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Review Article

Leptospirosis is an invasive infectious and systemic inflammatory disease

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ABSTRACT

Pathogenic *Leptospira* species are the causative agents of leptospirosis, a world-spreading zoonotic infectious disease. The pathogens possess a powerful invasiveness by invading human body through mucosal/skin barriers, rapid entry into bloodstream to cause septicemia, diffusion from bloodstream into internal organs and tissues to cause aggravation of disease, and discharge from urine through renal tubules to form natural infectious sources. Leptospirosis patients present severe inflammatory symptoms such as high fever, myalgia and lymphadenectasis. Hemorrhage and jaundice are the pathological features of this disease. Previous studies revealed that some outer membrane proteins of *Leptospira interrogans*, the most important pathogenic *Leptospira* species, acted as adherence factors to binding to receptor molecules (fibronectin, laminin and collagens) in extracellular matrix of host cells. Collagenase, metallopeptidases and endoflagellum contributed to the invasiveness of *L. interrogans*. Except for lipopolysaccharide, multiple hemolysins of *L. interrogans* displayed a powerful ability to induce pro-inflammatory cytokines and hepatocyte apoptosis. vWA and platelet activating factor acetylhydrolase-like proteins from *L. interrogans* could induce severe pulmonary hemorrhage in mice. *L. interrogans* utilized cellular endocytic recycling and vesicular transport systems for intracellular migration and transcellular transport. All the research achievements are helpful for further understanding the virulence of pathogenic *Leptospira* species and pathogenesis of leptospirosis.

Leptospirosis caused by pathogenic *Leptospira* species is a zoonotic infectious disease of global importance [1]. Every year, there are approximate one million new patients and ten-thousand fatal cases of leptospitrosis in the world [2,3]. This disease is endemic in Asia, Oceania and South America [4–6], but in recent years it is frequently reported in Europe, North America and Africa [7–10]. Therefore, leptospirosis has been considered as an

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emerging or re-emerging infectious disease in many areas of the world [11].

Leptospira is classified as pathogenic and saprophytic species [12]. Furthermore, according to the diversity of molecular genetics and pathogenic ability, all leptospiral strains are divided into pathogenic, intermediate and saprophytic types [13–17]. The pathogenic type contains *Leptospira alexanderi*, *Leptospira alstonii*, *Leptospira borgpetersenii*, *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira kmetyi*, *L. mayottensis*, *L. noguchii*, *Leptospira santarosai* and *Leptospira weilii* genospecies, in which *L. interrogans* is the most prevalent genospecies in the world but the pathogenicity of *L. kmetyi* has been disputed. The intermediate type contains *Leptospira broomii*, *Leptospira fainei*, *Leptospira inadai*, *Leptospira licerasiae* and *Leptospira wolffii* genospecies that occasionally cause disease in human and animals. The saprophytic type contains *Leptospira biflexa*, *L. meyeri*, *Leptospira vanthielii*, *L. wolbachii* and *Leptospira yanagawae* genospecies that are living in natural water and never cause disease.

Approximate 200 animals including livestock and dogs have been confirmed as the hosts of pathogenic *Leptospira* species [12]. The infected animals present mild or no symptoms but can persistently discharge leptospires from urine to contaminate environments. Animal kidneys are the preferential organs in residence as a reservoir of pathogenic *Leptospira* species [18]. Human individuals are infected by contact with the *Leptospira*-contaminated natural water or wet soil [19]. However, nearly all of the infected individuals suffer from serious leptospirosis [19,20]. Pathogenic *Leptospira* species are able to rapidly invade into human body through mucosal and skin barriers and fast enter bloodstream to cause a septicemia and all the patients present severe inflammatory symptoms such as high fever, myalgia and superficial lymphadenectasis [20,21]. In many cases, the pathogens are diffused from bloodstream into lungs, liver, kidneys and cerebrospinal fluid to cause lethal pulmonary diffuse hemorrhage, severe jaundice-induced renal failure and meningoencephalitis [19–21]. In the course of leptospirosis, jaundice and hemorrhage are served as the most important clinical features [12,20,22]. In addition, partial leptospirosis patients also present a short period of leptospiral discharge from urine at convalescence stage and many pathogen-unknown patients with chronic kidney diseases were found due to infection of pathogenic *Leptospira* species [23,24]. Recently, leptospirosis has been considered as a systemic inflammatory response syndrome (SIRS) due to the storm of cytokines in the patients [25–27]. However, until now, the molecular basis of pathogenic *Leptospira* remains limitedly understood [28].

Leptospira has a cell wall similar to that of Gram-negative bacteria. However, many previous studies revealed that lipopolysaccharide (LPS) of *Leptospira* has a lower endotoxic activity than that of enteric bacilli such as *Escherichia coli* LPS [12,29–31]. Except of expression of many hemolysins, no any typical exotoxin-encoding genes can be found in genomes of pathogenic *Leptospira* species [32–34]. It is well known that pathogenic ability of prokaryotic microbes is dependent on invasiveness and toxins that decide the infected state formation and tissue injury. In this review, we summarize the recent achievements in the virulence factors and their effective mechanisms of pathogenic *Leptospira* species.

Adherence factors

Adherence is a process of pathogenic microbes attaching to surface of host cells by binding of microbial ligands to cellular receptors and it is considered as the first step for further colonization or invasion into hosts of microbial pathogens during infection. Adherence factors are the surface molecules or components of microbes such as teichoic acids of Gram-positive bacteria and pili of Gram-negative bacteria. In previous view, the cellular receptors binding to microbial adherence factors should be located in surface of host cells. However, many recent studies revealed that the molecules in extracellular matrix (ECM) of host cells, such as fibronectin (FN), laminin (LN) and different types of collagens (COLs), act as the major receptors of adherence factors from many bacteria including spirochetes [35–37]. *L. interrogans* was able to adhere to the surface of mouse macrophages and monkey renal fibroblasts [38]. We found that *L. interrogans* can adhere to the surface of cells with one or two terminals of leptospiral body (Fig. 1). However, the adherence mechanism of *L. interrogans* has not been revealed.

Leptospira has a Gram-negative cell wall with inner and outer membranes but never been found to produce pilus. Therefore, some outer membrane proteins (OMPs) of pathogenic *Leptospira* species have been considered as the potential adherence factors. Endostatin-like OMPs of *L. interrogans* were the first identified adherence factors binding to the FN and LN in ECM of host cells [39]. Subsequently, a series of *L. interrogans*

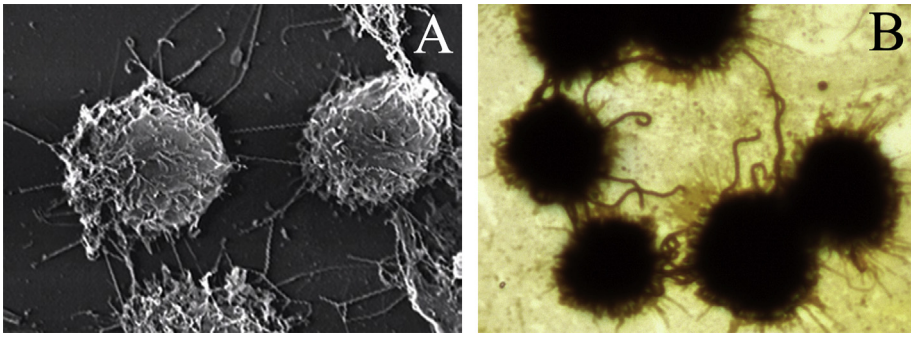

Fig. 1 Adherence of *L. interrogans* to mouse J774A.1 macrophages, observed by scanning electron microscopy (A) and ordinary microscopy after silver-staining (B).

OMPs and outer membrane lipoproteins (OMLPs), such as Lsa21/32/63 and LipL32/53, were reported to be involved in the leptospiral adherence to cellular ECM and binding to FN, LN and/or COL4 [40–44]. In particular, LigA and LigB of *L. interrogans*, the two members of bacterial immunoglobulin superfamily, were also confirmed to bind to ECM proteins in attachment of host cells [45]. Interestingly, the OMPs and OMLPs of *Leptospira santorosai* have been shown to increase the accumulation of ECM through TGF-β/Smad signaling pathway and the expression of FN through Toll-like receptor 2 (TLR2) pathway [46,47].

However, these ECM protein-binding proteins of pathogenic *Leptospira* species need a further determination about their function as adherence factors using more reliable and accurate methods such as surface plasmon resonance (BiaCore) and atomic force microscopy because nearly all of the studies only ELISA was used to detect the combination of the leptospiral OMPs and OMLPs with cellular ECM proteins. Moreover, the ECM proteins expressed by different host cells have a large diversity. For example, FN is the unique ECM protein expressed by human macrophages while human vascular endothelial cells express FN, LN and COL3/4 [48].

Invasive enzymes

Except of adherence factors, invasive enzymes also play important roles in invasiveness of bacteria during infection. Pathogenic *Leptospira* species s possess a powerful invasive ability, for example, rapid invading into human body and bloodstream, diffusion from bloodstream into internal organs and tissues, and discharge from urine.

Collagenase has been confirmed to play an important role in invasiveness of many pathogenic bacteria during infection. A recent study demonstrated that *L. interrogans* serovar Lai strain Lai produce a collagenase named as ColA that hydrolyzed COL1/3/4 with a high hydrolytic ability in *vitro* [49]. When the spirochete was co-incubated with human umbilical vein endothelial cells (HUVEC) and renal epithelial cells (HEK293), the expression and secretion of ColA collagenase were significantly increased. Compared to the wild-type strain, the *colA* gene-knockout mutant presented a remarkably attenuated transcytosis through the monolayers of HUVEC and HEK293 cells as well as a notably decreased leptospire-loading in tissues and discharge from urine in hamsters.

Metalloprotease/metallopeptidase (MP) from eukaryotes and prokaryotes is a large group of Zn^{2+}-dependent protein/peptide hydrolases that are classified into at least 60 types. The extracellular MP has been reported to contribute to the invasiveness of bacterial pathogens [50]. In the genome of *L. interrogans*, there are numerous MP-encoding genes [33]. We found that *L. interrogans* serovar Lai strain Lai expressed three M16-type MPs that could hydrolyze FN, LN and COL1/3/4. The deletion of the M16-MPs-encoding genes caused a significant decrease of the leptospiral loading in lungs, liver and kidneys as well as a significantly attenuated virulence in hamsters. Besides, a mammalian cell entry (Mce) protein from *L. interrogans* was reported to mediate the leptospiral internalization into mouse macrophages by its RGD motif binding to α5β1/$\alpha_V\beta3$ integrin pathway and the endoflagellum of *L. interrogans* was also demonstrated to contribute to in leptospiral invasiveness [51,52].

Toxins

Endotoxin and exotoxin are the two major types of toxins produced by bacterial pathogens. Pathogenic *Leptospira* species have no any typical exotoxin-encoding genes but possess a complete set of LPS (i.e. endotoxin) synthesis genes in their genomes [33,34]. Phagocytosis plays a crucial role in innate and adaptive anti-infection immunity to kill and eliminate invaded microbes in hosts [53]. Therefore, anti-phagocytosis has been considered as an important agent in virulence of microbial pathogens. However, an earlier study firstly found that *L. interrogans* was able to induce apoptosis of mouse macrophages [54]. *L. interrogans* was then found to cause apoptosis of human and mouse macrophages through cytomembrane Fas/FasL-triggered caspase-8/3-dependent and ROS/p53-triggered caspase-independent mitochondrial AIF/EndoG pathways [55–57]. However, until now, the toxins of pathogenic *Leptospira* species have not been completely characterized yet.

1. LPS LPS is also called endotoxin probably due to it is a structural component in cell wall of Gram-negative bacteria and has an extensive toxicity to mammalian cells. LPS is a biomacromolecule composed of lipid A, core polysaccharide and O-antigenic polysaccharide, in which lipid A decides the toxicity while O-specific polysaccharide determines antigenicity [58]. In many pathogenic Gram-negative bacteria, such as *E. coli*, *Shigella* and *Salmonella* spp, LPS is the major toxin to cause pathological changes [59]. In 1986, LPS from *L. interrogans* serovar Copenhageni was first identified [60]. The basic structure of lipid A in LPS from *L. interrogans* serovar Pomona was similar to that from *E. coli* but presented some significant differences [61]. The lipid A of *E. coli* contains two phosphate groups at C1 and C4 sites of diaminoglucose backbone linked by one lauric acid (C_{12}) and five myristic acids (C_{14}), while that of the spirochete displayed a lack of the phosphate group at C4 site and a methylation of the phosphate group at C1 site as well as its diaminoglucose backbone is linked by two lauric acids (C_{12}) and one lauric olefinic acid ($C_{12:1}$), one myristic olefinic acid ($C_{14:1}$) and two palmitic acids (C_{16}) [61,62]. The structure of leptospiral lipid A and its comparison with lipid A of *E. coli* were summarized in Fig. 2. Previous studies confirmed that the phosphate groups in lipid A, especially that at C1 site, are closely associated with endotoxic activities such as pyrogenicity, Shwartzman reaction and limulus amebocyte lysate solidification as well as the fatty acids in lipid A play an important role in toxicity in which myristic acid is more toxic than other fatty acids [63–65]. Although the clinical symptoms and pathological changes in leptosirosis patients are similar to endotoxicosis such as endotoxin-like inflammatory reaction, capillary endothelial injury, microthrombosis and decreased blood coagulation [12,20,22], previous studies still demonstrated that the general endotoxic activities of *L. interrogans* LPS were lower than those from enteric bacilli such as *E. coli* LPS [12,29–31].

Differing from *E. coli* LPS recognized by TLR4, LPS of *L. interrogans* activated mouse macrophages through TLR2-dependent mechanism [66]. In particular, a recent study

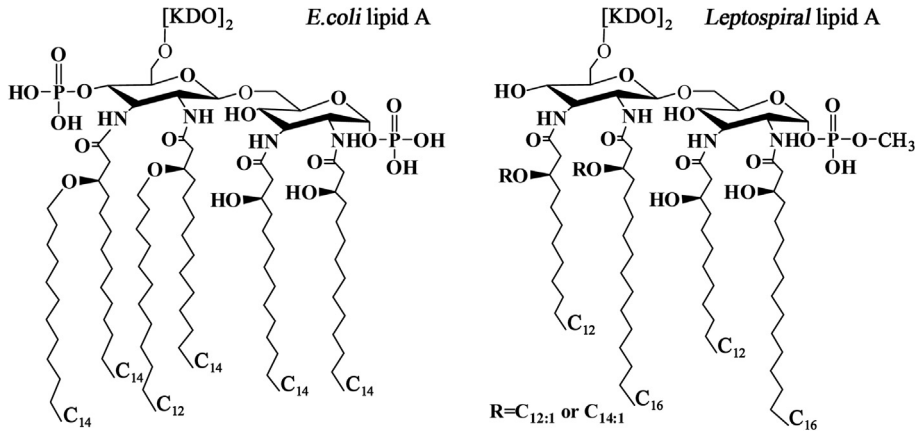

Fig. 2 Comparison between lipid A structures from *L. inetrrogans* and *E. coli* (summarized from Que-Gewirth NLS et al. J Biol Chem 2004; 279:25420–9 and Hinckley MB et al. J Biol Chem 2005; 280:30214–24).

found that LPS of *L. interrogans* induced the expression and cytomembrane translocation of Fas/FasL proteins in human and mouse macrophages through JNK/p38MAPK signaling pathways to promote the macrophage apoptosis [67]. LPS from *L. interrogans* presented a higher molecular weight and contained additional fucose, two different types of dideoxy N-acetyl-hexosamines and extended O-antigenic polysaccharide compared to that from *L. licerasiae*, an intermediate type of *Leptospira* [68].

2. Hemolysins Some of bacterial pathogens produce several hemolysins that can be classified as sphingomyelinases and non-sphingomyelinases [69,70]. However, in the genomes of several *L. interrogans* serovars, there are at least nine hemolysin-encoding genes, in which *sph*1-4 and *sphH* were annotated to encode sphingomyelinase-type hemolysins while *hlpA*, *hlyC*, *hlyX* and *tlyA* were predicted to encode non-sphingomyelinase hemolysins [33,34]. The first reported leptospiral hemolysin, SphH, was confirmed as a pore-forming toxin on human pulmonary epithelial and monkey renal epithelial cells [71]. Another leptospiral hemolysin, Sph2, was identified as a Mg^{2+}-dependent sphingomyelinase and was increased in the expression during infection of cells [72,73]. The Sph1-3, HlpA and TlyA of *L. interrogans* were secreted and displayed a hemolytic activity in *vitro*. Importantly, these five secreted leptospiral hemolysins induced the strong production of IL-1β, IL-6 and TNF-α of human and mouse macrophages through TLR2/4-JNK/NF-κB signaling pathways [32]. In particular, a recent study reported that Sph2 of *Leptospira intertrogans* damaged cytomembrane of human blood vessel endothelial, lung epithelial and liver cells, invaded into the cells through clathrin-mediated endocytosis and translocated onto mitochondria to induce the increase of intracellular reactive oxygen species (ROS) and decrease of the mitochondrial membrane potential (MMP). The high ROS and low MMP levels caused the apoptosis of the three types of cells [74]. Except for erythrocatalysis, these hemolysins of *L. interrogans* seem to induce inflammatory reaction, cytomembrane injury and cell apoptosis.

3. Hemorrhage inducers Leptospirosis was initially called Weil's disease with the clinical features such as jaundice, hemorrhage, conjunctival congestion and renal failure [8]. Therefore, hemorrhage is one of the important pathological changes of leptospirosis. For example, pulmonary diffuse hemorrhage (PDH), a severe type of leptospirosis, usually causes the death of over 50% of the patients [12]. LPS from bacteria including *Leptospira* can cause blood vascular engorgement and permeability increase but nearly has no ability to directly induce hemorrhage in tissues [60].

Blood coagulation system has an important physiological function against hemorrhage [75]. In the blood coagulation process, platelet aggregation plays a crucial role by providing a platform for interaction and activation of blood coagulation factors while von Willebrand factor (vWF) initiates the platelet aggregation through its region-A binding to GPIbα receptor of platelets [76]. Platelet activating factor (PAF) promotes platelet aggregation by induction of free Ca^{2+} increase and coagulation factor-III secretion but it is inactivated by PAF acetylhydrolase (PAF-AH) [77]. *L. interrogans vwa*-I and *vwa*-II genes contain region-A domains and their products (vWA) caused pulmonary hemorrhage in mice by competitive binding to platelet GPIbα receptor competed with vWF but no ability to activate platelet aggregation-dependent PI3K/AKT-ERK and PLC/PKC signaling pathways [78]. In addition, the product of *L. interrogans* LA_2144 gene was proved to have PAF-AH activity in *vitro* [79]. Our recent experiments showed that the LA_2144 gene product caused the decrease of serum PAF and coagulation factor-III, coagulation time extension of peripheral blood and pulmonary and renal hemorrhage in mice.

4. Jaundice inducers Jaundice is another clinical feature of leptospirosis but its mechanism remains unexplored. Excess cholerythrin mainly due to low function of hepatocytes is a common causative agent of jaundice. An earlier study found that the hepatocytes of *L. interrogans*-infected guinea pigs were apoptotic [80]. LPS has an extensive toxicity to different types of mammalian cells. Although no experimental evidences have been obtained, LPS of *Leptospira* causing hepatocyte injury is reasonable presumption. For example, a recent study showed that Sph2, a hemolysin secreted from *L. intertrogans*, induced the apoptosis of human liver cells in *vitro* [74].

5. Others A recent study reported that OMP047 of *L interrogans* displayed a high affinity to bind to human and mouse Fas proteins to induce apoptosis of human and mouse macrophages in *vitro* [67]. Toxin-antitoxin (TA) systems/modules as

stress-response elements have been found in many bacteria [81]. The toxins in TA modules cause bacterial cycle growth arrest and programmed death while the antitoxins neutralize the toxins. In the TA modules of *E. coli*, MazEF and ChpIK, the toxic proteins acted as endoribonucleases and interferases to inhibit bacterial protein synthesis and cause bacterial death [82,83]. The MazF and ChpK, the toxins in MazEF and ChpIK TA modules of *L. interrogans*, were over-expressed and externally secreted during infection of human macrophages that caused the decreased viability and necrosis of macrophages [84]. Among the two leptospiral toxins, MazF was confirmed as a RNA lysase (RNase).

Inflammatory reaction in leptospirosis

Inflammation is an essential component of innate and adaptive immune responses in hosts against microbial pathogens. Leptospirosis patients present a strong inflammatory reaction and excessive inflammatory reaction can cause extensive tissue injury and multiple organ failure [25,26]. Monocyte-derived macrophages and neutrophils are the two major infiltrating phagocytes to kill invaded microbes and produce inflammatory cytokines during infection. However, macrophages but not neutrophils acted as the main infiltrating and leptospiral phagocytes in leptospirosis patients and *Lepospira*-infected mice [85]. In addition, the macrophage chemokines, MCPs, MIP-δ/MIP-1αs and RANTES, but not the neutrophil chemokines, IL-8 and KC, in the sera of patients and *Lepospira*-infected mice were significantly increased by detection of cytokine microarray.

1. Inflammatory cytokines in leptospirosis Many pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8 and IL-12, can be detectable in the sera from leptospirosis patients but TNF-α and IL-6 have been considered to be closely associated with the severity and mortality of this disease [86–89]. However, IL-1β, IL-6 and TNF-α were reported as the main pro-inflammatory cytokines in the sera of both leptospirosis patients and *L. interrogans*-infected mice detected by cytokine microarray [32].

2. Pro-inflammatory cytokine inducers LPS is known as a strong inducer of pro-inflammatory cytokines. However, some proteins from pathogenic *Leptospira* species have been reported as pro-inflammatory cytokine inducers. The OMPs, especially LipL32, from *L. santarosai* were found to induce the expression of pro-inflammatory cytokines from different renal cells and caused mouse nephritis through TLR2/p38MAPK signaling pathway [90–93]. As shown above, the secreted hemolysins of *L. interrogans* had a powerful ability to induce the IL-1β, IL-6 and TNF-α of macrophages through TLR2/4-JNK/NF-κB signaling pathways [32]. In particular, our recent experiments showed that the PAF-AH of *L. interrogans* displayed a low phospholipase A2 (PLA2) activity to cause the significant increase of serum prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) in mice, the two common lipid inflammatory factors.

Leptospiral diffusion in leptospirosis

In the course of leptospirosis, pathogenic *Leptospira* species are able to migrate from bloodstream into lungs, liver, kidneys and cerebrospinal fluid to cause aggravation of disease [20–22]. As described above, the animal hosts of pathogenic *Leptospira* species can persistently discharge leptospires from urine to form natural infectious source [19]. However, leptospirosis patients and infected animal hosts usually present one time of septicemia at early stage during infection, implying that the spirochetes should propagate in kidneys. Therefore, migration of pathogenic *Leptospira* species through small blood vessels and renal tubules is important for aggravation of leptospirosis in patients and transmission of the spirochetes from animals to humans.

A recent study revealed that *L. interrogans* entered human and mouse blood vessel endothelial and renal tubule epithelial cells and fibroblasts through caveolae/integrin-β1-PI3K/FAK-microfilament endocytosis pathways to form leptospiral vesicles that avoided fusion with lysosomes [94]. The leptospiral vesicles recruited Rab5/Rab11 and Sec/Exo-SNARE proteins in endocytic recycling and vesicular transport systems for intracellular migration and then release from the cells through a SNARE-complex-mediated FAK-microfilament/microtubule exocytosis pathway (Fig. 3). In the process of leptospiral vesicle transport, *L. interrogans* was found to propagate in mouse fibroblasts alone. Endocytosis is divided into clathrin-, caveolae- or lipid raft-dependent types and macropinocytosis. The filipin, a caveolae-dependent endocytosis inhibitor, blocked the endocytosis of *L. interrogans* into all the cells [94]. However, chlorpromazine, a clathrin-dependent endocytosis inhibitor, blocked the internalization of *L. interrogans* Sph2 hemolysin into human blood vessel endothelial, lung epithelial and liver cells [74].

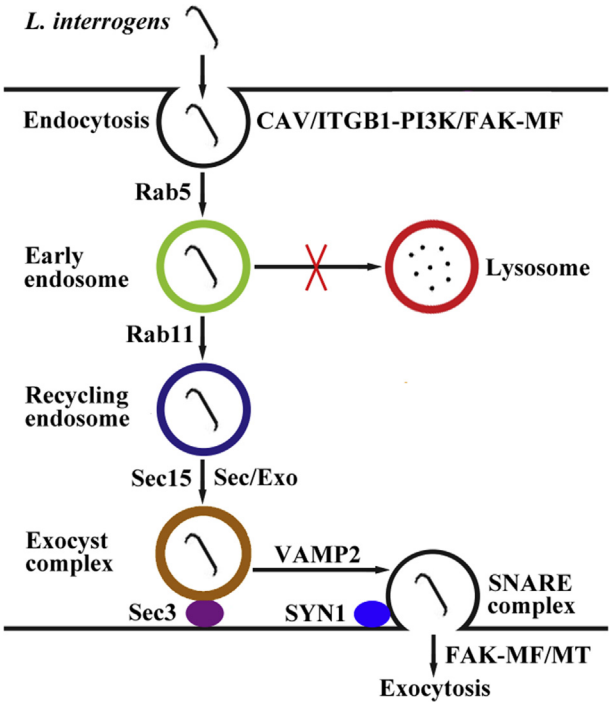

Fig. 3 Schematic diagram of *L. interrogans* transcytosis through blood vessel endothelial and renal tubule epithelial cells and fibroblasts (cited from Li Y et al. eLife 2019; 8:e44594-622).

Conclusion

The course of leptospirosis seem to be a process of continuous migration and transcytosis of pathogenic *Leptospira* species through mucosal and skin barriers to invade into hosts, blood vessel wall to enter or exit from bloodstream and renal tubule epithelium to discharge in urine. In the process, pathogenic *Leptospira* species produce many virulence factors to cause pathological changes in patients. Although leptospiral LPS possesses a lower endotoxic activity than typical bacterial LPS, so many secreted leptospiral hemolysins and their powerful pro-inflammatory cytokine-inducing ability play important roles in inflammatory clinical manifestation and tissue inflammatory injury in leptospirosis pateints. However, until now, the mechanisms about the migration of bacteria including *Leptospira* in *vivo* have been rarely reported. Endocytosis is the first step of leptospiral transcytosis and it is initiated by binding of leptospiral adherence factors to ECM molecule receptors. However, pathogenic *Leptospira* possesses many different adherence factors and ECM molecules expressed by different cells are obviously various. Furthermore, S100A10, a Ca^{2+}-binding protein in S100 family, and annexin A2 (AnxA2), a membrane phospholipid-binding protein, were reported to form S100A10-AnxA2 complex that induced endocytosis of *Salmonella typhimurium* by stimulation of cytoskeleton rearrangement [95]. Summarily, human leptospirosis can be considered as an invasive infectious and systemic inflammatory disease and the mechanisms of pathogenic *Leptospira* species migration in hosts need to be further studied.

Conflicts of Interest

The authors declare they have no conflicts of interest.

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