Proteoglycan-Dependent Endo-Lysosomal Fusion Affects Intracellular Survival of Salmonella Typhimurium in Epithelial Cells

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Proteoglycans (PGs) are glycoconjugates which are predominantly expressed on cell surfaces and consist of glycosaminoglycans (GAGs) linked to a core protein. An initial step of GAGs assembly is governed by the β-D-xylosyltransferase enzymes encoded in mammals by the XylT1/XylT2 genes. PGs are essential for the interaction of a cell with other cells as well as with the extracellular matrix. A number of studies highlighted a role of PGs in bacterial adhesion, invasion, and immune response. In this work, we investigated a role of PGs in Salmonella enterica serovar Typhimurium (S. Typhimurium) infection of epithelial cells. Gentamicin protection and chloroquine resistance assays were applied to assess invasion and replication of S. Typhimurium in wild-type and xylosyltransferase-deficient (ΔXylT2) Chinese hamster ovary (CHO) cells lacking PGs. We found that S. Typhimurium adheres to and invades CHO WT and CHO ΔXylT2 cells at comparable levels. However, 24 h after infection, proteoglycan-deficient CHO ΔXylT2 cells are significantly less colonized by S. Typhimurium compared to CHO WT cells. This proteoglycan-dependent phenotype could be rescued by addition of PGs to the cell culture medium, as well as by complementation of the XylT2 gene. Chloroquine resistance assay and immunostaining revealed that in the absence of PGs, significantly less bacteria are associated with Salmonella-containing vacuoles (SCVs) due to a re-distribution of endocytosed gentamicin. Inhibition of endo-lysosomal fusion by a specific inhibitor of phosphatidylinositol phosphate kinase PIKfyve significantly increased S. Typhimurium burden in CHO ΔXylT2 cells demonstrating an important role of PGs for PIKfyve dependent vesicle fusion which is modulated by Salmonella to establish infection. Overall, our results demonstrate that PGs influence survival of intracellular Salmonella in epithelial cells via modulation of PIKfyve-dependent endo-lysosomal fusion.

Keywords: Salmonella, proteoglycans, glycosaminoglycans, xylosyltransferase, PIKfyve, gentamicin
INTRODUCTION

Proteoglycans (PGs) are heavily glycosylated proteins facilitating cell-matrix and cell-cell interactions and are also playing an important role in bacterial adhesion, invasion, and immune response (1). All PGs consist of a core protein substituted with glycosaminoglycans (GAGs) – long linear polysaccharides comprised of repeating disaccharide units. Based on the structure with glycosaminoglycans (GAGs) – long linear polysaccharides important role in bacterial adhesion, invasion, and immune cell-matrix and cell-cell interactions and are also playing an important role in mammalian cells (21). While it is known that Salmonella can modulate phosphoinositide pathways in host cells (22, 23), limited knowledge exists on possible interactions of phosphoinositides with PGs.

To investigate the contribution of surface and intracellular PGs to Salmonella infection we utilized a proteoglycan-deficient Chinese hamster ovary (CHO) cell line (3). We demonstrate that absence of PGs in epithelial CHO cells results in an altered PIKfyve-dependent endo-lysosomal trafficking affecting intracellular Salmonella survival.

MATERIALS AND METHODS

Cell Lines

Chinese hamster ovary (CHO) CHO-K1 WT and CHO-K1 pgsA745 (aka ΔXylT2, referred to as ΔXylT) (3) cells were routinely cultured in DMEM/F-12 GlutaMAX growth medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Biochrom).

CHO ΔXylT Cell Line Complementation

CHO ΔXylT mutant was complemented with a plasmid expressing human XYLTI as described (24). Complementation of a G418-selected clone was confirmed by flow cytometry using the heparan sulfate-specific phage display antibody AO4B08 (25). The overlay histograms with the peak heights were normalized to mode (% of Max). The data were analyzed using FlowJo v.10 software (TreeStar).

Bacteria and Growth Conditions

Salmonella enterica serovar Typhimurium (S. Typhimurium) 14028s (26), S. Typhimurium SL1344 WT (27), S. Typhimurium SL1344 eGFP (pFPV25.1) (28), S. Typhimurium SL1344 ΔssaR (29), and S. Typhimurium SL1344 ΔsiF (30) were grown overnight at 37°C with shaking in lysogeny broth (LB) supplemented with 100 µg/mL streptomycin, 100 µg/mL ampicillin, or 50 µg/mL kanamycin, when appropriate. The reporter strain S. Typhimurium SL1344 p4889 (31) was grown in presence of carbenicillin 50 µg/mL. Listeria monocytogenes EGD strain (32) was grown at 37°C in Brain Hearth Infusion (BHI) broth. For infection, overnight cultures of bacteria were sub-cultured and grown for 3 h at 37°C to mid-log phase.

Generation of Acid Shock Reporter Plasmid

The acid shock-responsive promoter of asr was used to control expression of sfGFP. A dual fluorescence reporter was generated based on p4889, and P_{asr}T in p4889 was replaced by P_{asr} by Gibson assembly (GA) cloning. Primers VF-p4889 and VR-p4889 were used to PCR amplify the vector backbone of p4889, and 1f p4889-Pasr and 1r Pasr-sfGFP were used to amplify the P_{asr} region from genomic DNA of S. Typhimurium. GA resulted
in plasmid p5386 that was confirmed by DNA sequencing, and functional analyses of response of sgFP expression upon acid shock exposure in synthetic media buffered to various pH.

**Gentamicin Protection Assay**

CHO WT, CHO ΔXylT, or complemented CHO ΔXylT cells (CHO cx) were seeded in 24-well plates (10^5 cells/well) and incubated overnight in 5% CO₂ at 37°C. The next day, cells were infected with either *Listeria monocytogenes* EGD, or with different *Salmonella* strains at MOI 10, 50, or 100 (as indicated). For quantification of adherent bacteria, 30 min (or 60 min for *Listeria*) post infection (p.i.), cells were washed 3 times with PBS and lysed in PBS containing 1% (v/v) Triton X-100 and 0.1% (v/v) sodium dodecyl sulfate (SDS). The cell lysates were then serially diluted in PBS and plated on LB agar or on BHI agar for colony-forming unit (CFU) count. For later time points, upon washing, culture medium was replaced with a medium supplemented with 100 µg/mL gentamicin (Sigma) to kill extracellular bacteria. The number of invaded bacteria was determined by plating the cells lysates 1.5 h p.i. (2.5 h p.i. for *Listeria*). Medium was replaced by medium containing the indicated concentrations of gentamicin and bacterial intracellular survival or replication was assessed 4 and 24 h p.i.

**Gene Expression Analysis**

Total RNA was extracted from CHO cells using the High Pure RNA Isolation Kit (Roche) following the manufacturer’s guidelines. Reverse transcription of 1 µg RNA of each sample was done with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was assessed by qPCR using the Power SYBR® Green PCR Master Mix (Applied Biosystems) using gene specific primers (Supplementary Table S1). Relative gene expression was calculated by the ΔΔCt method (33) and normalized to Gapdh and Rps9 housekeeping genes.

**Gentamicin ELISA**

To determine levels of intracellular gentamicin, CHO WT and CHO ΔXylT cells were washed four times with PBS and lysed. The concentration of gentamicin in cell lysates was measured using the GEN ELISA Kit (Cusabio) according to the manufacturer’s protocol.

**Gentamicin Cy3 Conjugation and Cell Labeling Experiments**

Gentamicin sulfate salt (Sigma-Aldrich) was mixed with the Sulfo-Cyanine3 NHS ester (Lumiprobe) in 50:1 molar ratio and incubated for 1 h at room temperature. The conjugate (Gen-Cy3) was isolated by reversed-phase chromatography (column C18), aliquoted, dried, and stored in the dark at -20°C. Prior to usage, the conjugate was resuspended in sterile water, absorbance at 548 nm was measured, and a concentration was calculated using the molar attenuation coefficient of the Sulfo-Cyanine3 NHS ester. In the cell labeling experiments, gentamicin sulfate used in a protection assay was replaced with Cy3-conjugated gentamicin (GEN-Cy3) at the indicated concentrations. CHO WT and CHO ΔXylT cells incubated with GEN-Cy3 were fixed at different time points post infection.

CHO WT and CHO ΔXylT cells were seeded on coverslips and then infected with *S. Typhimurium* eGFP at an MOI of 50. Upon bacterial invasion, CHO cells were incubated for 2, 7, or 24 h in presence of 50 nM Lysotracker Red DND-99 (Sigma-Aldrich). Then, CHO cells were extensively washed with PBS, fixed with 4% paraformaldehyde (PFA) and stained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) (1:1000) to visualize nuclei. Images were recorded on a Zeiss Apotome.2 microscope using Axiovision 4.9.1 software (Zeiss).

**Immunocytochemistry**

CHO WT and CHO ΔXylT cells were seeded on coverslips in 24 well plates, fixed with 4% PFA, washed 3 times with PBS, and permeabilized with Triton-X100 (0.1%). Unspecific binding was blocked using 2% normal goat serum (NGS), cells were then incubated with the A04B08 antibody (25) (1:100) recognizing both heparin and HS. Infected cells were additionally stained with rabbit anti-*Salmonella* antibody (1:100). Upon washing, bound A04B08 antibodies were detected by incubation with mouse anti-VSV tag IgG antibody P5D5 (1:400), followed by Alexa 488-conjugated goat anti-mouse IgG (1:1000), Alexa 568-conjugated goat anti-rabbit IgG (1:1000) (Thermo Fisher Scientific®). Phalloidin-iFluor 647 (1:1000) (Abcam) and DAPI (Invitrogen) were applied to visualize F-actin and nuclei, respectively. For a list of antibodies used see Supplementary Table S2.

**Chloroquine Resistance Assay**

To determine the number of cytosolic *S. Typhimurium* within the CHO cells, chloroquine (CHQ) resistance assay was performed as described (34). Briefly, CHO WT and CHO ΔXylT cells were infected as described above. 24 h p.i., the cells were incubated for 1 h in the presence of CHQ (400 µM) and gentamicin (cytosolic bacteria) or with gentamicin only (total intracellular bacteria). CHO cells were then lysed, and serial dilutions plated on LB agar plates.

**Infection With *S. Typhimurium* Reporter Strains**

CHO WT and CHO ΔXylT cells seeded on coverslips were infected with *S. Typhimurium* p4889 reporter strain at MOI 100. Upon bacterial invasion, CHO medium was supplemented with 100 µg/mL gentamicin. 24 h p.i., the infected cells and uninfected controls were fixed with 4% PFA, and then incubated with DAPI (1:1000). Images were recorded with a Zeiss Apotome.2 microscope using Axiovision 4.9.1 software (Zeiss). Bacteria in cytoplasm and in *Salmonella*-containing vacuole were enumerated in 20 random fields of view (FOV) using the Fiji software (35).

To test exposure of intracellular bacteria to acidic endosomal environments, CHO WT and CHO ΔXylT were infected with *S. Typhimurium* harboring p5386 at MOI of 10. Cells were incubated with or without gentamicin as indicated. CHO cells were detached 2 h after infection, chloramphenicol was added
in a final concentration of 200 µg/mL to stop further bacterial protein biosynthesis, and incubation was continued at 4°C in order to allow full maturation of all synthesized sfGFP molecules. To control the effect of host cell endosomal acidification on intracellular S. Typhimurium, acidification was abrogated by vATPase inhibitor bafilomycin added after infection in a final concentration of 100 nM. For quantification, at least 50,000 CHO cells were analyzed by flow cytometry on an Attune NxT (Thermo Fisher) instrument. Cells were gated for DsRed-positive, i.e., S. Typhimurium-infected population, and sfGFP fluorescence of this population was determined as proxy for the level of acidification.

Transfection
10^6 CHO WT and CHO ΔXylT cells were resuspended in 100 µl Nucleofector Solution and mixed with 5 µg of p4605 plasmid encoding human ARL8B (ADP Ribosylation Factor Like GTPase 8B) (36) and transfected using the Nucleofector Program U-027 (Lonza). Immediately after transfection, cells were resuspended in CHO medium, seeded onto cover slips in 24 well plates, and incubated for at least 18 h prior to infection.

Antibody Uptake Assay
CHO WT and ΔXylT cells seeded on coverslips were infected with S. Typhimurium WT or with S. Typhimurium WT eGFP at an MOI of 50. 1.5 h p.i., medium was replaced with medium containing 10 µg/mL gentamicin and rabbit anti-Salmonella antibody (Difco). 24 h p.i., cells were fixed with 4% PFA, and bacteria were stained with the Alexa 546-conjugated donkey anti-rabbit IgG. In case of WT bacteria, samples were additionally stained with mouse anti-Salmonella antibody (Meridian Life Science) and Alexa 488-conjugated donkey anti-mouse IgG secondary antibody (Thermo Fisher Scientific). Bacteria were enumerated in 20 random FOVs.

Endo-Lysosomal Fusion Assay
CHO WT and CHO ΔXylT cells were seeded on coverslips for pulse-chase experiments. In brief, CHO cells were first incubated with dextran-Alexa568 (10,000 MW, 0.4 mg/mL) (Invitrogen) for 4 h, washed and incubated in dextran-free CHO medium for 18 h. Cells were then pulsed with dextran-Alexa568 (10,000 MW, 0.4 mg/mL) for 10 min, washed and incubated in CHO medium for 30 min. CHO WT and CHO ΔXylT cells were fixed with 4% PFA, and stained with phalloidin-iFluo 647 Reagent (1:1000) (Abcam) and DAPI. Images of 20 random FOVs were acquired on Zeiss Apotome.2 microscope with 63 × oil immersion objective using AxioVision 4.9.1 software (Zeiss). Spatial resolution of images was 9,7674 pixels per micron, pixel size: 0.1024 × 0.1024 micron^2. Endo-lysosomal fusion was scored by quantifying colocalization between the two labeled dextrans using ImageJ version 1.52e and JACoP plugin for pixel intensity spatial correlation analysis (37). Pearson’s correlation coefficient and Manders split coefficients (M1 and M2, thresholds set manually for both channels) were calculated.

Cytotoxicity Assay
CHO cells were infected with S. Typhimurium WT strain as described previously. 24 h p.i., supernatants were collected and an activity of the lactate dehydrogenase (LDH) was measured using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) following the manufacturer’s instructions.

Statistical Analysis
Data were analyzed using Prism V7.0d software (GraphPad). Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, or Dunnett’s multiple comparison test, the Kruskal-Wallis test followed by Dunn’s multiple comparison test, unpaired two-tailed t-test, or Wilcoxon-Mann-Whitney test as indicated. The results were considered statistically significant when p-values were smaller than 0.033. Graphs display the mean values ± SD and represent three independent biological repetitions unless stated otherwise.

RESULTS
Proteoglycans Are Crucial for Salmonella Survival Within CHO Cells
Proteoglycans were shown to contribute to Salmonella invasion via interaction with the bacterial adhesin PagN (7). However, PagN is only expressed under intracellular (SPI2)-inducing conditions and when PagN is not expressed, invasion of host cells is PG-independent. Furthermore, it is not known if PGs are important for intracellular survival or replication. To test this, CHO WT and proteoglycan-deficient CHO ΔXylT cells were infected with S. Typhimurium WT strains. Bacterial adhesion (30 min p.i.), invasion (1.5 h p.i.), and early replication (4 h p.i.) were comparable between CHO WT and CHO ΔXylT cells. However, 24 h p.i., we detected a significant reduction of intracellular bacteria in CHO ΔXylT compared to CHO WT cells incubated in presence of 100 µg/mL gentamicin (Figure 1A). Gentamicin-mediated killing of S. Typhimurium 14028S and SL1344 strains CHO ΔXylT cells was dose-dependent (Figure 1B). In contrast, when ampicillin was applied instead of gentamicin, a dose-dependent reduction of bacterial intracellular numbers was detected in the both CHO WT and CHO ΔXylT cells infected with S. Typhimurium 14028S at 24 p.i. (Supplementary Figure S1). To determine whether the effect on intracellular survival is Salmonella-specific, we infected CHO WT and CHO ΔXylT cells with another intracellular pathogen, Listeria monocytogenes. In contrast to Salmonella, reduction of intracellular Listeria was dependent on the gentamicin dose but not dependent on the presence of proteoglycans (Figure 1C).

Next, we asked whether proteoglycan can affect the uptake of gentamicin into CHO cells as increased uptake of gentamicin by CHO ΔXylT cells could contribute to increased killing of intracellular Salmonella. CHO cells were infected with S. Typhimurium WT as described above and intracellular gentamicin concentrations were measured by ELISA. No differences in intracellular gentamicin levels were detected.
Proteoglycans influence survival of intracellular S. Typhimurium. (A) CHO WT and CHO ΔXYLT cells were infected with S. Typhimurium WT at MOI 10 for 30 min, washed three times with PBS and then lysed to assess adherence. Remaining wells were incubated for 1 h with 100 µg/mL gentamicin to kill extracellular bacteria, and then either lysed immediately to evaluate invasion or at various time points post infection (p.i.) following incubation with media supplemented with 100 µg/mL gentamicin, as indicated. Lysates were collected, serially diluted and plated on agar plates. CHO ΔXYLT (open circles), CHO WT cells (closed circles).

(B) CHO WT and CHO ΔXYLT cells were infected with S. Typhimurium SL1344 or with S. Typhimurium 14028s at MOI 50. Inhibition of bacterial growth (of both strains) in CHO ΔXYLT cells was dependent on the gentamicin concentration used. (C) CHO WT and CHO ΔXYLT cells were infected with either S. Typhimurium WT for 30 min., or with L. monocytogenes for 1 h (MOI 50) followed by treatment with 100 µg/mL gentamicin for 60 min. At 1.5 h (Salmonella) or 2.5 h (Listeria) medium was replaced with medium containing the indicated concentration of gentamicin. (D) Total cell lysates of the CHO WT and CHO ΔXYLT cells at 24 h p.i. (non-infected, or infected with S. Typhimurium) incubated with 10 mg/mL or 100 µg/mL gentamicin were collected and levels of the intracellular gentamicin were measured by ELISA. Data points, means and SD of representative results of three independent experiments are depicted. One-way ANOVA with Tukey’s multiple comparison test, n.s. (not significant), ***p < 0.001.

between CHO WT and CHO ΔXYLT cells, or between uninfected and infected cells (Figure 1D). Previous studies emphasized a role of the transient receptor potential channels (Trpv), Trpv1 (38) and Trpv4 (39), and the multidrug resistance protein 2 (Mrp2 or Abcc2) (40) in the cellular uptake and transport of gentamicin, respectively. Expression of Trpv1, Trpv4, and Mrp2 genes was comparable in non-infected and infected CHO WT and CHO ΔXYLT cells (Supplementary Figures S2A–C). Collectively, these findings indicate that a lack of proteoglycans in CHO cells does not affect active or passive gentamicin uptake.

To verify that the observed phenotype is indeed due to the proteoglycan deficiency, we complemented the CHO ΔXYLT cells with the human XXYLT2 gene. When compared to the proteoglycan-deficient CHO ΔXYLT cells, complemented CHO cXYLT cells harbored similar levels of Salmonella after invasion (at 1.5 h p.i.), but significantly more bacteria at 24 h p.i. (Figure 2A). However, while compared to CHO WT cells, complemented CHO cXYLT cells still had lower S. Typhimurium loads 24 h p.i., which correlated with lower amounts of proteoglycans present on CHO cXYLT cells, as assessed by flow cytometry (Supplementary Figure S3A), indicating only partial complementation of PGs. Next, we tested if addition of proteoglycans to the medium could also complement Salmonella survival in CHO ΔXYLT cells. Addition of heparin (a structural analog of heparan sulfate) to the medium increased bacterial survival in CHO ΔXYLT cells in a dose-dependent manner, but did not affect Salmonella survival in CHO WT cells (Figure 2B). In contrast, addition of equimolar amounts of chondroitin sulfate A (Figure 2C) or 2-fucosyllactose (Figure 2D) did not affect intracellular bacterial numbers in either CHO cell line. Notably, heparin did not support or inhibit growth of S. Typhimurium in LB medium and did not affect killing of Salmonella in LB broth supplemented with 100 µg/mL gentamicin (Supplementary Figure S3B). To summarize, these results indicate that host proteoglycans are important for the
FIGURE 2 | Proteoglycan-dependent phenotype can be rescued by an addition of the external GAGs as well as by XylT2 gene complementation. CHO WT and CHO ΔXylT cells were infected with S. Typhimurium WT and incubated for 24 h with 100 µg/mL gentamicin or lysed immediately to evaluate invasion. (A) Infection of complemented of CHO ΔXylT cells (indicated as cXylT, in black squares) resulted in significantly higher numbers of intracellular Salmonella in comparison to CHO ΔXylT cells. Mann–Whitney U test, *p < 0.033. CHO WT and CHO ΔXylT cells were infected with S. Typhimurium WT at MOI 50. Gentamicin (100 µg/mL) was added 30 min p.i. Heparin sodium salt (B), chondroitin sulfate (C) or 2′-fucosyllactose (D) was added at indicated concentrations 1.5 h p.i. Data points, means and SD of representative results of three independent experiments are depicted. One-way ANOVA with Dunnett’s multiple comparison test, not significant differences are not indicated, ***p < 0.001.

CHO Cells Lacking Proteoglycans Display a Lower Abundance of SCV-Associated Salmonella

To investigate if a lack of proteoglycans might affect subcellular localization of intracellular bacteria, CHO WT and CHO ΔXylT cells were infected with a S. Typhimurium reporter strain expressing DsRed protein constitutively and sfGFP only when bacteria are located in the cytosol (31). Microscopy revealed that CHO ΔXylT cells incubated with 100 µg/mL gentamicin had significantly lower numbers of total bacteria, but significantly higher numbers of GFP-expressing, cytosolic Salmonella compared to CHO WT at 24 h p.i., as shown by an elevated ratio of cytosolic/total bacteria in infected cells (Figures 3A,B). Addition of chloroquine selectively kills Salmonella within SCVs (34). Therefore, we used a combination of chloroquine resistance assay and gentamicin protection assay to determine cytosolic and intra-SCV bacteria corroborating our results obtained with the reporter strains (Figure 3C). To further investigate the subcellular localization of Salmonella, S. Typhimurium ΔsifA strain was utilized. This mutant is not able to maintain SCV integrity upon infection, which results in an extensive cytosolic replication of bacteria (30). Intracellular replication was analyzed by gentamicin protection assay, and expressed as a ratio of replicated (CFU at 24 h p.i)/invaded (CFU at 1.5 h p.i) bacteria. Intracellular proliferation of S. Typhimurium ΔsifA in CHO WT cells in the presence of 100 µg/mL gentamicin was about two times higher when compared to S. Typhimurium WT. In contrast, in CHO ΔXylT cells, S. Typhimurium ΔsifA intracellular replication was about 50 times higher compared to S. Typhimurium WT strain (Figure 3D). The differences in late replication were even more pronounced in CHO and CHO ΔXylT cells incubated with 200 µg/mL gentamicin (Figure 3D). Overall, these data indicate that the reduction of bacterial burden in CHO ΔXylT cells was due to a diminished number of bacteria in SCV, while cytosolic bacteria were largely unaffected by increasing concentrations of gentamicin.

Recently, it has been shown that Salmonella-induced filaments (SIFs) can increase the exposure of bacteria to internalized antibiotics in the SCV (41). To evaluate a contribution of the SIF network to the observed phenotype, CHO WT and CHO ΔXylT cells were infected with either S. Typhimurium WT or S. Typhimurium ΔssaR (a SPI-2 mutant lacking SIFs) (29). In
agreement with the findings by Liss et al. (41), incubation of the infected CHO WT cells with 200 µg/mL gentamicin for 24 h resulted in a significantly higher intracellular proliferation of S. Typhimurium ΔssaR compared to the WT strain. In contrast, numbers of both intracellular Salmonella WT and ΔssaR were strongly decreased in CHO ΔXylT cells (Figure 3E) indicating that the gentamicin-mediated inhibition of bacterial growth in CHO ΔXylT cells is independent of SIFs.

Proteoglycans Are Important for PIKfyve-Dependent Endo-Lysosomal Fusion

Our observation that CHO WT and CHO ΔXylT cells had similar levels of intracellular gentamicin was based on ELISA measurements of whole cell lysates. Next, we tested whether intracellular localization of gentamicin is altered in the absence of proteoglycans. Indeed, in infected CHO ΔXylT cells Cy3-labeled gentamicin was found close to Salmonella, or bacterial debris, while in CHO WT cells gentamicin-Cy3 was distributed more randomly (Figure 4A). Of note, uninfected CHO WT and ΔXylT cells were similar in terms of a distribution of labeled gentamicin (Supplementary Figure S4). Such re-distribution of an antibiotic may enhance Salmonella killing within modified compartments of CHO ΔXylT cells. Association of bacteria with vacuolar markers such as LAMP-1 or ARL8B was similar in CHO WT and CHO ΔXylT cells (Figure 4B).

We hypothesized that a lack of PGs may also alter intracellular routing of cargo other than antibiotics. To test this, we employed an antibody uptake assay. Cells were infected with GFP-expressing S. Typhimurium, and an anti-Salmonella antibody was added to cell culture medium 1.5 h p.i. (after invasion of
bacteria). 24 h p.i., we observed that significantly higher numbers of intracellular S. Typhimurium were stained with the anti-Salmonella antibody in infected CHO ΔXylT cells compared to CHO WT cells (Figures 5A,B). Addition of heparin to the cell culture medium 1.5 h p.i. reduced the number of double-positive bacteria in the infected CHO ΔXylT cells, while the total number of S. Typhimurium increased. These data indicates an important role of PGs in proper vesicle trafficking (Figure 5C).

Vacuole acidification is sensed and manipulated by Salmonella. To test whether endo-lysosomal trafficking and acidic vacuole formation is affected by proteoglycans we stained acidic organelles by incubation with Lysotracker. Strikingly, CHO ΔXylT cells (both non-infected and infected) were less stained than CHO WT cells when incubated with Lysotracker (Figure 6A). Interestingly, complemented CHO cXylT cells displayed an intermediate degree of Lysotracker staining (Supplementary Figure S5). To test if a lack of PG affects SCV acidification, we used a Salmonella strain harboring dual fluorescence reporter p5386 to monitor exposure of intracellular Salmonella to acidic pH (Supplementary Figures S6AB). The reporter features constitutive expression of DsRed, allowing the localization of intracellular Salmonella, and sfGFP under control of the acid shock response-activated promoter P_{asr} (42, 43). In vitro analyses demonstrated the P_{asr} is activated if Salmonella is exposed to media of pH 5.0 or lower. Exposure to media with higher pH did not lead to synthesis of sfGFP under control of P_{asr} (Supplementary Figure S6C). Inhibition of acidification of the SCV by addition of vATPase inhibitor bafilomycin fully ablated expression of P_{asr}:sfGFP (Supplementary Figure S6D). Expression of P_{asr}:sfGFP at 2 h p.i. was not affected by presence of gentamicin in the cell culture medium (Figure 6B). However, at 8 h p.i., we observed lower expression of P_{asr}:sfGFP in CHO ΔXylT cells compared to CHO WT cells (Figure 6C). Taken together, the acidification of endosomal compartments is impaired in PG-deficient cells as indicated by the lower signal intensity.
Proteoglycans Are Important for Endo-Lysosomal Trafficking

FIGURE 5 | Endocytosed cargo in infected proteoglycan-deficient CHO ΔXylT cells co-localizes with Salmonella. CHO cells were infected with S. Typhimurium eGFP. Gentamicin (100 µg/mL) was added 30 min after infection and 1.5 h p.i. anti-Salmonella antibody was added to the medium. 24 h p.i. cells were fixed with 4% PFA, and stained with DAPI. (A) Double-positive bacteria are indicated with arrows in enlarged section. Scale bars, 10 µm. (B) Number of double-positive bacteria was counted in 20 FOVs. Mann–Whitney U test, **p < 0.002. (C) 1.5 h p.i. heparin (30 µM) was added to growth medium containing anti-Salmonella antibody. 20 random FOVs were counted, mean values with 95% CI are shown. Mann–Whitney U test, n.s., not significant.

of Lysotracker labeling and lower expression of Pars-sfGFP in ΔXylT cells.

To identify, at which stage trafficking of endocytosed cargo is affected by the lack of PGs, we utilized inhibitors of clathrin-mediated endocytosis (dynasore), phosphoinositide 3-kinase PI3K (wortmannin), as well as an inhibitor of FYVE finger-containing phosphoinositide kinase (PIKfyve) activity (YM201636). Application of dynasore 1.5 h p.i. (added after invasion of bacteria resulted in a significantly higher recovery of S. Typhimurium from CHO ΔXylT cells compared to the non-treated controls. In contrast, dynasore treatment had no significant effect on intracellular bacterial numbers in CHO WT cells (Figure 7A). In addition, treatment of either CHO WT or CHO ΔXylT cells with wortmannin had no significant effect on intracellular S. Typhimurium numbers (Supplementary Figure S7). When PIKfyve activity in CHO WT cells was inhibited with YM201636, S. Typhimurium numbers were reduced in a dose-dependent manner in agreement with the results by Kerr et al. (44). However, YM201636 treatment of CHO ΔXylT cells resulted in significantly more intracellular bacteria compared to non-treated cells (Figure 7B). Heparin treatment abrogated the dose-dependent effect of YM201636 on Salmonella survival (Figure 7C) implying a direct effect of PGs on PIKfyve activity. Soluble heparin was detected within endosomal compartments and SCVs in the heparin-treated CHO ΔXylT cells as revealed by immunostaining (Supplementary Figure S10). Application of YM201636 also resulted in diminished numbers of double-positive bacteria in both CHO cell lines in an antibody uptake assay (Figure 7D). Furthermore, incubation of CHO WT and CHO ΔXylT cells with PIKfyve-inhibitor dramatically enhanced the size of acidic lysosomes (Supplementary Figure S8). These data demonstrate a critical role of proteoglycans in PIKfyve-mediated fusion events.

To assess if endo-lysosomal fusion is abrogated in CHO ΔXylT cells, we employed a modified pulse-chase assay using Alexa568- and Alexa488-labeled dextrans to label lysosomes/late
Acidic organelles in CHO ΔXylT cells display reduced labeling by Lysotracker. (A) Uninfected CHO WT and CHO ΔXylT cells, or cells infected with S. Typhimurium EGFP at MOI of 50, were incubated for 2 h with 50 nM Lysotracker Red. Cells were fixed with 4% PFA. Representative images of three biological repetitions, scale bars, 10 μm. (B,C) Acidification of Salmonella is dependent on the presence of PGs. CHO WT or CHO ΔXylT cells were infected with Salmonella WT harboring an acid shock sensor. Infection and analyses by flow cytometry were performed as described in Supplementary Figure S6. After infection for 30 min, cells were treated with 100 μg/mL gentamicin for 1 h followed by 10 μg/mL gentamicin for 1 h (B) or 7 h (C). The mean sfGFP fluorescence intensity is displayed for CHO cells harboring DsRed-positive Salmonella. X-means and standard deviations are shown for triplicate samples, and the data shown are representative for three biological replicates with similar outcome. Unpaired t-test, n.s., not significant, **p < 0.001.

DISCUSSION

In the present study, we report a novel role of PGs for endo-lysosomal fusion. PG deficiency abrogates endo-lysosomal fusion which affects Salmonella survival within epithelial cells in a context of gentamicin protection assay. Wild-type CHO cells exclusively utilize Xylt2 for PGs biosynthesis and lack detectable Xylt1 gene expression (45). CHO pgsA745 (ΔXylT2) cells lack the xylosyltransferase-II enzyme and thus, are PG-deficient (3). This cell line has been extensively used to investigate the contribution of PGs to the entry of bacterial and viral pathogens into host cells (46). Recently, it has been identified that CHO ΔXylT cells also carry a mutation in the Lama2 gene. The resulting deletion of the long isoform of the laminin subunit α2 significantly reduced invasion of group B Streptococcus in CHO ΔXylT compared to CHO WT cells (47). Indeed, while both the short and the long isoforms of laminin-2 were expressed in our CHO WT cells, CHO ΔXylT cells lacked the long isoform expression (Supplementary Figure S9). In addition, it was shown that when Salmonella is grown under pagN-inducing conditions there was a reduced uptake into CHO ΔXylT cells (7). However, in our study, no differences in terms of S. Typhimurium adhesion to and invasion into the CHO WT and CHO ΔXylT cell lines were detected. Upon invasion, Salmonella hijack endo-lysosomal trafficking and acquire host factors including LAMP1 and ARL8B in
Inhibition of endo-lysosomal fusion increases Salmonella burdens in CHO ΔXylT cells. CHO WT and CHO ΔXylT cells were infected with S. Typhimurium WT at MOI = 50 and incubated for 24 h in presence of 100 µg/mL gentamicin and increasing concentrations of (A) dynasore or (B) YM201636. One-way ANOVA with Dunnett’s multiple comparison test, n.s. (not significant), *p < 0.033, **p < 0.002, ***p < 0.001. (C) CHO WT and CHO ΔXylT cells were first infected with S. Typhimurium WT at MOI of 50. After 30 min, cells were incubated with 100 µg/mL gentamicin, heparin (30 µM), and different concentrations of YM201636 for 24 h. One-way ANOVA with Dunnett’s multiple comparison test, not significant differences are not indicated. (D) CHO cells were infected with S. Typhimurium eGFP. Gentamicin (100 µg/mL) was added 30 min after infection and 1.5 h p.i. anti-Salmonella antibody and 0.8 µM YM201636 were added to the medium. 24 h p.i., cells were fixed with 4% PFA. 20 random FOV were counted. Graph shows mean values with 95% CI. Mann–Whitney U test, **p < 0.002, n.s., not significant.

order to establish a Salmonella-containing vacuole (SCV) and later on, Salmonella-induced filaments (SIFs). Microscopy and chloroquine resistance assays revealed that in the absence of PGs, significantly less bacteria were associated with SCVs when compared to WT CHO cells, while similar numbers of cytosolic bacteria were found in CHO WT and CHO ΔXylT cells. It should be noted that no differences in the levels of a total intracellular gentamicin between CHO WT and CHO ΔXylT cells were detected. Thus, we reasoned that an intracellular localization of gentamicin might be altered in the infected CHO ΔXylT cells, which leads to an increased exposure of specific bacterial populations to the antibiotic. Although cell membranes are generally regarded to be impermeable to gentamicin, aminoglycosides can be transported into epithelial cells via endocytosis-dependent and -independent pathways (48). It was previously reported that endocytosis of gentamicin resulted in its accumulation within lysosomes and in increased lysosomal ROS production in kidney epithelial cells (49). CHO ΔXylT cells are not defective in terms of endocytosis/phagocytosis (47), which is supported by our data regarding uptake of gentamicin (Cy3-labeled and by ELISA). However, during infection, we observed an increased co-localization of gentamicin and SCV-associated bacteria in proteoglycan-deficient CHO cells. Indeed, the S. Typhimurium ΔsifA mutant, which cannot establish a functional SCV and therefore localizes to the cytoplasm (50), was significantly less affected by increasing gentamicin concentrations than WT Salmonella, in both CHO cell lines. To conclude, the intracellular re-distribution of gentamicin in proteoglycan-deficient CHO cells was associated with a drastic reduction of Salmonella counts.

In addition, we detected increased accumulation of an anti-Salmonella antibody in SCV/SIF compartments in CHO ΔXylT compared to CHO WT cells. This process could be blocked by addition of heparin to cell culture medium or by inhibiting of PIKfyve activity in CHO ΔXylT cells. CHO WT cells treated with a specific PIKfyve inhibitor
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(Figure 8) CHO ΔXylT cells display reduced degree of endo-lysosomal fusion. CHO WT and CHO ΔXylT cells were seeded on cover slips and pulsed for 4 h with dextran-Alexa568, followed by incubation with CHO medium. 18 h later dextran-Alexa488 was added for 10 min, cells were washed and 30 min later, cells were fixed with 4% PFA. (A) Co-localization of Alexa-labeled vesicles can be seen in the merged section. Scale bars, 5 µm. (B) Pearson correlation coefficient was calculated for 20 FOVs per cell line. (C) CHO ΔXylT cells have same fraction of late endosomes overlapping with early endosomes (M1), but smaller fraction of early endosomes co-localizing with late endosomes (M2). Mander’s overlap coefficient calculated for each of 20 FOVs, Mann–Whitney $U$ test, $^* p < 0.033$, $^{**} p < 0.002$. Data are representative of two biological repetitions, median values are indicated on graphs.

(YM201636) were characterized by significantly reduced bacterial loads compared to untreated controls which is in line with observations by Kerr et al. (44). In contrast, inhibition of PIKfyve in CHO ΔXylT cells increased S. Typhimurium counts. PIKfyve is a kinase that converts PtdIns3P into PtdIns(3,5)P$_2$. It has been suggested that PIKfyve orchestrates the fusion of Salmonella macropinosomes with organelles of the late endosomal/lysosomal system (44), and more recent data link PIKfyve activity with the recycling of tight junction proteins (51) and with a re-distribution of endocytosed cargo from/to lysosomes occurring at late stages of endocytic vacuole maturation (52). As we observed a different distribution of Cy3-labeled gentamicin and endocytosed antibody within the CHO WT and CHO ΔXylT cells, we speculated that proteoglycans are required for PIKfyve-dependent endo-lysosomal fusion and subsequent trafficking/recycling pathways. Interestingly, CHO ΔXylT cells displayed reduced labeling with Lysotracker, which was increased upon treatment with YM201636. The pH inside the SCV can affect bacterial survival in multiple ways: for example, phagosomal pH in macrophages is important for susceptibility of S. Typhimurium to gentamicin (53). In addition, there is evidence that autophagosome-lysosome fusion in CHO cells is affected by the pH in acidic compartments (54). While, in our experiments, early acidification of the SCV in CHO WT and CHO ΔXylT cells was similar as determined by acid shock response reporter strains, at the later time points acidification of SCV in CHO ΔXylT cells was impaired.

Our results raise the question of specific interactions between phosphoinositides and PGs in the context of infection. For example, it was shown that binding of the transmembrane heparan sulfate PG syndecan-4 to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) is required for formation of focal adhesions (55). Because phosphoinositides are essential for actin assembly, it is not surprising that Salmonella can deplete PtdIns(4,5)P$_2$ by the effector SigD which results in membrane fission during bacterial invasion (56). However, the role of PGs in SCV/SIF biogenesis and endo-lysosomal trafficking is less clear. Several studies showed that syndecans, along with the endosomal sorting complex required for transport (ESCRT) proteins, are involved in the formation of multivesicular endosomes or bodies...
(MVBs) (57, 58). MVBs can fuse with lysosomes or be exported as exosomes. It is known that *Salmonella* disturbs normal endosome to lysosome trafficking, affecting the ESCRT system (23) and exocytosis (16, 17). PtdIns(3,5)P₂ (hence, PIKfyve) regulates endosomal fission and fusion, and MVB formation (20).

Taken together, our data show that altered routes of endocytosed cargo in PG-deficient epithelial cells interfere with vesicle acidification and *Salmonella*-modulated PIKfyve-dependent fusion to establish a replicative niche and thereby elucidate a novel role of PGs in intracellular vesicle trafficking and SCV formation.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

AG, AS, HB, FR, MH, and GG: conceptualization. AG, AO, AS, HB, and WK: investigation. AG, AO, AS, LF, FR, HB, MH, and GG: data analysis. AG, AS, and GG: manuscript writing. AG, AO, AS, HB, FR, LF, MH, and GG: manuscript editing and approval.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00731/full?supplementary-material

**FIGURE S1 |** Intracellular *Salmonella* are sensitive to treatment with ampicillin. (A) CHO cells were infected with *S. Typhimurium* 14028s WT at MOI 10, and ampicillin was used instead of gentamicin in a protection assay. 24 h p.i., cells had similar low CFU counts. Dotted line indicates limit of detection, ND – not detected. (B) Infected CHO WT and CHO Δ*xyfT* cells had comparable levels of released lactate dehydrogenase as measured by LDH assay kit. Dotted line indicates limit of detection. Kruskal–Wallis test with Dunn’s multiple comparison test, n.s. (not significant).

**FIGURE S2 |** CHO WT and Δ*xyfT* cells had comparable expression levels of gentamicin transporters genes. Gene expression was measured in CHO WT and CHO Δ*xyfT* cells, non-infected and infected with *S. Typhimurium* WT. Control CHO cells were incubated without gentamicin. Gene expression was normalized to Gapdh and Rps9, and control CHO WT data used as a calibrator. Comparable levels of Abcb2 (A), Tmpr2 (B), and Top4 (C) expression were observed in both CHO WT and Δ*xyfT* cells, regardless infected or not. One-way ANOVA with Tukey’s multiple comparison test and Mann-Whitney test, not significant differences are not indicated.

**FIGURE S3 |** Presence of PGs in CHO cells. (A) CHO WT, CHO Δ*xyfT*, and complemented CHO Δ*xyfT* cells (indicated as CHO Δ*xyfT*+Tyr9) were stained with heparin sulfate-specific antibody and analyzed by flow cytometry. (B) *S. Typhimurium* WT was grown for 6 h in LB broth in presence/absence of 30 µM heparin, and OD600 was recorded every 10 min. Mean values of three technical replicates of a representative experiment out of two are shown.

**FIGURE S4 |** Gentamicin uptake in CHO WT and CHO Δ*xyfT* cells. CHO cells were incubated for 7 h with gentamicin-Cy3 conjugate and then fixed with 4% PFA. Microscopy revealed a similar distribution of the labeled antibiotic within the CHO WT and CHO Δ*xyfT* cells.

**FIGURE S5 |** Labeling by Lysotracker correlates with PGs expression in CHO cells. CHO WT, CHO Δ*xyfT*, and complemented CHO Δ*xyfT* cells were infected with *S. Typhimurium* EGFP at MOI 50 and incubated for 24 h with 50 nM Lysotracker Red added upon invasion of bacteria. CHO Δ*xyfT* cells had an intermediate staining (compare to Supplementary Figure S3A). Representative images of two biological replications. Scale bars, 10 µm.

**FIGURE S6 |** A dual fluorescence reporter for acid shock exposure of *Salmonella*. (A) Plasmid map of p5386, encoding DsRed constitutively under control of promoter P₂₃₃₃, and sfGFP under control the acid shock-inducible promoter P₄₃₃. (B) Flow cytometry and gating of *Salmonella* without fluorescent protein expression, or constitutive expression of DsRed or sfGFP. (C) Acid shock of cultured bacteria induces sfGFP expression. *Salmonella* WT harboring p5386 (S.Tm WT) was grown in PCN, pH 7.5 to mid-log phase. Bacteria were pelleted, washed twice in sterile saline, and resuspended in PCN buffered to the indicated pH. Culture was continued for 1 h, bacteria were harvested by centrifugation and resuspended in PCB containing 200 µg/mL chloramphenicol to stop further protein biosynthesis. The bacteria were incubated for at least 2 h at 4 °C to allow full maturation of sfGFP, and subjected to flow cytometry. (D) For in vivo analyses, S.Tm WT harboring p5386 was subcultured for 3 h and used to infect ca. 2 × 10⁵ CHO WT or CHO Δ*xyfT* cells at MOI of 10. If indicated (+ Bac), bafilomycin was added to a final concentration of 100 nM. Cells were infected for 30 min, washed three times to remove non-internalized bacteria and incubated 2 h with or without gentamicin addition as indicated in Fig 6B. A representative example of an assay with a constant concentration of 10 µg/mL gentamicin is shown. After washing, cells were detached using biotase, chloramphenicol was added to a final concentration of 200 µg/mL and incubated for at least 4 h at 4 °C for allow full maturation of sfGFP. Flow cytometry was performed by gating of CHO cells and the level of DsRed and sfGFP fluorescence was determined for at least 50,000 infected host cells.

**FIGURE S7 |** Inhibition of PI3K does not affect intracellular *Salmonella*. CHO WT and CHO Δ*xyfT* cells were infected with *S. Typhimurium* WT at MOI of 50 and incubated for 24 h with 100 µg/mL gentamicin and increasing concentrations of specific inhibitors. Addition of wortmannin had no effect on survival of *Salmonella* survival in either CHO cell line. One-way ANOVA with Dunnett’s multiple comparison test, only significant differences are indicated.

**FIGURE S8 |** PIKfyve kinase inhibition increased labeling by Lysotracker in both CHO WT and CHO Δ*xyfT* cells. CHO cells infected with *S. Typhimurium* EGFP were incubated for 24 h with 50 nM Lysotracker Red added upon invasion, in presence/absence of 0.8 µM YM201636. Microscopy revealed enlarged lysosomes/endosomes in YM201636-treated CHO cells. Representative images, scale bars, 10 µm.

**FIGURE S9 |** CHO Δ*xyfT* cells lack Lama2 expression. cDNA of the uninfected CHO cells was used to screen for an expression of Lama2 isoforms. ND – not detected.
FIGURE S10 | Addition of heparin to the medium results in intracellular accumulation of heparin in the endo-lysosomal system. CHO ΔXYT cells, uninfected and infected with S. Typhimurium WT at MOI of 50 were incubated for 24 h with 100 μg/mL gentamycin and with 30 μM heparin. Heparin (green) was detected inside CHO ΔXYT cells, in the same compartment as bacteria (red). In CHO WT cells, in the absence of added heparin, HS staining shows localization of HS at the cell surface but also in endo-lysosomal compartments. Representative images of two biological repetitions, scale bars, 10 μm or 5 μm (in enlarged sections).

TABLE S1 | Primers used in this study.

TABLE S2 | Antibodies used in this study.
36. Reuter T, Vorwerk S, Liss V, Chao T-C, Hensel M, Hansmeier N. Proteomic analysis of Salmonella-modified membranes reveals adaptations to macrophage clearance. *Mol Cell Proteomics*. MCP. (2020) [epub ahead of print]. doi: 10.1074/mcp.RA119.001841

37. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. (2006) 224:213–32. doi: 10.1111/j.1365-2818.2006.01706.x

38. Myrdal SE, Steyer PS. TRPV1 regulators mediate gentamicin penetration of cultured kidney cells. *Hear Res*. (2005) 204:170–82. doi: 10.1016/j.heares.2005.02.005

39. Karasawa T, Wang Q, Fu Y, Cohen MD, Steyer PS. TRPV4 enhances the cellular uptake of aminoglycoside antibiotics. *J Cell Sci*. (2008) 121:2871–9. doi: 10.1242/jcs.023705

40. Notenboom S. Increased apical insertion of the multidrug resistance protein 2 (MRP2/ABCC2) in renal proximal tubules following gentamicin exposure. *J Pharmacol Exp Ther*. (2006) 318:194–202. doi: 10.1124/jpet.106.104547

41. Liss V, Swart AL, Kehl A, Hermanns N, Zhang Y, Chikkaballi D, et al. Salmonella enterica remodels the host cell endosomal system for efficient intravascular nutrition. *Cell Host Microbe*. (2017) 21:390–402. doi: 10.1016/j.chom.2017.02.005

42. Allam US, Krishna MG, Sen M, Thomas R, Lahiri A, Gnanadhas DP, et al. Acidic pH induced STM1485 gene is essential for intracellular replication of Salmonella. *Virology*. (2012) 3:122–35. doi: 10.4161/viru.19029

43. Seputiene V, Motiejûnas D, Suziedelis K, Tomenius H, Normark S, Melefors O, et al. Molecular characterization of the acid-inducible ars gene of *Escherichia coli* and its role in acid stress response. *J Bacteriol*. (2003) 185:2475–84. doi: 10.1128/JB.185.8.2475-2484.2003

44. Kerr MC, Wang JTH, Castro NA, Hamilton NA, Town L, Brown DL, et al. Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of *Salmonella enterica*. *EMBO J*. (2010) 29:1331–47. doi: 10.1038/emboj.2010.28

45. Cuellar K, Chuong H, Hubbell SM, Hinsdale ME. Biosynthesis of chondroitin and heparan sulfate in Chinese hamster ovary cells depends on xylosyltransferase II. *J Biol Chem*. (2007) 282:5195–200. doi: 10.1074/jbc.M611048200

46. Rostand KS, Eko JD. Microbial adherence to and invasion through proteoglycans. *Infect Immun*. (1997) 65:1–8.

47. van Wijk XM, Döhrmann S, Hallström BM, Li S, Voldborg BG, Meng BX, et al. Whole-genome sequencing of invasion-resistant cells identifies laminin α2 as a host factor for bacterial invasion. *mBio*. (2017) 8:e2128-16. doi: 10.1128/mBio.02128-16

48. Nagai J, Takano M. Entry of aminoglycosides into renal tubular epithelial cells via endocytosis-dependent and endocytosis-independent pathways. *Biochem Pharmacol*. (2014) 90:331–7. doi: 10.1016/j.bcp.2014.05.018

49. Denamur S, Tyteca D, Marchand-Brynaert J, Van Bambeke F, Tulkens PM, Courtoy PJ, et al. Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic. *Free Radic Biol Med*. (2011) 51:1656–65. doi: 10.1016/j.freeradbiomed.2011.07.015

50. Brumell JH, Tang P, Zaharik ML, Finlay BB. Disruption of the Salmonella-containing vacuole leads to increased replication of *Salmonella enterica* serovar typhimurium in the cytosol of epithelial cells. *Infect Immun*. (2002) 70:3264–70. doi: 10.1128/IAI.70.6.3264-3270.2002

51. Dukes JD, Whiteley P, Chalmers AD. The PIKfyve inhibitor YM201636 blocks the continuous recycling of the tight junction proteins claudin-1 and claudin-2 in MDCK cells. *PLoS One*. (2012) 7:e28659. doi: 10.1371/journal.pone.0028659

52. Krishna S, Palm W, Lee Y, Yang W, Bandypadhyay U, Xu H, et al. PIKfyve regulates vacuole maturation and nutrient recovery following engulfment. *Dev Cell*. (2016) 38:536–47. doi: 10.1016/j.devcel.2016.08.001

53. Menashe O, Kaganskaya E, Bazsov T, Yaron S. Aminoglycosides affect intracellular *Salmonella enterica* serovars typhimurium and virchow. *Antimicrob Agents Chemother*. (2008) 52:920–6. doi: 10.1128/AAC.00382-07

54. Kawai A, Uchiyama H, Takano S, Nakamura N, Ohkuma S. Autophagosome-lysosome fusion depends on the pH in acidic compartments in CHO cells. *Autophagy*. (2007) 3:154–7. doi: 10.4161/auto.3634

55. Oh E-S, Woods A, Lim S-T, Theibert AW, Couchman JR. Syndecan-4 proteoglycan cytoplasmic domain and phosphatidylinositol 4,5-bisphosphate coordinately regulate protein kinase C activity. *J Biol Chem*. (1998) 273:10624–9. doi: 10.1074/jbc.273.17.10624

56. Terebiznik MR, Vieira OV, Marcus SL, Slade A, Yip CM, Trimble WS, et al. Elimination of host cell PtdIns(4,5)P2 by bacterial SigD promotes membrane fusion during invasion by *Salmonella*. *Nat Cell Biol*. (2002) 4:766–73. doi: 10.1038/ncb854

57. Friand V, David G, Zimmermann P, Syntenin and syndecan in the biogenesis of exosomes: syndecan-syntenin pathway in exosome biogenesis. *Biol Cell*. (2015) 107:331–41. doi: 10.1111/boc.201500010

58. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci*. (2018) 75:193–208. doi: 10.1007/s00018-017-2595-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.