Yersinia enterocolitica exploits different pathways to accomplish adhesion and toxin injection into host cells

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Summary

The current paradigm suggests that Yersinia enterocolitica (Ye) adheres to host cells via the outer membrane proteins Yersinia adhesin A (YadA) or invasin (Inv) to facilitate injection of Yops by the type III secretion system. In this process Inv binds directly to β1 integrins of host cells while YadA may bind indirectly via extracellular matrix proteins to β1 integrins. Here we challenged this paradigm and investigated the requirements for Yop injection. We demonstrate that Inv- but not YadA-mediated adhesion depends on β1 integrin binding and activation, and that tight adhesion is a prerequisite for Yop injection. By means of novel transgenic cell lines, shRNA approaches and RGD peptides, we found that YadA, in contrast to Inv, may use a broad host cell receptor repertoire for host cell adhesion. In the absence of β1 integrins, YadA mediates Yop injection by interaction with αV integrins in cooperation with yet unknown cofactors expressed by epithelial cells, but not fibroblasts. Electron microscopic and flow chamber studies revealed that a defined intimate contact area between Ye and host cells resulting in adhesion forces resisting shear stress is required for Yop injection. Thus, the indirect binding of YadA to a broad extracellular matrix (ECM) binding host cell receptor repertoire of different cell types makes YadA a versatile tool to ensure Yop injection. In conclusion, given the differential expression of the outer membrane proteins Inv and YadA in the course of Ye infection and differential expression of integrins by various host cell populations, the data demonstrate that Ye is flexibly armed to accomplish Yop injection in different host cell types, a central event in its immune evasion strategy.

Introduction

Yersinia enterocolitica (Ye) is an enteric pathogen causing enteritis and enterocolitis as well as extraintestinal manifestations such as mesenteric lymphadenitis, reactive arthritis and septicaemia (Koornhof et al., 1999; Smego et al., 1999). One important mechanism of Ye to evade host defence by innate immunity and to replicate extracellularly in the host depends on type III secretion system (T3SS) consisting of an injectisome and effector proteins, the latter of which are injected into host cells...
The T3SS injectisome is a complex, adenosine triphosphate driven protein export machinery. The basal body of the injectisome is composed of proteins forming a ring-like structure, spanning through the bacterial membranes, the periplasm and the peptidoglycan wall. The injectisome terminates in a needle-like structure that protrudes from the bacterial surface (Ghosh et al., 2004; Marlovits and Stebbins, 2010; Loquet and Higuchi, 1958; Forsberg and Wolf-Watz, 1988; Hakansson et al., 1993) at the area of host cell contact. However, Yop secretion can also be triggered by Ca\(^{2+}\) chelation or extracellular amino acids such as glutamate (Hocking et al., 2012). Pore-forming proteins enable injection of effector proteins such as Yop proteins through the membrane of host target cells (Hakansson et al., 1993) at the area of host cell contact. However, Yop secretion can also be triggered by Ca\(^{2+}\) chelation or extracellular amino acids such as glutamate combined with a temperature shift to 37°C (Kupferberg et al., 1958; Forsberg and Wolf-Watz, 1988; Straley et al., 1993; Forsberg et al., 1994; Lee et al., 2001; Lee and Scheunewind, 2002). Yersinia virulence is dependent on the T3SS (Trütsch et al., 2004), which injects at least six effector Yops into host cells. The Yops inhibit proinflammatory host responses and antigen uptake/presentations (Erfurth et al., 2004; Viboud and Bliska, 2005; Autenrieth et al., 2007), induce apoptosis, disrupt the actin cytoskeleton or inhibit phagocytosis (Cornelis, 2002; Viboud and Bliska, 2005).

In recent studies, a β-lactamase reporter system has been applied to detect Yop injection both in cell culture and in an experimental mouse infection model (Marketon et al., 2009; Körberle et al., 2009; Durand et al., 2010). Cell culture studies using HeLa cells, fibroblasts and phagocytes have demonstrated that the outer membrane proteins of Yersinia spp. invasin (Inv), Yersinia adhesin A (YadA) and attachment-invasion locus (Ail) are required for cell adhesion and Yop injection by the T3SS (Sory and Cornelis, 1994; Mejia et al., 2008; Felek and Krukonis, 2009; Maldonado-Arocho et al., 2013). YadA is a trimeric autotransporter adhesin consisting of a membrane anchor, a stalk and a head domain (Hoizyk et al., 2000). It is expressed at 37°C and regulated by the transcriptional regulator of the Yersinia virulence regulon VirF (Skurnik and Toivanen, 1992; Tahir et al., 2000). YadA has been demonstrated to be essential for the virulence of Ye in mice (Di Genaro et al., 2003; Schütz et al., 2010). Specifically, YadA mediates cell adhesion, autoagglutination and serum resistance, and provokes host cell responses including the production of cytokines (El Tahir and Skurnik, 2001). Inv is a member of the intimin/Inv protein family (Tsai et al., 2010) consisting of a membrane-associated β-barrel and extracellular C-terminal domains. The last two C-terminal domains are involved in host cell binding (Lee et al., 2000). In contrast to YadA, Inv is regulated by RovA and expressed at a low temperature (26°C) and low pH (Ellison et al., 2004). In cell culture experiments, Inv has been demonstrated to promote host cell adhesion and invasion. In experimental mouse infection, Inv plays a role in pathogenicity in the early phase of infection; however, it is not essential for virulence (Pepe and Miller, 1993; Pepe et al., 1994; Leo and Skurnik, 2011). The Ail protein is a 17 kDa and due to its small size it is easily masked by other surface structures. Deletion of Ail in Ye has no impact on the course of orogastric Ye infection of mice (Wachtel and Miller, 1995). In cooperation with YadA, Ail ensures a high level of serum resistance by binding of factor H (Bialas et al., 2012). Ail of Ye mediates adhesion/invasion when expressed in Escherichia coli (Wachtel and Miller, 1995). Comparison of Y. pestis and Y. pseudotuberculosis (Yps) Ail expressed in E. coli revealed a reduced fibronectin binding and reduced adhesion/invasion capacity of Yps Ail due to subtle differences in the amino acid sequence of Ail. In contrast to Y. pestis, expression of Ail in Yps YpIII strain does not lead to measurable adhesion or invasion, suggesting that other surface structures like lipopolysaccharide (LPS) interfere with Ail cell-binding activity (Tsang et al., 2013). Thus, variations in the amino acid sequences as well as the bacterial surface context might strongly influence adhesion ability of Ail. Studies (Felek et al., 2009; Tsang et al., 2010; Ho et al., 2012) with Y. pestis Ail suggested that it interacts with the centrally located 120-kDa fragment containing type III Fn (FNIII) repeats of fibronectin (Kolodziejek et al., 2007; Felek and Krukonis, 2009; Tsang et al., 2010; 2012; Ho et al., 2012).

Recent work using fibroblasts has demonstrated that both Inv and YadA require β1 integrins for Yop injection (Körberle et al., 2009). Detailed studies have determined that Inv is a high-affinity ligand for the β1 integrins (α3β1, α4β1, α5β1, αβ8) and (αβ1) (Isberg and Leong, 1990; Leong et al., 1993; Hamburger et al., 1999), while YadA binds to extracellular matrix (ECM) proteins such as collagen, laminin and fibronectin (El Tahir and Skurnik, 2001; Heise and Dersch, 2006; Leo et al., 2008; 2010). As ECM proteins bind to β1 integrins, it is assumed that adhesion of YadA to β1 integrins might occur indirectly via ECM proteins.

Previous studies addressing the interaction of Yps with host cells showed that in the absence of both YadA and Inv, constitutive expression of pH 6 antigen (psaA), an adhesion factor expressed by Yps and Y. pestis and mediating binding to glycosphingolipids (Payne et al., 1998; Makoveichuk et al., 2003), rescues adhesion but is not sufficient to promote Yop injection (Mejia et al., 2008). These data suggest a specific role of Inv and YadA to initiate Yop injection. In addition, these studies suggest that in Yps infection Src kinases, Rho GTPases and the
actin cytoskeleton are also involved in promoting Yop injection into HeLa cells (Meija et al., 2008). Although β1 integrins are thought to be crucial for Yop injection, previous studies did not differentiate between adhesion promoted via Inv or YadA in this context.

Integrins represent a large family of transmembrane α/β heterodimeric receptors that mediate cell–cell and cell–matrix interaction (Hynes, 1992; van der Flier and Sonnenberg, 2001). They are subdivided in integrin families according to the ECM proteins they mainly bind to, such as collagen receptors (α1β1, α2β1, α10β1, α11β1), laminin receptors (α3β1, α6β1, α7β1, α6β4), RGD receptors (binding to vitronectin and fibronectin: α5β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8, cllβ3) and according to specific expression on leukocytes (αDβ2, αLβ2, αMβ2, αXβ2, αEβ7, α4β1, α9β1, α4β7, αEβ7) (Barczyk et al., 2010). As for β1 integrins, they play a crucial role in development by regulating several important cellular functions, including cell adhesion, spreading and cell migration (Fassler and Meyer, 1995; Fässler et al., 1996; Brakebusch et al., 1997; Stupack and Cheresh, 2002). The binding of integrins to ECM proteins leads to integrin clustering, the recruitment of adaptor and signalling proteins and finally to the formation of focal adhesions, which connect integrins to the actin cytoskeleton and mediate signalling (outside–in signalling). Prior to ligand binding integrins have to shift from an inactive to an active conformation (inside–out signalling) (Hynes, 1992; Moser et al., 2009), which is triggered by the recruitment of talin and kindlin to the cytoplasmic domain of β integrin subunits (Calderwood, 2004; Bouaouina et al., 2008; Montanez et al., 2008; Moser et al., 2009).

In the presented study, we addressed which host cell surface receptors and which signalling pathways are specifically required to promote YadA- and Inv-mediated adhesion of Ye to and subsequent Yop injection into host cells. Although leukocytes are the main target for Yop injection (Köberle et al., 2009; Autenrieth et al., 2010; Durand et al., 2010; Maldonado-Arocho et al., 2013), we used as an easy to manipulate and commonly used simplified model fibroblasts and epithelial cell lines to study Yop injection (Eitel and Dersch, 2002; Meija et al., 2008; Wolters et al., 2013). To this end, we employed novel transgenic cell lines and demonstrate that both YadA and Inv contribute to Yop injection. Although Inv strictly interacts with β1 integrins and largely requires β1 integrin cytoplasmic domains and a linkage to the actin cytoskeleton, YadA-mediated adhesion and Yop injection was found to be β1 integrin dependent in fibroblasts, but β1 integrin independent in epithelial cells. Studies using RGD peptides as well as shRNA approaches defined RGD receptors of the αV integrin family as alternative receptors for YadA to promote a tight adhesion that is required to inject Yops into epithelial cells.

### Results

#### Role of β1 integrins for Inv- and YadA-mediated Yop injection into fibroblasts and epithelial cells

To test whether β1 integrins play an essential role for Yop injection and whether and how β1 integrin mediated signalling contributes to Yop injection, we used different transgenic cell lines. We infected β1 integrin-deficient GD25 mouse embryo fibroblasts and β1 integrin-transfected GD25 cells (GD25β1A) (Retta et al., 1998) as well as β1 integrin-deficient epithelial cells (CDβ1−/−) and β1 integrin-transfected epithelial cells (CDβ1A) with the Ye wild-type strain containing a β-lactamase–YopE hybrid protein (Ye pBla), or Ye mutant strains lacking either both Inv and YadA (ΔInvΔYadA-pBla), YadA (ΔYadA-pBla) or Inv. Successful Yop injection into viable host cells was determined by means of a Bla reporter assay. In this assay, injected YopE-Bla converts the green fluorescence of CCF4 into blue fluorescence due to its β-lactamase activity (Charpentier and Oswald, 2004; Köberle et al., 2009). In addition, we determined adhesion of bacteria to host cells by microscopic assays.

Infection of both fibroblasts and epithelial cells with Ye pBla (wild type) revealed Yop injection into GD25β1A, CDβ1−/− and CDβ1A, but not into β1 integrin-deficient GD25 cells, indicating that β1 integrins are apparently required for Yop injection into fibroblasts, but are dispensable for Yop injection (blue cells) into epithelial cells (Fig. 1A). Ye ΔYadAΔInv-pBla did not result in adhesion (data not shown) and consequently no Yop injection was observed (Fig. 1B). Adhesion and subsequent Yop injection thus depend on either YadA or Inv, and that adhesion seems to be a prerequisite for Yop injection.

After infection of cells with Ye ΔYadA-pBla expressing Inv, but not YadA, adhesion was only found for β1 integrin-expressing GD25β1A and CDβ1A cells, but not for β1 integrin-deficient GD25 or CDβ1−/− cells indicating that Inv-mediated adhesion strictly depends on β1 integrins (Fig. 1C). Consistently, Yop injection could specifically be detected in β1 integrin-expressing cells.

After infection of cells with Ye ΔInv-pBla expressing YadA, but not Inv, Ye adhered to all tested cell lines (Fig. 1D) irrespective of β1 integrin expression (Fig. S1). Surprisingly, Yop injection was detected in GD25β1A, CDβ1−/− and CDβ1A, but not in GD25 cells, indicating that despite obvious adhesion, YadA-mediated Yop injection into fibroblasts required the presence of β1 integrins (Fig. 1D). In contrast, YadA-mediated Yop injection into epithelial cells occurred also in the absence of β1 integrins. Comparable results were obtained with other fibroblast (KFβ1fl/fl) and epithelial (GE11β1) cell lines and their β1 integrin-deficient derivatives (KFβ1−/−, GE11) (Fig. S2), suggesting that the observations are representative for fibroblasts and epithelial cells.
To define the dose-dependent relationship between adhesion and infection dose or Yop injection, cells were infected with different multiplicity of infection (MOI) of YeΔInv-pBla or YeΔYadA-pBla, and adhesion and Yop injection were investigated by flow cytometry and adhesion assays (Fig. 2). After infection with YeΔYadA-pBla, adhesion infection dose dependently increased to GD25β1A and CDβ1A cells (but not to GD25 or CDβ1−/− cells) (Fig. 2, left panel). Increased adhesion to GD25β1A and CDβ1A cells was paralleled by an infection dose-dependent increase in Yop injection into the β1 integrin-expressing GD25β1A and CDβ1A cells, but not into GD25 or CDβ1−/− cells (Fig. 2, middle panel). An infection dose-dependent increase of

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adhesion was found for all cell lines after infection with Ye ΔInv-pBla (Fig. 2, left panel), supporting the notion that YadA-mediated adhesion can also occur in the absence of β1 integrins. Yop injection occurred in the presence of β1 integrins into both epithelial CDβ1A cells and GD25β1A fibroblasts; in the absence of β1 integrins, however, Yop injection occurred only in CDβ1−/− cells but not in GD25 fibroblasts (Fig. 2, middle panel). Inv-mediated Yop injection (upon infection with Ye ΔYadA-pBla) into CDβ1A cells was highly effective even at low MOI as compared with YadA-mediated Yop injection (upon infection with Ye ΔInv-pBla). This most likely reflects the high affinity of Inv for β1 integrins. Analysis of the β1 integrin expression levels of tested cell lines by flow cytometry demonstrated higher expression levels in CDβ1A compared with GD25β1A cells (Fig. S2A), which is in keeping with the fact that Inv-mediated Yop injection into CDβ1A cells occurs very efficiently even at low MOI as compared with GD25β1A cells. In conclusion, these data demonstrate that Inv-mediated host cell adhesion depends on β1 integrins and precedes Yop injection whereas YadA-mediated

Fig. 2. Association between Ye adhesion to cells and Yop injection. GD25 and GD25β1A fibroblasts as well as CDβ1−/− and CDβ1A epithelial cells were infected with ΔInv-pBla (red lines connected with triangles) and ΔYadA-pBla (blue cells connected by circles), respectively, at different MOI (10, 50, 100) and then stained with fuchsine to determine adhesion by microscopy (left panel), or stained with CCF4 to detect Yop injection by flow cytometry (middle panel). The association between MOI, adhesion and Yop injection (right panel) summarizing at least three independent experiments.
host cell adhesion may also occur in the absence of β1 integrins and does not necessarily result into Yop injection.

**YadA-triggered Yop adhesion is mediated by RGD receptors of the β1 and αV family of integrins**

YadA-mediated adhesion and Yop injection into epithelial cells also occurs in the absence of β1 integrins (Fig. 2B), leading to question which are alternative receptor(s) involved in these processes. Beside members of the β1 integrin family (α3β1, α5β1, α6β1), the GD25 and CD cell lines also express members of the αV integrin family (αVβ1, αVβ3, αVβ5, αVβ6, αVβ8) as determined by immunostaining and flow cytometry (Fig. S2 B, C). The αV integrin family members together with α5β1 belong to the RGD receptor family known to bind ECM proteins such as vitronectin and fibronectin (Ruoslahti, 1996; Takada et al., 2007).

The total expression level of the αV integrin chain was comparable in GD25, GD25Δ1A, CDΔ1A and CDΔ1A cells while the various β chains (β1, β3, β5, β6, β8) were variably expressed in different cell lines (Fig. S2A and C). The cell receptor repertoire of the different cell lines is summarized in Fig. S2D. Although the β3 chain was not expressed in GD25Δ1A cells, the β5 integrin chains were expressed in all cell lines. The β6 integrin was only expressed in epithelial cells, but not in fibroblasts (Fig. S2C), and β8 chain was not detectable in any cell line (data not shown). Therefore, we hypothesized that RGD receptors of the αV family may contribute to Ye adhesion and Yop injection.

To tackle this issue, we used RGD peptides and heparin as tools. RGD peptides inhibit interaction of the RGD-binding receptors (α5β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8) with some of their assigned ligands such as fibronectin harbouring RGD motifs (Ruoslahti, 1996; Goligorsky et al., 1998). Although Inv does not contain a RGD sequence, the RGD recognition site of the α5β1 integrin appears to be important for binding to Inv as fibronectin and RGD peptides inhibit the binding of Inv to the integrin (Van Nhieu and Isberg, 1991). Heparin binds to integrin receptors including RGD-binding receptors (αVβ3, α5β1, αVβ3, αVβ5) and ECM proteins, and interferes with the function of heparin sulfate proteoglycans (Ori et al., 2008; Faye et al., 2009; Ballut et al., 2013); in addition, heparin interferes with Yop injection (Boyd et al., 1998).

To assess whether heparin interferes with Inv- or YadA-mediated adhesion and Yop injection, fibroblasts and epithelial cells were pretreated with heparin sulfate and then infected with different Ye mutant strains. Inv-mediated adhesion and Yop injection by Ye ΔYadA-pBla was not affected by heparin (Fig. 3A, left panel). In contrast, heparin almost completely inhibited YadA-mediated adhesion and Yop injection by Ye ΔInv-pBla into both GD25 and CD cells irrespective of the presence of β1 integrins (Fig. 3A, right panel). Thus, the impact of heparin was thus independent of the presence of β1 integrins but specific for YadA and might result from inhibition of either YadA–ECM interaction or by affecting ECM–host cell receptor interaction. These data confirm that obviously different mechanisms are involved in YadA–β1 integrin and Inv–β1 integrin interaction, which may predominantly reflect the direct (Inv) and indirect (YadA) mode of adhesion binding to integrins. Moreover, the simultaneous binding of heparin to both heparin-binding domains of ECM proteins and to heparin-binding sites of RGD receptors might contribute to the strong inhibition of YadA-mediated adhesion.

To investigate whether RGD motifs are involved in Ye–host cell interaction, GD25, GD25Δ1A, CDΔ1A and CDΔ1A cells were incubated with RGD or control (RAD) peptides prior to infection with Ye mutant strains (Fig. 3B). Treatment with RGD peptide prior to infection with Ye ΔYadA-pBla did not significantly reduce Inv-mediated adhesion to the cells, but significantly reduced Yop injection into GD25Δ1A cells (Fig. 3B, left panel). YadA-mediated adhesion was significantly reduced by RGD peptide only in CDΔ1A cells. However, RGD peptide significantly reduced Yop injection only into GD25Δ1A and CDΔ1A cells compared with RAD-treated cells after infection with ΔInv-pBla (Fig. 3B, right panel). These data suggest that RGD receptors found on CDΔ1A cells (Fig. S2D), most likely αV integrins, may contribute to YadA-mediated adhesion and Yop injection.

To pursue this hypothesis and to explore whether αV integrins contribute to Yop injection, GD25, CDΔ1A as well as CDΔ1A cells were transduced with shRNA specific for αV integrins. This treatment led to a significant depletion of αV integrins in all cell lines (Fig. 4A). Adhesion of Ye ΔYadA-pBla to CDΔ1A cells and Yop injection was not affected (Fig. 4B, left panel), demonstrating that αV integrins are not involved in Inv-mediated adhesion and Yop injection.

Adhesion of Ye ΔInv-pBla to GD25 and CDΔ1A cells was significantly reduced upon αV depletion; in addition, Yop injection into CDΔ1A cells was also significantly reduced (Fig. 4B, right panel).

As αV shRNA treatment did not totally abrogate Yop injection in any of the cell lines, αV-depleted CDΔ1A cells or αV-depleted CDΔ1A cells were additionally treated with RGD peptides to block the remaining RGD receptors, namely αV integrins or αV integrins and α5β1 respectively (Fig. 4C). The results show that Yop injection was significantly reduced by RGD treatment in αV-depleted CDΔ1A cells and abrogated by RGD treatment in αV-depleted

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Fig. 3. Heparin and RGD peptides interfere with YadA-mediated Yop injection.
Indicated cell lines were treated with (A) heparin (1 mg ml⁻¹), (B) RGD or RAD peptide (500 μg ml⁻¹) for 1 h and then infected with ΔYadA-pBla (left panel) or ΔInv-pBla (right panel) (MOI 100), and adhesion (30 min after infection) and Yop injection (1 h after infection) were determined as described earlier. Graphs depict means + standard deviation from at least three independent experiments. Significant differences between groups are indicated by asterisks (⁎ P < 0.05, ⁎⁎ P < 0.01, ⁎⁎⁎ P < 0.001) as determined by one-way analysis of variance with Bonferroni corrections. nd, not determined.
Fig. 4. Inhibition of αV integrin expression affects YadA-mediated adhesion and Yop injection.

A. Flow cytometry analysis of αV integrin expression of shGFP RNA or shαV RNA transduced cell lines after immunostaining. GD25 and GD25β1A fibroblasts as well as CDβ1−/− and CDβ1A epithelial cells were infected with (B; right panel) and (C) ΔInv-pBla or (B; left panel) ΔYadA-pBla (MOI 100), and adhesion rate (30 min after infection) and Yop injection (1 h after infection) were determined. Cell lines were stably transduced with shGFP RNA or shαV RNA. Cells were treated with RAD or RGD peptides (500 μg ml−1) prior to infection. Graphs depict means ± standard deviation (three experiments). Significant differences are indicated by asterisks (* P < 0.5, ** P < 0.01, *** P < 0.001). nd, not determined.
CD|β1−/−| cells, underlining the importance of RGD receptors (αV integrins and α5β1) for YadA-mediated Yop injection into epithelial cells.

We conclude that YadA-mediated adhesion to fibroblasts and epithelial cells is achieved in the presence of αV integrins alone or in the presence of both αV integrins and β1 integrins. For productive Yop injection into fibroblasts, both αV and β1 integrins are required. In contrast, for productive Yop injection into epithelial cells only αV integrins are required while β1 integrins seem to be principally dispensable for this process.

Comparison of the integrin receptor repertoire of GD25 and CD|β1−/−| cells revealed that αV|β6 is only expressed by epithelial CD cells, but not by GD25 fibroblasts as summarized in Fig. S2D, which led to the hypothesis that β6 integrin expression might explain the differences in Yop injection between fibroblasts and epithelial cells. However, overexpression of β6 integrin in GD25 cells (GD25 pMSCV|β6) or depletion of β6 integrin in CD|β1−/−| cells by shRNA treatment (CD|β1−/−| sh|β6) did not alter Yop injection after infection of the cells with Ye-pBla or Ye Δinv-pBla (Fig. S3A and B). Thus, the different β6 integrin expression of fibroblasts and epithelial cells does not explain the found differences in Yop injection. Similarly, depletion of β3 integrin in CD|β1−/−| cells by shRNA (CD|β1−/−| sh|β3) had no impact on Yop injection (Fig. S3C). These data suggest that depletion of two family members of the αV integrin family (αV|β1 or αV|β3 or αV/αV|β5) is either compensated by other members of the αV integrin family or they are not involved in interaction with Ye. GD25 and CD|β1−/−| sh|β6 as well as GD25|β6 and CD|β1−/−| cells express comparable αV integrin receptors, suggesting that additional yet unknown cofactors exist that are required to promote Yop injection into CD|β1−/−| cells that are absent in GD25 cells.

Together, these data demonstrate that YadA, in contrast to Inv, may exploit a broad host cell receptor repertoire for host cell adhesion; in the absence of β1 integrins, YadA mediates Yop injection by interaction with αV integrins in cooperation with yet unknown cofactors expressed by epithelial cells but not by fibroblasts.

An intimate contact of Ye to host cells is required for Yop injection

Interaction of YadA and host cell receptors is supposed to involve ECM proteins such as fibronectin, laminin, and collagen (El Tahir and Skurnik, 2001).

Differential expression of these ECM proteins by the cell lines used in our study might be causative for the different ability of YadA to mediate adhesion and Yop injection. Therefore, we analysed the expression of these ECM proteins on the cell surface by immunostainings and subsequent flow cytometry, or ECM protein of whole cell lysates by immunoblot analyses. The flow cytometry analyses demonstrate that all cell lines were comparably covered with laminin (Fig. 5A). GD25 cells, however, displayed a significantly stronger fibronectin staining compared with the other cell lines (Fig. 5A). Collagen I/III was not detectable on the surface of any tested cell lines. Immunoblot analyses of ECM expression revealed similar results for fibronectin and laminin, while immunoblot analyses of all tested cell lines suggested similar high collagen I/III expression (Fig. 5B). The inability of YadA promoting Yop injection was apparently not due to the lack of laminin, collagen I/III and fibronectin in the cell lines. However, whether the increased fibronectin exposure of GD25 cells inhibits Yop injection is yet unclear.

Therefore, we explored the Ye–host cell contact in more detail by electron microscopy (Fig. S4). According to the electron micrographs, there was a close contact between Ye-pBla and the host cell membrane of both GD25 and CD|β1−/−| cells (Fig. 6). This close contact zone was associated with cell protrusions (Fig. 6A, black arrowheads), suggesting that upon attachment signalling events and actin rearrangement occurred at the host cell surface despite the absence of β1 integrins (Fig. 6A). Interestingly, quantitative analysis revealed that the total area and distance between Ye and GD25 cells was significantly larger compared with the space between Ye-pBla and CD|β1−/−| cells (Fig. 6B).

Because pretreatment with RGD peptides significantly altered Yop injection, but not adhesion, the question arose whether RGD peptides have an impact on the quality of adhesion. Indeed, pretreatment of CD|β1−/−| cells with RGD peptides significantly increased the space of the contact area between Ye and CD|β1−/−| cells (Fig. S5). In contrast, this treatment did not affect the space between Ye and GD25 cells. These data lead us to the hypothesis that an intimate contact between Ye and cells is necessary to accomplish Yop injection and that the disturbance of this intimate contact may prevent Yop injection.

Therefore, to further explore this issue, adhesion of Ye to cells was tested with flow culture conditions under mild shear stress (0.5 dynes) as recently reported by our group (Baur et al., 2014). To this end GD25, GD25|β1A, CD|β1−/−| and CD|β1A cells were seeded in a flow chamber, and the cells were exposed to culture medium containing a defined number of Ye-pBla GFP. Bacterial suspensions were pumped through the channels with a constant flow resulting in shear stress of 0.5 dynes/cm². Fluorescence microscopy was carried out to quantify the number of Ye adherent to cells. In contrast to the outcome of a classical static adhesion assay (as demonstrated, e.g., in Fig. 1), the number of Ye adhering to GD25 cells was significantly lower compared with GD25|β1A, CD|β1−/−| and CD|β1A cells (Fig. 7), indicating that the adhesion of Ye to GD25 cells is less stable.
Fig. 5. ECM expression of fibroblast and epithelial cell lines.
A. Indicated cell lines were harvested, stained with antibodies directed against fibronectin, laminin or collagen and an APC-conjugated secondary antibody and subsequently analysed by flow cytometry. Mean fluorescence of one experiment is given by numbers. The bar chart depicts the mean of the MFI and standard deviation of three experiments. Asterisks indicate significant differences.
B. Whole cell extracts were generated and immunoblot analysis was performed for detection of fibronectin, laminin or collagens. Actin levels served as loading control. One representative out of three experiments is shown.
Fig. 6. Analysis of Ye-host cell interaction by electron microscopy.
GD25, GD25β1A, CDβ1−/− and CDβ1A cells were infected with Ye-pBla for 30 min (MOI 100). Thereafter, cells were fixed and subjected to transmission electron microscopy.
A. Electron micrographs of Ye-Bla adhering to cells. Cell protrusions that are often found at the contact zone between Y. enterocolitica and host cells are labelled by arrow heads.
B. Mean + standard deviation calculated for the contact area between Ye and host cell. Values were calculated for at least 40 cells and normalized as described in Experimental procedures. Asterisks indicate significant differences (**P < 0.001).
Taken together, these data demonstrate that YadA may require a complex interaction via ECM proteins with host cell receptors such as β1 and αV integrins to achieve an intimate contact between Ye and host cells resulting in a distinct quality of adhesion, which is able to resist at least mild shear stress, are required to accomplish Yop injection by Ye (summarized in Fig. S6).

**Downstream signalling events involved in adhesion are required for subsequent Yop injection**

β1 integrins can switch between an active and an inactive conformation, and can transduce signals if they are active and ligand occupied (Takagi et al., 2003; Luo and Springer, 2006; Montanez et al., 2008; Moser et al., 2009; Campbell and Humphries, 2011). β1 integrin activation is regulated by talin1 and kindlin2 binding to NPXY motifs in the cytoplasmic domain of β1 integrins (Tadokoro et al., 2003; Montanez et al., 2008). The cytoplasmic domain of β1 integrins comprises binding sites for many proteins of the focal adhesion complex and is decisive for integrin activation (Tadokoro et al., 2003; Montanez et al., 2008). In order to test whether activation of integrins via the cytoplasmic domain is required for Ye adhesion and Yop injection, we used GD25 cells transfected with β1 integrin deletion mutants that lack distinct parts of the cytoplasmic domains (GD25β1COM, GD25β1TR) (Fig. 8A) (Retta et al., 1998; Liu et al., 2000; Meves et al., 2011). GD25β1COM cells lack the C-terminal amino acids 783–798 of the cytoplasmic domain. This part contains the kindlin and the distal talin binding site. GD25β1TR cells lack C-terminal amino acids 763–798, which in addition comprises the proximal talin binding site. Expression levels of β1 integrins of all transfected GD25 cells were comparable (Fig. 8B). Both cell lines were infected with Ye mutant strains and compared with GD25β1 cells (Fig. 8C). Adhesion of Ye ΔYadA-pBla was abrogated in GD25β1COM or GD25β1TR cells. Consequently, Yop injection was abolished. This indicates that the cytoplasmic domain of β1 integrins is critical for Inv-mediated host cell contact and Yop injection. In contrast, adhesion of Ye ΔInv-pBla to GD25β1TR cells was only slightly and not significantly affected, while Yop injection by Ye ΔInv-pBla was not abrogated but significantly reduced. This observation confirms our finding that YadA promotes adhesion also in the absence of β1 integrins. Therefore, the impact of the cytoplasmic domain of β1 integrins on adhesion cannot be clearly addressed with these cells. The partial reduction of YadA-mediated Yop injection into GD25β1COM and GD25β1TR cells suggests that the cytoplasmic domain of β1 integrins is not necessarily required for some basal Yop injection but crucial for efficient Yop injection. Inside–out signalling of β1 integrins facilitated by their cytoplasmic part seems to be important for Inv-mediated adhesion and Yop injection and may support YadA-mediated Yop injection.
A factor that decisively regulates the activity state of β1 integrins is their association with the actin cytoskeleton. The pulling force of the actin cytoskeleton on integrins enables high-affinity ligand-receptor interaction (Campbell and Humphries, 2011). Previous studies in which the cytoskeleton was disturbed by, for example, cytochalasin D (CyD) suggested that an intact actin cytoskeleton is critical for Yop injection (Mejia et al., 2008). To investigate whether this holds true for both Inv- and YadA-mediated host cell interaction, cells were treated with CyD, and Ye adhesion and Yop injection were determined (Fig. 9A). Inv-mediated adhesion and Yop injection by Ye ΔYadA-pBla were significantly reduced only in GD25β1A, but not CDβ1A cells. The β1 integrin cytoskeleton linkage thus seems to play a critical role for Ye adhesion and Yop injection in fibroblasts, but not in epithelial cells. In contrast, YadA-mediated adhesion and Yop injection by Ye ΔInv-pBla was not affected...
Fig. 9. Role of actin cytoskeleton and Rho GTPases for adhesion and Yop injection. Indicated cell lines were treated with (A) CyD (3.9 μM), (B) TcdB (50 ng ml⁻¹) or (C) CNF1 (1 μg ml⁻¹) prior to infection with ΔYadA-pBla (left panel) or ΔInv-pBla (right panel) mutant strains, and Yop injection (β-lactamase reporter assay) and adhesion were determined as described earlier. Asterisks indicate significant differences between groups as calculated by one-way analysis of variance with Bonferroni corrections (**P < 0.01, ***P < 0.001).
by CyD, demonstrating that the association of β1 integrins with the actin cytoskeleton in this case is not necessarily required.

Actin cytoskeleton remodelling is regulated by Rho GTPases, and might directly influence its association with integrins and thereby their activation status. Rho GTPases are critically involved in Yop injection as inhibition of Rho GTPases by toxin B (TcdB) or genetic inhibition of Rac1 reduces Yop injection (Mejia et al., 2008; Köberle et al., 2009; Wolters et al., 2013), while activation of Rho GTPases by the cytosolic necrotizing factor 1 (CNF1) boosts this process (Schweer et al., 2013; Wolters et al., 2013). To further explore the role of Rho GTPases for adhesion and Yop injection, cells were treated with TcdB in order to deactivate Rho GTPases prior to Ye infection. Subsequent infection with Ye ΔYadA-pBla revealed that Inv-mediated adhesion to GD25β1A was even increased by TcdB, while adhesion to CDβ1A cells was not affected (Fig. 9B). Inv-mediated Yop injection into both GD25β1A and CDβ1C cells was significantly reduced by TcdB. Infection with Ye ΔInv-pBla revealed that YadA-mediated adhesion was not affected by TcdB treatment, while Yop injection was significantly reduced in all cell lines irrespective of β1 integrin expression. In contrast to previous published results, these data indicate that the activation of Rho GTPases is dispensable for Ye host cell adhesion, but involved in both, Inv- and YadA-mediated Yop injection utilizing the β1 integrin or the αvβ3 integrin pathway.

We previously hypothesized that the contact of Ye to GD25 cells may not be intimate enough to initiate signalling events required for Yop injection. Therefore, we wondered whether this could be circumvented by CNF1 treatment that leads to activation of Rho GTPases (Wolters et al., 2013) (Fig. 9C). Treatment of cells with CNF1 significantly increased Inv-mediated Yop injection into GD25β1A cells and YadA-mediated Yop injection into GD25β1A, CDβ1C and CDβ1A cells. However, CNF1 was not able to increase YadA-mediated Yop injection into GD25 cells. This suggests that tight adhesion might be a prerequisite for Rho GTPase regulation of Yop injection in a secondary step.

Finally, we addressed signalling events downstream of integrin activation that have been reported to be important for Inv-mediated internalization into epithelial cells or inflammasome activation (Bruce-Staskal et al., 2002; Owen et al., 2007; Thinhwa et al., 2014). To this end, Yop injection was analysed in cells lacking the Src kinases Src, Yes and Fyn (SYF), and Fak-deficient cells as well as Fak/Pyk2 deficient cells (Fig. S7). The results show that, in contrast to the importance for Ye uptake, neither Src deficiency nor Fak/Pyk2 deficiency had an impact on Yop injection, indicating that these downstream signalling events are not required for Yop injection by Ye.

Discussion

Different Y. enterocolitica adhesins determine appropriate adhesion to host cell receptors to promote Yop injection into host cells

Previous studies emphasized that adhesion of Ye to host cells via the adhesins Inv and YadA is crucial for subsequent injection of Yops into host cells by the T3SS, with adhesion being accomplished by the binding of host cell β1 integrins (Köberle et al., 2009). Studies by Isberg and Leong (1990) demonstrated that Inv binds directly to, and is a high-affinity ligand for, several αβ1 integrin heterodimers. By contrast, a direct binding of YadA to β1 integrins has not yet been demonstrated. However, YadA binds to ECM proteins, such as collagen, laminin and fibronectin (Eitel and Dersch, 2002; Heise and Dersch, 2006; Leo et al., 2008; 2010). Furthermore, it has been shown that binding of Yps YadA to fibronectin is partially inhibited by RGD peptides (Heise and Dersch, 2006). Fibronectin consistently increases adhesion of Ye to host cells in the presence of YadA or both YadA and Inv, but surprisingly decreases adhesion in the presence of Inv and absence of YadA (Hudson et al., 2005). Despite some variations of the YadA head domain of various Yersinia spp. and strains, which determine different ECM protein-binding properties (Heise and Dersch, 2006), all these data support the hypothesis that ECM proteins bound to YadA build a bridge by which YadA indirectly binds to β1 integrins. This model is widely accepted despite it has not yet been experimentally verified.

In the present study, we demonstrate that integrins are critical for adhesion, and that adhesion is a prerequisite for Yop injection. Both fibroblast and epithelial cells with different integrin receptor repertoires were used to determine the integrin repertoire required for adhesion and Yop injection. Adhesion of Ye to host cells was either mediated by Inv or YadA, and deletion of both adhesins totally abrogated adhesion and Yop injection, demonstrating that in Ye infection at least in fibroblasts and epithelial cells no further adhesins play a major role in these processes. This is also supported by recent in vivo findings indicating that in experimental Ye infection in mice predominantly YadA, and to a minor degree Inv, are essential for Yop injection into leukocytes (Deuschle et al., unpubl. data). In contrast, in Yps infection, Ail, in addition to Inv and YadA, has been shown to be involved in Yop injection into leukocytes (Maldonado-Arocho et al., 2013). The different roles of adhesins in Ye and Yps infection may be due to structural differences of both Inv and YadA. Inv of Ye lacks the enhancer domain D2 that has been claimed to mediate homodimerization of Inv, which in turn increases host cell binding (Dersch and Isberg, 2000). YadA of Yps and some Ye strains display a domain consisting of approximately 31 amino acid residues within the head
region that contributes to the uptake of *Yersinia* by host cells (Heise and Dersch, 2006). Whether and how these domains of Inv and YadA also affect the ability of Yps or Ye to inject Yops into host cells is yet unclear.

The host cell receptor repertoire determines the success of YadA- and Inv-mediated adhesion and Yop injection

The data reported herein demonstrate that Inv and YadA exploit different integrin receptor repertoires for adhesion and subsequent Yop injection. Although Inv-mediated adhesion and subsequent Yop injection requires the β1 integrin family, we clearly demonstrate that YadA can also exploit αV integrins for adhesion. The use of β1 integrin-deficient fibroblasts shows that YadA-mediated adhesion resulting in productive Yop injection requires both αV and β1 integrins.

Depletion of αV integrins in CDβ1A cells did not affect adhesion, but significantly reduced Yop injection, suggesting that β1 integrins and αV integrins synergize also for optimal Yop injection into epithelial cells. This conclusion is in agreement with reports indicating synergy between αV and β1 integrins. It has been shown that α5β1 integrins determine adhesion to fibronectin whereas αVβ3 integrins enable mechanotransduction modulating cell stiffness (Roca-Cusachs *et al.*., 2009). In cells harbouring a receptor repertoire consisting only of αV integrins (GD25 cells, CDβ1−/− cells), Ye adheres via YadA only (Fig. 4). In this situation, however, subsequent Yop injection occurred only into epithelial cells (CDβ1−/− cells), but not into GD25 fibroblasts. The only difference in the integrin repertoire between epithelial cells and fibroblasts is the additional expression of αVβ6 integrins by epithelial cells; the hypothesis that αVβ6 integrin is responsible for the different adhesion and Yop injection pattern of GD25 and CDβ1−/− cells was not supported by the data as neither depletion of β6 integrins in CDβ1−/− cells nor overexpression of β6 integrins in GD25 cells affected Yop injection. Thus, a yet unknown further receptor expressed by or a signalling event operating in epithelial cells, but not in fibroblasts, is essential for appropriate adhesion and Yop injection promoted by αV integrins.

The quality of adhesion is decisive for Yop injection

Electron microscopy analysis revealed a strong association between a tight adhesion between Ye and host cells and effective Yop injection indicated by a significantly smaller area at the contact zone between bacteria and host cells. In addition, the distances at the contact zone between GD25 cell membranes and Ye cell walls compared with the distance between CDβ1−/− cells and Ye were clearly different (nearest distance 16 ± 11 vs. 10 ± 6 nm; *P* > 0.05 to farthest distance 71 ± 40 vs. 36 ± 29 nm; *P* < 0.001). Therefore, we assume that such a tight adhesion is critical for productive Yop injection.

Previous studies showed that the length of the injectisome has to be adapted to the distance between Ye and host cells. Reduction of the needle length by mutating the ruler YscP reduces Yop injection while the increase of the needle length shows minor effects (Journet *et al.*, 2003; Mota *et al.*, 2005). Reduced Yop injection by a shorter injectisome can also be compensated by adapting the length of YadA. Assuming the length of YadA (approximately 23 nm) (Hoiczky *et al.*, 2000; El Tahir and Skurnik, 2001; Mikula *et al.*, 2012) and an activated integrin (19–28 nm) (Nermut *et al.*, 1988; Xiong *et al.*, 2001; Campbell and Humphries, 2011) with uncertainty of the size of ECM attached to cells and Ye layer, one would assume a distance of approximately 40–50 nm and a needle length of 55 ± 11 nm in the process of host–cell interaction during Yop injection. However, more detailed analysis including superresolution microscopy is required to explore this hypothesis.

Nevertheless, the strong association between a tight adhesion between bacteria and host cells and effective Yop injection might reflect that the probability of an injectisome to be close enough for Yop injection might be higher in a situation where Ye adheres more tightly. However, experiments with the Ye E40 (plJM4001) [yscP680]-pBlA mutant strain, which expresses injectisomes with increased needle length of about 88 ± 23 nm (Mota *et al.*, 2005), failed to improve Yop injection into GD25 cells (data not shown), suggesting that tight adhesion per se might be essential to promote effective Yop injection.

Although tight adhesion between pathogen and host cell plays a critical role for subsequent Yop injection, recent studies demonstrated that serum factors may also influence Yop injection into host cells (Maldonado-Arocho *et al.*, 2013; Merritt *et al.*, 2014). Thus, pretreatment with serum significantly reduced Yop injection in particular in B and T cells after Yps infection. In contrast, Yop injection into phagocytes was less affected (Maldonado-Arocho *et al.*, 2013). A study addressing Yop injection by *Y. pestis* demonstrated that heat labile mouse serum factors strongly affect Yop injection into leukocytes and redistribute targeting for Yop injection towards neutrophils (Merritt *et al.*, 2014). In line with these data, in the presence of 10% inactivated serum, Yop injection by Ye into both GD25β1A cells and CDβ1A cells was strongly reduced in a YadA− but not in an Inv-dependent manner (data not shown). Thus, serum factors may specifically interfere with the indirect binding of YadA via ECM proteins to host cells. Although fibronectin that is abundant in serum was excluded as a putative inhibitor of Yop injection by Yps (Maldonado-Arocho *et al.*, 2013), the nature of the serum...
inhibitors is so far elusive. In addition, heat labile factors may modulate Yop injection by *Y. pestis* (Merritt et al., 2014). Nevertheless, further studies are required to identify serum factors involved in Yop injection by *Ye*.

**Signalling events involved in Yop injection**

A previous study showed that the actin cytoskeleton plays a role for Yop injection by *Yps* into HeLa cells (Mejia et al., 2008). Our data show, however, that this is not necessarily the case in *Ye* infection and suggest that the requirement of the actin cytoskeleton rearrangement for *Ye* adhesion and Yop injection varies depending on the cell type. In fact, while Inv-mediated adhesion and Yop injection into fibroblasts was abrogated by CyD, this was not the case in epithelial cells or in YadA-mediated adhesion to fibroblasts or epithelial cells. This suggests that the cytoskeleton rearrangement is dispensable if appropriate receptors such as β1 integrins are highly expressed and in turn mediate the critical firm adhesion allowing Yop injection.

Previous studies highlighted the importance of Rho GTPases for Yop injection (Adkins et al., 2007; Mejia et al., 2008; Wolters et al., 2013). Recently, it was reported that Rho activation triggered by the YopB/YopD translocon or by binding of adhesins to β1 integrins stimulates actin polymerization and activates Yop injection (Mejia et al., 2008). Our data suggest that Rho GTPases are not essentially required for adhesion but relevant in subsequent signalling events involved in Yop injection. Consistently, Rho GTPases may control pore formation by YopB/YopD as recently suggested (Mejia et al., 2008). Thus, the actin cytoskeleton and Rho GTPases act on different levels to promote Yop injection.

Inv-mediated adhesion to fibroblasts lacking the cytoplasmic domain of β1 integrins was totally abrogated. This domain is required for, for example, talin binding required for inside–out signalling (Harburger et al., 2009; Wang, 2012), indicating that inside–out signalling is important for Ye adhesion and Yop injection. Because YadA-mediated adhesion can also be achieved by αV integrins, the role of the cytoplasmic domain of β1 integrins for YadA-mediated adhesion could not be addressed. However, the significant inhibition of YadA-mediated Yop injection due to lack of cytoplasmic domain of β1 integrins clearly demonstrates that efficient YadA-mediated Yop injection nevertheless requires inside–out signalling of integrins.

**Pathways determining the efficiency of Yop injection**

According to so far published and the herein presented data, we propose the following scenarios for Inv- and YadA-determined adhesion and Yop injection by Ye (Fig. 10).

(i) The Inv pathway: Inv binds to β1 integrins with high affinity; subsequent inside–out signalling and Rho GTPase activation is required; these events lead to interaction of the integrin receptors with the actin cytoskeleton further strengthening the Inv-β1 integrin binding. In turn, these events lead to firm adhesion followed by Yop injection. Alternatively, high integrin expression levels may render the requirement of actin rearrangement dispensable for adhesion. Subsequent to adhesion, the process of Yop injection is regulated by Rho GTPases.

(ii) The YadA pathway: YadA binds to ECM and subsequently to β1 and αV integrins. A common feature of these pathways is that they are heparin sensitive. This leads to the assumption that ECM proteins as well as heparin-binding receptors are involved. Moreover, these pathways do not require actin cytoskeleton rearrangement or Rho GTPase activation for adhesion; however, Rho GTPase activation is important for efficient Yop injection.

(ii-a) YadA-β1 integrin-dependent pathways: YadA binds indirectly via ECM to laminin receptors (α3β1, α6β1) or the RGD receptor α5β1 to host cells. For optimal Yop injection, β1 integrin inside–out signalling is required. This pathway may operate with much less efficacy also without inside–out signalling of the β1 integrins. YadA-mediated Yop injection into fibroblasts is strictly β1 integrin dependent and requires in addition αV integrins. Depletion of αV integrins in β1 integrin-expressing epithelial cells does not affect adhesion but partially Yop injection, indicating that αV integrins synergize with β1 integrins for productive Yop injection.

(ii-b) YadA-β1 integrin-independent pathways: YadA binds via ECM proteins to RGD receptors of the αV integrin family, which leads to adhesion. For productive Yop injection additional non-integrin receptors have to be postulated, which might also be involved in ECM binding, cooperate with αV integrins and be functionally redundant to β1 integrins.

In addition to the herein presented concept, *Yersinia* spp. may additionally exploit leukocyte-specific factors for Yop injection. Evidence for this hypothesis was provided by a recent study addressing host cell targeting of *Y. pestis*. *Y. pestis* exploits CD14, which acts as a co-receptor of TLR4 for LPS binding as well the leukocyte-specific integrin αMβ2 (CD11b, CR3) for targeting of and Yop injection into neutrophils (Merritt et al., 2014).

Although *Y. pestis* do not express Inv or YadA and uses a widely different set of adhesins (Ail, PsaA, Pla) and as compared with enteric yersiniae (Ail, YadA and Inv) for

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Yop injection into host cells (Felek et al., 2010), in further studies it has to be addressed whether during Ye infection host factors like CD14 and CD11b are also critical for Yop injection into leukocytes.

**Experimental procedures**

**Bacterial strains and growth conditions**

_Yersinia enterocolitica_ E40 strains used in this study are listed in Table 1. All _Yersinia_ strains were grown overnight in Luria-Bertani broth at 27°C supplemented with antibiotics or Na-arsenite (Na-arsenite and nalidixic acid from Sigma Chemical, kanamycin, streptomycin and tetracycline from Applichem) in combinations according to the indicated resistances and supplementation needs (Table 1). A 1:20 dilution of the bacterial culture was incubated for additional 3 h at 37°C. The bacteria were washed once with phosphate-buffered saline (PBS; Life Technologies) and the optical density at 600 nm was determined.

**Plasmid constructions and generation of bacterial strains.** All plasmid constructs and bacteria used are listed in Table 1. E40

Fig. 10. Different pathways including various integrins and signalling events are exploited by Ye Inv and YadA to accomplish adhesion to and Yop translocation into host cells.

A. Inv-mediated internalization requires binding of invasin to β1 integrins. This integrin activation induces recruitment and stimulation of FAK and Src, which target Rho GTPases, thereby controlling uptake via regulation of the actin cytoskeleton.

B. Adhesion leading to Inv-mediated Yop injection requires binding to and inside-out signalling of β1 integrins and the engagement of the actin cytoskeleton. Yop injection is regulated by Rho GTPases but does not require FAK or Src kinases. Alternatively, higher expression levels of β1 integrins may compensate a necessary involvement of the actin cytoskeleton for Yop injection.

C. YadA-mediated adhesion is achieved by indirect binding via ECM proteins to αv integrins or β1 integrins. This requires integrin activation but not necessarily involvement of the actin cytoskeleton. Cooperation of either αv integrins and β1 integrins or αv integrins with putative unknown co-receptors promotes tight adhesion required for operative Yop injection. Subsequent Yop injection is regulated by Rho GTPases but does not require Fak or Src kinases.
### Table 1. Plasmids and bacterial strains used in this study.

| Designation | Genotype or description | Reference or source |
|-------------|-------------------------|---------------------|
| **Plasmids** |                         |                     |
| pMK-Bla     | HindIII yopE53-BamHI-rls-cre-Sall fragment of pMK4 was replaced by a HindIII yopE53-BamHI-rls-Sall fragment of pBM53-Bla. KanR expression of YopE1-53-lambda lactamase hybrid protein under control of the YopE promotor | (Köberle et al., 2009) |
| pBM-Bla     | BglII-Tet6-NcoI cassette amplified from pACYC184 was inserted into BglII NcoI sites of pMK-Bla leading to loss of kanamycin resistance. Tet6 expression of YopE1-53-lambda lactamase hybrid protein under control of the YopE promotor | This study |
| pACYC184    | DsRed-express from pDsRed-express was cloned into pACYC184 as described by (Oellerich et al., 2007) under the control of a tac promoter | This study |
| pASK-IBA2   | Expression vector with AHTC inducible promoter (AmpR) | IBA technologies |
| pASK-IBA2/invA | invA from Ye WA-314(O:8) cloned into pDSRed-express was cloned into pACYC184 as described by (Oellerich et al., 2007) | (Oellerich et al., 2007) |
| pASK-IBA2INV | Inv-pBla expression vector designed as Gateway® destination vector and for TetR-inducible expression of target genes in cell lines (NeoR) | This study |
| pMK-Bla     | ΔpMK-Bla strain lacking pYV40 virulence plasmid (NalR, KanR) | Köberle et al., 2009 |
| pLJM4029    | pYV40 with deletion in the yadA gene (ArsR, Strept) | (Meerbrey et al., 1995) |
| pMS154      | Suicidal plasmid, constructed by ligation of a 1.4 kb inv fragment with pKNG160 (TetR) | (Sory and Cornelis, 1994) |
| pASK-IBA2   | Expression vector with AHTC inducible promoter (AmpR) | IBA technologies |
| pASK-IBA2/invA | invA from Ye WA-314(O:8) cloned into XbaI-HindIII sites of pASK-IBA2 (AmpR) | (Oellerich et al., 2007) |
| pACYC184INV | Inv-pBla expression vector designed as Gateway® destination vector and for TetR-inducible expression of target genes in cell lines (NeoR) | This study |
| pASDR-INV   | Inv-pBla expression vector designed as Gateway® destination vector and for TetR-inducible expression of target genes in cell lines (NeoR) | This study |
| pLJM4029INV | Inv-pBla expression vector designed as Gateway® destination vector and for TetR-inducible expression of target genes in cell lines (NeoR) | This study |
| pMK-Bla     | ΔpMK-Bla strain lacking pYV40 virulence plasmid (NalR, KanR) | Köberle et al., 2009 |
| pASK-IBA2   | Expression vector with AHTC inducible promoter (AmpR) | IBA technologies |
| pASK-IBA2/invA | invA from Ye WA-314(O:8) cloned into XbaI-HindIII sites of pASK-IBA2 (AmpR) | (Oellerich et al., 2007) |
| pACYC184INV | Inv-pBla expression vector designed as Gateway® destination vector and for TetR-inducible expression of target genes in cell lines (NeoR) | This study |
| pASDR-INV   | Inv-pBla expression vector designed as Gateway® destination vector and for TetR-inducible expression of target genes in cell lines (NeoR) | This study |
| pLJM4029INV | Inv-pBla expression vector designed as Gateway® destination vector and for TetR-inducible expression of target genes in cell lines (NeoR) | This study |

**Y. enterocolitica** E40 strains

- **E40**
  - Serotype O:9 patient isolate (NalR, ArsR)
  - This study
- **E40 Δasd**
  - E40 strain with asd gene knockout, deficient in L-aspartate-dehydrogenase expression (NalR, ArsR, DAP+) | (Köberle et al., 2009) |
- **Ye:pBla**
  - E40 Δasd strain transformed with pMK-Bla (NalR, ArsR, KanR) | (Köberle et al., 2009) |
- **Ye:Bla**
  - E40 Δasd strain transformed with pACYC184 (Köberle et al., 2009) |
- **E40 Δasdsinv**
  - inv mutant strain obtained by recombinatorial integration of suicide plasmid pMS154 into E40 Δasds (NalR, ArsR, TetR) | This study |
- **YadA-pBla**
  - E40 Δasdsinv strain transformed with pMK-BlA (NalR, ArsR, KanR) | This study |
- **YedA-pBla**
  - E40 Δasdsinv strain transformed with pLJM4029 (YadA-) and with pMK-Bla (NalR, ArsR, KanR, Strept) | This study |
- **YE1-pOVA**
  - E40 Δasdsinv strain transformed with pMK-Ova (NalR, ArsR, KanR) | This study |
- **E40-pOVA**
  - E40-pOVA strain transformed with pACYC184 (Köberle et al., 2009) |
- **E40-pOVA**
  - E40-pOVA strain transformed with pACYC184 (Köberle et al., 2009) | This study |
Δascl1Δnv was obtained by integration of suicide plasmid pMS154 into E40 Δascl Nas6, Ars6 and Tet6 according to previous descriptions (Sory et al., 1995).

Cell culture and in vitro infections. The murine GD25 cells were derived from the embryonic stem cell clone G201 that is deficient in the β1 integrin subunit (Fassler et al., 1995; Retta et al., 1998). The stably transformed cell lines GD25β1A, GD25β1COM and GD25β1TR were obtained by electroporation of wild-type human integrin β1A CDNA or β1A mutants truncated at threonine residue 762 or isoleucine residue 762 (Retta et al., 1998) into the β1-deficient GD25 cells. To obtain GD25 cells expressing αVβ6 integrin (GD25pMSCV + β6) or as a respective control cells (GD25pMSCV), mouse β6 integrin coding sequence was amplified by PCR and integrated into pMSCVpuro (Clontech) using the restriction sites Xhol and EcoRI. The resulting vector pMSCV ltb6 or as a control a sole pMSCVpuro were transduced into GD25 cells using a retrovirus produced by phoenix cells (ATCC® CRL-3213™). For this purpose, 3 × 10⁶ phoenix cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS; Sigma), 100 U ml–1 penicillin G and 0.1 mg ml–1 streptomycin (Life Technologies) in a 10-cm plate to 50–60% confluency. Medium was changed and 25 μM chloroquine added. Transfection cocktail using 20 μg DNA, 125 mM CaCl2 in 2 ml 1× Hank’s balanced solution solution (140 mM NaCl, 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) and 1.42 mM piperazineethanesulfonic acid (HEPES) and 1.42 mM piperazineethanesulfonic acid (HEPES)) was added to the cells. After 48 h of incubation, the medium without chloroquine was replaced with fresh medium and 20 μg psLVαVβ6 transduction virus was added. After 48 h, the cells were washed with PBS and then serum-free medium was added, and the cells were cultured in the presence of 4 μg ml–1 puromycin (TOKU-E). The amount of αVβ6 depletion was detected by flow cytometry after staining with anti-mouse αVβ6 (Millipore). In case of CDβ1Δ−Δ shV cells, single clones were prepared to check the cell clone with best αV depletion.

GE11 cells are epithelial-like β1 integrin knockout cells, which were generated as described previously (Gimond et al., 1999). The GE11β1 cells were obtained by transduction with the murine β1 integrin integrated into a pMSCVpuro vector using a retrovirus produced by phoenix cells. Mouse β1 integrin coding sequence was amplified by PCR and cloned into pMSCVpuro (Clontech) using the restriction sites Xhol and EcoRI. GE11 cells were cultivated in DMEM (Life Technologies) supplemented with 10% FCS, 100 U ml–1 penicillin G and 0.1 mg ml–1 streptomycin. For GE11β1 cells, 2 μg ml–1 of puromycin (TOKU-E) was added. SYF (ATCC CRL-2459; deficient in Src, Yes and Fyn), SYF + cSrc (ATCC CRL-2498), Fak−/− (ATCC CRL-2644) and Fak+/− (ATCC CRL-2645) were obtained by ATCC. Cells deficient for Fak−/− and Pyk2 were obtained by stable transduction of Fak−/− cells with shRNA against Pyk2 (Sigma).

For in vitro infections, cells were detached from culture vessel with EDTA/Trypsin (Life Technologies). Cells were then counted, and a defined number of cells were seeded in the respective cell culture vessels and cultured overnight. One hour before infection the medium was replaced with medium devoid of FCS and antibiotics. The cells were then infected with different Yersinia or E. coli strains with indicated MOI and period of time. The bacteria were spun down at 300 g for 2 min to establish contact to the host cells.

Detection of β-lactamase activity by flow cytometry. For the detection of β-lactamase activity by flow cytometry, 125 × 10⁵ cells were seeded in 24-well plates and cultured overnight at 37°C. After 1 h infection with different Ye E40 strains (MOI 100), cells were detached from culture vessels and resuspended in 1× CCF4-AM staining solution (Life Technologies) supplemented with probenecid (Sigma Chemicals) prepared according to the manufacturer’s instructions (Life Technologies) and incubated 40 min in a dark place at room temperature prior to FACS analysis. Flow cytometry was performed on a LSR II (Becton Dickinson) using Summit 4.3 software (Dako) or FlowJo 7.6.3 (Tree Star).

Antibody staining. For staining of cell surface integrin subunits or heterodimers 1.25 × 10⁵ cells were suspended in PBS with 1% FCS (Sigma) and stained with indicated antibodies [rat anti-mouse CD29 directly conjugated with allophtocyanin (APC; BioLegend), unconjugated rat anti-human CD29 (AILB2), Armenian hamster anti-mouse CD29 (HM3-1) directly conjugated with APC (BioLegend), Armenian hamster anti-mouse CD61 directly conjugated with APC (Hm3-31; BioLegend), unconjugated rat anti-mouse integrin β4 (346-11A, BD Pharmaning), unconjugated mouse anti-β5 (KN52, eBioscience), unconjugated mouse anti-mouse αVβ6 (10D5; Millipore), unconjugated rat anti-mouse CD51/αV (RMV-7; BD Pharmaning), unconjugated goat anti-mouse CD49c/Integrin α3

supplemented with 10% FCS, 100 U ml–1 penicillin G and 0.1 mg ml–1 streptomycin.

CDβ1A shV, CDβ1Δ−Δ shV and GD25Δ shV all as well as respective control cells (shGFP) were generated by treating CDβ1A, CDβ1Δ−Δ and GD25 cells with lentiviral particles transducing shRNA targeting the integrin αV subunit (Sigma Mission). Transduced cells were selected by addition of medium with 4 μg ml–1 puromycin (TOKU-E). The amount of αV depletion was detected by flow cytometry after staining with anti-mouse αVβ6 (Millipore). In case of CDβ1Δ−Δ shV cells, single clones were prepared to check the cell clone with best αV depletion.

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Adhesion assays. To determine adhesion of Ye E40 strains. ΔInv-pBla, ΔYadA-pBla and ΔInv,ΔYadA-pBla to fibroblasts and epithelial cells, 1 × 10^5 cells were seeded on coverslips in a 24-well plate and cultured overnight at 37°C. When cells were treated with CyD, TcdB or RGD peptides prior to infection (see below) cell culture vessels were coated with 0.01% polyethylenimine (PEI) for 2 min before seeding to prevent detachment of cells from cover slips during washing steps. One hour before infection the culture medium in the wells was replaced with medium devoid of FCS and antibiotics. Then cells were infected with indicated MOI (MOI 10, 50 or 100) and bacteria were spun down at 300 g for 2 min to establish contact to the cells. Following a 30-min infection time, cells were washed three times with PBS, fixed with 4% PFA and stained with fuchsin and analysed by microscopy. The number of adherent bacteria per cell was determined by counting 100 cells plus adherent bacteria per sample. Samples were prepared in triplicate.

To analyse adhesion and Yop injection after Ye infection with immunofluorescence microscopy, 8 × 10^4 cells per well were seeded on an 8-well ibidi μ-slide (ibidi) and cultured overnight at 37°C. One hour before infection the culture medium was replaced with medium devoid of FCS and antibiotics. Then cells were infected with either E40-pBla DsRed or E40-pOVA DsRed (MOI 100) for 1 h. Cells were washed three times with PBS, overlaid with 1× CCF4-AM staining solution and incubated 40 min at room temperature in the dark. Stained cells were analysed for adherent bacteria and β-lactamase activity with a laser scanning microscope 710 NLO (Carl Zeiss).

To measure adhesion under dynamic conditions, a dynamic flow chamber assays were performed in principal as recently described (Müller et al., 2011). Cell lines (1 × 10^6 per 50 μl) were seeded in growth medium without antibiotics on poly-L-lysine-coated multichannel slides (ibidi) to 1.6 × 10^6 E40-GFP per ml under constant flow conditions for 10 min (shear stress, 0.05 dyne/cm²). Cells were fixed with 4% paraformaldehyde in PBS and subsequently stained with phalloidin-Tritc and DAPI and analysed by immunofluorescence microscopy. Images were digitally processed with Photoshop (Adobe Systems). Bacterial adhesion was quantified by counting cell-adherent bacteria on 100 cells per bacterial strain.

Exposure of cells to substances. To dissect if certain cell functions are required for Yop injection, cultured cells were incubated with different substances interfering with the particular cellular functions prior to infection with Ye E40 strains. These substances were supplied to cells as following: to inhibit actin polymerization, 3.9 μM CyD (Sigma Aldrich) was supplied to host cells 2 h before infection. CyD prevents polymerization of actin monomers, thereby disrupting actin filaments. The RGD peptide H-Gly-Arg-Gly-Asp-Ser-Pro-OH (Merck Millipore) was shown to inhibit the binding of ECMs with Arg-Gly-Asp binding motifs to integrins. Cells were incubated with 500 μg ml⁻¹ RGD or as a control the respective RAD peptide H-Gly-Arg-Ala-Asp-Ser-Pro-OH (Merck Millipore) 30 min prior to infection. Heparin was used at a concentration of 1 mg ml⁻¹ and supplied to cells 1 h before infection. To inhibit activation of Rho GTPases, Clostridium difficile TcdB was applied at a concentration of 50 ng ml⁻¹ 3 h prior to infection. TcdB is a broad spectrum inhibitor of Rho GTPases targeting RhoA, Rac1 and Cdc42 subtypes and functions as a threonine residue glycosylating agent leading to GTP hydrolysis and inactivation of GTPases. For constitutive activation of Rho GTPases, E. coli CNF1 (kindly provided by Martin Apelbelbacher) (Wolters et al., 2013) was applied to cells at a concentration of 1 μg ml⁻¹ 2 h prior to infection. CNF1 possesses transglutaminase activity and deamidates glutamine residues in Rho GTPases blocking GTP hydrolysis. Exposure of host cells to substances was continued during infection with Ye.

Requirements for Yersinia-mediated Yop injection. To analyse bacterial adhesion with transmission electron microscopy 5 × 10^5 cells were seeded on PEI precoated polycarbonate cell culture inserts (Millipore, 0.4 μm pore size, 12 mm diameter) in a 24-well plate and cultured overnight at 37°C. One hour before infection the culture medium in the inserts was replaced with medium devoid of FCS and antibiotics. Then cells were infected with E40-pBla (MOI 100) for 30 min. Cells were washed three times with PBS and then fixed with Karnovsky fixative (3% paraformaldehyde, 3.6% glutaraldehyde, pH 7.2). Post-fixed samples (1% OsO₄ containing 1.5% potassium ferrocyanide in aqua bident, 2 h) were rinsed with distilled water, block stained with uranyl acetate (2% in distilled water), dehydrated in alcohol (stepwise 50–100%) and embedded in glycid ether (polymerized for 48 h at 60°C, Serva). Ultrathin sections were examined with a transmission electron microscope (Carl Zeiss) operating at 120 kV. The area between adherent Ye and host cells at a contact zone per 100 nm was determined using ImageJ (Fig. S4).

ECM expression. To determine ECM protein expression, whole cell lysates were generated by lysing the cells in 1% Triton X-100 in PBS containing protease inhibitors. One hundred micrograms of protein extract was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After Western blot transfer on a polyvinylidene fluoride (PVDF) membrane, ECM proteins were detected with Li-Cor Odyssey (Li-Cor Biosciences) after labelling with fluorescent antibodies using rabbit anti-mouse collagen I + III (Merck Millipore), rabbit anti-human/mouse collagen IV (LifeSpan Biosciences), rabbit anti-human/mouse laminin (LifeSpan Biosciences), rabbit anti-mouse/ rat fibronectin (Merck Millipore) and goat anti-rabbit IgG conjugated with DyLight800 as a secondary antibody. For flow cytometry analysis of ECM decoration on the cells, cultured cells were harvested and stained with above-described antibodies. As a secondary antibody an APC-conjugated goat anti-rabbit IgG (Pharmingen-BD). Flow cytometry was performed on LSR II (Becton Dickinson) using Summit 4.3 software (Dako).

Statistics. If not stated otherwise, the means and standard deviations of data derived by cell culture experiments were calculated from at least three independent experiments. Statistical analyses were performed using one-way analysis of variance.
with Bonferroni corrections (comparison of all groups) as indicated in the figure legends or the text using GraphPad Prism software (GraphPad Software).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Integrin expression levels in fibroblasts and epithelial cells. Expression of indicated integrin α or β subunits, or αβ heterodimers in GD25, GD25β1A, CDβ1− and CDβ1A cells was detected by flow cytometry after staining with (A) anti-human CD29 AIIB2 (GD25, CD); anti-mouse CD29 (KF, GE11); antibodies against integrin chains (B) αV, α3, α5, α6 and (C) β3, β5 and αVβ6. Representative histograms with means + SD of at least three experiments are depicted. (D) Integrin receptor repertoire of the different cells was compiled using the following criteria: (i) determination of expression of integrin single chains; (ii) integrins are only expressed on the surface as heterodimers; and (iii) distinct single chains form only distinct heterodimers.

**Fig. S2.** Integrin αβ subunits are essential for Inv- but not YadA-mediated Yop injection.

KF1α− or KF1β6 fibroblasts and GE11 or GE11β1 epithelial cells were infected with (A) Ye-pBla (B) ΔinvYadA-pBla, (C) ΔYadA-pBla or (D) Δinv-pBla (MOI 100) for 1 h, stained with CCF4 and analysed by flow cytometry for the percentage of blue cells indicating Yop injection. The data represent means + SD of at least four experiments. Asterisks indicate significant differences determined by one-way ANOVA with Bonferroni corrections (**P < 0.001).

**Fig. S3.** Impact of αVβ6 integrins on Yop injection. Cells as indicated were stably transduced (A) pmSCV-β6 or pmSCV, (B) sh β6 or sh GFP and (C) sh β3 or sh GFP and clonal cell lines generated. (Left panels) Expression of αVβ6 or β3 integrins was determined by antibody staining and flow cytometry. Representative histograms of at least two experiments are depicted. (Right panels) Cells were infected with indicated Ye strains stained with CCF4 and analysed by flow cytometry for percentage of blue cells indicating Yop injection. Data represent means + SD of at least three experiments.
**Fig. S4.** Quantification of the contact area between adherent yersiniae and host cells. The area between host cells and bacteria within quadrants with a length of 250 nm was marked and then quantified using ImageJ. For comparison the areas of the contact zone were normalized to a length of 100 nm per analysed cell.

**Fig. S5.** Adhesion of Ye-pBla is affected by RGD peptides. A. GD25 and CDβ1−/− cells were treated with 500 μg ml−1 RGD or RAD peptide for 30 min, then infected with Ye-pBla (MOI 100) for 30 min and samples were analysed by transmission electron microscopy. B. The area between single adherent Ye and host cells per 100 nm contact zone was calculated with ImageJ. The data represent means + SD for at least 20 single adherent Ye.

**Fig. S6.** Summary of findings to define relationship between adhesion and Yop injection. A. Summary of results of Figs. 1–7 and Fig. S2. B. Illustrates relationship between adhesion and Yop injection: green Ye indicate firm adhesion and Yop injection, yellow Ye indicate unstable adhesion without Yop injection; red Ye indicate no adhesion, no Yop injection.

**Fig. S7.** Src kinases and Fak are dispensable for Yop injection. A. SYF (deficient for src, lyn and yes), SYF + c-src and control cells as well as (B) Fak−/−, Fak+/+ cells and Fak−/− transduced with shRNA against Pyk2 (Fak−/− shPyk2) were infected and Yop injection was analysed by flow cytometry. The data represent means + SD of at least three experiments. C. Immunoblot analysis of Fak and actin expression.