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A new role for host annexin A2 in establishing bacterial adhesion to vascular endothelial cells: lines of evidence from atomic force microscopy and an in vivo study

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Abstract

Understanding bacterial adhesion is challenging and critical to our understanding of the initial stages of the pathogenesis of endovascular bacterial infections. The vascular endothelial cell (EC) is the main target of *Rickettsia*, an obligately intracellular bacterium that causes serious systemic disease in humans and animals. But the mechanism(s) underlying bacterial adherence to ECs under shear stress from flowing blood prior to activation are unknown for any bacteria. Although host surface annexin a2 (ANXA2) has been identified to participate in efficient bacterial invasion of epithelial cells, direct evidence is lacking in the field of bacterial infections of ECs. In the present study, we employ a novel, anatomically based, in vivo quantitative bacterial-adhesion-to-vascular-EC system, combined with atomic force microscopy (AFM), to examine the role of endothelial luminal surface ANXA2 during rickettsial adherence to ECs. We also examined whether ANXA2 antibody affected binding of *Staphylococcus aureus* to ECs. We found that deletion of ANXA2 impeded rickettsial attachment to the ECs in vitro and blocked rickettsial adherence to the blood vessel luminal surface in vivo. The AFM studies established that EC surface ANXA2 acts as an adherence receptor for rickettsiae, and that rickettsial adhesin OmpB is

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the associated bacterial ligand. Furthermore, pretreatment of ECs with anti-ANXA2 antibody reduced EC surface-associated *S. aureus*. We conclude that the endothelial surface ANXA2 plays an important role in initiating pathogen–host interactions, ultimately leading to bacterial anchoring on the vascular luminal surface.

Introduction

The ability of invasive bacteria to adhere to host cell surfaces is a pivotal initial step in successfully establishing infection of an obligate or facultative intracellular parasitic bacterium [1–10]. In bloodstream infections, for most bacterial microorganisms that are generally not considered intracellular pathogens, the establishment of metastatic endovascular infections after adhering to vascular endothelial cells (ECs) is one of the major determinants in disease outcome [11–17]. Understanding how bacteria attach to host cells is a crucial challenge in microbiology, and in medicine is a key for controlling bacterial infections at early stages [18]. Remarkably, in animal models mimicking sepsis, several *Staphylococcus aureus* proteins have been shown to mediate the adhesion of *S. aureus* to activated ECs that were stimulated by an initial infection with a low dose of bacteria 24 h before reinfection with a higher dose [11, 12, 19]. However, the mechanism(s) underlying bacterial adherence to ECs under shear stress from flowing blood prior to activation are completely unknown. This knowledge is critical to our understanding of the initial stages of the pathogenesis of endovascular bacterial infections, which is needed for developing therapeutics that could limit infection at this initial stage.

Obligately intracellular bacteria in the genus *Rickettsia* (*R.*) are the causative agents of spotted fever group (SFG) rickettsioses [20–22], including *R. rickettsii* [22, 23], *R. conorii* [24], and *R. australis* [25, 26]. Typically, rickettsiae are transmitted through the bite of infected ticks, and ECs are the primary vertebrate host target cells [11, 27]. The initial step in establishing a productive intracellular infection is for the bacterium to recognize and establish an adhesive interaction with specific cellular receptor(s) to firmly anchor itself on the host EC luminal surface, thus overcoming detachment by shear stress from blood flow prior to invasion into the EC [11, 28]. Therefore, rickettsial infection is a suitable model to employ for studying endovascular bacterial adhesion. Remarkable insights into the rickettsial components involved in this initial interaction have come from identification of rickettsial adhesins [29–35], although comparatively little is known about host surface receptor(s) and the mechanism for establishing the connection between the host cell surface and rickettsiae. Host proteins Ku70 [36], α2β1 integrin [29], clathrin [37], caveolin 2 [37], and exchange protein activated by cAMP (EPAC) [38] have been identified as being involved in rickettsial invasion into nonphagocytic host cells via endocytic mechanisms [36]. Yet, as analyzed by an immunofluorescence (IF)-based assay, only the α2β1 integrin heterodimer [29] and EPAC1 [38] were shown to be involved in rickettsial adhesion to the host cell surface. α2β1 integrin mainly serves as a endothelial receptor for extracellular matrix molecules [39]. EPAC is an intracellular cAMP receptor [40] and is speculated to play a regulatory role, rather than as a direct receptor for rickettsial adherence on the host cell surface. Using a functional antibody specific to KU70, it was shown that rickettsial

invasion into Vero cells was effectively blocked, but there was no effect on rickettsial adhesion to Vero cell surfaces [36].

ECs express abundant plasminogen (Plg), and Plg activator binding sites on their vascular luminal surfaces serve plasmin-based fibrinolytic functions [41], among which the annexin A2 (ANXA2) is the best recognized and is emerging as the focus of research on a growing spectrum of biologic and pathologic processes [42, 43]. ANXA2 is a Ca^{2+}-regulated and phospholipid-binding protein that associates with cell membrane lipid rafts and the actin cytoskeleton [42, 44, 45]. It is detected on endothelial surfaces in the form of a complex with S100A10, $(ANXA2\text{-}S100A10)_2$. Of note, there is in vitro evidence that ANXA2 participates in efficient invasion of *S. aureus* [46], *Escherichia coli* [47, 48], *Pseudomonas aeruginosa* [49], *Salmonella typhimurium* [50], and *Mycoplasma pneumoniae* [51] in epithelial cells via regulation of cytoskelton remodeling in the vicinity of lipid rafts. Neutralization of the ligands on *P. aeruginosa* by incubation of the bacteria with recombinant, soluble ANXA2 prevents bacterial entry into human epithelial cells, suggesting ANXA2 may be a receptor for bacterial adherence and/or invasion [49]. Although ANXA2 has been identified as a binding partner of *S. aureus* adhesin clumping factor A in a protein–protein binding assay [46], direct in vitro or in vivo evidence are completely lacking in the field of endovascular infections after adherence to ECs.

In the present study, we employ a novel, anatomically based, in vivo quantitative bacterial-adhesion-to-vascular-EC analysis system, combined with atomic force microscopy (AFM), to examine the role of endothelial luminal surface ANXA2 during rickettsial adherence to ECs. We identified endothelial surface ANXA2 as a receptor for SFG rickettsial adhesion in vivo using AFM probes coated with recombinant *R. conorii* adhesin OmpB (reOmpB) to assess dynamic interactions during rickettsial adhesion. Our results demonstrated that recombinant ANXA2 (reANXA2), compared to bovine serum albumin (BSA) and Plg, has the most binding ability to reOmpB during measurement of protein–protein interaction forces. We also used reOmpB-coated probes with single living human umbilical vein endothelial cells (HUVECs) and demonstrated that reOmpB exerted an ANXA2-dependent binding strength to the living endothelial surface. Moreover, an ANXA2 monoclonal antibody could attenuate adhesion of *S. aureus*, a versatile facultative intracellular pathogen, to human ECs.

Materials and methods

Mice

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch (UTMB). Wild-type (WT) mice (C57BL/6J) were obtained from Jackson Laboratory (Bar Harbor, ME). *Anxa2*-null mice, on the C57BL/6J background, were a generous gift from Dr Katherine Hajjar (Weill Cornell Medicine, New York, NY) [52]. All mice used in this study were 8 to 12-week-old males. C57BL/6J mice are highly susceptible to *R. australis*. Therefore, this organism was chosen as the SFG rickettsial agent of choice [25].

Rickettsiae and *S. aureus*

R. australis (strain Cutlack) was prepared as described [38]. Uninfected Vero cells were processed as mock control material using the same procedure. All biosafety level (BSL) 3 or ABSL3 experiments were performed in CDC-certified facilities in the Galveston National Laboratory at UTMB, Galveston, TX, using established procedures and precautions. *S. aureus* (ACTT25923) was maintained in laboratories of the Department of Laboratory Diagnostics, Changhai Hospital, Shanghai, China.

Mouse in vivo, anatomically based, quantitative rickettsial adhesion measuring system

The C57BL/6 mouse—*R. australis* model is an established animal model of human SFG rickettsiosis because the pathology involves disseminated endothelial infection and pathological lesions, including vasculitis in multiple organs, similar to what is observed in human SFG rickettsiosis [25, 38]. To quantitatively assess rickettsial adhesion in vivo, after an ordinarily lethal dose of *R. australis* (1×10^7 PFU/0.2 μl; the LD_{50} is 1×10^6 PFU) was injected through the tail vein [38], rickettsial virulence (by plaque assay) was measured in a 1 μl blood sample collected from the orbital venous sinus (OVS) at different times until 1 h post infection (p.i.). Taking advantage of this anatomically based inoculation model, the principle concept is that the more rickettsiae adhered to the luminal surfaces of the blood vessels, the fewer rickettsiae could be detected in blood samples. Multiple visceral organs were then fixed without perfusion rinse (to keep blood in the vessel lumens) for IF-based histological studies to identify unattached rickettsiae, which were trapped in clots in the vessel lumens. Since borders between blood and ECs were invisible in some capillaries, IF-based assessment will exclude capillaries in all organs. For the IF assay, tissue samples of brain and liver were fixed in a 4% neutral buffered solution of for-maldehyde, embedded in paraffin, sectioned at 5-μm thickness, and processed by IF microscopy for detection of SFG rickettsiae as described previously [53]. Briefly, deparaffinized and rehydrated sections were incubated with rabbit or guinea pig polyclonal antibody against SFG rickettsiae (1:1000) overnight at 4 °C. Rickettsial antigens were detected with AlexaFluor 594-conjugated goat anti-rabbit or DyLight 594-conjugated goat anti-guinea pig antibodies. Nuclei were counterstained with DAPI. Fluorescent images were analyzed using an Olympus BX51 epifluorescence or Olympus IX81 confocal microscope.

AFM system

The biomechanical properties of interactions between reOmpB and reANXA2 at protein–protein and protein–cell levels, respectively, were studied using an AFM system (Flex-AFM, Nanosurf AG, Liestal, Switzerland). Colloidal cantilevers with a 5-μm polystyrene bead were used (SHOCON-G-PS, Applied NanoStructures, Mountain View, CA). Published protocols were employed to coat polystyrene beads and polystyrene slides (Electron Microscopy Sciences, Hatfield, PA) with streptavidin [54] and biotinylize recombinant proteins [55]. The cantilevers were functionalized by incubation with streptavidin-reOmpB at 50 μg/ml overnight at 4 °C. The polystyrene slides were functionalized by incubation with streptavidin-reANXA2 or Plg at 50 μg/ml overnight at 4 °C. Unbound proteins were rinsed away using PBS. The exposed surface of the bead was blocked by BSA (Sigma, St. Louis, MO) at 500 μg/ml in PBS [53]. AFM imaging and measurements were generally taken

within 1 h after blocking. The spring constant of the cantilever was calibrated using the Sader method in air [56]. The cantilever spring constant varied between 0.10–0.15 N/m. Force spectroscopy was done in static force mode operating on 25 μm^2 areas on a living cell surface or on a recombinant protein-functionalized slide to measure the specific unbinding force during rupture of the protein–cell or protein–protein interaction. The maximum compression force was set to 150 pN. The contact time was kept constantly at 500 ms before the cantilever was retracted at a constant pulling speed of 1 μm/s to measure the force–extension curve. In order to evaluate the effect of the nonspecific unbinding force, confluent cell monolayers were pretreated with anti-ANXA2 antibody at 3 μg/ml for 30 min to block ANXA2 on the cell surface before the AFM measurements. Normal mouse IgG was employed as negative control. We scanned five cells per group, each with a different cantilever. FD curves were analyzed using the open source software Atomic J [57].

All other methods are detailed in the Supplementary Materials section.

Statistical analysis

Values are reported as mean ± SEM. The data were analyzed using Student's *t* test or one-way ANOVA analysis (Sigmaplot, Sigma Stat, Jandel Scientific Software, San Rafael, CA). *P* values are as follows: $^{**}P < 0.01$ and $^{*}P < 0.05$. Statistical significance was considered as $P < 0.05$.

Results

Rickettsiae associated with ANXA2 on EC surfaces upon initial infection

ANXA2 on the surfaces of HUVECs at the beginning of infection with *R. australis* at 10 MOI was visualized in vitro using IF confocal microscopy on nonpermeabilized, fixed cells [38, 58, 59]. IF staining revealed that ANXA2 (Figs. 1a and S1) colocalized with rickettsiae on plasma membrane external surfaces. Immunoelectron microscopy (IEM) studies using anti-ANXA2 antibodies demonstrated that ANXA2 was detected between the surfaces of rickettsiae and the external surfaces of ECs shortly after infection (Fig. 1b).

These data demonstrate that ANXA2 on apical surfaces of ECs appears to participate in establishing rickettisal attachment on EC surfaces.

Deletion of *ANXA2* impeded rickettsial attachment to the ECs in vitro

Growing evidence indicates that mammalian cell surface ANXA2 participates in heterotypic cell-to-cell adhesion [60, 61]. It is further emerging that ANXA2 is involved in bacterial invasion [47, 49, 51]. These finding prompted us to determine the potential role of ANXA2 during rickettsial adhesion.

First, we examined whether ANXA2 antibody affects rickettsial adhesion to the endothelial surface. Following pretreatment with an ANXA2 antibody (clone 666316), HUVEC surface-associated rickettsiae were markedly decreased at 15 min p.i. in a dose-dependent manner, compared to irrelevant normal IgG controls as observed by fluorescent microscopy (Fig. 2a).

To confirm whether lack of ANXA2 directly impedes bacterial infection, we utilized mouse brain microvascular endothelial cells (BMECs) from WT and *Anxa2*-null mice. Endothelial surface-associated rickettsiae examined by impermeable IF microscopy were decreased in *Anxa2*-null BMECS, compared to WT BMECs, at 15 min p.i. with 10 MOI rickettsiae (Fig. 2b).

This in vitro evidence suggests that host ANXA2 plays an imperative role during rickettsial adhesion to host EC surfaces.

Global depletion of *ANXA2* blocked rickettsial adherence to the blood vessel luminal surface in vivo

After 10 LD50 doses of *R. australis* (1×10^7 PFU) were injected through the tail vein, viable rickettsiae in circulating blood collected from the OVS at different times p.i. was measured by plaque assay (Fig. 3a). Viable bacteria that were not adhered to the endothelium were not detected in blood samples as shown by plaque assay at 15 min p.i., but they became detectable as early as 30 min p.i. Plaque assays showed that the number of viable rickettsiae in circulating blood was higher in *Anxa2*-null mice ($n = 10$) than WT mice ($n = 10$) at 1 h p.i. (Fig. 3b).

To keep blood in the vessel lumens in tissue samples, multiple visceral organs were fixed without perfusion for IF-based histological studies to identify unattached rickettsiae that otherwise become trapped in clots in the vessel lumens. IF staining showed that rickettsiae were located in two major blood vessel compartments, the luminal surface or tunica intima of the vascular wall and postmortem artifacts of fixed blood clots that show evidence of trapped circulating bacteria (Fig. 3c). In WT mice ($n = 10$), rickettsiae were detected on the luminal surface or tunica intima of the vascular wall (Figs. 3c and S2–5). However, in *Anxa2*-null mice ($n = 10$), significantly fewer rickettsiae were detected in the same areas, while unattached rickettsiae were visible in the blood clots in the lumen of these blood vessels (Figs. 3c and S2–5).

The observation that the vascular endothelium in *Anxa2*-null mice does not contain SFG rickettsiae shortly after infection (adherent and invading), coupled with in vitro evidence that deletion of host *Anxa2* impedes rickettsial attachment to the endothelial surface, suggests that host ANXA2 plays an important role during rickettsial adhesion to the EC surface.

Interestingly, between the WT and *Anxa2*-null groups, there was no difference in the quantity of rickettsial antigen-positive white blood cells that were isolated from peripheral blood samples 1 h following a lethal dose of rickettsiae injected via the tail vein, as detected using flow cytometry (Supplemental Fig. S6).

reANXA2 showed strong interaction with reOmpB

It remains unclear whether ANXA2 functions as a receptor for direct rickettsial binding or only as a signaling pathway modulator that regulates the mechanical interaction between rickettsial adhesin(s) and other unidentified endothelial surface receptor(s). AFM has been increasingly employed in the investigation of the biomechanical basis of bacterial adhesion to host surfaces at the single molecule level, providing deeper insights into the binding

mechanism [18, 62, 63]. We functionalized the AFM cantilever probe with reOmpB (amino acids 1363–1655). We conducted the force spectroscopy to directly measure the specific unbinding force during rupture of the interaction between reOmpB and human reANXA2 and Plg. BSA was used as a control. Representative force–distance (FD) curves exhibit rupture events during separation of protein–protein interactions (Fig. 4a). As shown in Fig. 4b, the AFM study using the reOmpB-functionalized probe established that the strongest binding interaction was observed with reANXA2, compared to BSA or Plg.

reOmpB showed an ANXA2-dependent binding strength on the surface of a live EC

We employed AFM to directly measure the rickettsial adhesin molecule's binding force to a living host cell surface. Using relevant antibodies as specific functional blockers, the mechanical strength of the interactions between reOmpB and potential receptor(s) expressed at the apical surface of a single living EC can be detected. We applied a reOmpB-coated cantilever probe directly on a single, live HUVEC and found that reOmpB establishes stronger binding to the endothelial surface (Fig. 4c), compared to the BSA-coated probe. Pretreating the HUVEC with an ANXA2 antibody (clone 666316) in media significantly reduced the unbinding force between the reOmpB functionalized AFM probe and the EC surface (Fig. 4c), suggesting that reOmpB binds to the surface of a live EC in an ANXA2-dependent manner.

The data from these experiments indicate that ANXA2 on the host EC surface acts as an adherence receptor for SFG rickettsia, and that OmpB is the associated bacterial ligand.

Pretreatment of ECs with anti-ANXA2 antibody reduced EC surface-associated *S. aureus*

To determine the roles that ANXA2 plays in establishing infection of ECs by other bacteria, we examined whether ANXA2 antibody affected binding of *S. aureus*, a facultative intracellular bacterium, to human ECs. We found that, following pretreatment of HUVECs with anti-ANXA2 antibody (clone 666316), the number of endothelial surface-associated *S. aureus* were decreased at 15 min p.i., compared to irrelevant normal IgG controls, in a dose-dependent manner (Fig. 5).

Discussion

Rickettsial infection can be controlled by appropriate broad-spectrum antibiotic therapy after early diagnosis [20, 22, 23, 64]. Nevertheless, untreated or misdiagnosed rickettsioses are frequently associated with severe morbidity and mortality [20, 23, 65–67]. An initial flu-like, non-specific, clinical febrile presentation partially explains the underdiagnosis of these diseases [68]. A case-fatality rate as high as 32% has been reported in hospitalized patients with Mediterranean spotted fever [67]. Disseminated endothelial infection and endothelial barrier disruption with increased microvascular permeability are the central pathophysiologic features of rickettsial infection. Rickettsial pathogens exert their pathologic effects after adhering to and then invading the endothelial lining of blood vessels. The bacteria must establish adhesive interactions to firmly anchor on the endothelial luminal surface to successfully resist detachment by hemodynamic shear stress from flowing blood prior to invasion of the EC [11, 27]. This adherence requires recognition and interaction with

specific cellular receptors, and is thought to be dependent mainly on rickettsial outer membrane proteins OmpA (Sca0), Sca1, Sca2, and OmpB (Sca5), which are encoded by surface cell antigen (*sca*) family genes [29, 58] and expressed on the surface of SFG rickettsiae [69]. These proteins are considered to play important roles as adhesins by mediating interactions with mammalian cells during rickettsial adhesion and subsequent invasion [29, 30, 37]. Our current study unveiled an association between rickettsiae and host ANXA2 on EC surfaces at the beginning of infection. By developing a novel, anatomically based, quantitative bacterial adhesion measuring system, which couples plaque assays and IF, we revealed that global depletion of *ANXA2* blocked rickettsial adherence to the blood vessel luminal surface in vivo. Using AFM, we demonstrated that the binding strength of reOmpB with surface ANXA2 in living ECs reflects a direct interaction. Interestingly, an anti-ANXA2 antibody also attenuated *S. aureus* adhesion to the EC surface. Taken together, these findings support that host ANXA2 can serves as an adherence receptor for both rickettsiae and *S. aureus*.

The establishment of bacterium–endothelium adhesion is firm enough to withstand shear forces from blood flow, and requires specific host cell surface receptors that recognize and interact with bacterial adhesin(s). Different elements on the host plasma membrane external surfaces, including cadherins [70], CD31 [71], fibrinogen [72–74], ICAM-1 [75], integrins [7, 29, 76], Plg [77], platelet activating factor receptor [78], selectins [79], and von Willebrand factor [11, 27] are recognized as participating in adhesion by various nonobligately intracellular bacterial pathogens. Our previous study combining genetic and pharmacological manipulations demonstrated that inhibition of intracellular cAMP receptor EPAC1 suppressed rickettsial adherence to ECs [38], although the underlying mechanism remains unclear. Host α2β1 integrin heterodimer has been identified to be involved in the initial adhesion of *R. conorii* to the host cell [29]. Using IF assays on nonpermeabilized cells, host α2β1 integrin heterodimer has been identified as an adherence receptor for OmpA heterologously expressed in *E. coli*. In contrast, OmpB-mediated adhesion is independent of α2β1 integrin [29]. Furthermore, either functional blocking using antibodies against α2β1 integrin or small interfering RNA (siRNA) transfection that silences α2β1 integrin synthesis in host cells perturbs *R. conorii* invasion, but does not affect adhesion [29]. Therefore, there must be an unknown host mechanism(s) that allows rickettsiae to interact with host cell surfaces by establishing adhesion prior to invasion.

A critical feature of any host cell receptor for initial bacterial adhesion is its integral location and availability on the vascular luminal surface of ECs, which are the primary targets of rickettsiae present in the circulation following inoculation and subsequent multiplication. Identified host receptors for adhesion of other bacteria are distributed predominantly on the basolateral surfaces of ECs for homologous cell–cell adhesion or cell–extracellular matrix interactions, or reside inside the cell and are translocated to the cell surface during an inflammatory response [7, 11, 27, 29, 70–76, 79]. For rickettsiae, proteins in host subcellular compartments may potentially participate in host–pathogen interactions during subsequent invasion, rather than during initial attachment.

ANXA2 is an important receptor that allows Plg and tPA to assemble host fibrinolytic machinery on the endothelial luminal surface, and plays a critical effector role in vascular

patency [45, 80, 81]. Lines of evidence from the present study unveiled a novel role for endothelial luminal surface ANXA2, which serves as a host cell-binding unit for rickettsial adhesion. Global abolishment of *ANXA2* in vivo decreases the number of rickettsiae attached to the vascular luminal surfaces, but increases the number of bacteria in circulating blood at the initial stages of infection following intravenous inoculation. Pretreatment of ECs with anti-ANXA2 antibody also reduced endothelial surface-associated *S. aureus.* An AFM study provided direct evidence that endothelial ANXA2 is an adherence receptor for rickettsial binding on the endothelial surface.

In bacteriology, it has been reported that in vitro experiments utilizing static conditions and dynamic experimental flow conditions can lead to different outcomes for bacterial adherence to ECs [74, 82]. To the best of our knowledge, in rickettsiology there have been no reports on approaches that take into account hemodynamic forces [83]; there have been no mechanistic studies to identify adherence receptors by examining the rickettsia–host receptor binding force that is able to withstand hemodynamic shear stresses in vitro or in vivo. These facts highlight the importance of studying rickettsial adhesion mechanisms using real-time measurements of nanoscale microforces of interactions between rickettsial adhesins and endothelial surfaces. AFM techniques are particularly well suited to achieve this goal [62]. The key advantage of AFM over other imaging techniques in this study is that it provides a measurement of real-time, nanoscale-relevant information of the interactive forces between a single ligand and its receptor [63]. Over the last two decades, this technique has been developed to measure inter- and intra-molecular forces, revealing detailed insights into the functional mechanics of biomolecules. By using AFM on living cells infected with *Listeria monocytogenes*, the unbinding force between a bacterial ligand and its host receptor were measured [63]. In the present study, we employed AFM in protein–protein and protein–living cell models, and obtained nanobiomechanical information to help elucidate the rickettsiae–host cell surface interaction that allows the rickettsiae to overcome shear stress within a blood vessel and establish a productive infection.

Previous studies have identified specific rickettsial proteins as adhesins, particularly the OmpA and OmpB [29, 35, 84]. Antibodies to OmpA and OmpB, but not to lipopolysaccharide, can protect severe combined immunodeficiency mice against fatal SFG rickettsial infection [85]. OmpB is the most abundant protein that is expressed in the outer membrane, and occurs in all rickettsial species [86]. Rickettsial OmpB can be cleaved into a passenger domain (amino acids 451–1308) and the β-peptide (amino acids 1335–1704) [86, 87]. The passenger domain has been reported to be functional in bacterial invasion by interaction with Ku70 [37], while the β-peptide has been shown to interact with mammalian surface proteins [35]. Our AFM study on protein–protein interactions showed that reOmpB (amino acids 1363–1655) binds to reANXA2 significantly in vitro. By using AFM in a protein-living cell interaction model, we probed direct reOmpB binding to living endothelial apical surfaces in an ANXA2-dependent manner. These data suggest that the rickettsial OmpB–ANAX2 interaction exists as a mechanic force during rickettsial binding to the host EC surface. OmpB is the rickettsial effector in establishing binding to ANXA2. This has prompted us to plan experiments using the same approaches to identify potential adhesin(s) of *S. aureus*, which lacks outer membrane proteins, in the interaction with ANAX2.

Importantly, current state-of-the-art in vitro models have been successful for elucidating mechanisms regarding bacterial adhesion to host cells, and have significantly improved outcomes following genetic manipulation of selected targets [36, 38]. However, there are deficiencies using in vitro models to evaluate bacterial adhesion to endothelium [74, 82]. In order for rickettsiae to invade cells, the bacteria must first attach to the EC surface that is under hemodynamic shear stress, which occurs at the blood vessel luminal surface in the direction of blood flow [88]. Although a closed-pump system can be used as an in vitro model mimicking dynamic circulation, such an approach poorly reflects the physiological hemodynamic forces and the anatomical complexity of the endothelium system in vivo and imposes significant limitations for downstream experiments. To date, surgical-and intravital fluorescent microscopy-based rodent models have been used in kinetic studies of *Staphylococcus* adhesion to endothelium in vivo [11, 12]. In addition, mouse models are emerging as a useful model system in postmortem histopathology-or homogenized tissue-based in vivo studies on bacterial infection involving adhesion and invasion [13, 89–91]. Nevertheless, there have been no reports that describe mouse in vivo approaches that take into account physiological hemodynamic forces and genetic manipulation of biochemical targets using mutant strains [19]. Using established *Anxa2*-null mice, we have developed a novel anatomically based, in vivo quantitative bacterial adhesion measuring system, which couples plaque assays and IF, and provides a platform to meet the sampling requirements for more downstream experimental approaches, e.g., quantitative real-time PCR and electron microscopy. This system allows us to quantitatively evaluate bacterial adherence to blood vessel luminal surfaces under physiological hemodynamic shear forces using C57BL/6J WT mice and global *Anxa2*-null mice. To our knowledge, this is the first non-invasive mouse model for quantitative in vivo studies of pathogen adhesion to endothelium in a physiologically simulated hemodynamic environment.

In conclusion, our studies combined an AFM protein–protein and protein–living cell interacting force measuring system with an in vivo model that allowed us to study bacterial adhesion to the vascular EC. This work revealed a novel role for endothelial surface ANXA2 as a receptor for SFG rickettsial binding. Rickettsial OmpB is the bacterial ligand for this interaction. Endothelial ANXA2 is also involved in establishing *S. aureus* binding to the EC surface. Therefore, endothelial apical surface ANXA2 plays an important role in initiating pathogen–host interactions, ultimately leading to bacterial anchoring on the vascular luminal surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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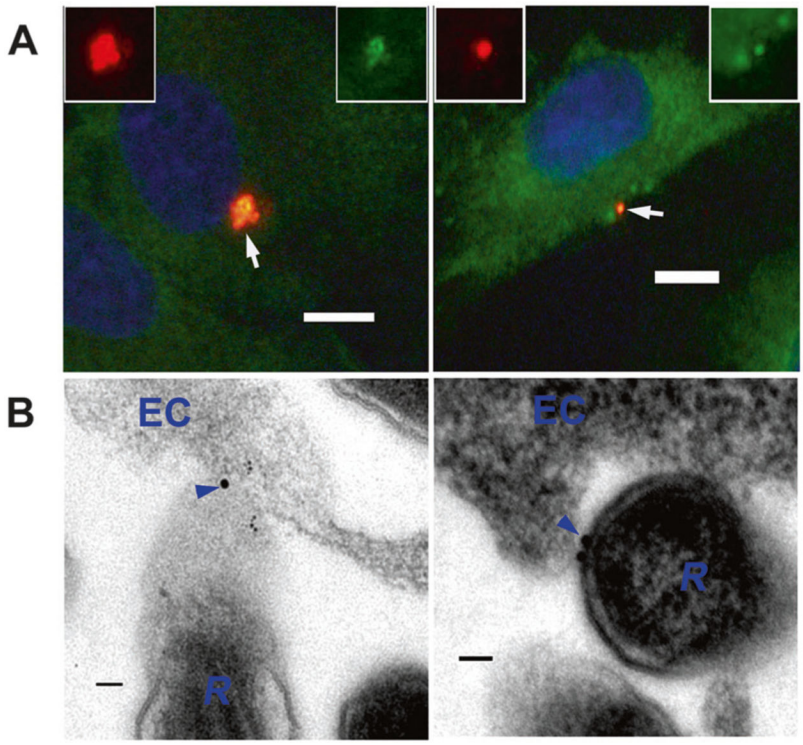

Fig. 1.

ANXA2 is involved in rickettsial attachment to nonphagocytic host ECs. **a** Representative dual-target IF staining of rickettsiae (red) and ANXA2 (green) in HUVECs 15 min post infection (p.i.) with *R. australis* at 10 MOI. HUVECs were fixed with 4% paraformaldehyde as an impermeable fixation. Nuclei of HUVECs were counterstained with DAPI (blue). The areas indicated by the arrows are enlarged and distinguish rickettsial (red) and ANXA2 (green) staining (boxed inserts). Scale bars, 10 μm. **b** Immunoelectron microscopic (IEM) detection of ANXA2 (arrowheads) in HUVECs (EC) infected with *R. australis* (R) 15 min p.i. with *R. australis* at 10 MOI. Scale bars, 100 nm

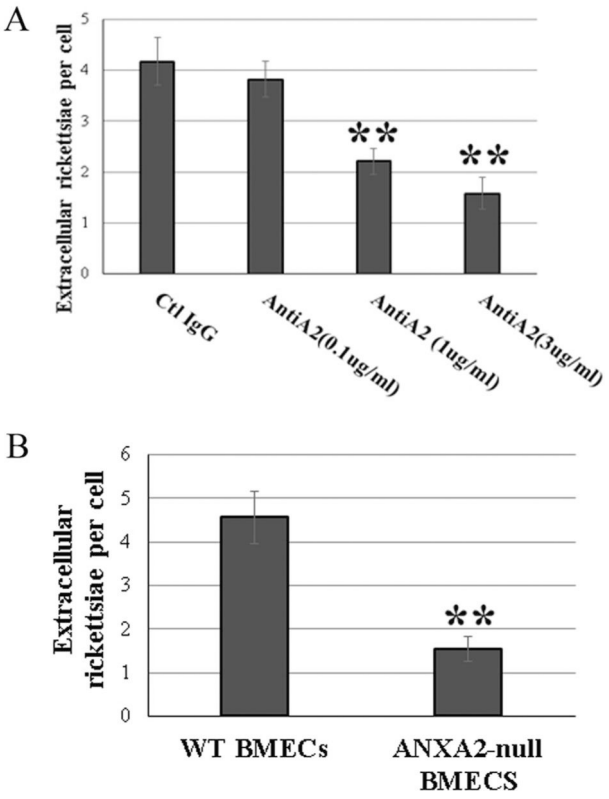

Fig. 2.

Inactivation of ANXA2 impeded rickettsial attachment to the endothelial cells in vitro. Extracellular adhesive bacteria in anti-ANXA2 (AntiA2)-treated HUVECs at 0.1 ($n = 5$), 1 ($n = 10$), and 3 ($n = 5$) μg/ml (**a**) or BMECs from WT ($n = 6$) and Anxa2-null ($n = 6$) mice (**b**) were enumerated by IF microscopy at 15 min p.i. with *R. australis* at 10 MOI. Normal mouse IgG was used as control IgG (Ctl IgG, 3 μg/ml) ($n = 5$). All ECs were fixed with 4% paraformaldehyde as impermeable fixation. Data are represented as mean ± SEM. **compared to the control group, $P < 0.01$

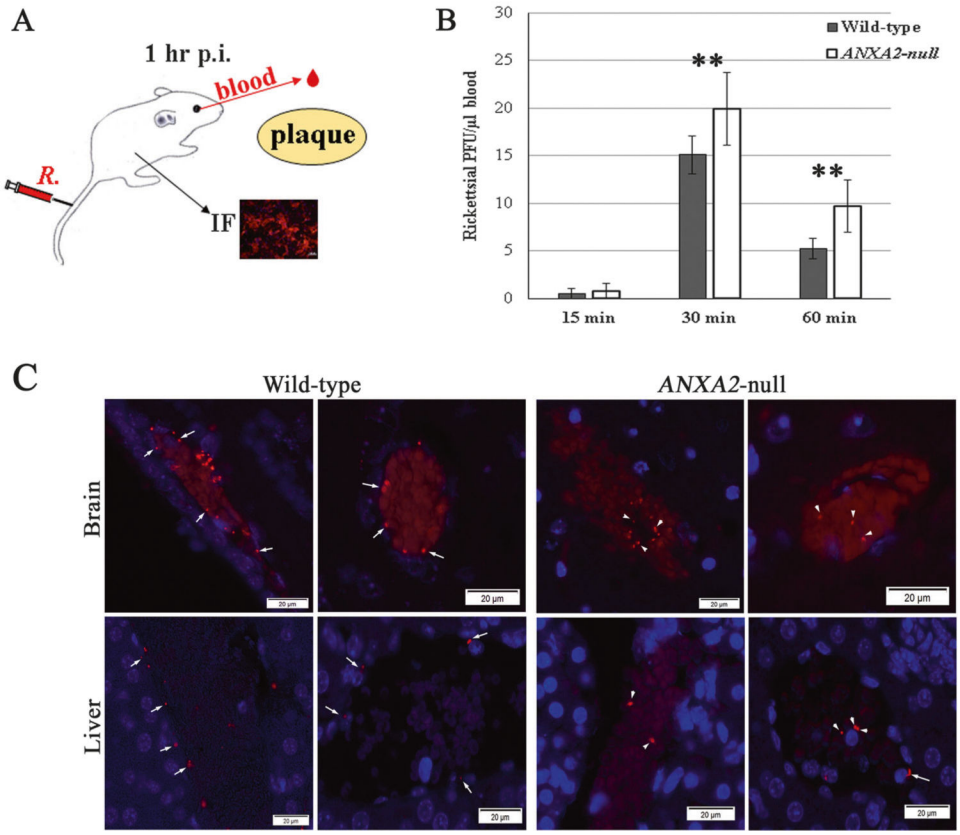

Fig. 3.

Global depletion of *ANXA2* blocked rickettsial adherence to the blood vessel luminal surface in vivo. **a** The anatomically based in vivo quantitative rickettsial (*R.*) adhesion analysis system. **b** Plaque assay for *R. australis* using blood samples collected from the orbital venous sinus of WT ($n = 10$) and ANXA2-null ($n = 10$) mice at different time p. i. with 10 LD50 doses of *R. australis* intravenously. Data are represented as mean ± SEM. **Compared to the control group, $P < 0.01$. **c** Representative IF-based identification of rickettsial (red) location in WT and *Anxa2*-null mice 60 min p.i. with 10 LD50 doses of *R. australis* intravenously. Rickettsiae are adhered to intima layer of blood vessels (arrows) or wrapped in blood clots (arrowheads) in the lumens of blood vessels. Nuclei are blue. Scale bars; 20 μm

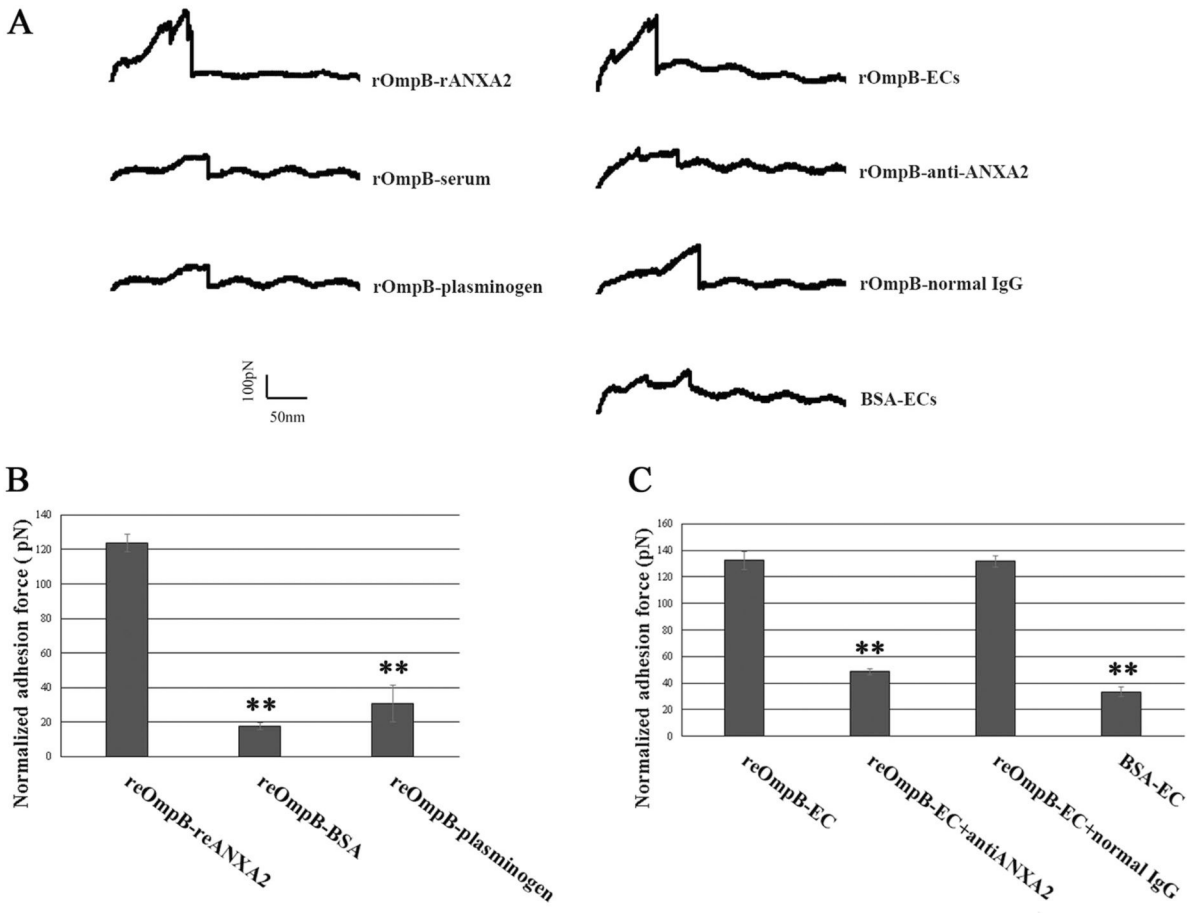

Fig. 4.

a Representative force–distance curves were recorded during protein–protein or protein–cell surface separation using an AFM system. The last unbinding event detected is a measure of the unbinding force of the interaction at single molecule level [62]. **b** AFM studies measured the binding forces between reOmpB and reANXA2, human plasminogen, and BSA, respectively. Data are represented as mean ± SEM. **Compared to the group of reANXA2, $P < 0.01$. **c** AFM studies measured the binding forces between reOmpB and HUVECs in normal media, media supplemented with ANXA2 antibodies or normal IgG. BSA was employed as a control. Each treatment group was measured in static mode operating on 1 × 1 μm areas (1024 data points) of one single living HUVEC for a given period (1 h). Data acquired during this time for a specific area was regarded as one group data. At least five different detection areas on five different living HUVECs were measured in one treatment group in one experiment. Each experiment was studied in triplicate. Data are represented as mean ± SEM. **Compared to the group of normal media, $P < 0.01$

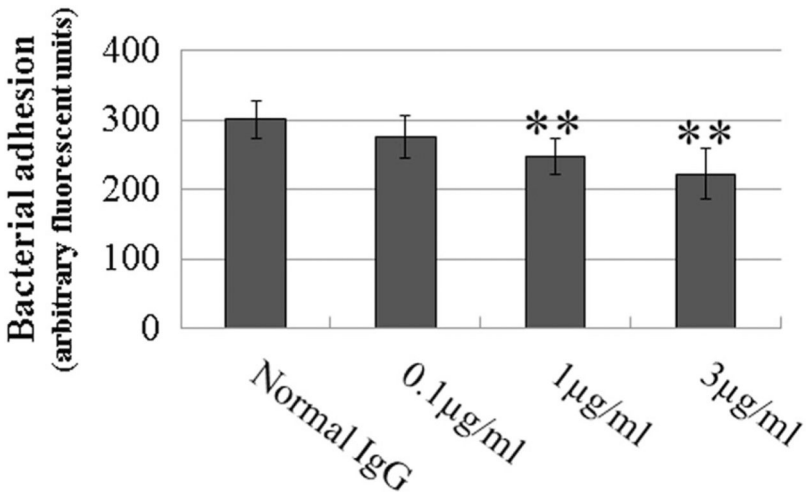

Fig. 5.

Pretreatment of HUVECs with ANXA2 antibody reduced endothelial surface-associated *S. aureus*. Extracellular adhesive FITC-d-Lys-labeled bacteria in anti-ANXA2 antibody-treated HUVECs at 0.1, 1, and 3 μg/ml were detected by a SpectraMax i3× fluorescence plate reader at 15 min p.i. with *S. aureus* at 100 MOI. Normal mouse IgG was used as control IgG (normal IgG, 3 μ g/ml). All ECs were fixed with 4% paraformaldehyde as impermeable fixation. All experiments were repeated three times. There are 35 samples in each group. Data are represented as mean ± SEM. **Compared to the control group, $P < 0.01$