Integrin-linked kinase is required for vitronectin-mediated internalization of *Streptococcus pneumoniae* by host cells

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Summary

By interacting with components of the human host, including extracellular matrix (ECM) proteins, *Streptococcus pneumoniae* has evolved various strategies for colonization. Here, we characterized the interaction of pneumococci with the adhesive glycoprotein vitronectin and the contribution of this protein to pneumococcal uptake by host cells in an integrin-dependent manner. Specific interaction of *S. pneumoniae* with the heparin-binding sites of purified multimeric vitronectin was demonstrated by flow cytometry analysis. Host-cell-bound vitronectin promoted pneumococcal adherence to and invasion into human epithelial and endothelial cells. Pneumococci were trapped by microspike-like structures, which were induced upon contact of pneumococci with host-cell-bound vitronectin. αvβ3 integrin was identified as the major cellular receptor for vitronectin-mediated adherence and uptake of pneumococci. Ingestion of pneumococci by host cells via vitronectin required a dynamic actin cytoskeleton and was dependent on integrin-linked kinase (ILK), phosphatidylinositol 3-kinase (PI3K), and protein kinase B (Akt), as demonstrated by gene silencing or in inhibition experiments. In conclusion, pneumococci exploit the vitronectin–αvβ3-integrin complex as a cellular receptor for invasion and this integrin-mediated internalization requires the cooperation between the host signalling molecules ILK, PI3K and Akt.

Key words: Pneumococci, Vitronectin, Invasion, Integrin, ILK, Akt, Microspikes

Introduction

The Gram-positive microorganism *Streptococcus pneumoniae* (pneumococcus) is a natural commensal of the human upper respiratory tract. In addition to colonization, pneumococci are the aetiological agent of severe local infections, including otitis media and sinusitis, and of life-threatening diseases such as community-acquired pneumonia, septicaemia and meningitis (Cartwright, 2002). During colonization of the nasopharyngeal cavity, pneumococci adhere to host epithelial cells; this is a dynamic process that requires the involvement of bacterial adhesins and eukaryotic cellular surface receptors. The major pneumococcal adhesin, PspC, recognizes directly and in a human-specific manner the ectodomain of the polymeric immunoglobulin receptor (Elm et al., 2004; Hammerschmidt et al., 1997; Hammerschmidt et al., 2000; Zhang et al., 2000). An adhesive function was also demonstrated for phosphorylcholine of the pneumococcal cell wall, which was shown to mediate internalization into endothelial cells via platelet-activating factor receptor (PAFr) (Cundell et al., 1995; Radin et al., 2005). The lipoprotein PsaA, which is part of an ABC transporter complex for manganese, is thought to interact specifically with E-cadherin (Anderton et al., 2007). In addition, a subclass of pneumococcal strains produces pili, which promote bacterial adherence to host cells (Barocchi et al., 2006). Other pneumococcal surface-exposed proteins, such as the lipoprotein SlrA, have been shown to contribute to colonization, but no further direct interactions with eukaryotic surface receptors have been demonstrated (Hermans and Hazelzet, 2005; Hermans et al., 2006).

Similar to other Gram-positive bacteria, the human pathogenic pneumococci interact with various components of the extracellular matrix (ECM) and serum proteins (Bergmann and Hammerschmidt, 2006). The relevance of adhesin–host-protein interactions for pneumococcal attachment to host cells or the infection process has recently been indicated for bacterial interactions with plasminogen, the matricellular protein thrombospondin and complement factor H (Bergmann et al., 2003; Hammerschmidt et al., 2007; Rennemeier et al., 2007). Moreover, pneumococci produce adhesins that interact specifically with other host matrix proteins, including fibronectin and vitronectin (Kostrzynska and Wadstrom, 1992; Holmes et al., 2001).

Vitronectin and fibronectin are common adhesive glycoproteins of the ECM and are abundant in plasma (Preissner et al., 1985). Vitronectin, which is predominantly synthesized in the liver,

interacts with a wide variety of ligands and is involved in cell-ECM adhesion, cell differentiation and proliferation, and blood coagulation, and is an inhibitor of complement pathway(s) (Seiffert, 1997; Preissner and Seiffert, 1998). Two forms of vitronectin exist under physiological conditions, with most of the circulating pool of vitronectin in a native (monomeric) form (M_r 75 kDa), whereas the multimeric form of vitronectin is found predominantly in the ECM or is tissue associated (Stockmann et al., 1993). The vitronectin molecule comprises several ligand-binding domains. The N-terminal region mediates binding to a subclass of integrin receptors on the surface of eukaryotic cells (Jenne et al., 1989; Ruoslahti et al., 1987). A heparin-binding site is localized in the C-terminal part of the molecule and mediates binding to proteoglycans such as dextran sulfate and heparan sulfate (Cardin and Weintraub, 1989; Francois et al., 1999). The interaction of vitronectin with integrin receptors, including the major receptor αvβ3 integrin, triggers integrin clustering and induces assembly of intracellular protein complexes that are relevant for various signalling cascades (Pankov and Yamada, 2002). Moreover, integrins serve as cellular receptors for bacterial pathogens, which engage adhesive glycoproteins of the ECM as molecular bridges. These indirect interactions with integrins tightly link bacteria with host cells and promote internalization of the pathogens by host cells upon induction of an outside-in signalling (Grashoff et al., 2004; Hynes, 2002).

Although several pathogenic bacteria interact with vitronectin (Chhatwal et al., 1987; Eberhard and Ullberg, 2002; Kostrzynska et al., 1992; Kostrzynska and Wadstrom, 1992; Liang et al., 1995; Paulsson and Wadstrom, 1990; Paulsson et al., 1992), the impact of this interaction on host-cell invasion was only demonstrated for *Neisseria gonorrhoeae* (Dehio et al., 1998). By contrast, the indirect engagement of α5β1 integrin receptors by fibronectin-binding protein (FnBP)-producing Gram-positive pathogens such as *Staphylococcus aureus* or *Streptococcus pyogenes* [group A streptococcus (GAS)] was shown to induce bacterial invasion and signal cascades in host cells (Agerer et al., 2003; Cue et al., 1998). Uptake of staphylococci requires the actin cytoskeleton and Src-family tyrosine kinases, and is triggered by the recruitment of focal-contact-associated proteins to the site of bacterial attachment (Agerer et al., 2005). The analysis of the induced host-cell response upon fibronectin-dependent *S. pyogenes* invasion revealed that the intracellular host-cell molecules phosphatidylinositol 3-kinase (PI3 kinase; also known as PI3K) and integrin-linked kinase (ILK) are essential for this invasion mechanism (Wang et al., 2006; Wang et al., 2007).

ILK is a scaffold protein and cross-links the integrin cytoplasmic domains with proteins of the cytoskeleton. As a consequence, ILK is implicated in integrin-dependent cytoskeletal reorganization and cellular activities such as the regulation of cell survival, proliferation and apoptosis (Brakebusch et al., 2002; Delcommenne et al., 1998; Giancotti and Ruoslahti, 1999; Hannigan et al., 1996; Huang and Wu, 1999; Nikolopoulos and Turner, 2002; Yamaji et al., 2001). ILK is ubiquitously expressed and consists of three ankyrin (ANK) repeats at the N-terminus – which interact with Pinch1 or Pinch2 – a pleckstrin homology (PH) domain capable of binding to phosphatidylinositol 3-phosphate, and a C-terminally located domain that shares high homology with serine-threonine protein kinases but also mediates the interaction with β1 integrin, β3 integrin, paxillin and the F-actin-binding protein parvin (Nikolopoulos and Turner, 2002; Olski et al., 2001; Tu et al., 2001). Together with the adaptor proteins Pinch1 and Pinch2, ILK and parvin form a ternary complex termed IPP (Legate et al., 2006). Although ILK lacks crucial consensus sequences of serine-threonine kinases, it is suggested to be a pseudokinase that regulates the activation of glycogen synthase kinase-3 (GSK3) and protein kinase B (Akt; also known as PKB). Akt is phosphorylated at Thr308 through the 3-phosphoinositide-dependent kinase PDK1, whereas phosphorylation at Ser473 was shown to depend on PI3K activity and mammalian target of rapamycin (mTOR) (Boudeau et al., 2006; Delcommenne et al., 1998; Lawlor and Alessi, 2001; Lynch et al., 1999; Persad and Dedhar, 2003; Sarbassov et al., 2004). A recent study indicated that ILK interacts with rictor, which is a component of mTOR complex 2, and that ILK and rictor are required for Akt phosphorylation at Ser473 (McDonald et al., 2008).

The facets of the pneumococci-vitronectin interaction and in particular the consequences for bacterial invasion of the host cell and induced signal cascades have not yet been addressed. The goal of this study was to assess the effect of vitronectin on pneumococcal adherence to eukaryotic cells and subsequent internalization of bacteria. In addition, the major host-surface integrin receptor and the impact of signal molecules on vitronectin-mediated pneumococcal invasion into host cells, with a special emphasis on ILK, were demonstrated for the first time.

Results

Pneumococci recruit multimeric vitronectin

S. pneumoniae were previously shown to bind human vitronectin (Kostrzynska and Wadstrom, 1992). Incubation of pneumococci with human plasma resulted in recruitment of human vitronectin, as shown by immunoblot analysis and flow cytometry (Fig. 1A,B). Flow cytometry indicated a dose-dependent binding of human, as well as mouse, vitronectin, albeit that mouse vitronectin was bound to a lesser degree (Fig. 1B). Direct interaction experiments revealed a dose-dependent binding of multimeric vitronectin to pneumococci, whereas binding of the native form of vitronectin was significantly lower (Fig. 1C-E). The presence of pneumococcal capsular polysaccharide greatly impaired the interaction of multimeric vitronectin with pneumococci (Fig. 1F), as was reported previously for the binding of human thrombospondin 1 and factor H (Hammerschmidt et al., 2005; Hammerschmidt et al., 2007; Rennemeier et al., 2007).

Vitronectin interconnects pneumococci and eukaryotic cells

Different host cell lines were pre-treated with vitronectin and adherence of pneumococci was monitored. The cell lines used in this study displayed no endogenous vitronectin under our culture conditions (data not shown). Flow cytometric analysis showed that the multimeric isoform binds more efficiently to eukaryotic cells compared with the native isoform (data not shown). Adherence of pneumococci to nasopharyngeal epithelial (Detroit 562) cells was significantly increased in the presence of host-cell-bound multimeric vitronectin, whereas host-cell-bound native vitronectin did not influence significantly pneumococcal attachment (Fig. 2A). The degree of bacterial adherence to Detroit 562 cells via vitronectin correlated well with the amount of host-cell-bound multimeric vitronectin (Fig. 2B).

Similar to in Detroit 562 cells, vitronectin promoted adherence of pneumococci to A549 alveolar epithelial cells and human brain-derived microvascular endothelial cells (HBMEC) (Fig. 2C,D). Determination of the CFU (colony-forming units per cell) after infection assays revealed that host-cell-bound vitronectin also promoted internalization of pneumococci in both human epithelial

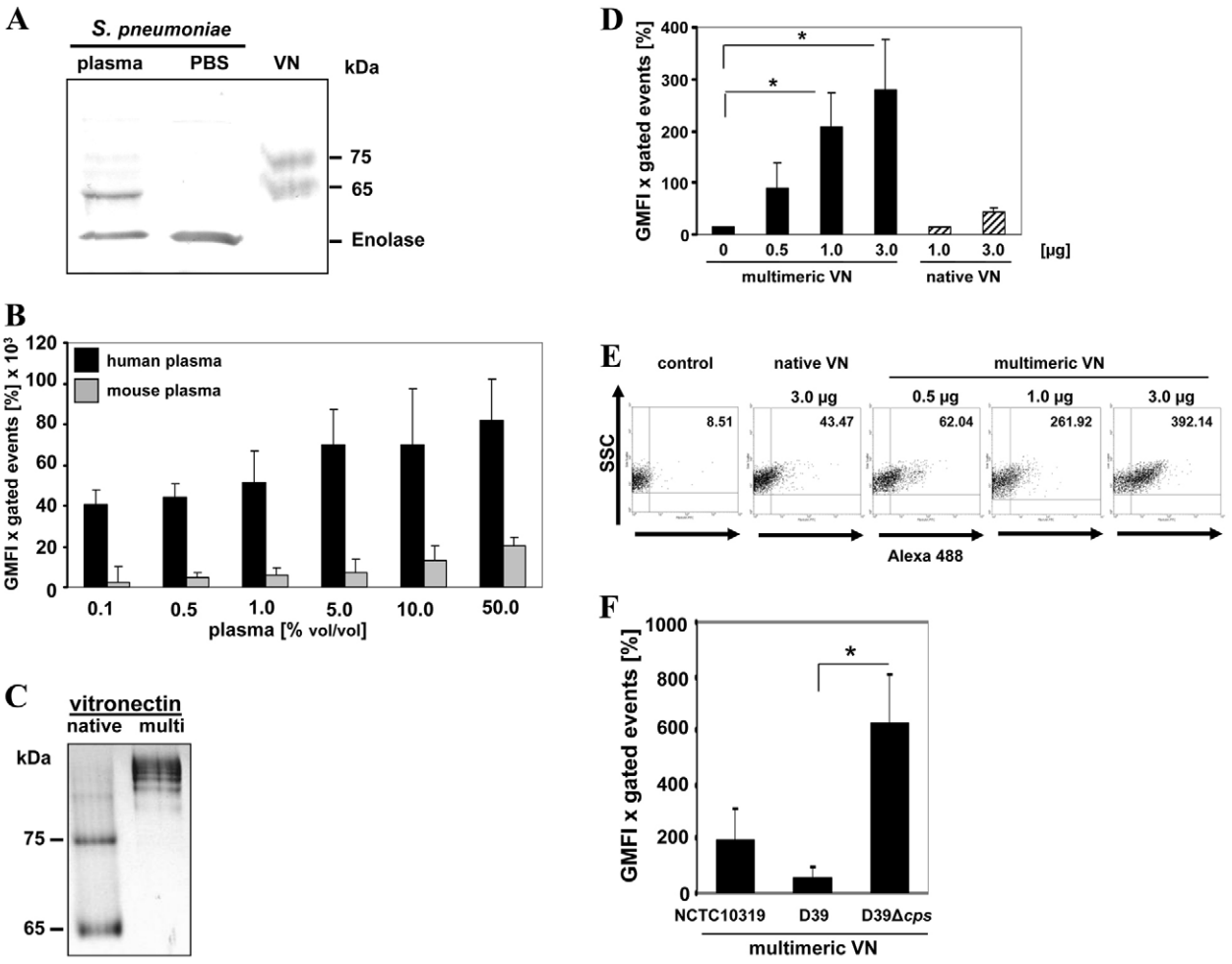

Fig. 1. Species-unspecific recruitment of vitronectin by *Streptococcus pneumoniae*. (A) *S. pneumoniae* (NCTC10319) binding of vitronectin derived from human plasma was detected by immunoblot analysis using a polyclonal anti-vitronectin antibody. Enolase protein of pneumococci was used as a loading control and detected with an anti-enolase IgG (Bergmann et al., 2003). Human plasma vitronectin was detected as 65- and 75-kDa protein bands. Incubation of bacteria with PBS instead of plasma was used as negative control (PBS). (B) *S. pneumoniae* (NCTC10319) recruitment of human or mouse plasma-derived vitronectin was monitored by flow cytometry analysis after incubation of pneumococci with various concentrations of plasma. Dose-dependent and species-unspecific recruitment of plasma-derived vitronectin by pneumococci was monitored by using monoclonal antibody VN7. Results are presented as geometric mean fluorescence intensity (GMFI) × percentage of gated events. (C) Documentation of purified native and multimeric (multi) human vitronectin by non-denaturating PAGE. (D) Recruitment of purified human vitronectin (VN) isoforms by *S. pneumoniae* was analyzed by flow cytometry after incubation of bacteria (NCTC10319: Cps+; serotype 35A) with indicated amounts of multimeric or native vitronectin. Binding data are presented as GMFI × percentage of gated events. (E) Vitronectin that is bound to bacteria is shown as dot plots of a representative flow cytometric analysis. The control dot plot shows the GMFI in the absence of vitronectin but after incubation of the sample with the antibodies. (F) The influence of pneumococcal encapsulation on the binding of purified multimeric vitronectin was analyzed by flow cytometry, and results are expressed as GMFI × percentage of gated events (means ± s.d. of at least three independent experiments, each done in triplicate; **P*<0.05).

and endothelial cells (Fig. 2E). These cell-culture infection experiments demonstrated that host-cell-bound vitronectin functions as a molecular bridge and mediates pneumococcal adherence to and invasion into host cells, and that this mechanism is independent of the type of host cell.

Influence of heparin on vitronectin-mediated adhesion and invasion of pneumococci

The impact of the heparin-binding site of the vitronectin molecule (Lynn et al., 2005; Xu et al., 2001) on vitronectin recruitment by pneumococci was investigated by using heparin as a competitor in binding experiments. Our flow cytometric analysis demonstrated a dose-dependent inhibition of vitronectin acquisition by heparin (Fig. 3A), suggesting that the heparin-binding site of vitronectin plays a key role for the pneumococci-vitronectin interaction. When host cells were pre-incubated with vitronectin and adherence of pneumococci was investigated in the presence of heparin, a significant reduction of vitronectin-mediated attachment of pneumococci to host cells was found (Fig. 3B). By employing the antibiotic protection assays, we further demonstrated that heparin inhibited in a dose-dependent manner the uptake of pneumococci into host cells (Fig. 3C).

Vitronectin-mediated pneumococcal invasion into host cells relies on αvβ3 integrins

To identify the relevant cellular receptors involved in vitronectin-mediated adherence and invasion of pneumococci into host nasopharyngeal epithelial cells (Detroit 562 cells), vitronectin-

mediated adherence of pneumococci was monitored by immunofluorescence microscopy in the presence of an RGD peptide. A significant reduction in vitronectin-dependent infection of nasopharyngeal cells with pneumococci was demonstrated (Fig. 4A). By contrast, association of vitronectin with host-cell surface was not significantly affected by the control peptide RGE (data not shown). In blocking experiments with purified integrins and anti-integrin antibodies, vitronectin-promoted internalization of pneumococci by Detroit 562 cells was significantly reduced after pre-incubation of vitronectin with αvβ3-integrin proteins, whereas no significant reduction of bacterial uptake was detected when α5β1 integrins were employed as inhibitors (Fig. 4B). Likewise, functional blocking antibodies against β3 integrins or the αvβ3 integrin abolished vitronectin-mediated invasion of pneumococci into host cells (Fig. 4C), whereas pneumococcal invasion was not affected by anti-β1-integrin or anti-HCAM antibodies.

Actin-cytoskeleton dynamic is essential for vitronectin-mediated pneumococcal invasion into host cells

The impact of the actin cytoskeleton on vitronectin-mediated pneumococcal invasion was investigated by infection studies in the presence of the pharmacological inhibitors cytochalasin D and latrunculin B, or jasplakinolide. Cytochalasin D and latrunculin B inhibit actin polymerization and jasplakinolide stabilizes actin filaments. The presence of these inhibitors significantly blocked pneumococcal invasion via vitronectin as determined by enumeration of recovered intracellular pneumococci (Fig. 5A), indicative for a role of actin dynamics in the uptake mechanism. Moreover, illustrations by confocal laser scanning microscopy (CLSM) and scanning electron microscopy of adherent pneumococci or pneumococci during the process of invading nasopharyngeal host cells demonstrated the induction of microspike-like structures that were involved in bacterial trapping (Fig. 5B-E).

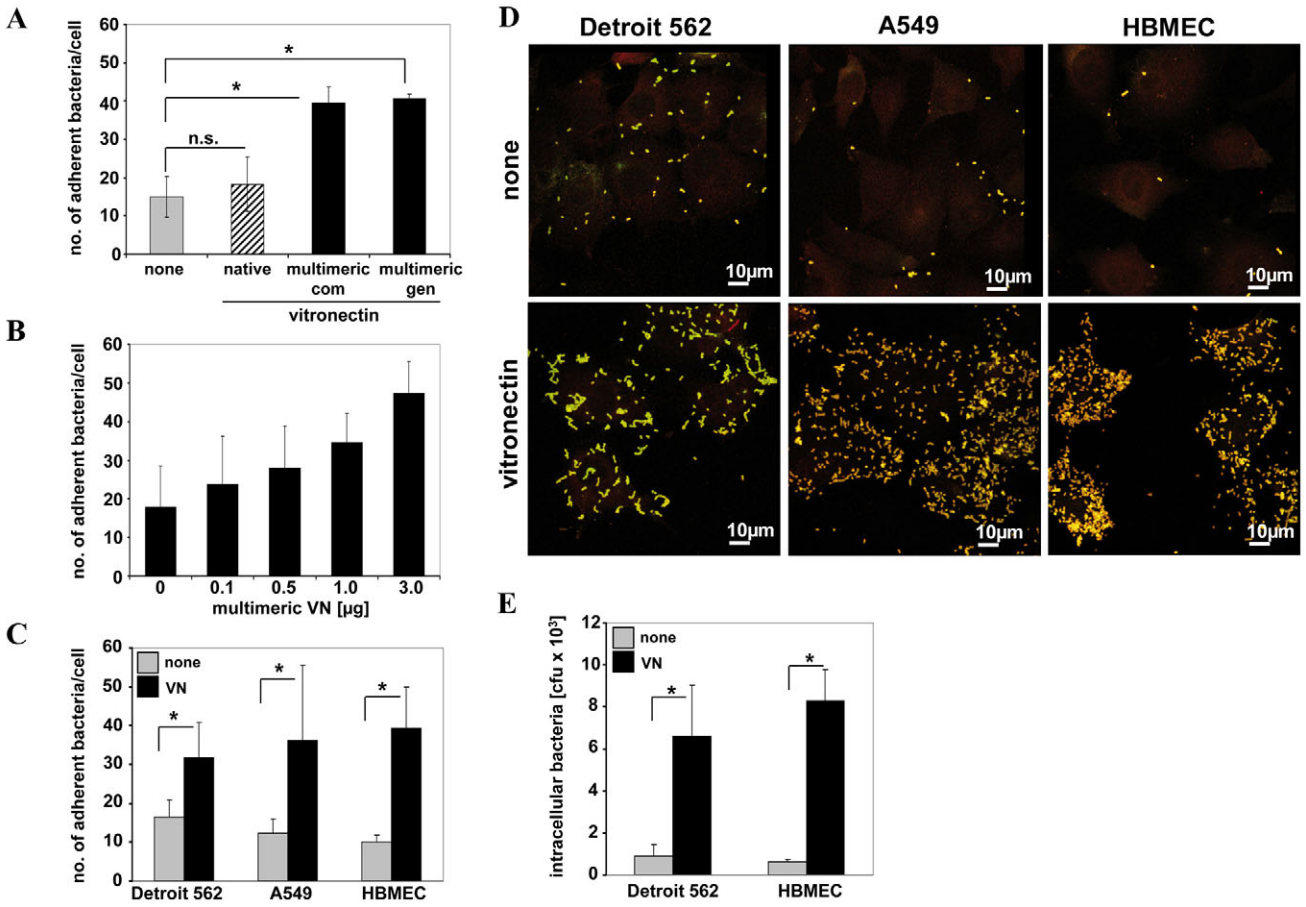

Fig. 2. Host-cell-associated multimeric vitronectin promotes adherence to and invasion of pneumococci into host tissue cells. (A) Pneumococcal (NCTC10319) adherence (at 4 hours) was investigated by immunofluorescence staining after pre-incubation of Detroit 562 cells with 3.0 µg native vitronectin or 3.0 µg of commercially purchased (com) or 3.0 µg self-generated (gen) multimeric vitronectin. The number of attached bacteria per cell in the absence of vitronectin (none) was used as control. Results represent the mean ± s.d. of at least three independent experiments, each done in duplicate. **P*<0.005; n.s., non-significant. (B) Multimeric vitronectin promotes the adherence of pneumococci to Detroit 562 cells in a dose-dependent manner. (C) Adherence of pneumococci to the human epithelial cells Detroit 562 or A549, and the endothelial cell line HBMEC after 4 hours of infection was determined by immunofluorescence in the absence (none) or presence of 3.0 µg multimeric vitronectin (VN). Results represent the mean ± s.d. of at least three independent experiments, each done in duplicate. **P*<0.05. (D) Immunofluorescence microscopy of pneumococci attached to host cells in the absence (none) or presence of multimeric vitronectin. (E) Invasion and intracellular survival of pneumococci in Detroit 562 epithelial cells and the endothelial cell line HBMEC in the absence (none) or presence of multimeric vitronectin (VN) was determined by the antibiotic protection assay. Results represent the mean ± s.d. of at least three independent experiments, each done in duplicate. **P*<0.03.

These structures were especially observed when vitronectin acts as a molecular bridge and thereby enhanced the number of host-cell-attached pneumococci (Fig. 5B,D). The actin-binding proteins frabin and profilin are essential for microspike formation (Suetsugu et al., 1998; Umikawa et al., 1999). CLSM showed that the presence of frabin and profilin in pneumococci induced microspikes after vitronectin-mediated adherence of the bacteria, suggesting that these actin-binding proteins contribute to the formation of bacterial-induced microspike-like structures (Fig. 5C).

Involvement of ILK in vitronectin-mediated internalization of pneumococci

In order to analyze whether ILK is involved in αvβ3-integrin-dependent internalization of pneumococci, we tested invasion of pneumococci into ILK-floxed ($ILK^{fl/fl}$) and ILK-deficient ($ILK^{-/-}$) fibroblasts. Internalization of pneumococci into $ILK^{fl/fl}$ fibroblasts expressing wild-type ILK was significantly increased in the presence of vitronectin (Fig. 6A). By contrast, substantial decrease of uptake of pneumococci was seen in $ILK^{-/-}$ cells, independent of the presence of vitronectin (Fig. 6A,B). To test the involvement of the kinase domain of ILK, ILK-ANK fibroblasts expressing the N-terminal ANK repeats and lacking the C-terminal part containing the ILK kinase domain were infected with pneumococci. Similar to ILK-deficient cells, the vitronectin effect was abolished, indicative of the essential role of the kinase domain in vitronectin-mediated uptake of pneumococci by host cells (Fig. 6C). The essential role of ILK was confirmed with lung epithelial cells A549, a host cell line relevant for pneumococcal infections,

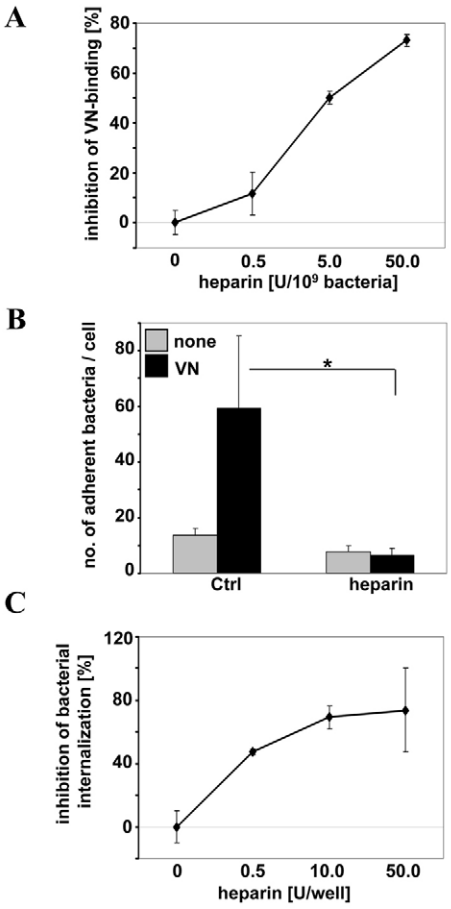

Fig. 3. Influence of heparin on the recruitment of vitronectin and invasion into host cells. (A) The dose-dependent influence of heparin on the binding of multimeric vitronectin to pneumococci was analyzed by flow cytometry using a polyclonal anti-vitronectin antibody and FITC-labelled secondary antibody. Results are presented as the mean ± s.d. of at least three independent experiments. (B) The influence of heparin on the adherence of pneumococci to Detroit 562 cells in the absence (none) or presence of 3.0 μg multimeric vitronectin (VN) was determined by immunofluorescence microscopy. Results are presented as the mean ± s.d. of at least three independent experiments. **P*<0.01. (C) Dose-dependent inhibition by heparin of vitronectin-mediated pneumococcal invasion into Detroit 562 cells was measured by the antibiotic protection assay, and the number of internalized bacteria was determined after 4 hours. Results are presented as mean ± s.d. of at least three independent experiments.

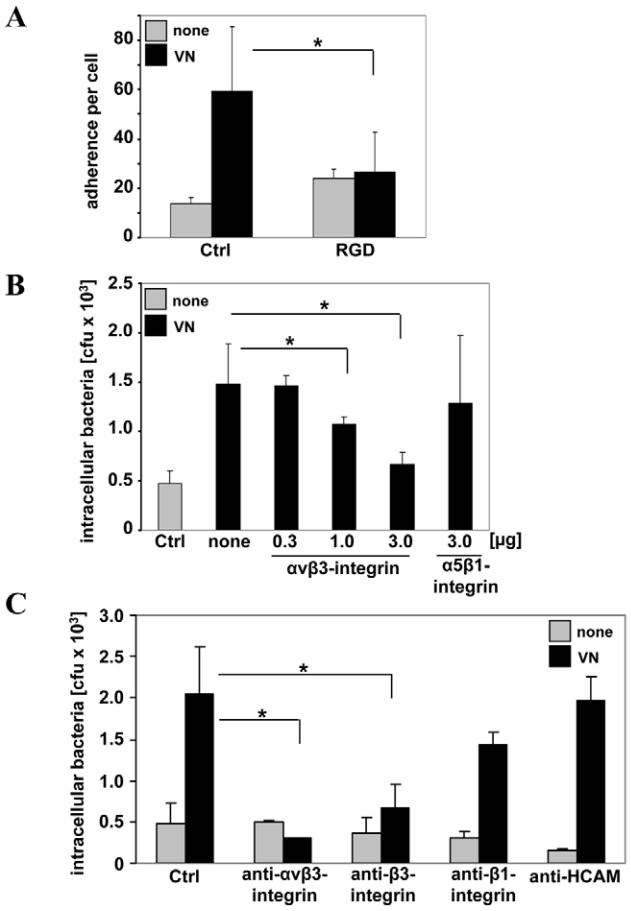

Fig. 4. Integrin-dependent vitronectin-mediated pneumococcal adherence to and invasion into host cells. (A) Vitronectin-mediated adherence of pneumococci to Detroit 562 cells in the absence (Ctrl) or presence of RGD-peptide (H-Gly-Arg-Gly-Asp-Asn-Pro-OH) was monitored after 4 hours of infection by immunofluorescence microscopy. Results are presented as the mean ± s.d. of at least three independent experiments. **P*<0.01. (B) Vitronectin-mediated invasion of pneumococci into host cells was followed in the absence (Ctrl) or presence of soluble αvβ3 integrin or α5β1 integrin as indicated after 4 hours using the antibiotic protection assay. Results are presented as the mean ± s.d. of at least three independent experiments. **P*<0.05. (C) Influence of various blocking antibodies directed against host adhesion receptors on vitronectin-mediated internalization of pneumococci into host cells was investigated by antibiotic protection assay. Results are presented as the mean ± s.d. of at least three independent experiments. **P*<0.05. none, infections performed in the absence of vitronectin; VN, infections performed in the presence of vitronectin.

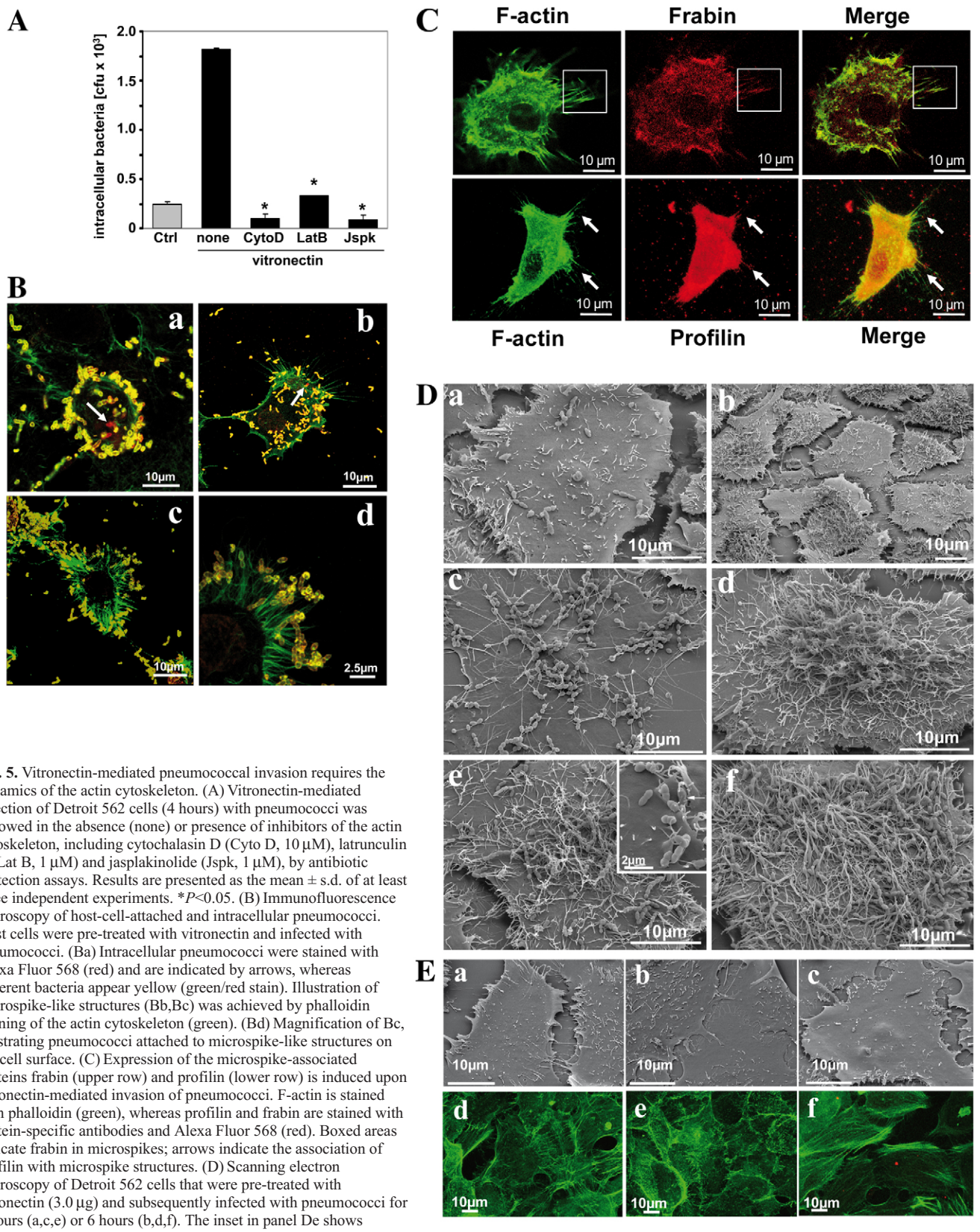

Fig. 5. Vitronectin-mediated pneumococcal invasion requires the dynamics of the actin cytoskeleton. (A) Vitronectin-mediated infection of Detroit 562 cells (4 hours) with pneumococci was followed in the absence (none) or presence of inhibitors of the actin cytoskeleton, including cytochalasin D (Cyto D, 10 µM), latrunculin B (Lat B, 1 µM) and jasplakinolide (Jspk, 1 µM), by antibiotic protection assays. Results are presented as the mean ± s.d. of at least three independent experiments. **P*<0.05. (B) Immunofluorescence microscopy of host-cell-attached and intracellular pneumococci. Host cells were pre-treated with vitronectin and infected with pneumococci. (Ba) Intracellular pneumococci were stained with Alexa Fluor 568 (red) and are indicated by arrows, whereas adherent bacteria appear yellow (green/red stain). Illustration of microspike-like structures (Bb,Bc) was achieved by phalloidin staining of the actin cytoskeleton (green). (Bd) Magnification of Bc, illustrating pneumococci attached to microspike-like structures on the cell surface. (C) Expression of the microspike-associated proteins frabin (upper row) and profilin (lower row) is induced upon vitronectin-mediated invasion of pneumococci. F-actin is stained with phalloidin (green), whereas profilin and frabin are stained with protein-specific antibodies and Alexa Fluor 568 (red). Boxed areas indicate frabin in microspikes; arrows indicate the association of profilin with microspike structures. (D) Scanning electron microscopy of Detroit 562 cells that were pre-treated with vitronectin (3.0 µg) and subsequently infected with pneumococci for 4 hours (a,c,e) or 6 hours (b,d,f). The inset in panel De shows pneumococci invading the host cell (arrow). (E) Scanning electron microscopy (top) and immunofluorescence microscopy (bottom) after staining the actin cytoskeleton of untreated Detroit 562 cells (a,d), Detroit 562 cells incubated with multimeric vitronectin (b,e) or host cells infected with pneumococci in the absence of vitronectin (c,f).

in which ILK expression was knocked-down using siRNA interference. The reduction of ILK expression was indicated by immunoblot analysis (Fig. 6E). Moreover, reduction of ILK expression in A549 cells impaired the formation of a normal F-actin cytoskeleton (Fig. 6F), as was recently demonstrated for other cell types such as fibroblasts (Grashoff et al., 2003; Lorenz et al., 2007; Sakai et al., 2003). Vitronectin-mediated uptake of pneumococci into A549 cells with a genetic *ILK* knockdown was significantly reduced compared with non-transfected host cells or A549 cells transfected with the control siRNA, as determined by the antibiotic protection assays (Fig. 6D). Immunofluorescence microscopy demonstrated that the attachment of pneumococci to transfected or non-transfected cells remained unaffected (data not shown).

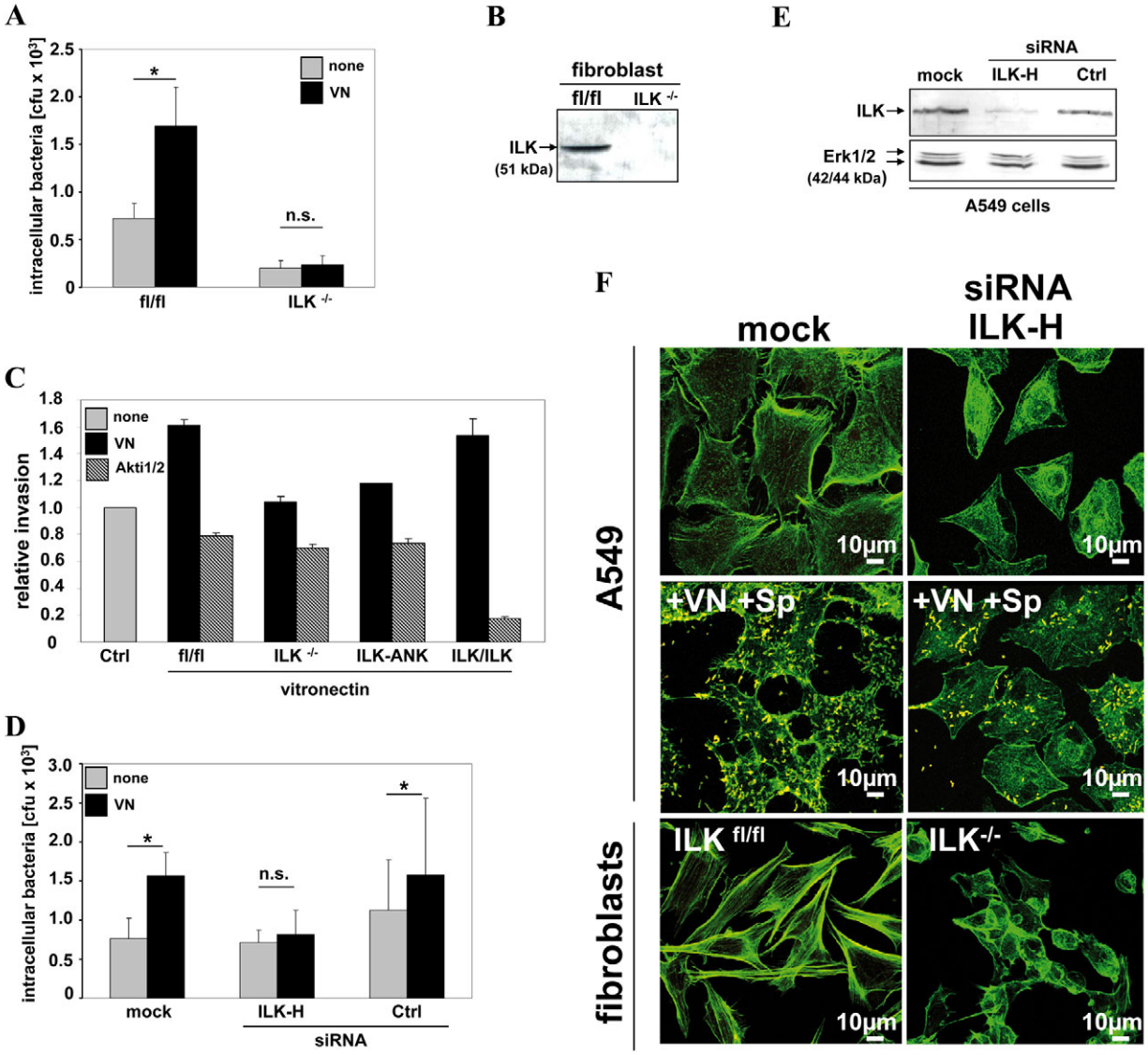

Fig. 6. Vitronectin–αvβ3-integrin-mediated invasion of host cells by pneumococci depends on ILK expression. (A) Vitronectin-mediated invasion of pneumococci into ILK-expressing fibroblasts (fl/fl) or *ILK*$^{-/-}$ fibroblasts was determined by the antibiotic protection assay. Results are presented as the mean ± s.d. of at least three independent experiments. **P*<0.004; n.s., non-significant. (B) Immunoblot analysis of ILK expression in ILK-expressing fibroblasts (fl/fl) or ILK-deficient cells (*ILK*$^{-/-}$). (C) Vitronectin-mediated pneumococcal internalization into fibroblasts overexpressing ILK (ILK/ILK) or fibroblasts expressing the N-terminal ankyrin repeats (ILK-ANK). ILK-ANK cells lack the C-terminal kinase domain. Wild-type (Ctrl), *ILK*$^{fl/fl}$ (fl/fl) and *ILK*$^{-/-}$ fibroblasts were used as controls, and pneumococcal invasion was examined in the presence and absence of the Akt1/2 inhibitor VIII (Akti1/2, 10 µM). Pneumococcal invasion indicates the relative number of intracellular bacteria compared with non-treated fibroblasts (Ctrl). Data are shown as mean ± s.d. of at least three independent experiments. (D) Vitronectin-dependent pneumococcal invasion of non-transfected A549 cells (mock) or cells transfected with the ILK-specific siRNA (ILK-H) or with a control siRNA (Ctrl) was performed 48 hours post-transfection. The number of intracellular and recovered pneumococci was monitored by the antibiotic protection assay. Results are presented as the mean ± s.d. of at least three independent experiments. **P*<0.004. (E) Reduction of ILK expression was controlled by immunoblot analysis of transfected cell lysates with specific anti-ILK antibodies to verify knock down of ILK by siRNA. Erk1/2 served as loading control. (F, top row) ILK ablation leads to an impaired F-actin cytoskeleton, as demonstrated by immunofluorescence microscopy of ILK-expressing A549 cells (mock) and ILK-knockdown A549 cells (siRNA ILK-H). F-actin is stained with phalloidin and the impact of ILK on the cytoskeleton is also shown for ILK-knockout fibroblasts (*ILK*$^{-/-}$; bottom-right panel). The middle row shows A549 cells treated with vitronectin and infected with pneumococci. Bacteria were stained with Alexa Fluor 488 and Alexa Fluor 568, and appear yellow (green/red stain).

PI3K- and Akt-dependency for the vitronectin–αvβ3-integrin-promoted invasion of pneumococci

As ILK is activated upon integrin engagement in a PI3K-dependent manner (Delcommenne et al., 1998), invasion of pneumococci into Detroit 562 cells via vitronectin was monitored in the presence of the PI3K-specific inhibitor Wortmannin (Fig. 7A). Wortmannin, as well as LY294002, significantly decreased vitronectin-mediated internalization of pneumococci into Detroit 562 cells (Fig. 7A). In addition, immunofluorescence microscopy indicated that pneumococcal adherence to host cells was not altered in the presence of these inhibitors (data not shown). The amount of DMSO used in these inhibition studies affected neither host-cell viability (trypan blue) nor the invasion of pneumococci (data not shown). Because Akt activity is thought to be regulated by ILK (Persad and Dedhar, 2003), blockade of this downstream reaction by a specific Akt inhibitor (Akti1/2 VIII) of vitronectin–αvβ3-integrin-mediated invasion of pneumococci was tested and was found significantly reduced (Fig. 7A). Similar to PI3K inhibitors, pneumococcal adherence was not affected (data not shown). Furthermore, phosphorylation of Akt was especially induced upon infection of host cells with pneumococci in the presence of host-cell-bound vitronectin (Fig. 7B). In host cells with ILK knockdown, significantly lower Akt phosphorylation at Ser473 was observed upon pneumococcal infection compared with wild-type cells (Fig. 7B). Phosphorylation was significantly lower in vitronectin-treated but non-infected cells and almost absent in untreated cells upon infection (Fig. 7B). To investigate whether the overexpression of ILK rescues the inhibitory effect of Akt inhibition and leads to vitronectin-mediated pneumococcal invasion, ILK/ILK (*ILK*$^{-/-}$/*ILK*-FLAG) fibroblasts were infected with pneumococci. However, the results showed that higher expression levels of ILK cannot rescue the deterioration of Akt that was induced by the Akt inhibitor, confirming the essential role of Akt during vitronectin–αvβ3-integrin-mediated invasion of pneumococci into host cells (Fig. 6C).

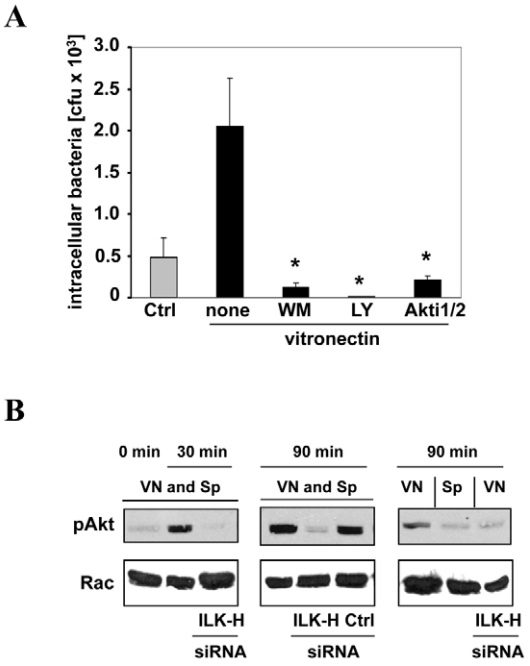

Fig. 7. PI3K- and Akt-dependent pneumococcal invasion via the vitronectin–αvβ3-integrin pathway. (A) Vitronectin-dependent internalization of pneumococci into Detroit 562 cells in the absence (none) or presence of the PI3K inhibitors Wortmannin (WM, 50 nM) or LY294002 (LY, 50 nM), or the Akt1/2 inhibitor VIII (Akti1/2, 10 μM) was measured after 4 hours of infection by using the antibiotic protection assay. Results are presented as mean ± s.d. of three independent experiments. **P*<0.05. (B) 48 hours prior to infection, A549 cells were transfected with *ILK*-specific siRNA (ILK-H) or control siRNA (Ctrl). The phosphorylation of Akt (pAkt) in A549 cells was analyzed after 30 minutes and 90 minutes of infection with pneumococci by western blot analysis in the absence or presence of host-cell-bound vitronectin (VN) and with or without bacteria (Sp), as indicated. Simultaneously, the protein Rac served as loading control.

Discussion

Pneumococci interact specifically with a variety of proteins of the ECM and serum (Hammerschmidt, 2006). However, the impact of these interactions on bacterial invasion into host cells, the cellular receptors themselves and the induced signalling cascades have not been explored. The exception is represented by the pneumococci-thrombospondin interaction. Recent work revealed that host-cell-bound thrombospondin recognizes the peptidoglycan of *S. pneumoniae* and other Gram-positive pathogens and promotes adherence of these microorganisms to host cells (Rennemeier et al., 2007). In addition to thrombospondin, pneumococci interact with the adhesive ECM glycoproteins fibronectin and vitronectin. In this study we have demonstrated that pneumococci interact especially with the multimeric form of vitronectin and that host-cell-bound vitronectin promotes pneumococcal adherence to and invasion into eukaryotic cells. Although pneumococci are exclusively human pathogenic bacteria, binding of vitronectin in physiologically relevant concentrations occurs in a species-unspecific manner. Our data demonstrate that pneumococci interact with host-cell-bound vitronectin and exploit αvβ3 integrin as the major cellular receptor, as is depicted in the schematic model in Fig. 8. Ingestion of pneumococci via vitronectin–αvβ3-integrin depends on actin dynamics and stimulates the formation of microspike-like structures on the surface of host cells. In addition, molecules of the intracellularly associated focal-adhesion (FA) complex seem to be essential to induce pneumococcal invasion into host cells. One of the key molecules of FA is ILK, which translates the vitronectin-mediated integrin binding of pneumococci into an uptake signal, as demonstrated by genetic ablation or knockdown of ILK. In addition, PI3K and Akt were shown to be essential for the vitronectin–αvβ3-integrin-promoted cellular invasion by pneumococci.

Vitronectin is a multifunctional glycoprotein of the ECM and serves as a potential docking target for pathogenic bacteria to cellular tissues. Specific interactions with vitronectin were reported for the human pathogenic microorganisms *Haemophilus influenzae*, *Staphylococcus aureus*, *N. gonorrhoeae*, *Escherichia coli*, *Pneumocystis carinii*, *Helicobacter pylori*, *S. pyogenes* and group C and G streptococci (Chhatwal et al., 1987; Dehio et al., 1998; Eberhard and Ullberg, 2002; Kostrzynska and Wadstrom, 1992; Kostrzynska et al., 1992; Liang et al., 1995; Paulsson and Wadstrom, 1990; Paulsson et al., 1992). Despite the identification of several bacterial vitronectin-binding proteins, including the *Haemophilus* surface fibrils (Hsf), the staphylococcal proteins Eap and Emp, and the gonococcal OpaA protein, the impact of the vitronectin interaction on adherence and invasion has only been studied for the human-specific pathogen *N. gonorrhoeae* (Dehio et al., 1998; Duensing and van Putten, 1997; Hallström et al., 2006; Hussain et al., 2001; Jonsson et al., 1995). Remarkably, binding of vitronectin to the gonococcal Opa protein requires the presence of

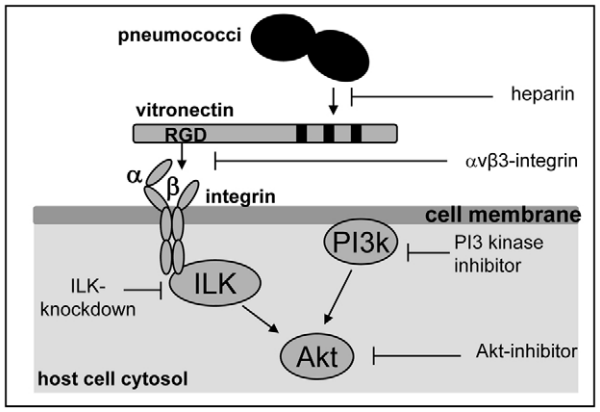

Fig. 8. Schematic model of the vitronectin–αvβ3-integrin-mediated invasion mechanism of *S. pneumoniae* and of the involved signalling pathway.

glycosaminoglycans, which act as a molecular bridge and link the vitronectin molecule and bacterial OpaA protein (van Putten et al., 1998). A similar mechanism was suggested for the fibronectin-mediated uptake of gonococci into eukaryotic host cells. Here, the glycosaminoglycans act as a bacterial co-receptor and facilitate fibronectin–integrin-mediated bacterial invasion (van Putten et al., 1998).

S. pneumoniae interacts especially with the multimeric form of vitronectin representing the ECM-associated vitronectin that was shown here to promote pneumococcal invasion. The pneumococci-vitronectin interaction was inhibited by heparin and heparan sulfate, which both bind to the C-terminally located heparin-binding domain of vitronectin. Multimeric vitronectin contains highly reactive heparin-binding sites and promotes multivalent interactions with various ECM components and cell-surface receptors. Previous studies demonstrated that binding activities of multimeric vitronectin to bacterial surfaces differ from binding activities of the monomeric isoform owing to additional availabilities of binding sites exposed on the multimeric form of vitronectin (Stockmann et al., 1993). Hence, this interaction resembles the pneumococcal interaction with fibronectin, which was suggested to occur via an interaction with the heparin-binding site in the C-terminal part of the molecule, whereas other pathogens interact with the heparin-binding sites in the N-terminal part of fibronectin (van der Flier et al., 1995).

To date, the pneumococcal vitronectin-binding protein is unknown. However, pre-treatment of pneumococci with proteolytic enzymes such as Pronase E abolished the interaction with vitronectin, whereas treatment with sodium periodate showed no effect (data not shown), suggesting a proteinaceous nature of the bacterial adhesin. Sequence comparisons with known vitronectin-binding proteins did not reveal significant homologies or domains with high similarities to pneumococcal proteins or peptides. Moreover, binding studies performed with pneumococci that were deficient for a subclass of surface proteins suggested that none of the choline-binding proteins function as a pneumococcal vitronectin receptor (data not shown), and these studies also excluded the pneumococcal fibronectin-binding protein PavA (Holmes et al., 2001; Pracht et al., 2005) as a putative adhesin (data not shown).

Integrins are essential for the interaction of cells with the ECM. Binding of extracellular ligands such as fibronectin or vitronectin to integrins induces integrin clustering and the recruitment of cytoplasmic proteins that mediate the connection to F-actin filaments and the activation of signalling proteins (Hynes, 2002). Importantly, integrins can also trigger signalling pathways that promote bacterial uptake (Hauck and Ohlsen, 2006). Ingestion of *Yersinia* species that produce the outer-membrane protein termed invasin is induced by a direct interaction of this *Yersinia* protein with β1 integrins (Isberg and Barnes, 2001). Similar to pneumococci, other pathogenic bacteria bind via ECM components to integrins and induce their ingestion, which has been shown to require the activity of several components of the FA, such as focal adhesion kinase or paxillin (Agerer et al., 2005; Wang et al., 2007). In addition to pathogenic *Neisseria* (Hauck and Meyer, 2003; Unkmeir et al., 2002), staphylococci and group A streptococci also bind to fibronectin, which indirectly connects the microorganisms with host-cell integrins (Agerer et al., 2003; Ozeri et al., 1998). The α5β1-integrin-dependent uptake of *S. pyogenes* via engagement of fibronectin leads to ILK activation and downstream signalling (Wang et al., 2006).

Here, we have shown for the first time that pneumococci have adopted this mechanism and we demonstrated that pneumococci engage vitronectin to adhere to αvβ3 integrins, which promotes bacterial internalization in an ILK-dependent manner. These data and our inhibition studies performed with peptides and antibodies demonstrated the predominant role of αvβ3 integrin as the docking site for vitronectin-mediated pneumococcal adherence to and invasion into host cells. Vitronectin–αvβ3-integrin-dependent pneumococcal invasion requires the actin cytoskeleton dynamic, and host-cell microspikes or microspike-like structures were formed, which trap the bacteria. Microspikes contain the actin-bundling protein fascin, and the actin-binding proteins profilin and frabin are essential for microspike formation (Suetsugu et al., 1998; Umikawa et al., 1999). The observed morphological changes upon activation of integrins through the concerted action of bacteria and vitronectin resemble the microspike formation observed during integrin-activated cell spreading on matrix proteins (Adams, 1995). Moreover, genetic ablation of ILK demonstrated the importance of ILK for this uptake mechanism. ILK transduces signals from integrins to subcellular compartments but also vice versa from the integrins to the ECM (Grashoff et al., 2004). Activation of ILK depends on PI3K activity (Delcommenne et al., 1998). Inhibition of PI3K activity with a specific inhibitor abolished the integrin-mediated invasion of pneumococci, which suggests that the direct inactivation of ILK and also the indirect inhibition of the regulatory activity of ILK are, individually, sufficient to inhibit pneumococcal invasion. Immunoblot analysis demonstrated phosphorylation of Akt upon pneumococcal invasion via the integrin pathway and, in addition, the influence of Akt during bacterial invasion was confirmed in inhibition studies with an Akt-specific inhibitor. ILK acts as a signal transducer and interacts with a variety of adaptor proteins that regulate actin-cytoskeleton dynamics. Similar to *S. pyogenes* (Wang et al., 2006), the ILK kinase domain is essential for integrin-mediated pneumococcal uptake by host cells. Therefore, a key aspect of future research activities will be the assessment of the role of other adaptor proteins, including paxillin, parvin and Pinch, and how these molecules cooperate with other molecules of FA during pneumococcal invasion.

The observed bacterial strategy is very similar to a signalling induced by ECM components alone, suggesting that a variety of pathogenic microorganisms are highly adapted to their host environment, which enables them to exploit the dynamic processes of the host for adherence to and invasion into eukaryotic host cells.

This points to a common mechanism employed by pathogens to promote colonization, persistence and/or invasive infections.

Materials and Methods

Bacterial strains and culture conditions

S. pneumoniae (NCTC10319; serotype 35A) was cultured in Todd-Hewitt broth (Oxoid, Basingstoke, UK) supplemented with 0.5% yeast extract (THY) to mid-log phase or grown on blood agar plates (Becton Dickinson). A pneumolysin-negative mutant of a serotype 35A strain was used in infection assays to avoid the cytotoxic effects of pneumolysin as described previously (Pracht et al., 2005). Pneumococcal strain D39 (Cps+, serotype 2) and its non-encapsulated mutant D39Δ*cps* (Rennemeier et al., 2007) were used in binding studies to indicate the effect of the CPS. D39 and NCTC10319 do not produce pili, as examined by PCR (data not shown).

Proteins, inhibitors and antibodies

Human multimeric vitronectin was purchased from Chemicon (Temecula, Canada) and the conformation was indicated in non-denaturing polyacrylamide gel electrophoresis and Coomassie brilliant blue stain. Purification of human native and multimeric vitronectin was performed as described previously (Preissner et al., 1985). Human vitronectin was detected using a polyclonal rabbit anti-vitronectin antibody or with a mouse monoclonal antibody (VN7) recognizing heparin-binding domains of vitronectin (Stockmann et al., 1993). Mouse vitronectin was detected by immunoblotting using polyclonal antibodies sc-15332 (Santa Cruz Biotechology, USA). To detect bacteria-bound vitronectin antibody, VN7 was used. The reaction of VN7 with human vitronectin is twofold higher compared with mouse vitronectin (data not shown). Humans and mice contain similar concentrations of plasma vitronectin (200-400 μg/ml versus 250-300 μg/ml) (Seiffert et al., 1991; Preissner et al., 1985). The peptide containing the RGD sequence for integrin binding (H-Gly-Arg-Gly-Asp-Asn-Pro-OH) and the RGE control peptide (H-Gly-Arg-Gly-Glu-Ser-Pro-OH) were purchased from Bachem (Switzerland). Cytochalasin D and heparin (potassium salt) were obtained form MP Biomedicals. Latrunculin B, jasplakinolide, LY294002, Wortmannin and Akt1/2 inhibitor VIII were from Calbiochem. αvβ3 integrin (CD51/CD61) and α5β1 integrin (CD49e/CD29), and monoclonal antibodies recognizing β1 integrins (anti-CD29; MAB2253Z, clone 6S6), β3 integrins (anti-CD61; MAB2023Z, clone B3A) or anti-αvβ3-integrin antibodies (anti-CD51/CD61; CBL544), all possessing blocking activities (Unkmeir et al., 2002), were obtained from Chemicon. The following other antibodies were used: mouse anti-HCAM (anti-CD44, Santa Cruz, sc-7297), rabbit anti-ILK (Sigma), mouse anti-Rac (Becton Dickinson), anti-p-Akt (Ser437), rabbit anti-Akt (Cell Signalling), rabbit anti-Erk1/2 (Santa Cruz, sc153), rabbit anti-profilin-1/2 (FL-140; Santa Cruz), mouse anti-frabin (clone 43; BD Bioscience, San Diego, CA), HRP-conjugated rabbit IgG (Dianova) and HRP-conjugated mouse IgG (Jackson Laboratories).

Flow cytometric analysis of vitronectin binding to pneumococci

To investigate the binding of vitronectin to pneumococci, 5×10^7 pneumococci in a volume of 100 μl phosphate-buffered saline supplemented with 0.5% foetal bovine serum (PBS/FBS) were incubated for 30 minutes with human plasma, mouse plasma, purified multimeric, or purified monomeric vitronectin at 37°C. Bacteria were washed with PBS and recruitment of purified human vitronectin was analyzed with the polyclonal anti-vitronectin IgG in PBS/FBS. Bound vitronectin from human plasma was analyzed using the monoclonal VN7 antibodies and bound mouse vitronectin was analyzed with the mouse-specific polyclonal IgG sc-15332. Detection of bound vitronectin was conducted after 20 minutes incubation with a 1:300 dilution of an Alexa-Fluor-488-conjugated mouse or rabbit IgG (MoBiTec GmbH, Goettingen, Germany). Finally, the treated bacteria were washed with PBS and fixed with 3.0% paraformaldehyde (PFA) in a total volume of 200 μl. Fluorescence intensity was measured by flow cytometry using a FACS-Calibur (Becton Dickinson) or FACS-Canto (Becton Dickinson) flow cytometer. Pneumococci were detected using log-forward and log-side scatter dot plots, and a gating region was set to exclude debris and larger aggregates of bacteria. At least 10,000 bacteria were analyzed. The geometric mean fluorescence intensity (GMFI) multiplied by the percentage of labelled bacteria was recorded as a measure for binding activity. Unspecific binding of the antibodies was subtracted from the vitronectin-containing samples. Evaluation of binding was performed using WinMDI (version 2.8) or FACSDiva (version 6.0, Becton Dickinson). At a minimum, three independent experiments were done and each sample in triplicate.

Immunodetection of vitronectin recruited from plasma by pneumococci

After 30 minutes incubation of 2×10^9 bacteria with human plasma, the whole pneumococcal cell lysates were subjected to SDS-PAGE with 4% stacking and 10% separating gel. Thereafter, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore) using a semi-dry blotting system (Bio-Rad). The membranes were blocked with 10% skim milk (Oxoid) in PBS (10 mM) prior to incubation with rabbit polyclonal anti-vitronectin antibodies (1:100). Detection of vitronectin was performed using HRP-conjugated anti-rabbit antiserum (1:5000) followed by incubation with a substrate solution containing 1 mg/ml 4-chloro-1-naphthol and 0.1% H_2O_2 in PBS.

Eukaryotic cell lines and ILK-knockout fibroblasts

The human nasopharyngeal carcinoma cell line Detroit 562 (ATCC CCL-138), human A549 cells (lung alveolar epithelial cells type II pneumocytes; ATCC CCL-185) and human brain-derived microvascular endothelial cells (HBMEC) were used for standard infection assays and cultured as described (Rennemeier et al., 2007). As indicated by flow cytometric analysis, the host cells did not capture ECM proteins derived from our serum (FBS Gold, PAA, Germany), including fibronectin and vitronectin (Rennemeier et al., 2007). Generation of ILK-deficient fibroblasts ($ILK^{-/-}$), which contain a homozygous deletion of ILK, and ILK-floxed ($ILK^{fl/fl}$) fibroblasts is described in Sakai et al. (Sakai et al., 2003). ILK/ILK fibroblasts ($ILK^{-/-}$/ILK-FLAG), which overexpress ILK and produce two- to six-times more ILK [Carsten Grashoff (University of Virginia Health System, Charlottesville, VA) unpublished] than wild-type fibroblasts $ILK^{fl/fl}$, and ILK-ANK ($ILK^{N\text{-terminal}}$) fibroblasts were a generous gift from R. Fässler. ILK-ANK fibroblasts express the N-terminal ANK repeat domain (amino acids 1-180) of murine ILK with a C-terminal triple FLAG-tag. The generation of ILK/ILK and ILK-ANK cell lines is described in Hehlgans et al. (Hehlgans et al., 2008). All fibroblasts were cultured in DMEM, 10% FBS.

Cell-culture infection experiments with pneumococci and blocking experiments

Pneumococcal adherence or invasion was quantified after infecting eukaryotic host cells. The infections were carried out as described previously (Pracht et al., 2005). Briefly, the host cells were seeded on glass coverslips (diameter 12 mm) or directly in the wells of a 24-well plate (Cellstar, Greiner, Germany) at a density of 5×10^4 cells per well and cultivated to confluent cell layers with approximately 2×10^5 cells per well. The cells were washed three times with Dulbecco's modified Eagle's medium containing HEPES (DMEM-HEPES, PAA Laboratories, Coelbe, Germany) supplemented with 1.0% foetal calf serum (FCS) and then infected with pneumococci. In a standardized assay we used a multiplicity of infection of 25 bacteria per host cell and infections were carried out for 3 hours at 37°C and 5.0% CO_2. Thereafter, unbound bacteria were removed by rinsing three times with DMEM-HEPES with 1.0% FBS. The infection dose (CFU) per well was controlled by serial plating of the bacteria on blood agar plates. To elucidate the effect of host-cell-bound vitronectin on pneumococcal adherence and invasion, the cells were treated for 30 minutes with 3.0 μg human vitronectin in 500 μl DMEM-HEPES supplemented with 1.0% FBS at 37°C and 5.0% CO_2. Thereafter, unbound vitronectin was removed by washing the cells once with DMEM-HEPES. In inhibition experiments with blocking antibodies, the host cells were incubated for 30 minutes with the antibodies (10 μg/ml of anti-β3-integrin, anti-αvβ3-integrin, anti-β1-integrin, or anti-HCAM) in a total volume of 500 μl followed by an incubation with multimeric vitronectin. Expression of β3 integrins, αvβ3 integrin, β1 integrins and HCAM by Detroit 562, A549 or HBMEC was confirmed by flow cytometry using the corresponding antibodies (data not shown). The purified αvβ3 integrins or α5β1 integrins were incubated for 10 minutes with equal amounts of purified human vitronectin in a volume of 20 μl and then added to the cells. The host-cell-unbound proteins were removed after 30 minutes incubation and the cells were infected with pneumococci. The pharmacological inhibitors used to study the impact of the cytoskeleton or signalling molecules were solved in DMSO and the cells were pre-incubated for 30 minutes with the inhibitors prior to the incubation with vitronectin. The infection assays were performed in the presence of the inhibitors. As a control, the cells were incubated with DMSO alone and infected as required. The amount of DMSO used in our assay had no influence on cell viability, cell morphology or pneumococcal adherence (data not shown).

Evaluation and visualization of pneumococcal host-cell adherence by immunofluorescence staining and microscopy

For immunofluorescence microscopy, the infected host cells were fixed on the glass coverslips with 3.0% paraformaldehyde. The immunofluorescence staining of pneumococci attached to the host cells and in some case also the differentiation between extracellular and intracellular bacteria was carried out as described recently (Rennemeier et al., 2007). This results in Alexa-Fluor-568-labeled intracellular bacteria (red fluorescence) and Alexa-Fluor-488/568-labeled extracellular bacteria (green/yellow) or only in green fluorescent adherent pneumococci. At least 50 cells were counted using a fluorescence microscope and a confocal laser scanning microscope (Leica TCS SP5 AOBS), and the LAS AF SP5 software were used for image acquisition. Each bar in the images represents 10 μm.

Quantification of pneumococcal invasion

Antibiotic protection assays were performed to quantify the total number of internalized and recovered pneumococci after the infection experiments, which were carried out under different conditions. The infections were performed for 3 hours as described and thereafter the cell layers were washed thoroughly to remove unbound bacteria. To kill the extracellular, adherent pneumococci, the host cells were incubated for 1 hour with DMEM-HEPES containing 100 μg gentamicin and 100 U penicillin G at 37°C and 5.0% CO_2. The intracellular pneumococci were released by a saponin-mediated host-cell lysis (1.0% w/v). The total number of invasive and recovered

pneumococci was monitored after plating sample aliquots on blood agar plates, followed by colony formation and enumeration. Each experiment was repeated at least three times and results were expressed as mean ± s.d.

siRNA silencing of ILK expression

Owing to high transfection efficiencies of A549 cells compared with Detroit 562 cells we selected lung epithelial cells A549 for siRNA knockdown experiments. siRNA silencing of *ILK* gene expression in A549 host cells was performed using 50 nM of validated stealth ILK-siRNA-Oligo (Hs_ILK_4HP, Qiagen). This sequence targets the PH-domain of ILK (Troussard et al., 2003). Host cells were transfected with the specific siRNA and the control siRNA with a GC content equal to the specific siRNA using the lipofectamine RNAiMax reagent (Invitrogen) according to the manufacturer's instructions. Prior to transfections, A549 cells were cultured in antibiotic-free medium and transfections were carried out with 50 nM of the siRNA-Oligo in OptiMEM serum-free medium (Invitrogen). After 48 hours, invasion of pneumococci was analyzed. The transfection efficiency was quantified microscopically by using 50 nM of a GFP-conjugated siRNA (Invitrogen). ILK silencing was controlled by immunoblot analysis with the anti-ILK antibody.

SDS-PAGE and immunoblot analysis

Host cells were removed from the cell culture dishes with a cell scraper and cells were lysed with lysis buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 1.0% Triton X-100, 10% glycerol, 0.5% Deoxycholate) containing a complete protease inhibitor cocktail tablet (Roche). The amount of protein in the samples was determined using the Bradford protein quantification method (Sigma) and equal amounts of protein lysates were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 10% skim milk (Oxoid) prior to antibody incubations. Expression of ILK was indicated by immunodetection using the ILK-specific antiserum (Sigma, 1:1000) together with the horseradish peroxidase (HRP)-conjugated antiserum (1:5000, Dianova). As a loading control, Erk or Rac were detected with an Erk-specific antibody (Santa Cruz, sc153) or anti-Rac antibody (Becton Dickinson). Phosphorylation of Akt or total Akt was analyzed by antibodies recognizing Akt-Ser473-*P* (Cell Signaling; no. 9271) and Akt (Cell Signaling; no. 9272), respectively. Incubation with the Akt-*P*-specific antibody was performed overnight at 4°C followed by washing steps with PBS/0.05% Tween 20 (Sigma). Antibody binding was detected with a substrate solution containing 1 mg/ml 4-chloro-naphthol and 0.1% H_2O_2 in PBS or by enhanced chemiluminescence (ECL, Amersham).

Field emission scanning electron microscopy (FESEM)

Infected cells were fixed with 4% paraformaldehyde, washed with TE buffer (20 mM TRIS, 1 mM EDTA, pH 6.9), dehydrated by incubating with a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 15 minutes, critical-point dried with liquid CO_2 (CPD 30, Bal-Tec, Liechtenstein) and covered with a gold film by sputter coating (SCD 50040, Bal-Tec, Liechtenstein) before being examined in a field emission scanning electron microscope (Zeiss DSM 982 Gemini, Carl Zeiss) using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

Statistical evaluation

The infection experiments were performed at three times, each in duplicate and the data were expressed as mean ± s.d. Differences in adherence and internalization of pneumococci were analyzed by the two-tailed unpaired Student's *t*-test. In all analyzes, *P* values of <0.05 were considered statistically significant.

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