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Modeling Persistent *Mycoplasma pneumoniae* Infection of Human Airway Epithelium

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Summary

Mycoplasma pneumoniae is a human respiratory tract pathogen causing acute and chronic airway disease states that can include long-term carriage and extra-pulmonary spread. The mechanisms of persistence and migration beyond the conducting airways, however, remain poorly understood. We previously described an acute exposure model using normal human bronchial epithelium (NHBE) in air-liquid interface culture, showing that *M. pneumoniae* gliding motility is essential for initial colonization and subsequent spread, including localization to epithelial cell junctions. We extended those observations here, characterizing *M. pneumoniae* infection of NHBE for up to four weeks. Colonization of the apical surface was followed by pericellular invasion of the basolateral compartment and migration across the underlying transwell membrane. Despite fluctuations in transepithelial electrical resistance and increased NHBE cell desquamation, barrier function remained largely intact. Desquamation was accompanied by epithelial remodeling that included cytoskeletal reorganization and development of deep furrows in the epithelium. Finally, *M. pneumoniae* strains S1 and M129 differed with respect to invasion and histopathology, consistent with contrasting virulence in experimentally infected mice. In summary, this study reports pericellular invasion, NHBE cytoskeletal reorganization, and tissue remodeling with persistent infection in a human airway epithelium model, providing clear insight into the likely route for extra-pulmonary spread.

Introduction

Mycoplasma pneumoniae is a bacterial pathogen of the human conducting airway, causing pharyngitis, tracheobronchitis, bronchiolitis, croup, and atypical pneumonia (Esposito et al., 2004; Waites and Talkington, 2004). *M. pneumoniae* is thought to account for up to 40% of all community-acquired pneumonia and is the leading cause of pneumonia in older children and young adults (Waites and Talkington, 2004), but the true prevalence of *M. pneumoniae* disease is difficult to determine due to the limitations of current detection methods (Daxboeck et al., 2003). Airway disease progression is typically slow in both onset and recovery, with mycoplasmas often persisting in the respiratory tract for long periods during

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convalescence. *M. pneumoniae* can also exhibit a prolonged carrier state where mycoplasmas are detected by PCR, and yet culture and serology are negative (Foy, 1993; Kraft et al., 2002; Meyer Sauter et al., 2014; Nilsson et al., 2008; Spuesens et al., 2013). Moreover, long-term sequelae such as asthma and chronic obstructive pulmonary disease, and extra-pulmonary spread to diverse sites, including neural and cutaneous tissue and synovial and pericardial fluid, are common (Waites and Talkington, 2004). However, the mechanisms of persistence and extra-pulmonary spread remain poorly understood and understudied.

Normal human airway epithelium is differentiated and pseudo-stratified, with tight junctions maintaining normal barrier function and cell polarity. Mycoplasma colonization initiates on the apical surface of airway epithelium, whereas epithelial nutrient acquisition occurs via the basolateral compartment. A variety of tissue and organ culture systems have been used to model *M. pneumoniae* pathogenesis *in vitro*. These include both human and other mammalian primary cultures, cell lines derived from respiratory or other tissue, and mammalian tracheal rings and tracheal explant cultures (Chen and Krause, 1988; Collier and Clyde, 1971; Gabridge, 1983; Gabridge, 1984; Krunkosky et al., 2007; Muse et al., 1976). However, these primary cells, cell lines, and tissue explants use submersion culture, hence in most cases host cells are not polarized and mycoplasma infection occurs at the same host cell surface as nutrient acquisition. Tracheal explants exhibit airway cell differentiation, albeit incomplete and poorly characterized. Organ culture models are fully differentiated but require submersion, which limits their value in reproducing the microenvironment that mycoplasmas normally encounter in the airways.

We utilize primary normal human bronchial epithelium (NHBE) cells in air-liquid interface (ALI) culture to model *M. pneumoniae* colonization of the airways (Jordan et al., 2007; Krunkosky et al., 2007; Prince et al., 2014). ALI-grown NHBE cells present a polarized, pseudo-stratified and fully differentiated mucociliary epithelium, allowing initiation of infection on the apical surface, which is exposed to air, in the same manner as occurs with aerosol transmission in the human airways. Using this model we previously demonstrated that mycoplasma gliding is essential to overcome mucociliary defenses and characterized temporal and spatial features of *M. pneumoniae* infection over the first four hours, where mycoplasmas initially localize to the tips of cilia, then accumulate transiently at the base of the cilia, and gradually spread laterally, with an affinity for intercellular junctions (Jordan et al., 2007; Prince et al., 2014).

In the current study we examined *M. pneumoniae* – NHBE cell interaction in ALI culture for up to four weeks post-infection, observing pericellular invasion of the basolateral compartment by *M. pneumoniae* with subsequent localization to the lower transwell chamber. Barrier function remained largely intact despite fluctuations in transepithelial electrical resistance and increased NHBE cell desquamation. Mycoplasmas were observed associated with desquamated cells, which is consistent with the presence of mycoplasma-infected epithelial cells in sputum from patients with mycoplasma airway disease (Collier and Clyde, 1974). Desquamation was accompanied by epithelial remodeling, including histochemical evidence of cytoskeletal reorganization and development of deep furrows in the epithelium. Finally, invasion and histopathology differed for *M. pneumoniae* clinical

isolates exhibiting contrasting virulence in experimentally infected mice (Techasaensiri et al., 2010).

Results

M. pneumoniae persistence and invasion of NHBE cultures

We assessed *M. pneumoniae* color-changing units (CCU) over time as a measure of persistence in NHBE ALI cultures (Fig. 1, **Apical**). We also assessed CCU levels in the lower transwell chamber for evidence of mycoplasma migration from the NHBE apical surface, across the epithelial barrier (Fig. 1, **Basal**). Epithelium-associated CCU data likely underestimated true *M. pneumoniae* numbers for two reasons. First, trypsin treatment to dissociate the monolayer resulted in some loss of mycoplasma viability (data not shown). Second, it was necessary to remove the apical gel layer mucus every third day to limit mucus accumulation and mimic the clearing effect of the mucociliary escalator *in vivo*. The mucus collected from infected NHBE cultures contained individual mycoplasmas and mycoplasmas associated with desquamated epithelial cells (see below). Nevertheless, the data clearly demonstrated *M. pneumoniae* persistence at high levels in association with epithelial cultures over the course of extended infections, with CCU levels for strains M129 (Fig. 1) and PO1 (data not shown) significantly higher than for S1.

CCU data for the lower transwell chambers also likely underestimated true mycoplasma numbers, as the NHBE growth medium in the lower chambers was replaced every third day. While CCU analysis does not discriminate between continuous mycoplasma migration to the lower chamber and subsequent growth therein, the non-permissive nature of the NHBE culture medium suggests that the latter is minimal. Mycoplasma strains M129 and PO1 were reproducibly detected in the lower chamber as early as 4h post-infection, and CCU levels generally increased over time (Fig. 1 **Basal**, and data not shown). In contrast, strain S1 was not detected in the lower transwell chamber until 24h post-infection and reached densities over time that were significantly lower, as much as 10^3 -fold, than were observed for M129 (Fig. 1) or PO1 (data not shown). This strain difference in migration to the lower transwell chamber prompted us to compare their gliding speeds. M129 and PO1 exhibited statistically similar speeds (0.318 ± 0.136 and 0.305 ± 0.103 $\mu\text{m}/\text{sec}$, respectively), while strain S1 was significantly slower (0.256 ± 0.104 $\mu\text{m}/\text{sec}$; $P < 0.01$).

NHBE barrier function remained largely intact during extended infection

This is the first published report of transepithelial migration by *M. pneumoniae in vitro*. In order to assess integrity of epithelial barrier function, we measured transepithelial electrical resistance (TEER) of control and *M. pneumoniae*-infected cultures (Fig. 2). TEER for uninfected NHBE cultures ranged from 550 to 1805 $\Omega\text{-cm}^2$ over time in multiple experiments employing primary cells originating from two different individual donors. TEER values for all strains, infection doses, and time-points up to two weeks post-infection were generally at least 90% of control values. Furthermore, we often noted TEER values 20-30% higher than corresponding uninfected controls for all strains at several time points (Fig. 2 and data not shown). However, by day 18 and beyond (Fig. 2 and data not shown), TEER values typically fell below corresponding uninfected controls, though still reflecting

substantial barrier function ($>400 \Omega\text{-cm}^2$) compared to chambers without epithelial cells ($150\text{-}180 \Omega\text{-cm}^2$). Differences between mycoplasma strains were not statistically significant.

NHBE cell desquamation

To mimic the mucociliary escalator *in vivo* and limit accumulation of gel-layer mucus, the apical surface of NHBE cultures was washed with Hank's Balanced Salt Solution (HBSS) every third day throughout the study. At each time point replicate apical washes were pooled and assessed by cytopsin for desquamated epithelial cells. Some desquamation is normal as a result of cell turnover, but microscopic analysis revealed that desquamation was considerably more severe for *M. pneumoniae*-infected cultures (Table 1). Desquamated cells for control cultures were generally larger in size individually and in small clusters, while *M. pneumoniae*-infected cultures were smaller in size with larger clusters and sheets of epithelia that often failed to exhibit normal nuclear staining (Fig. 3). In addition, the cytopsin of apical washes demonstrated the presence of both cell-free mycoplasmas (data not shown) and mycoplasmas adherent to desquamated epithelium, where they were frequently associated with the periphery of epithelial cells (Fig. 3, **arrows**). Mycoplasmas present in these luminal washings were viable, based on a color change within 4d in SP-4 growth medium, suggesting a high infection burden. Despite increasing levels of desquamation over time, at no point during the course of infection did we observe basal growth medium in the apical compartment, which is consistent with our TEER results and indicates retention of significant barrier function.

Epithelial remodeling during *M. pneumoniae* infection

We used immunohistochemical analysis of beta tubulin and F-actin to investigate topographical changes at the NHBE surface. Cultures were examined under low magnification to identify areas of irregular beta tubulin staining. These regions of interest were then analyzed by confocal microscopy for F-actin labeling using topographical volume display (Fig. 4). Topographical confocal microscopy revealed that both M129- and S1-infected cultures developed striking epithelial furrows as early as 7d post-infection. These epithelial furrows were not detected in control cultures and increased in size and depth through 28d. Optical sections closest to the upper surface of the transwell membranes revealed basal-like cells at the bottom of furrows at day 28 post-infection (data not shown). Cross-sectional z-plane image microscopy of these furrows for multiple immunohistochemical targets revealed a high density of *M. pneumoniae* in these regions for both S1 and M129 (Fig. 5A). The yellow reflects co-localization of *M. pneumoniae* (red) and beta tubulin (green), which represents the apical surface of the airway cells and corresponds primarily to cilia. This co-localization of *M. pneumoniae* and deep furrowing may facilitate mycoplasma migration across the epithelium. However, cross-sectional imaging of cultures as early as 4h post-infection also revealed evidence of *M. pneumoniae* penetration of the epithelium prior to furrow development (Fig. 5B, **red arrows**) and was consistent with the detection of *M. pneumoniae* in the lower transwell chambers at this early time point.

Cross-sectional z-plane imaging with immunohistochemical staining revealed substantial actin reorganization in mycoplasma-infected NHBE cultures, based on phalloidin staining of

F-actin (Fig. 5A, purple). We investigated actin reorganization in infected NHBE cultures further by using depth-coding analysis (Fig. 6). Color was assigned to F-actin fluorescence depth from the apical (magenta) to basolateral (blue) surface. The actin cytoskeleton in uninfected control cultures exhibited distinct polarity, localizing primarily near the luminal surface, as expected (magenta). In contrast, infected NHBE cultures exhibited substantial actin reorganization that extended 40-60µm inward, including prominent regions that spanned from the luminal to the basolateral surface.

Discussion

M. pneumoniae localization to the ciliated epithelium of the human airways is well documented (Collier and Clyde, 1971) and consistent with manifestations of mycoplasma respiratory disease (Waites and Talkington, 2004). While the advent of *in vitro* tissue and organ culture made it possible to explore the mechanisms of *M. pneumoniae* pathogenesis in the airways (Collier and Clyde, 1971; Dallo and Baseman, 2000; Kannan and Baseman, 2006; Lipman and Clyde, 1969; Muse et al., 1976; Somerson et al., 1965; Yavlovich et al., 2004), such studies typically employed primary or immortalized cells grown in submersion culture, which severely limits the extent to which they accurately reflect actual host-pathogen interactions in the human airways. We use NHBE cells in ALI culture to overcome this limitation, providing a superior model for *M. pneumoniae* pathogenesis under conditions that more closely mimic natural infections.

We recently described spatial and temporal features of *M. pneumoniae* interaction with NHBE cultures during the first 4h post-infection (Prince et al., 2014). Mycoplasmas infecting the luminal surface traverse the gel layer mucus, adhere to the tips of the cilia, and migrate rapidly to the cilia base (Prince et al., 2014). Lateral spread follows, more slowly than expected and perhaps influenced by tethered mucins or pattern-recognition receptors in the periciliary layer (Button et al., 2012; Kesimer et al., 2013; Shimizu et al., 2005; Shimizu et al., 2007). In the current study we followed *M. pneumoniae* infection of NHBE ALI cultures for up to 28d, revealing that initial colonization led to direct invasion of the basolateral compartment and was accompanied by increased epithelial desquamation and reorganization of the normal pseudostratified columnar epithelium. *M. pneumoniae* persistence for up to 28d in this model contrasts strikingly with the rapid loss of epithelial integrity when NHBE ALI cultures are infected with rhinovirus or influenza (T. Krunkosky, unpublished). Our findings thus underscore the value of NHBE ALI cultures to model early events in infection, to explore factors that may contribute to persistence, and to do so independent of potential influence from local inflammatory cells such as granulocytes, lymphocytes, or macrophages that would reside in the underlying lamina propria of the airway epithelium *in vivo*.

This is the first published report of *M. pneumoniae* invasion of the basolateral compartment of human airway epithelium. Our previous observation that *M. pneumoniae* often localizes to the cell periphery in the early stages of infection (Prince et al., 2014) suggests that mycoplasmas accessed the basolateral compartment, at least initially, via a pericellular route across epithelial tight junctions, which is consistent with cross-sectional z-plane images (Fig. 5B). A similar *M. pneumoniae* affinity for intercellular boundaries was likewise noted

with hamster tracheal rings in submersion culture (Muse et al., 1976). The concurrent, high TEER measurements suggest that any potential disruption of tight junctions to allow access to the basolateral compartment was localized rather than generalized. Alternatively, *M. pneumoniae* may exploit normal junctional remodeling that occurs with active extrusion of senescing epithelial cells, as reported for *Listeria monocytogenes* invasion at multicellular junctions (Pentecost et al., 2006). We saw no evidence of intracellular invasion or trans-cellular migration, although we did not explore this possibility exhaustively. Mycoplasma access to the basolateral compartment may also have been facilitated by epithelial remodeling and the coordinated formation of epithelial furrows, based on the high mycoplasma density commonly observed in these regions. Both mechanisms likely require mycoplasma gliding motility, which is supported by *in vivo* animal studies (Hara et al., 1974; Hardy et al., 2002; Szczepanek et al., 2012). Regardless of the route, migration to the basal lamina might provide a favorable site for nutrient acquisition and immune evasion and potentiate spread to extra-pulmonary sites, as occurs in a significant number of cases (Waites and Talkington, 2004).

Over the course of this study, *M. pneumoniae* strains M129 and S1 consistently differed both in the overall infection burden that persisted in NHBE cultures and the levels of mycoplasma migration to the basal transwell chamber (Fig. 1). The significantly slower gliding speed of S1 likely contributed to a lower colonization efficiency compared to M129 and PO1 (Fig. 1, **Apical**, and data not shown), consistent with our previous observations with the slow-gliding P200 mutant (Jordan et al. 2007; Prince et al., 2014). Lower colonization efficiency could in turn account for the difference in overall infection burden over time (Fig. 1, **Apical**). Potential differences in rate of desquamation could also account for lower apical CCU levels for S1, but this was not suggested by TEER results, where the two strains were comparable (Fig. 2). Finally, M129 and S1 differ by genotype (1 and 2, respectively), which might be significant given the difference in biofilms formed by these genotypes (Simmons et al., 2013). However, it is unlikely that genotype alone accounts for the strain differences observed here, because the PO1 strain (genotype 2) was indistinguishable from M129 in colonization of NHBE cultures (data not shown).

Desquamation of *M. pneumoniae*-infected airway epithelium has been reported previously, both with tracheal ring organ culture and in human sputum (Carilli et al., 1970; Collier and Clyde, 1974; Muse et al., 1976; Pierce and Hirsch, 1958; Woodruff et al., 1973), further underscoring the relevance of the NHBE ALI model. Desquamated cells in those reports exhibited a degenerating appearance, with cell pathology supporting a primary role for mycoplasma CARDS toxin (Kannan and Baseman, 2006). This was likewise the case here, where desquamated cells from infected cultures were observed in large clumps and sheets, with smaller cell size and abnormal nuclear staining compared to uninfected controls. Epithelial desquamation and remodeling impacts mucociliary barrier efficacy and is thus consistent with both altered mucociliary clearance for up to one year post diagnosis of *M. pneumoniae* respiratory disease (Jarstrand et al., 1974), as well as the frequency of *M. pneumoniae* co-infection with other respiratory tract pathogens (Chiu et al. 2015), including children with acute asthma perturbation (Meza et al., 2016). Collier and Clyde (1974) reported culture of up to 10^6 mycoplasma colony-forming units per ml of sputum from

patients with *M. pneumoniae* infections. Mycoplasma association with desquamated epithelium may facilitate horizontal transmission to new hosts, as well as spread to adjacent airway epithelium. Regarding the latter, it was common here to observe heavily infected epithelium adjacent to other regions largely free of mycoplasmas (data not shown). The reason for this pattern is currently not known but may reflect variability in the thickness of the overlying gel layer mucus.

TEER levels reflected retention of considerable barrier function over the course of these infection studies. Furthermore, we consistently observed transient increases in TEER to levels that were significantly higher than their corresponding uninfected controls. Epithelial tight junctions are, out of necessity, dynamic structures having the capacity for continual remodeling with no reduction in overall integrity (Steed et al., 2010). Whether the transient increases in TEER also correlate with upregulation of tight junction components in response to *M. pneumoniae* infection is not known. Recent studies suggest that one possible mechanism by which the epithelium actively responds to environmental stresses is through epithelial-to-mesenchymal transition (EMT) (Nowrin et al., 2014). EMT is a process by which epithelial cells lose their polarity and cell adhesions to gain migratory and invasive properties, becoming more like mesenchymal cells. Airway studies *in vivo* demonstrate that epithelial cell responses from microbes, pollutants and allergens can induce EMT, resulting in disruptions within the mucosal barrier (Hackett, 2012; Khalmuratova et al., 2017; Pain et al., 2014). The epithelial furrows seen here as a part of a coordinated epithelial remodeling during persistent mycoplasma infection may involve EMT. Confocal microscopy demonstrated co-localization of *M. pneumoniae* with the epithelial surface within the furrows. These regions also exhibited an increase in F-actin, as demonstrated by the increase in phalloidin staining. Two cell markers that change in association with EMT are F-actin and vimentin (Ponnusamy et al., 2013; Yan et al., 2010). Actin forms cytoskeletal microfilaments and is essential to many important cellular processes, including cellular migration. Actin exists in two forms in cells: monomeric globular G-actin and its polymerized counterpart, F-actin. Our observed increase in F-actin in epithelial furrows in *M. pneumoniae*-infected cultures is consistent with EMT. Vimentin is a cellular intermediate filament which also plays an active role in regulating and reorganizing epithelial cellular architecture during EMT. Our preliminary data suggest that vimentin mRNA is altered at 14 and 28 days post-infection (data not shown), consistent with the epithelial remodeling seen at these time points by confocal microscopy as possible EMT.

The vicious circle hypothesis for chronic respiratory infection suggests that insults to the mucociliary apparatus impair mucociliary clearance, leading to increased bacterial colonization. Bacterial persistence then leads to direct airway damage via bacterial byproducts and indirect damage via the inflammatory response, and this in turn impairs clearance and resolution (Cole, 1989). Our observations from extended *M. pneumoniae* infection of NHBE cultures are consistent with this hypothesis. Sustained desquamation and epithelial remodeling over the course of *M. pneumoniae* infection compromises further clearance, and perhaps in conjunction with invasion of the epithelium enables the persistence that is a hallmark of *M. pneumoniae* infections.

Experimental Procedures

Mycoplasma strains and culture conditions

Wild-type *M. pneumoniae* strains M129 (17th broth passage) (Lipman and Clyde, 1969); S1 (Kannan et al., 2005); and PO1 (Simmons et al., 2013) were each originally isolated from a human infection but differ in biofilm phenotype and virulence in experimentally infected mice (Simmons et al., 2013; Techasaensiri et al., 2010). Mycoplasmas were grown at 37° C in SP-4 medium (Tully et al., 1977) until the phenol red pH indicator turned orange, harvested in HBSS (Sigma-Aldrich), collected by centrifugation at $20,000 \times g$ at 4° C, and washed 1× in fresh HBSS. Mycoplasmas were then suspended in 6.0 ml HBSS, passed $10 \times$ through a syringe with a 25-gauge needle, and centrifuged $123 \times g$ for 5 min at 25° C to remove large clumps.

Mycoplasma gliding measurement

M. pneumoniae gliding was measured as described (Hasselbring et al., 2005) except that mycoplasmas were grown directly in chambered slides (Nunc Nalgene) in modified SP-4 medium (no phenol red; + 3% gelatin; pH 7.2) overnight at 37° C, then the spent medium was removed and replaced with fresh pre-warmed modified SP-4 medium. Motility of individual cells was assessed microscopically on a Leica DM IRB inverted microscope (Leica Microsystems) with a 100× oil-immersion objective, 1.4 numerical aperture, equipped with a Hamamatsu Orca ER CCD camera (Hamamatsu Photonics). Data were processed with Openlab version 5.5.0 (Perkin Elmer), using a minimum of 20 uninterrupted frames at a constant time interval along a collision-free path for 100 individual cells per strain. This software allows for movement tracking with time stamp capability for calculating distance traveled over time. Gliding frequency was determined as the number of motile cells per field divided by the total number of cells in the same field $\times 100\%$ ($n = 272$ cells per strain).

Mycoplasma infection of NHBE cultures

NHBE cells (Krunkosky et al., 2000) grown in 0.33 cm^2 transwells were cultured and infected as described previously (Prince et al., 2014) except that PneumaCult-ALI Medium (Stem Cell Technologies) was used at the beginning of ALI culture to accelerate terminal differentiation (LeDizet et al., 1998). Briefly, NHBE cells, considered terminally differentiated when $>70\%$ of the monolayer surface was ciliated as estimated visually (typically 15d after the shift to ALI culture) were washed apically with HBSS and incubated 4h at 37°C to allow mucus accumulation to a depth approximating that on intact airways. A 100 μl volume of HBSS alone (negative control) or HBSS with 10^4 - 10^6 *M. pneumoniae* color change units (CCU; Stemke and Robertson, 1982) was added apically to initiate infections (8 wells per strain, dilution, and time point). For some experiments, we included infections initiated with 10^3 or 10^9 CCU, as indicated. Mycoplasma suspensions were incubated on the apical surface of NHBE cultures for 4h at 37°C, after which the apical liquid was removed and the surface washed once with HBSS. Typically about 2% of the mycoplasmas in the inoculum remain NHBE cell-associated after 4h (Prince et al., 2014), the remainder being removed with the apical liquid and gel layer mucus at this point. Infected cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 ,

changing the cell culture medium in the basal chamber and washing the apical surface once with 200 μ l HBSS every 3d. Apical washes were collected and frozen (-20°C) for analysis of desquamation. At the indicated time points up to 28d post-infection the cell surface was washed with HBSS and barrier function measured as transepithelial electrical resistance (Prince et al., 2014).

Mycoplasma detection in transwells and basal chambers

Relative mycoplasma numbers in transwell chambers were measured as CCU. Briefly, transwells with infected airway cells were moved to a new lower chamber, and the upper chambers were washed with 200 μ l HBSS and incubated with 200 μ l of trypsin-EDTA (Sigma-Aldrich) per well for 15min at room temperature to disrupt the epithelium and dissociate the mycoplasmas. A 200- μ l volume of SP-4 medium was added per well, and the membrane surfaces were scraped to remove attached cells. The resulting suspensions were passed 10 $\times$ through a syringe with a 25 gauge needle to disperse cells, and replicate aliquots were analyzed for CCU, incubating for up to 10d at 37°C for CCU determination (Stemke and Robertson, 1982). The original basal chambers for each sample and time point were assessed similarly but did not require trypsin treatment to harvest the mycoplasmas.

Transepithelial electrical resistance

The integrity of the epithelial barrier was measured as TEER using the Epithelial Voltohmmeter (World Precision Instruments, Inc.) as described previously (Prince et al., 2014).

Assessment of NHBE cell desquamation

Apical surfaces for all treatments at each time point were washed with 200 μ l HBSS. These washes were pooled and collected for cytopins by centrifuging at 1,200 rpms for 5min in a Shandon cytospin (ThermoFisher Scientific). Following centrifugation, samples were air dried, stained with a Romanowsky stain and analyzed with an Olympus BX41 microscope. Slides were examined for individual epithelial cells and epithelial clusters/rafts, i.e. groups of cells that were adherent to each other and generally ranged in size from 5 to 20 cells. Slides were scored according to the number of individual cells and cell rafts. Alternatively, cells collected by cytospin were prepared for DAPI (4,6-diamidino-2-phenylindole; Sigma-Aldrich) stain and immunohistochemistry as described previously (Prince et al., 2014).

Confocal microscopy analysis of NHBE cultures

Cultures were prepared for immunohistochemistry as described previously (Prince et al., 2014) and probed apically as follows. DAPI was used for nuclear staining; beta tubulin, primarily in cilia, was detected with fluorescein isothiocyanate-conjugated mouse anti-beta tubulin (Abcam, Inc.; 1:100 or 1:250). *M. pneumoniae* cells were labeled with rabbit anti-*M. pneumoniae* serum (1:250) raised against whole organisms (Baseman et al., 1982) followed by goat anti-rabbit Ig conjugated with Cy3 (1:250; ThermoFisher Scientific); and F actin was labeled using Alexa Fluor 647 Phalloidin (1:200; ThermoFisher Scientific). Transwell supports were mounted on slides with 15 μ l of Slowfade Gold antifade mount (ThermoFisher Scientific), covered with no.1½, 22 mm square coverslip, and subsequently

sealed with nail polish. Slides were analyzed on a Nikon Instruments A1R confocal microscope using the CFI Plan Fluor 40× oil objective (1.3 numerical aperture) at 25°C on a floating stage. Optical sections were scanned and stacked for image reconstruction. Slides of uninfected cultures were used to set the lasers and determine background. Images were acquired and files saved in the Nikon Nd2 file format, and image analysis was conducted with the NIS-Elements viewer version 4.2 (Nikon Instruments) using the auto scale function for image enhancement. Analyzed images were saved as TIFF files in the viewer. Three-dimensional deconvolution in the NIS-Elements viewer version 4.2 was used to analyze 40 µm and 60 µm optical sections. Depth color coding was conducted on phalloidin-stained sections within the NIS-Elements viewer version 4.2 software following z-scan analysis with the luminal surface set to zero and the scan extending to a depth of 60 µm into the epithelium. Topographical volume displays of Z-series on control and infected NHBE cultures were generated with alpha blending using NIS-Elements.

Statistical analysis

Differences in CCU recovery and TEER were analyzed using multivariate ANOVA followed by Holm-Sidak *post hoc* pairwise comparisons of strains and doses per time point by using SigmaPlot (SYSTAT Software). Mycoplasma gliding velocities were assessed using the Student's t-test in SigmaPlot.

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Abbreviations

ALI	air-liquid interface
CCU	color-changing units
EMT	epithelial-to-mesenchymal transition
HBSS	Hank's Balanced Salt Solution
NHBE	normal human bronchial epithelium
TEER	transepithelial electrical resistance

Fig. 1. Mycoplasma CCU recovered from transwell chambers over extended NHBE infection
Mycoplasma CCU recovered from the apical (upper panel) and basal (lower panel) transwell chambers following NHBE cell infection with *M. pneumoniae* strains M129 or S1 at the inocula shown. Each bar represents the mean and standard deviation (n=6). *, P<0.05 between strains

Fig. 2. NHBE cell barrier function during extended infection with *M. pneumoniae*
TEER measurements for infected NHBE cells are presented relative to the corresponding uninfected NHBE control culture (dashed line) at that time point (% of Control). Each bar represents the mean and standard deviation (n = 6). *, P < 0.05

Fig. 3. Desquamated NHBE cells collected in apical washes from *M. pneumoniae*-infected and control NHBE cultures

The same field is shown in each row, as visualized by phase contrast microscopy (left panels); fluorescence microscopy after DAPI stain (center panels); and fluorescence microscopy after probing with *M. pneumoniae*-specific antiserum (right panels). In representative images from NHBE cultures 27 d post-infection with strain S1 (upper panels), desquamated cells generally appeared in large clusters and sheets, nuclear staining was faint and diffuse, and mycoplasmas were readily detected, often associated with the cell periphery (arrows). In contrast, desquamated cells from uninfected control epithelium at the same time point (lower panels) generally appeared individually or in small clusters, with distinct nuclear staining. Bar, 15 μ m.

Fig. 4. Topographical volume display of NHBE cultures with and without extended *M. pneumoniae* infection

Topographical volume displays of confocal Z-series for F-actin were utilized as an indicator of NHBE surface remodeling at the indicated time points during *M. pneumoniae* infection. Uninfected controls at the same time points were used as a baseline to assess topographical changes in the culture. Shown are representative apical views of NHBE control cultures, and cultures infected with *M. pneumoniae* strains M129 or S1 at 4h, 7-14d, and 28d post infection. Arrows indicate areas where furrows have developed.

Fig. 5. Immunohistochemical analysis of NHBE cultures with and without extended *M. pneumoniae* infection

(A) Representative cross-sections of control and infected NHBE cultures. The initial inoculum was 10^6 CCU for both strains. Green, beta tubulin (primarily associated with cilia); purple, F-actin; blue, DAPI; red, *M. pneumoniae*; yellow, co-localization of *M. pneumoniae* and beta tubulin staining; pink, co-localization of DAPI and *M. pneumoniae*. White ovals indicate examples of areas in control NHBE cultures with high cilia density and a well-organized apical actin cytoskeleton. White arrows indicate examples of areas of invagination. Note red and yellow staining in these areas, indicating *M. pneumoniae* alone and co-localizing with cilia, respectively. Yellow arrows, evidence of the actin cytoskeleton extending downward into the cells. Bar, 10 μ m. (B) Higher magnification illustrating mycoplasma (M129) migration to the basal lamina and beyond (red arrows).

Fig. 6. Depth-coding analysis of actin reorganization in NHBE cultures

F actin was primarily localized near the apical surface of uninfected NHBE cell control cells. In contrast, significant F actin reorganization was observed in NHBE cell cultures infected with strains M129, PO1, or S1. Color key to left indicates depth from the apical (magenta) to basal (blue) surface.

Table 1

NHBE cell desquamation levels for control and *M. pneumoniae* M129- or S1-infected cultures at the indicated times post-infection.

Group	Control	M129						S1					
		10 ⁵			10 ⁶			10 ⁵			10 ⁶		
Dose		Individual cells	Cell rafts	Individual cells	Cell rafts	Individual cells	Cell rafts	Individual cells	Cell rafts	Individual cells	Cell rafts	Individual cells	Cell rafts
4 h	+ ^a		+		+		+	+	+		+	+	+
1 d	+		+		+		+	+	+		+	+	+
2 d	+		+		+		+	+	+		+	+	+
5 d	+		+		+		+	+	+		++	++	+
15 d	+		+		++		++	++	++		++	++	+++
18 d	+		+		+++		+++	+++	+++		+++	+++	+++

^a +, < 10 individual cells or cell clusters present; ++, 10-50 individual cells or cell clusters present; +++, >50 cells or clusters present