OXFORD FEMS

Pathogens and Disease, 2023, **79**, 1–12

DOI: 10.1093/femspd/ftad035

Research Article

Bordetella pertussis targets the basolateral membrane of polarized respiratory epithelial cells, gets internalized, and survives in intracellular locations

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Editor: Laurence Luu

Abstract

The airway epithelial barrier is a continuous highly organized cell layer that separates the exterior from the underlying mucosal tissue, preventing pathogen invasion. Several respiratory pathogens have evolved mechanisms to compromise this barrier, invade and even reside alive within the epithelium. *Bordetella pertussis* is a persistent pathogen that infects the human airway epithelium, causing whooping cough. Previous studies have shown that *B. pertussis* survives inside phagocytic and nonphagocytic cells, suggesting that there might be an intracellular stage involved in the bacterial infectious process and/or in the pathogen persistence inside the host. In this study we found evidence that *B. pertussis* is able to survive inside respiratory epithelial cells. According to our results, this pathogen preferentially attaches near or on top of the tight junctions in polarized human bronchial epithelial cells and disrupts these structures in an adenylate cyclase-dependent manner, exposing their basolateral membrane. We further found that the bacterial internalization is significantly higher in cells exposing this membrane compared with cells only exposing the apical membrane. Once internalized, *B. pertussis* mainly remains in nondegradative phagosomes with access to nutrients. Taken together, these results point at the respiratory epithelial cells as a potential niche of persistence.

Keywords: *Bordetella pertussis*; respiratory epithelial cells; tight junctions; basolateral membrane; adenylate cyclase toxin; bacterial intracellular survival

Introduction

Whooping cough is a re-emerging respiratory disease caused by *Bordetella pertussis*. Despite years of vaccination, it is still a major cause of infant morbidity and mortality worldwide. One of the causes of its re-emergence is the suboptimal formulation of current vaccines that fail to prevent respiratory tract colonization and bacterial transmission (Warfel et al. 2014), eventually enabling circulation of the pathogen among highly vaccinated populations (Esposito et al. 2019). Because the human is the only host for this pathogen, the epidemiological situation seems to indicate the existence of asymptomatic carriers who eventually become sources of transmission (Cherry et al. 2005). The location of the pathogen in these reservoirs is still under investigation. *Bordetella pertussis* was historically considered as an extracellular pathogen, but previous studies from our group and others have shown that this bacterium might be an intracellular facultative pathogen (Hellwig et al. 1999, Lamberti et al. 2008, 2010, 2016, Jakub et al. 2020, Petráčková et al. 2020). We showed that *B. pertussis* can survive inside immune cells, such as neutrophils and macrophages, and within phagosomes that do not fuse with lysosomes (Lamberti et al. 2008, 2010). We further found evidence pointing at macrophages as a potential niche of bacterial persistence (Lamberti et al. 2010, 2016, Valdez et al. 2016, 2021).

Bordetella pertussis is transmitted by aerosolized droplets and the first interaction between the bacterium and the host takes place in the respiratory tract. The human respiratory epithelium constitutes a continuous polarized cell layer that plays a major role in host immunity and immune response modulation (Hasan et al. 2018). Cells in this layer are laterally joined to each other by an apical junctional complex comprising tight junctions and the underlying adherens junctions (Anderson and Van Itallie 2009). Beneath this apical junctional complex are desmosomes and gap junctions that provide mechanical strength and intercellular ion channels, respectively (Anderson and Van Itallie 2009). Tight junctions restrict the free diffusion of membrane components delineating the boundaries between the apical and the basolateral membrane in polarized epithelial cells (Hasan et al. 2018). They also guarantee the integrity and barrier function of the epithelium, preventing pathogen invasion from the respiratory tract lumen (Hasan et al. 2018). Several respiratory pathogens, however, have evolved mechanisms to compromise this barrier and infect the host through the epithelium. Depending on the pathogen, the outcome of these infections might lead to bacterial intracellular survival with or without intracellular replication, or to the spread of the infection (Bertuzzi et al. 2019). Among others, *Burkholderia* species were found to be able to disrupt the epithelial barrier (Duff et al. 2006) and survive in the intracellular location (Burns et al. 1996, Martin and Mohr 2000). *Pseudomonas aeruginosa* was found to be able to alter the epithelial barrier integrity (Halldorsson et al. 2010) and persist inside the cells (Malet et al. 2022). Nontypeable *Haemophilus influenzae* also disrupts barrier integrity (Clarke et al. 2011) and survives in lung epithelial cells, eventually developing

Received 29 May 2023; **accepted** 30 November 2023

an intracellular niche that plays a major role in chronic infections (Clementi et al. 2014). Although previous studies addressing the intracellular survival of *B. pertussis* in respiratory cells showed very low invasion, they also demonstrated that this pathogen is able to survive inside these cells for days (Bassinet et al. 2000, Gueirard et al. 2005, Lamberti et al. 2013) and even repopulate the extracellular medium once released from the intracellular compartment (Lamberti et al. 2013). These studies were performed in sub-confluent cultures without formed tight junctions and, therefore, with incompletely polarized cells that might lack relevant features of respiratory epithelial cells. The existence of basolateral and apical membranes in host cells usually plays a central role in the outcome of the interaction between the cell and the pathogen. For instance, *P. aeruginosa* binds to different receptors in basolateral and apical membranes through different virulence factors, resulting in different host cell response to the infection (Bucior et al. 2010, 2012). *Campylobacter jejuni* (Bouwman et al. 2013) and *H. influenzae* (Wegele et al. 2020) were also shown to differentially interact with apical and basolateral membranes in epithelial cells, promoting invasion, preferentially through the latter.

A better understanding of *B. pertussis* interaction with the respiratory epithelial barrier might provide some clues about the infectious process and persistence strategies of this pathogen. In this work we studied the initial interaction of *B. pertussis* with polarized respiratory epithelial cells in order to gain some insight into the eventual relevance of the apical and basolateral membranes in the bacterial attachment, internalization and intracellular fate.

Materials and Methods

Bacterial strain and growth conditions

Bordetella pertussis strain Tohama I was used in this study. In some experiments, BpGR4, a Tohama-derivative strain lacking filamentous hemagglutinin (FHA) expression (Locht et al. 1992), and BpΔCyaA, a Tohama-derivative strain lacking CyaA expression (Cerny et al. 2015), were used. Bacteria were stored at -70°C and recovered by growth on Bordet Gengou agar (BGA; Difco Laboratories, Detroit, MI, USA) plates supplemented with 15% (v/v) defibrinated sheep blood at 36°C for 3 days. Virulent (Bvg+) bacteria were subsequently seeded on Stainer-Scholte (SS) liquid medium, cultured for 20 h at 36°C and used in the experiments. Animal handling and all procedures were in compliance with the Argentinian animal protection Law 14346.

Cell culture and differentiation

The simian virus 40 large T antigen-transformed human bronchial epithelial cell line 16HBE14o- was kindly provided by Dieter Gruenert (Cozens et al. 1994). Cells were recovered from frozen stocks and maintained in Minimal Essential Medium (MEM; Gibco, Grand Island, NY, USA) supplemented with 10% v/v fetal bovine serum (Gibco) at 37°C in 5% CO_2 in fibronectin (Sigma Chemical Co., Saint Louis, MO, USA) and collagen (Sigma Chemical Co.) coated culture flasks (Jet Biofil, Guangzhou, China). Routine subcultures for the cells were performed at 1:6 split ratios by incubation with 0.05% (w/v) trypsin-EDTA (Gibco) for 10 min at 37°C. For monolayer differentiation, 5×10^5 cells were seeded on collagen-coated coverslips on 24-well tissue culture plates (Eppendorf, Hamburg, Germany) with MEM supplemented with 10% v/v fetal bovine serum for 7 days with culture medium renewed every second day. For the differentiation into small groups or islands, 5×10^3 cells were seeded on coated coverslips and cultured as stated for monolayers. Cell polarization was monitored by occludin staining. Cells were fixed in 4% paraformaldehyde, washed with PBS and incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl (Merck Millipore, Darmstadt, Germany). After two washing steps, the cells were further incubated overnight with mouse monoclonal antibodies against occludin (Invitrogen, Waltham, MA, USA) in PBS containing 0.1% (v/v) Triton X-100 and 0.2% (w/v) bovine seroalbumin (BSA; Sigma). After washing with PBS, the cells were incubated with Cy3-conjugated F(ab´)2 fragments of goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). Then coverslips were mounted with anti-fading agent (Invitrogen, Carlsbad, CA, USA) and microscopic analyses were performed using a confocal laser scanning microscope (model TCS SP5; Leica, Wetzlar, Germany).

Attachment assays

For attachment assays, 7-day-polarized island or monolayers grown on coated coverslips were incubated with wild type *B. pertussis* at a multiplicity of infection (MOI) of 20 bacteria per cell in MEM supplemented with 0.2% w/v BSA at 37°C with 5% CO_2. In some assays, cells were incubated with BpGR4 at a MOI of 200. After 5 h of infection, cells were washed three times with PBS to remove nonattached bacteria and fixed in 4% w/v paraformaldehyde, washed with PBS then incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl. Surface-bound and internalized bacteria were discriminated by a two-step labeling procedure followed by fluorescence microscopy analysis, as previously described (Lamberti et al. 2010). Briefly, surface-bound bacteria were detected by incubation of the cells with rabbit anti-*B. pertussis* serum for 30 min at room temperature, followed by incubation with FITC-conjugated goat F(ab')2 fragments of anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for another 30 min at room temperature. To determine the number of intracellular bacteria, after two washes with 0.2% BSA (PBS), the cells were permeabilized by incubation with PBS containing 0.1% w/v saponin (Sigma) and 0.2% w/v BSA for 30 min, followed by a further incubation for 30 min at room temperature with rabbit anti-*B. pertussis* serum in the presence of 0.1% w/v saponin and 0.2% (w/v) BSA. After three washes, the cells were incubated with Cy3-conjugated F(ab')2 fragments of goat anti-rabbit IgG (Jackson ImmunoResearch) for 30 min at room temperature. After washing, the samples were analyzed by fluorescence microscopy under a confocal laser scanning microscope (model TCS SP5; Leica). The numbers of extracellular (red and green) and intracellular (red) bacteria were evaluated by examination of at least 100 eukaryotic cells. When indicated, bacteria were preincubated for 15 min with heparin (1 $\mu g\ mL^{-1}$) (Sigma) prior to infection (Menozzi et al. 1994). The heparin was maintained throughout the experiment. At the concentration used, heparin treatment did not affect epithelial cell viability, as determined by trypan blue dye exclusion. In addition, the drug did not affect bacterial viability or growth rate under the conditions tested. When indicated, cells were incubated with the wild type *B. pertussis* or BpΔCyaA, a Tohama-derivative strain lacking CyaA expression (MOI: 0.2) in MEM supplemented with 0.2% w/v BSA at 37°C with 5% CO_2. After 24 h of infection, cells were washed three times with PBS to remove nonattached bacteria and were fixed in 4% w/v paraformaldehyde, washed with PBS and incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl. After two washing steps, the cells were further incubated overnight with mouse monoclonal antibodies against occludin in PBS containing 0.1% w/v saponin (Sigma) and 0.2% w/v BSA (Sigma). To determine total bacteria, cells were incubated for 30 min at room temperature with rabbit anti-*B.*

pertussis serum in the presence of 0.1% w/v saponin and 0.2% (w/v) BSA. After washing with PBS containing 0.1% w/v saponin and 0.2% w/v BSA, the cells were incubated with Cy3-conjugated F(ab′)2 fragments of goat anti-mouse IgG (Molecular Probes) and FITC-conjugated goat F(ab′)2 fragments of anti-rabbit IgG (Jackson ImmunoResearch) for another 30 min in PBS containing 0.1% w/v saponin and 0.2% w/v BSA. Then coverslips were mounted with anti-fading agent (Invitrogen) and microscopic analyses were performed using a confocal laser scanning microscope (model TCS SP5; Leica). In all experiments, a minimum of 150 eukaryotic cells were examined per sample.

In selected assays, the Tight Junction Organization Rate (TiJOR) was calculated using ImageJ software macro (Terryn et al. 2013) to assess the disruption of the tight junction network. TiJOR was calculated by evaluating entire series of images obtained by confocal microscopy.

Semipermeable membrane cell culture and infection

For monolayer integrity assays, 5×10^5 cells were seeded on coated 12-mm semipermeable Transwell inserts (3-μm pore size; Corning Costar, Bodenheim, Germany) in submerged conditions with MEM supplemented with 10% v/v fetal bovine serum for 7 days with culture mediumrenewed every second day both in apical and basolateral compartments. To confirm the functional integrity of the epithelial cell layers, transepithelial electrical resistance (TEER) was measured with a Millicell ERS-2 Volt-Ohm meter (Millipore, Billerica, MA, USA). Epithelial cell layers grown (as described above) consistently showed TEER values of ∼350 $\Omega.cm^2$ within 1 week of seeding. Cultured monolayers showing a minimum TEER value of 350 $\Omega.cm^2$ were incubated with wild type *B. pertussis* or BpΔCyaA, a Tohama-derivative strain lacking CyaA expression (MOI: 20) in MEM supplemented with 0.2% w/v BSA. After 5 h of infection, samples from basolateral compartments were taken and serial dilution plated to enumerate colony-forming units (CFU).

Intracellular trafficking

Bacterial intracellular trafficking was investigated by confocal laser scanning microscopy as described previously (Lamberti et al. 2010) with minor modifications. Briefly, 7-day-polarized monolayers grown on coated coverslips were incubated with wild type *B. pertussis* (MOI: 20) suspended in MEM supplemented with 0.2% w/v BSA 4 at 37ºC, washed three times with PBS to remove nonattached bacteria and incubated for 1 h at 37°C in MEM supplemented with 10% fetal bovine serum with 100 μg mL^{-1} of polymyxin B sulfate (Sigma), an antibiotic that cannot penetrate mammalian cells (Lee et al. 1990), to kill the remaining extracellular bacteria. Polymyxin B concentration was then reduced to 5 μg mL^{-1} until the end of the experiment. Samples of cells taken at 5 and 48 h post-infection were fixed in 4% w/v paraformaldehyde, washed with PBS and incubated for 10 min at room temperature with PBS containing 50 mM NH_4Cl. For immunofluorescence staining of intracellular and extracellular bacteria, surface-bound bacteria were detected by incubation of the cells with rabbit anti-*B. pertussis* serum for 30 min at room temperature, followed by incubation with FITC-conjugated goat F(ab′)2 fragments of anti-rabbit IgG for another 30 min at room temperature. For intracellular bacteria staining and colocalization with intracellular markers, after two washes with 0.2% BSA (PBS), the cells were permeabilized by incubation with PBS containing 0.1% w/v saponin and 0.2% w/v BSA for 30 min, followed by an incubation for 30 min at room temperature with either mouse anti-human LAMP-2 (CD107b) monoclonal antibodies (Pharmingen, San Diego, CA, USA) plus rabbit anti-*B. pertussis* serum or mouse anti-human cathepsin D monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) plus rabbit anti-*B. pertussis* serum in the presence of 0.1% w/v saponin and 0.2% (w/v) BSA. After three further washes, the cells were incubated with Cy3-conjugated F (ab′)2 fragments of goat anti-rabbit IgG and Alexa-647-conjugated donkey anti-mouse antibodies (Jackson ImmunoResearch) for 30 min at room temperature. Then coverslips were mounted with anti-fading agent (Invitrogen) and microscopical analyses were performed with a confocal laser scanning microscope (model TCS SP5; Leica). In all experiments, a minimum of 100 eukaryotic cells were examined per sample.

For transferrin uptake assays, infection was performed as described above and, at 5 and 48 h post-infection, the cells were depleted of transferrin by three washes and a subsequent incubation in MEM containing 1% BSA for 10 min and further incubated for 30 min with Alexa-Transferrin-594 (Molecular Probes) in an excess of BSA (1%) to saturate nonspecific endocytosis. Finally, the cells were fixed in 4% w/v paraformaldehyde and surface-bound and intracellular bacteria stained as described in this section. At least 50 intracellular bacteria per sample were analyzed for colocalization with transferrin in each experiment.

Colocalization analyses were conducted using ImageJ (http://rsb.info.nih.gov/ij/) and the "View5D" plugin (https://nanoimaging.de/View5D/) for manual determination. The analysis was performed for each phagosome, generating a histogram that combined information from the Cy3 (bacteria), and Alexa-647 (LAMP-1 or cathepsin D) or Alexa-594 (Transferrin) channels. Positive colocalization was established when the red histogram overlapped with the green histogram. Colocalization was further validated using the "Colocalization threshold" plugin (https://imagej.net/plugins/colocalization-threshold).

Fluorescence *in situ* hybridization and colocalization with lysosomes

Fluorescence *in situ* hybridization (FISH) studies were performed as previously described with minor modifications (Valdez et al. 2016). Briefly, 7-day-polarized monolayers were incubated with wild type *B. pertussis* (MOI: 20) in MEM supplemented with 0.2% w/v BSA at 37ºC with 5% CO_2. After 4 h of infection, cells were washed three times with PBS to remove nonattached bacteria and incubated for 1 h at 37°C in MEM supplemented with 10% fetal bovine serum with 100 μg mL^{-1} of polymyxin B sulfate (Sigma) to kill the remaining extracellular bacteria. After this incubation, polymyxin B concentration was reduced to 5 μg mL^{-1} until the end of the experiment. Forty-eight hours after infection, the cells were incubated with or without 200 nM LysoTracker DND-99 (Molecular Probes) for 15 min at 37°C and fixed in 4% w/v paraformaldehyde. Hybridization of intracellular bacteria in PFA-fixed infected cells with fluorescently labeled oligonucleotides was performed as described elsewhere (Valdez et al. 2016) with minor modifications. The Alexa 488-conjugated DNA probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) was designed for rRNA of eubacteria (Manz et al. 1993). A non-EUB338 DNA probe, complementary to EUB338, was used as a negative control. Bacterial detectability depends on the presence of enough ribosomes per cell, hence providing qualitative information on the physiological state of the bacteria on the basis of the number of ribosomes per cell. EUB338 or non-EUB388 was added to a final concentration of 10 nM each in the hybridization buffer. The hybridization

buffer contained 0.9 M NaCl, 20 mM Tris/HCl, pH 7.4, 0.01% (v/v) sodium dodecyl sulfate (SDS) and 35% (v/v) formamide. Hybridization was carried out for 2 h at 46ºC in a humid chamber, followed by a 30-min wash at 48°C. The washing buffer contained 80 mM NaCl, 20 mM Tris/HCl, pH 7.4, 0.01% (v/v) SDS and 5 mM EDTA (pH 8). The cells were mounted onto glass slides with anti-fading agent (Invitrogen) and microscopical analyses were performed with a confocal laser scanning microscope (model TCS SP5; Leica).

Intracellular survival

For intracellular survival, polymyxin B protection assays were performed as previously described (Lamberti et al. 2010). Briefly, 7-day-polarized monolayers were incubated with wild type *B. pertussis* (MOI: 20) in MEM supplemented with 0.2% w/v BSA at 37ºC with 5% CO_2. After 4 h of infection, cells were washed three times with PBS to remove nonattached bacteria and incubated for 1 h at 37°C in MEM supplemented with 10% fetal bovine serum with 100 μg mL^{-1} of polymyxin B sulfate to kill the remaining extracellular bacteria. After this incubation, polymyxin B concentration was reduced to 5 μg mL^{-1} until the end of the experiment. Five and 48 h after infection, the monolayers of cells were washed three times with PBS and incubated with trypsin-EDTA to detach the cells from the well. Next, the cells were lysed in sterile water, and serial dilutions of the lysates were rapidly plated onto bBGA plates to enumerate CFU. Viable intracellular bacteria were expressed as the number of viable bacteria per well.

Statistical analysis

The Student's t-test (95% confidence level) or ANOVA was used for statistical evaluation of the data. The significance of the differences between the mean values of the data analyzed by ANOVA was determined with the least-significant-difference test at a 95% confidence level. The results are shown as mean ± SD.

Results

Bordetella pertussis attachment to polarized epithelial cells

In order to investigate *B. pertussis* interaction with polarized epithelial cells we first set up the experimental model using 16HBE14o- cells. This immortalized human bronchial epithelial cell line has retained the characteristics of the original primary cells and is able to polarize *in vitro* (Cozens et al. 1994). As previously described (Grumbach et al. 2009), these cells developed a monolayer of cells with tight junctions between them after 7 days of *in vitro* culture on fibronectin and collagen-coated coverslips in submerged conditions (Fig. 1a). To investigate bacterial attachment, a monolayer of 7-day-polarized cells was incubated with *B. pertussis* at a MOI of 20 for 5 h at 37ºC, washed to remove nonattached bacteria, stained and analyzed by fluorescence microscopy. As can be seen in Fig. 1b, many bacteria (35 ± 4% of the bacteria, n = 3) were found attached near the intercellular boundaries and on top of the tight junctions, although this area is only a small fraction of the exposed surface.

Bordetella pertussis effect on the tight junctions

Previous studies have shown that purified adenylate cyclase toxin (CyaA) compromises tight junction integrity (Hasan et al. 2018). During infection, the presence of *B. pertussis* attached near the tight junctions might affect the integrity of these structures due to the local release of this virulence factor. In order to evaluate this hypothesis, we infected 7-day-polarized monolayers with wild type *B. pertussis* (Bp) or an isogenic mutant strain deficient for CyaA expression (BpΔCyaA) at a low multiplicity of infection (MOI: 0.2) and let the infection progress for 24 h. The monolayers were then washed, fixed and stained to address tight junction integrity by fluorescence microscopy. Fig. 2a shows that the wild type *B. pertussis* disrupted the staining pattern of tight junctions, whereas the CyaA-deficient mutant strain did not. These results were confirmed quantitatively by calculating the TiJOR for representative confocal images of each condition. As can be seen in Fig. 2b, the monolayer infection with the wild type strain caused the disruption of the occludin network, while the infection with the CyaA-deficient strain did not. These results indicate that *B. pertussis* has the ability to disrupt tight junction integrity in a CyaA-dependent manner.

We next investigated whether *B. pertussis* is able to cross the epithelial monolayer and the role of CyaA in the process. To this end, 16HBE14o- monolayers were grown in 3.0-μm pore-size semipermeable membranes and further incubated with either wild type *B. pertussis* or the isogenic strain defective for CyaA expression at a MOI of 20. The infecting bacteria were seeded in the apical compartment. Samples of the culture medium from the apical and the basolateral compartments were plated to enumerate CFU 5 h after infection. Fig. 2c shows that only the wild type strain was recovered from the basolateral compartment, indicating that *B. pertussis* was able to cross the epithelial monolayer in a CyaA-dependent way.

Bordetella pertussis internalization

Tight junction disruption exposes the basolateral membrane and the subcellular space of the epithelium. Previous studies have shown that many pathogens invade the epithelial cells more efficiently through the basolateral membrane compared with the apical membrane (Bucior et al. 2010, Bouwman et al. 2013, Wegele et al. 2020). Therefore, in order to address the eventual relevance of this tight junction disruption and basolateral membrane exposure, we next evaluated *B. pertussis* attachment to cells that expose this membrane. To this end, we used 7-day-polarized cells grown into small groups (islands) that allow evaluation of *B. pertussis* interaction with cells that expose apical and basolateral membranes (inner and outer cells of the island, respectively). The cells were incubated with *B. pertussis* (MOI: 20) for 5 h, washed and surface-attached, and internalized bacteria were discriminated by double-staining fluorescence microscopy. Fig. 3a shows the distribution of bacteria in these islands of polarized cells. Fig. 3b shows that the number of bacteria associated with the outer cells that expose the basolateral membrane was significantly higher than that associated with the inner cells. Taking into account that the outer cells expose a larger membrane surface area than the inner cells, the increased attachment might not necessarily mean a preference of *B. pertussis* for the basolateral membrane, although it does indicate an efficient attachment to this membrane. In addition to this high attachment, Fig. 3c further shows that the percentage of bacteria internalized by the outer cells was significantly higher than the percentage of bacteria internalized by the inner cells. Taken together, these results indicate that *B. pertussis* efficiently attaches to cells that expose basolateral membranes and that the bacterial internalization rate in these cells is higher than in those that only expose the apical membrane.

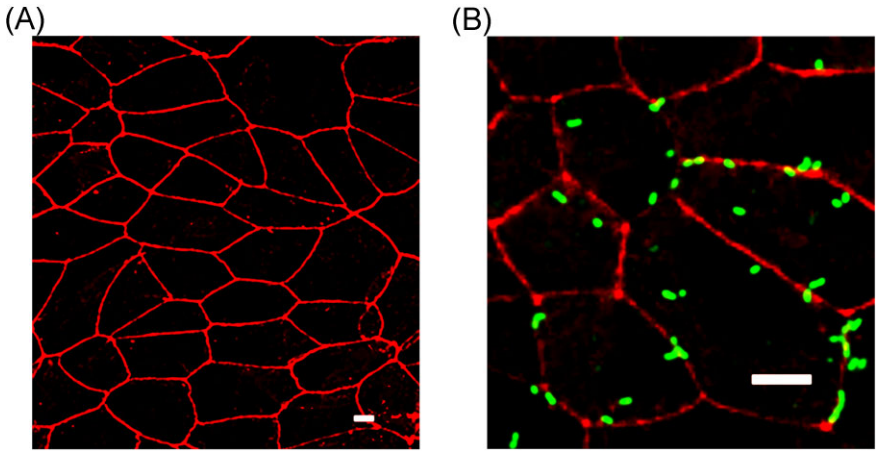

Figure 1. *Bordetella pertussis* attachment to polarized epithelial cells. Seven-day-polarized 16HBE14o- cells were incubated with wild type *B. pertussis* (MOI: 20) for 5 h at 37°C, washed with PBS and fixed with 4% paraformaldehyde. Bacteria were stained using rabbit polyclonal antibodies against *B. pertussis* followed by F(ab')2 fragments of FITC-conjugated goat anti-rabbit antibodies (green). Tight junctions were stained using mouse monoclonal antibodies against occludin followed by F(ab')2 fragments of Cy3-conjugated goat anti-mouse secondary antibodies (red). Panel (**A**) shows an uninfected monolayer with intact tight junctions. Panel (**B**) shows the bacteria attached to the monolayer. The images shown are representative of three independent experiments (scale bar: 5 μm)

Heparin inhibitable binding domain of FHA is involved in *B. pertussis* attachment to the basolateral membrane

FHA is the main adhesin of *B. pertussis*, and its importance in bacterial colonization of the respiratory tract is well known. In order to evaluate the relative contribution of FHA in the bacterial attachment to the basolateral and the apical membrane, islands of polarized cells were incubated with wild type *B. pertussis* (Bp) or an isogenic mutant strain lacking the expression of FHA (BpΔFHA). After 5 h of incubation at 37°C, the cells were washed to remove nonattached bacteria and stained to determine the number of attached bacteria by fluorescence microscopy. Because the attachment of the FHA-deficient strain proved to be significantly lower than the wild type strain, a higher MOI was used for BpΔFHA in order to get a reliable readout (namely, Bp MOI: 20; BpΔFHA MOI: 200). Fig. 4a shows that the bacterial attachment was significantly lower in the absence of FHA, even although the MOI used for this strain was 10 times higher than the MOI used for the wild type *B. pertussis*, confirming the central role of FHA in the bacterial attachment to these cells. In addition, Fig. 4a shows a greater decrease in bacterial attachment to the basolateral membrane compared with the apical membrane in the absence of FHA, highlighting the relevance of this adhesin for this interaction.

Previous studies identified different binding domains of FHA. Among them, a heparin-inhibitable lectin-like domain was found relevant in *B. pertussis* attachment to epithelial cells (Menozzi et al. 1991, Hannah et al. 1994, Menozzi et al. 1994). This binding domain interacts with sulfated polysaccharides that were found preferentially expressed in the basolateral membrane of polarized cells (Bucior et al. 2010). To gain some insight into the FHA-dependent *B. pertussis* attachment to basolateral membranes, we investigated the role of this domain in the bacterial attachment to polarized cells by competition assays using heparin. To this end, bacteria were incubated with 1 mg mL^{-1} heparin during 15 min prior to 5-h incubation (MOI: 20) with islands of 7-day-polarized cells in the presence of heparin. Control assays were performed incubating untreated bacteria with cells in the absence of heparin at the same multiplicity of infection. Bacterial attachment was analyzed by fluorescence microscopy. Fig. 4b shows that the presence of heparin determined a significant decrease in the bacterial attachment to the outer cells, while the bacterial attachment to the inner cells was not affected. This result suggests that the heparin-binding domain of FHA plays a central role in *B. pertussis* attachment to the basolateral membrane of epithelial cells. On the other hand, the absence of differences in bacterial attachment to inner cells in the presence or absence of heparin suggests that this domain is not particularly relevant for the bacterial attachment to the apical membrane.

Bordetella pertussis intracellular trafficking

In order to evaluate the fate of *B. pertussis* once internalized, we evaluated the bacterial intracellular trafficking by means of confocal microscopy. Seven-day-polarized cells were incubated with *B. pertussis* (MOI: 20) for 4 h, washed to remove nonattached bacteria and treated for 1 h with 100 μg mL^{-1} polymyxin B to kill extracellular bacteria. After this, antibiotic concentration was reduced to 5 μg mL^{-1} until the indicated time points. At 5 and 48 h post-infection, cells were fixed, stained and analyzed by confocal microscopy. Fig. 5 shows that ~90% of internalized bacteria colocalized with the late endosomal marker LAMP-2 5 h after infection. That percentage of colocalization with this specific marker remained without significant changes until the end of the experiment, at 48 h. The colocalization of *B. pertussis* with the lysosomal marker cathepsin D was low at 5-h post-infection and did not show significant changes over time. We then evaluated whether intracellular bacteria have access to extracellular nutrients via the recycling pathway. Forty-eight hours post-infection the cells were pulsed with Alexa-594-conjugated transferrin and its colocalization with *B. pertussis* was evaluated by confocal microscopy. Fig. 6 shows that *B. pertussis* colocalizes with transferrin 48 h post-infection, suggesting that the intracellular bacteria have access to extracellular nutrients through the host cell recycling pathway.

Taken together, these results suggest that the majority of intracellular bacteria avoid the trafficking to degradative lysosomes and remain in phagosomes with access to extracellular nutrients, even at long times post-infection.

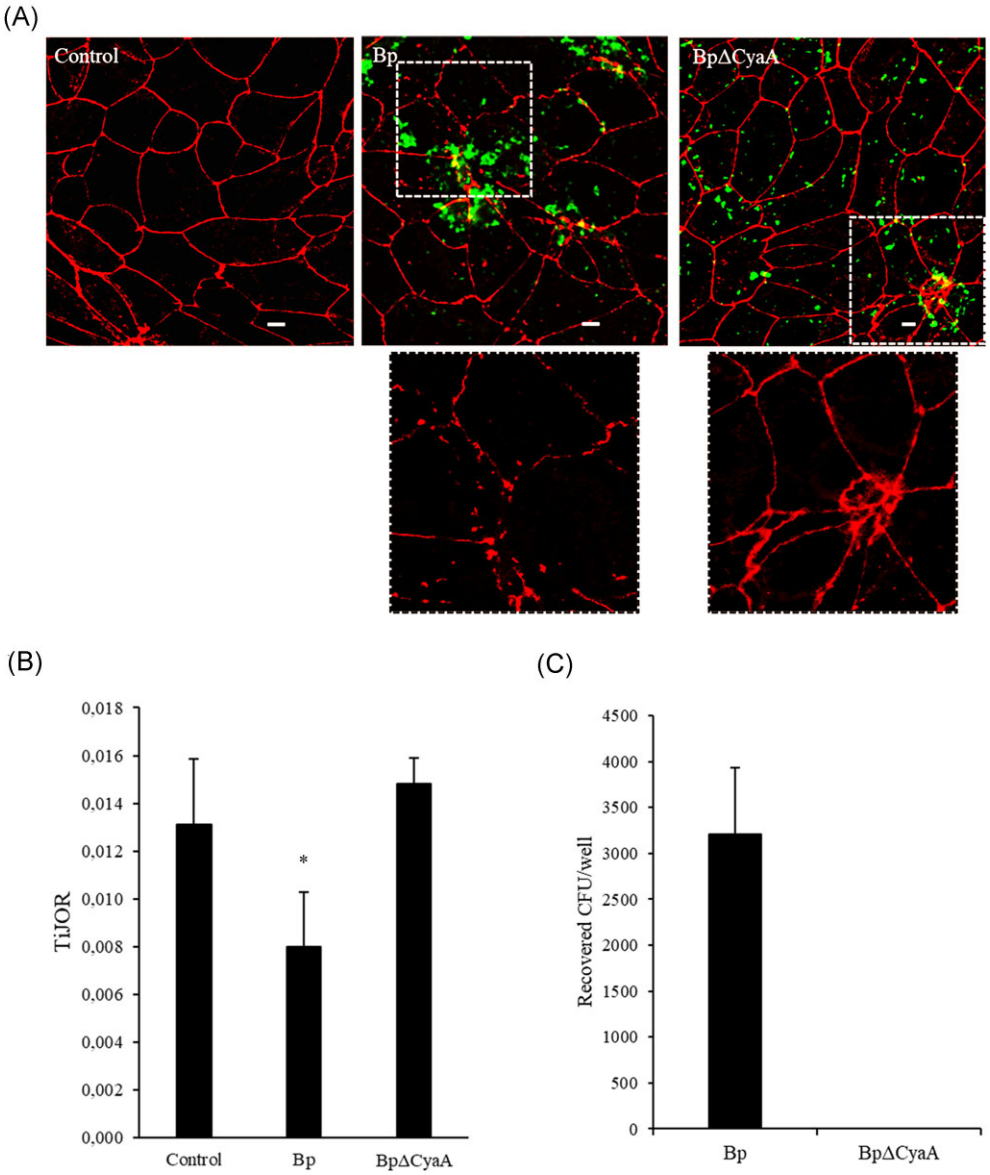

Figure 2. *Bordetella pertussis* effect on tight junctions. Seven-day-polarized 16HBE14o- cells were incubated with wild type *B. pertussis* (BpWT, MOI: 0.2) or an isogenic adenylate cyclase-deficient strain (BpΔCyaA, MOI: 0.2) for 24 h at 37°C, washed with PBS and fixed with 4% paraformaldehyde. Bacteria were stained using rabbit polyclonal antibodies against *B. pertussis* followed by $F(ab')_2$ fragments of FITC-conjugated goat anti-rabbit antibodies (green). Tight junctions were stained using mouse monoclonal antibodies against occludin followed by $F(ab')_2$ fragments of Cy3-conjugated goat anti-mouse antibodies (red). Panel (**A**) shows the occludin staining patterns and bacteria attached to the cells upon infection with wild type *B. pertussis* or the CyaA-deficient strain. Uninfected cells were used as the control. The images shown are representative of three independent experiments (scale bar: 5 μm). Panel (**B**) shows the tight junction organization rate (TiJOR) values for cells infected with wild type *B. pertussis* or the CyaA-deficient strain. Uninfected cells were used as the control. TiJOR values were calculated for representative images using ImageJ software. Seven-day-polarized 16HBE14o- grown on semipermeable inserts (3-μm pore size) were incubated with wild type *B. pertussis* or the CyaA-deficient strain (MOI: 20). Five hours after infection, samples from the basolateral compartment were plated to enumerate colony-forming units (CFU). Panel (**C**) shows the number of CFU/well recovered for each strain. Means ± SD of three independent experiments are shown in each case (∗ $P < 0.05$)

Bordetella pertussis intracellular survival

Because the results obtained in the intracellular trafficking studies suggested that *B. pertussis* might persist alive inside these epithelial cells, we next investigated the viability of internalized bacteria by means of FISH staining at 48 h post-infection. To this end, 7-day-polarized cells were incubated with *B. pertussis* (MOI: 20) for 4 h, washed to remove nonattached bacteria and treated for 1 h with 100 μg mL^{-1} polymyxin B to kill extracellular bacteria. After this, antibiotic concentration was reduced to 5 μg mL^{-1} until the end of the experiment. At 48 h post-infection, cells were pulsed with LysoTracker™, fixed and metabolically active bacteria were stained by FISH and analyzed by confocal microscopy. Fig. 7a shows that 48 h after infection there are intracellular bacteria metabolically active that do not colocalize with the acidotropic dye LysoTracker™.

We next evaluated the bacterial intracellular survival by polymyxin B protection assays. To this end, 7-day-polarized cells were incubated with *B. pertussis* (MOI: 20) for 4 h, washed to remove nonadherent bacteria and treated for 1 h with 100 μg mL^{-1} polymyxin B to kill extracellular bacteria. After this, antibiotic concentration was reduced to 5 μg mL^{-1} and cell samples were taken at 5, 24 and 48 h post-infection. At each time point, cells

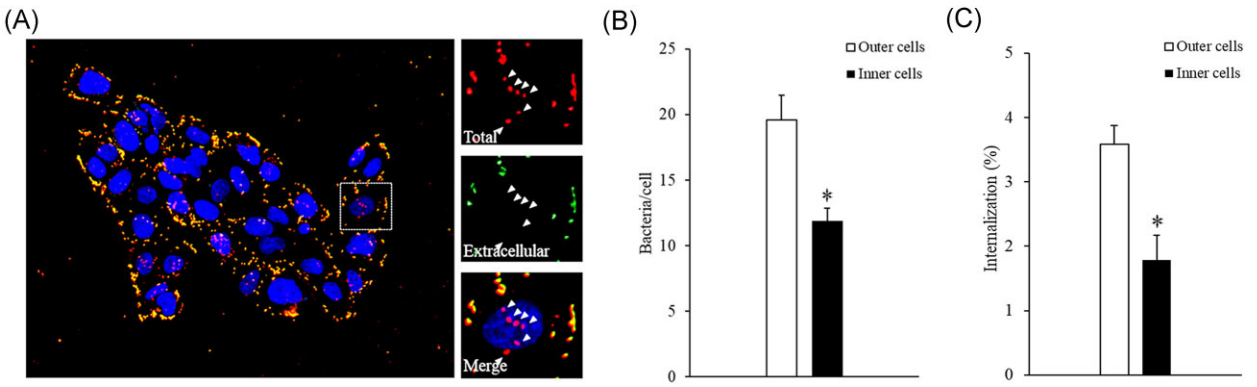

Figure 3. *Bordetella pertussis* internalization. Seven-day-polarized 16HBE14o- cells grown into islands were incubated with wild type *B. pertussis* (MOI: 20) for 5 h at 37°C, washed with PBS and fixed with 4% paraformaldehyde. Surface-bound bacteria were stained using rabbit polyclonal antibodies against *B. pertussis* followed by $F(ab')_2$ fragments of FITC-conjugated goat anti-rabbit antibodies (shown in green). To determine intracellular bacteria the cells were permeabilized and the bacteria were stained using rabbit polyclonal antibodies against *B. pertussis* followed by $F(ab')_2$ fragments of Cy3-conjugated goat anti-rabbit antibodies (shown in red). Cell nuclei were stained using DAPI (shown in blue). Panel (**A**) shows the distribution of cell-associated *B. pertussis*. In the right panels, internalized bacteria are indicated with arrowheads. Images representative of three independent experiments are shown. Panel (**B**) shows the number of total bacteria associated with the inner and outer cells. Panel (**C**) shows the percentage of total attached bacteria that were internalized by inner and outer cells. Means ± SD of three independent experiments are shown in each case. Significant differences in the number of bacteria/cell and in the internalization percentage were observed between the inner and outer cells (* $P < 0.05$)

were detached, lysed and serial dilutions were plated to enumerate CFU counts. Fig. 7b shows that the intracellular survival remained high over the whole time of the experiment, indicating that bacterial intracellular killing was very low. This result is in agreement with the low bacterial colocalization with lysosomal marker cathepsin D and the colocalization with transferrin over the time post-infection.

Discussion

Bordetella pertussis incidence has increased over recent years despite the high vaccination coverage. One of the reasons proposed for this resurgence is the transmission from infected, usually asymptomatic, individuals vaccinated with currently used vaccines (Althouse and Scarpino 2015, de Graaf et al. 2020). Although these vaccines efficiently prevent the symptoms of the disease, they fail to prevent host colonization and transmission (Warfel et al. 2014). Furthermore, the immunity induced by these vaccines wanes rapidly (Mooi et al. 2014) and adults who do not receive booster doses have low or undetectable antibody levels, unless recently exposed to the pathogen. The lack of antibodies allows *B. pertussis* attachment to the respiratory epithelium (Van Den Berg et al. 1999, Rodríguez et al. 2006) and prevents the induction of an efficient bactericidal response by immune cells (Lamberti et al. 2008, 2010, Kroes et al. 2022), eventually enabling the infection and the pathogen persistence within the host.

The airway epithelium is the first barrier against pathogen invasion and plays a major role in host immunity. Successful pathogens, however, developed different strategies to subvert these defense mechanisms and develop airway infections (Bertuzzi et al. 2019). The results of the present study reveal new aspects of *B. pertussis* interaction with respiratory epithelial cells that might play a relevant role in the infectious process. Our results suggest a potential mechanism of cellular infection comprising bacterial attachment next to the tight junctions followed by the disruption of these structures by bacterial toxins and the subsequent attachment to and internalization mainly through the basolateral membrane. After internalization, this pathogen seems able to survive intracellularly within nondegradative vesicles.

We here found that *B. pertussis* binds around or on top of the tight junctions in polarized monolayers. This attachment pattern has been observed in others such as *P. aeruginosas* (Lee et al. 1999) and enteropathogenic *Escherichia coli* (Aroeti et al. 2012). The factors that mediate this attachment pattern are not completely understood for those pathogens. In the case of *B. pertussis*, the documented lipid raft enrichment near tight junctions (Nusrat et al. 2000) might help to explain the observed tropism because they have been reported as *B. pertussis*-docking platforms in cellular membranes (Lamberti et al. 2009).

Our results showed that, after attachment, *B. pertussis* disrupts the tight junctions in a CyaA-dependent way. Previous studies performed with purified CyaA have already shown that this toxin affects the transepithelial electrical resistance of cell monolayers by disrupting these structures (Hasan et al. 2018). In this study, we found that CyaA delivered by the attached bacteria disrupts tight junctions. Studies performed in semipermeable inserts showed that the wild type *B. pertussis*, but not the CyaA-defective mutant, was able to cross the epithelial barrier at early times post-infection. Although we cannot rule out an eventual contribution of bacterial transcellular transport across the epithelial monolayer in this experimental set-up, these results are consistent with the tight junction disruption mediated by CyaA shown by our results and those obtained by others (Hasan et al. 2018). The bacterial binding near junctional complexes might enable a local concentration of CyaA high enough to disrupt cell-to-cell unions. Tight junction disruption exposes the basolateral membranes and the sub-epithelial space. The results presented in this study show that *B. pertussis* attaches to cells exposing the basolateral membrane and are internalized more efficiently through this membrane. In this scenario, CyaA opening of tight junctions might promote infection by increasing the exposed cell surface and by favoring internalization through the basolateral membrane. Although FHA was found to be involved in bacterial attachment to both the apical and basolateral membranes, our findings suggest a stronger dependence of bacterial attachment to the basolateral membrane on FHA compared with the attachment to the apical membrane. FHA contains different binding domains that mediate bacterial attachment to the different targets on the host cells. The better known domains are an Arg-Gly-Asp (RGD) tripeptide involved in

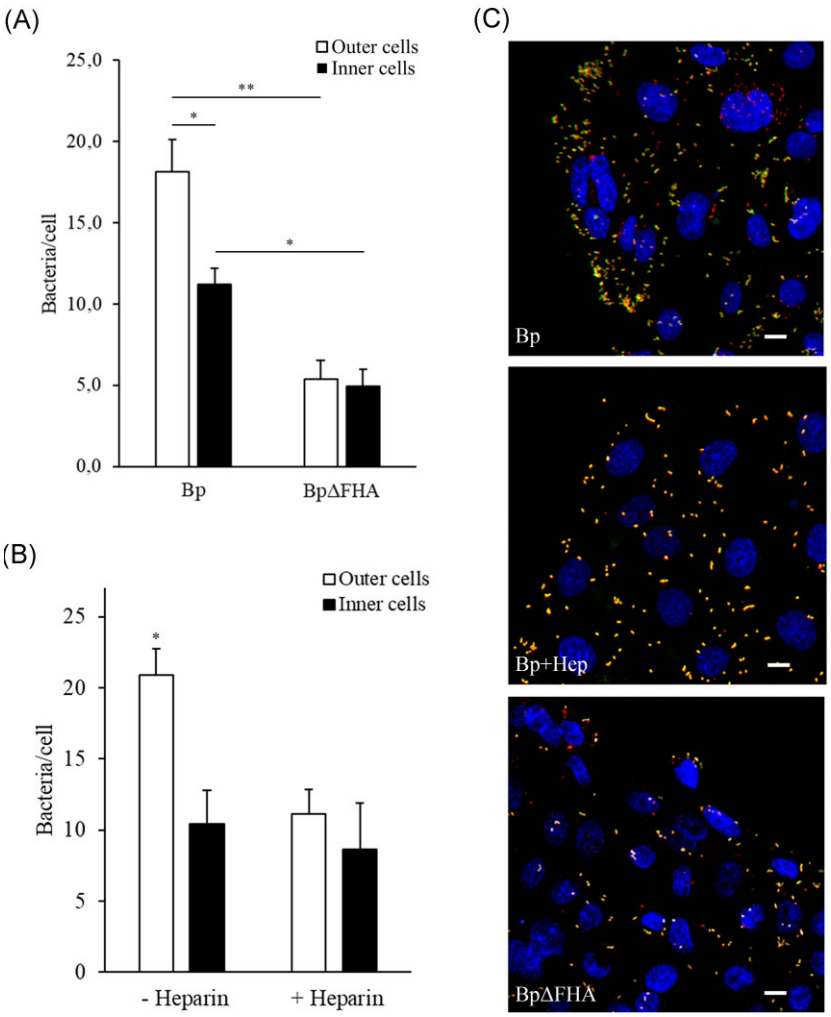

Figure 4. Heparin inhibitable binding domain of FHA is involved in *B. pertussis* attachment to the basolateral membrane. Seven-day-polarized 16HBE14o- cells grown in islands were incubated with wild type *B. pertussis* (BpWT, MOI: 20) or an isogenic FHA-deficient mutant (BpΔFHA, MOI: 200) for 5 h at 37°C, washed with PBS and fixed with 4% paraformaldehyde. Panel (**B**): *Bordetella pertussis* previously incubated with or without 1 mg mL^{-1} heparin for 15 min (+ Heparin and – Heparin, respectively) were incubated for 5 h at 37°C with islands of 7-day-polarized 16HBE14o- cells in the presence or absence of 1 mg mL^{-1} of heparin, respectively. The cells were then washed with PBS and fixed with 4% paraformaldehyde. In both cases, surface-bound bacteria were stained using rabbit polyclonal antibodies against *B. pertussis* followed by $F(ab')_2$ fragments of FITC-conjugated goat anti-rabbit antibodies (green). To determine intracellular bacteria, the cells were permeabilized and the bacteria were stained using rabbit polyclonal antibodies against *B. pertussis* followed by $F(ab')_2$ fragments of Cy3-conjugated goat anti-rabbit antibodies. Cell nuclei were stained using DAPI (shown in blue). Panel (**A**) shows the number of total bacteria per inner or outer cell for BpWT and BpΔFHA. Panel (**B**) shows the number of total bacteria per inner or outer cell for BpWT in the presence or absence of heparin. Means ± SD of four independent experiments are shown in each case (* $P < 0.05$; ** $P < 0.01$). Panel (**C**) shows representative images of the distribution of cell-associated *B. pertussis* in each case

attachment to macrophages and monocytes (Relman et al. 1990, Ishibashi et al. 1994); a carbohydrate recognition domain involved in attachment to macrophages and epithelial cells (Prasad et al. 1993); a mature C-terminal domain proposed to mediate attachment to immune and epithelial cells (Julio et al. 2009); and a domain that displays a lectin-like activity that targets heparin and other sulfated polysaccharides (Menozzi et al. 1991). Recent studies suggested that the FHA–heparin interaction could be relevant for *B. pertussis* attachment to macrophages (Golshani et al. 2022). Our results suggest that this interaction is also involved in the bacterial attachment to the basolateral membrane as the presence of heparin specifically reduced bacterial attachment to cells exposing this membrane, while attachment levels remained unchanged in cells only exposing apical membranes. This is in agreement with the reported polarized expression of sulfated polysaccharides that were found mainly present in the basolateral membrane (Bucior et al. 2010). Accordingly, the presence of heparin did not affect the bacterial attachment to the apical membrane. We assume that the bacterial attachment to the apical membrane is mainly mediated by other domains of FHA because, although the presence of heparin did not affect the bacterial attachment to this membrane, the lack of expression of FHA also determined a significant drop in bacterial attachment to cells, exposing only the apical membrane. As found for other pathogens (Bucior et al. 2010, Bouwman et al. 2013, Pedersen et al. 2017, Wegele et al. 2020), the bacterial internalization through the basolateral membrane was also higher compared with the internalization through the apical membrane. In the case of *B. pertussis*, the basolateral expression of β_1 subunit of $\alpha_5\beta_1$ integrin (VLA-5) in polarized epithelial cells (Pilewski et al. 1997, Roger et al. 1999) might be involved in this differential internalization rate. This integrin was found involved in *B. pertussis* internalization upon interaction with the RGD do-

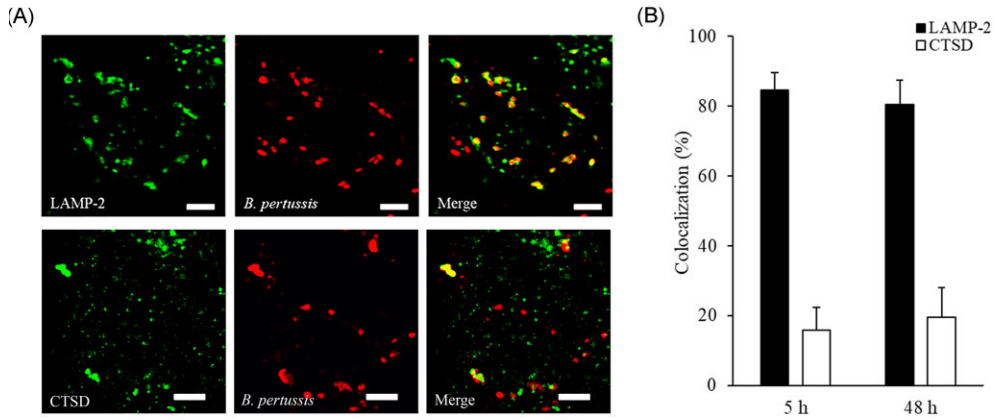

Figure 5. *Bordetella pertussis* intracellular trafficking. Seven-day-polarized 16HBE14o- cells were incubated with wild type *B. pertussis* (MOI: 20) for 4 h at 37°C, washed three times with PBS and further incubated with 100 μg mL^{-1} polymyxin B for another hour. After this, the cells were incubated with 5 μg mL^{-1} polymyxin B until the indicated time points. At these time points, samples of cells were washed with PBS and fixed with 4% paraformaldehyde and permeabilized. Bacteria were stained using rabbit polyclonal antibodies against *B. pertussis* followed by F(ab')$_2$ fragments of Cy3-conjugated goat anti-rabbit antibodies (shown in red). LAMP-2 (CD107b) was stained with mouse monoclonal antibodies followed by F(ab')$_2$ fragments of Alexa-647-conjugated donkey anti-mouse antibodies (shown in green). Cathepsin D (CTSD) was stained with mouse monoclonal antibodies followed by F(ab')$_2$ fragments of Alexa-647-conjugated donkey anti-mouse antibodies (shown in green). Panel (**A**) shows representative confocal images showing bacterial colocalization with LAMP-2 and cathepsin D 48 h post-infection. Panel (**B**) shows the percentage of internalized *B. pertussis* colocalizing with each marker. Means ± SD of five independent experiments are shown in each case (scale bar: 5 μm)

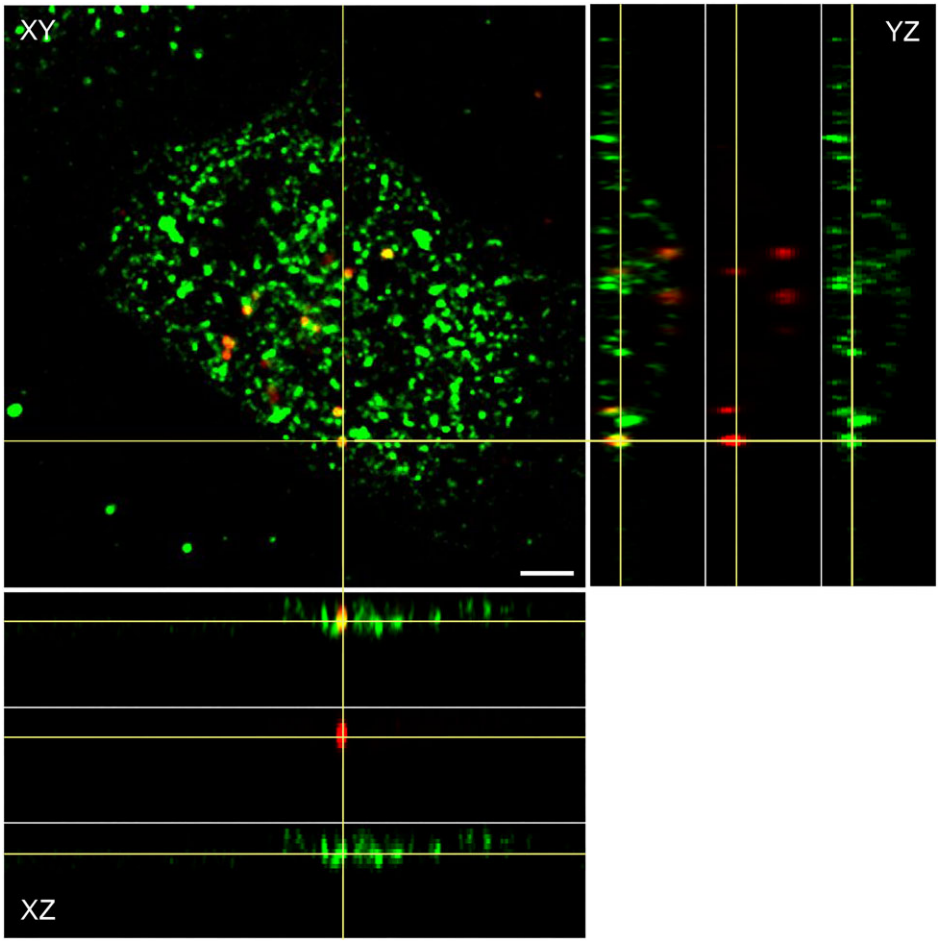

Figure 6. *Bordetella pertussis* access extracellular nutrients. Seven-day-polarized 16HBE14o- cells were incubated with wild type *B. pertussis* (MOI: 20) for 4 h at 37°C, washed three times with PBS and further incubated with 100 μg mL^{-1} polymyxin B for another hour. After this, cells were incubated with 5 μg mL^{-1} polymyxin B. Forty-eight hours after infection the cells were pulsed with Alexa-594-conjugated transferrin (green) for 30 min, fixed, and the bacteria were stained as stated in Fig. 5. *Bordetella pertussis* colocalization with transferrin 48 h post-infection is seen in yellow. Twenty-three confocal planes were acquired by confocal microscopy and processed using ImageJ software to obtain a Z-stack. Orthogonal views (XZ and YZ) show an intracellular bacteria colocalizing with transferrin (scale bar: 5 μm)

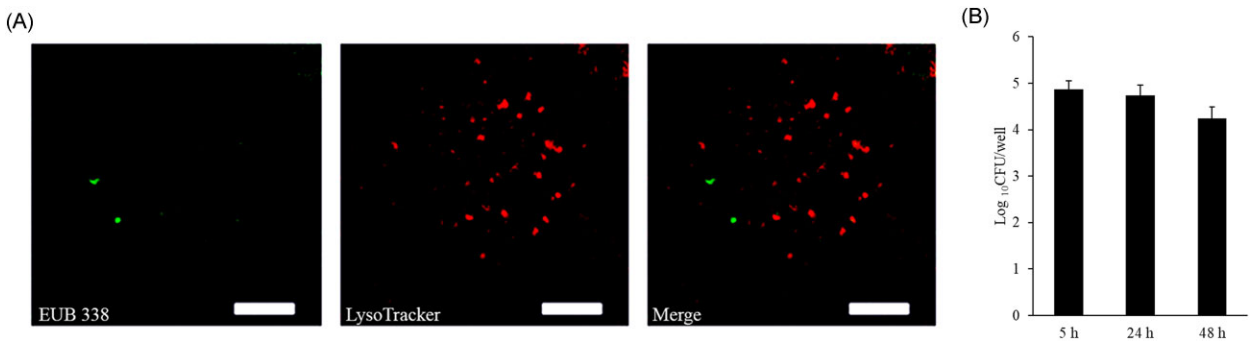

Figure 7. *Bordetella pertussis* fluorescence *in situ* hybridization and intracellular survival. Seven-day-polarized 16HBE14o- cells were incubated with wild type *B. pertussis* (MOI:20) for 4 h at 37°C, washed and further incubated with 100 μg mL^{-1} polymyxin B for another hour. The cells were then incubated with 5 μg mL^{-1} polymyxin B until the indicated time points. For fluorescence *in situ* hybridization and colocalization with LysoTracker™, samples of cells were pulsed with the acidotropic marker for 15 min and subsequently fixed with 4% paraformaldehyde. After this, intracellular viable bacteria were green fluorescent-labeled by fluorescent *in situ* staining with an Alexa-488-conjugated EUB-338 probe. For Polymyxin B protection assays, after antibiotic treatment, the cells were detached at the indicated time points by trypsin-EDTA treatment, lysed with 0.1% p/v saponin and serial dilution plated to determine CFU counts. Panel (**A**) shows metabolically active *B. pertussis* stained with EUB338-Alexa-488 probe (shown in green) and lysosomes labeled with LysoTracker™ (shown in red) at 48 h post-infection (scale bar: 5 μm). Panel (**B**) shows the mean number of intracellular CFU at each time point. Means ± SD of three independent experiments are shown

main of FHA in alveolar and bronchial epithelial cells (Ishibashi et al. 2001). Taking into consideration these previous results and ours, a two-step mechanism for basolateral attachment and internalization of *B. pertussis* in polarized epithelial cells might be proposed. This mechanism would comprise an initial bacterial–cell interaction mainly mediated by heparan sulfate-derived proteoglycans, followed by the bacterial internalization with the eventual involvement of $\alpha_5\beta_1$ integrin. A similar mechanism was proposed for *Chlamydia* spp. involving a first interaction with heparan sulfate proteoglycans that eventually leads to a subsequent binding to different host receptors, such as mannose receptor, epidermal growth factor receptor, ephrin receptor A2 and β_1 integrin, leading to the pathogen internalization (Di Pietro et al. 2019).

Once internalized, most of the bacteria followed a nonbactericidal pathway because 48 h after internalization a high percentage of the internalized *B. pertussis* were still alive, as determined by polymyxin B protection assays. Accordingly, our results showed that intracellular bacteria have access to extracellular nutrients and are metabolically active at long times post-infection. After internalization, most of the intracellular bacteria seem able to inhibit phagolysosomal fusion, as found before in immune cells (Lamberti et al. 2016, Valdez et al. 2016). Only ∼20% of the internalized bacteria were found in degradative lysosomes positive for cathepsin D 48 h after infection. Because most of the intracellular bacteria were found in LAMP-2 positive phagosomes, the high intracellular survival seems to indicate that the bacteria might remain alive in vesicles decorated only with late endosomal markers. It was previously shown that LAMP-1/2 and cathepsin D markers do not always colocalize (Cheng et al. 2018), suggesting the existence of nondegradative LAMP-1/2 positive vesicles. Many bacterial pathogens that survive within epithelial cells reside in late endosomal vesicles that do not fuse with lysosomes. Among them, *C. jejuni* (Bouwman et al. 2013), *Burkholderia cenocepacia* (Sajjan et al. 2006), *Helicobacter pylori* (Terebiznik et al. 2006) and uropathogenic *E. coli* (Mysorekar and Hultgren 2006) are examples of pathogens that are able to remain alive within epithelial cells inside vesicles that acquire late endosomal markers such as LAMP-1/2, but not lysosomal markers like cathepsins. The high intracellular survival observed in this study, together with previous studies reporting intracellular survival in cell lines (Bassinet et al. 2000, Gueirard et al. 2005, Lamberti et al. 2013) and in primary airway models (Sivarajan et al. 2021), seem to indicate that the respiratory epithelium might constitute a previously underestimated niche of persistence. The bacterial ability to survive inside these cells could favor the persistence in the host respiratory tract by providing a safe environment where this pathogen can avoid immune defense mechanisms during the development of the infectious process. Because *B. pertussis* was previously described as being able to repopulate the extracellular medium once released from the intracellular compartment (Lamberti et al. 2013), it is tempting to speculate that this safe intracellular environment could be a stage in the infection process after which *B. pertussis* could infect other cells such as macrophages or even another host.

As stated, apart from constituting a physical barrier, the airway epithelium is also a relevant player in host immunity. While both the phagocytic capacity and the antimicrobial activity of epithelial cells are considerably lower than those displayed by professional phagocytes, the vast number of epithelial cells would determine a rather relevant contribution to pathogen uptake and degradation, unless the pathogen is able to hijack the host defensive response. The results presented here suggest that *B. pertussis* is able to evade intracellular killing, therefore exploiting internalization by the airway epithelial cells as a means to persist and/or disseminate the infection. This ability to survive in the intracellular location could be relevant during respiratory tract colonization, contributing to explaining the difficulties in controlling *B. pertussis* infection and highlighting the need for better vaccines to prevent the development of intracellular infections of this pathogen.

Author contributions

Carlos Manuel Baroli (Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing), Juan Pablo Gorgojo (Conceptualization, Funding acquisition, Investigation, Writing – review & editing), Bruno Martín Blancá (Investigation), Martina Debandi (Investigation) and Maria Eugenia Rodriguez (Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing)

Acknowledgments

JPG and MER. are members of the Scientific Career of CONICET. CMB and MD are doctoral fellows of CONICET and BMB is a researcher of UNLP.

Conflict of interest: The authors declare no conflicts of interest.

Funding

This work was supported by the National Agency for Scientific and Technological Promotion (ANPCyT-MINCyT) [PICT 2016 2363 to MER, PICT-2019-04296 to JPG] and La Plata National University (www.unlp.edu.ar) [Grant 11/X811 to MER].

References

Althouse BM, Scarpino SV. Asymptomatic transmission and the resurgence of bordetella pertussis. *BMC Med* 2015;**13**:146.

Anderson JM, Van Itallie CM. Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol* 2009;**1**:a002584.

Aroeti B, Friedman G, Zlotkin-Rivkin E *et al.* Retraction of enteropathogenic E. coli type IV pili promotes efficient host cell colonization, effector translocation and tight junction disruption. *Gut Microbes* 2012;**3**:267–71.

Bassinet L, Gueirard P, Maitre B *et al.* Role of adhesins and toxins in invasion of human tracheal epithelial cells by Bordetella pertussis. *Infect Immun* 2000;**68**:1934–41.

Bertuzzi M, Hayes GE, Bignell EM. Microbial uptake by the respiratory epithelium: outcomes for host and pathogen. *FEMS Microbiol Rev* 2019;**43**:145–61.

Bouwman LI, Niewold P, van Putten JPM. Basolateral invasion and trafficking of Campylobacter jejuni in polarized epithelial cells. *PLoS One* 2013;**8**:e54759.

Bucior I, Mostov K, Engel JN. Pseudomonas aeruginosa-mediated damage requires distinct receptors at the apical and basolateral surfaces of the polarized epithelium. *Infect Immun* 2010;**78**:939–53.

Bucior I, Pielage JF, Engel JN. Pseudomonas aeruginosa Pili and Flagella mediate distinct binding and signaling events at the apical and basolateral surface of airway epithelium. *PLoS Pathog* 2012;**8**:e1002616. https://doi.org/10.1371/journal.ppat.1002616.

Burns JL, Jonas M, Chi EY *et al.* Invasion of respiratory epithelial cells by Burkholderia (Pseudomonas) cepacia. *Infect Immun* 1996;**64**:4054–9.

Cerny O, Kamanova J, Masin J *et al.* Bordetella pertussis adenylate cyclase toxin blocks induction of bactericidal nitric oxide in macrophages through cAMP-dependent activation of the SHP-1 phosphatase. *J Immunol* 2015;**194**:4901–13.

Cheng XT, Xie YX, Zhou B *et al.* Characterization of LAMP1-labeled nondegradative lysosomal and endocytic compartments in neurons. *J Cell Biol* 2018;**217**:3127–39.

Cherry JD, Grimprel E, Guiso N *et al.* Defining pertussis epidemiology: clinical microbiologic and serologic perspectives. *Pediatr Infect Dis J* 2005;**24**:S25–34.

Clarke TB, Francella N, Huegel A *et al.* Invasive bacterial pathogens exploit TLR-mediated downregulation of tight junction components to facilitate translocation across the epithelium. *Cell Host Microbe* 2011;**9**:404–14.

Clementi CF, Håkansson AP, Murphy TF. Internalization and trafficking of nontypeable Haemophilus influenzae in human respiratory epithelial cells and roles of IgA1 proteases for optimal invasion and persistence. *Infect Immun* 2014;**82**:433–44.

Cozens AL, Yezzi MJ, Kunzelmann K *et al.* CFfR expression and chloride secretion in polarized immortal Human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1994;**10**:38–47.

de Graaf H, Ibrahim M, Hill AR *et al.* Controlled human infection with bordetella pertussis induces asymptomatic, immunizing colonization. *Clin Infect Dis* 2020;**71**:403–11.

Di Pietro M, Filardo S, Romano S *et al.* Chlamydia trachomatis and Chlamydia pneumoniae interaction with the host: latest advances and future prospective. *Microorganisms* 2019;**7**:140. https://doi.org/10.3390/microorganisms7050140.

Duff C, Murphy PG, Callaghan M *et al.* Differences in invasion and translocation of Burkholderia cepacia complex species in polarised lung epithelial cells in vitro. *Microb Pathog* 2006;**41**:183–92.

Esposito S, Stefanelli P, Fry NK *et al.* Pertussis prevention: reasons for resurgence, and differences in the current acellular pertussis vaccines. *Front Immunol* 2019;**10**:1–11.

Golshani M, Rahman WU, Osickova A *et al.* Filamentous hemagglutinin of Bordetella pertussis does not interact with the $\beta(2)$ integrin CD11b/CD18. *Int J Mol Sci* 2022;**23**:12598. https://doi.org/10.3390/ijms232012598.

Grumbach Y, Quynh NVT, Chiron R *et al.* LXA4 stimulates ZO-1 expression and transepithelial electrical resistance in human airway epithelial (16HBE14o-) cells. *Am J Physiol-Lung Cell Mol Physiol* 2009;**296**:L101–8.

Gueirard P, Bassinet L, Bonne I *et al.* Ultrastructural analysis of the interactions between Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica and human tracheal epithelial cells. *Microb Pathog* 2005;**38**:41–6.

Halldorsson S, Gudjonsson T, Gottfredsson M *et al.* Azithromycin maintains airway epithelial integrity during Pseudomonas aeruginosa infection. *Am J Respir Cell Mol Biol* 2010;**42**:62–8.

Hannah JH, Menozzi FD, Renauld G *et al.* Sulfated glycoconjugate receptors for the Bordetella pertussis adhesin filamentous hemagglutinin (FHA) and mapping of the heparin-binding domain on FHA. *Infect Immun* 1994;**62**:5010–9.

Hasan S, Kulkarni NN, Asbjarnarson A *et al.* Bordetella pertussis adenylate cyclase toxin disrupts functional integrity of bronchial epithelial layers. *Infect Immun* 2018;**86**. https://doi.org/10.1128/IAI.00445-17.

Hasan S, Sebo P, Osicka R. A guide to polarized airway epithelial models for studies of host–pathogen interactions. *FEBS J* 2018;**285**:4343–58.

Hellwig SMM, Hazenbos WLW, Van de Winkel JGJ *et al.* Evidence for an intracellular niche for bordetella pertussis in broncho- alveolar lavage cells of mice. *FEMS Immunol Med Microbiol* 1999;**26**:203–7.

Ishibashi Y, Claus S, Relman DA. Bordetella pertussis filamentous hemagglutinin interacts with a leukocyte signal transduction complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). *J Exp Med* 1994;**180**:1225–33.

Ishibashi Y, Relman DA, Nishikawa A. Invasion of human respiratory epithelial cells by Bordetella pertussis: possible role for a filamentous hemagglutinin Arg-Gly-Asp sequence and $\alpha 5\beta 1$ integrin. *Microb Pathog* 2001;**30**:279–88.

Jakub N, David J, Irena L *et al.* A mutation upstream of the rplN-rpsD ribosomal operon downregulates bordetella pertussis virulence factor production without compromising bacterial survival within human macrophages. *Msystems* 2020;**5**:e00612–20.

Julio SM, Inatsuka CS, Mazar J *et al.* Natural-host animal models indicate functional interchangeability between the filamentous haemagglutinins of Bordetella pertussis and Bordetella bronchiseptica and reveal a role for the mature C-terminal domain, but not the RGD motif, during infection. *Mol Microbiol* 2009;**71**:1574–90.

Kessie DK, Lodes N, Oberwinkler H *et al.* Activity of tracheal cytotoxin of bordetella pertussis in a human tracheobronchial 3D tissue model. *Front Cell Infect Microbiol* 2021;**10**:1–15.

Kroes MM, van Vliet LC, Jacobi RHJ *et al.* Long lasting antibodies from convalescent Pertussis patients induce ROS production and bacterial killing by human neutrophils. *Front Cell Infect Microbiol* 2022;**12**:888412.

Kügler S, Böcker K, Heusipp G *et al.* Pertussis toxin transiently affects barrier integrity, organelle organization and transmigration of monocytes in a human brain microvascular endothelial cell barrier model. *Cell Microbiol* 2007;**9**:619–32.

Lamberti Y, Cafiero JH, Surmann K *et al.* Proteome analysis of bordetella pertussis isolated from human macrophages. *J Proteomics* 2016;**136**:55–67.

Lamberti Y, Gorgojo J, Massillo C *et al.* Bordetella pertussis entry into respiratory epithelial cells and intracellular survival. *Pathog Dis* 2013;**69**:194–204.

Lamberti Y, Hayes JA, Perez Vidakovics ML *et al.* Cholesterol-dependent attachment of human respiratory cells by Bordetella pertussis. *FEMS Immunol Med Microbiol* 2009;**56**:143–50.

Lamberti Y, Perez Vidakovics ML, van der Pol LW *et al.* Cholesterol-rich domains are involved in Bordetella pertussis phagocytosis and intracellular survival in neutrophils. *Microb Pathog* 2008;**44**:501–11.

Lamberti YA, Hayes JA, Perez Vidakovics ML *et al.* Intracellular trafficking of bordetella pertussis in human macrophages. *Infect Immun* 2010;**78**:907–13.

Lee A, Chow D, Haus B *et al.* Airway epithelial tight junctions and binding and cytotoxicity of Pseudomonas aeruginosa. *Am J Physiol-Lung Cell Mol Physiol* 1999;**277**:L204–17. https://doi.org/10.1152/ajplung.1999.277.1.l204.

Lee CK, Roberts AL, Finn TM *et al.* A new assay for invasion of HeLa 229 cells by Bordetella pertussis: effects of inhibitors, phenotypic modulation, and genetic alterations. *Infect Immun* 1990;**58**:2516–22.

Locht C, Geoffroy MC, Renauld G. Common accessory genes for the Bordetella pertussis filamentous hemagglutinin and fimbriae share sequence similarities with the papC and papD gene families. *EMBO J* 1992;**11**:3175–83.

Malet JK, Hennemann LC, Hua EM-L *et al.* A model of intracellular persistence of Pseudomonas aeruginosa in airway epithelial cells. *Cell Microbiol* 2022;**2022**:1.

Manz W, Szewzyk U, Ericsson P *et al.* In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. *Appl Environ Microb* 1993;**59**:2293–8.

Martin DW, Mohr CD. Invasion and intracellular survival of Burkholderia cepacia. *Infect Immun* 2000;**68**:3792.

Menozzi FD, Gantiez C, Locht C. Interaction of the Bordetella pertussis filamentous hemagglutinin with heparin. *FEMS Microbiol Lett* 1991;**78**:59–64.

Menozzi FD, Mutombo R, Renauld G *et al.* Heparin-inhibitable lectin activity of the filamentous hemagglutinin adhesin of Bordetella pertussis. *Infect Immun* 1994;**62**:769–78.

Mooi FR, Van Der Maas NAT, De Melker HE. Pertussis resurgence: waning immunity and pathogen adaptation—two sides of the same coin. *Epidemiol Infect* 2014;**142**:685–94.

Mysorekar IU, Hultgren SJ. Mechanisms of uropathogenic Escherichia coli persistence and eradication from the urinary tract. *P Natl Acad Sci USA* 2006;**103**:14170–5.

Nusrat A, Parkos CA, Verkade P *et al.* Tight junctions are membrane microdomains. *J Cell Sci* 2000;**113**:1771–81.

Pedersen GA, Jensen HH, Schelde ASB *et al.* The basolateral vesicle sorting machinery and basolateral proteins are recruited to the site of enteropathogenic E. coli microcolony growth at the apical membrane. *PLoS One* 2017;**12**:1–21.

Petráčková D, Farman MR, Amman F *et al.* Transcriptional profiling of human macrophages during infection with Bordetella pertussis. *RNA Biology* 2020;**17**:731–42.

Pilewski JM, Latoche JD, Arcasoy SM *et al.* Expression of integrin cell adhesion receptors during human airway epithelial repair in vivo. *Am J Physiol* 1997;**273**:L256–63.

Prasad SM, Yin Y, Rodzinski E *et al.* Identification of a carbohydrate recognition domain in filamentous hemagglutinin from Bordetella pertussis. *Infect Immun* 1993;**61**:2780–5.

Relman D, Tuomanen E, Falkow S *et al.* Recognition of a bacterial adhesin by an integrin: macrophage CR3 (αMβ2, CD11b CD18) binds filamentous hemagglutinin of Bordetella pertussis. *Cell* 1990;**61**:1375–82.

Rodríguez ME, Hellwig SMM, Pérez Vidakovics MLA *et al.* Bordetella pertussis attachment to respiratory epithelial cells can be impaired by fimbriae-specific antibodies. *FEMS Immunol Med Microbiol* 2006;**46**:39–47.

Roger P, Puchelle E, Bajolet-Laudinat O *et al.* Fibronectin and alpha5beta1 integrin mediate binding of Pseudomonas aeruginosa to repairing airway epithelium. *Eur Respir J* 1999;**13**:1301–9.

Saha C, Nigam SK, Denker BM. Involvement of Gαi2 in the maintenance and biogenesis of epithelial cell tight junctions*. *J Biol Chem* 1998;**273**:21629–33.

Sajjan US, Yang JH, Hershenson MB *et al.* Intracellular trafficking and replication of Burkholderia cenocepacia in human cystic fibrosis airway epithelial cells. *Cell Microbiol* 2006;**8**:1456–66.

Sivarajan R, Kessie DK, Oberwinkler H *et al.* Susceptibility of human airway tissue models derived from different anatomical sites to bordetella pertussis and its virulence factor adenylate cyclase toxin. *Front Cell Infect Microbiol* 2021;**11**:797491.

Terebiznik MR, Vazquez CL, Torbicki K *et al.* Helicobacter pylori VacA toxin promotes bacterial intracellular survival in gastric epithelial cells. *Infect Immun* 2006;**74**:6599–614.

Terryn C, Sellami M, Fichel C *et al.* Rapid method of quantification of tight-junction organization using image analysis. *Cytometry Pt A* 2013;**83A**:235–41.

Valdez HA, Marin Franco JL, Gorgojo JP *et al.* Human macrophage polarization shapes B. pertussis intracellular persistence. *J Leukoc Biol* 2022;**112**:173–84. https://doi.org/10.1002/JLB.4A0521-254R.

Valdez HA, Oviedo JM, Gorgojo JP *et al.* Bordetella pertussis modulates human macrophage defense gene expression. *Pathog Dis* 2016;**74**:1–14.

Van Den Berg BM, Beekhuizen H, Mooi FR *et al.* Role of antibodies against Bordetella pertussis virulence factors in adherence of Bordetella pertussis and Bordetella parapertussis to human bronchial epithelial cells. *Infect Immun* 1999;**67**:1050–5.

Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *P Natl Acad Sci USA* 2014;**111**:787–92.

Wegele C, Stump-Guthier C, Moroniak S *et al.* Non-typeable haemophilus influenzae invade choroid plexus epithelial cells in a polar fashion. *Int J Mol Sci* 2020;**21**:1–24.

Received 29 May 2023; **accepted** 30 November 2023