

Force Nanoscopy Demonstrates Stress-Activated Adhesion between *Staphylococcus aureus* Iron-Regulated Surface Determinant Protein B and Host Toll-like Receptor 4

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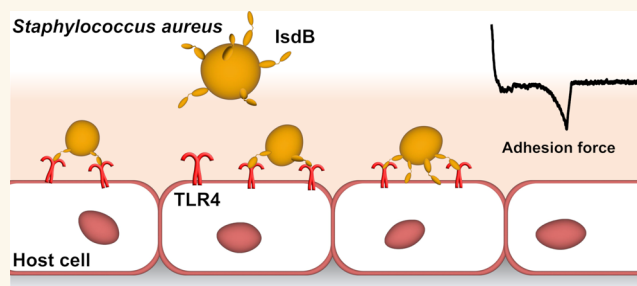
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ABSTRACT: The *Staphylococcus aureus* iron-regulated surface determinant protein B (IsdB) has recently been shown to bind to toll-like receptor 4 (TLR4), thereby inducing a strong inflammatory response in innate immune cells. Currently, two unsolved questions are (i) What is the molecular mechanism of the IsdB-TLR4 interaction? and (ii) Does it also play a role in nonimmune systems? Here, we use single-molecule experiments to demonstrate that IsdB binds TLR4 with both weak and extremely strong forces and that the mechanostability of the molecular complex is dramatically increased by physical stress, sustaining forces up to 2000 pN, at a loading rate of 10^5 pN/s.

We also show that TLR4 binding by IsdB mediates time-dependent bacterial adhesion to endothelial cells, pointing to the role of this bond in cell invasion. Our findings point to a function for IsdB in pathogen–host interactions, that is, mediating strong bacterial adhesion to host endothelial cells under fluid shear stress, unknown until now. In nanomedicine, this stress-dependent adhesion represents a potential target for innovative therapeutics against *S. aureus*-resistant strains.

KEYWORDS: single-cell force spectroscopy, single-molecule force spectroscopy, *Staphylococcus aureus*, IsdB, TLR4, endothelial cells, bacterial adhesion



Staphylococcus aureus causes life-threatening diseases in humans such as endocarditis, pneumonia, and sepsis and is also a common cause of foreign body-associated infections, chronic osteomyelitis, and ectopic reactions of the skin.^{1,2} Such pathogenic potential is induced by an arsenal of secreted^{3,4} and cell surface⁵ virulence factors. *S. aureus* expresses up to 24 cell wall-associated (CWA) proteins, the most prevalent ones being microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), a family of proteins defined by tandemly arrayed Ig-like folded domains.⁶ Several CWA proteins play an essential role in adhesion and invasion of host cells, a process enabling protection of the pathogen from host immune defenses and from antibiotics. Adhesion can occur indirectly when extracellular proteins (fibronectin, vitronectin, or fibrinogen) are used to form a bridge between host receptors, usually integrins, and specific CWA proteins. Key players in triggering indirect adhesion are fibronectin-binding proteins (FnBPs)⁷ and clumping factor A (ClfA).⁸ In addition, there are also direct adhesion mechanisms that do not rely on a linker, as observed for the *S. aureus* autolysin Atl.⁹

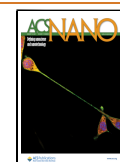
Innate immunity is an essential system in humans whereby immune cells, macrophages, and dendritic cells participate in phagocytosis and inflammatory processes. Immune cells recognize specific molecules on the surface of pathogens using pattern recognition receptors (PRRs). Among these, toll-like receptors (TLRs) are transmembrane proteins expressed in both immune and nonimmune (e.g., endothelial cells) cells. TLRs are composed of an extracellular leucine-rich repeats (LRRs) domain that mediates the recognition of pathogen-associated molecular patterns (PAMPs), followed by a transmembrane domain and a cytoplasmic tail containing a Toll/IL-1b receptor (TIR) domain that interacts with downstream signaling proteins.^{10,11} TLR4, a member of this

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large protein family, binds staphylococci cytotoxins, such as phenol soluble modulins PSM α 1, PSM α 2, PSM α 3, PSM β 1, and PSM β 2,¹² providing the pathogen with a mechanism to block inflammation and enhance its survival in the host. While a major role in Gram-negative bacterial recognition is played by TLR4, through binding to lipopolysaccharide (LPS),^{13,14} its role in host innate immune response against Gram-positive bacteria has only been recently identified.^{15,16}

Iron-regulated surface determinant (Isd) proteins (IsdA, IsdB, IsdC, and IsdH) are a group of *S. aureus* CWA proteins primarily involved in the acquisition of iron from hemoglobin. Each Isd molecule contains one or more structurally conserved near iron transporter (NEAT) motifs, namely, two in the case of IsdB (Figure 1A). Structurally, each NEAT domain adopts a β sandwich fold that consists of 2 five-stranded antiparallel β sheets.^{17,18} It has recently been shown that IsdB influences immune response and innate immunity by triggering a strong inflammatory response in innate immune cells via interaction with TLR4.¹⁶ IsdB causes the release of proinflammatory cytokines in innate immune cells through high-affinity binding to TLR4 ($K_D = 98$ nM). Essential TLR4 binding residues on IsdB include valine189 and tyrosine192 in the NEAT1 motif, as well as three glutamic acid residues in the linker segment between NEAT1 and NEAT2 domains.¹⁶ Binding of recombinant IsdB to TLR4 occurs in the absence of the small protein MD-2, which is strictly required for the interaction of LPS with TLR4.¹⁹ IsdB also induces IL-1 β release by monocytes and bone marrow-derived dendritic cells via the TLR4-dependent NLPR3-caspase-1 inflammasome signaling cascade.¹⁶ Finally, IsdB mediates indirect invasion of epithelial and endothelial cells, through a vitronectin bridge between IsdB and $\alpha_v\beta_3$ integrin.²⁰

In this study, we address two issues, *i.e.*, (i) what is the molecular mechanism of TLR4 binding by IsdB? and (ii) Could this interaction represent a previously undescribed mechanism of direct host adhesion and invasion, knowing that TLR4 is expressed by both immune and nonimmune cells? As expression of IsdB is favored when bacteria are grown under iron starvation conditions,^{20,21} experiments were performed with cells grown in either iron-poor (RPMI) or iron-rich (BHI) medium. In body fluids like the blood, free iron concentrations are very low, meaning that in bacteremia, the pathogen finds ideal conditions to express IsdB. Single-molecule force spectroscopy experiments demonstrate that IsdB binds TLR4 with pN range forces and that the strength of the molecular complex is dramatically enhanced by physical stress. Hence, our results highlight the role of IsdB in promoting bacterial adhesion to human cells under fluid shear stress.

RESULTS AND DISCUSSION

IsdB Mediates Bacterial Adhesion to TLR4-Coated Surfaces. A macroscopic optical assay was used to test the ability of SH1000 Δspa cells to adhere to the TLR4-coated substrates. This strain has been employed in order to avoid potential interference of protein A with the inhibitory activity of antibodies, such as anti-TLR4, used further on in the study. Bacteria cultured in RPMI largely adhered (Figure 1b), unlike those grown in BHI, indicating that IsdB is largely expressed in iron-poor conditions, that immobilized TLR4 receptors were functional, and that the adhesin is the main surface component mediating bacterial adherence. The forces driving IsdB-dependent *S. aureus* adhesion to TLR4 surfaces were studied

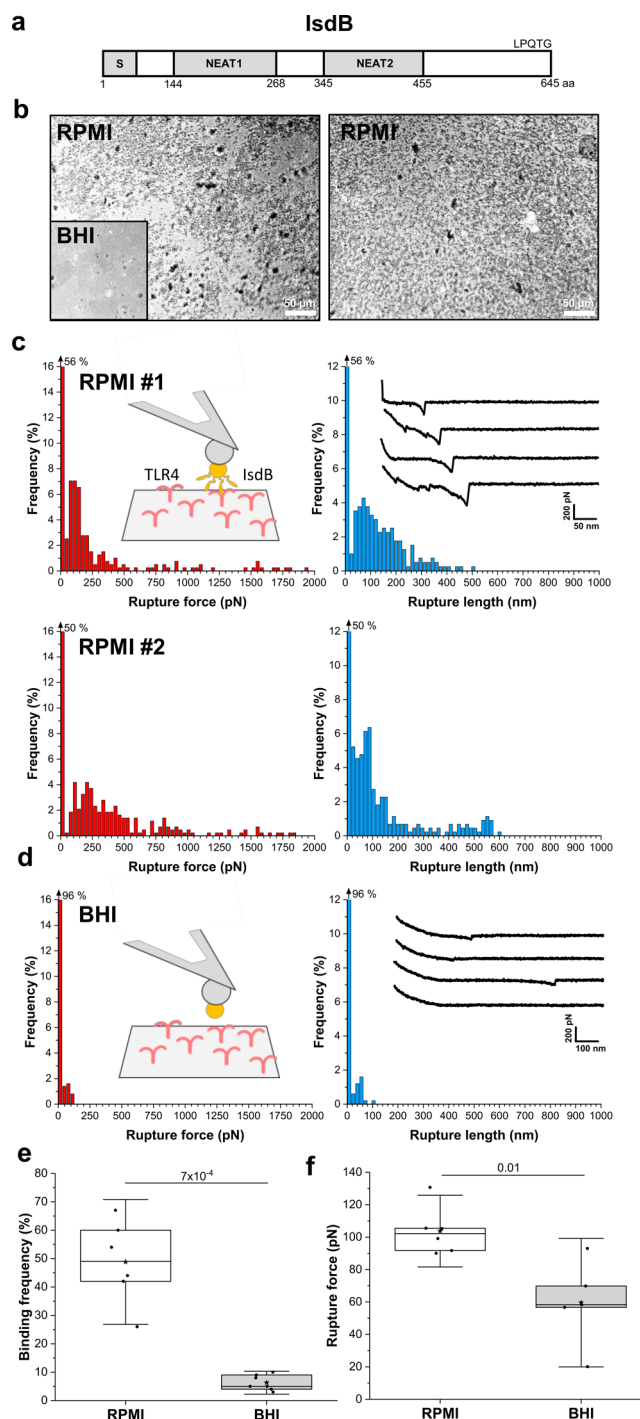


Figure 1. IsdB mediates bacterial adhesion to TLR4-coated substrates. (a) Schematic representation of the modular structure of *S. aureus* IsdB. Signal sequence (S) and near iron transporter domains (NEAT1 and 2) with respective positions are represented. (b) Optical microscopy images of TLR4-coated surfaces incubated with *S. aureus* Δspa cells grown in RPMI and BHI (inset) media. Bacteria grown in BHI do not adhere to the surfaces, showing the role of IsdB in mediating bacterial adhesion to TLR4. For RPMI cells, images of 2 independent surfaces are shown and the scale bars are 50 μ m. (c–f) Adhesion of *S. aureus* Δspa cells to TLR4-coated surfaces assessed by single-cell force spectroscopy (SCFS). Rupture force and rupture length histograms of (c) 2 representative cells grown in RPMI medium ($n = 175$ and 225 adhesive curves for cell #1 and cell #2, respectively) and (d) 1 representative cell grown in BHI medium ($n = 21$ adhesive

Figure 1. continued

curves), acquired by recording force–distance curves in PBS between single bacteria and TLR4 substrates, at a retraction speed of 1 $\mu\text{m/s}$. Schemes of the SCFS setups and representative retraction force profiles are shown as insets. (e) Box plot comparing the binding frequency of bacteria grown in RPMI and BHI culture media ($n = 6$ and 7 cells, respectively). (f) Box plot showing the rupture force obtained for bacteria grown in RPMI and BHI media ($n = 6$ and 5 cells, respectively). In both (e) and (f), means are represented by stars, medians by lines, boxes indicate the 25–75% quartiles, and whiskers the standard deviation. P -values were determined by a two-sample t -test.

by means of single-cell force spectroscopy (SCFS).^{22,23} Force–distance curves between bacterial probes and TLR4 substrates showed numerous signatures with single adhesion peaks for RPMI-grown cells (Figures 1c and S1) with a rupture force of 104 ± 15 pN (Figure 1f; mean $\pm$ SD total of $n = 1048$ adhesive curves; 6 cells) and a rupture length of 73 ± 18 nm. By contrast, BHI-grown cells (Figures 1d and S2) featured a dramatic drop of binding frequency, from 49% (Figure 1e; $n = 1271$ adhesive curves from 6 cells) to 6% ($n = 211$ adhesive curves from 7 cells), and poor adhesion with weak rupture forces, 59 ± 26 pN (Figure 1f; $n = 122$ adhesive curves, 5 cells), and short rupture lengths, 42 ± 17 nm. Being a 645 amino acid-long protein, a contour length of 232 nm is expected for IsdB when fully unfolded, assuming that each amino acid residue contributes with 0.36 nm. The rupture length value of 73 ± 18 nm in RPMI suggests a high

mechanostability of IsdB, which hardly unfolds before bond rupture.

Mechanical Strength of Single IsdB-TLR4 Interactions. To further study single IsdB-TLR4 interactions, SH1000 Δspa cells were probed with AFM tips functionalized with TLR4 (single-molecule force spectroscopy, SMFS).²² Force–distance curves and representative rupture force histograms (Figures 2a, S3, 2b, and S4) revealed that RPMI-grown bacteria adhered to TLR4 tips with a frequency of 26% (Figure 2c; $n = 2011$ adhesive curves from 8 cells), while this frequency dropped to 7% for BHI-grown bacteria ($n = 664$ adhesive curves from 10 cells). Rupture forces of 100 ± 16 pN and 72 ± 11 pN (Figure 2d; $n = 3193$ and 665 adhesive curves, 10 cells) were measured in both conditions. Interestingly, even though the mean forces fell within a weak force regime, most of the RPMI-grown cells probed by SCFS and SMFS featured much stronger adhesion forces, up to 2000 pN. This unusual adhesion force signature, comprising an extremely wide range of forces, agrees well with those reported for the interaction with vitronectin (Vn)²⁴ and $\alpha_v\beta_3$ integrin,²⁵ two additional host partners of this bacterial adhesin. Overall, it strongly suggests that SCFS and SMFS data under low iron conditions reflect the direct interaction between IsdB and TLR4. Similar rupture length ranges were observed for both low- and high-force events (Figure S5).

Specificity was further supported by probing RPMI-bacteria with TLR4 tips preincubated with anti-TLR4 monoclonal antibody (Figures 3a and S6). In these conditions, the binding frequency significantly dropped to 6% (Figure 3d; $n = 436$ adhesive curves from 8 cells), with a decrease of the rupture forces (Figure 3e). By contrast, the binding frequency

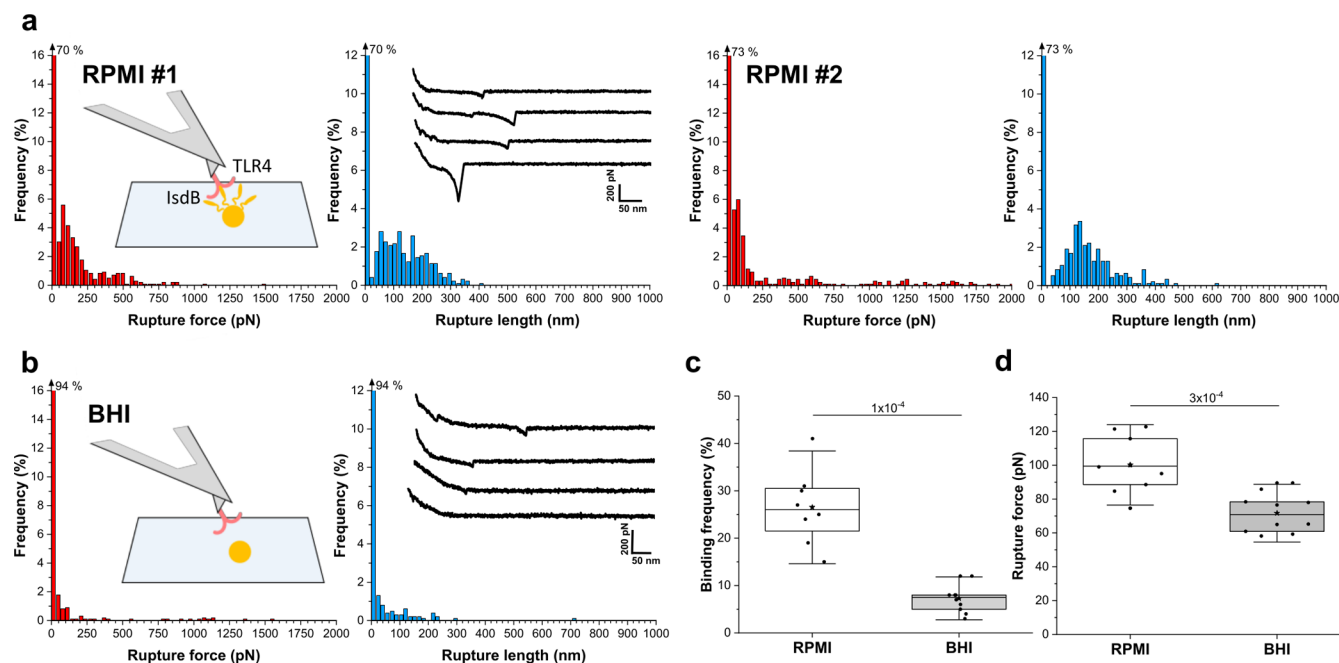


Figure 2. Strength of single IsdB-TLR4 interactions assessed by single-molecule force spectroscopy (SMFS). Rupture force and rupture length histograms acquired by recording force–distance curves in PBS between TLR4-functionalized AFM tips and (a) 2 representative Δspa cells grown in RPMI medium ($n = 289$ and 259 adhesive curves, for cell #1 and cell #2, respectively) and (b) 1 representative Δspa cell grown in BHI medium ($n = 59$ adhesive curves), at a retraction speed of 1 $\mu\text{m/s}$. Schemes of the SMFS setups and representative retraction force profiles are shown as insets. (c) Box plots comparing the binding frequency of bacteria grown in RPMI and BHI culture media ($n = 8$ and 10 cells, respectively). (d) Box plot showing the rupture force obtained for bacteria grown in RPMI and BHI media ($n = 10$ cells, for both cases). Means are represented by stars, medians by lines, boxes indicate the 25–75% quartiles, and whiskers the standard deviation. P -values were determined by a two-sample t -test.

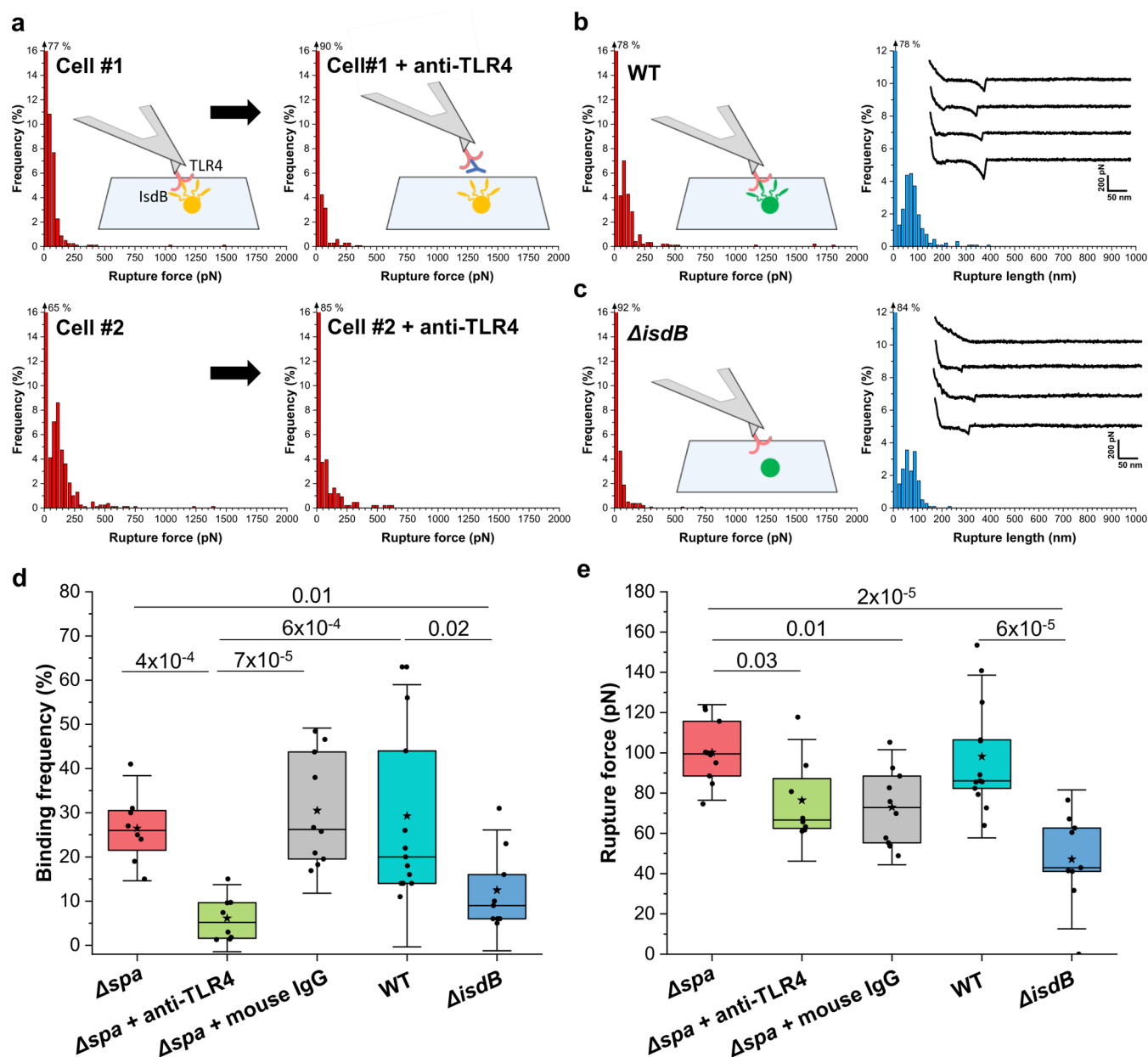


Figure 3. IsdB-TLR4 strength confirmed by anti-TLR4 blocking and *S. aureus* strain lacking IsdB. (a) Rupture force histograms of 2 representative *Δspa* cells, cultured in RPMI medium, obtained by recording force–distance curves in PBS at a retraction speed of 1 $\mu\text{m/s}$ between TLR4-functionalized AFM tips before ($n = 188$ and 276 adhesive curves, for cell #1 and cell #2, respectively) and after blocking with 100 $\mu\text{g/mL}$ of anti-TLR4 monoclonal antibody ($n = 98$ and 65 adhesive curves, for cell #1 and cell #2, respectively). (b) Rupture force and rupture length histograms of a representative *S. aureus* cell expressing IsdB (WT, $n = 200$ adhesive curves) cultured in RPMI. (c) Force data of a representative *S. aureus* cell lacking IsdB (*ΔisdB*, $n = 95$ adhesive curves) cultured in RPMI. Schemes of the SMFS setups and representative retraction force profiles are shown as insets. Box plots comparing (d) binding frequency and (e) rupture forces obtained for *Δspa* cells before and after blocking with anti-TLR4 monoclonal antibody, after tip treatment with monoclonal mouse IgG as a negative control, WT and *ΔisdB* strains ($n = 8, 8, 10, 13$, and 9 cells, respectively). Means are represented by stars, medians by lines, boxes indicate the 25–75% quartiles, and whiskers the standard deviation. *P*-values were determined using Kruskal–Wallis test followed by post hoc Dunn’s test.

remained unchanged when using a control nonspecific monoclonal mouse IgG (Figures 3d and S7).

We note that rupture lengths of 101 ± 44 nm were detected for RPMI cells (vs 58 ± 34 nm for BHI cells) and thus not very different from SCFS values. In addition to showing that IsdB is mechanically stable, this indicates that TLR4 is not fully unfolded upon pulling the tip away from the bacterial surface (unfolded TLR4 = 218 nm; 607 amino acids). This behavior is easily explained by the fact that IsdB binds to several amino

acid residues, such as lysine and glutamic acid,¹⁶ located along distinct positions within the TLR4 sequence, and would also account for the relatively broad distributions of rupture lengths.

We then compared the adhesion behavior of *Δspa* cells with that of the isogenic SH1000 WT strain expressing IsdB and of the SH1000 *ΔisdB* strain lacking IsdB, all grown in RPMI. While similar binding frequencies were observed for WT cells (29%, $n = 3167$ adhesive curves from 13 cells, Figures 3b,d and

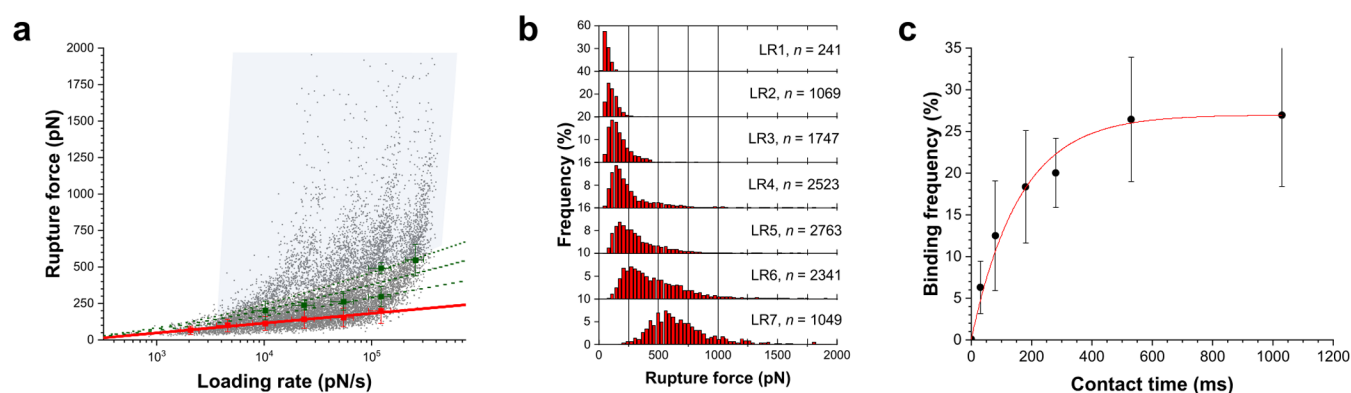


Figure 4. Physical stress strengthens the IsdB-TLR4 interaction. (a) Dynamic force spectroscopy plot of the IsdB-TLR4 interaction acquired on 6 Δspa cells cultured in RPMI growth medium ($N = 11700$). Shown are Bell-Evans fit of the single-bond data (red line) and Williams-Evans predictions for multiple simultaneous uncorrelated interactions (green dashed lines) until quadruple bonds. The blue background region includes the set of data points that are beyond the multiple bonds prediction. Bell-Evans model yielded $k_{off} = 6.52 \pm 1.44 \text{ s}^{-1}$ and $x_{\beta} = 0.14 \pm 0.01 \text{ nm}$, the complex off-rate constant and distance along the reaction coordinate of the transition between bound and unbound states, respectively. Solid circles represent the mean values, and error bars the standard deviations. (b) Rupture force histograms as a function of discrete loading rate ranges: LR1 $< 2.7 \times 10^3$, $2.7 \times 10^3 < \text{LR2} < 6.3 \times 10^3$, $6.3 \times 10^3 < \text{LR3} < 1.5 \times 10^4$, $1.5 \times 10^4 < \text{LR4} < 3.4 \times 10^4$, $3.4 \times 10^4 < \text{LR5} < 8.0 \times 10^4$, $8.0 \times 10^4 < \text{LR6} < 1.9 \times 10^5$, $1.9 \times 10^5 < \text{LR7} < 4.3 \times 10^5 \text{ pN/s}$. A major shift toward high forces with increasing LRs is observed. (c) Plot of binding frequency as a function of contact time obtained for Δspa cells ($n = 7$ cells) cultured in RPMI medium, and associated pseudo-first-order kinetics fit (red line), yielding $k_{on} = (0.8 \pm 0.2) \times 10^4 \text{ M}^{-1}\text{s}^{-1}$, the kinetic on-rate constant of the IsdB-TLR4 complex. Means and standard deviations are indicated by solid circles and error bars, respectively.

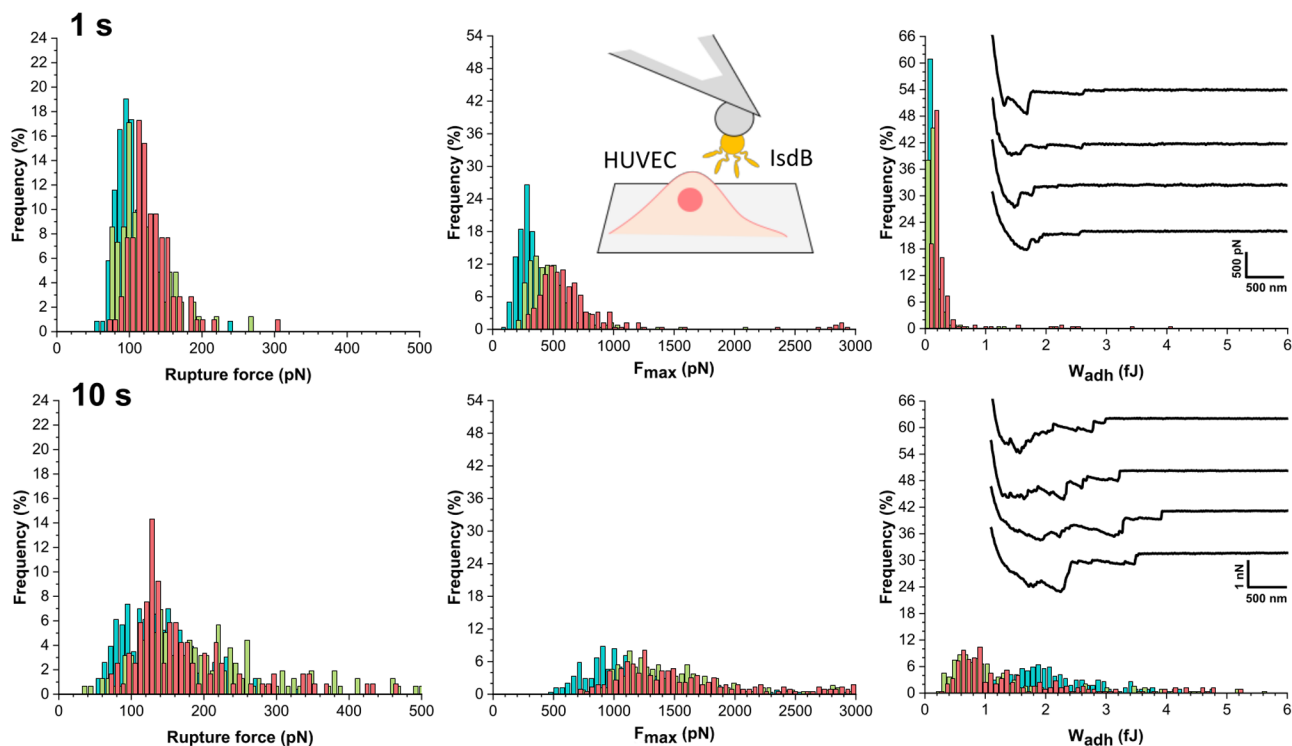
S8) and Δspa cells, $\Delta isdB$ cells showed much lower binding probability, 12% (Figures 3c,d and S9, 139 adhesive curves from 9 cells). Rupture forces of 98 ± 27 and $47 \pm 23 \text{ pN}$ were detected for the two strains, respectively (Figure 3e), matching the magnitude of the forces measured for Δspa (Figures 2a and S3). These results provide direct evidence that, under low (moderate) mechanical tension, IsdB specifically interacts with TLR4 with a strength of $\sim 100 \text{ pN}$. We argue that this value is the unit force corresponding to a single bond as (i) the same force was observed both in SCFS and SMFS using different cells from independent cultures; (ii) it was not dramatically decreased upon anti-TLR4 blocking (expected if multiple bonds were probed in parallel); and (iii) similar forces were observed in both Δspa and WT bacteria expressing IsdB.

Physical Stress Strengthens the IsdB-TLR4 Interaction. *S. aureus* adhesion can be strongly enhanced by fluid shear stress,²⁶ and several recent studies have shown that this phenomenon originates from the stress-activation of several CWA proteins (SdrE, ClfA, and ClfB), i.e., their ability to bind their target ligands with ultrastrong forces when they are exposed to high mechanical force.^{27–30} We wondered whether the IsdB-TLR4 complex follows such a stress response by measuring rupture forces while varying the loading rate (LR, the rate at which force is applied, estimated from the linear slope immediately preceding each rupture event on the force vs time curves). Data corresponding to single interactions were fitted with the Bell-Evans model,^{31,32} from which we extracted a k_{off} value of $6.5 \pm 1.4 \text{ s}^{-1}$ (Figure 4a). The parameters from the Bell-Evans fit were used to estimate the rupture forces of multiple simultaneous parallel bonds, according to the Williams-Evans prediction.^{33,34} For forces below 500 pN, a good correlation was obtained between the prediction and the rupture forces extracted from the rupture force distributions. However, a large amount of high and very high forces (500–2000 pN) could not be fitted, implying they do not result from the rupture of multiple uncorrelated bonds. Examination of the rupture force distributions plotted for discrete LR ranges (Figures 4b and S10), revealed a progressive shift toward high

forces with increasing LRs. While at low LR ($< 6.3 \times 10^3 \text{ pN/s}$), a single narrow distribution of weak forces, lower than 250 pN, was observed, at high LR ($> 1.9 \times 10^5 \text{ pN/s}$), a broad distribution composed of high and very high forces, up to 2000 pN, was seen. Hence, under high LRs, the population of lower forces becomes depleted, and only strong forces dominate. As these forces cannot be described by multiple bonds, we believe that they originate from an atypical catch-bond mechanism, where the IsdB-TLR4 interaction strengthens with tensile loading. Interestingly, the appearance of single narrow rupture length distributions at high LR ranges, as opposed to multiple broad distributions at low LR ranges (Figure S11), strongly supports the notion of a stress-activated mechanism involving conformational changes in the structure of the complex. Additionally, no strong correlation was observed between the force and rupture length for the different LR ranges studied (Figure S12).

This force-enhanced mechanism that we believe is used by the bacteria to more efficiently adhere to TLR4 under high shear stress is reminiscent to those described for the staphylococcal adhesins SdrG, SdrE, SpsD, ClfA, and ClfB, all belonging to the MSCRAMMs family and all forming DLL-like complexes with their multiple ligands.^{27,29,30,35,36} In the particular case of IsdB, not only similar mechanisms have been described for its interaction with the extracellular matrix component Vn²⁴ and host cell $\alpha_v\beta_3$ integrin,²⁵ but more recently, the hypothesis of a catch-bond mechanism for the interaction with the oxidized form of hemoglobin (metHb) has been pointed out.³⁷ In fact, IsdB is known to bind hemoglobin for heme extraction through its NEAT2 domain, an essential process for bacteria to efficiently capture iron during the host infection process, circumstances where most of the time free iron is scarce, and moreover, bacterial cells are exposed to extremely high shear forces, the same *in vivo* conditions faced while interacting with TLR4. It is worth mentioning that the loading rates we tested are biologically relevant since they span the range of shear forces encountered by bacterial cells in flowing blood.²⁷

a RPMI



b BHI

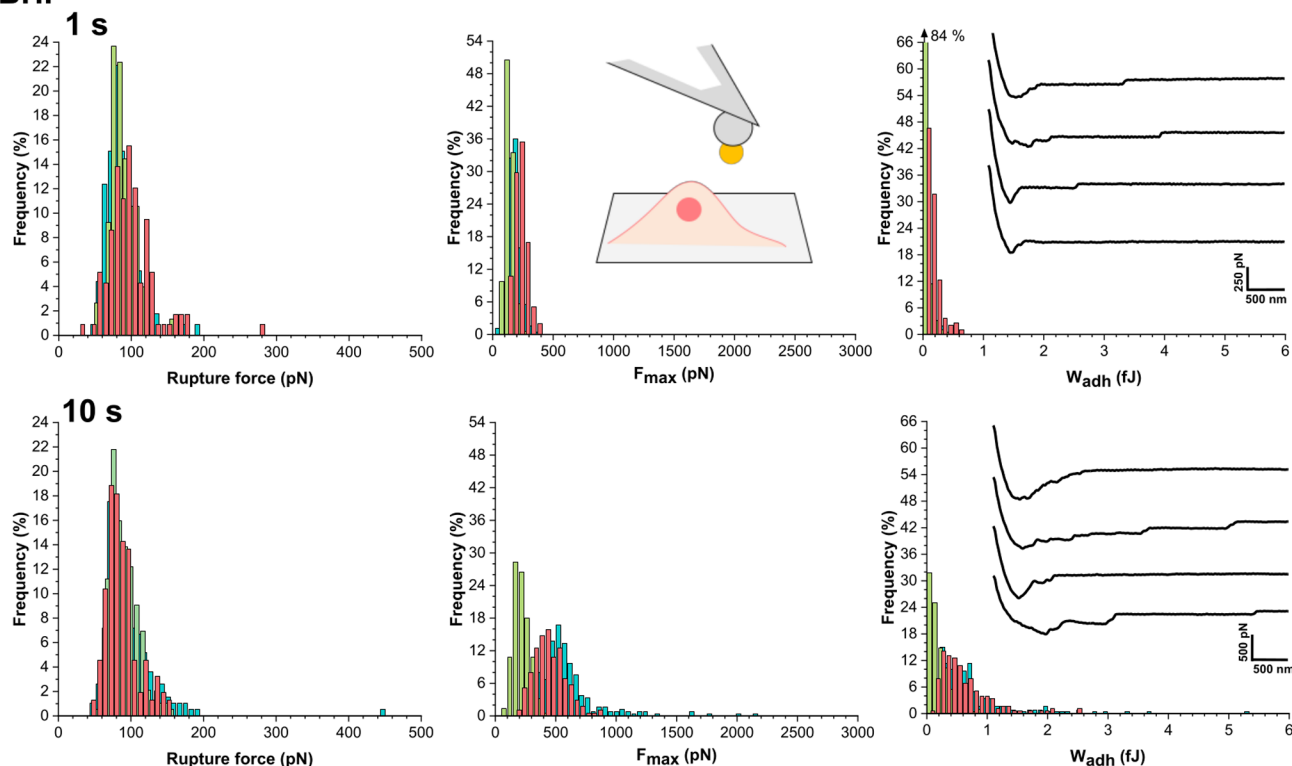


Figure 5. IsdB mediates bacterial adhesion to human endothelial cells via TLR4. (a, b) Adhesion of *S. aureus* Δspa cells to human umbilical vein endothelial cells (HUVEC) expressing TLR4, assessed by single-cell force spectroscopy (SCFS). Rupture force, maximum adhesion force (F_{max}), and work of adhesion (W_{adh}) histograms of three different cell pairs acquired by recording force–distance curves in HEPES, at a retraction speed of $20 \mu\text{m/s}$, after 1 and 10 s of interaction between single bacteria grown in (a) RPMI and (b) BHI media and TLR4-expressing endothelial cells. 25 mM glucose was added to EGM-2 endothelial cell growth medium to induce TLR4 expression by HUVEC. Schemes of the SCFS setups and representative retraction force profiles are shown as insets. Data from a total of 256 curves for each cell pair.

Finally, we observed that the binding frequency increased with interaction time (Figure 4c), and from this relationship the kinetic on-rate constant of the complex (k_{on}) was extracted assuming a pseudo-first-order kinetics,^{38,39} $k_{\text{on}} = (0.8 \pm 0.2) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, yielding, from the relation $k_{\text{off}}/k_{\text{on}}$, a dissociation constant $K_d \sim 800 \mu\text{M}$. Even though this value differs from the K_d previously determined by ensemble measurements (98 nM),¹⁶ this is not surprising due to the different nature of these parameters. While in our experiments the IsdB-TLR4 complex was dissociated under high force, i.e., far from equilibrium, biochemical affinity values are estimated at equilibrium, in the absence of force. This is consistent with the notion that under mechanical stress, protein complexes dissociate through a path different from that occurring in thermal dissociation⁴⁰ and, this is particularly important in the case of bacterial adhesins engaged in catch bonds, as reported here for IsdB-TLR4. This has been shown for the case of the prototypical adhesin SdrG and its Fg ligand,^{35,41} where two distinct dissociation pathways emerge depending on whether the dissociation occurs at high or low forces. These differences highlight the importance of studying the behavior of such receptor–ligand systems under mechanical force, as *in vivo*, *S. aureus* cells are exposed to different shear stress conditions, which are known to activate the pathogen–host interactions. Future investigations using *in silico* and *in vitro* techniques capable of mimicking forces acting tangentially along the bacterial cells, e.g., steered molecular dynamic simulations and microfluidic flow chamber assays, will certainly complement our findings, by providing further insight into how IsdB-mediated bacterial adhesion to TLR4 is modulated by shear stress.

IsdB Mediates Bacterial Adhesion to Host Cells via TLR4. Does the IsdB-TLR4 bond support bacterial adhesion to, and possibly invasion of, human endothelial cells? We tested this hypothesis by recording the forces between Δspa cells and human vein endothelial cells (HUVEC) expressing TLR4, cultured in the presence of high glucose concentration.^{42,43} Retraction force profiles revealed complex adhesion force features with discrete jumps and force plateaus (Figure 5), reflecting the rupture of intermolecular bonds and the extraction of membrane tethers, respectively. The last rupture event, corresponding to force plateaus for most of the curves, was registered at mean forces of $108 \pm 8 \text{ pN}$ (Figure 5a, $n = 757$ adhesive curves from 3 cells) and $86 \pm 2 \text{ pN}$ (Figure 5b, $n = 645$ adhesive curves from 3 cells), for bacteria grown in RPMI and BHI, respectively, after 1 s of interaction with TLR4-expressing HUVEC, with a length up to $6 \mu\text{m}$ in both cases (Figure S13). The presence of membrane tethers confirms that bacteria successfully established contact with the host cell membrane, and furthermore, this contact is strengthened for bacterial cells expressing IsdB. Maximum adhesion forces, $F_{\text{max}} = 415 \pm 105$ and $179 \pm 38 \text{ pN}$, were observed for Δspa cells grown in RPMI and BHI, respectively, for the same interaction time. Again, distinct adhesion behavior observed for bacteria culture in the two different conditions shows that bacterial interaction with endothelial cells is mediated by IsdB. Finally, work of adhesion, W_{adh} , was estimated from the area under the retraction curves,⁴⁴ yielding similar values, up to 1 fJ, for the bacteria grown in the two culture media.

We then tested the effect of time on the bacterial adhesion to HUVEC. When increasing the duration of bacterial–host adhesion to 10 s, we observed that both rupture and maximum forces increased for Δspa cells grown in RPMI, $143 \pm 22 \text{ pN}$

and $1201 \pm 154 \text{ pN}$, respectively (Figure 5a, 737 adhesive curves from 3 cells) while much less pronounced differences were observed for BHI-cultured bacteria, $83 \pm 6 \text{ pN}$ and $390 \pm 178 \text{ pN}$, respectively (Figure 5b, 638 adhesive curves from 3 cells). Strikingly, not only average rupture force and F_{max} markedly increased for bacteria grown in RPMI, but their range also increased, up to 500 pN and 3 nN, respectively. Similarly, W_{adh} values were seen to substantially increase, up to 5 fJ, only for Δspa cells grown in RPMI, while BHI-cultured bacterial cells interact with HUVEC with a W_{adh} value in the 0–2 fJ range. Altogether, these data show that the IsdB-TLR4 complex plays an important role in bacterial adhesion and, with time, the deformable host cell membrane increasingly covers the bacterial cells, leading ultimately to their internalization.

As mentioned above, high concentrations of glucose induce expression and activation of TLR4 in endothelial cells and this effect may lead to the pathogenesis of diabetic retinopathy.⁴³ Recently, Liu et al.⁴⁵ reported that *S. aureus* can block insulin function through LtaS, a membrane-bound enzyme involved in lipoteichoic acid biosynthesis. Thus, TLR4 can also be upregulated in endothelial cells during *S. aureus* infection, generating ideal conditions, where both IsdB and TLR4 are highly expressed, for these two molecules to interact. We can speculate that adhesion of *S. aureus* to endothelial cells via the IsdB-TLR4 axis can be operational following the occurrence of two events: (i) the increased expression of TLR4 induced by high levels of glycemia in pathological conditions and (ii) the enhanced exposure of IsdB on the staphylococcal surface in iron starvation conditions in the blood.

It has been previously shown that TLR4 directly binds to IsdB *in vitro*¹⁶ and we confirmed this point in our experimental conditions. However, we do not exclude the possibility that, *in vivo*, the IsdB-TLR4 interaction may be modulated by other biochemical-associated partners, such as MD-2. As already reported, IsdB induces cytokine release in human monocytes and murine bone marrow-derived dendritic cells.¹⁶ Thus, it is worth exploring the possibility that IsdB binding to TLR4 on endothelial cells could elicit a signaling pathway that triggers innate immune mechanisms.⁴⁶

CONCLUSIONS

S. aureus expresses a plethora of virulence factors capable of interacting with multiple host ligands through very diverse mechanisms that determine adhesion to and invasion of various types of mammalian cells, notably endothelial cells, allowing the pathogen to evade the host defense and antibiotics action. For instance, binding to endothelial cells can occur via (i) FnBPA, requiring fibronectin as bridging molecule and $\alpha_5\beta_1$ integrin as host receptor;⁴⁷ (ii) ClfA through the ternary complex, formed between ClfA, fibrinogen and $\alpha_v\beta_3$ integrin;⁸ (iii) IsdB, via interaction with the extracellular matrix protein vitronectin (Vn), which acts as a bridge between IsdB and $\alpha_v\beta_3$ integrin.²⁰ While in such cases adhesion to endothelial cells requires the formation of ternary complexes, in the present study we report a distinct adhesion mechanism based on the formation of a stress-activated complex between IsdB and TLR4. In stringent conditions, such as hyperglycemia, and low iron concentration in the blood, endothelial cells deploy increased expression of TLR4 and, in the meantime, Staphylococcal cells are able to express high levels of IsdB, with consequent *S. aureus* firm adhesion to the host cells and initiation and spreading of the infective process. To unravel the details of this interaction, IsdB binding to

TLR4 was examined under physical stress using single-cell and single-molecule atomic force microscopy. As a result of this study, strong bonds in the nano-Newton range were detected, providing the pathogen with a specific mechanism of adhesion to endothelial cells. Thus, as well as being a pathogen-associated molecular pattern capable of triggering innate defense mechanisms,¹⁶ IsdB appears to be a potent virulence factor and an attractive target for the development of therapeutics against this formidable bacterium. In conclusion, it may be worth exploring the functioning of such an atypical mechanism of adhesion of *S. aureus* to other tissues and cells of the host.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *S. aureus* SH1000 Δspa , SH1000 WT, and SH1000 $\Delta isdB$ strains^{20,21} were grown overnight, either in Roswell Park Memorial Institute (RPMI) or brain heart infusion (BHI) medium, at 37 °C under shaking. The resultant stationary phase cultures were then harvested by centrifugation at 2000g for 5 min, washed twice in PBS and 50× diluted in the same buffer.

HUVEC Culture Conditions. Human Umbilical Vein Endothelial Cells (HUVEC) from pooled donors (Lonza) were cultured in commercial endothelial cell growth medium-2 (EGM-2 BulletKit, Lonza) in T25 flasks at 37 °C, 5% CO₂, and 100% humidity. Cells from passages 3–6 were seeded on 50 mm culture-treated polymer coverslip bottom dishes (ibidi), in the presence of 25 mM glucose to induce TLR4 expression,^{42,43} for at least 48 h prior to the experiments and used after reaching confluence.

Functionalization of Substrates and AFM Cantilevers. Prior to functionalization, gold-coated glass coverslips and cantilevers (PNP-Tr-Au, Nanoworld) were thoroughly rinsed with water and ethanol, dried with nitrogen flow, and cleaned in a UV-ozone chamber for 15 min. These were then immersed overnight in a solution containing a mixture of 1 mM 10% 16-mercaptododecahexanoic acid (Sigma-Aldrich) and 90% 1-mercapto-1-undecanol (Sigma-Aldrich) in ethanol, protected from light, rinsed with ethanol, and dried with nitrogen flow afterward. After that, substrates and cantilevers were immersed in an aqueous solution containing 10 mg/mL N-hydroxysuccinimide (NHS) and 25 mg/mL 1-ethyl-3-(3-dimethylamino)propyl)-carbodiimide (EDC) for 30 min and further rinsed with water. A last 1 h incubation step with a 0.1 mg/mL solution of recombinant human TLR4 (R&D Systems) in PBS was performed, before rinsing the substrates and cantilevers with PBS.

Macroscopic Adhesion Assay. TLR4-coated substrates were incubated with bacterial suspensions at 37 °C for 2 h and further washed with PBS to remove loosely attached cells. Optical microscopy images were acquired using an inverted microscope (Zeiss AXIO Observer Z1).

Single-Cell Force Spectroscopy. Colloidal probes for single-cell experiments were prepared as previously described.²³ In short, triangular tipless cantilevers (NP-O10, Bruker) were first mounted on the AFM and brought into contact with a thin layer of UV light-curable glue (NOA 63, Norland Edmund Optics). The glue-covered part of the cantilever was then approached to a silica bead of 6.1 μ m diameter (Bangs Laboratories) to form a colloidal probe, that was afterward taken out of the AFM, and the glue was cured under UV light for 20 min. Cantilevers were then immersed for 1 h in Tris-buffered saline solution (50 mM Tris, 150 mM NaCl, pH 8.5) containing 4 mg/mL of dopamine hydrochloride (Sigma-Aldrich), rinsed with Tris-buffered saline solution, and mounted on the AFM setup. 200 μ L of the diluted bacterial suspension was left to adhere to a polystyrene dish for 20 min, and the dish was further washed three times and filled with 2 mL of PBS for measurements. Experiments were conducted at room temperature using a JPK NanoWizard 4 NanoScience AFM. Colloidal probe calibration was performed by the thermal noise method, yielding a spring constant of $\sim$ 0.08 N/m. The colloidal probe was gently brought into contact with a single bacterial

cell, and once the probe was retracted, the attachment of the cell to the colloidal probe was monitored with an inverted optical microscope. This freshly prepared cell probe was then positioned over the TLR4-coated substrate, previously glued onto the dish, and 16 $\times$ 16 force–distance curves were recorded in force mapping mode on multiple 10 μ m $\times$ 10 μ m spots, using a contact force set point of 250 pN, constant approach and retraction speeds of 1 μ m/s, and a dwell time of 1 s. For SCFS experiments on HUVEC, 25 μ L of a diluted bacterial suspension were deposited on a dish containing confluent HUVEC monolayers, at a distinct location, and bacterial probes prepared as described above. Force measurements were performed in a HEPES-buffered saline solution at 37 °C. 16 $\times$ 16 force–distance curves were acquired across 1 μ m $\times$ 1 μ m areas of the HUVEC, using a contact force set point of 750 pN, constant approach and retraction speeds of 20 μ m/s, and a dwell time of 1 or 10 s.

Single-Molecule Force Spectroscopy. For single-molecule experiments, 200 μ L of the diluted bacterial suspension were deposited on a polystyrene dish, for 20 min, and the dish was further washed three times and filled with 2 mL of PBS for measurements. Experiments were conducted at room temperature using a JPK NanoWizard 4 NanoScience AFM. TLR4-functionalized cantilevers were calibrated by the thermal noise method, yielding a spring constant in the 0.03–0.06 N/m range. 32 $\times$ 32 force–distance curves were recorded in force mapping mode on 500 nm $\times$ 500 nm areas on top of single bacterial cells, using a contact force set point of 250 pN, constant approach and retraction speeds of 1 μ m/s, and a dwell time of 0.5 s. For dynamic force spectroscopy experiments, the retraction speed was varied from 1 to 2.5, 5, and 10 μ m/s. For blocking experiments, TLR4-functionalized cantilevers were exposed to a 100 μ g/mL anti-TLR4 monoclonal antibody (MAB14782, R&D Systems) solution in PBS for 1 h. A monoclonal mouse IgG antibody (Human MD-2 Antibody, MAB1787, R&D Systems) was used as negative control. All force data were analyzed with the JPK Data Processing software, and statistical analysis was carried out using Origin software (OriginPro 2021).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.4c12648>.

Additional rupture force and rupture length data of SCFS and SMFS experiments performed with *S. aureus* SH1000 Δspa strain as well as respective force vs rupture length plots; blocking experiments with anti-TLR4 antibody; and SH1000 WT and SH1000 $\Delta isdB$ control strains (Figures S1–S13) (PDF)

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Author Contributions

T.O.P., P.S., and Y.F.D. designed the experiments. T.O.P. performed the experiments and collected the data. T.O.P. analyzed the data. T.O.P., P.S., and Y.F.D. wrote the article.

Notes

The authors declare no competing financial interest.

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