Vitronectin Binds to a Specific Stretch within the Head Region of Yersinia Adhesin A and Thereby Modulates Yersinia enterocolitica Host Interaction

Mühlenkamp, Melanie C.; Hallström, Teresia; Autenrieth, Ingo B.; Bohn, Enwin; Linke, Dirk; Rinker, Janina; Riesbeck, Kristian; Singh, Birendra; Leo, Jack C.; Hammerschmidt, Sven; Zipfel, Peter F.; Schütz, Monika S.

Published in:
Journal of Innate Immunity

DOI:
10.1159/000449200

2017

Document Version:
Peer reviewed version (aka post-print)

Link to publication

Citation for published version (APA):

Total number of authors:
12

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Vitronectin binds to a specific stretch within the head region of Yersinia adhesin A and thereby modulates Yersinia enterocolitica host interaction

Running title: YadA-mediated interaction of Ye with vitronectin

Melanie C. Mühlenkamp*, Teresia Hallström†, Ingo B. Autenrieth*, Erwin Bohn*, Dirk Linke‡, Janina Rinker*, Kristian Riesbeck§, Birendra Singh§, Jack C. Leo‡, Sven Hammerschmidt¶, Peter F. Zipfel†‖ and Monika S. Schütz*

*) Institute for Medical Microbiology and Hygiene, University Hospital Tübingen, Elfriede-Aulhorn-Str. 6, 72076 Tübingen, Germany
†) Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute, Jena, Germany
‡) Department of Biosciences, University of Oslo, P.O. Box 1066 Blindern, 0316 Oslo, Norway
§) Clinical Microbiology, Dept. of Translational Medicine, Lund University, Jan Waldenströms gata 59, SE-205 02 Malmö, Sweden
¶) Department Genetics of Microorganisms, Interfaculty Institute for Genetics and Functional Genomics, Ernst-Moritz-Arndt-Universität, Greifswald, Greifswald, Germany
‖) Friedrich Schiller University Jena

Corresponding author: Dr. Monika Schütz, Institute for Medical Microbiology and Hygiene, University Hospital Tübingen, Elfriede-Aulhorn-Str. 6, 72076 Tübingen, Germany, email: monika.schuetz@med.uni-tuebingen.de, phone: +49-7071-2981527 fax: +49-7071-294972

Keywords: bacterial infection, cell surface molecules, complement, YadA, vitronectin;
ABSTRACT

Complement resistance is an important virulence trait of Yersinia enterocolitica (Ye). The predominant virulence factor expressed by Ye is the Yersinia adhesin A (YadA), which enables bacterial attachment to host cells and extracellular matrix (ECM) and additionally allows acquisition of soluble serum factors. The serum glycoprotein vitronectin (Vn) acts as an inhibitory regulator of the terminal complement complex (TCC) by inhibiting the lytic pore formation. Here, we show YadA-mediated direct interaction of Ye with Vn and investigated the role of this Vn-binding during mouse infection in vivo. Using different Yersinia strains, we identified a short stretch in the YadA head domain of Ye O:9 E40, similar to the “uptake region” of Y. pseudotuberculosis (Yps) YPIII YadA, as crucial for efficient Vn binding. Using recombinant fragments of Vn, we found the C-terminal part of Vn, including the heparin-binding domain (HBD) 3, to be responsible for binding to YadA. Moreover, we found that Vn bound to the bacterial surface is still functionally active and thus inhibits C5b-9 formation. In a mouse infection model, we demonstrate that Vn protects Ye from complement-mediated lysis and thus improved bacterial survival. Taken together, these findings show that YadA-mediated Vn binding influences Ye pathogenesis.
**INTRODUCTION**

*Yersinia enterocolitica* (*Ye*) and *Yersinia pseudotuberculosis* (*Yps*) are enteropathogens causing enteric and systemic diseases [1,2]. Besides the chromosomally encoded adhesins Invasin and Ail [3-5], the trimeric autotransporter adhesin (TAA) YadA is the decisive factor that determines pathogenicity of *Ye* [6]. YadA forms rigid fibrous structures which protrude ~23 nm from the cell surface [7,8] and mediates adhesion to ECM proteins such as collagen, fibronectin and laminin, and to complement factors [9]. Being the prototype of the TAA family of proteins, YadA is characterized by a modular composition of several domains: the extracellularly located N-terminal head domain is followed by a connector element (also called the neck region) leading into a coiled-coil stalk. The stalk is connected to the C-terminal translocator or membrane anchor domain, consisting of 4 β-strands per monomer [9]. To form a functional adhesin on the bacterial surface, three YadA monomers trimerize and form the pore of the translocator domain, which is inserted into the outer membrane [10]. The translocator enables the transport of the passenger domains onto the bacterial surface, where they also form obligate trimers [9].

YadA knockout strains of *Ye* are avirulent and do not cause infection in a mouse infection model [11-13]. This striking effect has been attributed mainly to the reduced efficiency of effector protein (Yop) delivery by a dedicated type three secretion system (T3SS), which requires proper adhesion to host cells and loss of adherence results in the inability to resist phagocytosis [14,15]. However, in *Yps*, which is more closely related to *Yersinia pestis*, YadA is dispensable for virulence and Yop injection [16]. YadA of *Yps* and *Ye* do not only differ in their role during infection, but also in sequence and binding repertoire of host ECM proteins and cellular receptors. YadA of *Yps* carries an additional stretch within its head region that enables entry into host cells [17]. This important stretch is absent in YadA of several *Ye*
serotypes and strains. Moreover, the binding capacities of YadA differ between Ye, which binds collagen and laminin, and Yps, which binds fibronectin [18].

By interacting with several complement factors, serum resistance is an important virulence trait of Ye. It has been shown that factor H, C4b-binding protein (C4BP) and C3 bind to the YadA stalk domain and thus inhibit complement killing [19,20]. Recently, we demonstrated a novel mechanism that contributes to serum resistance in Ye O:8 WA-314 and amended the current model of direct factor H-binding to YadA\(^{0.3}\) and YadA\(^{0.9}\). We have shown that Ye binds C3b or iC3b and thereby attracts high amounts of factor H to the bacterial surface [21]. This is different to the direct binding of factor H, which was shown earlier [19,20,22]. Importantly, by binding these complement regulatory factors, Ye is able to interfere with complement activity by inhibiting complement-mediated killing at an early stage of the cascade.

The human glycoprotein Vn is synthesized in the liver and secreted into plasma [23], where it is present as a monomer (65 and 75 kDa) at high concentrations (200 – 400 µg/mL) [24]. Vn also exists as an extravascular cell-bound multimeric form in several tissues, and Vn mRNA can be detected in high concentrations in the liver, brain, heart, and adipose tissue but is rare or absent in the kidney and spleen [25]. It comprises an N-terminal somatomedin-binding domain (SMB) consisting of 43 amino acid (aa) residues, followed by the host cell integrin receptor-binding motif RGD (Arg-Gly-Asp). In addition to four hemopexin-like domains with unknown function, Vn also contains 3 HBDs which span aa 82-137 (HBD-1), aa 175-219 (HBD-2) and aa 348-361 (HBD-3) [26,27]. Vn is an important regulator of complement activity at the level of TCC formation, a component of the ECM and also fulfills functions in cell migration and tissue repair [27].

At the level of TCC formation, Vn regulates complement activity by directly binding to the protein complex C5b-7 or to C9 [28]. The exact mode of regulation is not fully understood. It
has been postulated, however, that Vn binds the nascent precursor complex C5b-7, resulting
in a Vn-C5b-7 complex that is unable to insert into the cell membrane [27,28]. Vn can also
directly bind C9 and thereby inhibit C9 polymerization. This binding takes places through
HBD-3, whereas the binding site for the nascent C5b-7 is still unknown [27-29].

A wide variety of bacteria bind Vn via various surface proteins. The respiratory pathogens
Moraxella catarrhalis (Mc) and Haemophilus influenzae (Hi) as well as the urogenital
pathogen H. ducreyi express proteins belonging to the TAA family. These proteins are the
ubiquitous surface protein A2 (UspA2) of Mc, the Haemophilus surface fibrils (Hsf) and the
Haemophilus adhesin (Hia) of Hi or the H. ducreyi serum resistance protein A (DsrA) [9,30-
36]. In the invasive bacterial pathogen Neisseria meningitidis the three proteins Opc, Opa and
Msf interact with Vn [37-40]. However, to date no enteropathogenic bacteria have been
described to use Vn to escape complement-mediated attack and thus mediate serum
resistance.

Ye has evolved a multitude of mechanisms in order to evade the host immune system.
Amongst these, serum resistance is of uttermost importance. The significance of the
complement regulator Vn in complement evasion and modulation of host cell interaction with
bacterial and fungal pathogens has recently been recognized [27,30-32,37,39-44]. Ye is able to
bind several regulators of complement activity; however, the role of Vn in the Ye-host cell
interaction and pathogenicity has not yet been addressed in detail, but it was shown in
previous studies that YadA from Ye O:8 does not bind Vn under stringent assay conditions
[45]. In this study, we systematically investigated i) Vn binding of different Ye strains, ii)
which components of Ye might enable this binding and iii) how this interaction modulates Ye
serum resistance, host cell interaction and overall pathogenicity. Importantly, we were able to
demonstrate a novel mechanism facilitating Ye serum resistance mediated by the surface
adhesin YadA binding to Vn. We found that subtle differences within the YadA head domain
of different Yersinia strains determine the efficacy of the Vn binding. An additional stretch in Ye YadA<sup>O:9</sup>, which is similar to the “uptake region” of Yps YadA<sup>YPIII</sup> [18], was identified as a crucial region for high affinity binding of Vn. Moreover, we located HBD-3 within Vn as the YadA-binding site. Notably, bound Vn is active on the bacterial surface and protects bacteria from complement-mediated lysis by inhibition of C9 polymerization. This mechanism allows enhanced survival of Ye O:9 E40 during early phase of a mouse infection <i>in vivo</i>.
MATERIALS AND METHODS

Mice

C57BL/6 wild-type mice were purchased from Harlan Winkelmann (Horst, Netherlands). B6.129S2(D2)-Vtn<sup>tm1Dgi</sup>/J mice (http://jaxmice.jax.org/strain/004371.html) with a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, USA). All mice were bred under specific pathogen-free conditions in individually ventilated cages with access to water and food ad libitum. Experiments were performed with six to eight-week-old female mice according to German law with permission of the Regierungspräsidium Tübingen (permission number H4/15).

Plasmids

Plasmids used in this study are listed in Table 1. [46,47]

Bacterial strains and culture conditions

All Yersinia strains were cultivated in lysogeny broth (LB) medium with supplements (antibiotics as indicated in Table 2) overnight at 27°C. To promote YadA expression, a 1:20 dilution of the overnight culture was made with fresh medium and incubated for 3h at 37°C. Moraxella strains were grown overnight at 37°C in brain-heart infusion (BHI) medium. All bacteria were washed twice with PBS and subsequently the optical density at 600 nm was determined. The number of bacteria used for the individual experimental set ups are indicated in the respective sections. All bacterial strains used in this study are listed in Table 2. [48-53]

Serum

Normal human serum (NHS) was collected from at least 4 healthy volunteers and pooled. Aliquots were stored at -80°C and thawed only once. Heat inactivated serum (HIS) was generated by incubating at 56°C for 30 min immediately before use.
Antibodies

Antibodies used in this study are listed in Table 3.

Purified proteins used in this study

Purified monomeric and multimeric Vn was purchased from BD Bioscience (Heidelberg, DE) and Millipore (Schwalbach, DE), respectively. Vn fragments were expressed and purified as previously described [35,54].

Binding assay with serum or purified proteins analysed by flow cytometry

To analyse binding of purified Vn or Vn and factor H from HIS, a total of $1 \times 10^7$ bacteria per assay were incubated with 5-50 % HIS (as indicated in figures and/or figure legends) or purified Vn (1-10 µg/ml) diluted with PBS (Life Technologies, Darmstadt, DE) in a total volume of 100 µl for 30 min at 37°C. As an internal control, each strain was also treated with PBS only. Recombinant Vn fragments were used at 4 µg/ml. After washing with 1 % BSA in PBS (washing buffer), bacteria were spun down and the pellet was resuspended in 200 µl 4 % paraformaldehyde (PFA) in PBS for 1 h at room temperature. Bacteria were washed once again and finally incubated with primary polyclonal antibodies (pAb) directed against Vn or factor H overnight at 4°C. The next day, bacteria were washed once and incubated with suitable secondary antibodies for 1 h at room temperature. After a final washing step bacteria were transferred to FACS tubes and analysed with a Fortessa LSR II instrument. Data analysis was carried out using WinMDI version 2.8. The PBS only control was used to determine background staining using the same primary and secondary antibodies as for all other samples. Values obtained for the control samples were subtracted from the values obtained for the corresponding samples that were incubated in serum or purified Vn. All flow cytometry figures show background subtracted values.
Detection of Vn-binding or YadA expression by Western blot

To analyse Vn binding by immunoblotting, 5x10^8 bacteria (bacterial numbers were determined photometrically by measuring the optical density at 600 nm; a volume corresponding to the desired number of bacteria was harvested by centrifugation and the bacterial pellets were then used to carry out the assay) were incubated in 100 µl 50 % HIS diluted in PBS as described above. Thereafter, bacteria were washed twice with washing buffer, once with PBS and finally resuspended in 50 µl deionized water. For detection of YadA, bacteria were simply washed after harvest. After addition of 25 µl 4x Laemmli buffer (Bio-Rad Laboratories, München, DE), samples were boiled for 5 min at 95°C and separated in a 10 % acrylamide SDS gel (Bio-Rad Laboratories, München, DE). Each lane was loaded with an equal number of bacteria. After blotting, membranes were blocked with 3 % BSA, 5 % milk powder in TBS for 1 h at room temperature. Membranes were then incubated with desired antibodies (a complete list of antibodies and working dilutions is given in Table 3) for 1 h at room temperature or at 4°C overnight, washed with 0.1 % TBS-T and then incubated with the suitable secondary antibody (Table 3). Fluorescence signals were recorded using a LICOR Odyssey imaging system.

Detection of Vn-binding by Blot Overlay Assay

Bacterial lysates were prepared as described above, separated by SDS-PAGE and blotted. After blocking with 5 % milk, 3 % BSA in PBS for 3 h at room temperature, the membrane was incubated with 7 µg/ml purified monomeric Vn in 3 % BSA in PBS-T overnight at 4°C. After washing with 0.1% TBST, Vn was detected with rabbit anti-Vn pAb and a secondary DyLight 680-conjugated goat-anti-rabbit pAb. Fluorescence signals were recorded using a LICOR Odyssey imaging system.
Purification of DNA from Yersinia colonies

Yersinia strains were streaked on LB agar plates without antibiotics. Next day a single colony was used for DNA extraction using the Qiagen QIAmp DNA Mini kit according to the manufacturers protocol. DNA was finally eluted in 100 µl ultrapure water.

PCR amplification of the YadA head region

To test Yersinia YadA for the presence of the additional stretch (enabling recruitment of Vn) within its head region, we used the primers YadA_Seroseq_435F (5’-gatcagtgtctctgcgccat-3’) and YadA_Seroseq_435R (5’-gccccataagtaactgcgcga-3’) that bind to highly conserved regions up- and downstream of the uptake region (see Fig. S1). According to the sequence alignment the PCR reaction should yield a fragment of 442 bp with Ye O:9 E40 or 451 bp with Yps YIII (both harboring the uptake region of ~ 90 bp) or 337 bp with Ye O:8 WA-314, 346 bp with Ye O:3 6471/76 and Ye O:5,27 (all three lacking the uptake region) and thus allow us to discriminate between YadA with and without “uptake region”. 1 µl of DNA was used as template. The PCR program we used was the following: 2 min 95°C (initial denaturation), 30 sec 95°C→1 min 55°C→ 30 sec 68°C (repeated 29x), 5 min 72°C (final extension) and cooling at 4°C until further processing.

Separation of PCR products by capillary gelelectrophoresis

To determine the size of PCR products they were analysed using a QIAxcel capillary gelelectrophoresis system according to manufacturer’s protocol.

DNA sequencing

PCR products were purified using the Promega Wizard® SV Gel and PCR Clean-Up System according to the manufacturer’s protocol. Subsequently Sanger sequencing was performed by GATC using the same primers as for the PCR reaction.
Sterile glass coverslips were coated with purified Vn (10 µg/ml) at 4°C overnight and air-dried. Coverslips were then placed in a 24-well plate and either incubated with PBS or 100 µM Heparin in PBS. 5x10⁷ bacteria (Ye O:9 E40 pBla EGFP) were added to each well, spun down for 5 min at 300 g and incubated for 1 hour at 37°C in humidified atmosphere. Afterwards, the supernatant was removed, cells were washed two times and finally fixed by the addition of 4 % PFA in PBS. After washing, slides were mounted in Mowiol and micrograph pictures were acquired using a ZEISS LSM 510. To quantify adhesion, the number of bacteria for a given field of view (representative for the entire coverslip) was counted.

**Analysis of C5b-9 deposition by flow cytometry**

To analyse whether bound Vn was functionally active, bacteria were incubated with Vn (10-50 µg/ml) or C4BP (10-50 µg/ml) for 30 min at 37°C. After washing, bacteria were incubated with C5b-6 (1 µg/ml) and C7 (1 µg/ml) for 10 min and thereafter C8 (0.4 µg/ml) and C9 (1 µg/ml) were added for 30 min at 37°C. All complement components except for Vn were from Complement Technology (Tyler, TX, USA). Deposited C5b-9 was detected by mouse anti-human C5b-9 mAb followed by Alexa fluor®-647-conjugated goat anti-mouse pAb. After two additional washes, bacteria were analysed by flow cytometry (EPICS XL-MCL; Coulter, Hialeah, FL). All incubations were kept in a final volume of 100 µl 1 % BSA in PBS and washes were performed with the same buffer. Primary and secondary pAb were added separately as negative controls for each strain analysed.

**In vitro serum killing assay**

To analyse the susceptibility of Ye and Yps against complement-mediated killing in human serum, 5x10⁶ bacteria were incubated in 100 µl 20 % NHS or HIS for 30 min at 37°C.
Complement activity was stopped by adding 100 µl BHI medium and placing the samples for 5 min on ice. Afterwards, a serial dilution of the samples were prepared, plated on selective agar plates and incubated at 27°C for 48 h. The colony forming units (CFU) were determined. The serum bactericidal effect was calculated as the survival percentage, taking the bacterial counts obtained with bacteria incubated in HIS as 100 %.

**In vivo serum killing assay**

To analyse lytic activity of serum complement against Ye in C57BL/6 and B6.129S2(D2)-Vtn^tm1Dgi/J mice, animals were infected intravenously with 1x10^7 bacteria. After 30 min, mice were sacrificed by CO₂ asphyxiation and blood was withdrawn from the heart. Heparin (100 µl at 100 µg/ml) (Sigma-Aldrich, Steinheim, DE) was mixed with the blood to avoid coagulation. Serial dilutions of the samples were plated on selective agar and incubated at 27°C for 48 h. The CFU were determined by counting the colonies.

**Bioinformatics and statistical analysis**

The GI numbers or the references of the sequences used in this work are listed in Table 2. Alignments were produced with Kalign or Muscle, and further edited manually [55,56]. Data are means ± SD and were analysed with the Student t test, or with one-way ANOVA for multiple comparison as described in the figure legends. GraphPad Prism 6.0 was used to analyse the data (GraphPad Software, La Jolla, CA). Differences were considered significant when p ≤ 0.05. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
RESULTS

*Yersinia enterocolitica* O:9 E40 efficiently binds Vn

Vn plays an important role in the complement resistance of *e.g.* *Mc, Hi* and *Streptococcus pneumoniae* [32,34,44,54,57]. In order to test if *Ye* is able to bind Vn and if there are differences in the binding capacity of various *Ye* strains and serotypes, we incubated a set of strains in 50% HIS, washed the cells and detected Vn bound to bacteria by immunostaining with antibodies directed against Vn and subsequent flow cytometry analysis (Fig. 1A). Upon incubation with HIS we found very diverse binding properties of *Ye* strains compared to *Mc RH4* and *Yps YPIII*. *Mc RH4* served as a positive control [30,34], whereas *Yps YPIII* was used as an additional comparator. It has been recognized earlier that *Yps YPIII* YadA differs from YadA sequences of other strains and that this difference coincides with a change in preferred ECM binding partners and this might possibly also affect interaction with Vn (*Yps YPIII* YadA preferentially binds to Fibronectin instead of collagen and laminin as observed with *Ye*) [18]. *Ye O:9 E40* was able to bind exceptionally high amounts of Vn which led to a mean fluorescence intensity ~2.8 times higher compared to that measured with *Mc RH4* (133.9 ± 33.9 vs. 47.4 ± 19.6). Also *Yps* bound Vn, but at concentrations comparable to that of the *Mc RH4* positive control (56.7 ± 11.0 vs. 47.4 ± 19.6). *Ye O:8 WA-314* and *Ye O:3 6471/176* also bound Vn, though to a lesser extent compared to *Mc RH4* (~54.1 or 70.7% of *Mc RH4* signal). *Ye O:8 8081* bound only residual amounts of Vn (6.7 ± 0.6). Interestingly, the binding of Vn to *Ye O:9 E40* depended on the presence of the plasmid of *Yersinia virulence (pYV)* and was dose-dependent (Fig. 1C and D). In a plasmid-deficient strain (*Ye O:9 E40 ΔpYV*) Vn binding was almost abolished (6.2 ± 2.8). To test if the strain-specific binding-pattern of Vn (*O:9 E40 > YPIII > RH4 > O:3 > O:8 WA-314 > O:9 E40 ΔpYV = O:8 8081*) is exclusive compared to other serum factors, we also tested the binding of factor H (Fig. 1B). Factor H has been shown to interact with several discontinuous stretches within the
stalk domain of YadA [20-22,58]. Our data corroborate previous findings that binding of factor H by Yersinia strains relies on the presence of YadA, but in contrast to Vn, there is no significant difference in binding efficiency in the various serotypes tested. This indicates different mechanisms of binding of Vn and factor H. Taken together, we found that Ye O:9 E40 is able to bind high amounts of serum-derived as well as purified Vn but only in the presence of the pYV plasmid, in a dose-dependent manner. In contrast, Ye O:8 WA-314, 8081 and Ye O:3 6471/76 are weak Vn binders, although they also carry the pYV plasmid. This partially substantiates earlier findings that YadA-dependent Vn binding is at least weak if not non-existent for Ye O:8 WA-314 in whole-cell adhesion assays under specific flow conditions [45].

**Binding of Vn is YadA-dependent**

In order to assess whether YadA is the determinant for binding of Vn to Yersinia we used flow cytometry to compare Vn binding in a Ye O:9 E40 wildtype strain (WT), a mutant deficient for YadA (ΔYadA), a mutant deficient for the chromosome-encoded adhesin Invasin (ΔInv), the corresponding double mutant (ΔInvΔYadA; ΔΔ), and again the cured strain lacking the pYV plasmid (ΔpYV) (Fig. 2A left panel). We used Mc RH4 as positive control and a Mc ΔUspA2H [32] knockout strain as a negative control (Fig. 2A right panel). Our data indicate that the presence of YadA, but not of Inv, is decisive for binding of Vn to Ye O:9 E40. Thus, in contrast to Ye O:9 E40 wildtype or ΔInv, Vn did not bind to ΔYadA, the ΔInvΔYadA double mutant or the pYV-cured strain. We could corroborate these findings by blot overlay assays and Western blot (Fig. 2B, C). Analysis of the influence of YadA and more specifically a distinct region within YadA of Yps for Vn binding revealed that also in Yps, YadA is the Vn binding determinant (Fig. 2A middle panel). Moreover, the deletion of 30 aa (Δ53-83) corresponding to the “uptake region” in the head domain of Yps YadA VPIII abolishes Vn binding (Fig. 2A middle panel). Thus, our data demonstrate that YadA is
essential for mediating Vn binding in Ye and that a stretch of 31aa within the head region of
YadA<sup>YPIII</sup> is decisive for binding of Vn in Yps.

A specific stretch within YadA discerns Vn-low-binding from Vn-high-binding

We found that Ye expressing YadA derived from the O:8 WA-314 strain is a relatively weak
binder compared to Ye O:9 E40 (Fig. 1A). Therefore, we aimed to determine if also other
strains carry the uptake region and also what actually discerns YadA<sup>O:9 E40</sup> from YadA<sup>O:8</sup> and
if this difference might be causative for the discriminative Vn binding behavior. The head
domain of YadA<sup>YPIII</sup> contains a stretch of sequence (“uptake region”), which is crucial for cell
adhesion and efficient internalization of <i>Yersinia</i> via YadA [18]. This motif is absent in YadA
of Ye O:8 but present in the Ye O:9 E40 strain (aa 56-88) (Fig. 3A). It is rich in prolines and
charged residues, suggesting an undefined loop structure (Fig. 3B), inserted in a shorter loop
that is not resolved in the crystal structure of the Ye O:3 YadA head (PDB: 1P9H) [59].
To investigate whether this motif is present exclusively in Ye O:9 E40 or can be found also in
other <i>Yersinia</i> strains and especially strains isolated from clinical specimens we carried out
PCRs. We designed primers binding to rather conserved regions within the YadA sequence
flanking that part of the head domain which comprises the uptake region (Fig. S1). The size
of the PCR products allowed us to easily detect the presence of the uptake region. Predicted
lengths of the YadA head fragments were: 346 bp (Ye O:3), 337 bp (Ye O:8), 346 bp (Ye
O:5,27), 451 bp (Ye O:9) and 442 bp (Yps YPIII). Strikingly, the additional stretch present in
YadA of Ye O:9 E40 and Yps YPIII was present in all tested clinical isolates of serotype O:9,
but absent in all other strains (belonging to the indicted serotypes; Fig. 3C) we tested. Ye O:9
E40 <i>Δ</i>YadA and water control were included as negative controls (Fig. 3C). All strains
depicted in (C) were also tested for Vn binding. Cell-surface associated Vn after incubation in
HIS was quantified by flow cytometry (Fig. 3D). Whereas all strains belonging to serotype
O:9 (No. 08-14) and possessing the uptake region within YadA bound Vn in comparable high
amounts as Ye E40 O:9, Ye strains of serotype O:3 (No. 01, 02, 03), O:8 (No. 04) and O:5,27
(No. 06, 07) turned out to be rather weak binders. Thus we assume that the presence of the
uptake region is the major determinant that allows binding of Vn and (at least in the strains we
have tested) is present exclusively in YadA of Ye strains of serotype O:9.

To test this hypothesis, we generated a YadA hybrid where we replaced the N-terminus of the
head domain of YadA<sup>O:8</sup> by that of YadA<sup>O:9 E40</sup> (including the uptake region) and a YadA<sup>O:9</sup>
E40 deletion mutant lacking the uptake region (aa 56-88) (Fig. 4A). We then compared Vn
binding by flow cytometry. The strains Ye O:9 ∆YadA expressing YadA<sup>O:8 WA-314</sup> or YadA<sup>O:9</sup>
E40 were also included in this analysis. In addition, we used Ye O:8 WA-314, Ye O:9 E40 and
Ye O:9 E40 ∆YadA as controls (Fig. 4B). Ectopic expression of YadA<sup>O:9 E40</sup> was able to
rescue Vn-binding of Ye O:9 E40 ∆YadA. This was also true for the O:9/O:8 hybrid YadA.

Additionally, deletion of the “uptake region” from YadA<sup>O:9</sup> led to significantly reduced Vn
binding (Fig. 4B). Our data show that the “uptake region” of YadA<sup>O:9 E40</sup> significantly
enhances recruitment of Vn. Of note, a sequence alignment of YadA from different Yersinia
strains revealed also insertions in the stalk regions of Ye YadA<sup>O:9 E40</sup> and YadA<sup>O:3 6471/76</sup> that
are not found in YadA<sup>YPIII</sup> and YadA<sup>O:8 WA-314</sup> (Fig. S2). However, these regions show no clear
association with Vn or factor H binding (Fig. 1A). Finally, we wanted to assess whether
cofactors expressed by Yersinia are necessary or if YadA containing the “uptake region”
alone is sufficient to mediate efficient binding of Vn. We tested Vn binding of E. coli omp2
[60] which ectopically expressed the YadA version described above (Fig. S3). We found that
expression of YadA<sup>O:9</sup> or the hybrid YadA<sup>O:9/O:8</sup> is sufficient to mediate binding of Vn. Thus
we conclude that the decisive factor for Vn binding is YadA comprising the “uptake region”.

Vn interacts with YadA via its C-terminal heparin-binding-domain 3 (HBD-3)

Previous work with Mc and Hi revealed HBD-3 as the decisive part of Vn for interaction with
UspA2 or Hsf [34,35]. Therefore, we wanted to know if this domain might also mediate the
interaction of Vn with YadA. In order to test this we first analysed whether Heparin might
block the binding of Ye to Vn by occupying the HBDs. This would be a clear indicator for the
involvement of one of the HBDs in the interaction with YadA. Coverslips were coated with
Vn and afterwards incubated with Ye O:9 E40 (expressing enhanced green fluorescent protein
(eGFP) for easier detection of binding) either in the presence or absence of Heparin.
Thereafter coverslips were washed, fixed, mounted and analysed by fluorescence microscopy
(Fig. 5A). Our results demonstrate that, in the presence of Heparin, binding of bacteria to Vn-
coated coverslips is significantly reduced. Therefore, we conclude that at least one of the
HBDs is involved in mediating the binding of Vn to YadA0:9 E40.
To locate the sites within Vn that actually determine YadA binding, we used a set of
recombinant Vn fragments (Fig. 5B). These fragments essentially comprise C-terminally
truncated Vn molecules as well as deletion mutants lacking parts of HBD-3 (comprising aa
348-361) or adjacent regions. All fragments were tested for appropriate quality (Fig. S4). Our
binding assay (Fig. 5C) demonstrates that the fragments Vn 80-396, 80-379, 80-373 and 80-
363 are efficiently bound by Ye O:9 E40. However, further C-terminal truncation comprising
either parts or the entire HBD-3 (80-353, 80-339) led to a reduction of binding. Fragments
lacking the entire HBD-3 plus the adjacent N-terminal region (80-330, 80-229) bound only
weakly to Ye O:9 E40 (Fig. 5C). Thus, we assume that not only HBD-3 but also the adjacent
N-and especially the C-terminal ~10-20 aa are important for a stable interaction of Vn with Ye
O:9 E40. These findings are in agreement with the fact that also a Vn molecule lacking the C-
terminal part of HBD-3 plus the adjacent C-terminal region (∆352-374) is impaired in binding
to Ye O:9 E40, whereas deletion of either only part of HBD-3 (∆352-362) or the adjacent C-
terminal region only (∆362-374) does not significantly influence binding. In conclusion, aa
331-363 are decisive for stable interaction of Vn with Ye O:9 E40.
Vn is functionally active and inhibits the terminal pathway when bound to the surface of Ye

Besides modulating the adhesive properties of pathogens, Vn regulates the terminal complement pathway and blocks TCC formation. In order to test if Vn bound to Ye is functionally active and inhibits the terminal complement pathway, we assayed C5b-9 deposition in the presence of Vn bound to intact bacteria. To this end, Ye O:9 E40 was preincubated with Vn or C4BP followed by addition of C5b-6, C7, C8 and C9. C5b-9 deposition was determined by using an anti-C5b-9 mAb and flow cytometry.

We clearly demonstrate that Vn bound to the surface of Ye O:9 E40 was functionally active and inhibited C5b-9 deposition in a dose-dependent manner (Fig. 6A, B). Vn (50 µg/ml) inhibited C5b-9 deposition by 61%. C4BP, the C3 convertase inhibitor of the classical/lectin pathways, did not influence the C5b-9 deposition and thus the terminal pathway. From this we conclude that Vn when bound to intact Ye is functionally active and inhibits the terminal complement pathway and C5b-9 deposition.

Binding of Vn decreases the susceptibility to complement mediated killing by human serum

YadA-mediated serum resistance is an important virulence trait of Ye [21,61,62]. To analyse the importance of Vn binding for preventing complement mediated killing, we performed serum killing assays. We incubated Ye O:9 E40, the corresponding YadA-deficient mutant (ΔYadA) and Ye O:8 WA-314 in normal human serum (Fig. 7A, “control strains”). Their survival was calculated as the survival percentage, taking the bacterial counts obtained with samples incubated in HIS as 100%. Our data show that Ye O:9 E40 – a strong Vn binder - is resistant to complement-mediated killing (% survival in NHS compared to HIS 119.1 ± 40.39) whereas the Ye O:9 E40 ΔYadA mutant strain was highly susceptible for killing by the complement system (16.74 ± 9.83). Compared to Ye O:9 E40, the weak Vn binder Ye O:8 WA-314 was significantly more susceptible to complement-mediated killing (39.21 ± 7.11)
compared to Ye O:9 E40 (Fig. 7A). Furthermore, we also tested Ye O:9 E40 ΔΔ expressing either YadA<sup>O:9</sup>, YadA<sup>O:9/O:8</sup>, YadA<sup>O:9 Δuptake region</sup> or YadA<sup>O:9</sup> for serum resistance. We found that the expression of YadA<sup>O:9</sup> (103.6 ± 3.42) and also of the O:9/O:8 hybrid YadA (105.7 ± 27.56) conferred serum resistance comparable to that of the Ye O:9 E40 wildtype strain. In contrast the serum survival was significantly reduced upon expression of YadA<sup>O:8</sup> (48.55 ± 9.36). Compared to all these strains, a strain expressing the YadA O:9 lacking the “uptake region” showed the greatest sensitivity towards serum treatment (16.8 ± 7.57). These data clearly indicate that the YadA-dependent binding of Vn plays an important role in preventing the lysis of Ye by the complement system.

**Mice deficient for Vn expression eliminate Ye more rapidly in short-term systemic infection**

It is known that YadA is decisive for survival of Ye upon contact with serum [6,12]. This is one reason why YadA-deficient strains of Ye are avirulent in the mouse model [12]. However, the contribution of YadA-dependent recruitment of Vn to survival of Ye in a mouse model has not been addressed so far. In order to test if the presence of Vn has an influence on the survival of Ye in vivo we infected Vn<sup>-/-</sup> and wildtype mice with Ye O:9 E40, sacrificed mice 30 min post-infection and determined the bacterial burden in the blood. We found that the bacterial load in the blood was significantly reduced (log<sub>10</sub> CFU per g blood = 2.7 ± 0.8) for the Vn<sup>-/-</sup> mice compared to wildtype mice (log<sub>10</sub> CFU per g blood = 4.2 ± 1.0) (Fig. 7B). In line with the reduction of C5b-9 deposition on Ye by Vn, these data would suggest that Vn protects Ye from early killing in the blood stream.

Compared to YadA of Ye O:9 E40, the YadA of Ye O:8 WA-314 shows low Vn binding capacity. Therefore, we hypothesized that due to this low Vn binding capacity and in contrast to our findings with Ye O:9 E40, the availability of Vn should only marginally impact the outcome of an early bloodstream infection with the Ye O:8 WA-314 strain. However, since the Ye O:9 and O:8 strains exhibit additional differences with regards to sequence and also
virulence mechanisms [63-66], this experiment may not solve the question whether the uptake region actually contributes to better clearance of infection by mediating more efficient binding of Vn specifically. Therefore, we used a slightly different approach. To clearly assess the role of the uptake region and to exclude that other differences between the Ye O:9 and the Ye O:9 strain tamper the result of our experiments we infected mice with Ye harboring pYadA^{O:9/8} hybrid or pYadA^{O:8} in the same strain background (Ye O:9 E40 ΔΔ). The basic sequence of the YadA of these strains is identical with exception of the part encoding the uptake region. Surprisingly, the infection of C57BL/6 wildtype or Vn^{−/−} mice with Ye O:9 E40 ΔΔ + pASK-IBA4c_yadAO:8 led to a small but significant difference in bacterial counts (Fig. S5A; 5.9 ± 0.3 log_{10} CFU per g blood in wildtype mice vs. 6.4 ± 0.3 in Vn^{−/−} mice). As observed previously with Ye O:9 E40, also infection with Ye O:9 E40 ΔΔ harboring pASK-IBA4c_yadAO:9/O:8 hybrid revealed a significantly reduced bacterial load in the blood for the Vn^{−/−} mice (log_{10} CFU per g blood = 4.9 ± 0.2) compared to wildtype mice (log_{10} CFU per g blood = 5.5 ± 0.2) (Fig. S5B). This leads to the assumption that the binding of Vn to different regions of YadA may have various implications on YadA function. While binding of Vn to the uptake region seems to increase virulence, binding of Vn to other regions of YadA might also reduce virulence.

**DISCUSSION**

Complement inhibitor recruitment by bacterial cell surface proteins and adhesins is an important virulence mechanism used by many pathogens. Accordingly, several complement regulators (factor H, Factor H like protein-1, C4BP) and complement proteins (C3b, iC3b) have been identified that interact with the Gram-negative enteropathogen Ye [19-22,61,62,67]. Here, we describe a novel mechanism that contributes to Ye complement resistance and overall virulence of Ye. We show that the TAA YadA of different *Yersinia* species binds Vn and we demonstrate that especially a part of the YadA head domain of YadA^{O:9 E40} comprising...
aa 56-88 binds Vn with high efficiency. Recruitment of Vn to YadA led to reduced surface formation and deposition of C5b-9 (TCC) and thus enhanced complement resistance. Moreover, Ye O:9 E40 was completely resistant to complement mediated killing in human serum in contrast to the YadA-deficient strain. In addition, it turned out that in comparison to Ye O:8 WA-314, Ye O:9 E40 is significantly more serum resistant. Using Vn-deficient mice we were also able to demonstrate reduced survival of Ye O:9 E40 in the absence of Vn in an in vivo serum killing assay. Thus, binding of Vn to the surface of Ye has great impact on the interaction of Ye with the host.

In our experiments, we found that different strains of Ye and Yps bind Vn in a YadA-dependent manner although different Yersinia strains exhibited divergent Vn binding capacities. Previous studies with different Mc wildtype strains show that Mc also binds Vn with different affinities via UspA2 [30]. The N-terminus of the UspA2 head domain sequence displays two different conserved regions that may explain these Vn-binding differences [68]. Furthermore, we show for the first time that Ye strains of serotype O:9 - unlike all other Ye strains we tested - exhibit an additional stretch in their YadA head domain. These strains, and to a lesser extent Yps YPIII, showed high-affinity binding to Vn while other tested Ye strains showed only low-affinity binding. Unfortunately, we were not able to correlate the ability to bind Vn and the pathogenic potential of clinical isolates due to the low frequency of Ye infection (and thus available isolates) and the fact that systemic infection with Ye happens only on rare occasions. The stretch in YadA^{O:9} is highly similar to the uptake region described for Yps YPIII [18], which is important for the ability of YadA to promote invasion of Ye into host cells. Yps binds preferentially to fibronectin, but has low affinity for laminin or collagen type I, which is in contrast to the ECM protein binding capacity of Ye which preferentially associates with collagen type I and laminin. This indicates that the uptake region may modulate the overall affinity to different ECM proteins. Sequence comparison of YadA^{O:9} E40
also revealed additional amino acid stretches in the YadA stalk domain lacking in some other Ye strains. However, comparison of the Vn binding capacity of different Ye and Yps strains show no clear indication that this region may also contribute to the differences in Vn binding since YadA<sup>0.3 6471/76</sup> has the same insertion in the stalk region. In contrast to Vn binding, the interaction with factor H, which was shown to bind to the stalk region of YadA in Ye and Yps strains, revealed no differences [20]. This indicates that the presence or absence of the uptake region modulates affinity to Vn.

The site of interaction between Mc and Vn was mapped to the N-terminal residues 30-177 within UspA2 [34]. This region is located in the head domain of UspA2, which is similar with YadA<sup>0.9 E40</sup>. Our data show that subtle differences within the YadA protein sequence can significantly influence the protein interaction repertoire of Ye. The recruitment of such proteins to the surface of Ye may exert a significant influence on serum resistance and host cell interaction.

Localization of the Vn binding domain within the YadA protein is a crucial step when analyzing the function of YadA in complement evasion. In contrast to complement regulators factor H or the complement component C3, which bind to the stalk domain of YadA [20], we found that Vn is bound via the YadA head domain. In Ye, the neutrophil binding domain is located at the N-terminal part of YadA, whereas the collagen binding domain is located at the central and C-terminal part of the YadA head domain [59,69-72]. The inhibition of Vn binding with heparin was already shown for Mc and Hi. In both species the interaction of Vn with UspA2 or Hsf was assigned to the HBD-3 [34,35]. In contrast, for Ye O:9 E40 not only the HBD-3 but also the adjacent N- and C-terminal portion of Vn are decisive for the efficient interaction with YadA. We conclude that complement evasion of Ye is not limited to interactions mediated by the stalk domain, but can involve the head domain of YadA,
depending on the strain in question. Furthermore, the “uptake region” in Ye O:9 seems to provide a binding domain for Vn which strongly amplifies binding of Vn.

Previous studies showed that recruitment of Vn by Mc or Hi inhibits C5b-9 formation to block pore formation [27]. However, analyzing the TCC formation in Ye with purified complement proteins (C5b-6, C7, C8 and C9) we showed that bound Vn inhibits the deposition of C5b-9 on the bacterial surface. Consequently, these data show that Vn bound to the bacterial surface via YadA is functionally active and inhibits the terminal pathway and thus contributes to complement resistance. Indeed, in in vitro serum killing assays we showed that Ye O:9 E40 is the strain that sustains treatment with serum most efficiently compared to Ye O:8 and Yps YPIII. In contrast, a YadA-deficient strain of Ye O:9 E40 was susceptible to serum killing. Thus YadA-mediated binding of Vn in Ye O:9 E40 is decisive for survival of serum treatment in vitro. The situation is different in Ye O:8 WA-314. This strain is much more sensitive to serum treatment compared with Ye O:9. We know that in Ye O:8 serum resistance is mediated by YadA-dependent recruitment of C3b/iC3b, factor H and C4BP [21,33]. As all these factors bind to YadA and at least for C4BP the binding site(s) within YadA is unknown, there might be competition for binding sites and this might lead to binding of low levels of Vn. Still, binding of all the other negative regulators of complement can mediate serum resistance to a certain extent. A decisive role of YadA for serum resistance of Yps YPIII is rather unlikely as it has been shown that Yps serum resistance occurs independently of the presence of a virulence plasmid (that encodes YadA; [73]). Known mechanisms involved in serum resistance of Yps are binding of C4BP and factor H via Ail [74,75]. Nevertheless, we have shown that also Yps binds Vn via YadA. We think that in this case the recruitment of Vn has a function different from mediating serum resistance and speculate that it might be involved e.g. in modulation of host cell targeting [66] and interaction [24].
Consequently, this should also improve survival of Ye in vivo. Indeed, short-term infection of Vn-deficient mice with Ye O:9 E40 revealed that Vn protects Ye from being killed by the immune system. A short-term infection of mice was used to avoid (as far as possible), the action of other virulence mechanisms such as those provided by the T3SS. According to ex vivo measurements the injection of Yops should efficiently show its action at later time points. Therefore, the short-term mouse experiments should reflect predominantly the impact of Vn on complement killing as the complement system is activated within seconds after infection. Thus, the mouse infection experiments provide evidence that the inhibition of the TCC formation by Vn via binding to YadA indeed has biological relevance. These findings clearly demonstrate the importance of Vn binding to the “uptake region” for the pathogenicity of Ye. However, binding of Vn may also counteract YadA-mediated virulence, which is indicated by the slightly increased bacterial load after infection of Vn-deficient mice with Ye O:9 E40 ΔΔ expressing YadAO:8. We assume that the weak binding of Vn outside of the uptake region might interfere with binding of other factors to YadA which are critical for YadA as a virulence factor. From an evolutionary point of view the acquirement of the uptake region converts Vn from a factor protecting against infection into a factor mediating immune evasion.

Although persons lacking terminal complement components are known to be more susceptible to Neisseria meningitidis [76] but not especially to Ye infections, Vn binding is one important mechanism contributing to the overall serum resistance of Ye. Ye YadA interacts with a multitude of complement regulatory factors (C4bp, C3b, iC3b and factor H), that all contribute to serum resistance of Ye in a true infection situation. These interactions in sum finally determine the success of Ye within the host. Taken together, our data add a novel mechanism how YadA mediates immune evasion. By binding the HBD-3 domain of Vn, YadA containing the “uptake region” mediates efficient
inhibition of TCC formation and thus contributes to complement resistance and better survival of Ye. YadA is a multifunctional protein mediating complement resistance and also adhesion, which in turn is critical for subsequent injection of Yops into the host cells via the T3SS. Beyond bacteriolysis mediated by the assembly of the TCC, the even more important effect of Vn may be to modulate the interaction of Ye with immune cells [66] and further studies will now address how Vn may influence adhesion, invasion and Yop injection during mouse infection.

ACKNOWLEDGEMENTS

We thank T. Späth for excellent technical assistance, A. Fruth (Wernigerode), E. Carniel (Paris), M. Skurnik (Helsinki), J. Heesemann (Munich) and D. Villinger (Frankfurt) for providing us clinical isolates of Yersinia enterocolitica and P. Dersch (Braunschweig) for the gift of the Yersinia pseudotuberculosis strains as well as the antibody against Yps YadA. This work was funded by grants from the German Research Council (DFG) within the SFB 766 to M.S. and I.B.A., the Swedish Medical Research Council (grant number K2015-57X-03163-43-4, http://www.vr.se) and Anna and Edwin Berger Foundation to K.R., and supported by the gender equality program E.05.00390 of the University Clinics Tübingen to M.S.
REFERENCES


5 Miller VL, Falkow S: Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infection and immunity 1988;56:1242-1248.


17 Eitel J, Dersch P: The YadA protein of *Yersinia pseudotuberculosis* mediates high-efficiency uptake into human cells under environmental conditions in which invasin is repressed. Infect Immun 2002;70:4880-4891.

18 Heise T, Dersch P: Identification of a domain in *Yersinia* virulence factor YadA that is crucial for extracellular matrix-specific cell adhesion and uptake. Proc Natl Acad Sci USA 2006;103:3375-3380.


**FIGURE LEGENDS**

**Fig. 1 Vn is efficiently bound by Ye O:9 E40 and Yps.** (A) Several strains of Ye (serotype O:9 with (O:9 E40) and without virulence plasmid (O:9 E40 ΔpYV), serotype O:3 (O:3 6471/76) and serotype O:8 (O:8 8081; O:8 WA-314) and one Yps (Yps YPIII) wildtype strain were incubated with heat-inactivated whole human serum, washed and subsequently analysed for the presence of Vn on the bacterial surface by flow cytometry. Mc (Mc RH4), which is known to bind Vn and Yps which we supposed to also bind Vn (see main text) were included as a positive control for Vn binding. Ye O:9 E40 which was cured from the virulence plasmid (plasmid of *Yersinia* virulence; pYV) that encodes for the Ye T3SS, effector proteins and YadA was included as negative control as we surmised that Vn-binding is pYV-dependent. YadA protein levels were analysed by western blot analysis in whole cell lysates and are shown below the bar chart (one representative western blot is shown). RNA polymerase protein was used as loading control. YadA<sup>O:3</sup> 6471/76 has a calculated molecular weight of approximately 141 kDa (455 aa), YadA<sup>O:8</sup> 8081 of 132 kDa (422 aa), YadA<sup>O:8</sup> WA-314 of 132 kDa (422 aa), YadA<sup>O:9</sup> E40 of 153 kDa (487 aa), YadA<sup>YPIII</sup> of 135 kDa (434 aa) and UspA2H of about 272 kDa (876 aa). (B) To test if strain-specific differences in binding of Vn are exclusive, we compared Vn binding levels to that of Factor H. In contrast to Vn, Factor H is bound in comparable amounts by all *Yersinia* strains tested, except for the negative control strain (O:9 E40 ΔpYV). The protein levels of YadA and the RNA polymerase as a loading control were analysed by western blot analysis in whole cell lysates and are shown below the bar chart (one representative western blot is shown). (C) Binding of serum-derived Vn to Ye O:9 E40 is dose dependent. Ye O:9 E40 and the pYV-cured version thereof were incubated with increasing serum concentrations. Afterwards cell-surface associated Vn was quantified by flow cytometry. (D) Ye O:9 E40 and the pYV-cured version thereof were incubated with increasing amounts of purified Vn. Afterwards cell-surface associated Vn was quantified by...
flow cytometry. Binding of purified Vn to Ye O:9 E40 is dose dependent. Data are means ± SD of at least four (A, B, C, D) individual experiments. (A, B) The main p values were determined by one-way ANOVA (A, B: p < 0.0001). Multiple comparisons were performed by one-way ANOVA with a Dunnett’s multiple comparisons test and the p values are indicated with asterisks. (C, D) The p values were determined by student t test. The error bars denote the SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

**Fig. 2 Vn binding to Ye is YadA-dependent.** (A, left panel) A Ye O:9 E40 wildtype strain (WT) or strains carrying individual deletions for the adhesins Invasin (ΔInv) or YadA (ΔYadA) and a respective double knockout strain (ΔΔ) as well as a virulence plasmid-cured strain (ΔpYV) were incubated with serum, washed and Vn binding was quantified by flow cytometry. (A, middle panel) Yps YPIII wildtype (WT) and corresponding strains lacking expression of the surface adhesin YadA (ΔYadA) or expressing a YadA version lacking part of the head domain (Δ53-83) were included as controls. (A, right panel) A Mc wildtype strain (WT) known to bind Vn via the surface adhesin UspA2 and a corresponding strain lacking expression of UspA2 (ΔUspA2) were included as positive and negative controls. YadA protein levels were analysed by western blot analysis in whole cell lysates and are shown below the bar chart (one representative blot is shown). (B) A selection of the strains used in (A) was tested for Vn binding in a blot overlay assay. Vn and YadA were detected on the identical blot with specific antibodies and differently labeled secondary antibodies (emission max. at 680 and 800 nm, respectively) simultaneously. Vn is bound only in presence of YadA (Ye) or UspA2, respectively. (C) In a direct binding assay, essentially performed as in (A), Vn can be detected in the expected molecular weight (65 and 75 kDa) by western blot only in those Ye strains expressing YadA. Data are means ± SD of at least four individual experiments (A), or one representative experiment out of three is shown (B, C). The main p value was determined by one-way ANOVA (A: p < 0.0001). Multiple comparisons were
performed by one-way ANOVA with a Dunnett’s multiple comparisons test and the p values are indicated with asterisks. The error bars denote the SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p > 0.0001.

**Fig. 3** A specific region in the YadA head domain is decisive for efficient binding of Vn.

(A) Alignment of the head of various YadA variants. White letters on gray background: signal peptide, black letters on gray background: the canonical “SVAIG” head repeats of YadA, italics: the neck region that links the head to the coiled-coil stalk of YadA. The originally proposed insertion of *Yps* by Heise & Dersch [18] is displayed in bold, and is slightly shifted towards the N-terminus of YadA. The dashed line on top shows the corrected position of the insertion, based on improved alignments and on the structure of the YadA head from *Ye O*:3, where the short insertion is not resolved (underlined region). This, and the unusually high number of prolines in this region suggests that it is not structured. The long version of insertion carries a strongly positive net charge (+5 for *Yps* YPIII, +4 for the *Ye O*:9 E40), which probably plays a role for the binding to fibronectin and Vn. (B) Schematic view of the differences in the YadA heads. The *Yps* YPIII and *Ye O*:9 E40 variants have long insertions in a unstructured loop region close to the N-terminus of the head. (C) PCR products comprising the YadA head region of *Ye O*:8 WA-314, *Ye O*:9 E40 with and without YadA, *Yps* YPIII, and clinical isolates derived from fecal samples (*Ye O*:3, No. 01-03; *Ye O*:8, No. 04; *Ye O*:5,27, No. 06-07 and *Ye O*:9, No. 08-12) or blood (No. 13) were separated by capillary gelelectrophoresis. Predicted length of PCR products was: *Ye O*:3 346 bp; *Ye O*:8 337 bp; *Ye O*:9 451 bp; *Ye O*:5,27 346 bp; *Yps* YPIII 442 bp. Water control and a YadA-deficient strain were included as negative controls. (D) The strains shown in (C) were tested for Vn binding. Cell-surface associated Vn after incubation in HIS was quantified by flow cytometry. One representative experiment out of three is shown (D).
**Fig. 4** The uptake region is decisive for YadA-mediated Vn binding. Schematic representation of different YadA versions that were expressed from a plasmid in Ye O:9 E40 and analysed for Vn binding capacity. The YadA versions tested comprise YadA\(^{O:8}\), YadA\(^{O:9}\), a YadA\(^{O:9/O:8}\) hybrid consisting of the O:9 head domain fused to the corresponding Ye O:8 head/stalk and membrane anchor domain (see Material and Methods for details) and Ye YadA\(^{O:9}\) with the uptake region deleted (Δ uptake region). (B) Flow cytometry analysis of Vn binding to different Ye strains carrying plasmids for inducible expression of the YadA versions depicted in (A). As control strains we used Ye strains expressing wildtype YadA from the endogenous pYV plasmid (Ye O:9 = positive control, Ye O:8) and a Ye O:9 E40 YadA-deficient strain (Ye O:9 ΔYadA = negative control). YadA protein levels were analysed by western blot analysis in whole cell lysates and are shown below the bar chart. Data are means ± SD of at least three individual experiments (B, flow cytometry) or one representative experiment out of three is shown (B, western blot). The main p value was determined by one-way ANOVA (B, flow cytometry: p < 0.0001). Multiple comparisons were performed by one-way ANOVA with a Dunnett’s multiple comparisons test and the p values are indicated with asterisks. The error bars denote the SD. * p < 0.05; ** p < 0.01, *** p < 0.001, **** p > 0.0001.

**Fig. 5** Vn interacts with YadA via its C-terminal heparin-binding-domain 3 (HBD-3).

(A) Adhesion of Ye to Vn-coated coverslips can be blocked by heparin. (B) Schematic representation of Vn, the C-terminally truncated Vn-molecules [54] and the Vn molecules carrying deletions within and adjacent to the Heparin-binding-domain 3 (HBD-3) [35] that were used for a direct binding assay. (C) Western blot of a binding assay of Ye O:9 E40 with full length Vn and all fragments depicted in (B). Vn fragments appear in green, YadA, which was detected simultaneously, appears in yellow bands (trimer runs at ~ 200 kDa). (D) Flow cytometry analysis of Vn binding to Ye O:9 E40 with full length Vn and all fragments.
depicted in (B). Data are means ± SD of at least three individual experiments (A, D), or one representative experiment out of three is shown (C). The p value for the comparison of – Heparin and +Heparin was determined by Students t test. The main p value was determined by one-way ANOVA (D: p < 0.0001). Multiple comparisons were performed by one-way ANOVA with a Dunnett’s multiple comparisons test and the p value is indicated with asterisks. The error bars denote the SD. * p < 0.05; ** p < 0.01, *** p < 0.001, **** p > 0.0001.

**Fig. 6 Vn is functionally active and inhibits the terminal pathway when bound to the surface of Ye.** (A) Histogram overlay of flow cytometry analyses of TCC formation (detected by formation of the neoepitope C5b-9) on the surface of Ye O:9 E40 wildtype after preincubation of bacteria with PBS or different concentrations of Vn in PBS (10, 25, 50 µg/ml). Preincubation with Vn reduces the amount of TCC that is formed. (B) Bar chart depicting C5b-9 deposition as percent of the amount of C5b-9 that was formed on the surface of bacteria preincubated with PBS only compared to bacteria preincubated with either Vn or C4BP at different concentrations (10, 25, 50 µg/ml). Vn, but not C4BP is able to reduce formation of C5b-9. Ab control indicates background signal that was obtained using secondary antibody only for detection. Data are means ± SD of at least three individual experiments. The main p value was determined by one-way ANOVA (B: p < 0.001). Multiple comparisons were performed by one-way ANOVA with a Dunnett’s multiple comparisons test and the p value is indicated with asterisks. The error bars denote the SD. * p < 0.05; ** p < 0.01, *** p < 0.001.

**Fig. 7 Ye O:9 E40 is resistant to complement mediated killing in vitro and in an in vivo serum killing assay, Ye is more efficiently eliminated in the absence of Vn.** (A) In vitro serum killing assay using Ye O:9 E40, Ye O:9 E40 ΔYadA, Ye O:8 WA-314, Yps YPII, Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:8, Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9, Ye O:9 E40
ΔΔ + pASK-IBA4C_yadAO:9/O:8 hybrid, Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9

Δuptake region. The serum bactericidal effect was calculated as the survival percentage. (B)

Wildtype and Vn/- mice were infected intravenously with 1x10⁷ Ye O:9 E40 for 30 min.

After that, mice were killed, blood was withdrawn and plated on selective agar plates. CFU
was determined by counting colonies the next day and is shown as log₁₀ CFU per g blood.

Data are means ± SD of at least three individual experiments (A). The main p value was
determined by one-way ANOVA (A, p < 0.0001). Multiple comparisons were performed by
one-way ANOVA with a Dunnett’s multiple comparisons test and the p values are indicated
with asterisks. The p value for the comparison of C57BL/6 and Vn/- mice was determined by
Student t test (B). The horizontal lines denote the mean, the error bars the SD. * p < 0.05; **
p < 0.01, *** p < 0.001, **** p > 0.0001, , n=6
Table 1. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Description</th>
<th>Resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBla</td>
<td>expression of YopE aa1-53-β-lactamase hybrid protein under control of the YopE promoter</td>
<td>Kanamycin</td>
<td>(46)</td>
</tr>
<tr>
<td>pACYC184 EGFP</td>
<td>EGFP expressed under control of a constitutive tac/lac promoter</td>
<td>Chloramphenicol</td>
<td>(47)</td>
</tr>
<tr>
<td>pASK-IBA4C_yadAO:8</td>
<td>yadA from Ye O:8 WA-314 cloned into pASK-IBA4C; expression under control of an anhydrotetracycline inducible promoter</td>
<td>Chloramphenicol</td>
<td>this study</td>
</tr>
<tr>
<td>pASK-IBA4C_yadAO:9</td>
<td>yadA from Ye O:9 E40 cloned into pASK-IBA4C; expression under control of an anhydrotetracycline inducible promoter</td>
<td>Chloramphenicol</td>
<td>this study</td>
</tr>
<tr>
<td>pASK-IBA4C_yadAO:9/O:8 hybrid</td>
<td>Plasmid for inducible expression of a hybrid protein consisting of the N-terminal aa 1-89 of yadA from Ye O:9 E40 fused to aa 55-422 of yadA from Ye O:8 WA-314; expression under control of an anhydrotetracycline inducible promoter</td>
<td>Chloramphenicol</td>
<td>this study</td>
</tr>
<tr>
<td>pASK-IBA4C_yadAO:9/Auptake region</td>
<td>Plasmid for inducible expression of yadA from Ye O:9 E40 lacking aa 60-86 comprising the uptake region; expression under control of an anhydrotetracycline inducible promoter</td>
<td>Chloramphenicol</td>
<td>this study</td>
</tr>
<tr>
<td>Bacterial strain</td>
<td>Description</td>
<td>Resistance</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Ye O:3 6471/76</td>
<td>Serotype O:3, fecal isolate, wildtype</td>
<td>-</td>
<td>(48) GI:48607</td>
</tr>
<tr>
<td>Ye O:8 8081</td>
<td>Serotype O:8, fecal isolate, wildtype</td>
<td>-</td>
<td>(49) GI:122815846</td>
</tr>
<tr>
<td>Ye O:8 WA-314 YadAwt</td>
<td>Coding sequence of YadA WA-314 O:8 was reinserted into a YadA0 strain</td>
<td>Nal, Kan, Spec</td>
<td>(12) GI:310923211</td>
</tr>
<tr>
<td>Ye O:9 E40 pBla</td>
<td>Ye O:9 E40 Δasd transformed with pMK-Bla</td>
<td>Nal, Kan, Ars</td>
<td>(46) GI:972903261</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔpYV pBla</td>
<td>Ye O:9 E40 Δasd without virulence plasmid transformed with pMK-Bla</td>
<td>Nal, Kan, Ars, Tet</td>
<td>(47)</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔInv pBla</td>
<td>Inv mutant strain obtained by recombinational integration of suicide plasmid pMS154 into E40 Δasd, transformed with pMK-Bla</td>
<td>-</td>
<td>(47)</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔYadA pBla</td>
<td>pYV- Δasd strain was transformed with pLJM4029 (YadA-) and with pMK-Bla</td>
<td>Nal, Kan, Ars, Strep</td>
<td>(47)</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔInv ΔYadA pBla</td>
<td>pYV- Δasd Δinv strain was transformed with pLJM4029 (YadA-) and with pMK-Bla</td>
<td>Nal, Kan, Ars, Tet, Strep</td>
<td>(47)</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:8</td>
<td>Ye O:9 E40 Δasd lacking expression of both YadA and Invasin transformed with pASK-IBA4C_yadAO:8</td>
<td>Nal, Kan, Ars, Strep, Cm</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9</td>
<td>Ye O:9 E40 Δasd lacking expression of both YadA and Invasin transformed with pASK-IBA4C_yadAO:9</td>
<td>Nal, Kan, Ars, Strep, Cm</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9/O:8 hybrid</td>
<td>Ye O:9 E40 Δasd lacking expression of both YadA and Invasin transformed with pASK-IBA4C_yadAO:9/O:8 hybrid</td>
<td>Nal, Kan, Ars, Strep, Cm</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9/Auptake region</td>
<td>Ye O:9 E40 Δasd lacking expression of both YadA and Invasin transformed with pASK-IBA4C_yadAO:9/Auptake region</td>
<td>Nal, Kan, Ars, Strep, Cm</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 E40 pBla eGFP</td>
<td>Ye O:9 E40 pBla transformed with pACYC184 EGFP</td>
<td>Nal, Kan, Ars, Cm</td>
<td>(47)</td>
</tr>
<tr>
<td>Ye O:3 01</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:3 02</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:3 03</td>
<td>Clinical isolate derived from swine (tongue)</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:8 04</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:5,27 06</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:5,27 07</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 08</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 09</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 10</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 11</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 12</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Ye O:9 13</td>
<td>Clinical isolate derived from blood sample</td>
<td>-</td>
<td>(51)</td>
</tr>
<tr>
<td>Ye O:9 14</td>
<td>Clinical isolate derived from fecal sample</td>
<td>-</td>
<td>this study</td>
</tr>
<tr>
<td>Yps YPIII</td>
<td>Yersinia pseudotuberculosis wildtype strain, pIB1</td>
<td>-</td>
<td>(45)</td>
</tr>
<tr>
<td>Yps YP46 pIB1</td>
<td>yadAΔ53-83</td>
<td>Kan, Amp</td>
<td>(18)</td>
</tr>
<tr>
<td>Yps YP47 pIB1</td>
<td>yadA−</td>
<td>Kan</td>
<td>(17)</td>
</tr>
<tr>
<td>Ec omp2 + pASK-IBA4C</td>
<td>Ec BL21 lacking expression of ompF transformed with pASK-IBA4C</td>
<td>Cm</td>
<td>this study</td>
</tr>
<tr>
<td>Ec omp2 + pASK-IBA4C_yadAO:8</td>
<td>Ec BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:8</td>
<td>Cm</td>
<td>this study</td>
</tr>
<tr>
<td>Ec omp2 + pASK-IBA4C_yadAO:8</td>
<td>Ec BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:8</td>
<td>Cm</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>with pASK-IBA4C_yadAO:9</td>
<td>Cm</td>
<td>this study</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------------</td>
<td>------------------</td>
<td>------------</td>
</tr>
<tr>
<td><em>Ec omp2</em> + pASK-IBA4C_yadAO:9/O:8 hybrid</td>
<td><em>Ec</em> BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:9/O:8 hybrid</td>
<td>Cm</td>
<td>this study</td>
</tr>
</tbody>
</table>

|                  | *Ec* BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:9Δuptake region | Cm               | this study |

| **Mc RH4 WT** | *Moraxella catarrhalis* wildtype strain | - | (52) |
| **Mc RH4 ΔUspA2H** | *Moraxella catarrhalis* lacking expression of UspA2H | Zeo | (53) |

Table 3. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugate</th>
<th>Clone</th>
<th>Manufacturer</th>
<th>Working dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti factor H</td>
<td>-</td>
<td>polyclonal</td>
<td>Complement Technology</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti Vn</td>
<td>-</td>
<td>polyclonal</td>
<td>Complement Technology</td>
<td>FACS 1:100; WB 1:1000</td>
</tr>
<tr>
<td>Rabbit anti Ye YadA</td>
<td>-</td>
<td>polyclonal</td>
<td>Lab antibody; I. Autenrieth</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit anti Yps YadA</td>
<td>-</td>
<td>polyclonal</td>
<td>Lab antibody; P. Dersch</td>
<td>1:200</td>
</tr>
<tr>
<td>Sheep anti Vn</td>
<td>-</td>
<td>polyclonal</td>
<td>AbD Serotech</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse anti human C5b-9</td>
<td>-</td>
<td>aE11</td>
<td>NeoClone Biotechnology</td>
<td>1:2000</td>
</tr>
<tr>
<td>Mouse anti β subunit of E.coli RNA-Polymerase</td>
<td>-</td>
<td>8RB13</td>
<td>Jackson ImmunoResearch</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donkey anti rabbit</td>
<td>APC</td>
<td></td>
<td>Jackson ImmunoResearch</td>
<td>1:200</td>
</tr>
<tr>
<td>Goat anti rabbit</td>
<td>DyLight 800</td>
<td></td>
<td>Thermo Scientific</td>
<td>1:10000</td>
</tr>
<tr>
<td>Goat anti rabbit</td>
<td>DyLight 680</td>
<td></td>
<td>Thermo Scientific</td>
<td>1:10000</td>
</tr>
<tr>
<td>Goat anti mouse</td>
<td>DyLight 680</td>
<td></td>
<td>Thermo Scientific</td>
<td>1:10000</td>
</tr>
<tr>
<td>Rabbit anti sheep</td>
<td>DyLight 800</td>
<td></td>
<td>Thermo Scientific</td>
<td>1:10000</td>
</tr>
<tr>
<td>Rabbit anti goat</td>
<td>Alexa-488</td>
<td></td>
<td>Jackson ImmunoResearch</td>
<td>1:200</td>
</tr>
<tr>
<td>Goat anti mouse</td>
<td>Alexa-647</td>
<td>polyclonal</td>
<td>Jackson ImmunoResearch</td>
<td>1:2500</td>
</tr>
</tbody>
</table>
Figure 1, Mühlenkamp et al.
Figure 2, Mühlenkamp et al.
Figure 3, Mühlenkamp et al.
Figure 4, Mühlenkamp et al.
Figure 6, Mühlenkamp et al.
revised Figure 7, Mühlenkamp et al.
Fig. S1 DNA sequence alignment of YadA from Ye strains of different serotypes. DNA alignment of YadA of Ye O:9 E40 (E40), O:8 WA-314 (O:8), O:3 6471/76 (O:3) and Yps YPIII (YPIII). Alanine is given in yellow, tyrosine in red, guanine in blue and cytosine in green. Amino acid conservation among the different Yersinia strains is shown in the grey bars above. The “uptake region” is highlighted in lilac. Primers that were chosen for PCR amplification of the YadA head region are indicated by black boxes.

Supplemental Figure 1

Mühlenkamp et al.
**Fig. S2 Alignment of YadA sequences.** Kalign/Muscle alignment of YadA of *Yps YPIII, Ye O:9 E40, Ye O:8 WA-314, Ye O:8 8081 and Ye O:3 6471/76*. The signal peptide is given in blue, the neck region is given in green and the membrane anchor domain is given in yellow. Canonical YadA head repeats are highlighted in red.
Fig. S3 Vn binding assay analysed by flow cytometry. YadA constructs were expressed in *E. coli* omp2 (Prilipov et al., 1998) and Vn binding from serum was quantified by staining with antibodies directed against Vn and flow cytometry. YadA that contains the “uptake region” (i.e. YadA O:9 and the hybrid YadAO:9/O:8) mediates efficient binding of Vn to the surface of *E.coli* (− = not induced; + = induced with anhydrotetracycline (AHTC) 1:10000). Data are means ± SD of three individual experiments.
**Fig. S4 Quality control of Vn fragments.** Integrity and amount of purified Vn fragments was tested by a Coomassie gel and a Western Blot. All fragments have the expected size and concentration and can be detected with the used anti-Vn antibody in comparable sensitivity. One representative experiment out of three is shown.
Fig. S5 Comparison of Ye O:9 E40 expressing YadAO:8 or the YadAO:9/O:8 hybrid protein in an in vivo serum killing assay. Wildtype and Vn<sup>−/−</sup> mice were infected intravenously with 1x10<sup>7</sup> Ye O:9 E40 for 30 min. After that, mice were killed, blood was withdrawn and plated on selective agar plates. The bacterial load was determined by counting colonies the next day and is shown as log<sub>10</sub> CFU per g blood. The p value for the comparison of C57BL/6 and Vtronectin<sup>−/−</sup> mice was determined by Student t test (B). The horizontal lines denote the mean, the error bars the SD. * p < 0.05; ** p < 0.01, *** p < 0.001, **** p > 0.0001, n=6.