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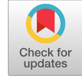
vWA proteins of *Leptospira interrogans* induce hemorrhage in leptospirosis by competitive inhibition of vWF/GPIb-mediated platelet aggregation



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ABSTRACT

Backgroud: *Leptospira interrogans* is the major causative agent of leptospirosis, a worldwide zoonotic disease. Hemorrhage is a typical pathological feature of leptospirosis. Binding of von Willebrand factor (vWF) to platelet glycoprotein-Ibα (GPIbα) is a crucial step in initiation of platelet aggregation. The products of *L. interrogans vwa*-I and *vwa*-II genes contain vWF-A domains, but their ability to induce hemorrhage has not been determined.
Methods: Human (Hu)-platelet- and Hu-GPIbα-binding abilities of the recombinant proteins expressed by *L. interrogans* strain Lai *vwa*-I and *vwa*-II genes (rLep-vWA-I and rLep-vWA-II) were detected by flowcytometry, surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). Hu-platelet aggregation and its signaling kinases and active components were detected by lumiaggregometry, Western analysis, spectrophotometry and confocal microscopy. Hu-GPIbα-binding sites in rLep-vWA-I and rLep-vWA-II were identified by SPR/ITC measurements.
Findings: Both rLep-vWA-I and rLep-vWA-II were able to bind to Hu-platelets and inhibit rHu-vWF/ristocetin-induced Hu-platelet aggregation, but Hu-GPIbα-IgG, rLep-vWA-I-IgG and rLep-vWA-II-IgG blocked this binding or inhibition. SPR and ITC revealed a tight interaction between Hu-GPIbα and rLep-vWA-I/rLep-vWA-II with K_D values of 3.87×10^{-7}-8.65×10^{-8} M. Hu-GPIbα-binding of rL-vWA-I/rL-vWA-II neither activated the PI3K/AKT-ERK and PLC/PKC kinases nor affected the NO, cGMP, ADP, Ca^{2+} and TXA_2 levels in Hu-platelets. G13/R36/G47 in Lep-vWA-I and G76/Q126 in Lep-vWA-II were confirmed as the Hu-GPIbα-binding sites. Injection of rLep-vWA-I or rLep-vWA-II in mice resulted in diffuse pulmonary and focal renal hemorrhage but this hemorrhage was blocked by rLep-vWA-I-IgG or rLep-vWA-II-IgG.
Interpretation: The products of *L. interrogans vwa*-I and *vwa*-II genes induce hemorrhage by competitive inhibition of vWF-mediated Hu-platelet aggregation.
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1. Introduction

Leptospirosis caused by *Leptospira* is a common global zoonotic infectious disease [1,2]. The disease is endemic in Asia, Oceania and South America, but in recent years it has been considered as an emerging infectious disease in Europe, North America and Africa, due to frequent case reports and several outbreaks [3–10].

Many animals such as rats and livestock serve as the natural hosts of pathogenic *Leptospira* genospecies and can persistently shed the spirochetes from their urine for a long period of time [11]. Human individuals

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Research in context

Evidence before this study

Leptospira interrogans is the most common causative agent of leptospirosis, a worldwide zoonotic infectious disease. Hemorrhage is a typical pathological feature and common lethal cause of leptospirosis, but its pathogenesis remains unknown. von Willebrand factor (vWF) triggers the platelet-mediated blood coagulation in humans and mammalian animals by binding platelets with its region A (vWF-A). The LB054 (*vwa*-I) and LB055 (*vwa*-II) genes of *L. interrogans* strain Lai contain vWF-A superfamily domains, but no reports have addressed their possible role in hemorrhage of leptospirosis.

Added value of this study

Our study demonstrated that the recombinant products of the *vwa*-I and *vwa*-II genes (rLep-vWA-I and rLep-vWA-II) are able to bind human or mouse platelets but block the vWF/ristocetin-mediated human platelet aggregation *in vitro* in which the platelet aggregation-associated PI3K/AKT-ERK and PLCγ_2/PKC signaling pathways are not activated. Moreover, rLep-vWA-I and rLep-vWA-II presented a high-affinity binding ability with glycoprotein-Ibα, the vWF receptor on platelets to initiate platelet aggregation and the G13/R36/G47 in Lep-vWA-I and G76/Q126 in Lep-vWA-II acted as the Hu-GPIbα-binding sites. rLep-vWA-I and rLep-vWA-II caused the diffuse pulmonary and focal renal hemorrhage in mice as well as the dysfunction of animal peripheral blood coagulation.

Implications of all the available evidence

The products of the *L. interrogans vwa*-I and *vwa*-II genes are the inducers of hemorrhage by competitive inhibition of vWF-mediated platelet aggregation in blood coagulation.

are infected by contact with the animal urine-contaminated water or wet soil. After invading into the human body through the skin and mucosa, pathogenic *Leptospira* genospecies can promptly enter the bloodstream to cause toxic septicemia and then spread into internal organs and tissues such as lungs, liver and kidneys to cause tissue injury [11,12].

Except for non-specific common symptoms such as high fever, headache and myalgia, severe leptospirosis patients are characterized by hemorrhage and jaundice and can die of septic shock, pulmonary diffuse hemorrhage (PDH) and acute renal failure [13,14]. In particular, PDH has a high mortality that usually causes rapid death of leptospirosis patients following frank hemoptysis and mouth-nose bleeding due to extensive intra-alveolar and interstitial hemorrhage [13–15]. Histopathological examination has confirmed that the hemorrhage in leptospirosis is diapedetic through small blood vessels, which occurs in nearly all tissues of the patients [15]. However, the molecular basis and mechanism of hemorrhage during leptospirosis remain unknown.

The blood coagulation system has an important physiological function to prevent hemorrhage [16]. In the blood coagulation process, platelet aggregation plays a crucial role by providing a platform for interaction and activation of blood coagulation factors and von Willebrand factor (vWF) initiates the platelet aggregation by binding to glycoprotein-Ib-α in the GPIb-IX-V complex in platelet membrane [17]. vWF contains twelve regions designated D'-D3-A1/2/3-D4-B1/2/3-C1/2-CK, of which the A1-A3 regions contain GPIbα-binding domains [18]. After ligation of vWF with GPIb-IX-V, the PI3K/AKT-ERK and PLC/PKC signaling pathways in platelets are activated by phosphorylation to cause an increase in NO, cGMP and free Ca^{2+} levels, which promote synthesis of thromboxane A_2 (TXA_2) and release of ADP from granules in platelets. High levels of TXA_2 and ADP induce talin and αIIbβ3 integrin polymerization to cause platelet aggregation [17–19]. Therefore, vWF is a key factor in human blood coagulation.

Among pathogenic *Leptospira* genospecies, *Leptospira interrogans* is the most predominant global genospecies [1,13]. *L. interrogans* has many serogroups and serovars but *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai is responsible for disease in over 60% of leptospirosis patients in China [3,14,20]. In the genomic DNA of *L. interrogans* serovar Lai strain Lai, we found that LB054 and LB055 genes contain several vWF type-A (vWF-A) superfamily domains and the two genes were named as *vwa*-I and *vwa*-II [21]. Previous studies reported that the vWF-A peptide segment from human vWF can bind to the GPIbα of human platelets, but it does not evoke platelet functional responses and result in blockage of vWF-induced platelet aggregation [22,23]. Therefore, we hypothesized that the products of *L. interrogans vwa*-I and *vwa*-II genes may induce hemorrhage in leptospirosis by competitive inhibition of vWF binding to GPIbα which blocks platelet aggregation.

In the present study, we therefore investigated the distribution of *vwa*-I and *vwa*-II genes in different pathogenic or saprophytic *Leptospira* strains. Subsequently, the platelet GPIbα-binding and aggregation-inhibiting ability of *L. interrogans* serovar Lai strain Lai recombinant *vwa*-I and *vwa*-II gene products containing vWF-A superfamily domains (rLep-vWA-I and rLep-vWA-II), as well as the expression and secretion of Lep-vWA-I and Lep-vWA-II of the spirochete during infection of human vascular endothelial cells were determined. Moreover, rLep-vWA-I- and rLep-vWA-II-induced hemorrhage in mice was also demonstrated. The results of this study identify the products of *L. interrogans vwa*-I and *vwa*-II as hemorrhage inducers in leptospirosis by competitive inhibition of vWF-mediated platelet aggregation.

2. Materials and methods

2.1. Ethics statement

All subjects (peripheral blood samples from three volunteers in our laboratory) gave written informed consent, and the study was approved by the Human Ethics Committee of Zhejiang University School of Medicine, and complied with the Declaration of Helsinki. Animal experiments were performed in accordance with the National Regulations for the Administration of Experimental Animals of China (1988–002) and the National Guidelines for Experimental Animal Welfare of China (2006–398). All the animal experimental protocols were approved by the Ethics Committee for Animal Experiment of Zhejiang University School of Medicine.

2.2. Leptospiral strains and culture

Thirteen strains of pathogenic *Leptospira* belonging to three genospecies for the serological diagnosis of human leptospirosis in China and two strains of non-pathogenic saprophytic *L. biflexa* were shown in Supplementary Data [3]. All the leptospiral strains were cultured in EMJH liquid medium at 28 °C [20].

2.3. Cell line and culture

Human umbilical vein endothelial cell (HUVEC) line was provided by the Cell Bank of Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, USA) at 37 °C in an atmosphere of 5% CO_2.

2.4. Animals

Female C3H/HeJ mice (15 ± 1 g, three-weeks old), female C57BL/6 mice (18 ± 2 g, four weeks old) for generating *Leptospira*-infected animal model [24], and New Zealand rabbits (3.0–3.5 kg) for preparing rLep-vWA-I-IgG and rLep-vWA-II-IgG were provided by the Laboratory Animal Center of Zhejiang University.

2.5. Detection of vwa-I and vwa-II genes in leptospiral strains

Genomic DNAs from the fifteen leptospiral strains were extracted using a Bacterial Genomic DNA Extraction Kit (Axygen, USA). The entire LB054 (*vwa*—I) or LB055 (*vwa*-II) gene was amplified from the DNA templates by PCR with the primers vwa-I-1F/vwa-I-1R or vwa-II-1F/vwa-II-1R (Table 1) using a High-Fidelity PCR Kit (TaKaRa, China). All the PCR products were examined by 1.5% ethidium bromide pre-stained agarose gel electrophoresis and then cloned into pMD19-T plasmid using a PCR Product T-A Cloning Kit (TaKaRa) for sequencing by Invitrogen Co. at Shanghai in China. The sequence identities were analyzed and compared with those in GenBank using BLAST software.

2.6. Bioinformatic analysis of vwa-I and vwa-II genes

The structures and functional domains in the *vwa*-I and *vwa*-II genes of *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai were analyzed using TMHMM, PsortB, Octopus, SignalP-4.1 and NCBI-Batch CD-Search softwares.

2.7. Expression of vwa-I and vwa-II gene segments

Expression of the *vwa*-I and *vwa*-II gene segments containing the entire vWF-A superfamily domains (*vwa*-I528 and *vwa*-II603) from *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai in *Escherichia coli* and extraction of the expressed recombinant proteins (rLep-vWA-I and rLep-vWA-II) were shown in Supplementary Information.

2.8. Removal of lipopolysaccharide in rLep-vWA-I and rLep-vWA-II

Possible contaminated *E. coli* LPS in the rLep-vWA-I and rLep-vWA-II extracts were removed with a Detoxi-gel endotoxin removing column chromatography (Thermo Scientific, USA) using pyrogen-free water for elution and then detected using a Limulus Amebocyte Lysate Test Kit (Lonza, Switzerland) as previously described [24,25].

Table 1
Information of primers used in this study.

Primer	Sequence (5′ to 3′)	Purpose	Size (bp)
vwa-I-1	F: ATGAATTTTCAATATCCTTAC R: TCATACATAATATCTCAGAAA	Detection of emtire *vwa*-I gene	939
vwa-II-1	F: TTGATTTCTAAAATAAGGGAA R: TCATATTACAGAAACGCATTTC	Detection of entire *vwa*-II gene	957
vwa-I-2	F: GCGCATATG(*Nde* I)GAAGGAGTA GATATATTA R: GCGCTCGAG(*Xho* I)CATTTCCTCCG GATCTTC	Expression of *vwa*-I528 segment	528
vwa-II-2	F: GCGCATATG(*Nde* I)GATATACTCTT TTTAGTG R: GCGCTCGAG(*Xho* I)TTGTAGAAT TGTAGAATC	Expression of *vwa*-II603 segment	603
vwa-I-3	F: TTGCAGGTGCGGCTTATT R: CGATTGCGGTCCCTTGTT	Detection of *vwa*-I-mRNA	111
vwa-II-3	F: TTCGTGGATTGGATGTGG R: GGAAATCGCTTGTGGATC	Detection of *vwa*-II-mRNA	161
16S-RNA	F: CTTTCGTGCCTCAGCGTCAGT R: CGCAGCCTGCACTTGAAACTA	Inner reference used in qRT-PCRs	145

F: forward primer. R: reverse primer. Underlined areas indicate the sites of endonucleases.

2.9. Preparation of rLep-vWA-I-IgG and rLep-vWA-II-IgG

The preparation of rabbit anti-rLep-vWA-I-IgG or rLep-vWA-II-IgG was shown in Supplementary Data.

2.10. Preparation of human and mouse platelets

Peripheral blood samples from healthy volunteers and C3H/HeJ or C57BL/6 mice were mixed with 0.1 volume of ACD buffer (75 mM sodium citrate, 39 mM citric acid, and 135 mM dextrose, pH 6.5) to prevent pellet self-aggregation, and then diluted with 2 volumes of MTH buffer (20 mM HEPES, 137 mM NaCl, 13.8 mM $NaHCO_3$, 2.5 mM KCl, 0.36 mM NaH_2PO4, 5.5 mM glucose, pH 7.4). The diluted blood samples were centrifuged at 180 ×*g* for 10 min at room temperature. The human (Hu) or mouse (Ms) platelet rich plasmas were suspended in ACD buffer and then centrifuged at 750 ×*g* for 15 min at room temperature. The Hu-platelets and Ms-platelets were suspended in MTH buffer for immediate use with no self-aggregation using a lumiaggregometer (type Model-700, Chrono-Log, USA) [26].

2.11. Determination of Hu/Ms-platelet-binding ability of rLep-vWA-I and rLep-vWA-II

Hu/Ms-platelet-binding ability of rLep-vWA-I or rLep-vWA-II was determined by flow cytometry [27]. Briefly, the Hu/Ms-platelets (10^7) were incubated with 2 μg rLep-vWA-I or rLep-vWA-II at 22 °C for 0.5, 1, 2 or 4 h, and then thoroughly washed with 0.01 M phosphate buffered saline (PBS, pH 7.4). Using 1:200 diluted rabbit anti-rLep-vWA-I- or rLep-vWA-II-IgG as the primary antibody and FITC-labeled goat anti-rabbit-IgG (Abcam, USA) as the secondary antibody, the Hu/Ms-platelet-binding percentages of rLep-vWA-I and rLep-vWA-II were detected using a flow cytometer (type FC-500MCL, Beckman Coulter, USA) with 485/538 nm excitation/emission wavelengths. In the detection, 10 μg rHu/rMs-vWF (Abcam) plus 150 μM ristocetin (Sigma), a common cofactor of vWF for binding GPIbα to induce platelet aggregation *in vitro* [26,28], and rHlpA, a recombinant hemolysin of *L. interrrogans* [24], were used as the controls.

2.12. Antibody blockage tests

Hu-platelets were incubated with rabbit rHu-GPIbα-IgG (Abcam) at 22 °C for 3 h while rLep-vWA-I or rLep-vWA-II was incubated with rLep-vWA-I-IgG or rLep-vWA-II-IgG at 37 °C for 1 h. The percentages of rLep-vWA-I or rLep-vWA-II binding to the GPIbα-blocked Hu-platelets and the IgG-combined rLep-vWA-I or rLep-vWA-II binding to Hu-platelets were detected by flow cytometry as above.

2.13. Determination of Hu-GPIbα-binding ability of rLep-vWA-I and rLep-vWA-II

Hu-GPIbα-binding ability of rLep-vWA-I or rLep-vWA-II was determined by SPR and ITC [29,30]. For SPR detection, 1 nM rHu-GPIbα (R&D, USA) was cross-linked on the activated CM5 sensing array (GE, USA) and then 0.05–0.8 nM rLep-vWA-I or rLep-vWA-II in PBS flowed through the surface of rHu-GPIbα-binding array. The combination of rLep-vWA-I or rLep-vWA-II with rHu-GPIbα was detected using a SPR-based detector (Type-T200, GE) and quantified by the values of equilibrium association constant (K_D). For ITC detection, 1 μM rLep-vWA-I- or rLep-vWA-II in PBS was added in the titration pool while 0.1 μM rHu-GPIbα in PBS was added in the sample pool. The K_D values reflecting the combination of rLep-vWA-I or rLep-vWA-II with the rHu-GPIbα in titrating process were detected using a type VP-ITC microcalorimeter (MicroCal, USA) and then analyzed using Origin software. In the detection, rHlpA, a recombinant hemolysin of *L. interrrogans* [24], was used as the negative array-linking fixed and mobile phase controls in SPR and the negative titration control in ITC.

2.14. Co-precipitation assay

The products of *vwa*-I and *vwa*-II genes in total leptospiral proteins were pulled down with rHu-GPIbα (R&D) by co-precipitation assay. Briefly, freshly-cultured *L. interrogans* strain Lai was precipitated by a 10,000 ×*g* centrifugation at 4 °C for 30 min. After washing twice with PBS and centrifugation again, the leptospiral pellet was suspended in distilled water and then ultrasonically broken on ice. The lysate was centrifuged at 10,000 ×*g* for 10 min (4 °C) to remove leptospiral debris and the supernatant containing total leptospiral proteins was collected to detect protein concentration using a BCA Protein Assay Kit (Thermo Scientific). 20 μg mouse anti-rHu-GPIbα-IgG (Abcam) in 500 μL PBS was mixed with 100 μL of 6 mg/mL protein-A-coated agarose beads (Millipore, USA) for incubation in a 90 rpm rotator (4 °C) overnight to form protein-A-GPIbα-IgG beads. After a 10-min centrifugation at 14,000 ×*g* (4 °C) and washing with PBS, the beads were suspended in 500 μL PBS and then incubated with 20 μg rHu-GPIbα (R&D) as above to form protein-A-GPIbα-IgG-GPIbα beads. After centrifugation and washing with PBS as above, the beads were suspended in 500 μL PBS and then incubated with 200 μg total leptospiral proteins in a 90 rpm rotator (4 °C) for 2 h. After centrifugation and washing thoroughly with PBS, the beads were suspended in Laemmili SDS-PAGE sample buffer for a 5-min water-bath at 100 °C to release protein-A-GPIbα-IgG-binding proteins. After a 10-min centrifugation at 14,000 ×*g* (4 °C), the supernatant was subjected to SDS-PAGE to examine the released proteins.

2.15. Identification of rHu-GPIbα-binding leptospiral proteins

The released proteins from protein-A-GPIbα-IgG beads in co-precipitation assay were identified by liquid chromatography plus a type LC1000-LTQ tandem mass spectrometry (LC-MS/MS, Thermo Scientific). The obtained data were automatically searched against the genomic database of *L. interrogans* strain Lai in GenBank (accession No.: NC_004342.2) using Proteome Discoverer 1.4 software.

2.16. Platelet aggregation and inhibition tests

In the platelet aggregation test, 10 μg rHu-vWF plus 150 μM ristocetin (rHu-vWF/ristocetin) were used as the inducers of Hu-platelet aggregation *in vitro* [18,26]. Hu-platelets (10^8) were incubated with 5 μg rLep-vWA-I or rLep-vWA-II in MTB at 37 °C for 1 h and then the rHu-vWF/ristocetin-mediated rLep-vWA-I- or rLep-vWA-II-treated Hu-platelet aggregation was detected using a lumiaggregometer (type Model-700, Chrono-Log, USA). On the other hand, rLep-vWA-I or rLep-vWA-II was pretreated with rLep-vWA-I-IgG or rLep-vWA-II-IgG at 37 °C for 1 h and then the rLep-vWA-I-IgG or rLep-vWA-II-IgG to reverse the role of rLep-vWA-I or rLep-vWA-II on inhibition of rHu-vWF/ristocetin-mediated Hu-platelet aggregation was detected as above.

2.17. Detection of signal kinase phosphorylation in platelet aggregation

Hu-platelets (10^8) were incubated with 5 μg rLep-vWA-I or rLep-vWA-II in MTB at 37 °C for 1 h. The Hu-platelets were precipitated by a 750 ×*g* centrifugation for 15 min at room temperature. After washing thoroughly with PBS and centrifugation, the Hu-platelet pellets were lysed with RIPA lysis buffer (Beyotime BioTech, China). The lysates were centrifuged at 3000 ×*g* for 10 min to remove Hu-platelet debris. The supernatants were collected to detect protein concentration using a BCA Protein Assay Kit (Thermo Scientific) and then were submitted to SDS-PAGE and electro-transferring onto PVDF membrane (Millipore). The phosphorylation levels of PI3K/AKT, ERK, PLC and PKC were detected by Western Blot assay using AKT, ERK1/2, PLC and PKC Phosphorylation Detection Kits (Cell Signaling, USA). In the assays, 10 μg rHu-vWF plus 150 μM ristocetin (rHu-vWF/ristocetin) were used as the control [26,28].

2.18. Measurement of nitric oxide, cGMP, TXA_2 and ADP in platelets

Hu-platelets (10^8) were incubated with 5 μg rLep-vWA-I or rLep-vWA-II in MTB at 37 °C for 1 h. The Hu-platelets were lysed and then centrifuged as above. The OD_{540} or OD_{450} values reflecting the nitric oxide (NO) or cGMP levels in the supernatants were measured using a Griess's Diazotization NO Assay Kit (Promega, USA) or a cGMP Assay Kit (R&D) by spectrophotometry. The supernatants were diluted at 1:50 with the assay buffer and then the OD_{450} values reflecting TXB_2 levels were measured using a TXB_2 Assay Kit (R&D) by spectrophotometry. Besides, the Hu-platelets were precipitated as above for homogenization in the assay buffer and then centrifuged at 10,000 ×*g* for 10 min. The supernatants were diluted with 50-fold volumes of the assay buffer for detecting the released ADP at the OD_{570} using an ADP Assay Kit (Abcam) by spectrophotometry. In the detection, 10 μg rHu-vWF plus 150 μM ristocetin (rHu-vWF/ristocetin) were used as the control [26,28].

2.19. Measurement of free Ca^{2+} in platelets

Hu-platelets (10^8) were incubated with 5 μg rLep-vWA-I or rLep-vWA-II in MTB at 37 °C for 1 h. The Hu-platelets were precipitated and washed as above. The Hu-platelet pellets were suspended in the assay buffer and then incubated with free Ca^{2+} Fluo-4 AM fluorescence probe at 37 °C for 30 min, followed by an additional incubation at room temperature for 30 min. The fluorescence intensity (FI) values reflecting free Ca^{2+} levels in the Hu-platelets using a Fluo-4 AM Calcium Assay Kit (Molecular Probes, USA) by laser confocal microscopy (type LSM510, Zeiss, Germany) with 494/516 nm excitation/emission wavelength. In the detection, 10 μg rHu-vWF plus 150 μM ristocetin (rHu-vWF/ristocetin) were used as the control [26,28].

2.20. Functional determination of products of vwa-I528 and vwa-II603 mutants

Previous studies confirmed that the point-mutation of some certain amino acid residues at the GPIb-binding sites in vWF-A domains of vWF caused the decrease of vWF-platelet combination and vWF-induced platelet aggregation [31,32]. The generation of point-mutated *vwa*-I and *vwa*-II gene segments containing entire vWF-A superfamily domains (*vwa*-I528 and *vwa*-II603), the expression of *vwa*-I528 and *vwa*-II603 segments in *E. coli* and the extraction of target recombinant products were shown in Supplemental Information. The Hu-GPIbα-binding and Hu-platelet aggregation-inhibiting abilities of point-mutated rLep-vWA-I and rLep-vWA-II proteins were determined by flow cytometry, SPR and ITC detection, and Hu-platelet aggregation inhibition test as described above.

2.21. Measurement of vwa-I and vwa-II mRNAs during infection of HUVEC

HUVEC (10^6 per well) was seeded in 6-well culture plates (Corning, USA) for a pre-incubation in an atmosphere of 5% CO_2 at 37 °C overnight. Freshly-cultured *L. interrogans* strain Lai was centrifuged at 13,800 ×*g* for 15 min at 15 °C and then washed twice with PBS. The harvested leptospires were counted under a dark-field microscope with a Petroff-Hausser counting chamber (Fisher Scientific) [33]. The cells were infected with the spirochete at a multiplicity of infection (MOI) of 100 (100 leptospires per host cell) for 1, 2, 4, 8 or 12 h [33,34], and then lysed with 0.05% NaTDC-PBS. The lysates were centrifuged at 350 ×*g* for 5 min (4 °C) to remove cell debris, and the supernatants were centrifuged at a 10,000 ×*g* for 30 min (4 °C) to precipitate leptospires. Using rabbit anti-*L. interrogans* strain Lai-IgG as the primary antibody and Alexa-Fluor488-conjugated goat anti-rabbit-IgG (Abcam), the integrity of leptospires were observed under a laser confocal microscope (LSM510-Meta, Zeiss, Germany) (495/519 nm excitation/emission wavelengths for Alexa-Fluor488 detection) [25], in

which the leptospires from culture in EMJH medium was used as the control. Total leptospiral RNAs were extracted using a TRIzol® Max™ Bacterial RNA Isolation kit (Invitrogen, USA) plus a gDNA Eraser Kit (TaKaRa) and then quantified by ultraviolet spectrophotometry. cDNAs from the RNAs were synthesized using a RTase cDNA Synthesis Kit (TaKaRa). Using the cDNAs as templates, the *vwa*-I-mRNA and *vwa*-II-mRNA levels were assessed by real-time fluorescence quantitative RT-PCR (qRT-PCR) using a SYBR® Premix Ex-Taq™ Kit (TaKaRa) in an ABI 7500 Real-Time PCR System (ABI, USA). The primers used are listed in Table 1. In the qRT-PCR, 16S rRNA gene of the spirochete was used as the internal reference while the spirochetes from EMJH medium (28 °C) and incubated in 2.5% FCS RPMI-1640 medium (37 °C) were used as the controls. The qRT-PCR data were analyzed using the $\Delta\Delta$Ct model and randomization test in REST 2005 software [20,34].

2.22. Detection of Lep-vWA-I and Lep-vWA-II expression and secretion of L. interrogans during infection of HUVEC

HUVEC was infected with *L. interrogans* strain Lai as described above. The co-cultures were lysed and then centrifuged as above to separate supernatants and leptospires. Subsequently, the integrity of leptospires from the co-cultures after lysis were detected by confocal microscopy as described above. The total proteins in the supernatants were extracted by trichloroacetic acid-acetone precipitation method [24], while the total leptospiral proteins were extracted by ultrasonical breakage and centrifugation as above, followed by detection of protein concentration as above. Using rabbit rLep-vWA-I-IgG or rLep-vWA-II-IgG as the primary antibody and HRP-conjugated goat anti-rabbit-IgG (Abcam) as the secondary antibody, Western Blot assay was used to detect Lep-vWA-I and Lep-vWA-II in the two protein specimens. The immunoblotting signals were quantified for analysis by densitometry (gray scale determination) using an image analyzer (Bio-Rad, USA) [33]. In the assays, LipL41 or Sph2 and FliY, a stable transmembrane lipoprotein or a secreted hemolysin and cytosolic protein of *L. interrogans*, were used as the expression or secretion controls [20].

2.23. Secretion inhibition test

L. interrogans strain Lai was pre-treated with 0.1 mM PAβN, a T1SS inhibitor, 2.5 mM NaN3 and 30 mM NaSCN, the T2SS inhibitors, or 10 mM Aurodox (Sigma), a T3SS inhibitor, for 6 h at 37 °C [35]. The secretion of Lep-vWA-I and Lep-vWA-II of the spirochete during infection of HUVEC cells was detected by Western Blot assay as above.

2.24. Detection of rLep-vWA-I- or rLep-vWA-II-induced hemorrhage in mice

Each of C3H/HeJ or C57BL/6 mice was intravenously injected with 100 μg rLep-vWA-I or rLep-vWA-II in 0.1 mL autoclaved PBS and eight animals were used per group. The animal lung, liver and kidney tissues as well as the peripheral blood plasma samples were collected on days 3 and 7 after injection for histopathological examination after HE-staining as well as for detection of prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT), thrombin generation time (TGT), fibrinogen (F—I) and fibrin degradation product (D-dimer) concentrations using an Auto-Blood Coagulation Analyzer (Sysmex, Japan) [16,36]. In addition, the coagulation time (CT) using Lee-White method, prothrombin (F-II) and prothrombin fragments 1 + 2 (F1 + 2) concentrations using ELISA, and thrombin generation time (TGT) using a Hemostasis System Analyzer (Haemoscope, USA) of the blood plasma samples were also detected as previously described [16]. On the other hand, the same concentration of rLep-vWA-I or rLep-vWA-II were pre-incubated with rLep-vWA-I-IgG or rLep-vWA-II-IgG at 37 for 1 h and then intravenously injected into the mice for histopathological examination as above. In the detection, the mice injected with the same volume of autoclaved PBS were used as the control.

2.25. 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 $p < .05$.

3. Results

3.1. Extensive distribution of vwa-I and vwa-II genes in pathogenic Leptospira genospecies

The PCR and sequencing data revealed that all the 13 tested strains of pathogenic of *L. interrogans*, *L. borgpetersenii* or *L. weilii* but not the 2 tested strains of saprophytic *L. biflexa*, possess both the LB054 (*vwa*—I) and LB055 (*vwa*-II) genes (Fig. 1A) with the nucleotide or amino acid sequence identities of 98.9%–99.8% or 98.9%–100% and 98.7%–99.9% or 98.0%–100% (GenBank accession No.: MG744315-MG744327 and MG744328-MG744340) compared to the reported same genes (GenBank accession No.: NC_004342.2). Moreover, the *vwa*-I or *vwa*-II genes of *L. interrogans* strain Lai had higher amino acid sequence identities (85.6%–100% or 74.4%–100%) compared to those from all the 13 strains belonging to 9 serogropus and 10 serovars of 5 pathogenic *Leptospira* genospecies in GenBank (Table 2). Bioinformatic analysis indicated that the *vwa*-I and *vwa*-II genes contain vWF-A superfamily domains (Fig. 1B). Although PsortB software predicted that the product of *vwa*-I gene was located in cytoplasmic membrane and the position of *vwa*-II gene product was unknown, TMHMM software predicted the two products as exoproteins without signal peptide sequences and transmembrane regions while Octopus software presented the similar possibility of the two products located the inside or outside of leptospiral cells (Supplementary Fig. S1). The data suggested that *vwa*-I and *vwa*-II genes are required by pathogenic but not non-pathogenic saprophytic *Leptospira* strains.

3.2. Characterization of recombinant expression products of vwa-I and vwa-II genes

The generated prokaryotic expression systems could express the target recombinant proteins (rLep-vWA-I and rLep-vWA-II) encoded by the wild-type or point-mutated *vwa*-I and *vwa*-II genes of *L. interrogans* strain Lai (Supplementary Fig. S2A and S4B). The spectrophotometric limulus amebocyte lysate test showed that no lipopolysaccharide (LPS) was detectable in all the rLep-vWA-I and rLep-vWA-II extracts after endotoxin-removing treatment (Supplementary Fig. S2C and S4D).

3.3. Hu-Platelet- and GPIbα-binding ability of rLep-vWA-I and rLep-vWA-II

The flow cytometric examination confirmed that both the rLep-vWA-I and rLep-vWA-II rapidly combined with Hu-platelets with the 94.7% and 92.4% maximal human (Hu)-platelet-binding percentages (Fig. 2A and Table 3). When the Hu-platelets were blocked with rHu-GPIbα-IgG, as well as the rLep-vWA-I or rLep-vWA-II was blocked with rLep-vWA-I-IgG or rLep-vWA-II-IgG, the Hu-platelet-binding ability of rLep-vWA-I or rLep-vWA-II was absent (Fig. 2A and Table 3). The surface plasmon resonance (SPR) and isothermal titration calorimetric (ITC) detection, the two sensitive and reliable methods to determine protein-protein binding [29,30], revealed that the equilibrium association constant (K_D) values reflecting the binding ability of rLep-vWA-I or rLep-vWA-II with rHu-GPIbα were 5.56×10^{-8} and 3.87×10^{-7} M or 8.65×10^{-8} and 6.42×10^{-7} M (Fig. 2B and C). In particular, the co-precipitation test showed that the rHu-GPIbα captured five protein bands from total proteins of *L. interrogans* strain Lai (Fig. 2D), and the LC-MS/MS identified two of the captured proteins (~36 kDa) as the

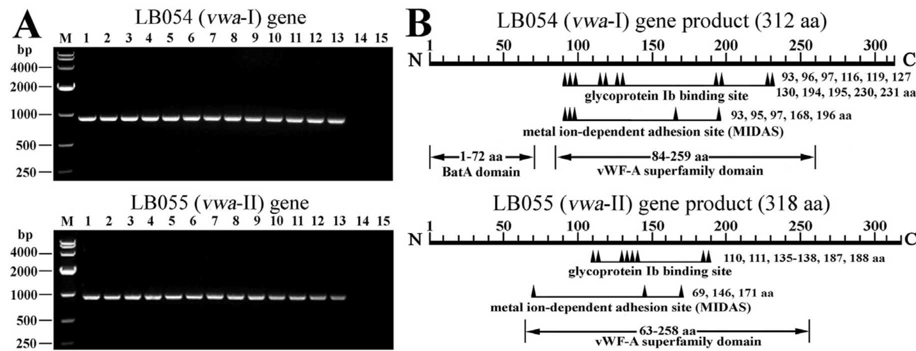

Fig. 1. Distribution and domains of LB054 and LB055 genes in leptospiral strains. (A). The PCR products of leptospiral LB054 (*vwa*-I) and LB055 (*vwa*-II) genes, determined by PCR. Lane M: DNA marker. Lanes 1–13: amplicoms of the *vwa*-I or *vwa*-II genes from thirteen pathogenic strains belonging to *L. interrogans*, *L. borgpetersenii* or *L. weilii*. Lanes 14–15: no amplicoms of *vwa*-I or *vwa*-II genes from two saprophytic strains of *L. biflexa*. (B). Predicted functional domains in LB054 (*vwa*—I) and LB055 (*vwa*-II) genes of *L. interrogans* strain Lai.

products of *vwa*-I and *vwa*-II genes according to their cleaved peptide sequences (LGLVVFAGAAYLQAPLTGDR, SKVIVLITDGVSNTGK IDPVTATDLAEQIGAK and EDGSYEINFEILQELSANTGGR for vWA-I, and GLDVDM VGDRGTDLSQAFTK, GTDLSQAFTKAEALLR and DGTLTSNSNSPGIIHSK for vWA-II) (Supplementary Fig. S3A and 3B). The other three captured proteins were identified as the products of LA2066 (~32 kDa, hypothetical protein), LA4255 (~21 kDa, FolE) and LA2836 (~19 kDa, hypothetical protein) (Supplementary Fig. S3C). The data suggested that the products of *L. interrogans vwa*-I and *vwa*-II genes have a specific Hu-platelet- or Hu-GPIbα-binding ability.

3.4. Inhibition of rLep-vWA-I and rLep-vWA-II on vWF-mediated platelet aggregation

Lumiaggregometer is commonly used to detect platelet aggregation *in vitro* [26]. Ristocetin, a co-coagulation factor, is necessary for vWF to induce platelet aggregation *in vitro* [37]. The platelet aggregation test showed that rLep-vWA-I and rLep-vWA-II did not cause Hu-platelet aggregation, but could inhibit the rHu-vWF/ristocetin-induced Hu-platelet aggregation (Fig. 3A). However, the inhibition of rLep-vWA-I or rLep-vWA-II could be removed by rLep-vWA-I-IgG or rLep-vWA-II-IgG pre-blockage (Fig. 3B). Moreover, no phosphorylation of the AKT, ERK, PLC and PKC as well as no increase of the NO, cGMP, TXA_2, free Ca^{2+} and ADP levels in the rLep-vWA-I- or rLep-vWA-II-binding Hu-platelets could be found (Fig. 3C and D). The data suggested that the products of *L. interrogans vwa*-I and *vwa*-II genes can not induce human platelet aggregation but block the vWF-mediated platelet aggregation.

3.5. GPIbα-binding sites in Lep-vWA-I and Lep-vWA-II

Previous studies reported that the point-mutation of some certain amino acid residuals in the region-A of human vWF caused the decrease of vWF-platelet binding and attenuation of vWF-mediated platelet aggregation [31,32]. The flow cytometric examination revealed that the recombinant single point-mutated products of *L. interrogans vwa*-I and *vwa*-II genes, rLep-vWA-I-G13S, rLep-vWA-I-R36Q, rLep-vWA-I-G47S, rLep-vWA-II-G76S and rLep-vWA-II-Q126R but not rLep-vWA-I-

Table 2
Identity of the amino acid sequences of leptospiral *vwa*-I/II-like genes.

Genospecies	Serogroup	Serovar	Strain	Genes	GenBank No.	Sequence Identity (%)
L. interrogans[*]	Icterohaemorrhagiae	Lai	Lai	*vwa*-I/*vwa*-II	MG744315/744328	100/100[***]
	Canicola	Canicola	Lin	*vwa*-I/*vwa*-II	MG744316/744329	99.7/99.0
	Pyrogenes	Pyrogenes	Tian	*vwa*-I/*vwa*-II	MG744317/744330	100/99.0
	Autumnalis	Autumnalis	Lin4	*vwa*-I/*vwa*-II	MG744318/744331	99.7/99.7
	Australis	Australis	65–9	*vwa*-I/*vwa*-II	MG744319/744332	100/99.7
	Pomona	Pomona	Luo	*vwa*-I/*vwa*-II	MG744320/744333	99.7/98.0
	Grippotyphosa	Grippotyphosa	Lin6	*vwa*-I/*vwa*-II	MG744321/744334	99.0/98.7
	Hebdomadis	Hebdomadis	56,069	*vwa*-I/*vwa*-II	MG744322/744335	99.7/99.0
L. interrogans[**]	Icterohaemorrhagiae	Lai	IPAV	RS18585/18590	NC_017552.1	100/100
	Icterohaemorrhagiae	Copenhageni	Fiocruz L1–130	RS18185/18190	NC_005824.1	100/99.4
	Icterohaemorrhagiae	Copenhageni	Piscina	RS19075/19080	NZ_CP018147.1	99.0/99.4
	Canicola	Canicola	114	19,360/19365	CP022884.1	99.7/98.4
	Pyrogenes	Manilae	UP-MMC-NIID	RS17985/17990	NZ_CP011932.1	99.7/99.7
	Australis	Bratislava	PigK151	RS18650/18655	NZ_CP011411.1	100/99.4
	Grippotyphosa	Linhai	56,609	RS18625/18630	NZ_CP006724.1	99.7/98.4
	Sejroe	Hardjo-prajitno	Hardjoprajitno	RS18285/18290	NZ_CP013148.1	100/99.7
L. borgpetersenii[*]	Javanica	Javanica	M10	*vwa*-I/*vwa*-II	MG744323/744336	99.3/100
	Ballum	Ballum	Pishu	*vwa*-I/*vwa*-II	MG744324/744337	99.7/100
	Tarassovi	Tarassovi	55–52	*vwa*-I/*vwa*-II	MG744325/744338	99.7/99.7
	Mini	Mini	Nan10	*vwa*-I/*vwa*-II	MG744326/744339	99.7/100
L. borgpetersenii[**]	Ballum	Ballum	56,604	RS16395/16400	NZ_CP012030.1	86.2/76.7
	Sejroe	Hardjo	BK-6	RS16740/16745	NZ_CP015045.1	85.6/74.4
L. weilii[*]	Manhao	Manhao 2	L105	*vwa*-I/*vwa*-II	MG744327/744340	100/99.3
L. santarosai[**]	/	Shermani	LT 821	RS17415/17420	NZ_CP006695.1	87.8/73.5
L. alstonii[**]	/	/	GWTS #1	RS19530/19535	NZ_CP015218.1	83.0/66.9
L. mayottensis[**]	/	/	MDI272	19,315/19320	CP030148.1	87.5/75.8

* The sequences from the present study by PCR and sequencing.
** The sequences from GenBank.
*** The amino acid sequence identity from LB_054 (*vwa*—I) and LB_055 (*vwa*-II) genes in GenBank (NC_004343.2).

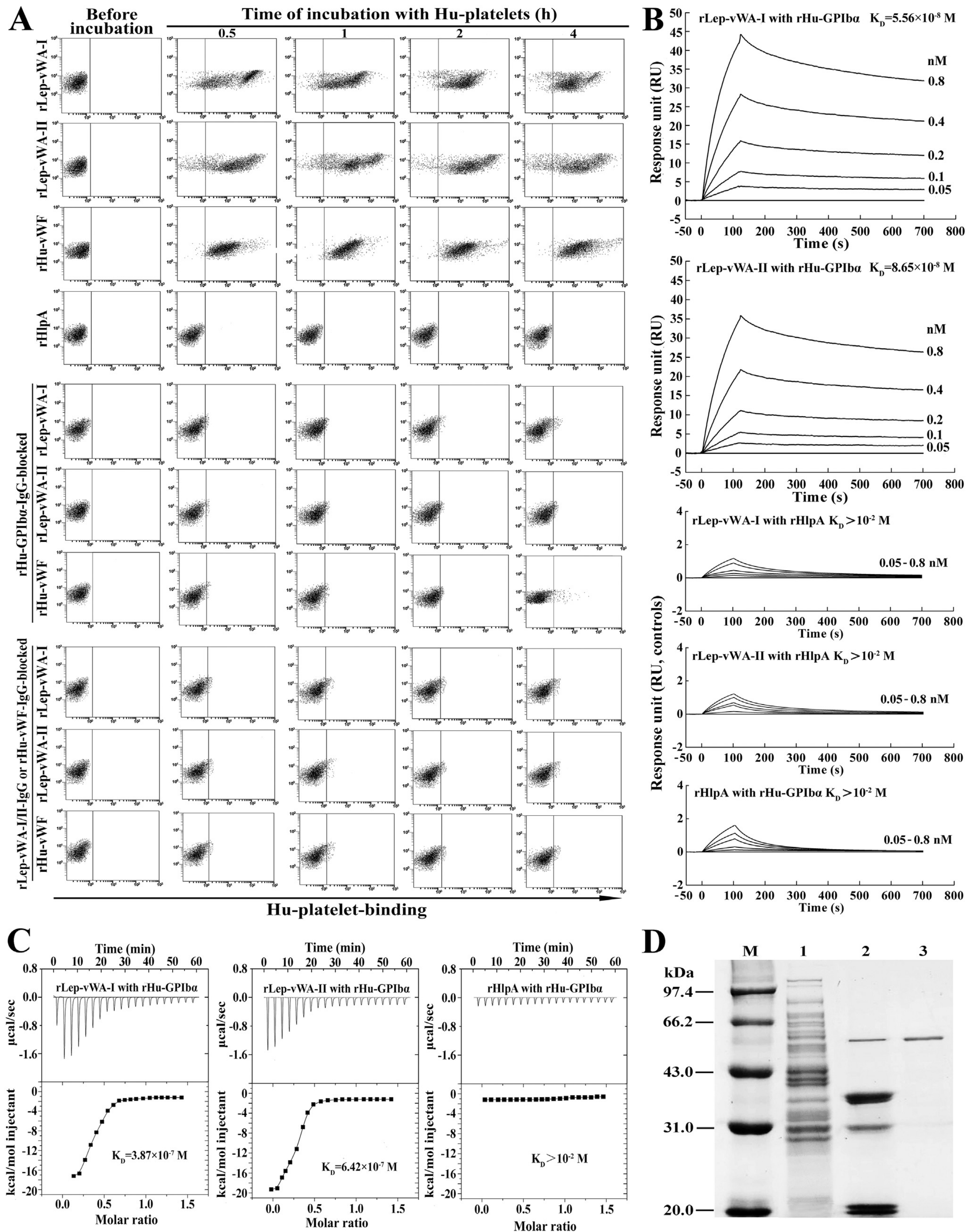

Fig. 2. Platelet- and GPIbα-binding ability of rLep-vWA-I and rLep-vWA-II. (A). Hu-platelet-binding ability of rLep-vWA-I and rLep-vWA-II, determined by flow cytometry. rHu-vWF and rHlpA, a commercial recombinant human vWF and a recombinant hemolysin of *L.* interrrogans, were used as the controls. (B). rHu-GPIbα-binding ability of rLep-vWA-I and rLep-vWA-II, determined by SPR. rHlpA, a recombinant hemolysin of *L.* interrrogans, was used as the control. (C). rHu-GPIbα-binding ability of rLep-vWA-I and rLep-vWA-II, determined by ITC. The legend is the same as shown in C. (D). rHu-GPIbα-captured leptospiral proteins, determined by co-precipitation test. Lane M: protein marker. Lane 1: total proteins from *L. interrogans* strain Lai. Lane 2: proteins released from protein-A-GPIbα-IgG beads. Lane 3: rHu-GPIbα control.

Table 3
Hu-platelet-binding percentages of rLep-vWA-I and rLep-vWA-II[△].

Group ($n = 3$)	Platelet-binding percentage (%, 10^4 platelets)			
	0.5 h	1 h	2 h	4 h
rLep-vWA-I	89.9 ± 3.6	90.7 ± 3.8	94.7 ± 4.2	93.3 ± 5.6
rLep-vWA-II	88.2 ± 3.1	90.2 ± 2.9	92.4 ± 3.5	90.7 ± 2.8
rHu-vWF	93.7 ± 3.5	94.4 ± 2.5	95.2 ± 2.1	93.5 ± 2.9
rHu-GP1bα-IgG-blocked rLep-vWA-I	3.5 ± 0.5*	3.7 ± 0.4*	4.2 ± 0.4*	5.4 ± 0.5*
rHu-GP1bα-IgG-blocked rLep-vWA-II	3.9 ± 0.5*	4.2 ± 0.6*	4.4 ± 0.6*	5.5 ± 0.5*
rHu-GP1bα-IgG-blocked rHu-vWF	2.5 ± 0.1*	2.7 ± 0.1*	3.0 ± 0.2*	3.1 ± 0.2*
rLep-vWA-I-IgG-blocked rLep-vWA-I	1.5 ± 0.1*	1.7 ± 0.1*	1.9 ± 0.1*	2.0 ± 0.2*
rLep-vWA-II-IgG-blocked rLep-vWA-II	1.7 ± 0.1*	1.8 ± 0.1*	2.0 ± 0.1*	2.1 ± 0.2*
rHu-vWFIgG-blocked rHu-vWF	1.4 ± 0.1*	1.7 ± 0.1*	2.1 ± 0.2*	3.9 ± 0.2*

△ The data from experiments such as shown in the Fig. 2A.
* $p < .05$ *vs* the Hu-platelet-binding percentages of specific IgG-unclocked rLep-vWA-I, rLep-vWA-II or rHu-vWF.

T112A, rLep-vWA-II-G48S and rLep-vWA-II-V75D, presented a significant decrease of Hu-platelet-binding and rHu-vWF/ristocetin-induced Hu-platelet aggregation-inhibiting abilities, compared to the prototypes of rLep-vWA-I and rLep-vWA-II (Fig. 4). In addition, the multiple point-mutated rLep-vWA-I-G13S/R36Q/G47S and rLep-vWA-II-G76S/Q126R could not bind to Hu-platelets and inhibit rHu-vWF/ristocetin-induced Hu-platelet aggregation (Fig. 4). The SPR and ITC detection also confirmed the significant attenuation of rHu-GPIbα-binding ability of the five single point-mutated rLep-vWA-I or rLep-vWA-II proteins and absence of rHu-GPIbα-binding ability of the two multiple point-mutated rLep-vWA-I or rLep-vWA-II protein (Supplementary Fig. S5). The data suggested that the G13/R36/G47 in Lep-vWA-I and G76/126R in Lep-vWA-II act as human platelet GPIbα-binding sites.

3.6. Increase of expression and secretion of Lep-vWA-I and Lep-vWA-II during infection

The cofocal microscopic examination confirmed the integrity of leptospires from the lysates of *Leptospira*-cell co-cultures (Fig. 5A). The qRT-PCR and Western Blot assay showed that the Lep-vWA-I or Lep-vWA-II mRNA level and protein expression of *L. interrogans* strain Lai were significantly increased during infection of human umbilical vein endothelial cells (HUVEC) (Fig. 5B and C). In particular, the secretion of Lep-vWA-I and Lep-vWA-II was also observed during infection (Fig. 5D). The Aurodox, a type 3 secretion system (T3SS) inhibitor, but not the T1SS and T2SS inhibitors (PAβN, NaN3 and NASCN) [38], caused a significant decrease in Lep-vWA-

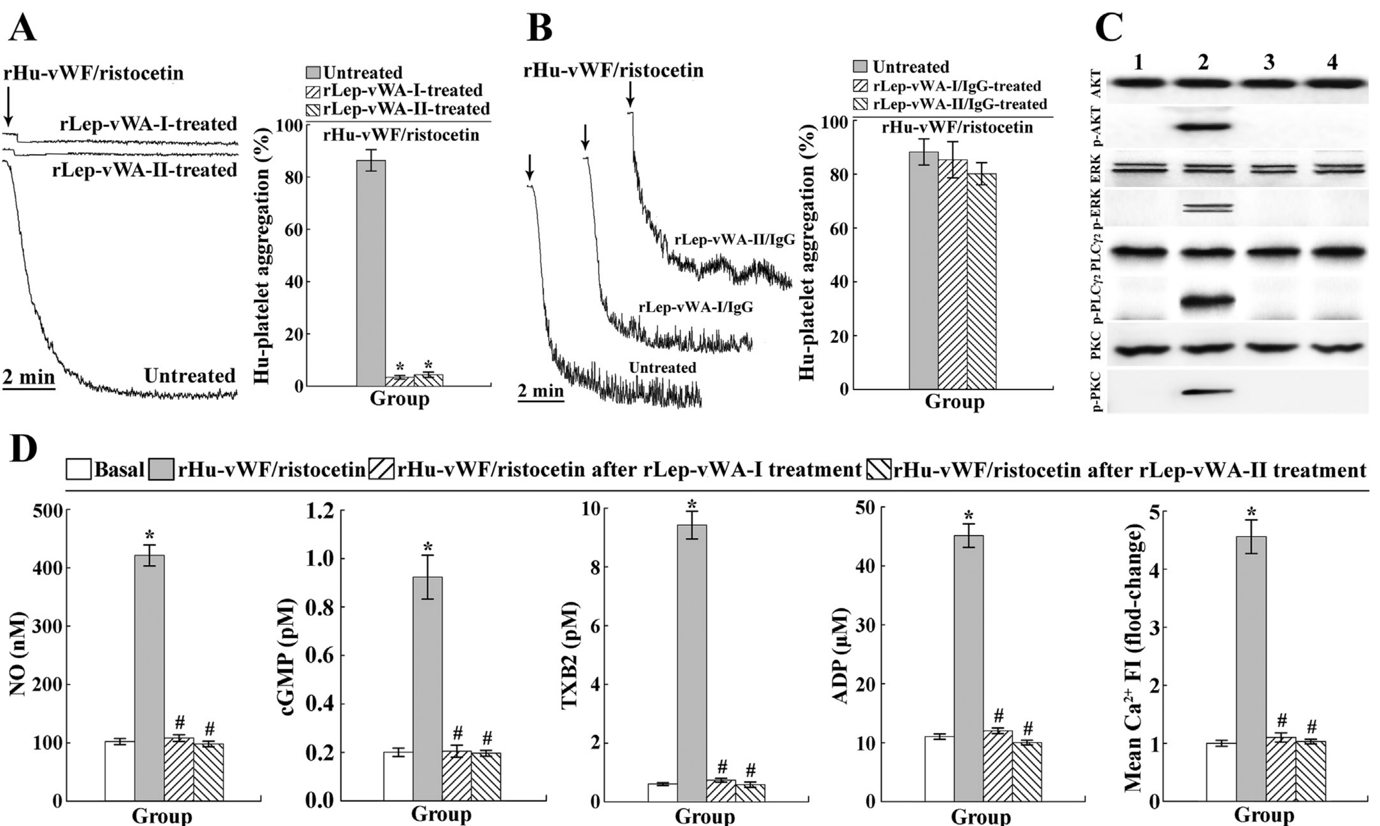

Fig. 3. Inhibition of rLep-vWA-I and rLep-vWA-II on vWF-mediated Hu-platelet aggregation. (A). Inhibition of rLep-vWA-I and rLep-vWA-II on vWF/ristocetin-induced Hu-platelet aggregation, determined by platelet aggregation test. (B). Reversed inhibition of rLep-vWA-I-IgG and rLep-vWA-II-IgG on rLep-vWA-I and rLep-vWA-II blockage of vWF/ristocetin-induced Hu-platelet aggregation, determined by platelet aggregation test. (C). No phosphorylation of the AKT, ERK, PLC and PKC in rLep-vWA-I- or rLep-vWA-II-treated Hu-platelets, determined by Western Blot assay. Lane 1: normal Hu-platelets. Lane 2: vWF/ristocetin-treated Hu-platelets. Lanes 3 and 4: rLep-vWA-I- or rLep-vWA-II-treated Hu-platelets. (D). No increase of the NO, cGMP, TXA2, free ADP and Ca2+ levels in rLep-vWA-I- or rLep-vWA-II-treated Hu-platelets, determined by spectrophotometry and confocal microscopy.

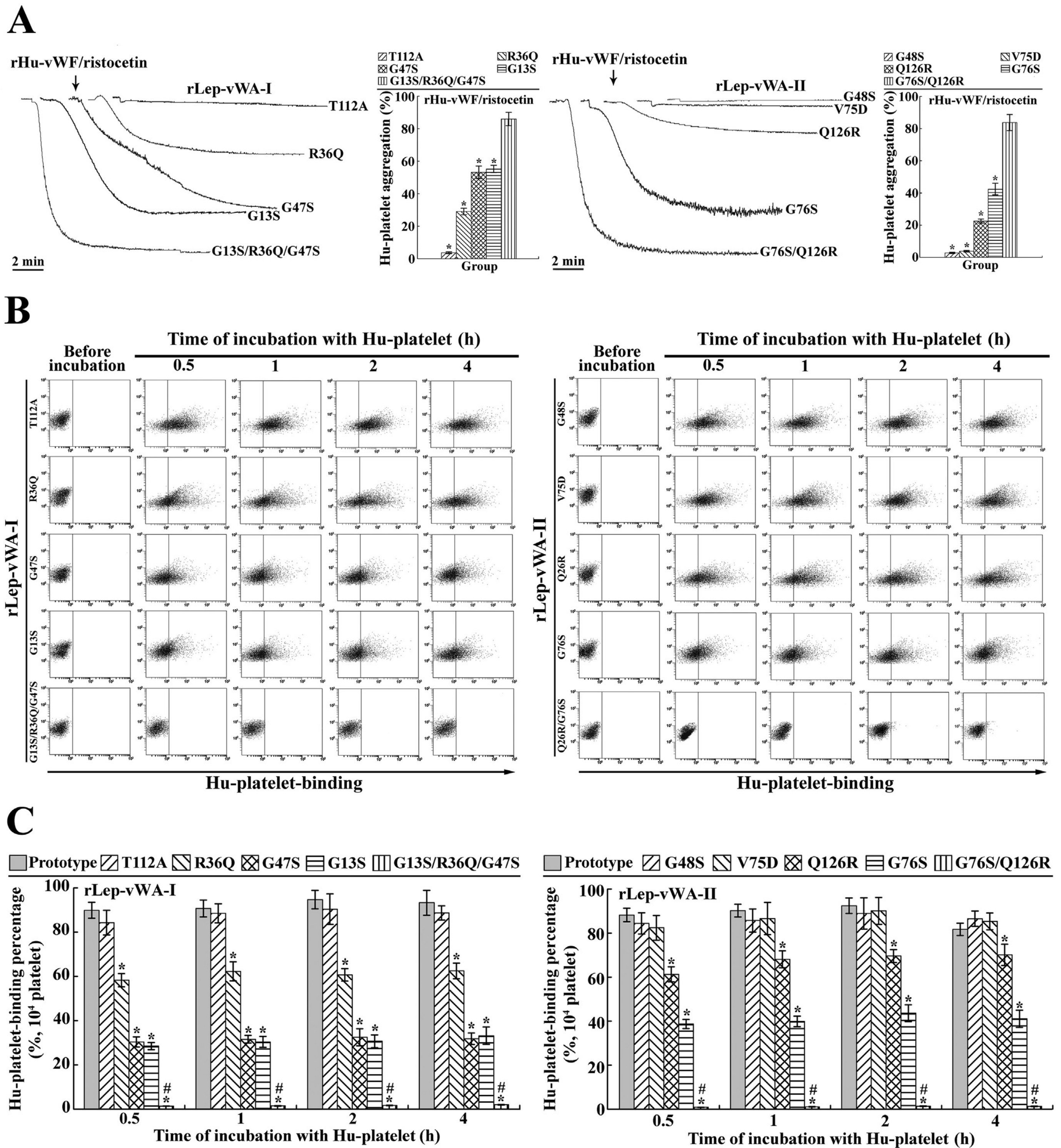

Fig. 4. GPIbα-binding sites in products of *vwa*-I528 and *vwa*-II603 mutants. (A). Decreased inhibition of point-mutated rLep-vWA-I and rLep-vWA-II on vWF/ristocetin-induced Hu-platelet aggregation, determined by platelet aggregation test. (B). Decreased Hu-platelet-binding ability of point-mutated rLep-vWA-I and rLep-vWA-II, determined by flow cytometry. (C). Statistical summary of the Hu-platelet-binding percentages of point-mutated rLep-vWA-I and rLep-vWA-II. Statistical data from experiments such as shown in B. Bars show the means ± SD of three independent experiments. *: $p < .05$ vs the Hu-platelet-binding percentages of prototypic rLep-vWA-I or rLep-vWA-II. #: $p < .05$ vs the Hu-platelet-binding percentages of single point-mutated rLep-vWA-I or rLep-vWA-II.

I and Lep-vWA-II secretion (Fig. 5E). The data suggested that *vwa*-I and *vwa*-II genes are required by *L. interrogans* during infection and the *vwa*-I and *vwa*-II gene products can be secreted through leptospiral T3SS.

3.7. Hemorrhage induced by rLep-vWA-I and rLep-vWA-II in mice

The gross pathological examination showed that all the lungs of rLep-vWA-I- or rLep-vWA-II-injected mice presented visible hyperemia

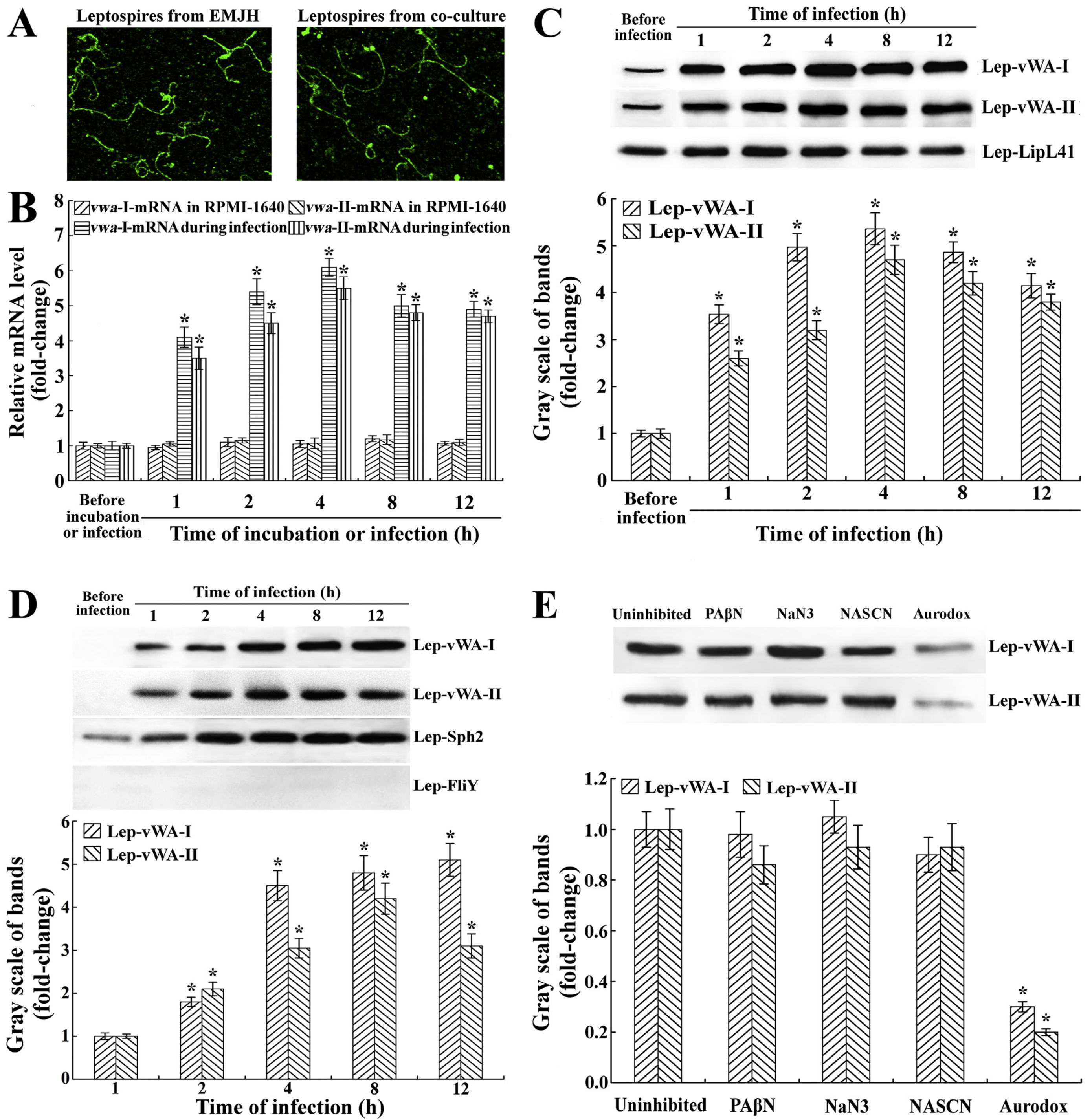

Fig. 5. Increase of *vwa*-I and *vwa*-II gene expression and secretion during infection. (A) The integrity of leptospires from the lysates of Leptospira-cell co-cultures, examined by cofocal microscopy. (B) Increase of vwa-I- or vwa-II-mRNA levels in *L. interrogans* strain Lai during infection of HUVEC, determined by qRT-PCR. Bars show the mean ± SD of three independent experiments. The vwa-I- or vwa-II-mRNA levels in the spirochete from EMJH medium (before infection) were set as 1.0. *: $p < .05$ vs the vwa-I- or vwa-II-mRNA levels in the spirochete before infection or in incubation with RPMI-1640 medium at 37 °C. (C). Increase of Lep-vWA-I and Lep-vWA-II expression in *L. interrogans* strain Lai during infection of HUVEC, determined by Western Blot assay. LipL41, a leptospiral outer membrane lipoprotein, was used as the control. The immunoblotting bands reflecting Lep-vWA-I and Lep-vWA-II expression levels during infection were quantified by gray scales. Bars show the mean ± SD of three independent experiments. The gray scale values of immunoblotting bands from the spirochete in EMJH medium (before infection) were set as 1.0. *: $p < .05$ vs the gray scale values reflecting the expression levels of Lep-vWA-I and Lep-vWA-II of the spirochete before infection. (D). Secretion of Lep-vWA-I and Lep-vWA-II of *L. interrogans* strain Lai during infection of HUVEC, determined by Western Blot assay. Sph2, a secreted leptospiral hemolysin, and FliY, a leptospiral cytosolic protein, were used as the controls. The rest of legend is the same as in B but for detection of Lep-vWA-I and Lep-vWA-II secretion. *: $p < .05$ vs the gray scale values reflecting the secreted Lep-vWA-I and Lep-vWA-II levels of the spirochete before infection. (E). Lep-vWA-I and Lep-vWA-II secretion through T3SS during infection of HUVEC, determined by Western Blot assay. PAβN or Aurodox is the T1SS or T3SS inhibitor while NaN3 and NaSCN are T2SS inhibitors. The rest of legend is the same as in B but for determination of Lep-vWA-I and Lep-vWA-II secretion pathways. *: $p < .05$ vs the gray scale values reflecting the secreted Lep-vWA-I and Lep-vWA-II levels of the spirochete before infection.

and swelling but the livers and kidneys had no visible changes compared to those of normal mice (Fig. 6A). The pathological examination showed that both rLep-vWA-I and rLep-vWA-II caused the pulmonary diffuse hemorrhage with interstitial pneumonia and extensive renal focal hemorrhage (Fig. 6B and C). When rLep-vWA-I or rLep-vWA-II was pre-blocked by rLep-vWA-I-IgG or rLep-vWA-II-IgG, the

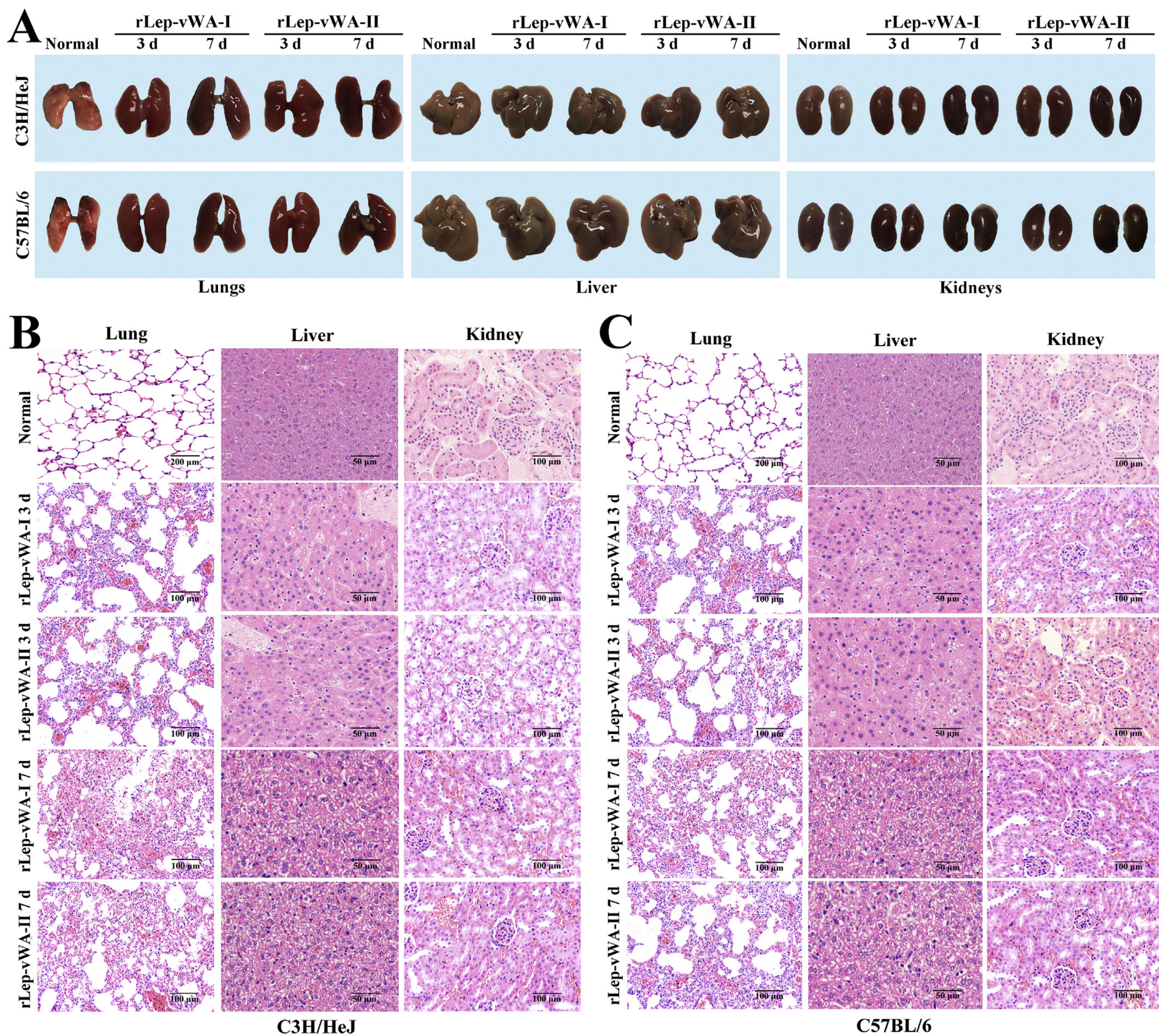

Fig. 6. rLep-vWA-I- and rLep-vWA-II-induced hemorrhage in mice. (A). The gross pathological changes of lungs, livers and kidneys of rLep-vWA-I- or rLep-vWA-II-injected C3H/HeJ or C57BL/6 mice. (B). rLep-vWA-I- or rLep-vWA-II-induced hemorrhage in C3H/HeJ mice, observed by microscopy after HE-staining. (C). rLep-vWA-I- or rLep-vWA-II-induced hemorrhage in C57BL/6 mice, observed by microscopy after HE-staining.

hemorrhagic phenomenons in the lung and kidney tissues of mice were absent (Supplementary Fig. S6). The data suggested that the products of *L. interrogans vwa*-I and *vwa*-II genes act as hemorrhage inducers in mice.

3.8. Ms-platelet-binding ability of rLep-vWA-I and blood coagulation dysfunction

The flow cytometric examination confirmed that rLep-vWA-I and rLep-vWA-II could rapidly bind to Ms-platelets form C3H/HeJ or C57BL/6 mice with the 91.9% and 90.5% or 92.1% and 91.6% maximal human (Hu)-platelet-binding percentages (Fig. 7A and B). On the other hand, the coagulation time (CT), prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT) and thrombin generation time (TGT) of peripheral blood specimens from the two rLep-vWA-I- or rLep-vWA-II-injected mice presented a 2.36–8.81 fold extension but the fibrinogen (coagulation factor I, F—I), prothrombin (coagulation factor II, F-II), prothrombin degradation fragments (F1 + 2) and fibrin degradation product (D-dimer) levels had no significant changes, compared to the normal mice (Fig. 7C). The data suggested that the products of *L. interrogans vwa*-I and *vwa*-II induce hemorrhage in mice due to attenuation or dysfunction of platelet aggregation.

4. Discussion

Leptospira is a large group of Gram-negative helical prokaryotic microbes that can be classified into pathogenic and non-pathogenic saprophytic *Leptospira* genospecies [1,2,11]. Both the pathogenic and saprophytic *Leptospira* genospecies express surface LPS (*i.e.* endotoxin) [39], but only the former is pathogenic due to expression of many invasive and virulence factors such as the ColA collagenase, the LigB adhensin, the Mce invasive protein and the OMP047 apoptotic inducer [20,25,34,40]. Unlike saprophytic *Leptospira*, which live in the natural environment, pathogenic *Leptospira* need to invade into animal hosts for propagation. This requires certain genetic diversity between the saprophytic and pathogenic *Leptospira* genospecies. In the present study,

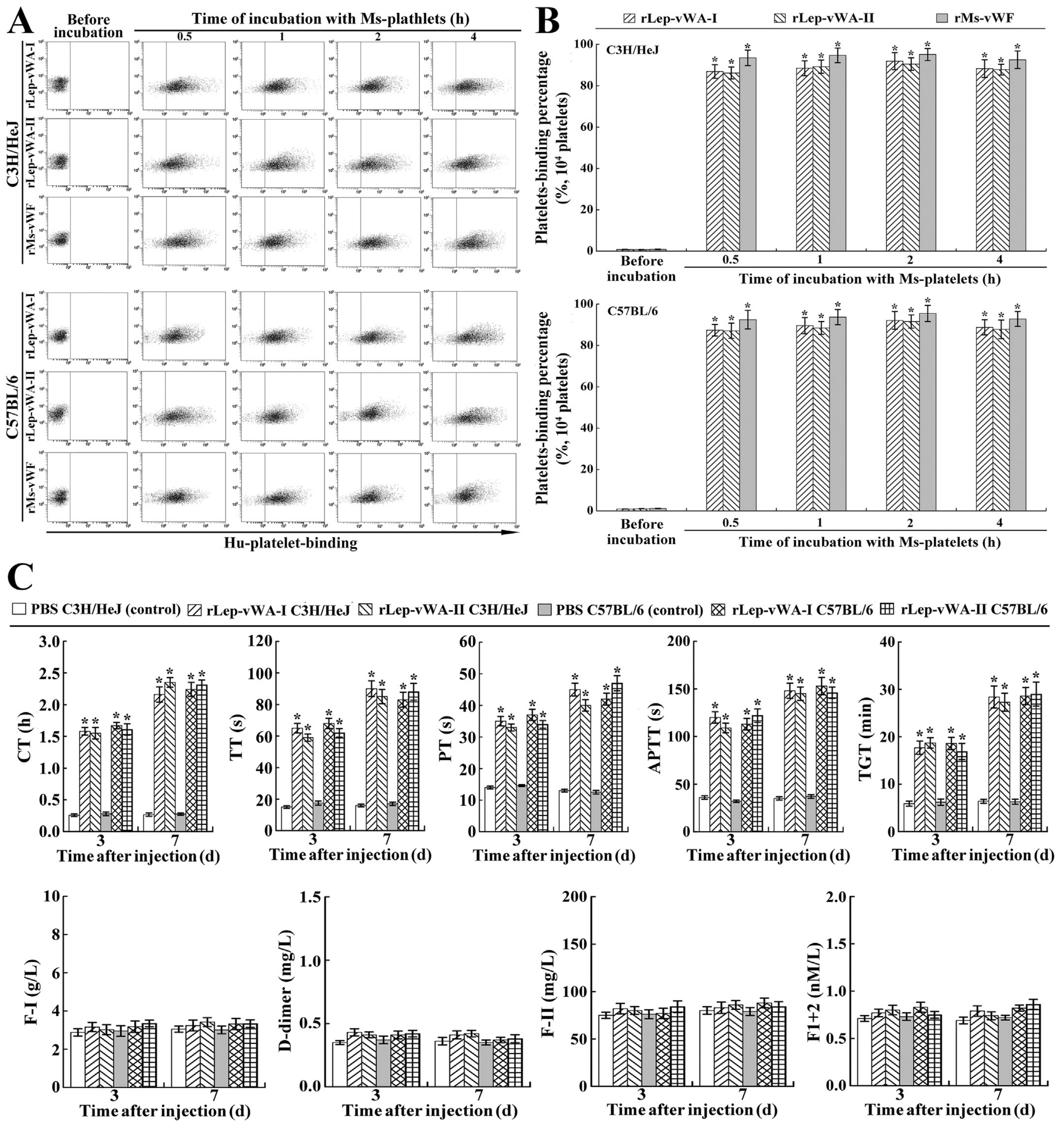

Fig. 7. Ms-platelet-binding ability of rLep-vWA-I and blood coagulation dysfunction. (A). Ms-platelet-binding ability of rLep-vWA-I and rLep-vWA-II, determined by flow cytometry. rHu-vWF, a commercial recombinant mouse vWF, was used as the control. (B). Statistical summary of the Ms-platelet-binding percentages of rLep-vWA-I and rLep-vWA-II. Statistical data from experiments such as shown in A. Bars show the means ± SD of three independent experiments. *: p < .05 vs the normal Ms-platelets. (C). Extension of the CT, PT, TT, APTT and TGT as well as no change of the F—I, D-dimer, F-II and F1 + 2 levels of peripheral blood specimens from rLep-vWA-I- or rLep-vWA-II-injected mice, determined by using an Auto-Blood Coagulation Analyzer and ELISA. *: p < .05 vs the CT, PT, TT, APTT and TGT of peripheral blood specimens from normal mice.

we found that only the strains from pathogenic *Leptospira*, but not from saprophytic *Leptospira* genospecies, possess sequence-conserved *vwa*-I and *vwa*-II genes. In particular, the expression of *L. interrogans vwa*-I and *vwa*-II genes as well as the secretion of their products (Lep-vWA-I and Lep-vWA-II) were observed during infection of human vascular endothelial cells. However, the T3SS inhibitor (Aurodox) but not the T1SS and T2SS inhibitors could inhibit the secretion of Lep-vWA-I and Lep-vWA-II. Earlier studies reported that *L. interrogans* may possess an incomplete type III secretion system (T3SS) because no genes could be predicted to encode a transmembrane channel in the T3SS for transport of proteins [41,42]. However, a recent study showed that the product of the *gspD* gene in *L. interrogans* strain Lai was predicted as a YscC-like protein, a porin for protein secretion in T3SS of *Yersinia pestis* [43]. Our data indicate that the pathogenic but not saprophytic *Leptospira*

genospecies possess *vwa*-I and *vwa*-II genes and the products of *vwa*-I and *vwa*-II genes can secrete through T3SS during infection.

Many prokaryotic pathogens, such as *Escherichia coli*, *Shigella dysenteriae*, *Helicobacter pylori*, *Clostridium difficile* and *Rickettsia* species, can cause hemorrhagic injury of infected hosts [44–48]. LPS has been confirmed as a strong causative agent of hemorrhage due to the inflammation-mediated increase of vascular permeability, damage of vascular endothelial cells and basement membrane, and inhibition of platelet adhesion and aggregation [49–51]. However, the biological activity of leptospiral LPS is much lower than that of *E. coli* LPS, while hemorrhage in leptospirosis is much more severe [6,13,25,39]. Therefore, pathogenic *Leptospira* genospecies must produce some factors other than leptospiral LPS in order to induce hemorrhage during infection.

GPIb is a platelet transmembrane protein composed of two α-subunits and two β-subunits [52]. During the process of vWF-induced platelet aggregation *in vivo*, vWF first binds to platelets through its vWF-A domain with the leucine-rich repeat N-terminal (LRRNT) domain of GPIbα to form a vWF-GPIb-IX-V complex that further initiates PI3K/AKT-PLC/PKC signaling to cause talin/integrin-dependent platelet aggregation [19,28]. Therefore, the GPIbα-binding ability of rLep-vWA-I and rLep-vWA-II, the products of *L. interrogans vwa*-I and *vwa*-II containing vWF-A superfamily domains, is the key feature of these two proteins for their ability to induce hemorrhage by competitive blockage of vWF. In the present study, both rLep-vWA-I and rLep-vWA-II were able to interact with Hu-platelets but also bind to rHu-GPIbα with K_D values of 3.87×10^{-7}-8.65×10^{-8} M. K_D values lower than 10^{-6} M generally indicate high-affinity protein-protein interactions [29,30]. In particular, latelet aggregation and aggregation inhibition tests confirmed that rLep-vWA-I and rLep-vWA-II blocked rHu-vWF/ristocetin-induced Hu-platelet aggregation *in vitro*. Moreover, this study also identified G13, G47 and R36 in Lep-vWA-I and G76 and Q126 in Lep-vWA-II as the rHu-GPIbα-binding residues. All the data demonstrate that the *L. interrogan vwa*-I and *vwa*-II gene products exhibit human platelet GPIbα-biding ability and can cause hemorrhage during leptospirosis by competitive inhibition of vWF-mediated platelet aggregation.

Hemorrhage can be classified as disruptive hemorrhage due to vessel wall injury and diapedetic hemorrhage due to thrombocytopenia and platelet dysfunction and is usually caused by heredopathia, malnutrition, leukemia or microbial infections [16,53]. Younger guinea pigs and Syrian hamsters are more suitable for leptospirosis animal models [54,55], but C3H/HeJ and C57BL/6 mice are also commonly used as the experimental hosts of *L. interrogans* [56,57]. In particular, commercial experimental reagents for mice but not for guinea pigs and Syrian hamsters are readily available. Animal experiments in our study showed that 100 μg rLep-vWA-I or rLep-vWA-II was able to cause severe diffuse diapedetic hemorrhage in lung tissues and extensive focal diapedetic hemorrhage in kidney tissues of mice, while hemorrhage could be blocked by rLep-vWA-I or rLep-vWA-II-IgG. CT, TT, PT, APTT and TGT are common assays to assess coagulation function of blood as well as the prothrombin degradation fragments (F1 + 2) and fibrin degradation product (D-dimer) levels reflect the generation of thrombin from activated prothrombin and formation of fibrin from cleaved fibrinogen [16,36]. Compared to the normal mice, all the coagulation times of peripheral blood specimens from rLep-vWA-I- and rLep-vWA-II-injected mice were extended, but no changes of the fibrinogen (F—I), prothrombin (F-II), F1 + 2 and D-dimer levels were found. In the process of blood coagulation, the aggregated platelets induced by vWF can produce coagulation factor V and combine with coagulation factor X to form a complex that cleaves prothrombin into thrombin to cause the conversion of fibrinogen into fibrin [17,58,59]. Moreover, the rLep-vWA-I- or rLep-vWA-II-injected mice also presented interstitial pneumonia. Previous studies revealed that heme, heat shock protein-60 (HSP60) and adenosine 5′ triphosphate released from erythrocytes can induce inflammation through NLRP3 inflammasome, NF-κB and Ca^{2+}/PKC signaling pathways of macrophages and vascular endothelial cells [60–62]. Therefore, the interstitial pneumonia observed in the mice may be caused by the released components of erythrocytes. All the data indicate that the products of *L. interrogan vwa*-I and *vwa*-II genes can cause hemorrhage in mice probably due to dysfunction and disorder of platelet aggregation.

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Declaration of interests

We declare no competing interests.

Contributors

J.Q.F, X.A.L. and J.Y. contributed to the design of this study. J.Q.F., M.I. and W.L.H. performed the experiments. J.Q.F., Y.L., Y.M.G. and K.X.L. analyzed the experimental data. J.Q.F., D.M.O. and J.Y. wrote and modify the manuscript. All authors reviewed and approved the final version of the manuscript.

Potential conflicts of interest.

No reported conflicts.

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