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LEPTOSPIRA INTERROGANS ENTERS DIFFERENT HOST CELLS BY BINDING TO DISTINCT MOLECULES IN THE EXTRACELLULAR MATRIX

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Abstract - Leptospirosis caused by *Leptospira* is a worldwide zoonotic disease. The spirochete can efficiently invade host cells and spread from blood into internal organs, but the mechanisms of leptospiral internalization into different host cells remain poorly understood. Adhesion of leptospires to the ECM (extracellular matrix) initiated leptospire internalization in macrophage cell-lines THP-1, J774A.1, and in human umbilical epithelial cells (HUVEC). We assessed the distribution of ECM , including fibronectin (FN), laminin and collagens 1-4 (COL1-COL4) in different cells. FN was expressed in THP-1 and J774A.1 cells, and FN, LN, COL3 and COL4 were expressed in HUVEC. Then, we found leptospires adhered to FN, LN, COL3 and COL4. The most important intracellular leptospires in macrophages (THP-1 and J774A) significantly decreased when leptospires were pre-blocked with FN This indicates that FN has an important role in the adherence to and entry of leptospires in macrophages, while in HUVEC, FN, LN, COL3 and COL4 assume this role.

Key words: Leptospira interrogans; extracellular matrix; adhesion; internalization

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INTRODUCTION

Leptospirosis is a worldwide disease that is lethal to some mammals. Leptospires penetrate the mucosal tissues and skin of mammals such as humans and mice, and are transported by the blood to different organs, including the liver, lungs and kidneys (Goncalves-de-Albuquerque et al., 2012). Leptospirosis is frequently reported in Asia, America and Europe. Mice are the most common host for transmitting leptospires to humans, because of their numbers and close contact with humans. The symptoms of leptospirosis in humans vary from mild to fatal, ranging from high fever with muscle pain to severe symptoms such as pulmonary hemorrhage, jaundice and meningitis, which lead to respiratory failure and renal failure with high mortality rates (Zhang et al., 2012a; Pappas et al., 2008). In mammals, different cell-types play different roles when infected by pathogens. Macrophages assume a crucial role in phago-

cytosis and killing pathogens by degradation. Vascular endothelial cells are responsible for the transport of pathogens from the blood stream to target organs as both macrophages and vascular endothelial cells uptake leptospires during infection.

The adhesion of pathogens to cells is the first step of invasion. Patti et al. (1994) first proposed the adhesion mode by which bacteria adhere to ECM and initiate invasion. ECM molecules play important roles in invasion through binding to integrins (Amano, 2003). Although leptospires are known to recognize FN, LN and COL4 during mammalian infection (Hauk et al., 2008; Stevenson et al., 2007), systematic studies on ECM distribution on cells and the roles of ECMs in leptospire internalization were few. The purpose of this study was to discover the mechanisms of leptospire internalization in different kinds of cells.

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), a human monocytic cell line (THP-1) and a human umbilical vein endothelial cell line (HUVEC) were 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. In particular, THP-1 cells were pre-treated with 10 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma) at 37°C for 48 h to differentiate them into macrophages before use (Wang et al., 2012).

Detection of cellular ECM molecules by confocal microscopy

THP-1, J774A.1 cells and HUVEC (1×10^5 cells per well) were seeded in 12-well culture plates (Corning, USA) containing a 12×12 mm coverslip per well for a 24 h at 37°C. After washing with 10 mM PBS (pH 7.4), the coverslips with cell monolayers were fixed with 4% paraformaldehyde-PBS for 30 min at room temperature, followed by a thorough washing with PBS and a 1-h blockage with 5% BSA-PBS at 37°C. Using rabbit anti-FN, LN, COL1, COL2, COL3 or COL4-IgG (Santa Cruz, USA; Abcam, UK) as the primary antibody, and Alexa Fluor594-conjugated donkey anti-rabbit-IgG as the secondary antibody (Invitrogen, USA), the ECM molecules expressed by the cells were examined under a laser confocal microscope (type FV1000, Olympus, Japan) (590 nm excitation and 617 nm emission wavelengths for Alexa Fluor 594 detection). In the detection, rabbit non-specific IgG (Abcam) instead of the IgG anti-ECM molecules as the primary antibody was used as the control.

Detection of cellular ECM molecules by quantitative ELISA

FN (Invitrogen), LN (Invitrogen), COL3 (BD, USA) or COL4 (R&D, USA) at different concentrations was coated in 96-well culture plates (Corning) at 4°C for 24 h. The plates were washed with PBST (0.1% Tween 20, 0.01 M PBS, pH7.4) and 0.1% TritonX-100, then blocked with 5% BSA-PBS. Using rabbit anti-FN, anti-LN, anti-COL3 or anti-COL4-IgG (Santa Cruz or Abcam) as the primary antibody, HRP-conjugated goat anti-rabbit-IgG (Cell Signaling Technology, USA) as the secondary antibody and TMB (Sigma) as the substrate, the OD_{450} value per well was detected using a Microplate Reader (Model 550, Bio-Rad, USA).

According to the OD_{450} values vs. the corresponding concentrations of each of the ECM molecules,

the standard ECM molecule concentration curves were established (see Standard concentration curves for quantitative ELISA). THP-1 or J774A.1 cells or HUVEC (1×10^4 per well) were seeded in 96-well culture plates (Corning) for a 24-h incubation at 37°C. After fixation with 4% paraformaldehyde-PBS and washing with PBS, the OD_{450} value per well was detected as above and the expressed yield of FN, LN, COL3 or COL4 per cell was calculated according to the standard curves. In the assay, the bovine serum albumin (BSA, Sigma) instead of ECM molecules as the coated protein was used as the control.

Standard concentration curves for quantitative ELISA

Detections were performed independently five times, in which three parallel wells per experiment for each of the ECM molecule samples with different protein concentrations were detected. Using the OD_{450} values as the Y-axis and the concentrations of each of the ECM molecules as X-axis, the standard concentration curves including calculation formulas were established for quantitative detection of FN, LN, COL3 and COL4 molecules in J774A.1 and THP-1 cells and HUVEC. In the assay, the BSA (Sigma) instead of ECM molecules as the coated proteins was used as the control.

Leptospiral adherence test

An ELISA-based adherence test was performed to detect the ECM molecules that act as the receptors of leptospiral adherence as previously reported (Choy et al., 2007). Briefly, fresh cultured *L. interrogans* strain Lai was centrifuged at 17 200×g for 15 min (4 °C), and the precipitated leptospires were suspended in PBS for counting with a Petroff-Hausser counting chamber (Fisher Scientific, USA) under a dark field microscope (Schreier et al., 2009). One μg per well in a 96-well plate (Corning) of FN (Invitrogen), LN (Invitrogen), COL1 (Sigma), COL2 (Sigma), COL3 (BD) or COL4 (R&D) was used as the coated protein. After blockage with 5% BSA-PBS for 1 h at 37°C, 100 μl PBS containing 5×10^6 leptospires per well were added for an 1-h incubation at 37°C. After washing with PBST, the leptospires in the wells were fixed with paraformaldehyde as above and then washed thoroughly with PBST. Using rabbit anti-*L. interrogans* strain Lai-IgG prepared by our laboratory as the primary antibody, HRP-conjugated goat anti-rabbit-IgG (Cell Signaling Techology) as the secondary antibody and TMB (Sigma) as the substrate, the OD_{450} value per well was detected using a Microplate Reader (Model 550, Bio-Rad). In the ELISA, the BSA (Sigma) instead of ECM molecules as the coated protein was used as the control. An OD_{450} value over the mean plus 3×SD of the controls was defined as positive (Dong et al., 2008).

Leptospiral adherence inhibition test

An ELISA-based adherence inhibition test was performed to determine the ECM molecules that served as the receptors of leptospiral adherence as previously reported (Choy et al., 2007; Trafny et al., 1995). Briefly, 100 μl BPS containing 5×10^6 *L. interrogans* strain Lai was pre-incubated with 100 μl PBS containing 5 μg FN (Invitrogen), LN (Invitrogen), COL3 (BD) or COL4 (R&D) at 37 °C for 1 h. The mixtures were centrifuged at 17 200×g for 15 min at 4°C, and the precipitated leptospires were suspended in 100 μl PBS. The subsequent steps were the same as in the adherence test above.

Detection of intracellular leptospires by confocal microscopy

THP-1 or J774A.1 cells or HUVEC (1×10^5 per well) were seeded in 12-well culture plates (Corning) for a 24-h incubation at 37°C. Leptospires were pre-blocked with FN, LN, COL3, COL4 separately and four kinds of ECMs (FN+LN+COL3+COL4) (1:500 dilution); leptospires were not pre-blocked in controls. The cell monolayers were infected with *L. interrogans* strain Lai at an MOI of 100 at 37°C for 1 h (Jin et al., 2009). After treatment with 50 μg ml^{-1} gentamicin for 15 min to kill the extracellular leptospires and digestion with 0.25% trypsin-PBS for 5 min to detach the cell-adhered leptospires, the cells were centrifuged at 100×g for 10 min at 4°C. The cell pellets were fixed with 4% glutaraldehyde-PBS for 30 min, and then permeabilized with 0.1% Triton X100-

PBS for 10 min to allow antibody penetration into the cells (Zhang et al., 2012b). After washing with PBS and blockage with 5% BSA-PBS, the cells were 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. After washing again with PBS, the cells were incubated with 1 μg ml^{-1} DAPI (Sigma) for 10 min to stain the cell nucleus. Finally, the cells were smeared on glass slides and observed under a laser confocal microscope (type FV1000, Olympus) (590 nm excitation and 617 nm emission wavelengths for Alexa Fluor594 detection, and 355 nm excitation and 460 nm emission wavelengths for DAPI detection).

Calculation of fluorescence intensity in cells

Fluorescence intensity of intracellular leptospires in 50 cells was calculated using Metamorph software.

Statistical analysis

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

RESULTS

Distribution of ECM molecules in different cells

Confocal microscopic examination confirmed that the THP-1 and J774A.1 cells expressed FN alone, while the HUVEC expressed FN, LN, COL3 and COL4. However, LN, COL1, COL2, COL3 and COL4 in the THP-1 and J774A.1 cells, and COL1 and COL2 in the HUVEC were undetectable (Fig. 1).

Standard concentration curves for quantitative ELISA

The standard concentration curves, including calculation formulas used in ELISA for quantitative detection of FN, LN, COL3 and COL4 molecules in J774A.1 and THP-1 cells and HUVEC, are shown in Fig. 2 and Table.1.

Content of ECM molecules in different cells by quantitative ELISA

Quantitative ELISA demonstrated that the number of FN molecules per cell expressed by the THP-1 and J774A.1 cells was about 4.2×10^7 and 3.8×10^7, respectively, while the HUVEC expressed 1.5×10^7 FN, 1.2×10^6 LN, 2.7×10^6 COL3, and 3.1×10^7 COL4 molecules per cell (Fig. 3).

L. interrogans adherence to ECM molecules

The adherence test showed that *L. interrogans* strain Lai could adhere to all of the six tested ECM molecules (FN, LN and COL1 to COL4) (Fig. 4). The leptospiral adherence was significantly attenuated when the leptospires were pre-blocked with different ECM molecules (Fig. 4). The data reveal the large diversity of ECM molecules expressed by macrophages and vascular endothelial cells as well as the strong ability of *L. interrogans* to adhere to host cells through a combination of different ECM molecules.

Internalization of L. interrogans into different host cells with pre-blocked ECMs

Leptospires were pre-blocked with FN, LN, COL3, COL4 and four kinds of ECMs (FN+LN+COL3+COL4) for 1 h; the control were leptospires not pre-blocked with ECMs. THP-1, J774A.1 cells and HUVEC were infected with *L. interrogans* strain Lai at an MOI of 100 at 37°C for1 h; confocal microscopy was used to detected the intracellular leptospires. After pre-blocking with FN, intracellular leptospires in macrophages (THP-1 or J774A) significantly decreased; in HUVEC, leptospires pre-blocked with four kinds of ECM separately (FN, LN, COL3 or COL4) significantly decreased intracellular leptospires. The effects were much more obvious in leptospires pre-blocked with four kinds of ECMs (FN+LN+COL3+COL4). The results indicate that FN assumed an important role in the adherence to and entry of leptospires in macrophages, while in

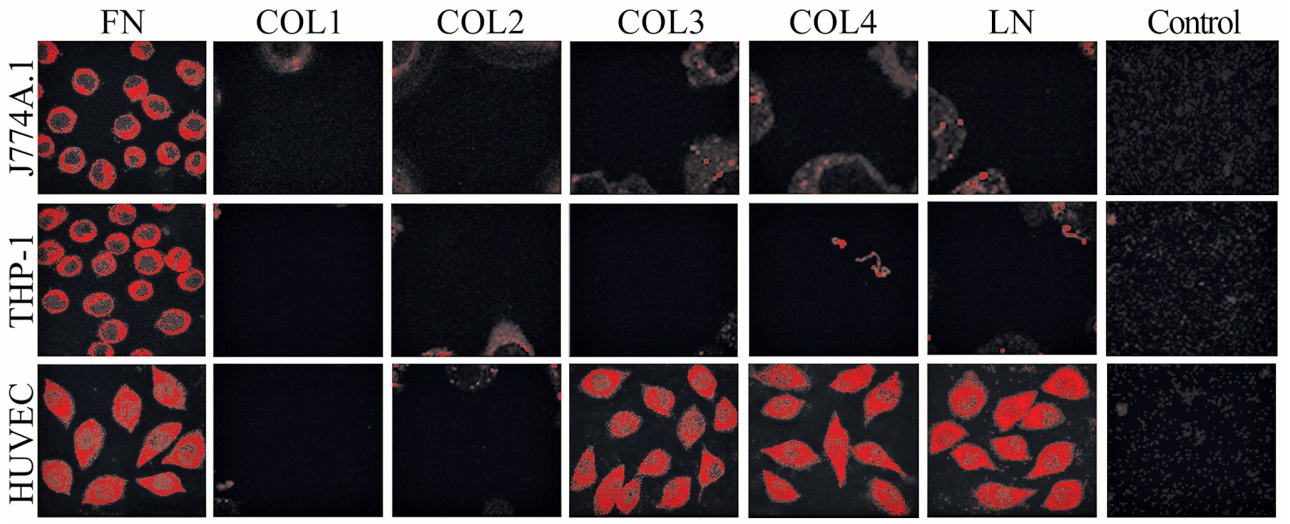

Fig. 1. ECM molecules expressed in THP-1 and J774A.1 cells and HUVEC determined by laser confocal microscopy. The red fluorescence indicates the FN expressed in THP-1 and J774A.1 cells, and FN, LN, COL3 or COL4 expressed in HUVEC.

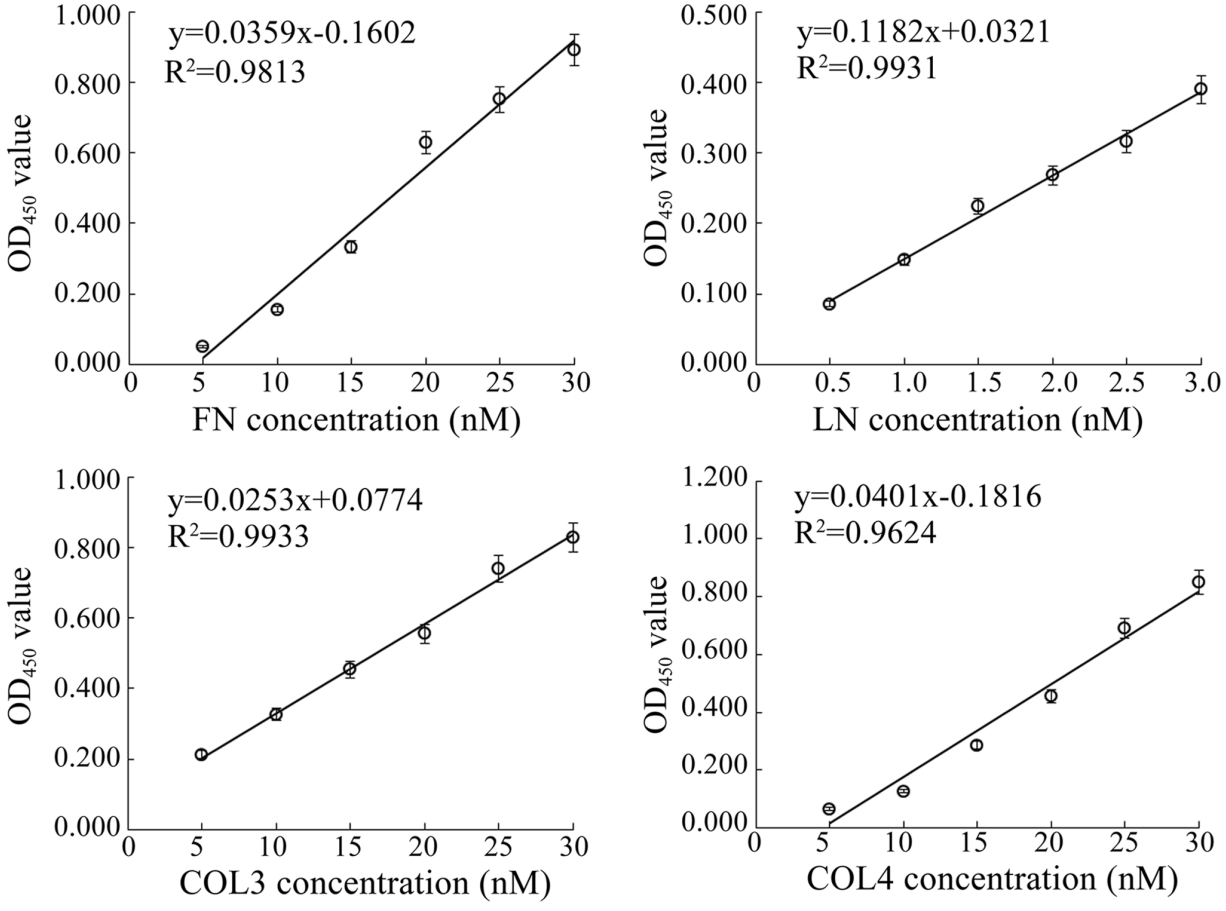

Fig. 2. Standard concentration curves for quantitative ECM molecule detection by ELISA. The curves were obtained from five independent experiments by quantitative ELISA to detect the FN, LN, COL3 or COL4 molecules. Each of the points indicates the OD_{450} values, shown as the means ± SD.

Table 1. Concentrations of ECM molecules and OD_{450} value.

ECM molecules	**concentration** (nmol/L)	**OD_{450} value±*s***
FN	30	0.891±0.114
	25	0.751±0.045
	20	0.628±0.043
	15	0.333±0.013
	10	0.155±0.018
	5	0.051±0.001
LN	3	0.39±0.008
	2.5	0.316±0.009
	2	0.268±0.022
	1.5	0.225±0.007
	1	0.148±0.008
	0.5	0.086±0.005
COL3	30	0.829±0.036
	25	0.74±0.031
	20	0.555±0.038
	15	0.454±0.051
	10	0.327±0.037
	5	0.213±0.02
COL4	30	1.04±0.047
	25	0.691±0.042
	20	0.457±0.118
	15	0.286±0.018
	10	0.126±0.042
	5	0.012±0.005

HUVEC, FN, LN, COL3 and COL4 assumed this role.

DISCUSSION

In this study, we found that the internalization mechanisms of leptospires in THP-1 cells, J774A.1 cells and HUVEC were varied. Many previous studies have investigated leptospire internalization by macrophages, such as THP-1 and J774A.1 cells (Wang et al., 1984; Liu et al., 2007), but the mechanism is still unknown. We investigated leptospire internalization not only by THP-1 and J774A.1 cells but also by HUVEC. The first step in invading a mammal is adherence to cells; pathogens adhere to ECM components, such as laminins, collagens

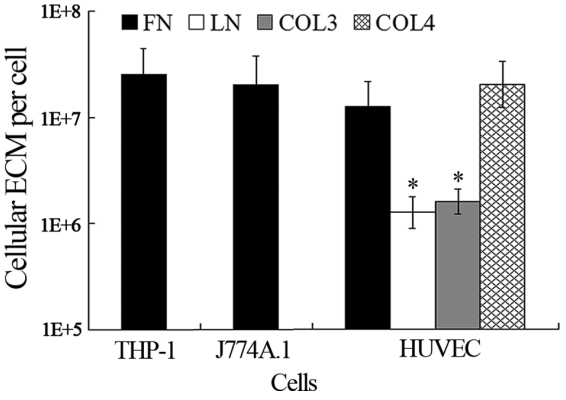

Fig. 3. Quantitative analysis of different ECM molecules expressed by THP-1 and J774A.1 cells and HUVEC, determined by ELISA. Bars show the means ± SD of three independent experiments. *p <0.05 vs. the OD_{450} values reflecting the expression yields of FN and COL4 in HUVEC.

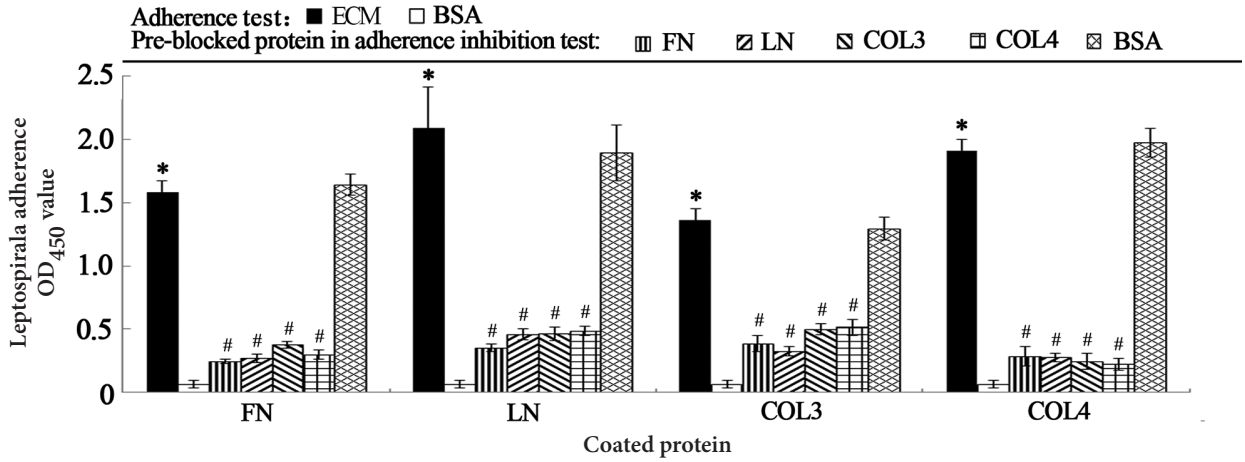

Fig. 4. Adherence of *L. interrogans* strain Lai to different ECM molecules and competitive adherence inhibition of the molecules. Bars show the mean ± SD of three independent experiments. The BSA is used as the negative control. *p <0.05 vs. the OD_{450} values reflecting the adherence level of the spirochete before blockage with different ECM molecules in adherence inhibition test of the spirochete.

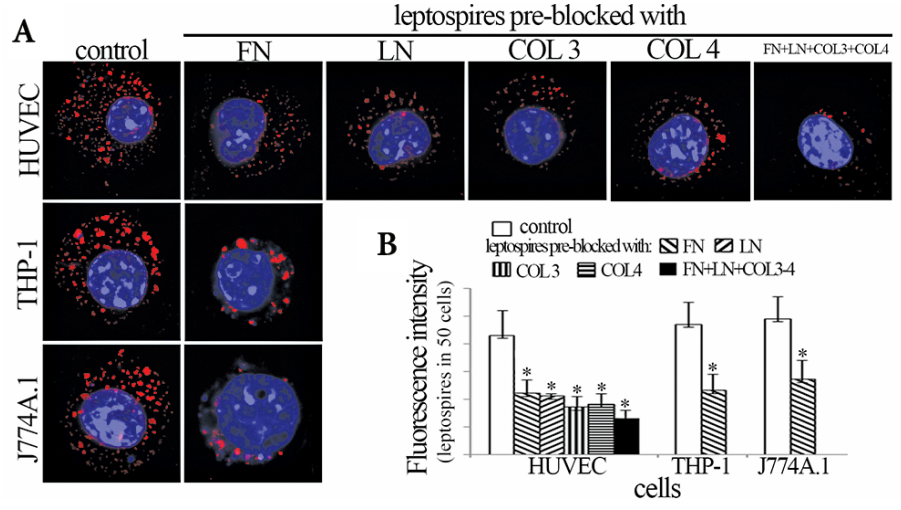

Fig. 5. Internalization of *L. interrogans* into different host cells after pre-blocking the ECM. (A). Leptospires were pre-blocked with FN, LN, COL3, COL4 separately and leptospires in THP-1 and J774A.1 cells and HUVEC were observed by laser confocal microscopy after infection with *L. interrogans* 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 THP-1 and J774A.1 cells and HUVEC during infection with *L. interrogans* strain Lai for 1 h. Bars show the means ± SD of three independent experiments. 50 cells were analyzed for each of the samples. *p < 0.05 vs. the red fluorescence intensity in the control before infection.

and fibronectin, which trigger a series of intracellular signals to promote internalization by the host cell (Pizarro-Cerda et al., 2006). However, ECM distribution varies in different cells. To date, little information about the ECM molecule distribution in mouse or human macrophages and vascular endothelial cells was available. Thus, we investigated the ECM molecules expressed by THP-1 and J774A.1 macrophages and HUVEC. Six kinds of ECMs (FN, LN, COL3 and COL4) were used in this study. The results showed that both macrophage types express FN alone while HUVEC express FN, LN, COL3 and COL4. The data indicate that there is a large diversity of expressed ECM molecules in dif-

ferent host cells. Some pathogens, by binding to the ECM, engage integrin receptors to invade host cells (Scibelli et al., 2007). Adherence tests showed that leptospires adhered to all four ECM molecules used in this study (FN, LN, and COL3-4). Pre-incubation of *L. interrogans* strain Lai with each of the tested ECM molecules displayed a competitive inhibitory effect on the adherence of leptospires to THP-1 and J774A.1 macrophages and HUVEC. In order to determine if and which kinds of ECM were involved in the internalization, leptospires were blocked with ECM. Internalization of leptospires in cells was detected by confocal microscopy.

In macrophages (THP-1 or J774A), intracellular leptospires were decreased when leptospire FN-binding sites were blocked. In HUVEC, leptospires pre-blocked with four kinds of ECM separately (FN, LN, COL3 or COL4) significantly decreased intracellular leptospire; the effects were much more obvious after pre-blocking with FN, LN, COL3 and COL4. The results showed that FN is responsible for adherence to and entry of leptospires in macrophages, while in HUVEC, FN, LN, COL3 and COL4 play that role.

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Authors' contributions

XZ and AS contributed equally to this work. JY conceived and designed the experiments. XZ and HW performed the experiments. XZ and AS analyzed the data. AS and YMG contributed reagents/materials/analysis tools. XZ wrote the paper.

Conflict of interest disclosure

The authors declare that they have no competing interests.

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