor LY294002 (Calbiochem) (35), 2 μM microfilament inhibitor cytochalasin D (Sigma) or 10 μM microtubule inhibitor colchicine (Sigma) at 37°C for 1 h (36), and then infected with *L. interrogans* strain Lai at an MOI of 100 for 1 h (Jin et al., 2009). The subsequent steps as well as observation of cytoskeletal rearrangement and intracellular leptospires by confocal microscopy were the same as described above. In this assay, the normal J774A.1 cells without treatment of any inhibitors were used as the controls.

Statistical analysis

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

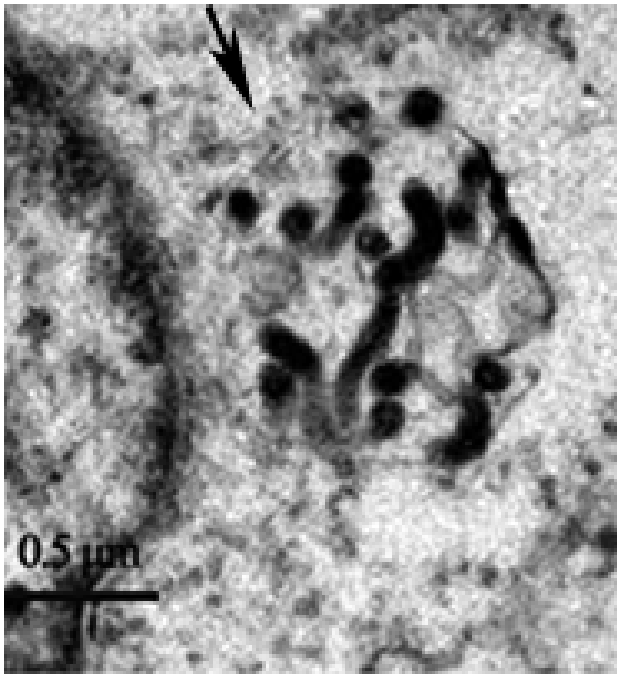
RESULTS

Internalization of L. interrogans through endocytosis

When *L. interrogans* strain Lai was incubated with J774A cells for 1 h, leptospires within phagocytic vesicles could be found in the cytosol of J774A.1 cells under the transmission electron microscope (Fig. 1).

Expression of integrins on J774A.1 cells

The flow cytometric examination demonstrated that J774A.1 cells expressed ITGB1, ITGB2 and ITGB3 (Fig. 2A).



J774A.1

Fig. 1. Internalization of *L. interrogans* into different host cells. Leptospires in J774A.1 cells under transmission electron microscope after infection with *L. interrogans* strain Lai for 1 h. The arrows indicate the leptospires within phagocytic vesicles of the host cells.

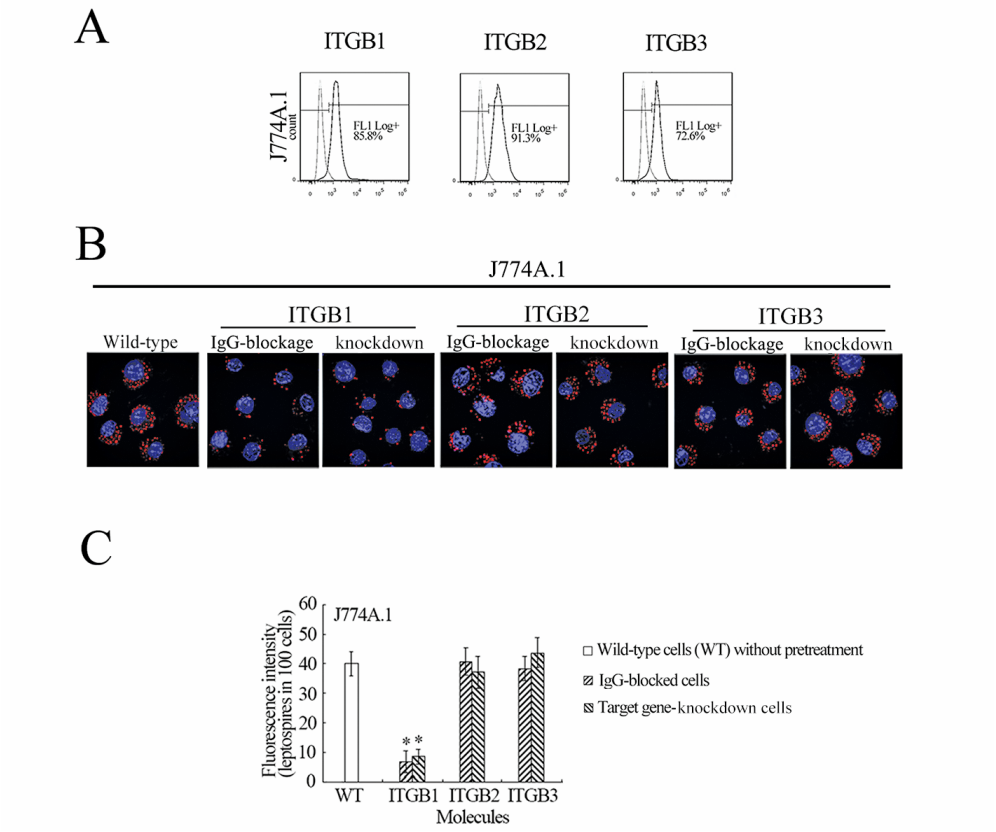


Fig. 2. Role of ITGB1 of J774A.1 cells in internalization of *L. interrogans*. (A) Expression of ITGB1, ITGB2, ITGB3 in J774A.1 cells, determined by flow cytometry. Ten thousand cells were analyzed for each of the samples. (B) Leptospires in J774A.1 cells and ITGB1-, ITGB2-, or ITGB3- knockdown J774A.1 cells infection with *L. interrogans* strain Lai for 1 h, observed by laser confocal microscope. The blue plaques in the middle of cells indicate the nucleus. The red spots around the nucleus indicate the intracellular leptospires. (C) Statistical summary of red fluorescence intensity reflecting the leptospires in J774A.1 cells after infection with *L. interrogans* strain Lai for 1 h. Statistical data from experiments such as shown in D. Bars show the means ± SD of three independent experiments. One hundred cells were analyzed for each of the samples. $^{*}p < 0.05$ vs. the red fluorescence intensity reflecting the leptospires in wild-type J774A.1 cells during infection with the spirochete.

Role of ITGB1 in internalization of L. interrogans

The ITGB1-, ITGB2- or ITGB3-knockdown J774A.1 cells obtained by RNA interference had growth kinetics similar to the wild-type cells (Fig S1. A). The qRT-PCR and Western blot confirmed the significant decrease of both mRNA and protein ITGB1, ITGB2 and ITGB3 in the target gene-knockdown cells (Fig S1. B and C).

When ITGB1 in J774A.1 cells was blocked with ITGB1-IgG or the expression of ITGB1 gene was inhibited with RNA interference, the number of leptospires in the cells during infection with *L. interrogans* strain Lai were significantly decreased compared to the wild-type cells (Fig. 2B and C). However, neither blockage of ITGB2 and ITGB3 protein or knockdown of ITGB2 and ITGB3 genes affected the internalization of leptospires. Moreover, the transfection with non-specific negative siRNAs and blockage with goat IgG of irrelevant specificity did not affect the internalization of the leptospires into J774A.1 cells (Fig S2. A and B). The data suggest that ITGB1 mediated the internalization of *L. interrogans* in the J774A.1 cells.

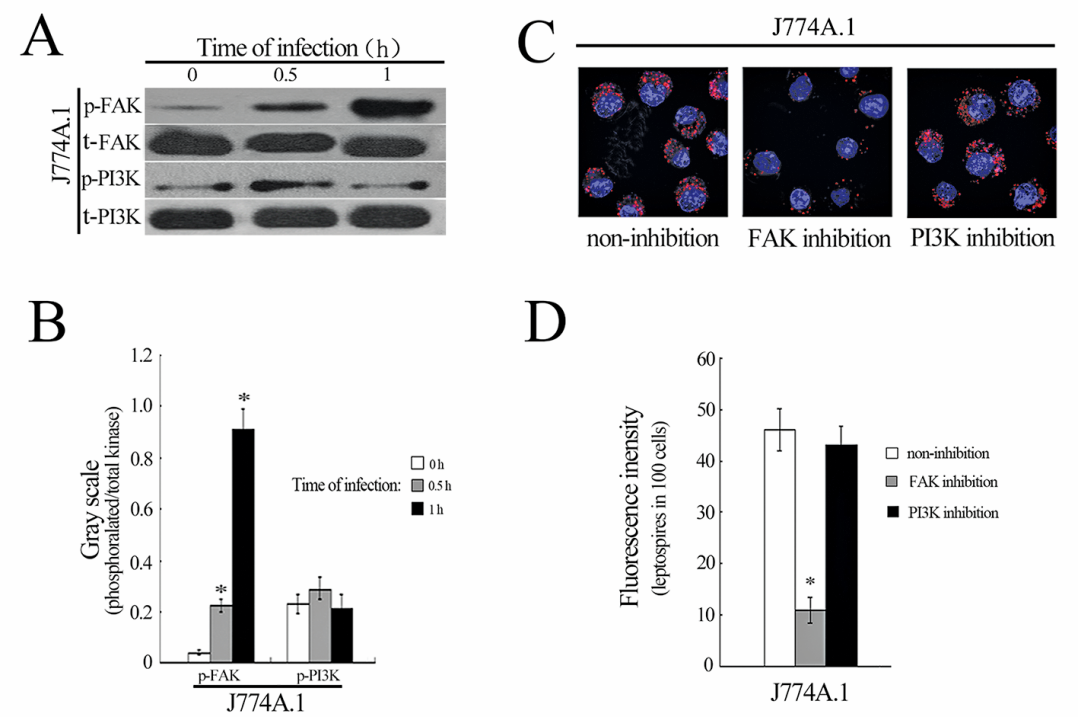


Fig. 3. Activation of FAK or PI3K in host cells during infection with *L. interrogans.* (A) Phosphorylation of FAK in J774A.1 cells during infection with *L. interrogans* strain Lai for the indicated times. The images indicate the levels of total FAK and PI3K or phosphorylated FAK (p-FAK) and PI3K (p-PI3K) in J774A.1 cells. The values at "0 h" indicate J774A.1 cells before infection. (B) Quantification of immunoblotting bands by gray scale determination reflecting FAK and PI3K phosphorylation in the leptospire-infected J774A.1 for the indicated times. Statistical data from experiments shown in A. Bars show the means ± SD of three independent experiments. The values at "0 h" indicate J774A.1 cells before infection. *$p < 0.05$ vs. the FAK or PI3K phosphorylation levels (gray scale) of the J774A.1 cells before infection (0 h). (C) Leptospires in FAK- or PI3K-inhibited J774A.1 cells, detected by laser confocal microscopy. The blue plaques in the middle of cells indicate the nucleus. The red spots around the nucleus indicate the intracellular leptospires. (D) Statistical summary of red fluorescence intensity reflecting the leptospires in the FAK or PI3K-inhibited leptospire-infected cells. Statistical data from experiments such as shown in C. Bars show the means ± SD of three independent experiments. One hundred cells were analyzed for each of the samples. *: $p < 0.05$ vs. the red fluorescence intensity reflecting the leptospires in the FAK-uninhibited or PI3K-uninhibited J774A.1 cells.

Activation of FAK or PI3K in different cells during infection

FAK and PI3K signaling pathways play crucial roles in cell membrane function such as phagocytosis and pathogen internalization through regulation of cytoskeletal rearrangement (Doherty et al., 2009). However, our Western blot revealed that the phosphorylation levels of FAK, but not PI3K in J774A.1 cells were increased during infection with *L. interrogans* strain Lai (Fig. 3A and B). When J774A.1 cells were pretreated with FAK inhibitor-I, the internalization of the spirochete was significantly decreased (Fig 3C and D). However, the inhibition of PI3K in J774A.1 cells had no influence on the internalization of leptospires.

Microfilament rearrangement in cells during leptospiral internalization

Our results showed that only microfilaments in the J774A.1 cells were aggregated during infection with *L. interrogans* strain Lai (Fig. 4A and B). When the microfilaments were inhibited with cytochalasin D, the number of intracellular leptospires in cells was significantly decreased, but the inhibition of microtubules with colchicine did not affect the internalization of leptospires (Fig. 4C and 5D). The data suggest

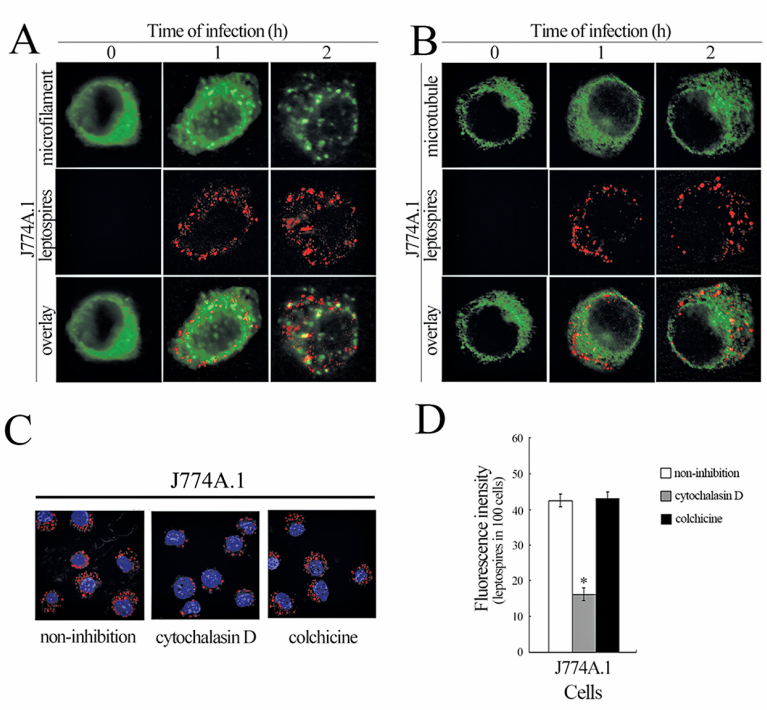


Fig. 4. Microfilament rearrangement in host cells during infection with *L. interrogans*. (A) Microfilament aggregation in J774A.1 cells during infection with *L. interrogans* strain Lai for the indicated times, determined by laser confocal microscopy. The images at "0 h" indicate J774A.1 cells before infection. The red spots in cells indicate the intracellular leptospires. (B) No microtubule aggregation in J774A.1 cells during infection with *L. interrogans* strain Lai for the indicated times, determined by laser confocal microscopy. The images at "0 h" indicate the J774A.1 cells before infection with the spirochete. The red spots in cells indicate the intracellular leptospires. (C) Leptospires in microfilament- or microtubule-blocked J774A.1 cells after infection with *L. interrogans* strain Lai for 1 h, detected by laser confocal microscopy. The blue plaques in the middle of cells indicate the nucleus. The red spots around the nucleus indicate the intracellular leptospires. Cytochalasin D is a microfilament inhibitor, while colchicine is a microtubule inhibitor. (D) Statistical summary of red fluorescence intensity reflecting the leptospires in the microfilament- or microtubule-blocked leptospire-infected cells. Statistical data from experiments such as shown in C. Bars show the means ± SD of three independent experiments. One hundred cells were analyzed for each of the samples. *: $p < 0.05$ vs. the red fluorescence intensity reflecting the leptospires in the microfilament-uninhibited leptospire-infected J774A.1 cells.

that rearrangement of microfilaments rather than microtubules in J774A.1 is responsible for internalization of *L. interrogans*.

DISCUSSION

Leptospirosis is a typical systemic infectious disease that depends on the invasive ability of pathogenic *Leptospira* species (McBride et al., 2005; Levett et al., 2001). Macrophages play a crucial role in the innate immune response against infection by pathogenic *Leptospira* species. Adherence is a prerequisite for bacterial invasion into host cells (Pizarro-Cerda et al., 2006). Fibronectin (FN), laminin (14), collagen I (COL1) and COL4 in the ECM of cells have been shown to act as the receptor molecules for the adherence of pathogenic *Leptospira* species (Barbosa et al., 2006; Choy et al., 2007; Stevenson et al., 2007; Hauk et al., 2008; Atzingen et al., 2008; Vieira et al., 2010). Ligation of ECM molecules with integrins has been shown to mediate the internalization of bacterial pathogens (Secott et al., 2004; Scibelli et al., 2007). Our results also demonstrated that J774A.1 cells express all three integrin subfamily proteins (ITGB1, ITGB2 and ITGB3). In particular, only the inhibition of ITGB1 in J774A.1 cells caused a significant decrease of leptospiral internalization. The data indicate that ITGB1 is responsible for the in-

ternalization of *L. interrogans* into mouse J774A.1 macrophages,

During bacterial internalization, the binding of integrin dimers to RGD-motif-containing ECM molecules has been shown to activate FAK and PI3K signaling pathways (Owen et al., 2007). Both FAK and PI3K pathways can mediate internalization of bacteria into different host cells through microfilament- and/or microtubule-dependent cytoskeletal rearrangements (Liu et al., 2007;, Wang et al., 2012). However, in this study, only FAK activation and microfilament aggregation in the leptospire-infected J774A.1 cells could be found. More importantly, inhibition of FAK and blockage of microfilament aggregation resulted in noticeable attenuation of leptospiral internalization.

Taken together, our findings indicated that ITGB1/FAK/microfilament-signaling mediate the internalization of *L. interrogans* in mouse J774A.1 macrophages, documenting one potential host-dependent factor of the virulence of *L. interrogans* in mice.

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Authors' contribution

XZ and AS contributed equally to this work.

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SUPPLEMENTARY MATERIAL

RESULTS

Characterization of the target gene-knockdown cells

The J774A.1 cells whose ITGB1, ITGB2 or ITGB3 gene had knocked down with RNA interference showed growth kinetics similar to the wild-type cells (Fig S1. A). The qRT-PCR and Western Blot assay confirmed the significant decrease of both mRNA and protein expressed by the ITGB1, ITGB2 or ITGB3 in the target gene knockdown J774A.1 cells (Fig S1. B and C).

Internalization of L. interrogans into non-specific siRNAs-transfected or goat IgG-blocked host cells

The transfection with non-specific negative siRNAs and blockage with irrelevant specific goat-IgG did not affect the internalization of the spirochete into the three cell types (Fig S2. A and B).

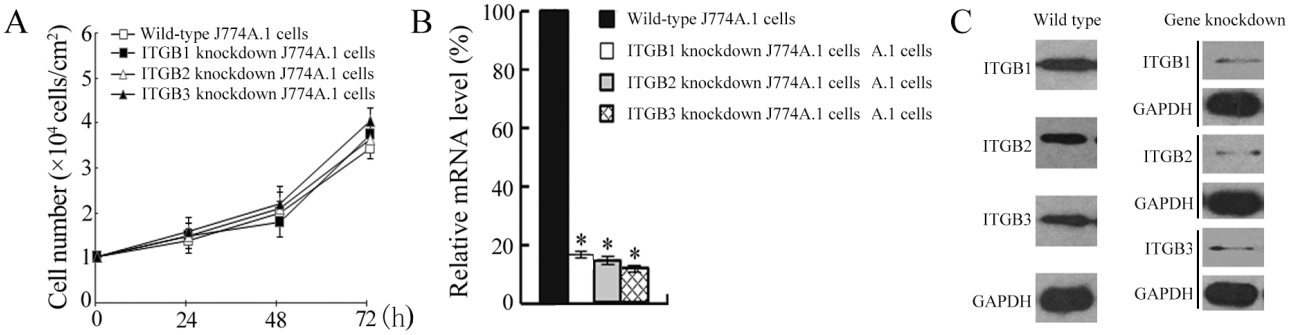

Fig. S1. Characterization of target gene knockdown cells. (A) Growth kinetics of the target gene knockdown cells determined by cell enumeration. The wild-type and gene knockdown cells were seeded in cell plate at 1×10^4 separately, cell number were counted at 24h, 48h and 72h. The data show the means ± SD of three independent experiments. (B) Significant decrease of ITGB1-, ITGB2- or ITGB3-mRNA levels in the target gene-knockdown cells determined by qRT-PCR. Bars show the means ± SD of three independent experiments. The ITGB1-, ITGB2- or ITGB3 mRNA levels in the wild-type J774A.1 was set as 100%. *: $p < 0.05$ vs. the ITGB1-, ITGB2- or ITGB3-mRNA levels in the wild-type J774A.1 cells. (C) Significant decrease of ITGB1, ITGB2 or ITGB3 protein expressed by the target gene-knockdown cells determined by Western blot assay.

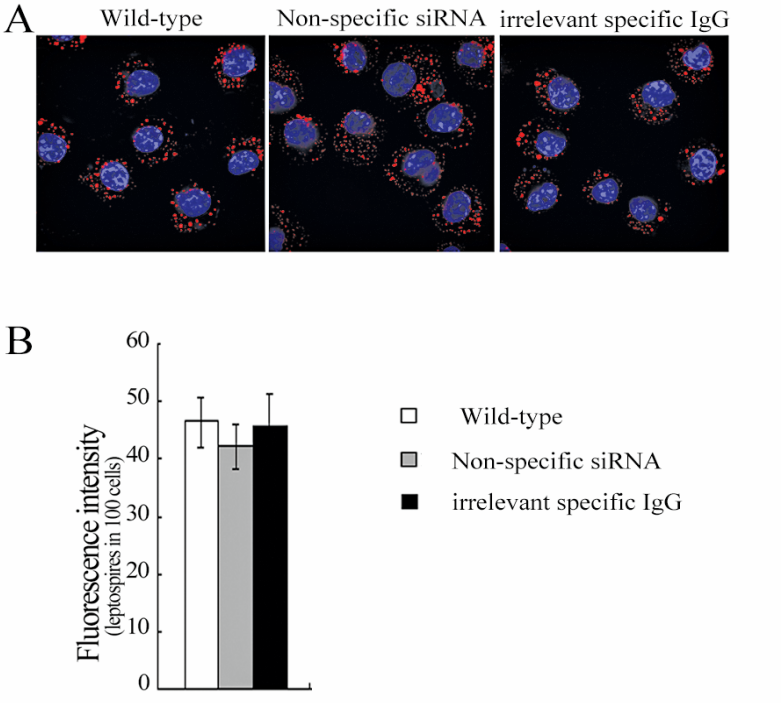

Fig. S2. Internalization of *L. interrogans* into non-specific siRNAs-transfected or irrelevant specific goat IgG-blocked host cells. (A) Leptospires in the non-specific siRNAs-transfected or irrelevant specific goat IgG-blocked J774A.1 cells under laser confocal microscope after infection with *L. interrogans* strain Lai for 1 h. The blue plaques in the middle of cells indicate the nucleus. The red spots around the nucleus indicate the intracellular leptospires. (B) Statistical summary of red fluorescence intensity reflecting the leptospires in the non-specific siRNAs-transfected or irrelevant specific goat IgG-blocked J774A.1 cells after infection with *L. interrogans* strain Lai for 1 h. Bars show the means ± SD of three independent experiments. One hundred cells were analyzed for each of the samples. There is no significant difference in the fluorescence intensity of the intracellular leptospires between the wild-type and non-specific siRNAs-transfected or irrelevant specific goat IgG-blocked J774A.1 cells.