Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Bacillus anthracis spores germinate extracellularly at air–liquid interface in an *in vitro* lung model under serum-free conditions

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Keywords
anthrax, *Bacillus anthracis*, germination, lung epithelial, spore, Sterne.

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2015/0511: received 10 March 2015, revised 2 May 2015 and accepted 29 May 2015

doi:10.1111/jam.12872

Abstract

Aims: To better understand the parameters that govern spore dissemination after lung exposure using *in vitro* cell systems.
Methods and Results: We evaluated the kinetics of uptake, germination and proliferation of *Bacillus anthracis* Sterne spores in association with human primary lung epithelial cells, Calu-3 and A549 cell lines. We also analysed the influence of various cell culture medium formulations related to spore germination.
Conclusions: We found negligible spore uptake by epithelial cells, but germination and proliferation of spores in the serum-free extracellular environment was evident. Spore germination was appreciably higher in immortalized cell cultures than in primary epithelial cells. Additionally, spores still germinated apically at a mucus-secreting air–liquid interface lung barrier that was devoid of cell culture medium much earlier than medium-only controls.
Significance and Impact of the Study: The role of lung epithelial cells in *B. anthracis* spore dissemination after inhalation remains poorly defined and rather controversial. These results are novel as they show spore germination is appreciably enhanced in the presence of lung cells *in vitro*, however, the cell line and cell state (air–liquid interface *vs* submerged in medium) dictates the extent of germination and in some cases proliferation.

Introduction

Bacillus anthracis (BA) is a Gram-positive spore forming bacterium and the aetiological agent of anthrax, a tier 1 select agent. Endospores measuring 1–2 microns in diameter pose a significant threat for bioterrorism misuse through delivery by inhalation, which is best represented by the 2001 anthrax letter attacks (Jernigan *et al.* 2002). Anthrax occurs after respiratory, gastrointestinal, cutaneous and injectable (drug-use) exposure. Even though cutaneous infection is much more common than respiratory routes of anthrax infection, the case-fatality rate is much higher after inhalation of anthrax spores *vs* skin infection (Sweeney *et al.* 2011). Following the anthrax letter attacks, the mortality rate among patients with known inhalation disease was 45% (5/11) even with aggressive antibiotic intervention (Jernigan *et al.* 2002). Despite decades of research that have defined detailed molecular mechanisms of BA pathogenesis at the cellular level, we still lack a basic understanding of critical steps after inhalation that lead to systemic disease.

BA sporulation is triggered under growth-limiting conditions and enables the organism to remain dormant and highly resistant to degradation (Dragon and Rennie 1995; Spotts Whitney *et al.* 2003). Conversely, germination of spores, referred to as spore outgrowth, occurs under favourable growth conditions and is initiated by unique nutrient combinations of amino acids and purine nucleosides by BA-encoded germination (Ger) receptors (Ireland and Hanna 2002; Moir *et al.* 2002; Gut *et al.* 2008; Luu *et al.* 2011). Spore germination induces rapid degradation of the protective spore coat layers, produces vegetative bacilli capable of expressing toxin, and is required for anthrax pathogenesis (Jagtap *et al.* 2006). Even though

inhalation of spores can lead to entrapment in the alveolar spaces so that spores evade mucociliary clearance, proliferation of spores outside the lung through the lymphatic system is a prerequisite for systemic virulence (Russell *et al.* 2008a; Twenhafel 2010). In spite of the role phagocytic cells play in clearing bacteria (alveolar macrophages and dendritic cells), BA spores supposedly require these cell types to reach lymph nodes (Guidi-Rontani 2002). Unresolved aspects of human anthrax infection include but are not limited to the following: (i) the location of initial germination after spore inhalation; (ii) the length of time for germination to occur postinhalation; and (iii) which cell types, if any, facilitate dissemination within or across the lung barrier (Tournier *et al.* 2009; Weiner and Glomski 2012).

Two different conceptual models have been proposed to describe possible mechanisms for BA dissemination in the lung. The Trojan horse model states that alveolar macrophages engulf spores and transports them to the more favourable environment of the germinal lymph nodes (Guidi-Rontani 2002). This Trojan horse model was later expanded to include dendritic cells, which have also been proposed as a conduit for spore translocation based on their phagocytic activity towards BA and the cell's demonstrated ability to transport spores to the lymph node (Brittingham *et al.* 2005; Cleret *et al.* 2007; Shetron-Rama *et al.* 2010). The recently proposed Jailbreak model states that spore germination occurs at the site of spore contact, the lung epithelium. Under the Jailbreak model, germination followed by BA-encoded toxin and protease production degrades tissue to facilitate transport of bacteria across the lung barrier (Weiner and Glomski 2012). Additionally, the Jailbreak model further proposes that spores do not require phagocyte engulfment by cells for transport, but that bacteria travel within bulk lymphatic flow to regional draining lymph nodes enabling systemic infection of the host. It should be emphasized that both Jailbreak and Trojan horse models are in agreement that both excessive toxin production during proliferation and failure of the immune system to clear bacteria leads to mortality. However, the differences between these models in where spores initially germinate has implications for therapeutic intervention strategies and in refining existing models of inhalation exposure.

Because the Trojan horse and Jailbreak models differ significantly regarding how BA interacts with the lung epithelium, we sought to better define the relationship of BA spores with primary human lung epithelial cells. While spore entry within lung epithelial cells has been observed both *in vitro* and *in vivo*, to what extent this is relevant during infection remains unresolved(Russell *et al.* 2008a; Tournier *et al.* 2009; Xue *et al.* 2010; Gut *et al.* 2011; Silva 2012). We present data related to uptake, germination and proliferation of spores in primary normal human bronchial epithelial cells (NHBE) cultured in two different manners: at air–liquid interface (ALI) which leads to differentiation along with mucus production, and as a traditional undifferentiated monolayer that is continuously submerged in culture medium. Comparative profiling of spores in the commonly used immortalized lung epithelial cells A549 and Calu-3 was also carried out (Foster *et al.* 1998; Harcourt and Haynes 2013). It was our intent that by assessing spore exposure outcomes within various lung cell lines under undifferentiated and differentiated conditions, we could better define parameters relating to lung spore exposure *in vitro* that are presently difficult to measure *in vivo*.

Materials and methods

Epithelial cell culture conditions

NHBE cells (CC-2540; Lonza) were cultured with 50% BronchiaLife B/T medium with LifeFactors kit (LL-0023; Lifeline Cell Technology, Frederick, MD, USA) and 50% B-ALI medium with Bulletkit™ (193514; Lonza, Basel, Switzerland). For ALI experiments, 0·4 μmol l^{-1} permeable transwell supports in 12-well format were preseeded with 30 $\mu g \times (cm^2)^{-1}$ rat-tail collagen (C3867; Sigma Aldrich, St. Louis, MO, USA). At 100% confluence, cells were brought to ALI and supplemented with differentiation activator supplement according to suggested guidelines by the B-ALI Bulletkit™ in respective 50/50 cell media mixtures. Cells at ALI allowed apical cell sides to be maintained in a semidry environment but basal cell sides were still in contact with medium. Basal medium was changed every 48–72 h for 14–18 days with mucus visible beginning at day 3 on apical cell surfaces. A549 (CCL-185, ATCC) and Calu-3 (HTB-55, ATCC) were cultured in undifferentiated and differentiated ALI states also resulting in mucus production at ALI. Calu-3 and A549 culturing was performed in recommended Dulbecco's modified eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS). For all cell lines gentamycin and Amphotericin B antimycotic were added to media during culturing according to manufacturers suggestions for ALI Bulletkit™(GA-1000 supplement, 193514; Lonza).

Culture conditions for BA spore infection

Beginning 48 h prior to BA infection antibiotics and antimycotic were not supplemented during media changes from cells. Two hours prior to infection only 10% of respective NHBE, Calu-3 and A549 cell growth medium was retained and 90% serum-free DMEM + L-glutamine was added to precondition cells to serum deprivation. One hour prior to infection cells were washed and

incubated 2 × 30 min DMEM + 2 mmol l^{-1} L-glutamine. For Fig. 4 phosphate buffered saline (PBS)-only infections an additional 3 × 5 min PBS washes were performed before spores were introduced so that residual DMEM would not influence BA germination outcomes.

Tested media formulations for spore germination and proliferation

For clarity, cell medium formulations are embedded within Fig. 1. For Fig. 3 findings, L-glutamine supplements were used at 2 mmol l^{-1} and were the following: L-glutamine (25030149; Life Technologies, Carlsbad, CA, USA), Glutamax™ (35050; Life Technologies) and Ultraglutamine (BE17-605E; Lonza).

Spore preparation and enumeration

Avirulent *Bacillus anthracis* Sterne strain 34F2 (pX01 + pX02-) was kindly provided by Dr. David Wunschel (Pacific Northwest National Laboratory). Spores were prepared as described previously (Buhr *et al.* 2008) in nutrient broth with CCY salts at 37°C for 72 h. Spores were harvested by centrifugation for 10 min at 10 000 ***g*** at 4°C and were resuspended in sterile water and stored at 4°C for 7 days to enhance vegetative cell lysis. Spores were then washed three times in sterile water prior to use. Analysis of spore purity by phase contrast microscopy demonstrated all preparations contained >95% phase bright spores. Spore stocks were enumerated throughout the study before infection in PBS supplemented with 0·02% Tween 80 (PBS-T).

Quantification of spores *vs* germinated BA

Samples were subjected to no heat treatment to quantify total bacteria (germinated + spores) *vs* heat treatment at 80°C for 30 min to kill vegetative cells, allowing for the quantification of heat-resistant spores only. For all

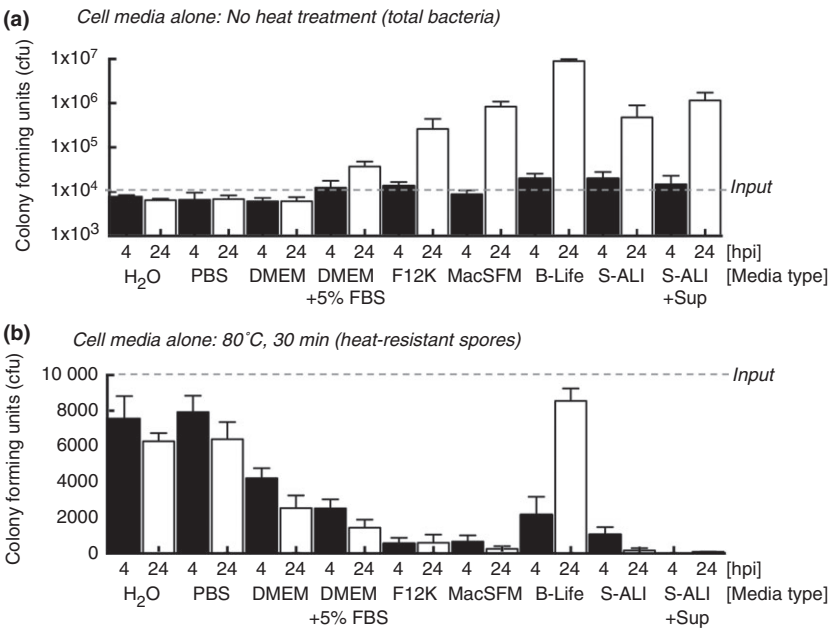

Figure 1 Elucidation of cell-free culture medium conditions that facilitate spore germination and subsequent proliferation. 10 000 spores (dashed line), confirmed at initial time of treatment were incubated at 37°C and 5% CO_2 and assessed for (a) total bacterial counts without heat treatment and then subjected to (b) 80°C for 30 min to deduce remaining heat-resistant spores in medium. Standard deviation at 4 h (■ black bars) and 24 h (□ white bars) were calculated from triplicate wells with $n \geq 5$ plates. Media formulations noted in figure are the following: H_2O-Nuclease-free water Life Technologies: 4387936; PBS-Phosphate buffered saline (1×) Life Technologies: 10010; DMEM-Dulbecco's Modified Eagle medium with L-glutamine Life Technologies: 21068-028; DMEM + 5% FBS-Dulbecco's Modified Eagle medium with L-glutamine and 5% FBS Life Technologies: 21068-028 and 26140; F12K-Ham's F-12K (Kaighn's) medium with L-glutamine Life Technologies: 21127; MacSFM-Macrophage serum-free medium with L-glutamine Life Technologies: 12065, B-Life-BronchiaLife: primary epithelial medium with L-glutamine and No Bulletkit supplements Lifeline Cell tech: LL-0023; S-ALI-Small airway liquid interface medium with L-glutamine no supplements Lonza: CC-4539; S-ALI + Sup-Small airway liquid interface medium with L-glutamine and Bulletkit supplements Lonza: CC-4539. Additional media tested (not shown) found to not be compatible with normal human bronchial epithelial cells was: RPMI 1640 Medium with L-glutamine Life Technologies: 11875; RPMI-1640 Medium- ATTC modification with L-glutamine Life Technologies: A10491; Gibco Minimal Essential Medium (EMEM) with L-glutamine Life Technologies: 11095, Lymphocyte Growth Medium-3 (LGM-3) with L-glutamine Lonza: CC-3211.

reported heat treatment and nonheat-treated CFU plate counts, 10-fold serial dilutions of samples in PBS-T were plated on TSA agar plates with overnight incubation at 37°C. For all CFU plate counts no fewer than three plates with 2–250 colonies were used in calculations. CFU standard deviation was calculated using GRAPHPAD PRISM 5.0 software (La Jolla, CA, USA). Asterisks noting significance in some figures was based on GRAPHPAD default parameters for one-sided nonparametric t-test with $P < 0{\cdot}05$ noting significance.

Spore uptake assays

Spores were deposited on undifferentiated and differentiated cells at a multiplicity of infection (MOI) of 1. After 1 h incubation cells were washed 3 × 1 ml with PBS and 1 × 1 ml DMEM. Germination was visualized by both confocal and bright field microscopy at various time points over an additional 23 h period with additional 3 × 1 ml PBS washes to collect supernatant. In some instances, additional more stringent washes were also performed with PBST and visually assessed (images shown in Fig. 5). For cell lysis studies as shown in Fig. 5, cells were freeze-thawed 3× at −80°C in either ultrapure water or PBS supplemented with 0·5% Triton X-100 (PBS-X) and vigorous pipetting with 3 × 1 ml washes in water or PBS-X buffer. Cell supernatant and lysate was transferred to 15 ml conical tubes and were subjected to either no sonication, gentle water bath sonication as previously described (Gutting *et al.* 2012), or harsher microtip pulse-sonication for 4 × 30 s with a minimum 5 min rest on ice between pulses.

Microscopy and immunofluorescence

Images of cells in associated with BA were acquired with either 20× or 40× objectives on an inverted bright field microscope (12-275-250; Thermo Fischer Scientific, Waltham, MA, USA). For immunofluorescence, differentiated cells at ALI were fixed, stained and washed directly in transwells that were later excised with a scalpel. All cells were first fixed in 4% paraformaldehyde (PFA) for 20–30 min. Cells were permeabilized for 15 min in PBS supplemented with 0·5% Triton X-100. Blocking and antibody staining was performed in Blockaid™ Blocking Solution (B-10710; Life Technologies) under standard primary and secondary antibody staining techniques with appropriate PBS-wash de-staining steps. Primary antibodies were added to final concentration of 2 μg ml^{-1} and were the following: mouse monoclonal MUC5AC (MS145; Thermo Fischer Scientific), mouse monoclonal β-tubulin (sc-5274, Santa Cruz Biotechnology), mouse monoclonal β-actin (AM4302; Life Technologies). Rabbit polyclonal antibody specific to *B. anthracis* (TC-7009; Tetracore, Rockville, MD, USA) was used at 5 μg ml^{-1}. Secondary antibodies for fluorescent staining were Alexa Fluor® 594 Goat Anti-Rabbit IgG (A-1101; Life Technologies) and Alexa Fluor® 488 Goat Anti-Mouse (A-10680; Life Technologies) both at 2 μg ml^{-1} that was subsequently followed with 1 μg ml^{-1} DAPI nuclear blue stain (D1306; Life Technologies). All cells were mounted in ProLong® Antifade (P-7481; Life Technologies) overnight and coverslips sealed the next morning with Cytoseal 60 (8310-4; Thomas Scientific). All images were acquired with a Zeis LSM 710 confocal microscope located at the Environmental Molecular Sciences Laboratory (EMSL) user facility at Pacific Northwest National Laboratory (PNNL) with either a 40× or 67× objective (400× or 670× total magnification) using 405, 488 and 594 nm laser settings.

Results

Rapid germination of BA spores is induced by serum that is commonly supplemented in cell culture growth formulations; however, some serum-free growth media formulations can also induce BA germination (Gut *et al.* 2011; Bensman *et al.* 2012). In the light of these observations, we set out to minimize the pitfalls and caveats of unwanted cell culture-based BA germination by elucidating appropriate serum-free cell culture incubation conditions that enable primary lung cells to survive at ALI but minimally impact germination of spores in the first 24 h of incubation. Shown in Fig. 1 are cell-free media-only results after treatment with 10 000 spores for those formulations that we found to be visibly compatible with NHBE cells (no cell rounding or dislodgement) for at least 48 h incubation. Of note, RPMI-1640 and EMEM media were not found to be compatible with NHBE, therefore, they were not tested with spores and are not included in Fig. 1a,b findings. Water and PBS were also not compatible with NHBE cells but are still shown in Fig. 1 results as they serve as appropriate negative controls. We added 10 000 spores to cell-free media (dashed line: input) that were incubated in 5% CO_2 at 37°C for either 4 or 24 h. and colony forming units (CFU) determined for the respective sample in triplicate. Figure 1a depicts total bacterial CFU counts, while Fig. 1b are heat-resistant spore-only counts. In summary, data presented in Fig. 1 show that DMEM with 2 mmol l^{-1} L-glutamine, referred to hereafter as DMEM + L-glut, was the only compatible serum-free media formulation that did not significantly induce proliferation of BA spores (increases in CFU), while keeping cells viable. Further experiments, therefore, focus on the use of DMEM + L-glut as it relates to studying epithelial cell contributions to BA germination and proliferation.

During the course of our medium studies, we noticed that Brochialife (B-life) media, which was also used in the culturing of primary lung cells up until cells reached 100% confluency, appeared to regenerate spores from proliferated vegetative bacteria based on the higher spore counts at 24 h *vs* 4 h (Fig. 1b, B-life). We, therefore, wanted to determine if spores in DMEM + L-glut without serum would also germinate, proliferate and resporulate BA if incubated for longer durations than the 24 h findings shown in Fig. 1. DMEM + L-glut was inoculated with either 10^5 or 10^2 spores that resulted in noticeable germination starting at 36 h post incubation and proliferation later at 60 h based on CFU plate counts for the higher 10^5 dose (Fig. 2). For the lower 10^2 spore dose visible germination and proliferation was not detected until 4 days after treatment. Lack of BA proliferation in DMEM + L-glut within the first 24 h for spores is in agreement with previous reports that have found DMEM can facilitate some spore outgrowth but not proliferation at 24 hpi (Gut *et al.* 2011). However, our results for both germination and resporulation in serum-free DMEM medium over a multiple day study are significant and to our knowledge have not been reported in the literature.

While L-glutamine is not expected to be circulating in the lung in liquid form, it is the most abundant amino acid in the human body with its concentration higher than that of all other 19 amino acids combined (Krebs 1935; Aledo 2004; Huang *et al.* 2013). L-glutamine is essential for immortalized cell line survival (Eagle 1955; Yuneva *et al.* 2007) and we found was also essential for all the lung cell lines we planned on testing, therefore it was important to determine if the addition of 2 mmol l^{-1} L-glutamine altered germination outcomes in DMEM. We found no significant differences in spores in DMEM with and without L-glutamine as spore germination was identified to similarly occur at approx. 32 h after incubation (Fig. 2c). We show additional supplementary data in Fig. S1 confirming similar spore outcomes during a 24 h time course and no differences in

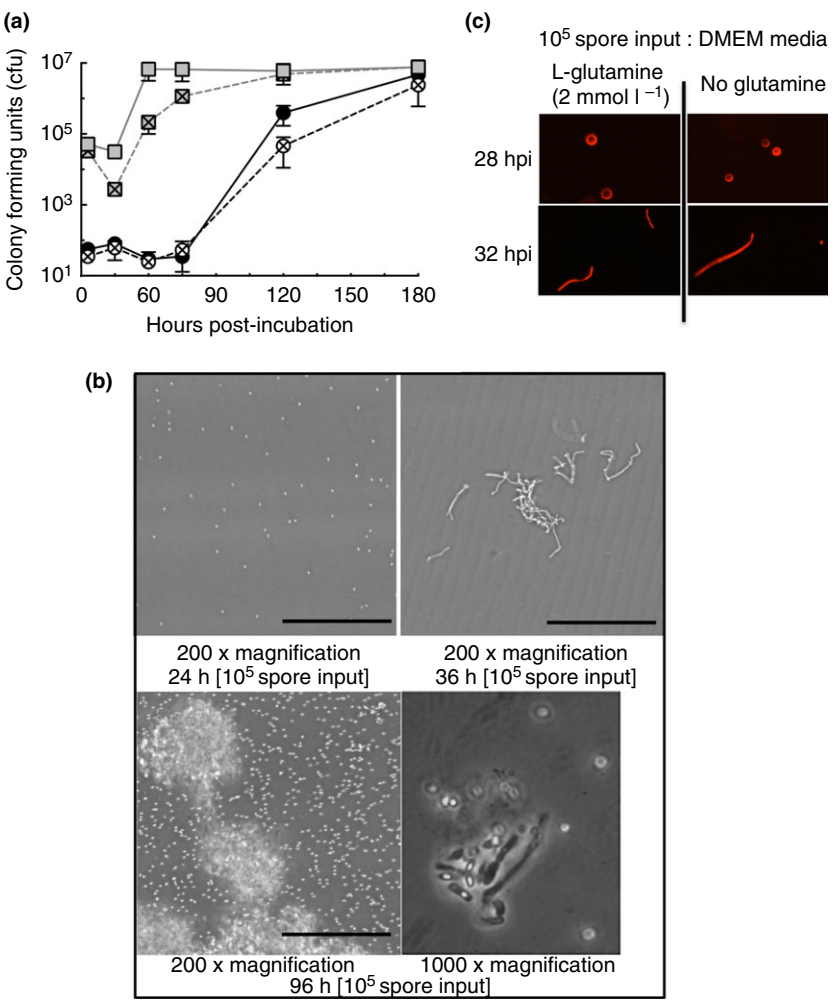

Figure 2 (a) Multiday-time course of heat-resistant spore formation *vs* total bacterial count at initial 100 and 100 000 spore doses. Total bacteria from 10^5 input-(□), heat-resistant spore only from 10^5 input-(⊠), total bacteria from 10^2 input-(●), heat-resistant spore only from 10^2 input-(⊗). were incubated in Dulbecco's modified eagle medium (DMEM) + 2 mmol l^{-1} L-glutamine and no serum at 37°C and 5% CO_2 for a total of 7·5 days (180 h). Standard deviation represents triplicate wells harvested for each time point and no <5 collective plate counts. (b) Microscopy images at 24 h show spore only morphology but by 36 h germination was visible. At 96 h resporulation after germination was evident (left) and at higher 1000× magnification (right) the remaining germinating bacteria appeared to also contain phase-bright spores suggesting they were close to completing resporulation. Scale bar is 50 microns. (c) Further analysis through immunofluorescence of spore sample from DMEM incubation indicates that germination began after 28 h but before 32 h and was not influenced by the addition of glutamine, which is critical to ensure cells survive *in vitro*.

supplementing or not supplementing DMEM medium with L-glutamine (Fig. S1).

Of note, the 'dip' in spore numbers reflected in Fig. 2a and loss of spores in Fig. 1b was concerning as we observed no germination until 32 h (Fig. 2c). Towards the end of this study, we found that while spores should have remained viable at 80°C (Spotts Whitney *et al.* 2003; Montville *et al.* 2005) if we reduced the temperature by only 5°C for spore treatment to 75°C we now only lost 25% of spores instead of 50% (Fig. S1, part B). However, the loss of 15–20% spores that still cannot be accounted for even at lower heating temperatures remains unexplained, we speculate that this phenomenon could be due to a subset of spores sticking to the tissue culture plastic and therefore not harvested, or the fact that incubation at 37°C in 5% $C0_2$ allows a subset of spores to shed their protective spore coat layer before bona fide germination of BA takes place after 28 h of incubation (Fig. 2b,c).

To further investigate the fate of spores in cell-free DMEM, we tested various glutamine supplement types that are commonly added to cell culture medium to determine if they too would alter germination of BA. We observed rapid germination and significant proliferation in DMEM formulations that were supplemented with Glutamax™ and Ultra-glutamine but not L-glutamine (all at recommended 2 mmol l^{-1}) (Fig. 3a). Based on previous reports confirming L-alanine as a pro-germinant of spores (Ireland and Hanna 2002), and the fact that both Glutamax™ and Ultra-glutamine are dipeptides with alanyl-derived structures conjugated to L-glutamine to enhance stability and uptake, it appears that these two supplements act as co-germinants in the presence of other DMEM growth nutrients. Proliferation caused by these commonly used glutamine derivatives was not solely dictated by the glutamine supplement as there was no proliferation for L-glutamine, Glutamax™ and Ultra-glutamine when supplement in PBS alone (Fig. 3a). We conclude from these results that both Glutamax™ and Ultra-glutamine should be avoided when performing BA studies in tissue culture.

While L-glut does not facilitate germination of spores, we next wanted to test to what extent DMEM L-glut medium previously used to culture NHBE impacted germination of spores. Used media from overnight cultured cells induced a 2-log increase in bacterial counts *vs* fresh DMEM L-glut that was not pre-treated with cells (Fig. 3b). The increased proliferation in used media was also found to be largely independent of glucose and glutamine from DMEM based on similar CFU levels when these components were omitted from the medium (Fig. 3b, right lanes). Unsurprisingly, when we visualized by bright field microscopy outgrowth and germination of BA spores in fresh (Fig. 3c, top) *vs* used media (Fig. 3c, bottom) in glutamine components tested in Fig. 3a, we found similar BA proliferation trends. Of note, one interesting caveat we found from Fig. 3 results was spores in association with Glutamax™ in fresh DMEM lead to significant visible outgrowth of *Bacilli* chains >100 microns in length (Fig. 3c, arrows), suggesting that there are inherent differences to BA germination for the glutamine supplement used. Figure 3 data for BA from already used cell-associated culturing *vs* media that was also in the incubator overnight but without lung cells suggests that: (i) cell-secreted metabolites from NHBE cells promote BA proliferation; (ii) as already mentioned, Glutamax™ and Ultra-glutamine should be avoided for these studies, but L-glutamine is still acceptable; however, for *in vitro* cell culture experiments with BA treatments should be performed in media that is fresh and not used. We further address the ability of NHBE cells to induce BA germination in the following results sections.

To better elucidate the influence that various lung epithelial cell lines have in directly inducing spore germination and proliferation, the lung cancer cell lines A549 and Calu-3, along with NHBE were comparatively assessed for BA proliferation. We added spores to cells in either serum-free DMEM L-glut (Fig. 4a) or PBS (Fig. 4b) that was tested over a MOI ranging from an MOI of 0·001 (10^2 deposited spores) to higher MOI of 1·0 (10^5 deposited spores). CFU plate counts 48 h after infection revealed a noticeable proliferation for spores in all cell types and all MOI concentrations after DMEM incubation for 48 hpi (Fig. 4a) but no BA proliferation when lung cells were not present (Fig. 4a, white bars). Proliferation of spores in DMEM cell cultures that had lung cells was so robust that it appears saturation was reached based on similar CFU plate counts for low and high MOI. Interestingly, the $>10^6$ CFU counts after infection for higher MOI 0·1 and 1·0 infection in PBS-treated cells (Fig. 4b), particularly for A549 and Calu-3 (Fig. 4b, black/chequered bars) *vs* NHBE cells (Fig. 4b, grey bars), suggest differences between these three lung cell lines in the ability to promote spore proliferation even when cell culture medium is not present.

The presence of cell-derived germination factors was further confirmed when spores were incubated in 'used' PBS in which cells had been incubated overnight but from which the lung cells themselves were removed by centrifugation prior to spore addition (Fig. 4c). PBS supernatant from our immortalized A549 and Calu-3 cell types induced both higher total CFU at 24 h (Fig. 4c) and BA outgrowth at earlier time points than NHBE (Fig. S2). Altogether, Fig. 4 results suggest that: (i) lung epithelial cells secrete one or multiple factors the promote

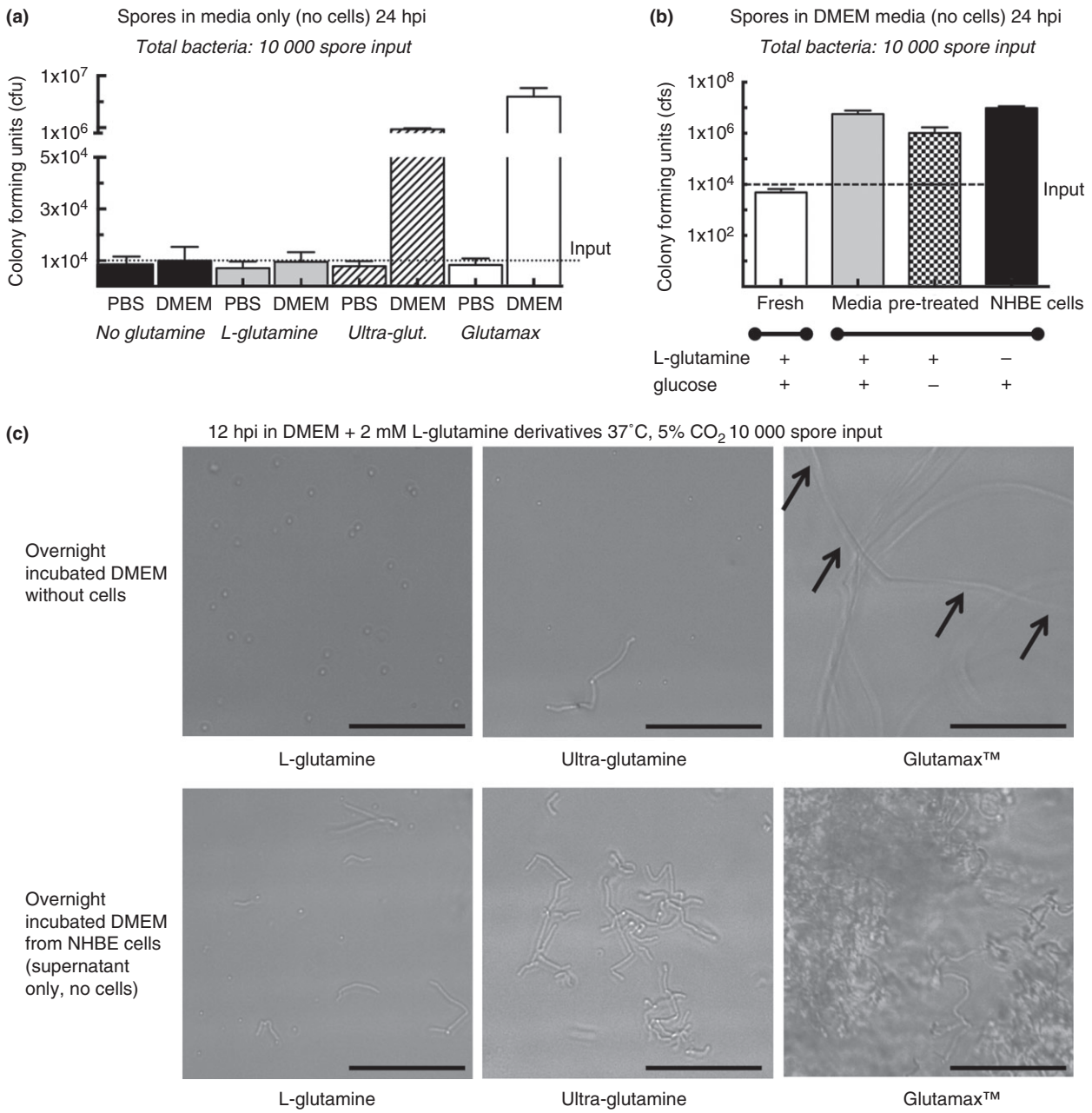

Figure 3 Assessment of Dulbecco's modified eagle medium (DMEM) media-only conditions that alter spore proliferation rates after 24 hpi at 37°C, 5% CO_2. Dashed line represents initial 10 000 spore input. (a) L-glutamine derivatives that include No glutamine (black bars-■), L-glutamine (grey bars-▒), Ultra-glutamine (diagonal-lined bars-▨) and Glutamax™ (white bars-□) all supplemented at 2 mmol l^{-1} in either phosphate buffered saline or DMEM. (b) DMEM media preincubated overnight without cells (lane 1, white bar-□) *vs* DMEM media preconditioned overnight in normal human bronchial epithelial cells (lane 2, grey bar-▒) in addition to DMEM that lacks either D-glucose (lane 3, chequered bar-▩) or L-glutamine (lane 4, black bar-■). In all bar graphs standard deviation represents triplicate wells and no less than five collective plate counts. (c) Microscopy images 12 h of after addition of either fresh (top row) or used (bottom row) DMEM supplemented with either 2 mmol l^{-1} L-glutamine, Ultra-glutamine (top row) or Glutamax™. Scale bar = 100 microns imaged with 40× objective for 400× total magnification. Arrows denote irregular bacilli structures measuring >100 microns in length.

BA germination. (ii) both supernatant and cells from cancerous lung cell lines induce germination and proliferation of BA more strongly than primary lung cells.

Previous results have shown that lung epithelial cells have the capacity to internalize spores, suggesting a route *in vivo* for dissemination involving transcytosis across the

epithelial barrier (Guidi-Rontani 2002; Gut *et al.* 2011). In the light of these previous findings, we assessed spore uptake in undifferentiated (Fig. 5) and differentiated NHBEs on transwells at ALI (Fig. 6). First, spores were deposited on NHBE cells within a low volume of DMEM + L-glut for 1 h followed by a series of PBS washes to remove noninternalized spores. Incubation was allowed to proceed and samples were visualized hourly for spore germination and to assess cell viability after internalization. Lung cells were harvested at 24 hpi and analysed for BA proliferation inside and outside the cell. We found that no matter how many serial PBS washes we performed, even if we increased the stringency of PBS washes by adding 0·02% Tween 20, a small subset of spores remained based on the visible outgrowth at 4 hpi for BA that was firmly adhered to the lung cell surface (Fig. 5a). By 24 hpi, significant numbers of vegetative bacteria were in the supernatant and a majority of lung cells appeared to be unaffected by BA growth. Vegetative BA at 24 hpi was still associated with the cell surface but vigorous washing could now dislodge a majority of BA, although a few BA clusters remained firmly attached (Fig. 5b).

Even though our data suggested PBS was not effective at removing all non-endocytosed spores from lung cell surfaces, we still attempted to quantify uptake of spores by NHBE lung cells after infection. After spores were incubated on lung cells for 1 h, followed by vigorous

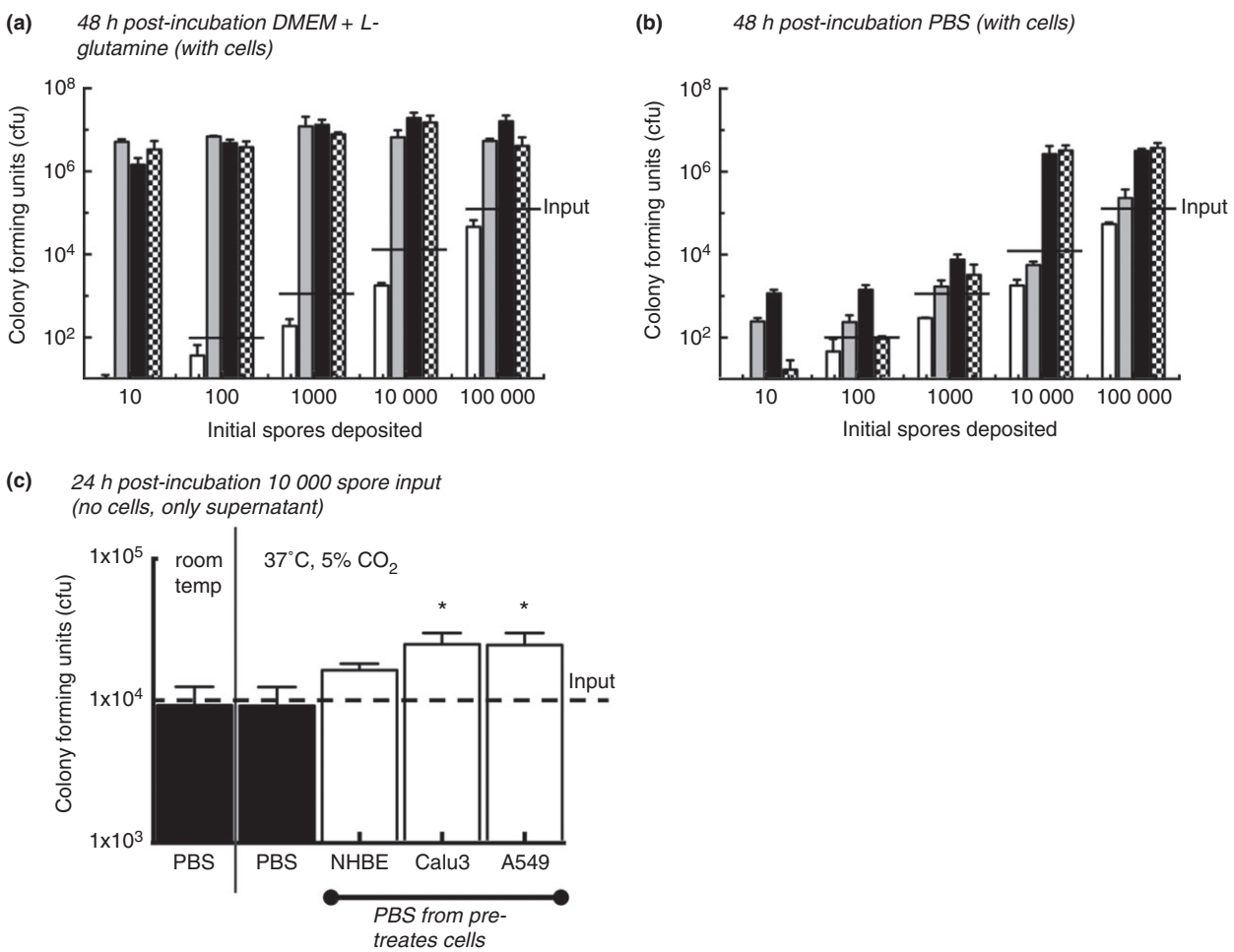

Figure 4 Lung epithelial cell lines normal human bronchial epithelial (NHBE), A549 and Calu-3 induce germination of spores. For (a) Dulbecco's modified eagle medium (DMEM) L-glutamine or (b) phosphate buffered saline (PBS) incubations, varying spore inocula ranging from 10 to 100 000 CFU input (depicted horizontal lines) were incubated in media alone (white bars-□) or in the presence of NHBE cells (grey bars-▒), Calu-3 cells (black bars-■) or A549 cells (chequered bars-▩) for 48 h. (c) Proliferation of spores in PBS media alone at room-temperature (lane 1, black bar-■), *vs* PBS alone incubated at 37°C, 5% CO_2 (lane 2, black bar-■) and PBS that came from prior overnight treatment of respective lung cell lines in which cells were removed before spore addition (lanes 2–5, white bars-□) incubated at 37°C, 5% CO_2 for 24 h, (*) asterisk denotes significance (*P*-value < 0·05) one-sided t-test for cell supernatants *vs* lane 2 no supernatant control.

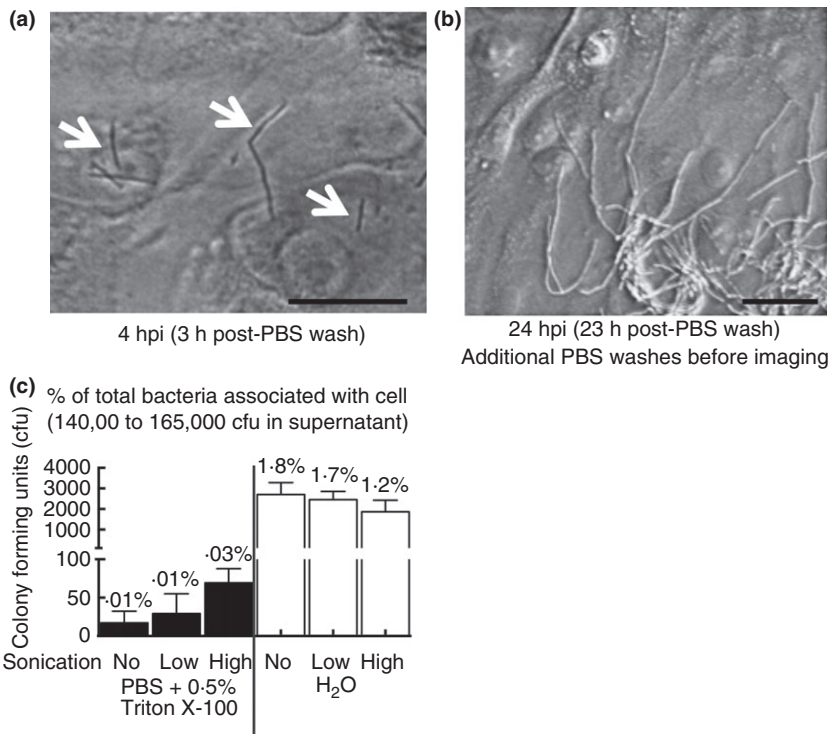

Figure 5 Newly germinated spores tightly adhere to the epithelial cell surface even after phosphate buffered saline (PBS) washes. (a) Outgrowth from spores for BA was evident at 4 hpi, PBS washes that were performed at 1 h could not dislodge bacteria as shown 3 h later (white arrows). Additional incubation to 24 hpi resulted in vegetative bacteria that could mostly be washed from surface, however, some bacterium (b) could also not be dislodged from surface. (c) Lung cells, as depicted in panel (b) after PBS washes, were additionally subjected to 3-cycles of freeze thaw at 24 hpi to release associated/endocytosed spores. Percentages for CFU plate counts are calculated *vs* the collected supernatant that ranged from 140 000 to 165 000 for a given well before the freezing process. Black bars represent sonication of postwashed cell in PBS supplemented with detergent and white bars represent similar lysis treatments but in water. Scale bars = 25 microns.

washing of lung cell surfaces using a series of three PBS washes, incubation was allowed to then proceed for an additional 23 h in DMEM L-glut. At 24 hpi, various lysis conditions were tested that entailed three freeze–thaw steps of lung cells in either water or PBS supplemented with 0·5% triton X-100 (PBS-X), followed by gentle or harsh sonication (Fig. 5c). Of the 140 000–165 000 germinated bacteria collected from the extracellular environment by CFU plate counts for each respective well 24 h after infection, a comparatively small fraction of BA representing 0·1–1·8% of total BA collected (Fig. 5c) was potentially still within lung cells, almost all of which were germinated (heat-sensitive, Fig. 5c) with only 0–10 total spores heat-resistant (data not shown). It should be noted that there was little NHBE cell death at 24 hpi. We can conclude from these spore uptake results that remaining BA after vigorous PBS washing that were stuck on the lung cell surface attributed to these rather minimal counts and there was in fact little to no actual endocytosis. Additionally, the fact that virtually no spores were identified along with an intact cell monolayer at 24 hpi implies little spore uptake potential for NHBE cells. We can conclude from these results that while using PBS to wash away noninternalized spores on lung cells is a highly ineffective means to quantify spore uptake/endocytosis.

Next we assessed if mucus-secreting NHBE cells differentiated at ALI would have similar adherence for spores along with minimal internalization as was found in Fig. 5 for NHBE cells submerged in medium. Bright field microscopy images of differentiated cells on transwells inserts at ALI were not possible due to the transwell membrane blocking adequate light passage and resolution of cells and spores. However, excision of transwell inserts and immunofluorescence on glass slides was possible. Germinated BA (bacillus structures) was noticeable at 4 hpi (Fig. 6a–c, red) with eventual proliferation indicated by higher levels of vegetative bacteria at 24 hpi (Fig. 6d, red). Additionally, we found from maximum density projections of immunofluorescence images that there was little (<1%) co-localization (yellow) of BA (red) with β-actin (Fig. 6a, green), β-tubulin (Fig. 6b, green) and even mucus-MUC5AC (Fig. 6c, green) cellular markers that would be suggestive of endocytosis. Finally, many of the spores that were not removed after fixing and subsequent washing were clearly in focus above the cell membranes and did not appear internalized. Little to no co-localization of BA with various cell markers (Fig. 6a–c, yellow merged colour) implies lung epithelial cells are likely refractory to spore uptake.

Proliferation and germination differences between NHBE and A549 as shown in Fig. 4b,c led us to try to better define the timing for germination of spores in these two lung cell types in differentiated ALI states. Unfortunately we found A549 cells to be delicate in nature when serum was not present and during PBS washing (Fig. S3 shows A549 lung cell dislodgement). Due to A549 instability on transwells without serum, we

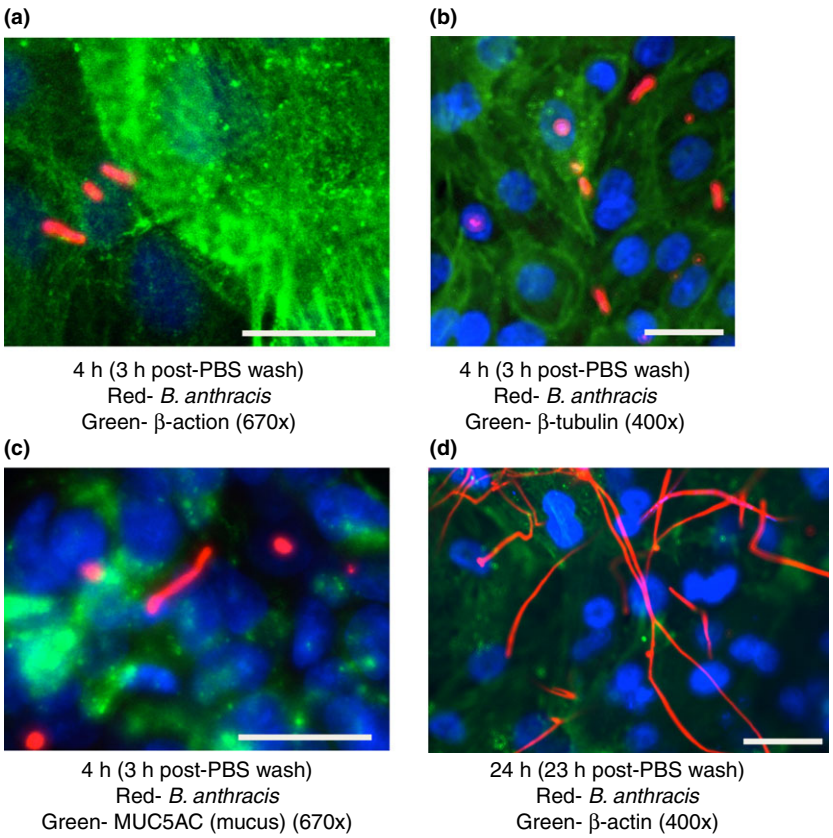

Figure 6 Immunofluorescence of transwell inserts of differentiated normal human bronchial epithelial cells after infection suggest minimal uptake as assessed by localization (yellow) with various cellular markers. DAPI nuclear stain- blue; *B. anthracis*- red; and cell markers (a) green-β-actin (b) green-β-tubulin (c) green-MUC5AC (mucus) all three imaged at 4 h postinfection. (d) 24 h infections, noticeable vegetative growth was evident for BA, green-β-actin for specified image. Images were acquired at either 40× or 67× objectives, Scale bar = 50 microns.

monitored immunofluorescence images 1–5 h after spore deposition on cells without attempting to remove unattached spores with PBS (Fig. 7). A549 spore-outgrowth began at 2 h and was prominent by 3 h, while that of NHBE was delayed relative to A549 by 1 h (Fig. 7a). Additionally, we observed what appeared to be a 'halo'-like staining structure indicative of phase-bright spores still in the ungerminated state by immunofluorescence more often in NHBE than A549 images at early time points after infection, this is reflected at the 2 hpi shown in Fig. 7a.

To assess the time course of BA proliferation in both NHBE and A549 cells in differentiated ALI states *vs* that of undifferentiated cells submerged in culture we performed a CFU time course after infection with 10 000 spores (Fig. 7b, dashed line input). NHBE cells in differentiated ALI states (Fig. 7b, black bars) and undifferentiated states submerged in medium (Fig. 7b, white bars) *vs* that of A549 cells that were also differentiated (Fig. 7b, horizontal-line bars) and undifferentiated (Fig. 7b, diagonal lines) were assessed 1–48 hpi. We found that due to transwell basal media leaking at ALI into the apical side where spores resided for A549 samples, we could not adequately measure proliferation at 12 h and later time points (Fig. 7b, NA). Instability of A549 cells at ALI again emphasized the differences in attachment abilities between lung epithelial cell types under serum-free conditions. Due to instability of A549 in serum-free DMEM, we recommend that this cell line not be used for pathogen assays or in studies at ALI unless serum is present. We discuss differences in cell types and cell states (submerged *vs* ALI) in the context of BA germination and proliferation in the discussion section.

Discussion

Mucus-secreting NHBE cells differentiated at ALI on transwells provide a more physiologically relevant *in vitro* model than traditional culturing methods to assess pathogens of the lung epithelium. Spores placed apically on ALI lung cells are in direct association with a mucus barrier, and are not submerged in cell culture medium. We have presented data that reveal the primary NHBEs at ALI facilitate germination of spores in the extracellular environment at 4 hpi with bacteria doubling (proliferation) in the extracellular environment by 14 hpi. This germination and proliferation for BA in the presence of lung cells is considerably earlier than our media-only controls. We have also shown that other factors besides serum heavily influence BA outcomes in cell culture and

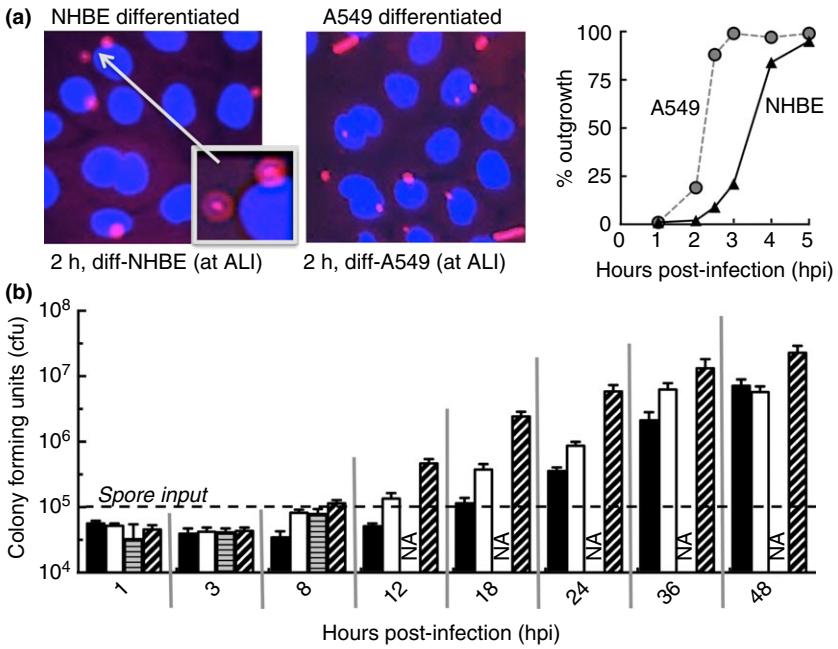

Figure 7 A549 *vs* normal human bronchial epithelial (NHBE) cells in differentiated ALI state induce faster BA germination and proliferation. (a) Immunofluorescence of transwells at 1–5 h assessed for spore out-growth indicative of germination. Shown is representative 2 hpi time points. DAPI nuclear stain- blue; *B. anthracis*- red. The 'halo' like structure (arrow) commonly seen for nongerminated spores by bright field was also more apparent for NHBE than A549 at early time points and that is reflected at 2 hpi by immunofluorescence. A minimum of 200 bacteria for each time point was counted and the percent of bacillus-shaped bacteria is shown (right). Spores in A549 cells (grey circles-●) and spores in NHBE cells (black triangles-▲) (b) CFU plates counts from transwells at ALI for differentiated NHBE (black bar-■) *vs* NHBE cells in traditional submerged 2D state (white bar-□) compared to A549 cell infections in differentiated state (horizontal striped bar-▤) *vs* A549 undifferentiated state (diagonal striped bar-▨). Differentiated A549 transwells leaked at 12 h and are therefore labelled as not applicable (NA) due to leaking of basal media at ALI into spore environment. All multiplicity of infections (MOI) were performed at 1·0 with 100 000 spore input.

therefore need to be carefully considered in the experimental design prior to introduction of BA, these include; (i) the type of glutamine supplement; (ii) time medium is in contact with cells (i.e. potential metabolites excreted by cells into the media; (iii) cell type; (iv) submersion of cells *vs* ALI; and (v) lysis methods to assess cell uptake of spores. Even with these five variables influencing BA outcomes, our findings still strongly suggest that spore uptake by primary lung epithelial cells is minimal and that a factor or factors secreted by lung epithelial cells induces BA germination. These *in vitro* studies suggest that the lung epithelium *in vivo* may also induce germination.

Germination and sporulation are tightly regulated processes that are physiologically taxing and require various signalling cascades to ensure successful morphological change (Liu *et al.* 2004). Committing to germination leaves the bacterium susceptible to immune detection, but is required for toxin production. Our observation of extensive and rapid transition of BA from heat-resistant to heat-sensitive bacteria, indicative of germination even without serum was unexpected and suggests epithelial cells excrete all the factors required to facilitate these processes. It should be highlighted, as we show in these results that spores will germinate if left long enough in virtually any cell culture medium in the warm environment of a CO_2 incubator. However, identifying the kinetics and key time points at which germination occurs while minimizing the caveats and potential pitfalls of artificial or unintended germination in cell culture medium is paramount if we are to study BA appropriately *in vitro*.

A handful of studies have looked at the role lung epithelial cells play in anthrax dissemination in tissue culture. These studies, most notably from Xu and collaborators have shown in a series of gentamicin protection assays that human small airway epithelial cells internalized ~1% of Ames (pX01+, pX02+) and Sterne (pX01+, pX02−) (Russell *et al.* 2008b; Xue *et al.* 2010, 2011). However, these studies were performed in DMEM + 10% FBS, known to germinate 85% of spores in as little as 10 min (Gut *et al.* 2008). Additionally we found that spores were rapidly susceptible to gentamicin killing even without serum in DMEM (data not shown), therefore it was difficult for us to perform the gentamicin protection assay with spores in our *in vitro* studies. It should be noted that it has been reported that Sterne

spores are largely dependent on serum for internalization within A549 (Xue *et al.* 2011), which we largely avoided. However, the rampant germination of spores in 10 min in FBS would be highly unfavourable for endocytosis based on the increased size of BA, therefore it is still unclear how germination is relevant in enhancing uptake. We can conclude from surveying the published literature that there is substantial variation in the assay conditions in studying BA spores within *in vitro* cell culture systems, this variation was a driving force in our own experimental design in attempting to address various cell culture variables as they relate to BA germination in lung cells.

In relation to the already mentioned previous studies assessing spore uptake in epithelial cells in tissue culture, our immunofluorescence co-localization experiments with NHBE cells at ALI revealed no noticeable interaction of spores with actin or tubulin and only minimal embedding within mucus of the extracellular surface. It is plausible that the infrequent spore uptake reported previously (~1%) did occur in our studies, in line with previous findings. However, under our assay conditions, which are the first to study BA endocytosis using primary lung cells in a mucus-secreting differentiated state at ALI, there was little evidence for spore uptake.

As we noted in the results, once spores began to germinate they became firmly associated with the epithelial surface for both ALI and submerged in medium studies and spores could not be removed even with extensive stringent washing. We conclude that using PBS to remove nonendocytosed BA should not be trusted as an effective washing treatment as it over estimates the levels of BA uptake for *in vitro* cell assays. While lung cells differentiated at ALI recapitulate many physiological aspects of the lung epithelium, Xu et al. have studied epithelial spore uptake within a mouse model at 2 and 4 h postinfection (Russell *et al.* 2008a), however, spore uptake of 0·62% ± 0·16% has been called strikingly low to infer a mechanism for transport across the epithelium (Tournier *et al.* 2009). We conclude from our findings and the low efficiency for spore uptake reported in mice that the lung epithelium likely forms a protective barrier that guards against spore internalization. Our results also infer that the lung epithelium may be well suited for germination of BA extracellularly, which argues in many regards for a Jailbreak model for BA dissemination in the lung.

Acknowledgements

Part of the research was performed using the Environmental Molecular Science Laboratory (EMSL), a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory under EMSL user proposal 48446. The Department of Homeland Security, Science and Technology Directorate provided funding for this research through contract HSHQPM-14-X-00037 to Pacific Northwest National Laboratory. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the United States Department of Energy under contract DE-AC06-76RLO.

Conflicts of Interest

Equipment and reagents reported in this research do not constitute an endorsement by Pacific Northwest National Laboratory or the United States Department of Homeland Security. The authors declare no conflict of interest related to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Characterization of spores (10 000 spore input: dashed line) after incubation in cell culture medium at 37°C, 5% CO_2.

Figure S2 Spore outgrowth/germination in PBS occurs at earlier time points for Calu-3 and A549 pretreated supernatants than for NHBE.

Figure S3 PBS washes of A549 (top) and NHBE (bottom) cells in serum-free media result in a compromised cell monolayer for A549 based on dislodging of cell (right half of respective image).