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Minireview

MINIREVIEW

Take my breath away: studying pathogen invasion of the human lung using primary tissue models

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One sentence summary: Utility of two human lung infection platforms, human alveolar macrophages and human precision-cut lung slices, to study multiple bacterial pathogens.

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ABSTRACT

The human pulmonary environment is complex, containing a matrix of cells, including fibroblasts, epithelial cells, interstitial macrophages, alveolar macrophages and neutrophils. When confronted with foreign material or invading pathogens, these cells mount a robust response. Nevertheless, many bacterial pathogens with an intracellular lifecycle stage exploit this environment for replication and survival. These include, but are not limited to, *Coxiella burnetii*, *Legionella pneumophila*, *Yersinia pestis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*. Currently, few human disease-relevant model systems exist for studying host–pathogen interactions during these bacterial infections in the lung. Here, we present two novel infection platforms, human alveolar macrophages (hAMs) and human precision-cut lung slices (hPCLS), along with an up-to-date synopsis of research using said models. Additionally, alternative uses for these systems in the absence of pathogen involvement are presented, such as tissue banking and further characterization of the human lung environment. Overall, hAMs and hPCLS allow novel human disease-relevant investigations that other models, such as cell lines and animal models, cannot completely provide.

Keywords: hAMs; hPCLS; pulmonary; lung; lung infection; intracellular bacteria

THE HUMAN ALVEOLAR ENVIRONMENT

The human lung is constantly exposed to airborne material that must be efficiently recognized, processed and removed to promote a healthy respiratory system. A multitude of cell types are present in the complex human alveolar environment (Fig. 1A), each serving distinct housekeeping and defense functions. These populations include resident type I and type II epithelial cells, fibroblasts, interstitial and alveolar macrophages, and neutrophils in addition to lymphocytes recruited during immune reactions (Franks *et al.* 2008). Epithelial cells are one of the most abundant cell types within this environment. Type I epithelial cells are a necessary structural component that form the alveolar wall and aid gas exchange across the air–blood barrier for proper respiration (Rackley and Stripp 2012). Type II epithelial cells produce surfactant proteins that reduce respiration-related surface tension and interact with foreign pathogens and allergens. These interactions can trigger immune cell activation or increase microbial uptake by phagocytic cells, ultimately degrading the pathogen (Lawson and Reid 2000; Rackley and Stripp 2012). Fibroblasts are prominent in the interstitium, forming the extracellular matrix for pulmonary structural support. Fibroblasts also aid wound repair by remodeling the extracellular matrix and healing the alveolar epithelium (Wuyts *et al.* 2013; Ito *et al.* 2014; White 2015). In addition to structural cells, macrophages are present as the first line of defense against

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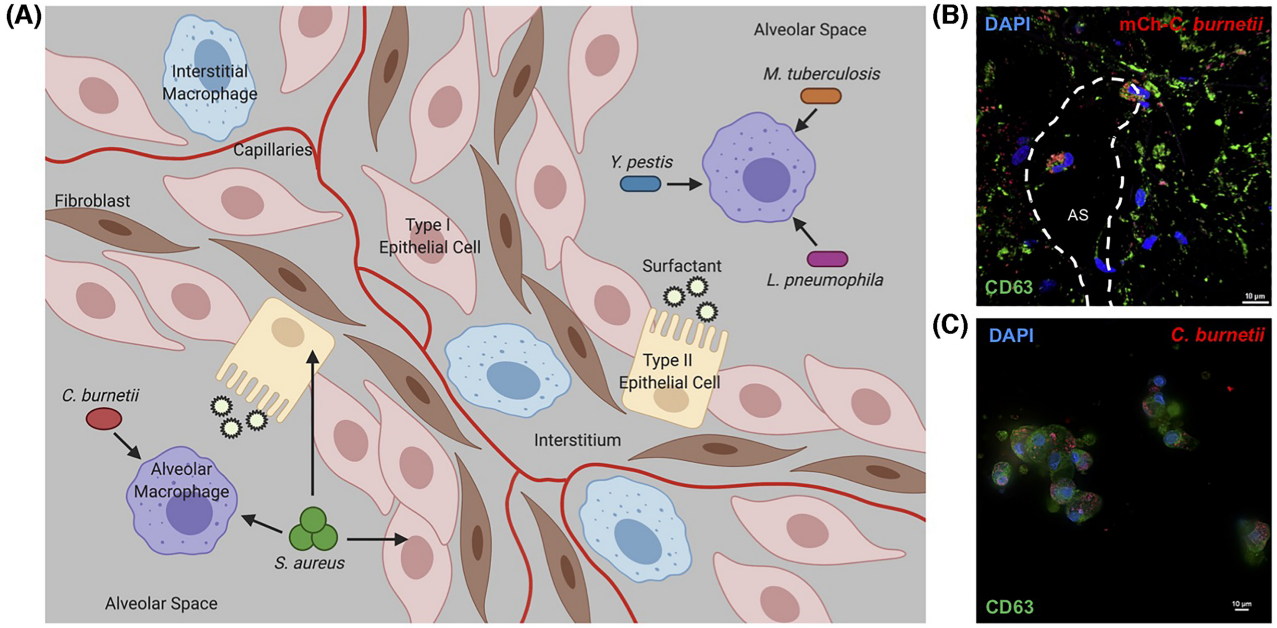

Figure 1. Preferential niche of bacteria in the lung. **(A)** Schematic of the human alveolar environment, cell types present and invading bacterial pathogens. Arrows = preferential niche(s) for each pathogen. Created with BioRender.com. **(B)** hPCLS were infected with NMII *Coxiella burnetii* expressing mCherry (red) for 96 h, then processed for immunofluorescence microscopy using CD63 antibody (lysosomes and PVs; green) and DAPI to stain DNA (blue). Substantially more hAMs contained *C. burnetii* compared with epithelial interstitial cells. AS = alveolar space. **(C)** Isolated hAMs were infected with NMI *C. burnetii* for 96 h, then processed for immunofluorescence microscopy using antibodies against CD63 (green), *C. burnetii* (red) and DAPI to stain DNA (blue). hAMs support PV expansion and robust *C. burnetii* growth.

foreign materials in the alveolus. Interstitial macrophages are present exclusively in the interstitial region, whereas alveolar macrophages are present within alveolar spaces, often lightly adhered to the epithelial lining of the alveolus (Lambrecht 2006). Macrophages are responsible for phagocytosis of foreign material, pathogen clearance, and inflammatory cytokine and chemokine production that stimulates a robust immune response (Rubins 2003; Schyns, Bureau and Marichal 2018). Neutrophils respond to inflammatory cytokine production, aid pathogen killing and represent an essential link between innate and adaptive immunity in the lung (Liu *et al.* 2017). Lastly, dendritic cells recognize foreign material and pathogens, and activate the adaptive immune response (Condon *et al.* 2011).

The complex alveolar environment is well equipped to proficiently respond to foreign inhaled material, including highly infectious microbes. Even so, many respiratory pathogens exploit cells in this environment for intracellular survival and replication required to cause debilitating disease. Due to the physiological relevance deficiency in using animal models of infection to mimic the human lung environment, it is essential to use the most relevant human-derived model systems to dissect mechanisms of microbial pathogenesis. In this review, we describe two recently developed infection platforms, human precision-cut lung slices (hPCLS) and primary human alveolar macrophages (hAMs), for the study of distinct bacterial pathogens and discuss the contribution of these systems to microbial pathogenesis.

PRIMARY HUMAN LUNG PLATFORMS

Many animal models of infection have been used to investigate the pulmonary response to microbial pathogens. Although these models provide valuable mechanistic information about specific diseases, translating these findings to humans is not always possible. Establishment of an *ex vivo* human lung system has provided researchers with a platform to assess mechanisms of pathogenesis in distinct alveolar and interstitial cell types (Graham *et al.* 2016). The system developed in our laboratory uses lungs obtained post-mortem from healthy donors with no presence of asthma, pneumonia or other infection (i.e. SARS-CoV-2). Lungs are injected with low-melting-temperature agarose and incubated at 4°C until solidified, after which they are sectioned into 2.5-cm-thick slices. Using a microtome, sections are cored and cut into hPCLS (diameter = 8 mm; thickness = 750 μm). hPCLS are cultured in standard cell culture medium containing antibiotics and antimycotics [Dulbecco's modified Eagle/F-12 (DMEM/F-12) medium with 10% fetal bovine serum, penicillin (50 U/mL), streptomycin sulfate (50 μg/mL), gentamicin sulfate (60 μg/mL) and amphotericin B (0.25 μg/mL)]. Prior to infection with a microbe, antibiotic/antimycotic-containing media is replaced with non-antibiotic/antimycotic media for 2–3 days. hPCLS can then be processed for multiple readouts, including microbial growth, microscopic analysis of replication vacuole formation and cytokine production.

Although this *ex vivo* platform can be used for many novel applications, important caveats must be considered. Neutrophils are present in low numbers in hPCLS, and influx as a result of pathogenic infection does not occur because the tissue has been separated from the circulatory system. The absence of influx hinders studies of the entire innate immune response, but allows detailed investigation of the role of macrophages in alveolar infection. Studies are currently underway to re-populate hPCLS with exogenous neutrophils and simulate influx. A second caveat to the hPCLS system is the lack of recruited adaptive immune cells, preventing investigation of the adaptive immune response to infection. While recognizing these limitations, hPCLS provide a platform to dissect the interaction between microbes and innate immune cells in the absence of confounding host inflammation-induced effects.

In addition to using hPCLS to study the entire alveolar region, cell signaling and intracellular infection can be assessed using isolated hAMs. These cells are easily harvested from donor lungs via bronchioalveolar lavage. Prior to agarose inflation of lungs, extracted bronchioalveolar lavage fluid (BALF) is incubated with a lysis buffer to eliminate red blood cells from the mixture. BALF samples are then plated and adherent hAMs cultured as above for hPCLS (Graham *et al*. 2016). Following isolation, hAMs do not proliferate, indicating terminal differentiation. The quantity and viability of harvestable hAMs per lung are variable, requiring experiments to be performed using lungs from at least three donors, which also provides statistically significant and biologically relevant results. Following isolation, hAMs can be infected with a microbe and processed for numerous cell biology assays, including protein analysis, cytokine/chemokine production and fluorescence microscopy (Fig. 1B and C). It should be noted that primary hAMs respond to bacterial pathogens in a more robust manner than human macrophage cell lines, highlighting the importance of assessing infection of primary cells to detect critical events that are below the limit of detection in cell lines (Graham *et al*. 2016). hAMs and hPCLS have now been used to study innate interactions with diverse microbial pathogens that exploit an intracellular niche as highlighted in the following sections (Table 1).

COXIELLA BURNETII

Q fever is a debilitating disease caused by the obligate intracellular bacterial pathogen *C. burnetii*. Acute Q fever is characterized by flu-like symptoms, whereas chronic Q fever can result in severe and potentially fatal endocarditis (Maurin and Raoult 1999; Mazokopakis, Karefilakis and Starakis 2010; Oyston and Davies 2011). *Coxiella burnetii* has an aerosol mode of transmission, and fewer than 10 inhaled bacteria can establish acute infection, underscoring the importance of this pathogen as a U.S. Centers for Disease Control and Prevention select agent (Oyston and Davies 2011). After human inhalation, *C. burnetii* is engulfed by an alveolar macrophage into a membrane-bound compartment, transitioning through the phagolysosomal maturation pathway and fusing with endosomes and acidic lysosomes that decrease the pH (pH ∼ 5.0) (Voth and Heinzen 2007; Samanta *et al*. 2019). During this process, *C. burnetii* is metabolically activated, employs a type IV secretion system (T4SS) and forms a host membrane-derived replication niche deemed the parasitophorous vacuole (PV) (Voth and Heinzen 2007; Crabill *et al*. 2018). Immortalized cell lines and murine models have been extensively used to study *C. burnetii* infection, although mice do not fully recapitulate human disease (Dragan and Voth 2020). Due to this deficiency in small animal model systems, hPCLS and hAMs provide reliable platforms for dissecting pathogenic events directly applicable to human disease.

Graham *et al*. (2013, 2016) established the hAM and hPCLS models of *C. burnetii* infection using multiple bacterial isolates. To characterize the hAM platform, cells were infected with one of three select agent isolates, Nine Mile I (NMI), a virulent strain that causes acute disease, G, a virulent strain that causes endocarditis, Dugway, a severely attenuated strain, or one avirulent isolate, Nine Mile II (NMII). All isolates form multiple PV that contain lipid droplets in hAMs and display robust growth similar to the well-established THP-1 human macrophage-like cell line. In response to *C. burnetii*, hAMs produce anti-apoptotic signals, including phosphorylated (activated) Akt and Erk1/2, and produce the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and anti-inflammatory interleukin-10 (IL-10) (Graham *et al*. 2013). Production of interleukin-1β (IL-1β) occurs throughout NMII infection of hAMs, but not during virulent isolate infection, suggesting isolate-specific immune signaling (Graham *et al*. 2013). Importantly, this study was the first to show cellular infection differences between isolates, while some infection events correspond with previously established cell models, highlighting the need for further *C. burnetii* studies using hAMs.

In addition to robust NMII growth within hAMs (Graham *et al*. 2016), IL-1β is produced by both hAMs and hPCLS, suggesting a potential role for activation of the inflammasome in response to avirulent *C. burnetii*. This activation was confirmed by assessing caspase-1 and caspase activation and recruitment domain (ASC) co-localization with IL-1β adjacent to *C. burnetii* in hAMs. Moreover, upregulation of genes such as *nlrp3* and *nod2* further indicates that infection of hAMs specifically elicits NLRP3 inflammasome activation (Graham *et al*. 2016). Collectively, these results enhance understanding of the innate response to the *C. burnetii*.

Further hPCLS investigation by Dragan *et al*. showed *C. burnetii* replication in hAMs, while no growth occurs within interstitial macrophages, fibroblasts or epithelial cells, further showcasing the cellular specificity of infection (Dragan, Kurten and Voth 2019). However, immortalized pulmonary cell lines, including A549 alveolar epithelial cells and human lung fibroblasts, support *C. burnetii* replication *in vitro*, although growth is significantly decreased in epithelial cell lines and more substantially in macrophage-like and fibroblast cell lines. These contrasting phenotypes further highlight the utility of the hPCLS system to define infection events and cell-specific responses in a natural setting. Furthermore, addition of surfactant protein-D, typically secreted by alveolar epithelial cells to eliminate pathogens, to hAMs does not prevent PV expansion or *C. burnetii* growth in contrast to mouse macrophage results (Soltysiak, van Schaik and Samuel 2015). In response to *C. burnetii*, hAMs produce substantial levels of interleukin-8 (IL-8), a neutrophil-attracting chemokine, further demonstrating a pro-inflammatory response and correlating with mouse studies that indicate a critical role for neutrophils in Q fever (Elliott, Peng and Zhang 2013). In addition to the immune responses above, a phenotypic switch in polarization from M1 (pro-inflammatory) to M2 (anti-inflammatory) occurs in hAMs during *C. burnetii* infection, suggesting a more permissive environment for replication is actively promoted by the pathogen (Dragan, Kurten and Voth 2019). Significant replication of *C. burnetii* in M2-polarized alveolar macrophages corroborates results previously noted in murine alveolar macrophage studies (Fernandes *et al*. 2016). Collectively, this study uncovered novel aspects of the *C. burnetii* preferential growth niche in humans using hAMs and hPCLS.

The hAM system is also useful for uncovering novel cellular signaling events, as primary cells often respond to pathogen stimuli more robustly than immortalized cells. Similar to other intracellular bacteria, *C. burnetii* manipulates numerous host signaling cascades to promote efficient growth and progression of disease. One pathway of importance in this pathogen setting is autophagy, the removal of damaged organelles and foreign material by relocation to lysosomes for degradation. The autophagosomal protein microtubule-associated light chain 3 (LC3) associates with roughly 70% of hAM PV during infection with NMII *C. burnetii* in a T4SS-dependent manner, indicating the organism actively recruits autophagosomes (Winchell *et al*. 2014). Levels of the cargo adaptor p62/Sequestosome-1, a protein that interacts with LC3 and targets material for degradation during selective autophagy, remain constant throughout

Table 1 Published uses of primary human lung infection systems.

Pathogen	Model	Techniques	Reference
Coxiella burnetii	hAMs	Bacterial growth analysis, fluorescence microscopy, electron microscopy, immunoblot, multiplex cytokine assay and cell viability	Graham *et al.* (2013)
	hAMs, hPCLS	Histology, confocal microscopy, immunoblot, ELISA, cell lysis and RT-PCR array	Graham *et al.* (2016)
	hAMs, hPCLS	Confocal microscopy, bacterial growth analysis and ELISA	Dragan, Kurten and Voth (2019)
	hAMs	Electron microscopy, confocal microscopy and immunoblot	Winchell *et al.* (2014)
	hAMs	Confocal microscopy and immunoblot	Winchell *et al.* (2018)
	hAMs	Immunoblot analysis and confocal microscopy	Colonne *et al.* (2016)
	hAMs	Fluorescence microscopy and immunoblot	MacDonald, Kurten and Voth (2012)
	hAMs	Fluorescence microscopy and immunoblot	MacDonald *et al.* (2014)
Legionella pneumophila	HLTEs	Histology, immunohistochemistry, bacterial growth analysis, transcriptomics, qRT-PCR and ELISA	Jager *et al.* (2014)
	HLTEs	Bacterial growth analysis	Hoppe *et al.* (2017)
Yersinia pestis	hAMs, hPCLS	Adherence assay, epifluorescence microscopy, flow cytometry analysis, cell viability, bacterial growth analysis, confocal microscopy and cytometric bead array	Banerjee *et al.* (2019)
	hAMs	Bacterial growth analysis	Crane *et al.* (2021)
Mycobacterium tuberculosis	hAMs	Cell viability, ELISA, DNA fragmentation, electron microscopy, epifluorescence microscopy and apoptosis analysis	Keane *et al.* (1997)
	hAMs	Flow cytometry, macrophage infection, cytokine bead array and RNA sequencing	Goenka *et al.* (2020)
	hAMs	Microarray, functional enrichment analysis and ingenuity pathway analysis (IPA)	Lavalett *et al.* (2020)
	hAMs	qPCR	Hackett *et al.* (2020)
	hAMs	Histology, confocal microscopy and PCR	Ufimtseva *et al.* (2018)
Staphylococcus aureus	hAMs, hPCLS	Fluorescence microscopy, confocal microscopy, proliferation assay, ELISA, immunoblot, cyttotoxicity assay and cell viability	Brann *et al.* (2019)
Alternative	hAMs	Phase contrast microscopy, phagocytosis analysis and bacterial killing	Cohen and Cline (1971)
	hAMs	ELISA, PGE_2 assay and nitric oxide (NO) assay	Menard, Turmel and Bissonnette (2007)
	hPCLS	Phagocytosis analysis, flow cytometry, fluorescence microscopy, proliferation analysis and two-photon fluorescence microscopy	Bai *et al.* (2016)
	hPCLS	WST-1 assay, confocal miscroscopy, live/dead staining, bronchioconstriction analysis, ELISA and histopathology	Neuhaus *et al.* (2017)

infection of hAMs with NMII, suggesting *C. burnetii* does not allow autophagic flux needed to revert the host cell to homeostasis (Winchell *et al.* 2014). p62 localizes to NMI and NMII PV, although silencing p62 expression does not significantly impact *C. burnetii* growth, suggesting a signaling role for the cargo adaptor. p62 contains multiple phosphorylation sites that direct protein activity and downstream signaling, with modification of S349 activating the Nuclear erythroid 2-related factor-2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) pathway, a major antioxidant mechanism in human cells. Nrf2 is also involved in activation of selective autophagy. Whereas total p62 levels remain constant throughout infection with NMII *C. burnetii*, S349-phosphorylated p62 levels increase and Nrf2 levels are stabilized, indicating activation the Nrf2-Keap1 pathway during infection (Winchell *et al.* 2018), which has not yet been studied using *C. burnetii*-infected murine models. These findings suggest important interplay between autophagy-related proteins and host signaling cascades during *C. burnetii* growth in hAMs.

In addition to Nrf2 signaling, *C. burnetii* activates the host cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) signaling pathway to form the PV and ensure host cell survival (MacDonald, Kurten and Voth 2012; MacDonald *et al.* 2014). The use of PKA inhibitor H-89 in hAMs infected with NMI or G *C. burnetii* results in defective PV formation compared with non-treated cells (MacDonald, Kurten and Voth 2012). Phosphorylated PKA levels increase from 72–96 hpi in NMI- and G-infected hAMs, suggesting PKA activity is required for optimal *C. burnetii* infection (MacDonald, Kurten and Voth 2012). Furthermore, a significant increase in apoptotic cell death occurs when *C. burnetii*-infected hAMs are treated with H-89 (MacDonald *et al.* 2014). Levels of phosphorylated Bad, a pro-apoptotic protein, also increase during *C. burnetii* infection, suggesting the pathogen uses PKA to inactivate Bad and prevent apoptosis. The use of hAMs confirmed the importance of PKA for optimal *C. burnetii* infection and prevention of apoptosis.

In addition to Bad, PKA activates vasodilator-stimulated phosphoprotein (VASP), which is a regulator of actin-based cytoskeleton assembly. Using hAMs, Colonne *et al.* showed VASP activity is essential for NMI PV formation, although *C. burnetii* does not use actin-based motility similar to related intracellular bacteria, but remains in the PV for replication (Colonne *et al.* 2016). VASP localization to the PV membrane suggests *C. burnetii*

uses VASP to coordinate actin organization around the PV during vacuole expansion. S157- and S239-phosphorylated VASP levels also increase throughout infection, further indicating activation of the protein (Colonne *et al.* 2016). Together, these results suggest VASP activation is necessary for optimal *C. burnetii* infection, and indicate an undefined role for additional actin-related proteins during infection.

Collectively, the hPCLS and hAM systems have allowed novel investigation of the *C. burnetii*-host dynamic not possible using immortalized cell lines and small animal models. Understanding of the targeted cellular niche, innate pro-inflammatory response, inflammasome activation and multiple cellular signaling pathways has been enhanced using these platforms to replicate *C. burnetii* interactions with the human lung. These systems have been extended to additional bacteria that exploit the intracellular host cell environment as described below.

LEGIONELLA PNEUMOPHILA

Legionella pneumophila is a Gram-negative bacterium that causes Legionnaires' disease, a type of pneumonia that presents with fever, headache, muscle pain, dyspnea and chest pain. It is difficult to distinguish this pneumonia from others, often resulting in misdiagnosis (Fields, Benson and Besser 2002; Muder and Yu 2002). *Legionella pneumophila* is typically found in natural fresh-water environments or can colonize man-made freshwater environments, such as cooling towers, and can replicate at a vast temperature range of 25–42°C. Here, *L. pneumophila* replicates within amoebae and is transmitted through infected aerosols (Castillo, Rajasekaran and Ali 2016). Once inhaled by a human, *L. pneumophila* is engulfed by alveolar macrophages and evades endocytic maturation that creates the degradative lysosomal environment. Instead, *L. pneumophila* fuses with endoplasmic reticulum-derived vesicles and deploys a T4SS to create a *Legionella*-containing vacuole in which to replicate (Newton *et al.* 2010; Castillo, Rajasekaran and Ali 2016). After replicating to high numbers, *L. pneumophila* causes pore formation and cell lysis to exit the host cell and infect bystander cells (Fields, Benson and Besser 2002; Newton *et al.* 2010). Infection systems used to model disease include cell lines, mice and guinea pigs. Primary hAMs and human lung tissue are underutilized *L. pneumophila* infection models, but remain the most disease-relevant systems to define pathogenic events.

Jager *et al.* used human lung tissue explants (HLTEs) from surgery patients to study *L. pneumophila* infection in the pulmonary environment (Jager *et al.* 2014). The HLTE model is different from the hPCLS model, as tissue samples are obtained from live patients compared with post-mortem hPCLS samples. Infection of HLTEs confirmed that alveolar macrophages are the primary target cell of *L. pneumophila* (Copenhaver *et al.* 2014). Dead macrophages and damaged tissue, with *L. pneumophila* close to damaged areas, are present after 48 h in HLTEs. To identify the cause of tissue damage, three conditions were used: wild type-infected, DotA$^-$-infected (T4SS-deficient) and *L. pneumophila*-shed outer membrane vesicle (OMV)-stimulated samples (Jager *et al.* 2014). Damage increases significantly in wild type-infected and OMV-stimulated samples after 48 h, with wild-type damage more substantial than DotA$^-$-infected samples, suggesting a functional T4SS is required for infection and resulting tissue damage. The use of multiple bacterial loads of wild type or T4SS-deficient *L. pneumophila* showed that bacterial inoculum size does not correlate to increased tissue damage. Bacterial colony counts confirmed HLTEs are permissible for intracellular replication of wild type but not DotA$^-$ bacteria, similar to previous studies using murine macrophage cell lines (Yan and Cirillo 2004). Transcriptional profiles of uninfected and infected HLTEs were compared and displayed a significant difference in the host response in these tissues. Expression of uteroglobulin, an airway secretory protein involved in immune cell recruitment, and the macrophage receptor with collagenous structure (MARCO), involved in bacterial uptake, decreases in wild type-infected HLTEs, though the importance of these events is unknown (Jager *et al.* 2014). Overall, this study opens the door to further use and characterization of disease-relevant human lung infection systems to model *L. pneumophila* infection.

Legionella pneumophila mutants with an insertion in Lpc2666 have decreased replication and increased association with lysosomal compartments (Shevchuk *et al.* 2014). Lpc2666 encodes the type IV pilus fimbrial biogenesis factor PilY1, which is necessary for proper T4SS assembly in *Pseudomonas aeruginosa*. Hoppe *et al.* expanded on this discovery by characterizing PilY1 in HLTEs (Hoppe *et al.* 2017). HLTEs were infected with wild type, *pilY1* knockout, *pilY1* complemented or T4SS-deficient *L. pneumophila*. A significantly decreased bacterial load is present in *pilY1* knockout-infected and T4SS-deficient-infected samples over a 50 h time course compared with wild type-infected samples. Complementation of PilY1 restores replication efficiency, confirming PilY1 is needed for proper *L. pneumophila* infection in a human lung system (Hoppe *et al.* 2017). Hoppe *et al.* further confirmed the importance and relevance of the HLTE infection model, defining a significant role for PilY1 during infection.

Collectively, use of HLTEs has enhanced understanding of *L. pneumophila* infection and pathogenesis in a human disease-relevant setting. In light of large numbers of mis-diagnosed cases of Legionnaires' disease, primary hAMs and human lung tissue represent new systems that can be used to define *L. pneumophila* infection events that are distinct from related pathogens that cause severe pneumonia.

YERSINIA PESTIS

Yersinia pestis is a Gram-negative facultative anaerobe that causes deadly pneumonic, bubonic and septicemic plague (Ditchburn and Hodgkins 2019). Bacterial transmission to humans occurs through an infected flea bite or contaminated aerosols, and *Y. pestis* is categorized as a tier 1 select agent due to the potential to cause widespread mortality. Pneumonic plague is the deadliest form of *Y. pestis* disease and presents with symptoms including fever, cough and shortness of breath (Bosio, Goodyear and Dow 2005). Pneumonic plague must be treated with antibiotics within 24 h after onset of symptoms to prevent lethality (Inglesby *et al.* 2000; Pechous *et al.* 2016; Ditchburn and Hodgkins 2019). After inhalation, *Y. pestis* can remain extracellular or infect pulmonary cells, and disease transitions from a pre-inflammatory to a pro-inflammatory phase within 2–4 days, with significant innate immune cell influx into the lungs (Bosio, Goodyear and Dow 2005). Unfortunately, early pre-inflammatory stages of *Y. pestis* pulmonary infection in humans are undefined. Therefore, hPCLS and hAMs represent important tools to characterize pre-inflammatory *Y. pestis* infection and model pneumonic plague in humans.

Recent work has established hPCLS and hAMs as reliable models of early *Y. pestis* infection. Plasminogen activator protease (Pla) is a virulence factor necessary for progression of pneumonic plague and is used throughout *Y. pestis* growth *in vivo*. *Yersinia pestis* Δ*pla* exhibits reduced adhesion to hAMs and decreased *Yersinia* outer protein (Yop) effector translocation via

the type III secretion system (T3SS) (Banerjee *et al.* 2019). In hPCLS, Yop effector translocation occurs at 2–4 hpi, demonstrating the utility of hPCLS for modeling early infection events. *Yersinia pestis* Δ*pla* also displays reduced Yop translocation in hPCLS compared with wild-type bacteria, particularly in alveolar macrophages, mirroring hAM results (Banerjee *et al.* 2019). These data suggest Pla plays a vital role in *Y. pestis* effector secretion in hAMs. Pro-inflammatory cytokine expression that is more difficult to assess in murine models, due to expression being below the limit of detection in *Y. pestis*-infected mice (Lathem *et al.* 2007), is altered during infection with *Y. pestis* Δ*pla* compared with wild-type bacteria. Secreted TNF-α, IL-6 and IL-8 all significantly increase during infection with *Y. pestis* Δ*pla*. This result is striking, insinuating Pla is vital for early inhibition of a pro-inflammatory response, permitting *Y. pestis* survival and replication (Banerjee *et al.* 2019). Together, these results demonstrate the use of hAMs and hPCLS in assessing host–pathogen interactions during *Y. pestis* infection.

Regarding additional potential virulence factors, BPI-inducible protein A (BipA), a conserved GTPase, is important for virulence of many pathogens such as *Escherichia coli* and *Pseudomonas aeruginosa*; however, the role of *Y. pestis* BipA during infection has not been defined (Scott, Diggle and Clarke 2003). Crane *et al.* assessed whether BipA impacts phagocytic cells, such as primary human neutrophils, hAMs and murine alveolar macrophages (MH-S) by infecting each cell type with wild-type *Y. pestis*, Δ*bipA* bacteria or Δ*bipA*::*bipA* bacteria. No difference in *Y. pestis* survival in hAM or MH-S cells occurs regardless of strain. However, survival of Δ*bipA Y. pestis* significantly decreases in neutrophils (Crane *et al.* 2021). Collectively, these results show BipA is critical for bacterial survival in neutrophils, but not macrophages.

Altogether, studies using hAMs and hPCLS during *Y. pestis* infection have further defined parameters required for efficient infection. Though use of these models is new to this field, disease-relevant systems are necessary to derive human disease-relevant conclusions for comparison to many years of small animal model studies.

MYCOBACTERIUM TUBERCULOSIS

Mycobacterium tuberculosis is the causative agent of human tuberculosis, one of the deadliest diseases worldwide. *Mycobacterium tuberculosis* has an aerosol mode of transmission and results in roughly 2 million deaths annually (Ufimtseva *et al.* 2018). Following inhalation, alveolar macrophages engulf *M. tuberculosis* wherein the pathogen survives and replicates (Huang *et al.* 2018). Infected alveolar macrophages secrete cytokines and chemokines, attracting innate immune cells such as neutrophils, lymphocytes, dendritic cells and T cells that collectively contribute to granuloma formation (Fogel 2015). Granuloma formation typically results in an inactive or 'latent' phase of disease, with no symptoms or disease transmission. However, the granuloma can be disrupted, allowing *M. tuberculosis* to disperse throughout the pulmonary environment and infect new cells (Smith 2003; Pai *et al.* 2016). Due to ease of transmission and infection of human alveolar macrophages, it is necessary to expand research on new therapies and targets of *M. tuberculosis* using disease-relevant models such as hPCLS and primary hAMs.

Keane *et al.* (1997) used *M. tuberculosis* H37Ra and H37Rv strains that are nonpathogenic and pathogenic in mice, respectively, to study apoptotic cell death in hAMs. Both strains cause significant cell death with or without addition of TNF-α, a potentially cytotoxic cytokine, though notably higher levels of death are triggered by H37Ra. Furthermore, both isolates elicit significant production of TNF-α at 2 and 24 h, suggesting TNF-α is not the sole reason for increased cell death during H37Ra infection. Visual fragmentation and condensation of DNA is evident in *M. tuberculosis* H37Ra-infected samples, indicative of apoptosis. TUNEL staining also reveals a significant number of apoptotic cells in samples infected with *M. tuberculosis* H37Ra (Keane *et al.* 1997). This study established hAMs as a reliable platform to better understand the role of apoptosis during early *M. tuberculosis* infection.

Infants are more susceptible to tuberculosis infection, making it necessary to compare infant hAMs to adult hAMs to define mechanistic aspects of infection severity. No discernible difference in macrophage phenotype marker expression or phagocytic capacity is evident between infant and adult hAMs (Goenka *et al.* 2020). Even so, infant hAMs support significantly increased *M. tuberculosis* replication at 24 and 48 h, with increased production of neutrophil-attracting CXCL8 compared with adult hAMs. Transcriptional analysis of infant hAMs revealed significantly altered gene expression and regulation of multiple cytokines and chemokines. Specifically, lower expression of mycobactericidal genes, IFN-γ response genes, and increased expression of polymorphonuclear attractant signals occur in infant hAMs (Goenka *et al.* 2020). Collectively, this study revealed novel age-dependent insights into *M. tuberculosis* infection.

hAMs have also been used to define differential gene expression in TB patients. Lavalett *et al.* (2020) isolated hAMs from healthy (AMCT) and tuberculosis-positive (AMTB) donors, infected cells *in vitro* with one of two clinical isolates of *M. tuberculosis*, UT127 or UT205, and determined the differential transcriptional profiles of these samples. Differentially expressed genes are similar between control hAMs and hAMs previously exposed to *M. tuberculosis* after infection with UT127 or UT205. However, specific pathways, including the NF-κB pathway, TNF signaling and the overall inflammatory response, are enriched in either healthy or tuberculosis-exposed hAMs after infection with *M. tuberculosis* (Lavalett *et al.* 2020). Infection of AMCTs with *M. tuberculosis* UT127 or UT205 increases immune response signaling, organelle assembly and IFN-γ response gene expression. In addition, infection of AMTBs with *M. tuberculosis* UT127 or UT205 elicits isolate-specific differences in gene expression. For example, UT127 increases the STAT- and IL-23-related gene expression while UT205 increases IL-10 and IL-17 production. Further analysis assessed the difference in canonical pathway expression between *M. tuberculosis* UT127 or UT205-infected AMCTs and AMTBs. AMCTs infected with either strain results in activation of pathways such as iNOS, PI3K/AKT and apoptosis signaling. Infected AMBTs show a striking difference, with activation of interferon and IRF cytosolic pattern recognition receptor pathways (Lavalett *et al.* 2020). Overall, this study demonstrated important differences in transcriptional profiles between healthy and tuberculosis-infected donor hAMs when infected with distinct clinical isolates of *M. tuberculosis*. Ultimately, these results can be used to inform isolate-specific therapy design.

Emerging research has identified a critical role for host cell metabolism in the response to intracellular pathogens, including *M. tuberculosis*. Glycolysis, an important metabolic process used by all human cells, is required for protection against *M. tuberculosis* (Gleeson *et al.* 2016). To demonstrate the metabolic reprogramming ability of the pathogen, hAMs were infected with *M. tuberculosis* H37Rv (virulent) or γ-irradiated H37Rv (inactive). Results showed γ-irradiated H37Rv-infected samples

have significantly increased production of *il-1b* mRNA and the glycolysis-related genes *SCL2A1* and *HK2* (Hackett *et al*. 2020). Strikingly, virulent *M. tuberculosis* H37Rv-infected hAMs have increased production of anti-inflammatory miRNA-21 (miR-21), suggesting a vital role for this miRNA during infection. This study then used bone marrow-derived macrophages to show miR-21 targets phosphofructokinase muscle (PFK-M) isoform to decrease glycolytic activity and program metabolism in favor of *M. tuberculosis* replication (Hackett *et al*. 2020). The use of primary hAMs strengthened these findings that show miR-21 reduces the pro-inflammatory immune response to *M. tuberculosis*.

In addition to defining cellular infection events, hAMs have been used to obtain information about infection severity. Ufimtseva *et al*. demonstrated an alternative method to obtain hAMs and other cell types from the pulmonary environment to study *M. tuberculosis* severity (Ufimtseva *et al*. 2018). Lung sections were surgically removed from tuberculosis-positive patients, processed and samples plated to define cell types within each area of the lung. Isolated cells consist of alveolar macrophages (AMs), dendritic cells, neutrophils, fibroblasts, lymphocytes, and multinucleated giant cells, although the most populous cell type is the AM (Ufimtseva *et al*. 2018). The majority of isolated AMs are infected with *M. tuberculosis*, whereas other cell types contain little to no bacteria. Some infected AMs are lipid rich, and *M. tuberculosis* co-localizes to these lipid body-positive regions. Strikingly, samples from patients with severe tuberculosis have increased populations of *M. tuberculosis*-infected AMs, suggesting a correlation between AM infection and disease severity. Infected macrophages are largely derived from cavity walls or distant sites within the pulmonary environment (Ufimtseva *et al*. 2018). Together, these results suggest a more direct route of AM isolation to study *M. tuberculosis* infection and indicate AM infection corresponds to disease severity.

Collectively, the studies above highlight the importance of using disease-relevant hAMs to fully understand *M. tuberculosis* pathogenesis in humans. Studies using this platform have provided apoptotic signaling discoveries, isolate-specific infection differences, age-dependent infection differences, the use of miRNA to reduce the pro-inflammatory response and an alternative route of cell isolation from tuberculosis-positive patients. The use of hAMs provides these results in a system that is immediately applicable to human disease and can be used to identify new therapeutic targets. We predict that hPCLS will also soon be used to further define early infection events in human lungs infected with *M. tuberculosis*.

STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is a Gram-positive pathogen that causes disease in most human organs, including severe pneumonia in the lungs. Although largely considered an extracellular pathogen, *S. aureus* can infect and survive within epithelial cells, macrophages and neutrophils, highlighting the versatility of the pathogen and ability to resist intracellular degradation (Almeida *et al*. 1996; Gresham *et al*. 2000; Flannagan, Heit and Heinrichs 2016). *Staphylococcus aureus* produces numerous virulence factors that elicit distinct disease symptoms and are often isolate specific. For example, the highly pathogenic isolate LAC produces the major cytotoxins Panton–Valentine leukocidin (PVL) and α-hemolysin (Hla), whereas the less cytotoxic isolate UAMS-1 produces neither PVL or Hla (Brann *et al*. 2019), yet can still cause major disease. Current models of *S. aureus* pulmonary infection include immortalized cell lines, primary cells, and mouse, rat and rabbit models (Martínez-Olondris, Rigol and Torres 2010; Flannagan, Heit and Heinrichs 2016). However, inconsistencies when comparing results from differing disease models, and distinct human events not replicated in small animal models of pneumonia, highlight the need for improved human infection models.

To address the inconsistencies noted above, Brann *et al*. (2019) characterized *S. aureus* infection of hPCLS and hAMs. Infection of primary hAMs by LAC or UAMS-1 *S. aureus* indicated survival of both isolates in a late phagosome; however, replication is not efficient in this intracellular niche, suggesting hAMs do not permit *S. aureus* replication, a striking contrast from murine macrophage studies (Brann *et al*. 2019). Further assessment found *S. aureus* largely does not reside in hAMs in hPCLS. The pathogen is present in higher numbers within the interstitium, which contains a large population of fibroblasts, suggesting non-macrophage cells are targeted by *S. aureus* during lung infection (Brann *et al*. 2019). Further investigation of hAMs showed there is a phenotypic switch from pro-inflammatory M1 to anti-inflammatory M2 polarization during *S. aureus* infection, indicating a more permissive environment similar to *C. burnetii* (described above). hAMs produce a significant pro-inflammatory response during infection with *S. aureus*, and increasing multiplicities of infection of LAC results in higher cytotoxicity than UAMS-1. The cytolysin Hla is produced during LAC infection, but presence of the toxin does not correlate to increased cytotoxicity, as a Hla-deficient LAC mutant efficiently kills hAMs (Brann *et al*. 2019). Collectively, these results indicate *S. aureus* does not use hAMs as an intracellular replication niche in human lung tissue, but targets cells in the interstitium. This study demonstrates hAMs and hPCLS can now be used to further advance understanding of *S. aureus* pneumonia in a system that is directly applicable to the human lung setting.

ALTERNATIVE USES

In the sections above, we highlighted pathogen-specific uses for primary hAMs and hPCLS. In addition to those examples, alternative uses of these platforms are important for further characterization of these primary cellular systems and lung physiology, and to investigate the effects of drug addition to pulmonary tissue. These alternative uses further define the hAM and hPCLS platforms while expanding use of each system for disease-relevant, pathogen-free studies. We provide a sampling of these alternative uses below.

A major use for primary lung cell systems is the ability to study host–macrophage function. Cohen and Cline used lavage fluid from resected human lungs to characterize the morphology and phagocytic ability of alveolar macrophages (Cohen and Cline 1971). Three varying sizes of mononuclear cell types were noted when cells were isolated and cultured. Using *Candida albicans*, the ability of alveolar macrophages to phagocytose organisms was assessed *in vitro*. This study determined phagocytosis increases over several days post-infection and is directly dependent on macrophage size. Comparing differing lung conditions, such as healthy lungs, smokers' lungs (cigarettes or marijuana), obstructed lungs or pneumonic lungs, researchers found smoker lung phagocytic capacity is comparable to healthy lungs whereas obstructed and pneumonic lung cells display increased phagocytosis. Specifically, hAMs display maximum ingestion ~45 min post-infection with heat-inactivated *C. albicans* or *Aspergillus fumigatus*. Researchers also compared the bactericidal ability of polymorphonuclear cells, hAMs and monocyte-derived macrophages infected with *Listeria monocytogenes*. These

comparative studies showed that polymorphonuclear cells have significantly increased bactericidal ability compared with hAMs and monocyte-derived macrophages (Cohen and Cline 1971). Collectively, this study revealed vital information about alveolar macrophage phagocytosis in distinct lung conditions that alters the response to foreign material.

Another nonpathogenic use for primary hAMs involves signaling molecules that regulate innate immune responses. For example, the role of serotonin in alveolar macrophage responses was first assessed in rat alveolar macrophage cell lines stimulated with lipopolysaccharide (LPS; Menard, Turmel and Bissonnette 2007). This study showed that addition of serotonin to cells decreases TNF secretion and increases IL-10 secretion. Importantly, the study was repeated and confirmed using hAMs, which display a similar decrease in TNF production with or without LPS stimulation after serotonin addition, and an increase in IL-10 with LPS stimulation after serotonin addition. Researchers also assessed the effect of indomethacin on cytokine production by hAMs, and noted indomethacin inhibits previously reported differences in TNF and IL-10 production (Menard, Turmel and Bissonnette 2007). These data suggest a major role for serotonin in regulating hAM cytokine production.

Routine use and storage of hPCLS begs the question of whether cryopreservation alters viability or functionality. Bai *et al.* (2016) addressed this question by evaluating cell viability and functional activities, including phagocytosis, airway contraction, intracellular calcium signaling and the effect of TAS2R agonists. These studies showed cryopreservation has no discernible effect on cell viability, lymphocyte presence or phagocytic capacity. Airway contraction in cryopreserved tissue is comparable to hPCLS that were never frozen using multiple agonists such as histamine, methacholine and β_2-adrenergic receptor agonist. Additionally, and vital for smooth muscle cell research, cryopreservation has no obvious effect on intracellular calcium regulation of contractions. To further pursue the use of hPCLS as a platform for smooth muscle cell research, this study determined calcium signaling is inhibited via addition of bitter-taste receptor TAS2R (Bai *et al.* 2016). Overall, these results show cryopreservation has no significant effect on hPCLS and is a viable option for long-term storage.

To further expand use of hPCLS, viability and functionality of long-term cultivated tissue was assessed by Neuhaus *et al.* (2017). After a 15-day incubation, results showed no significant difference in metabolic activity or cell viability compared with day 1. To replicate constriction, various concentrations of methacholine were added to hPCLS over 15 days. hPCLS constrict throughout the full 15-day cultivation, though higher concentrations of methacholine are required. To further assess hPCLS functionality, the production of TNF-α with or without LPS stimulation was evaluated. TNF-α production continuously decreases over a 15-day time period in LPS-treated samples. Histology demonstrated similar maintenance of pulmonary structural components in hPCLS at day 1 and day 15 (Neuhaus *et al.* 2017). This study further characterized functional aspects of hPCLS as a novel platform that can be maintained for several days *in vitro*.

hAMs and hPCLS are useful not only to further understanding of bacterial infection but also for alternative studies, such as tissue banking, macrophage function, the immune response to hormones and culturing techniques. These studies broaden the use of these novel lung model systems and further emphasize the relevance of primary platforms.

CONCLUSIONS

Overall, the hAM and hPCLS systems have enormous potential for studying human cell activity in a physiological setting. These systems are invaluable for studying the human pulmonary innate immune response, cellular signaling and lung infection by microbial pathogens. Obtaining these tissues from human donors post-mortem provides the most human disease-relevant *ex vivo* lung models to date. These infection systems are gaining traction in the bacterial pathogen field and are useful for all pulmonary infection research. For example, hAMs and hPCLS have been used for influenza research, providing the groundwork for the advantages these model platforms can provide in viral research. With these systems in place, the alarming circumstances of the SARS-CoV-2 global pandemic, and the need to rapidly respond to novel microbes, hAMs and hPCLS will be reliable platforms to further understand COVID-19 disease pathogenesis and future pathogen outbreaks. We predict these platforms will enhance understanding of human pulmonary disease and physiology in several research fields over the coming years.

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