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RESEARCH ARTICLE

Salmonella YrbD protein mediates invasion into the host by interacting with β2 integrin

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Highlights

- β2 integrin is an important host factor that promotes the invasion of *Salmonella* into host cells.
- The soluble protein in the extracellular domain of β2 integrin can neutralize *Salmonella* infection.
- The direct interaction between β2 integrin and *Salmonella* protein YrbD promotes the adhesion and internalization of *Salmonella* to host cells.

Abstract

Salmonella enterica serovar Typhimurium, the causative agent of gastroenteritis, is one of the most successful intracellular pathogens. Although certain host factors for *Salmonella* infection have been unveiled, the factors mediating *Salmonella* entry, particularly the invasion process, remain obscure. Here, we have unearthed β2 integrin, a crucial member of the integrin family, as an important host factor facilitating *Salmonella* invasion. It is demonstrated that overexpression of β2 integrin promotes *Salmonella* invasion, while the knockdown of β2 integrin significantly diminishes the extent of invasion. Moreover, *Salmonella* exhibits specific binding affinity towards β2 integrin, and the block of β2 integrin on cell surface substantially reduces the infection of cells *in vitro*. The ectodomain soluble protein of β2 integrin neutralized *Salmonella* infection both in cells (*in vitro*) and in mice (*in vivo*). Additionally, *Salmonella* protein YrbD directly interacts with β2 integrin to facilitate its invasion. To our knowledge, this study showed for the first time that the protein YrbD mediates *Salmonella* adhesion and internalization into host cells by interacting with β2 integrin. These findings not only broaden our understanding of the mechanisms underlying *Salmonella* entry, but also identify a prospective target for therapeutic control.

Keywords: β2 integrin, adhesion, internalization, *Salmonella*, protein YrbD

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1. Introduction

Salmonella enterica serovar Typhimurium (hereafter to be referred as *Salmonella*) is an enteroinvasive bacterial pathogen that represents one of the leading causes of foodborne illness worldwide, imposing a considerable public health burden and economic cost to society (Malik-Kale *et al.* 2011; Elhenawy *et al.* 2016). The burden of foodborne diseases is substantial, i.e., 550 million people fall ill every year and approximately 33 million deaths are reported globally (Aviv *et al.* 2019). Infection typically manifests as a localized gastrointestinal disease (enteritis and diarrhea); however, in immunocompromised individuals, the infection can become severe and life-threatening. *Salmonella* is ingested through contaminated food or water and is capable of surviving gastric acidic pH to reach intestinal epithelial cells, the prime targets of invasion (Brumell *et al.* 2001; Knodler 2015; Weber and Faris 2018). Currently, antimicrobial chemotherapy has become less effective due to rapidly increasing multidrug resistance, and both the U.S. Center for Disease Control and the WHO include *Salmonella* among the most serious infectious disease threats for human health (Steeb *et al.* 2013; Liu *et al.* 2023).

Invasion and survival within nonphagocytic intestinal epithelial cells are the crucial steps in *Salmonella* pathogenesis (Santos *et al.* 2015; Zhang *et al.* 2018; Sun *et al.* 2020). First, *Salmonella* attaches to the host cell surface; this process can involve reversible adhesion and irreversible docking *via* the type III secretion system 1 (T3SS-1), which is encoded by *Salmonella* (Misselwitz *et al.* 2011b). After attachment, *Salmonella* can enter cells by caveolin-1 or micropinocytosis-dependent pathways, and T3SS plays an important role in this process (Mallo *et al.* 2008; Lim *et al.* 2014; Teo *et al.* 2016). Among these *Salmonella* effectors, SopE, a G-nucleotide exchange factor for the Rho GTPases Rac1 and Cdc42, is the most potent trigger of actin polymerization and mediates epithelial cell invasion (Hardt *et al.* 1998; Bulgin *et al.* 2010; Misselwitz *et al.* 2011b). Upon internalization into the host cells, *Salmonella* establishes a niche known as a membrane-enclosed intracellular compartment called *Salmonella*-containing vacuole (SCV) that interacts with early endosomes and acquires markers such as Rab4, Rab5, and the early endosomal antigen-1 (EEA-1). Then, the maturing SCV undergoes extensive membrane remodeling and acquires late endosome/lysosomal markers such as Rab7 and lysosomal-associated membrane-associated protein-1 (LAMP-1). The SCV also acquires the vacuolar proton pump V-ATPase that is responsible for the acidification of this compartment (Hernandez *et al.* 2004; Spanò and Galán 2018). For successful invasion and replication inside host cells, the intestinal pathogen *Salmonella* likely exploits or hijacks the host cell machinery or pathways to its own profit. Recently, some host factors required for *Salmonella* invasion have been characterized, including MUC1, Myo1c, myosin II, myosin VI (MYO6), caveolin-1, and Arf GTPase (Brandstaetter *et al.* 2012; Davidson *et al.* 2015; Brooks *et al.* 2017; Li *et al.* 2019). These findings enhanced the understanding of the invasion process of *Salmonella*; however, the details involved in the *Salmonella* invasion process remain largely unknown.

Here, we identified β2 integrin, a crucial member of the integrin family, as an important host cellular factor for *Salmonella* invasion. We found that overexpression of β2 integrin promoted *Salmonella* invasion, and knocking down β2 integrin significantly reduces *Salmonella* invasion. We also found that *Salmonella* could specifically bind to β2 integrin. Antibodies against β2 integrin blocked *Salmonella* infection in cells. Moreover, The ectodomain soluble protein of β2 integrin neutralized *Salmonella* infection both *in vitro* and *in vivo*. More importantly, we confirmed that β2 integrin directly interacted with *Salmonella* protein YrbD. Furthermore, this is the first report that YrbD is a mediator of *Salmonella* adhesion and internalization into HeLa cells. Collectively, our findings suggested that β2 integrin is an important host cellular factor for *Salmonella* invasion.

2. Materials and methods

2.1. Cell culture

Human epithelial cell line HeLa (ATCC CCL-2) was grown in MEM supplemented with 10% FCS, 2 mmol L^{-1} L-glutamine, 1 mmol L^{-1} sodium pyruvate, and 100 U mL^{-1} penicillin-streptomycin (Hyclone, Logan, Utah, USA) at 37°C in a humidified atmosphere containing 5% (v/v) in CO_2. Human embryonic kidney (HEK293) (ATCC CRL-1573) cells and mouse macrophage RAW264.7 cells were maintained in DMEM supplemented with 10% FCS and 100 U mL^{-1} penicillin-streptomycin. Caco-2 cells were maintained in MEM supplemented with 20% FCS and 100 U mL^{-1} penicillin-streptomycin.

2.2. Bacterial strains, growth conditions, and genetic procedures

Salmonella strain SL1344 and its isogenic mutant derivatives were used in the experiments. In some experiments, *Salmonella* SL1344 carrying pM975 was

used, which expresses GFP under a SPI-2 promotor (pssaG). *Escherichia coli* DH5α (Tiangen, Beijing, China) was used for the construction and amplification of plasmids. All bacterial cells were grown in Luria-Bertani (LB) medium (BD, Lake Franklin, NJ, USA) supplemented with the standard antibiotic concentration dependent on the harboring plasmids (e.g., 100 μg mL^{-1} of ampicillin and/or 50 μg mL^{-1} of kanamycin). *Salmonella* SL1344 mutant (Δ*yrbD Salmonella*) and Δ*yrbD*::*yrbD Salmonella* strains were constructed by allelic exchange as previously described (Zhou *et al.* 2001; Liu *et al*. 2025) using plasmids pKD4, pKD46, pCP20 and pBAD30. All deletions were confirmed by PCR and DNA sequencing. For transformation, DNA was introduced into *Salmonella* by electroporation.

2.3. Plasmids, DNA manipulations, and oligonucleotides

The 28 host factors were amplified from total cDNA obtained from HEK293 and inserted into pCAGGS-Myc vector. Bacterial expression plasmid of β2 integrin was synthesized and cloned into pGEX-4T-1 vector. The gene for *yrbD* was amplified from the genome of *Salmonella* SL1344. For expression in mammalian cells, the gene was cloned into pCAGGS-Flag vector. Bacterial expression plasmid was constructed by inserting the gene into a pET-32a vector. All the plasmids were sequenced by Comate Biosciences for verification. All strains, plasmids and primers used in this study were listed in the Appendices A and B.

2.4. siRNA knockdown

To knock down the expression of the β2 integrin gene in HeLa cells, small inhibitory RNAs (siRNAs) (Dharmacon, Lafayette, CO, USA) were used. Briefly, 1×10^4 HeLa cells were seeded into 96-well cell carrier plates (PerkinElmer, Waltham, MA, USA). The following day, the cells were transfected using RNAiMAX (Invitrogen, Waltham, MA, USA) with either negative control siRNA (ON-TARGETplus Non-targeting Pool, D-001810-10-05) or siRNA directed against β2-integrin (ON-TARGETplus Human ITGB2 (3689) siRNA-SMARTpool, L-008010-00-0005) as detailed by the manufacturer. A concentration of 25 nmol L^{-1} of total siRNA was used in each knockdown.

2.5. Quantitative real-time PCR (qRT-PCR)

About 48 h after siRNA transfection, total RNA template was extracted using TRIzol (QIAGEN, Shanghai, China). Approximately 1 μg of total RNA was reverse transcribed into cDNA using a PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). Then, 10 μL of cDNA buffer was used as template for a 20 μL PCR reaction. qRT-PCR analysis was performed using the SYBR Premix *Ex Taq* (TaKaRa) on a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland); *GAPDH* was used as internal control gene.

2.6. Modified gentamycin protection assay

For the overexpression assays, HeLa cells were seeded onto 24-well plates at 1.0×10^5 cells per well. The cells were transfected with 1 μg of plasmids pCAGGS-Myc and pCAGGS-β2 integrin separately with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h of transfection, HeLa cells were infected with GFP-expressing *Salmonella* at multiple of infection (MOI) of 10 for 2 h at 37°C. After that, the extracellular bacteria were killed with 100 μg mL^{-1} of gentamicin for 2 h as above. The results of the overexpression assays were presented in 3 ways. First, the cells were thoroughly washed twice with PBS, and then analyzed using a fluorescence microscope (Carl Zeiss AG, Jena, Germany). Second, the cells were harvested by gentle scraping, and all cellular fluorescence density was measured using an FC500 flow cytometer (Beckman Coulter, Indianapolis, IN, USA). The results were analyzed using FlowJo software (FlowJo LLC, Ashland, OR, USA). Third, for CFU assays, the cells were lysed using 1% Triton X-100 (Sigma-Aldrich, MO, USA) in PBS. Samples were serially diluted in sterile PBS and plated onto LB agar plates with ampicillin. After incubation overnight at 37°C, the number of colonies was determined. siRNA-silenced cells were infected with GFP-expressing *Salmonella* at a multiple of infection (MOI) of 10. At 2 h post-infection, the extracellular bacteria were washed off with 1× Hank's balanced salt solution (HBSS) followed by incubation in HBSS containing 100 μg mL^{-1} of gentamicin to kill any extracellular bacteria for 2 h. Then, the cells were fixed with 4% paraformaldehyde (PFA) and stained with DAPI (Invitrogen) in PBS for 10 min. Stained cells were imaged using the PerkinElmer Operetta high-content system (PerkinElmer, Islington, London, UK). Uninfected cells served as the reference population for background fluorescence. Fifty fields per well were imaged at 20× magnification. Columbus software (PerkinElmer) was used to automatically identify and quantify green fluorescence and cell nuclei. The infection ratio was determined according to the numbers of infected cells versus the total number of cells per well. The assay was independently repeated 3 times.

2.7. Bacterial binding assays

HeLa cells grown on glass coverslips (NEST Biotech, Wuxi, China) placed in 12-well plates were transfected with desired plasmids or siRNA pool for 48 h as earlier described. Subsequently, the cells were washed with HBSS thrice and infected with *Salmonella* and incubated at 4°C for 1 h for the binding assays, leading to the attachment of *Salmonella* to the host-cell membrane rather than entry (de Tymowski *et al.* 2019). Cells were washed with ice-cold PBS thrice and fixed with 4% of PFA, permeabilized with 0.1% Triton X-100, and blocked with 5% bovine serum albumin (BSA) (Amresco, Ladner, PA, USA) in PBS. Then, immunostaining using anti-*Salmonella* antibody (FITC) (Abcam, Cambridge, UK), TRITC-Phalloidin (Sigma-Aldrich, St. Louis, MO, USA), and DAPI was performed. Coverslips were mounted using ProLong Diamond antifade mounting reagent (Life Technologies, Carlsbad, CA, USA). Samples were imaged using a Zeiss LSM880 laser scanning confocal microscope (Carl Zeiss AG) with appropriate filters for fluorescence detection. Since bacteria do not enter the cells, these attached onto the cell surface irregularly. Cells were scanned for about 6 layers using z-stacks (0.44 μm). A total of 24 images were used to assess *Salmonella* binding. The resolution of the acquired image was 1,024×1,024. The data were processed using Zeiss ZEN 2.3 blue edition software (Carl Zeiss AG) to generate images.

2.8. Antibody blocking

Experiments were performed on HeLa, Caco-2 and RAW264.7 cells with a mouse monoclonal antibody (mAb) (Santa Cruz Biotechnology, CA, USA) or a goat polyclonal antibody (pAb) (R&D Systems, CA, USA) against β2 integrin. Mouse IgG2a (Southern Biotech, AL, USA) and goat IgG control (R&D systems, Minneapolis, MN, USA) were used as isotype controls. For blocking studies, cells on 96-well carrier plates were pre-incubated with 0.1 mL of medium containing different concentrations of β2 integrin antibody at 4°C for 1 h before addition of the bacteria. Cells treated with isotype IgG2a or IgG were used as control. After washing with HBSS, GFP-expressing *Salmonella* at MOI of 10 containing the corresponding antibodies were incubated with the cells at 4°C for 1 h. After another round of 3 washes, the cells were again incubated with medium containing the corresponding antibodies at 37°C. At 2 h after incubation, the cells were fixed with 4% PFA and stained with DAPI, and the infection ratio was determined using the PerkinElmer Operetta High-Content System. The relative infection ratio of the antibody-treated groups was calculated according to the normalized isotype control infection ratio.

2.9. Infectivity neutralization assays in cells

The N-terminal GST-tagged soluble ectodomain of β2 integrin (β2 integrin-GST, amino acids 41–450) was expressed and purified by FriendBio Technology (Wuhan, Hubei, China). HeLa, Caco-2 and RAW264.7 cells were seeded onto 96-well carrier plates. *Salmonella* at MOI of 10 was mixed thoroughly with different concentrations of β2 integrin-GST in 0.1 mL of cell culture medium at 4°C for 1 h before being used to infect the cells. The *Salmonella* protein mix was added to the cells and incubated at 37°C. At 2 h post-infection, the extracellular bacteria were killed with 100 μg mL^{-1} of gentamicin for 2 h as earlier described. The cells were fixed with 4% PFA and stained with DAPI. Data were acquired using the PerkinElmer Operetta High-Content System and analyzed using Columbus software. The relative infection ratio was calculated as described above.

2.10. Infectivity neutralization assays in mice

For infectivity neutralization assays, 6- to 8-wk-old female C57BL/6 mice (Vital River, Beijing) maintained under specific pathogen-free conditions were used. Each mouse was inoculated intraperitoneally with 1×10^6 CFU of *Salmonella* SL1344 mixed with β2 integrin-GST (40 μg) or GST (40 μg) in 0.2 mL of PBS. Mice that were intraperitoneally inoculated with 0.2 mL of 40 μg β2 integrin-GST were used as toxicity controls. All mice were observed for 7 d for signs of sickness and death. Survival curves were generated using GraphPad Prism software. The log-rank (Mantel-Cox) test was used to analyze the statistical difference between the survival rates of the challenged mice.

2.11. Immunohistochemical analyses

Six- to 8-wk-old female C57BL/6 mice were used in the infection experiments. Water and food were withdrawn at 4 h before gavage with 20 mg of streptomycin (in 200 μL PBS), and then re-provided to the mice. After 20 h, water and food were withdrawn again for 4 h, then the mice were treated intragastrically (i.g.) with 1×10^8 CFU of *Salmonella* (in 200 μL PBS). Water was offered immediately and food was provided 2 h post infection. Five days post challenge, the infected mice were sacrificed. Tissue samples of caecum were collected and subjected to immunohistochemical analyses. Tissues

were removed and fixed in 10% formaldehyde/PBS (v/v) fixation buffer, dehydrated and embedded in paraffin wax, and sectioned at 1.5 μm thickness. After deparaffinization and rehydration, antigen retrieval was performed by immersing the sections in a citrate acid (pH 7.4)/sodium citrate buffer solution (pH 8.0) at 121°C for 30 min. Endogenous peroxidase activity was quenched by a 3% H_2O_2 methanol solution for 30 min at room temperature (RT). After thorough washing, the sections were blocked with 5% skim milk (Sigma-Aldrich, MO, USA) in PBS for 30 min at RT.

For immunohistochemistry, after incubating with 1:50 diluted rabbit anti-β2 integrin antibody (NBP1-88127, Novus, St. Charles, MO, USA) overnight at 4°C, the sections were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody for 30 min at RT, then rinsed thrice with PBS for 5 min each, stained with diaminobenzidine (DAB) (Sigma-Aldrich), and counterstained with hematoxylin. The sections were then dehydrated using ascending concentrations (70, 95, and 100%) of ethanol, cleared in xylene, and mounted with Entellan mounting medium (Sigma-Aldrich). Staining was assessed under a light microscope.

2.12. OMP extracts and SDS-PAGE

OMPs extraction was performed by means of the N-lauroylsarcosinate (also known as sarkosyl) method with modifications (Ferrer-Navarro *et al.* 2016). Briefly, *Salmonella* cells were harvested and lysed by sonication (3 s^{-1}/3 s^{-1}, 4°C) and collected by centrifugation at 15,000 r min^{-1} for 30 min at 4°C to remove any cell debris. The supernatants were collected and transferred into ultra-centrifugation tubes, and samples were centrifuged at 100,000×g for 1 h at 4°C. After centrifugation, the pellets were resuspended in 1% of freshly prepared sarkosyl solution and incubated for 60 min at RT with gentle agitation. After incubation, the samples were again centrifuged at 100,000×g for 1 h at 4°C, and the resulting pellets were washed twice. Finally, the pellets were carefully resuspended in 800 μL of MilliQ water. The protein concentrations were determined using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA).

2.13. Expression and purification of recombinant proteins

Escherichia coli BL21 (DE3) strain was used for bacterial expression of His-tagged proteins, including YrbD (YrbD-His, amino acids 1–183). To express YrbD-His, the bacterial strain was grown in LB medium at 37 °C until OD_{600}=0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was subsequently added to a final concentration of 0.5 mmol L^{-1}, and the czultures were incubated for further growth at 16°C for 16 h with continuous shaking. This protein was purified as previously described (Dang *et al.* 2016) with modifications.

2.14. Co-immunoprecipitation (Co-IP) and immunoblotting

For Co-IP, plasmid pCAGGS expressing YrbD-Flag (amino acids 1–183), β2 integrin-Myc (amino acids 1–769), Myc-tagged β2 integrin ectodomain (amino acids 1–723, β2 integrin-EX-Myc), β2 integrin I-like domain-Myc (amino acids 101–339), and β2 integrin ΔI-like domain (amino acids 1–100 and 340–769) were separately co-transfected into HEK-293 cells with Lipofectamine 2000. At 48 h post-transfection, the cells were washed with PBS and lysed with ice-cold 1% NP-40 PBS buffer and 1 mmol L^{-1} PMSF (Beyotime, Shanghai, China) for 1 h at 4°C. Then, debris was removed by centrifugation at 13,000 r min^{-1} for 20 min at 4°C. The supernatant was incubated with protein G agarose (Roche Diagnostics GmbH, Mannheim, Germany) at 4°C rotation for 4 h, followed by centrifugation to remove the beads and the non-specifically bound proteins. The supernatant was then collected and mixed with anti-Flag antibody-conjugated agarose beads (Sigma-Aldrich) by constant rotation for 6 h at 4°C. After conjugation, the beads were washed 5 times with pre-chilled 1% NP-40 PBS buffer. Finally, the beads were re-suspended in PBS, and bound protein was eluted using SDS-PAGE loading buffer. Protein samples were separated on 4–20% SDS-PAGE gels, transferred onto nitrocellulose membranes (Pall Corporation, New York, USA), and blocked in 5% skim milk solution at RT for 1 h. Then, these were hybridized with the following primary antibodies at 4°C overnight: anti-Flag mAb and anti-Myc mAb (Proteintech, Wuhan, Hubei, China). After 3 washes with PBST (PBS with 0.1% Tween-20), the membranes were incubated with the corresponding HRP-labeled secondary antibody.

2.15. GST pulldown assay

N-terminal GST-tagged β2 integrin protein ectodomain soluble protein (amino acids 23–700, β2 integrin-GST) was expressed and purified from *E. coli* by FriendBio Technology (Wuhan, Hubei, China). For the GST pulldown assay, 20 μg of purified β2 integrin-GST or GST protein was incubated with Glutathione Sepharose 4B beads (GE Healthcare, MO, USA) for 4 h at 4°C. The

beads were collected and washed thrice and incubated with pCAGGS expressing Flag-tagged YrbD protein for 6 h at 4°C with end to end rotation (or with 5 μg of YrbD-His). Following binding, the beads were washed 5 times, resuspended, and subjected to SDS-PAGE. The samples were followed by Western blotting using an anti-GST mAb and an anti-Flag mAb or an anti-His mAb (Proteintech, Wuhan, Hubei, China).

2.16. Bacterial attachment and invasion assay

The assay was performed as previously described (Misselwitz *et al.* 2011a). Briefly, 1×10^5 HeLa cells were seeded in 24-well plates and allowed to grow until 90% confluency. *Salmonella* was grown overnight, then subcultured (1:25) and grown for 3 h at 37°C. WT *Salmonella*, Δ*yrbD Salmonella*, or Δ*yrbD*::*yrbD Salmonella* were then added at MOI of 10, followed by incubation at 37°C. In the cell attachment assays, the cells were washed 5 times with 1×PBS at the indicated time point then lysed using 1% Triton X-100 in PBS. The samples were then serially diluted in PBS and plated on LB plates to determine the number of viable adherent bacterial cells. To determine *Salmonella* invasion, the unbound bacteria were washed off with PBS followed by incubation in HBSS containing 100 μg mL^{-1} of gentamicin to kill any extracellular bacteria for 1 h. The released intracellular bacteria were expressed as CFUs by plating on LB agar plates.

2.17. Coating of latex beads with proteins and invasion assays

About 1×10^9 latex beads (2.0 μm diameter, amine-modified polystyrene, Millipore, Burlington, MA, USA) were washed and resuspended in PBS. Purified YrbD-His fusion protein (5 μg) or GST was added and adsorbed onto the beads for 3 h at RT. The beads were then washed by PBS for 3 times and stored at 4°C. To determine the coupling efficiency, the beads were checked using immunoblotting. The latex beads coated with YrbD-His fusion protein were added to a near-confluent HeLa cell monolayer in a 6-well plate and incubated for 3 h at 37°C. Then, the unbound beads were removed by washing with PBS.

2.18. Immunoelectron microscopy

For immunoelectron microscopy, HeLa cells and *Salmonella* samples were all prepared using this protocol. Briefly, the samples were fixed with 4% PFA in 0.1 mol L^{-1} HEPES buffer at 4°C for 2 h and rinsed with PBS. Then, the specimens were dehydrated with N, N-dimethylformamide (DMF) at 4°C; 50% DMF, 15 min; 70% DMF, 15 min, 90% DMF, 15 min; and 100% DMF, 30 min. The samples blocks were then infiltrated with DMF and LR-White mixture at ratios of 2:1 and 1:2, respectively, at 4°C for 30 min each. These were successively incubated with pure LR-White for 1 h and with a fresh medium at 4°C overnight. Samples were transferred to fresh LR-White, infiltrated for 2 h at RT, and then polymerized by UV light at –20°C for 10 d.

Ultrathin cryosections, obtained by a Leica microtome, were collected on grids. Next, the sections were washed with PBS and treated with the blocking buffer (3% BSA in PBS) for 30 min at RT. The sections were then incubated with the primary antibodies diluted with the blocking solution for 40 min; the rabbit anti-YrbD antibody was used for detection of designated proteins on the grids. The sections were then washed with PBS and incubated with goat anti-rabbit 5-nm gold conjugates (Sigma-Aldrich) for 40 min. After washing with distilled water, the sections were stained with saturated uranyl acetate aqueous solution for 10 min and examined with a Hitachi H-7650 TEM.

2.19. Statistical analysis

All statistical tests were performed in GraphPad Prism version 7.0 (GraphPad software Inc. San Diego, CA, USA). An unpaired *t*-test was used to analyze differences between 2 groups. The one-way ANOVA was used to compare differences among multiple groups. The log-rank (Mantel-Cox) test was used to analyze the statistical difference between the survival rates of the challenged mice. Differences were considered significant when P-value<0.05.

3. Results

3.1. β2 integrin is essential to *Salmonella* infection

Recently, the genome-scale siRNA screen identified many candidate host factors that mediate *Salmonella* invasion into host cells. However, there are still many host factors contributing to the *Salmonella* invasion that have not yet been characterized (Misselwitz *et al.* 2011a). Here, we used an overexpression assay combined with a *Salmonella* infection strategy to screen 28 potential host factors for *Salmonella* infection based on the published reports (Misselwitz *et al.* 2011a; Hannemann *et al.* 2013). We observed that β2 integrin overexpression facilitates *Salmonella* infection (Appendix C). We then selected β2 integrin for further study. To assess the potential role of β2 integrin in *Salmonella* infection, we overexpressed β2 integrin in HeLa cells, which were then infected with

wild-type *Salmonella* (WT *Salmonella* (pM975); Appendix A). Here, WT *Salmonella* was expressing GFP under an SPI-2 promotor (pssaG). This GFP reporter was not expressed by extracellular bacteria, but strongly induced when *Salmonella* resides within the SCV. The results of overexpression assays were presented in 3 ways. First, the samples were observed using a fluorescence microscope (Fig. 1-A). Next, both flow cytometry measurement and colony-forming unit (CFU) assays verified that *Salmonella* infection was significantly higher in HeLa cells transfected with β2 integrin cDNA than in pCAGGS-Myc transfected cells 48 h after transfection (Fig. 1-B–D). To further verify the role of β2 integrin in *Salmonella* infection, we knocked-down β2 integrin expression by transfecting HeLa cells with siRNA si-β2 integrin, which specifically targets β2 integrin mRNA. Quantitative RT-PCR analysis showed that β2 integrin mRNA expression decreased by 90% at 48 h after transfection (Fig. 1-E). Compared to mock-transfected cells, Salmonella infection decreased by 25% in β2 integrin-silenced HeLa cells (Fig. 1-F and G). Together, these results indicated that β2 integrin is an important host factor for *Salmonella* infection.

3.2. β2 integrin is required for *Salmonella* binding

It is known that the first step of infection for any intracellular pathogen is to bind to the surface of the host targeted cell. Given that β2 integrin is mainly located in the plasma membrane and extracellularly and rarely expressed in other subcellular compartments, and β2 integrin has a relatively long extracellular domain (Streuli 2016; Kechagia *et al.* 2019), these characters prompted us to guess whether β2 integrin could affect the early infection process of *Salmonella*. To this end, β2 integrin-silenced HeLa cells were infected with *Salmonella* and incubated at 4°C for 1 h, leading to the attachment of *Salmonella* to the HeLa cell surface rather than entry of *Salmonella* into the HeLa cells. We found that binding of *Salmonella* to HeLa cells decreased in β2 integrin-silenced HeLa cells compared to mock-transfected cells (Fig. 2-A and B). The results were visualized using a Zeiss LSM880 laser-scanning confocal microscope (Carl Zeiss AG, Jena, Germany). In addition, consistent with this finding, overexpression of β2 integrin by transient transfection also led to an increase in the binding of *Salmonella* to HeLa cells

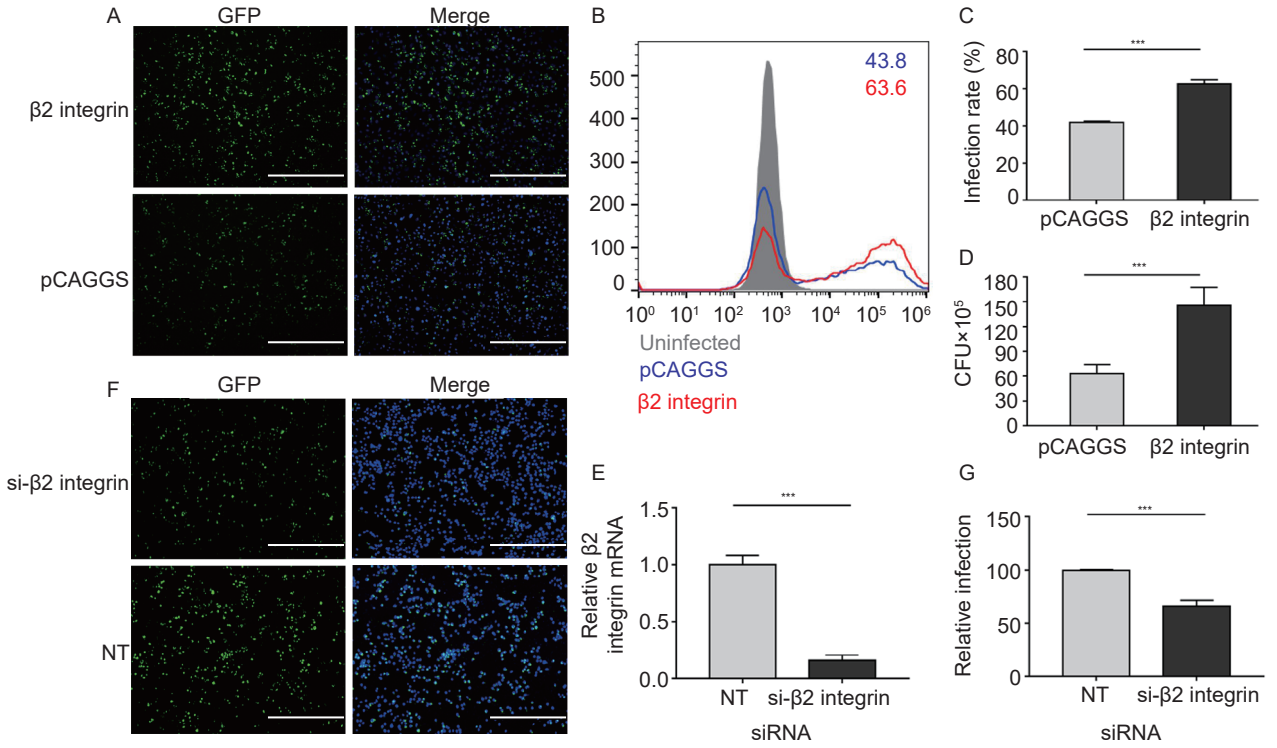


Fig. 1 Overexpression of β2 integrin or siRNA silencing affect *Salmonella* infection. A, overexpression of β2 integrin facilitated *Salmonella* infection in transfected HeLa cells. B and C, flow cytometry measurement. D, CFU assays. E, transfection with siRNA resulted in the downregulation of β2 integrin mRNA in transfected HeLa cells. NT, non-targeting siRNA. F and G, silencing of β2 integrin inhibited *Salmonella* infection of HeLa cells. Data are mean±SD. Unpaired *t*-test was used for the statistical analysis, and a *P*-value<0.05 was considered significant. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001. ns, not significant. Scale bars=400 μm in A and F.

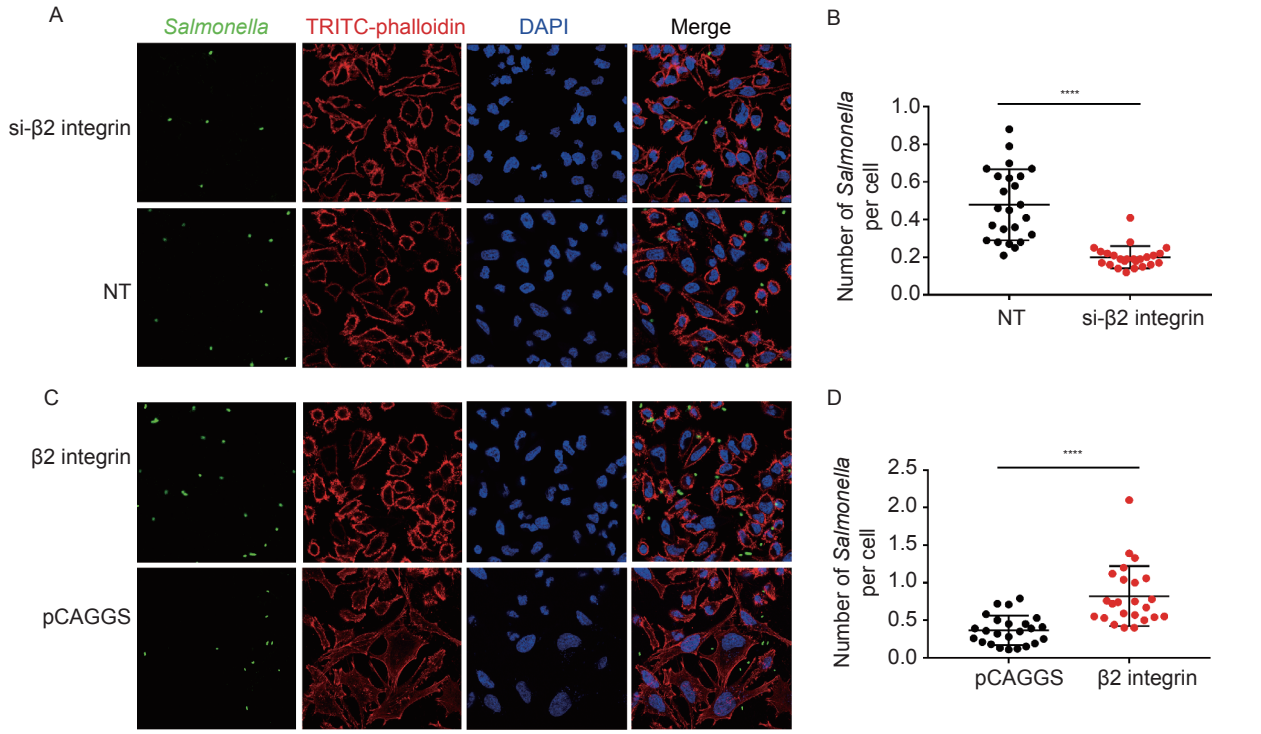


Fig. 2 β2 integrin is required for *Salmonella* binding. A, silencing of β2 integrin inhibited *Salmonella* binding of HeLa cells. HeLa cells were infected with *Salmonella* at 4˚C for 1 h. Cells were stained with anti-*Salmonella* antibody (green), actin-specific TRITC-phalloidin (red), and DAPI (blue). Samples were imaged using a Zeiss LSM880 laser-scanning confocal microscope and processed using Zeiss ZEN 2.3 blue edition software. Each image shown represents a set of 24 images. Presented are the combined z-stacks for each infected cell. B, number of *Salmonella* per cell with silencing of β2 integrin. C, overexpression of β2 integrin increased binding of *Salmonella* to HeLa cells. The samples were stained described as above. D, number of *Salmonella* per cell with overexpression of β2 integrin. Data are mean±SD. Statistical significance was determined using the Mann-Whitney test, and a *P*-value<0.05 was considered significant. ****, *P*<0.0001. ns, not significant. 63× objective lens.

(Fig. 2-C and D). Taken together, our findings verified that β2 integrin plays an essential role in the binding of *Salmonella* to HeLa cells.

3.3. β2 integrin ectodomain is necessary for *Salmonella* infection

Based on the fact that integrins are transmembrane proteins, and β2 integrins affect the binding of *Salmonella* to HeLa cells, we hypothesized that the β2 integrin ectodomain is the key factor that influences *Salmonella* infection. To test this hypothesis, we explored whether antibodies against β2 integrin could block *Salmonella* infection *in vitro*. HeLa cells were pre-incubated with a monoclonal antibody (mAb) or polyclonal antibody (pAb) recognizing β2 integrin ectodomain region to block the β2 integrin on membrane surface. The results showed that in the presence of mAb or pAb, *Salmonella* infection decreased in a dose-dependent manner. The inhibitory effect increased with increasing amounts of antibody (Fig. 3-A–D). The isotype antibodies IgG2a and IgG as control did not impart any inhibitory effect on *Salmonella* infection. To determine whether this effect was specific to HeLa cells, we also tested this in Caco-2 and RAW264.7 cells. The mAb against β2 integrin showed a similar inhibitory effect on *Salmonella* infection of Caco-2 cells (Fig. 3-E and F) and RAW264.7 cells (Fig. 3-G and H). These results further demonstrated that β2 integrin is specifically associated with *Salmonella* infection, and the ectodomain is indeed required for infection.

3.4. β2 integrin ectodomain soluble protein neutralizes *Salmonella* infection of cells and mice

Given that the β2 integrin ectodomain is closely involved in *Salmonella* infection, we investigated whether β2 integrin directly interacts with an unknown protein of *Salmonella*. If β2 integrin directly interacts with *Salmonella*, then disruption of the interaction should inhibit *Salmonella* infection. Thus, we performed neutralization assays *in vitro* using the β2 integrin ectodomain soluble protein. We found that soluble β2 integrin-GST effectively neutralized the *Salmonella* infection in HeLa cells in a dose-dependent manner (Fig. 4-A and B). In comparison, the GST protein did not seem to

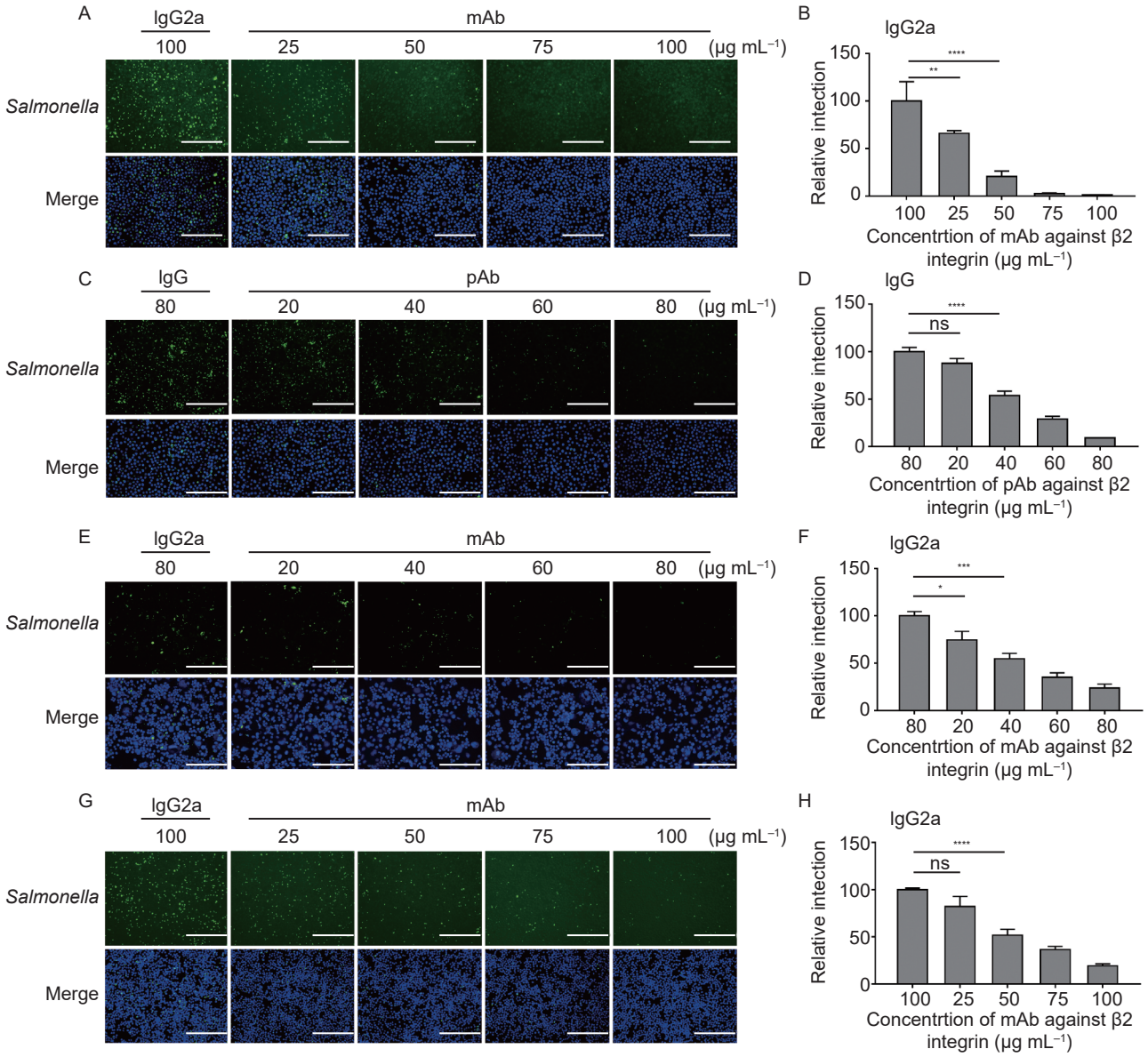


Fig. 3 Antibodies to β2 integrin block *Salmonella* infection of HeLa, Caco-2, and RAW264.7 cells in a dose-dependent manner. A and B, the mAb against β2 integrin blocked the *Salmonella* infection of HeLa cells. The isotype IgG2a at the highest concentration was used as control. C and D, the pAb against β2 integrin blocked the *Salmonella* infection of HeLa cells. The isotype IgG at the highest concentration was used as control. E and F, the mAb against β2 integrin blocked the *Salmonella* infection of Caco-2 cells. G and H, the mAb against β2 integrin blocked the *Salmonella* infection of RAW264.7 cells. Data are mean±SD. One-way ANOVA was used for the statistical analysis, and *P*-value<0.05 was considered significant. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. ns, not significant. Scale bars=200 μm.

impart any neutralizing effect on *Salmonella* infection of HeLa cells. The inhibitory effectiveness of β2 integrin-GST in Caco-2 (Fig. 4-C and D) and RAW264.7 cells (Fig. 4-E and F) were also dose-dependent. Considering the essential role of the β2 integrin ectodomain soluble protein in *Salmonella* infection *in vitro*, we next detected that whether β2 integrin-GST also plays a role *in vivo*. We mixed β2 integrin-GST (200 μg mL^{-1}) with *Salmonella* (1×10^6 CFU) for 2 h with rotation at 4°C, and then inoculated mice with the mixtures by intraperitoneal (i.p.) injection. Mice were observed for 7 d for signs of sickness or death. In contrast, the mice inoculated with β2 integrin-GST group started to die on day 4, and all had died within 5 d (Fig. 5-A), i.e., the β2 integrin-GST group survived for another 48 h compared with the controls. These results showed that β2 integrin-GST can attenuate *Salmonella* virulence *in vivo*. Overall, these results demonstrated that β2 integrin-GST neutralized the *Salmonella* challenge both *in vitro* and

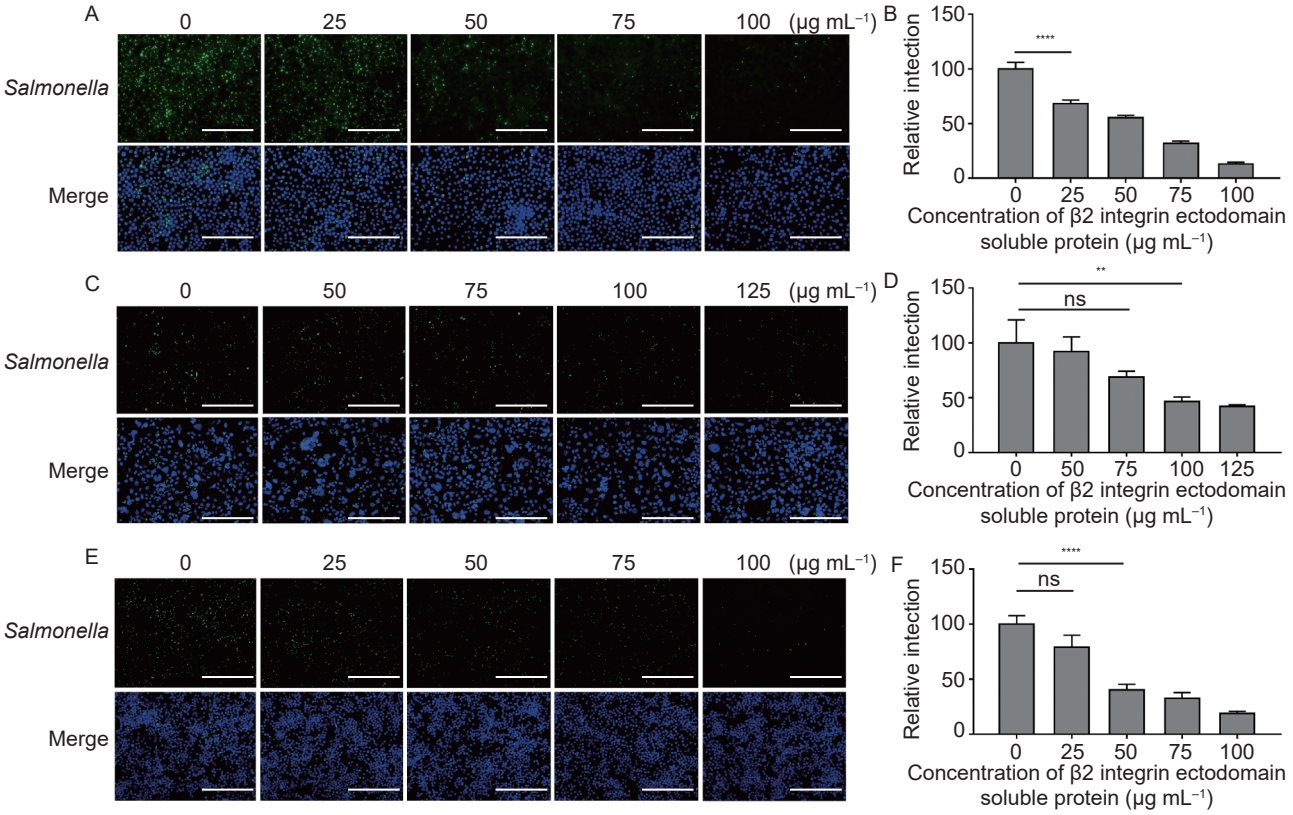
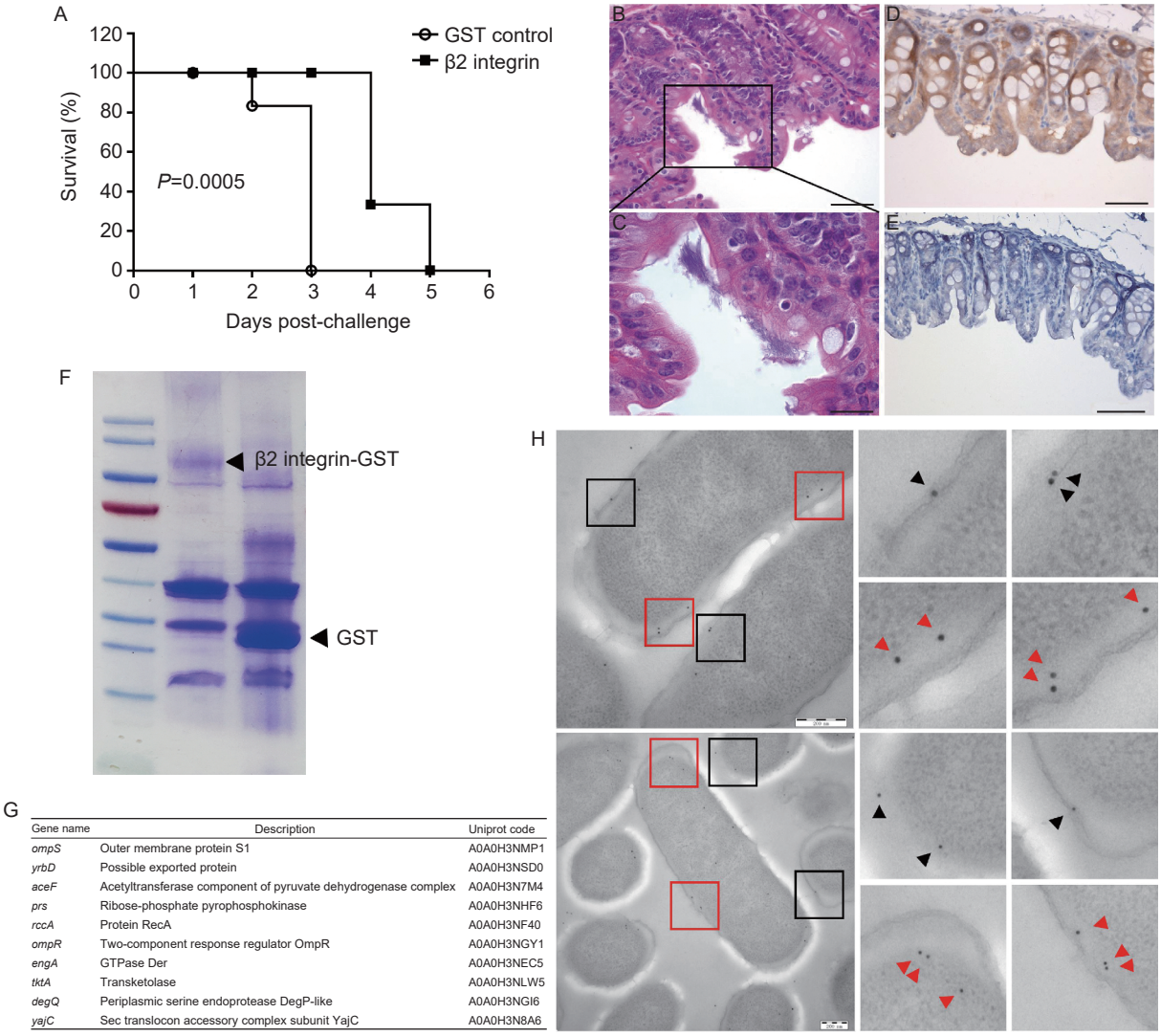


Fig. 4 The β2 integrin ectodomain soluble protein (β2 integrin-GST) neutralizes the *Salmonella* infection in HeLa, Caco-2, and RAW264.7 cells. A and B, β2 integrin-GST neutralizes the *Salmonella* infection in HeLa cells in a dose-dependent manner. C and D, β2 integrin-GST neutralizes the *Salmonella* infection in Caco-2 cells. E and F, β2 integrin-GST neutralizes the *Salmonella* infection in RAW264.7 cells. Data are mean±SD. One-way ANOVA was used for the statistical analysis, and *P*-value<0.05 was considered significant. **, *P*<0.01; ****, *P*<0.0001. ns, not significant. Scale bars=200 μm.

in vivo. These data indicated that β2 integrin probably directly interacts with some particular *Salmonella* proteins, leading to the inhibition of *Salmonella* infection. Integrins are cell surface receptors that are expressed in all cell types except for erythrocytes. To further address the importance of β2 integrin *in vivo*, C57BL/6 mice was intragastrically (i.g.) inoculated with 1×10^8 CFUs of *Salmonella*. Mice were euthanized 5 days post challenge, and caecum samples were subjected to haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) analyses. Representative images of H&E-stained sections clearly showed *Salmonella* invasion of caecum epithelial cells (Fig. 5-B and C). We also observed that β2 integrin was expressed and distributed throughout the caecum (Fig. 5-D). These findings further suggest that β2 integrin expressed in caecum may facilitate the invasion of *Salmonella* into intestinal epithelial cells.

3.5. β2 integrin directly interacts with *Salmonella* YrbD

Next, we tried to identify the *Salmonella* protein interacting with β2 integrin. Outer membrane proteins (OMPs) play an important role in the interaction of bacterial pathogens with host cells. We thus proposed using the GST pull-down assay to determine which *Salmonella* OMPs interact with β2 integrin. First, extraction of *Salmonella* OMPs was performed using the N-lauroylsarcosinate (also known as sarkosyl) (Ferrer-Navarro *et al*. 2016). According to current research, the sarkosyl extraction protocol has been shown to be the most effective and selective method compared to others. Then, we pooled the β2 integrin ectodomain soluble protein (β2 integrin-GST) with OMPs of *Salmonella* for a pull-down assay. Boiled samples were loaded onto a 4–20% SDS-PAGE gel followed by Coomassie staining (Fig. 5-F) for subsequent mass spectrometry analysis (Huada Gene Technology, Shenzhen, China), and approximately 150 differentially expressed proteins were identified (some proteins were shown in Fig. 5-G, and all differentially expressed proteins were shown in Appendix D). Among these proteins that interacted with β2 integrin, we identified the *Salmonella* YrbD protein to be the most interesting based on several reasons. First, studies on the *Salmonella*



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Gene name	Description	Uniprot code
ompS	Outer membrane protein S1	A0A0H3NMP1
yrbD	Possible exported protein	A0A0H3NSD0
aceF	Acetyltransferase component of pyruvate dehydrogenase complex	A0A0H3N7M4
prs	Ribose-phosphate pyrophosphokinase	A0A0H3NHF6
rccA	Protein RecA	A0A0H3NF40
ompR	Two-component response regulator OmpR	A0A0H3NGY1
engA	GTPase Der	A0A0H3NEC5
tktA	Transketolase	A0A0H3NLW5
degQ	Periplasmic serine endoprotease DegP-like	A0A0H3NGI6
yajC	Sec translocon accessory complex subunit YajC	A0A0H3N8A6

Fig. 5 The β2 integrin ectodomain soluble protein neutralizes *Salmonella* infection in mice. A, β2 integrin-GST neutralizes the *Salmonella* infection in mice. The log-rank (Mantel-Cox) test was used to analyze the statistical difference between the survival rates of the challenged mice. B and C, representative images of H&E-stained sections of caecum. D and E, immunohistochemical staining for β2 integrin in caecum of infected mice and uninfected mice (negative control). F, SDS-PAGE analysis of GST pull-down assay. G, some differentially expressed proteins based on mass spectrometry analysis. H, immunoelectron microscopy analysis for YrbD localization at both OM and IM of *Salmonella*. SL1344 was immunolabelled for YrbD with 5-nm gold particles. Black squares represent the labeling of YrbD on the OM of *Salmonella*. Red squares represent the labeling of YrbD on the IM of *Salmonella*. The zoom-in windows of the positive labeling were provided (right). Black arrows and red arrows represent the location of the OM and IM, respectively. Scale bars=200 nm.

YrbD protein are limited. Second, a homologous gene of *yrbD* in *E. coli* is the outer membrane lipid asymmetry maintenance protein MlaD, which forms a ring associated with an ABC transporter complex in the inner membrane (IM). Interestingly, the location of this protein in these two bacterial species varies. Thus, we first performed a localization analysis of YrbD using immunoelectron microscopy. The results confirmed that YrbD localized to both outer membrane (OM) and inner membrane (IM) of *Salmonella* (Fig. 5-H).

To investigate whether β2 integrin interacts with YrbD, we cloned and expressed YrbD in C-terminal Flag-tagged cells. We conducted Co-immunoprecipitation (Co-IP) with β2 integrin and YrbD. Myc-tagged β2 integrin protein (β2 integrin-Myc) was co-expressed with Flag-tagged YrbD in plasmid-transfected HEK293 cells for the Co-IP assay. The results showed that YrbD interacts with the β2 integrin protein in both its dimeric (~50 kDa) and monomeric (~25 kDa) forms. In contrast, β2 integrin did not interact with pCAGGS-Flag control (Fig. 6-A), further demonstrating the specificity of the interaction between β2 integrin and YrbD.

To determine whether β2 integrin directly interacts with YrbD, we pooled the β2 integrin-GST with YrbD-Flag in plasmid-transfected HEK293 cells for a pull-down assay. The results showed that YrbD-Flag was successfully pulled down by β2 integrin-GST but failed to be pulled down by GST (Fig. 6-B), thereby confirming that β2 integrin directly interacts with YrbD. In addition, we also pooled β2 integrin-GST with the purified recombinant His-tagged YrbD derived from the *E. coli* BL21 strain (YrbD-His) for a pull-down assay. Consistently, the YrbD-His protein had both monomer and dimer forms just as Flag-tagged YrbD expressed in eukaryotic cells. We found that

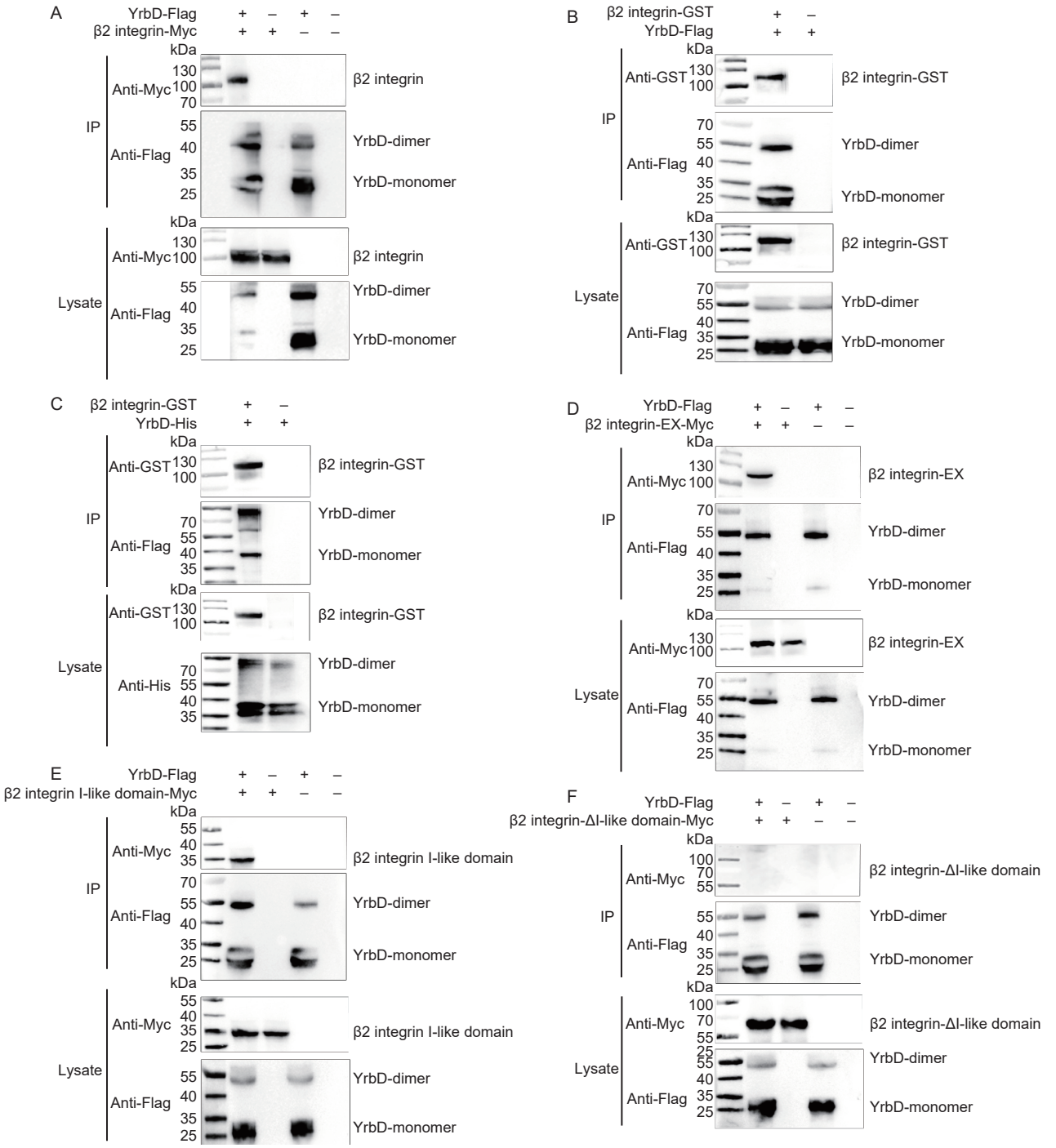

Fig. 6 Interactions between β2 integrin and *Salmonella* YrbD. A, the β2 integrin-Myc interacted with YrbD-Flag in Co-IP assays with plasmid-transfected HEK293 cell lysates. B, purified β2 integrin-GST pulled down YrbD-Flag. C, purified β2 integrin-GST pulled down purified YrbD-His. D, the extracellular domain of β2 integrin interacted with YrbD-Flag. E and F, deletion of β2 integrin I-like domain abolished the interaction between YrbD and β2 integrin.

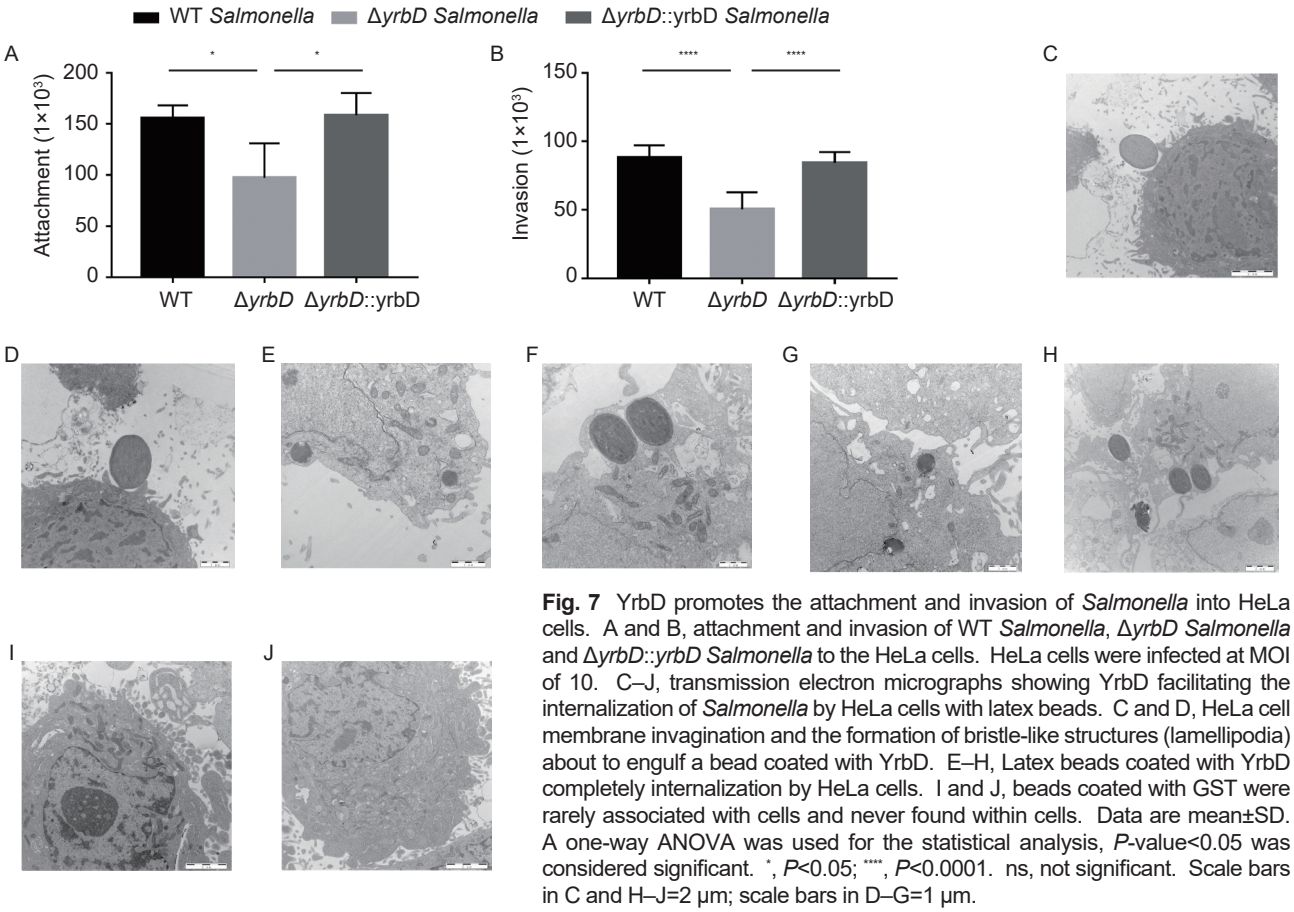

Fig. 7 YrbD promotes the attachment and invasion of *Salmonella* into HeLa cells. A and B, attachment and invasion of WT *Salmonella*, Δ*yrbD Salmonella* and Δ*yrbD*::*yrbD Salmonella* to the HeLa cells. HeLa cells were infected at MOI of 10. C–J, transmission electron micrographs showing YrbD facilitating the internalization of *Salmonella* by HeLa cells with latex beads. C and D, HeLa cell membrane invagination and the formation of bristle-like structures (lamellipodia) about to engulf a bead coated with YrbD. E–H, Latex beads coated with YrbD completely internalization by HeLa cells. I and J, beads coated with GST were rarely associated with cells and never found within cells. Data are mean±SD. A one-way ANOVA was used for the statistical analysis, *P*-value<0.05 was considered significant. *, *P*<0.05; ****, *P*<0.0001. ns, not significant. Scale bars in C and H–J=2 μm; scale bars in D–G=1 μm.

YrbD-His was successfully pulled down by β2 integrin-GST but failed to be pulled down by GST (Fig. 6-C). These results confirmed that β2 integrin directly interacts with *Salmonella* protein YrbD.

The typical domain of β2 integrin is divided into 3 parts: the extracellular region, the transmembrane region, and the intracellular region. We were particularly interested in which domain of the β2 integrin interacts with the YrbD. We constructed the corresponding recombinant plasmid, and the Co-IP result showed that the extracellular domain interacted with the YrbD (Fig. 6-D). As the I domain in the α subunit is implicated in ligand binding, the β2 integrin also possesses an extracellular I-like domain. We thus postulated that binding of YrbD to β2 integrin is attributable to the I-like domain. To test this hypothesis, Co-IP experiments were conducted, and the results showed that the I-like domain of β2 integrin alone was efficient in interacting with YrbD *in vitro*, and deletion of β2 integrin I-like domain completely abolished the interaction between YrbD and β2 integrin (Fig. 6-E and F). Taken together, these data clearly demonstrated that β2 integrin interacts directly with *Salmonella* YrbD, and the I-like domain is necessary for the interaction.

3.6. *Salmonella* YrbD promotes the attachment and invasion of *Salmonella* into HeLa cells

The role of YrbD in *Salmonella* is largely unknown. The invasion of host cells is a key step of *Salmonella* infections. We evaluated whether YrbD contributes to *Salmonella* to attach to and/or invade HeLa epithelial cells. First, *yrbD*-deletion (Δ*yrbD Salmonella*) and *yrbD*-complementary (Δ*yrbD*::*yrbD Salmonella*) strains were generated. A gentamicin protection assay revealed that the Δ*yrbD Salmonella* significantly reduced bacterial association and invasion to HeLa cells compared to WT *Salmonella*, and Δ*yrbD*::*yrbD Salmonella* restored the phenotype (Fig. 7-A and B). These findings indicated that YrbD contributes to *Salmonella* invasion of epithelial cells.

The effect of YrbD on *Salmonella* adherence and invasion was further confirmed using latex beads. We incubated HeLa cells with latex beads coated with GST protein or purified YrbD protein. Transmission electron microscopy confirmed that the latex beads coated with purified YrbD were taken up and internalized by non-phagocytic HeLa cells (Fig. 7-C–H). This entry was specific, as beads coated with GST were rarely associated

with cells and never found within cells (Fig. 7-I and J). In addition, when the latex beads coated with YrbD, pedestal-like structures and membrane invaginations were observed on the plasma membrane of HeLa cells, which were possibly formed by the elongation of microvilli surrounding the beads (Fig. 7-C and D). Furthermore, the uptake and complete internalization of beads coated with YrbD was also observed in HeLa cells (Fig. 7-E–H). These data showed that YrbD facilitated the uptake and internalization of latex beads by HeLa cells. These findings also showed, for the first time, the role of YrbD in facilitating the internalization of *Salmonella* by mammalian cells. Thus, our data indicated that the *Salmonella* membrane protein YrbD is sufficient to mediate *Salmonella* adherence to and invasion of HeLa cells.

4. Discussion

The microbial world is a competitive place, and bacterial pathogens have evolved sophisticated strategies to drive infection and to create a suitable niche for their survival and proliferation. It is known that pathogenic bacteria and their hosts are tightly associated with each another. Interactions between bacterial ligands and host cell factors are critical steps in the pathogenesis of bacterial infection. Identification of novel host cell factors may help elucidate the pathogenesis and also provide targets for the prevention and/or treatment of bacterial infections (Colonne *et al.* 2016; Yu *et al.* 2016; Tanner and Kingsley 2018). So far, the research on the pathogenesis of *Salmonella* has already made tremendous progress. Nevertheless, the mechanisms for *Salmonella* entry host cells remain unclear. Which host factors are involved in *Salmonella* entry host cells and whether a receptor takes part in the process of *Salmonella* invasion remain largely unknown. This study has shown that β2 integrin, a member of the integrin family, is an important host cellular factor for *Salmonella* invasion.

Integrins belong to the family of transmembrane cell surface molecules that mediate cell–cell, cell–extracellular matrix, and cell–pathogen interactions. In mammals, stable non-covalent interactions between 18 α-subunits and 8 β-subunits generate 24 functionally distinct integrin heterodimers. As a heterodimeric transmembrane receptor, β2 integrin may link with different a-subunits, such as CD11a (to generate LFA-1), CD11b (to produce Mac-1), CD11c, or CD11d (del Pozo *et al.* 2005; Gu *et al.* 2011). Here, we focused on β2 integrin and did not study the role of different α partners. Although previous studies have mostly focused on the immunological role of β2 integrin, recently, β2 integrin has been proposed to facilitate multi-bacterial infection of multiple bacteria. Sewald *et al.* (2008) demonstrated that β2 integrin is the T-lymphocyte receptor for the Helicobacter pylori vacuolating cytotoxin (Sewald *et al.* 2008). The extracellular domain of β2 integrin is sufficient for *E. coli* hemolysin and *Aggregatibacter actinomycetemcomitans* leukotoxin cytotoxic activity (Ristow *et al.* 2019). In addition, *Salmonella* has been shown to both colonize Peyer's patches *via* M cells and independently disseminate to extraintestinal sites *via* CD18-expressing phagocytes. Hence, the host can actively translocate enteric pathogens across the epithelium *via* CD18-expressing phagocytes (Vazquez-Torres *et al.* 1999). In this study, we showed that antibodies against β2 integrin could block *Salmonella* infection in HeLa, Caco-2 and RAW264.7 cells. Moreover, the β2 integrin ectodomain soluble protein neutralized *Salmonella* infection both in cells and mice. These findings indicated that the function of β2 integrin is similar to a receptor of *Salmonella*.

It is now clear that integrins transmit bidirectional signals across the plasma membrane, and ligand binding transduces signals from the extracellular domain to the cytoplasm in the classical outside-in direction. Furthermore, the β subunit of the integrin possesses an extracellular I-like domain specifying ligand binding (Margadant *et al.* 2011). Our results showed that the I-like domain of the β2 integrin ectodomain is necessary for interacting with YrbD (Fig. 6-E). We thus proposed that *Salmonella* interacting with the ectodomain of β2 integrin transmits signals that further promote *Salmonella* invasion. However, this question requires further investigation. Notably, integrins are constantly trafficked into cells through clathrin- or caveolin-mediated endocytosis or macropinocytosis routes, which then undergo endosomal sorting that determines degradation or recycled back to the plasma membrane (Bridgewater *et al.* 2012; Paul *et al.* 2015). Similarly, *Salmonella* enters the host cells *via* macropinocytosis or caveolae-mediated invasion (Lim *et al.* 2009; Liebl *et al.* 2017). Whether β2 integrin is internalized with *Salmonella* together or β2 integrin participates in SCV trafficking requires further investigation.

Pull-down assays were used to identify protein interactions with β2 integrin. Then, subsequent mass spectrometry identified about 150 differentially expressed proteins. We selected YrbD for further investigation. Many *Salmonella* effectors have been identified and elucidated to date, but mechanisms responsible for *Salmonella* invasion remain unclear. Hundreds of *Salmonella* proteins have their function annotated as "hypothetical", as they have never been experimentally characterized, including YrbD. We conducted immunoelectron microscopy analysis, which confirmed that YrbD localizes to both the OM and IM of *Salmonella*. Although YrbD expression is relatively

low in the OM, this does not automatically indicate that this protein does not have an important function, similar to G-protein-coupled receptors (GPCRs) that also exhibit low expression levels, yet these have crucial roles (Marchese *et al.* 2008; Colosimo *et al.* 2019). In addition, we demonstrated that β2 integrin directly interacts with YrbD. We also found that YrbD has cell-adhesive properties, and gentamicin protection experiments have demonstrated that YrbD is associated with *Salmonella* invasion. Importantly, transmission electron microscopy confirmed the uptake and internalization of latex beads coated with YrbD by non-phagocytic HeLa cells. For the first time, we have demonstrated the role of YrbD in facilitating the internalization of *Salmonella* by mammalian cells. Thus, our results suggested that the *Salmonella* surface protein YrbD is sufficient to mediate *Salmonella* adherence to and invasion of HeLa cells. Whether YrbD also has other functions remains unclear, and further studies of YrbD are warranted.

Given that β2 integrin-GST can neutralize the *Salmonella* challenge *in vivo*, but not induce complete abolition of *Salmonella* infection suggests that there were additional yet undefined factors in bacteria and host cells that mediate the *Salmonella* infection. As shown in Table 1 shows that there are multiple bacterial ligands that interact with β2 integrin, although in this study, only YrbD protein was selected for detailed study. Therefore, we cannot rule out the possibility that multiple bacterial ligands are involved with β2 integrin to facilitate *Salmonella* invasion. We also cannot exclude the possibility that *Salmonella* exploits multiple cellular membrane proteins (other than β2 integrin) in infecting cells *in vivo*. Several host factors have been reported to date (Brandstaetter *et al.* 2012; Davidson *et al.* 2015; Brooks *et al.* 2017; Li *et al.* 2019).

Indeed, *Salmonella* continue to be an important cause of the foodborne illness. Antimicrobial resistance is currently a global public health concern, and *Salmonella* has developed resistant serotypes. Unfortunately, current treatments for *Salmonella* infections are not sufficiently effective, partially due to the emergence of new multidrug-resistant strains (Angelo *et al.* 2016; Xie *et al.* 2022; Cuypers *et al.* 2023). It is important to point out that the present work focuses on the first stage of the infection, i.e., *Salmonella* entry into host cells. The underlying mechanisms on that are not completely understood. Therefore, we speculate that host factors that participate in *Salmonella* invasion may serve as potential therapeutic targets in treating *Salmonella* infections. Identifying such host factors could transform therapy and prevention. Importantly, these factors do not pose a problem of antimicrobial resistance. The present study has determined that disruption of the interaction between β2 integrin and *Salmonella* prolongs the survival of mice challenged with *Salmonella*. Clearly, our results showed that β2 integrin is a potential target for drug development. Given the importance of β2 integrin in *Salmonella* infections, it is essential to improve therapies against *Salmonella* and other pathogenic bacterial pathogens, it is important to note that the host factors involved in the invasion process should be paid more attention. *Salmonella* is an ancient successful intracellular pathogen that causes infections. Many questions remain to be addressed such as whether β2 integrin is internalized by cells together with *Salmonella*, how does β2 integrin participate in SCV vesicular trafficking in the host, and is β2 integrin detached from *Salmonella* and recycled back to the plasma membrane? The answers to these questions will improve our understanding of *Salmonella* pathogenesis. Our findings presented here open new doors for elucidating the fundamental molecular mechanism of *Salmonella* pathogenesis.

5. Conclusion

In this study, we revealed that *Salmonella* protein YrbD mediates invasion into host cells by interacting with β2 integrin. Overexpression of β2 integrin promoted *Salmonella* invasion, and either knocking down expression of β2 integrin or blocking β2 integrin on the cell surface significantly reduced the invasion. The ectodomain soluble protein of β2 integrin neutralized *Salmonella* infection both in cells (*in vitro*) and in mice (*in vivo*), and β2 integrin directly interacted with *Salmonella* protein YrbD. These findings not only expand our understanding of *Salmonella* entry mechanisms but also identify a potential target to therapeutically control it.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Ethical approval

All animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. The protocols were reviewed and approved by the Committee on the Ethics of Animal Experiments of Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences (CAAS). Mouse challenge experiments with *Salmonella* were conducted within animal biosafety level 2 facilities at Harbin Veterinary Research Institute (approval number IACUC-2017-197).

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2023.12.035

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